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Contamination with *Enterococcus* spp., strains of lettuces of different species acquired in Cumaná

Abstract

Lettuce is one of the most common crops in the world, with high index of vitamins, minerals and fiber. Raw food consumption is closely related to foodborne illness, of which *Escherichia coli* O157:H7, seems to be the prime perpetrator leading to undesirable consequences. However, *E. coli* is not the only microorganism that can be transmitted, as reports validating the involvement of *Aeromonas, Yersinia, Listeria, Staphylococcus, Campylobacter*, and *Salmonella* have surfaced. The objective of this work was to detect the contamination of lettuces of different species acquired in several places of Cumaná, with *Enterococcus* strains. Of 52 lettuces acquired in food retail centers, 38% were contaminated with *Enterococcus* spp., strains. The best-selling lettuce was *Lactuca sativa L*. (25/52), but the most contaminated was *Lactuca sativa* var. *capitata* (89%). The highest average for *Enterococcus* was obtained in lettuces from Municipal Market. The dominant species of *Enterococcus* were *E. faecalis* (40%), and *E. casseliflavus* (30%); and to a lesser extent *E. faecium* (15%), E. *gallinarum* (10%), and *E. avium* (5%). The susceptibility profile showed that there are strains resistant to glycopeptides, fluoroquinolones, ansamycins, macrolides, phenols and tetracyclines. The clonal dissemination of two strains of *E. faecalis* and one strain of *E. faecium* by antibiotyping was demonstrated in lettuces from the municipal market. These results demonstrate that the food chain is a route of dissemination of multidrug resistant *Enterococcus* to the human intestinal microbiota, turning the gastrointestinal tract into a reservoir of intractable bacteria with the available antibiotics.

Keywords: fecal contamination, foodborne illness, bacterial resistance, free antibiotics wastewater, irrigation water, agriculture, potential risk

Volume 11 Issue 1 - 2023

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Received: January 20, 2023 | **Published:** January 27, 2023

Introduction

Several studies have shown that vegetables grown in developing countries may be contaminated with pathogenic microorganisms, and the scope of contamination is high when wastewater is used for irrigation. Other sources of potential risk to health are organic fertilizers, methods of transporting products, handling in markets and at points of consumption.

Hygienic measures are an important for food safety, especially for vegetables eaten raw that grow close to the soil surface contaminated with human and animal fecal matter which include poultry manure. The survival of these bacteria in the soil after manure application can be up to 100 days. The transfer of pathogens to lettuce can occur through splashing effects caused by raindrops, sprinkler irrigation or by means of transport of soil particles by mechanical weeding.[1]

The consumption of vegetables contaminated with pathogenic microorganisms, especially in urban areas where wastewater is used to irrigate vegetable crops, has been the prime reason for the outbreaks of public health manifestations. To reduce the risk associated with the consumption of contaminated vegetables, people must know vegetable decontamination methods. [2] Increase in fast food consumption in street stalls, has been one of the main factor related to health problems associated with the proliferation of microorganisms due to unhygienic practices causing gastrointestinal problems,[3] and of these foods, the most contaminated are vegetables (including lettuce). In Argentina, a study was carried out in which it was demonstrated that resistant *Enterococcus* strains that were believed to be confined to the hospital setting were found in the community and that lettuce could be the vehicle for transmitting these pathogens to healthy individuals.[4]

The purpose of carrying out this work was to detect the contamination of lettuce of different species acquired in different places of Cumaná, with strains of *Enterococcus*, which will contribute to define its possible role as a reservoir of resistance genes to antibiotics for human clinical use.

Material and methods

Samples

The samples were 52 lettuces of different species which were previously identified as *Lactuca sativa L.* (Batavia), *Lactuca sativa* var. *longiofolia* (Roman), *Lactuca sativa* var. *capitata* (Icerberg), *Cichorium intybus* var. *foliosum* (Red chicory) and *Cichorium endivia* var. *crispum* (Endive), were acquired in various commercial establishments in Cumaná, Sucre state, in Venezuela. Sampling was carried out following the table of random numbers.

