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Lettuce for Human Consumption Collected in Costa Rica Contains Complex Communities of Culturable Oxytetracycline- and Gentamicin-Resistant Bacteria

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The present widespread use of antimicrobials in crop farming is based upon their successful application in human medicine. However, recent evidence suggests that the massive anthropogenic release of antimicrobials into the biosphere has selected for resistant bacteria and facilitated the transfer of resistance genes among them. This work deals with the examination of iceberg lettuce collected at 10 farms from two regions in Costa Rica. Farmers from nine sampling sites regularly apply commercial formulations containing gentamicin, oxytetracycline, streptomycin, or a combination of them without being able to indicate how often and how much of these products have been sprayed onto the crops. One organic farm was also investigated for comparative purposes. Oxytetracycline- and gentamicin-resistant bacteria were abundantly detected using selective enrichment cultures. Furthermore, colony mixtures from selective plates were characterized by chemotaxonomical and molecular fingerprinting methods. Both types of resistant communities accounted for a significant fraction of all culturable bacteria and included several resistance genes as well as factors for their potential horizontal transfer. Given the fact that lettuce is eaten raw, it may contribute to the dissemination of antimicrobial-resistant bacteria and/or their resistance genes from the environment to the microbial biota of the human intestine.

The present widespread use of antimicrobials in crop farming is based upon their successful application in human medicine. Estimations indicate that 50% of the total amount of antimicrobials marketed so far (37) have been employed as growth promoters in pig, poultry, and cattle production (34), as powder formulations for the control of bacterial phytopathogens (40), or as additives for aquacultural ponds (27).

McManus and coworkers previously discussed that antimicrobial application remains the most effective and economical method for managing certain plant diseases (21). Farmers in developing countries certainly profit from this beneficial potential but usually without knowledge of the appropriate dosage. Moreover, the lack of clear national policies and monitoring programs allows them to overlook the custody of qualitative and quantitative records of the applications done.

The practices mentioned above are not restricted only to countries whose economy predominantly relies on agriculture: they attain global relevance for the reason that bacteria are spread via the international trading of foodstuffs in an increasingly globalized world (36). In this context, our work aimed to evaluate whether crops treated with antimicrobials potentially expose consumers to antimicrobial-resistant bacteria from the environment.

This investigation describes the occurrence of oxytetracycline- and gentamicin-resistant bacteria in the culturable microbiota of lettuce collected at one organic farm and nine conventional farms located in Costa Rica. The work performed included plate counts of resistant and total bacteria as well as fatty acid methyl ester (FAME) and terminal restriction fragment length polymorphism (t-RFLP) analyses of mixtures of colonies recovered from selective media. In addition, plate community DNA was used as a template for the detection of multiple resistance genes and broad-host-range (BHR) plasmids of the incompatibility groups P-1 and Q. The presence of fecal bacteria in some irrigation water samples was also studied.

MATERIALS AND METHODS

Sample collection and preparation. Iceberg lettuce (*Lactuca sativa* var. *capitata*) samples were collected at five different farms from each of two important agricultural regions in Costa Rica. The first group of samples was obtained from Cartago (samples 1 to 5). The remaining five samples were collected in Zarcero (samples 6 to 10). One organic farm (sample 9) was included in the analysis to compare organic farming practices to conventional farming practices. On the organic farm, crops were fertilized with homemade compost prepared from production waste and sugar cane syrup. The owners of all conventional farms regularly protect their crops with diverse formulations containing gentamicin, oxytetracycline, streptomycin, or a combination of them. However, none of them could precisely recall the frequency of their use or the quantities sprayed onto the crops. Three fully grown lettuces of healthy appearance were selected ran-

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domly at each farm and pulled out of the soil using new plastic bags in substitution for gloves. After the removal of their roots with a disinfected knife at the field, lettuce samples were pooled and processed within 24 h. In the laboratory, external leaves were carefully removed to avoid cross-contamination with soil residues from the field. Next, all three specimens from a composite sample were aseptically cut up to obtain 10 g of their inner leaves. These materials were washed with 90 ml phosphate-buffered saline (PBS) in a sterile bag and homogenized in a stomacher.

Plate counts of total and resistant bacteria by a modified drop count method. Culturable bacteria were counted on glycerol-soil agar plates (DSMZ) (http://www.dsmz.de/media/med080.htm) modified by the addition of 100 $\mu g \cdot ml^{-1}$ cycloheximide and 0.001% crystal violet for the inhibition of fungi and gram-positive bacteria, respectively. Oxytetracycline or gentamicin was added to the same medium at a final concentration of 10 $\mu g \cdot ml^{-1}$ for enumeration of bacteria resistant to these antimicrobials. This latter type of plate will from now on be referred to as selective medium. Freshly made plates of modified glycerol-soil agar and selective medium were inoculated dropwise in triplicate with 10 μl of decimal dilutions prepared from the homogenized leaf suspensions mentioned above. These plates were incubated for 3 days at 30°C aerobically. The highest dilution that gave rise to bacterial growth in at least two of the triplicate inocula (D) was used for the computation of resistance coefficients according to the following formula: $(D_{\text{selective medium}}/D_{\text{modified glycerol-soil agar plates}}) \times 100$.

Recovery of resistant gram-negative bacteria. Oxytetracycline- and gentamicin-resistant bacteria were isolated by spreading 100 μl of the homogenized leaf suspensions onto selective medium (see above). After 5 days of incubation at 30°C under an aerobic atmosphere, 5 ml PBS was poured onto the plates, and glass pipettes were employed to scrape their surfaces until all colonies were removed. Biomass resuspended by this procedure was entirely transferred to sterile 15-ml tubes. These "culturable resistant communities" were washed twice with 5 ml PBS, thoroughly resuspended in 10 ml PBS, and processed immediately for FAME analysis and isolation of plate community DNA.