Isolation and recovery of *Enterococcus* spp., from lettuce of different species

10 g of lettuce leaves were placed in tubes with Brain Heart Infusion (BHI) broth; then they were incubated in a shaking water bath at 35ºC for 24 hours. An inoculum was taken from each tube and plated on *Enterococcus* Confirmatory agar (Biomark); the plates were incubated at 35ºC for 24 hours.[5]

Colonies with typical morphology for *Enterococcus* (yellow or cream) were sought on the selective agar plates with growth visible. All colonies were tested for catalase to verify that they were catalase negative. The selected colonies on the plates were transferred with a wooden toothpick to hemolysis tubes with 1 mL of BHI broth with 6.5 NaCl and placed in a water bath at 45ºC for 24 hours to confirm the presence of *Enterococcus*.[6]

Identification of *Enterococcus* species

Once the presence of *Enterococcus* strains was confirmed, the species were identified through the RAPID ID 32 STREP gallery (bioMérieux, Marcy-l'Étoile, France), which consists of 32 tests for streptococci and bacteria similar to this family. From a pure culture, a suspension of the strains was prepared at a turbidity equivalent to 4 McFarland. The gallery domes were inoculated with 55 µL of the suspension. The galleries were incubated at 35°C for 4 hours and read according to the manufacturer's instructions.

Detection of antibiotypes of strains of *Enterococcus* spp., from lettuce through antimicrobial susceptibility tests

The antimicrobial susceptibility profile was performed using the disk diffusion method. An inoculum corresponding to the 0.5 McFarland pattern of each strain was prepared, Müeller-Hinton (MH) agar plates were sown, according to the instructions in the M100-S26 manual. The antibiotics used were: vancomycin (30 µg), teicoplanin (30 µg), ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), tetracycline (30 µg), ciprofloxacin (5 µg), linezolid (30 µg), norfloxacin (10 µg), nitrofurantoin (300 µg), rifampin (5 µg). They were incubated at 35ºC for 24 hours, then the inhibition zones were read and correlated with the 2D interpretive tables published in the M100-S26 manual.[7]

The antibiotyping was carried out by comparing all the strains of the same species, from the point of view of the profile of the antimicrobial susceptibility tests, in order to distinguish whether the *Enterococcus* strains found in the lettuce were from the same strain or was it a different one.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) to ampicillin, vancomycin and ciprofloxacin was determined by the MH agar dilution method, according to the standards of the M100-S26 manual.[7] The strains used for quality control were: *S. aureus* ATCC 25923 (the negative control) and *E. faecalis* 77904 VanB (positive control). Agar plates containing different concentrations of antibiotic (0.5 – 512 µg/mL) were inoculated and incubated for 24 hours at 35ºC. The CMI is the first concentration where there is no visible growth. The presence of 1 to 3 colonies was not taken into account.

Statistical analysis

Once the necessary assumptions for the data obtained were verified, an analysis of variance (95%) was applied in order to obtain the possible existence of a difference in the frequency of the bacterial group between the types of lettuce, commercial warehouses and the times of sampling. The results obtained were presented in the form of tables and figures.[8]

Results

Fifty-two lettuces purchased in various commercial establishments in the city of Cumaná were studied. In the Municipal Market, five *Lactuca sativa L*, three *Lactuca sativa var. longiofolia* and three *Lactuca sativa* var. *capitata*; from the Mobile Stand: 16 *Lactuca sativa L*., from the Supermarket on the outskirts of the city: two *Lactuca sativa* var. *capitata* and two *Cichorium intybus* var. *foliosum*, from the Supermarket in the city center: four *Lactuca sativa L*. and two *Lactuca sativa* var. *capitata*, from the most popular supermarket: two *Lactuca sativa* var. *capitata*, from the exclusive Supermarket: three *Lactuca sativa var. longiofolia* and two *Cichorium endivia* var. *crispum*, from the new supermarket: three *Cichorium intybus* var. *foliosum* and five *Cichorium endivia* var. *crispum*.

Of the total lettuce studied, 20 were positive for *Enterococcus* (38%), of which the same number of strains of different species was obtained. According to the results obtained, it can be seen in table 1 that the highest frequency of isolation of *Enterococcus* spp., occurred in *Lactuca sativa* var. *capitata* (89%; 8/9), followed by *Cichorium intybus* var. *foliosum* (60%; 3/5). The lettuce acquired in the exclusive establishments and the street stall were not contaminated with *Enterococcus*.

When applying the simple ANOVA test to compare the amount of bacteria identified according to the place of purchase (Figure 1), significant differences were detected ($p < 0.05$), observing the highest average of *Enterococcus* in lettuce from the Municipal Market and the minimum average in the street stall and the exclusive supermarket. The a *posteriori* test shows the formation of two independent groups, presenting a higher average for the group represented by lettuce from the municipal market.

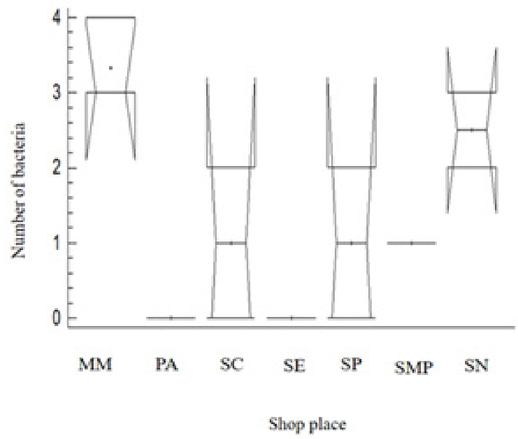

Figure 1 Analysis of Variance (ANOVA) of the lettuce retail centers studied and the frequency of *Enterococcus* isolates.

MM, municipal market; PA, street stall; SC, supermarket in the city center; SE, exclusive supermarket; SP, supermarket on the outskirts of the city; SMP, most popular supermarket; SN, new supermarket.

In the results obtained in general, the *Enterococcus* species mostly isolated in the lettuce samples were *E. faecalis* (40%) and *E. casseliflavus* (30%); while *E. faecium* (15%), *E. gallinarum* (10%) and *E. avium* (5%) were the least frequent.

The *Enterococcus* species recovered in the *Lactuca sativa L*. samples were *E. faecalis* (75%) and E. faecium (25%). Regarding *Lactuca sativa L*. var longifolia, only the species *E. faecalis* (100%) was isolated. In *Lactuca sativa* var. *capitata*, the most frequently isolated species was *E. casseliflavus* (75%), followed by *E. gallinarum* and *E. faecium* (12.5% respectively).

When applying the simple ANOVA test to compare the number of *Enterococcus* identified according to the lettuce class (Figure 2), no significant differences were found ($p > 0.05$). This is due to the fact that most types of lettuce present similar averages in terms of the frequency of isolated bacteria.

In *Cichorium intybus* var. *foliosum*, three species (*E. faecalis, E. gallinarum* and *E. avium*) were isolated with the same frequency (33.3%). In *Cichorium endivia* var. *crispum* two species were isolated, *E. faecalis* and *E. faecium* in equal percentage (50%).

Table 2 shows the antimicrobial susceptibility profile of the *Enterococcus* faecalis strains. The strains show resistance to

Citation: Abadía-Patiño L, Bastardo MF. Contamination with *Enterococcus* spp., strains of lettuces of different species acquired in Cumaná. *J Microbiol Exp.* 2023;11(1):13–18. DOI: 10.15406/jmen.2023.11.00380

fluoroquinolones (ciprofloxacin and norfloxacin), macrolides (erythromycin), and ansamycins (rifampicin).

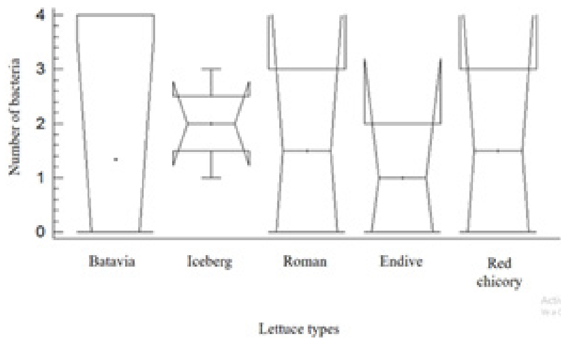

Figure 2 Analysis of Variance (ANOVA) of the types of lettuce studied and the frequency of *Enterococcus* isolates.

Batavia, *Lactuca sativa L*; Iceberg, *Lactuca sativa var*. capitata; Roman, *Lactuca sativa var. longifolia*; Endive, *Cichorium intybus* var. *foliosum*; Red chicory, *Cichorium endivia* var. *crispum*.

Table 3 shows the antimicrobial susceptibility of *E. casseliflavus* present in the samples of *Lactuca sativa* var. *capitata*. According to these results, these strains are 100% sensitive to the antibiotics teicoplanin and nitrofurantoin, but 100% resistant to rifampicin with intermediate susceptibility to erythromycin. Two strains resistant to linezolid.

Table 4 shows the antimicrobial susceptibility of *E. gallinarum* isolated from *Lactuca sativa* var. *capitata*, *Cichorium intybus* var. *foliosum* and *Cichorium endivia* var. *crispum* in which 100% resistance to rifampicin can be observed, as well as intermediate susceptibility to fluoroquinolones, linezolid, erythromycin and tetracycline.

Regarding the clonality of the strains, it was observed that in the *E. faecalis* species there are three clonal strains, two from *Lactuca sativa L*. (9E5 and 9E8) and one from *Lactuca sativa L*. var. longifolia (9E10), from three different lettuces, bought in different stalls on the same day in the Municipal Market. Two more strains of *E. faecalis* (9E6 and 9E9), one of *Lactuca sativa L*. and one of *Lactuca sativa L*. var. *longifolia*, also from the Municipal Market of the same day, but with another antibiotype. Regarding the species E. faecium, two clonal strains (9G8 and 11H8) of *Lactuca sativa L*. var. *capitata* were isolated on different days. and *Lactuca sativa L*. It is worth noting that the clonal strains were only in the lettuce purchased in the Municipal Market, which suggests that the source is the same, that is, it is a single supplier of lettuce for the vendors of the Cumaná Market, Sucre state.

Table 5 shows that the MIC of ampicillin against *Enterococcus* oscillates between 1 and 8 µg/mL, with which they are considered sensitive; the only resistant strain is *E. faecium*. Regarding the vancomycin MIC, the bulk of the population is in the sensitivity range (between 0.5 and 4 µg/mL).

Table 1 Frequency of *Enterococcus spp*. isolations, according to the type of lettuce studied

Lettuce	N° of samples	N° strains isolated	Frequency (%)
Lactuca sativa L.	25	4	16
Lactuca sativa L. var. longifolia	6	3	50
Lactuca sativa var. capitata	9	8	89
Cichorium intybus var. foliosum	5	3	60
Cichorium endivia var. crispum	7	2	28

N°, number; %, percentage

Table 2 Antibiotypes of *Enterococcus faecalis* strains isolated from different lettuces in Cumaná

	ANTIBIOTICS										
N	AMP	VAN	TEC	CIP	NOR	LZD	ERY	TET	C	RIF	F/M
1	S	S	S	R	R	S	I	S	R	R	S
3	S	S	S	I	S	S	I	S	S	S	S
1	S	S	S	I	I	S	I	S	S	R	S
2	S	S	S	S	S	S	I	S	S	R	S
1	S	S	S	S	S	S	R	S	S	R	S

N, number of strains; AMP, ampicillin; VAN, vancomycin; TEC, teicoplanin; CIP, ciprofloxacin; NOR, norfloxacin; LZD, linezolid; ERY, erythromycin; TET, tetracycline; C, chloramphenicol; RIF, rifampicin; F/M, nitrofurantoin

Table 3 Antibiotypes of *Enterococcus casseliflavus* strains isolated from different lettuces in Cumaná

	ANTIBIOTICS										
N	AMP	VAN	TEC	CIP	NOR	LZD	ERY	TET	C	RIF	F/M
1	S	S	S	S	S	S	I	I	I	R	S
1	S	S	S	I	I	S	I	S	S	R	S
1	R	I	S	R	R	R	I	S	S	R	S
1	S	S	S	I	I	S	I	S	S	R	S
1	S	I	S	I	I	S	I	S	S	R	S
1	S	S	S	I	S	R	I	S	I	R	S

N, number of strains; AMP, ampicillin; VAN, vancomycin; TEC, teicoplanin; CIP, ciprofloxacin; NOR, norfloxacin; LZD, linezolid; ERY, erythromycin; TET, tetracycline; C, chloramphenicol; RIF, rifampicin; F/M, nitrofurantoin

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Table 4 Antibiotypes of *Enterococcus gallinarum* strains isolated from different lettuces in Cumaná

	ANTIBIOTICS										
N	AMP	VAN	TEC	CIP	NOR	LZD	ERY	TET	C	RIF	F/M
1	S	R	S	R	I	R	I	I	S	R	S
1	S	S	S	I	S	I	I	S	S	R	S
1	S	S	S	I	I	S	I	S	S	R	S

N, number of strains; AMP, ampicillin; VAN, vancomycin; TEC, teicoplanin; CIP, ciprofloxacin; NOR, norfloxacin; LZD, linezolid; ERY, erythromycin; TET, tetracycline; C, chloramphenicol; RIF, rifampicin; F/M, nitrofurantoin

Table 5 Minimum inhibitory concentration (µg/ml) of *Enterococcus* spp. strains, isolated from lettuce samples studied

CMI	0,5	1	2	4	8	16	32	64	128
VAN	5	1	6	5	2	0	0	1	0
CIP	10	0	2	4	3	0	1	0	0
AMP	7	0	3	3	6	0	0	0	1

MIC, minimum inhibitory concentration; NPV, vancomycin; CIP, ciprofloxacin; AMP, ampicillin

Discussion

In industrialized countries, the daily consumption of fresh, ready-to-eat vegetables has increased in recent years, health concern. Today, ready to use bagged lettuce or mixed vegetables are available in supermarkets, to eat salads without having to prepare them at home, just by uncovering a bag and adding a little dressing. With this practice, a greater morbidity of foodborne diseases has arisen, especially these raw.[9]

Plants, as well as animals and humans, have their own microbiota. In a study carried out on *Lactuca sativa* var. *longifolia* found that the most predominant phyla of microbiota bacteria are: Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria. Among the main genera that comprise the nucleus of the composition of the phyllospheric microbiota of lettuce are: *Arthrobacter, Bacillus, Massilia, Pantoea* and *Pseudomonas*. The composition of the bacterial community on the surface of lettuce leaves varies according to the time of year in which they are cultivated as a function of time, space and environment.[10]

These habitual plant colonizing microorganisms are not harmful to humans. The problem arises when other microorganisms contaminate plants with irrigation water, manure, cross contamination with animals, dirty equipment or human manipulation.[11]

Not finding contamination by *Enterococcus* in the lettuce purchased at the street stall and the exclusive supermarket, it could be because they come from places where they do not use wastewater for irrigation or organic matter for compost, but it could also be that the vendors wash well lettuce before putting it on display for sale.

The lettuces purchased in the municipal market and the new supermarket were the most contaminated, and may have acquired this contamination in the farmland, due to the aforementioned factors, but also due to incorrect handling techniques. In a study carried out in Corrientes, Argentina, contamination by *Enterococcus* strains (42%) was demonstrated in 79 samples of *Lactuca sativa* acquired in three different centers.[5] In this investigation, it can be observed that *Lactuca sativa L.* is the most predominant; the one that was most contaminated by *Enterococcus* was *Lactuca sativa* var. *capitata* with 89% frequency (Table 1), acquired in seven food outlets in Cumaná, Sucre state.

The frequency of microorganisms in vegetables reflects the poor sanitary quality of the raw product at the time of consumption.[12] In Argentina, 42% *Enterococcus* strains were isolated from lettuce samples and among the most frequent species were *E. faecium* and *E. faecalis*.[5] In a study carried out in Côte d'Ivoire, both from lettuce (36 samples) and irrigation water from these crops (36 samples), 27 *Enterococcus* strains were isolated from lettuce and 29 from irrigation water. The main species was *E. faecalis* (75%), as well as strains of *E. faecium, E. gallinarum, E. casseliflavus* and *E. durans*.[13] Regarding the present work, *E. faecalis* was always the predominant species in these foods.

Most *Enterococcus* infections in humans are caused by *E. faecalis*, however, *E. faecium* is responsible for serious infections by multidrug-resistant strains and is the species that causes most deaths. Infections caused by other *Enterococcus* species are not very frequent and do not present multi-resistance problems either.[14] The results of this study reveal that most of the lettuces are colonized with *E. faecalis* as indicated by the international literature. Although *E. faecium* was not the predominant species, three strains resistant to all the antibiotics tested were isolated.

Regarding the susceptibility profile of the *E. faecium* strains isolated from fresh produce in the United States, they presented a high level of resistance to ciprofloxacin, tetracycline, and nitrofurantoin. In total, 91% of these strains were resistant to at least one antibiotic tested, as were 32% of the *E. faecalis* strains. These strains of *E. faecalis*, of the same origin, had a lower prevalence of resistance to human clinical antibiotics and those of agricultural relevance.[15]

These data reflect the serious problem of transmission of antibiotic-resistant pathogens through the food chain. In this work, all *E. faecalis* strains are 100% sensitive to nitrofurantoin. In the event that a person acquires this strain through the food chain and develops a urinary infection due to said strain (with a starting point due to fecal contamination), it is possible to treat them with nitrofurantoin. The fact that food is the vehicle for the transmission of *Enterococcus* to humans, that is, the reservoir for the horizontal transfer of resistance determinants between environmental and human strains, is a public health problem, since this species survives by passing the gastric barrier, multiplies and colonizes the intestinal tract, for long periods.[15] Patients with predisposing factors can develop infections by colonizing strains of their intestine, with mechanisms of resistance to antibiotics for human clinical use.

In countries like Argentina, Chile, and even Venezuela, there are very few studies or antecedents of antibiotic susceptibility of *Enterococcus* that have been carried out on strains from food, with the exception of those that cause gastrointestinal infections; For this reason, it is very difficult to compare these results. Continuous surveillance is necessary to know if the transmission of these bacteria occurs via the food chain or is generated in the environment.[16]

Immunosuppressed patients are those who can develop hospital-acquired infections due to strains of *E. gallinarum* and *E. casseliflavus*, causing bacteremia, endocarditis, septicemia, or urinary tract infection.[17] The danger of acquiring an infection from one of the strains isolated in this work is that some are resistant to antibiotics for human clinical use, as is the case with a strain of *E. faecalis* resistant to erythromycin, tetracycline, chloramphenicol, and rifampicin. More worrisome is that strains of *E. casseliflavus* are resistant to fluoroquinolones, linezolid, and ampicillin. This strain must be studied to determine if it produces penicillinase, since a difference greater than 5 mm was observed when the ampicillin-sulbactam disk was placed, compared to the ampicillin disk. They are usually colonizing strains and are uncommon in human infections.[18]

E. gallinarum and *E. casseliflavus* are not frequently isolated in clinical samples, but it must be recognized that they can cause severe invasive disease. Clinical experience with such strains has been limited. A review of the literature reveals that *E. gallinarum* or *E. casseliflavus/flavescens* can be isolated from a variety of patients who are chronically ill or immunosuppressed. Mainly, they are isolated from cases of bacteremia in patients with underlying conditions, such as renal failure, diabetes mellitus, hematological malignancy, organ or bone marrow transplant recipients, among other conditions.[18]

Three strains of *E. faecium* were isolated in this work; each of different lettuces (*Lactuca sativa L.*, *Lactuca sativa* var. *capitata*, *Chichorium endivia* var. *crispum*) resulting in high-level resistance to all antibiotics tested, while the only *E. avium* strain was totally resistant to linezolid and rifampicin, with intermediate sensitivity to ciprofloxacin, erythromycin and chloramphenicol. The latter is reflected in a study in which strains of *E. faecium* isolated from vegetables, soil, farm animals and manure were analyzed. Thirty-five strains out of 37 were resistant to aminoglycosides at a high level, confirmed by the CMI and by the detection of the resistance genes [aac(6')-Ie-aph(2''), aph(2')- Ic, aph(3')-IIIa and ant(4')-Ia].[1]

In a study with food of animal origin (shrimp), strains of *E. faecium* (49%), *E. faecalis* (29%), *E. gallinarum* (10%), *E. hirae* (5%), *E. casseliflavus* (3%), and *E. durans* (3%).[19] The strains were resistant to vancomycin (37%), tetracycline (46%) and erythromycin (49%), similar to what occurred in this study. In this study it was not possible to demonstrate the susceptibility profile of the isolated strains to aminoglycosides, due to the non-availability of high-load disks to these antibiotics in the country.

It is interesting to highlight that, in the literature, they refer to the importance that *Enterococcus* has acquired in recent times with respect to the high level of resistance to aminoglycosides, since they are intrinsically resistant at a low level. For example, in one study[16] they detected strains of *E. faecalis* (68%) and E. faecium (88%) resistant to gentamicin and both species 100% resistant to streptomycin, an important finding, since these strains were recovered from sewage, hence the importance of studying environmental bacteria and their resistance profiles.

Once these strains are consumed through the food chain, they are able to survive stomach acidity and pass to the gut to colonize it, becoming a human reservoir of potentially pathogenic strains, resistant to antibiotics for human clinical use, to treat serious infections.

This colonization can last from several months to a year and when the conditions are right, self-infect with gut strains,[20] such as long hospital stays, use of third-generation cephalosporins, hospitalization in intensive care, transplant patients, hematological disorders.[21] *Enterococcus* translocation occurs when an overgrowth of these happens in the gut lumen, which is very frequent in patients undergoing antibiotic treatments that are not effective on this genus,[22] and can perfectly cause serious infections. The results obtained in this investigation are of great relevance for public health, since *Enterococcus* strains with high percentages of resistance to antibiotics for human clinical use were found in bacteria from lettuce, a food widely consumed by the population.[23]

Conclusion

The food chain is a route of dissemination of multiresistant enterococci to the human gut microbiota, turning the gastrointestinal tract into a reservoir of bacteria that is intractable with available antibiotics.

Acknowledgments

None.

Conflicts of interest

The authors of the work declare that they have no conflict of interest with the distribution centers of lettuces.

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