FAME analysis. Five milliliters from each culturable resistant community was centrifuged at 4,000 rpm for 10 min. The pellet obtained was lyophilized overnight and transferred into clean, Teflon-lined, screw-cap Pyrex tubes rinsed with methyl tert-butyl ether. FAMEs were extracted, separated, and identified by gas chromatography-mass spectroscopy with a modification of the MIDI protocol described previously (18). Their abundance was expressed in mass percentages, and their names are given as recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (12).

Isolation of plate community DNA. Cells from each culturable resistant community in 500 μl were centrifuged and preserved in an equal amount of absolute ethanol. The DNeasy tissue kit (QIAGEN, Hilden, Germany) was used for DNA isolation according to the protocol recommended by the manufacturer for extraction of nucleic acids from gram-negative bacteria.

Amplification of 16S rRNA genes from plate community DNA. Bacterial 16S rRNA genes were amplified by PCR using primers 8-27F and 907-926R (19). The former oligonucleotide was labeled at its 5′ end with 6-carboxyfluorescein to facilitate fluorescence-based detection of the products. Reaction mixtures contained 1× PCR buffer II (Applied Biosystems, Darmstadt, Germany), 3.75 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates (Bioline, Berlin, Germany), 0.2 μM of each primer (Hybaid-Thermo Electron Co., Germany), and 5 U Amplitaq DNA polymerase Stoffel fragment (Applied Biosystems, Darmstadt, Germany). Amplification was done in a Mastercycler ep gradient thermal cycler (Eppendorf, Hamburg, Germany) with the following cycling program: 94°C for 5 min; 30 cycles at 94°C for 30 s, 54°C for 45 s, and 72°C for 1 min; and a final prolonged step at 72°C for 5 min for template extension. The quality and yield of the PCRs were checked by electrophoresis on 0.7% agarose gels.

Community fingerprinting by t-RFLP. PCR products were purified with a PCR purification kit (QIAGEN, Hilden, Germany) and quantified with the Quant-iT DNA assay kit (Molecular Probes, Invitrogen, Karlsruhe, Germany). Next, 200 ng of each reaction mixture was digested with 40 U HhaI according to the instructions of the manufacturer (New England Biolabs, Frankfurt am Main, Germany). Reactions were terminated after 3 h of incubation by heating the mixtures at 65°C for 20 min. A 1.25-μl volume of each restriction digest was mixed with loading buffer containing GS-1000 ROX size standard (Applied Biosystems, Darmstadt, Germany) and run on 5% polyacrylamide gels on an ABI 377 automated sequencer (Applied Biosystems, Darmstadt, Germany). Fragments of the internal standard coloaded in each lane were used for the generation of calibration curves, which were later used for determining the length of each terminal restriction fragment (t-RF) with the Genescan software (Applied Biosystems, Darmstadt, Germany). Aggregate peak heights were standardized to the lowest cumulative value represented among all profiles being compared (5), and the abundance of each t-RF was given in relation to the normalized aggregate peak height of its corresponding profile. Consequently, the relative abundance of a t-RF was expected to represent all colonies of a single taxon on the selective plate used for plate community DNA isolation. Abundance data in percentages were used for the calculation of a diversity index (Shannon's entropy) and for the computation of t tests on diversity with PAST software, version 1.34 (8). In addition, t-RF were identified by the ISPaR function of the web-based tool Microbial Community Analysis III (http://mica.ibest.uidaho.edu/).

Detection of tetracycline and gentamicin resistance genes in plate community DNA. The TET1 and Ribo primer systems described previously by Schnabel and Jones (32) and Aminov et al. (2) were used for the detection of *tet* genes coding for efflux and ribosomal protection proteins in pooled DNA samples. Positive pools were identified, and their corresponding individual samples were analyzed with a selection of oligonucleotides described previously (23). Primers aac3-1, aac3-2 plus aac3-6, aac3-3 plus aac3-4, aac6, and ant (9) were used for the detection of genes for gentamicin-modifying enzymes in pools of plate community DNA. PCR conditions were applied as indicated in the original descriptions of the oligonucleotides. Amplification products were separated by gel electrophoresis and transferred by capillarity to positively charged nylon membranes overnight. DNA was fixed to the membranes by incubation for 2 h at 80°C, and the membranes were hybridized for 16 h to digoxigenin-labeled probes (Roche Applied Science, Mannheim, Germany) generated from resistance genes of reference strains. Signals were detected by chemiluminescence after the addition of CDP-Star (Tropix; Applied Biosystems, Darmstadt, Germany) and exposure of the blots to ECL film (Amersham-Pharmacia, Freiburg, Germany). The same reference strains were used for probe synthesis and for validation of the amplifications.

Detection of IncP-1 and IncQ plasmids in plate community DNA. Conserved segments of plasmids belonging to the Inc P-1 and Inc Q incompatibility groups were amplified with the oligonucleotide systems *trfA2* and *oriV*, in that order (6). Purified plasmids RP4 (IncP-1_α), R751 (IncP-1_β), and RSF 1010 (IncQ) were included as positive controls in the amplifications and for probe synthesis. PCR, blotting, and hybridization conditions were performed as described above.

Collection and assessment of fecal contamination in irrigation water samples. Groundwater was more frequently used for irrigation. Additional sources included shallow ponds, tap water, or even small watercourses contaminated with solid waste. Samples of irrigation water were collected in 100-ml Whirl-Pak bags and maintained at 4°C until analysis. A most-probable-number (MPN) method involving the dilution of the samples in sets of five replicate aliquots was employed for the enumeration of total and fecal coliforms. Ten milliliters, 1 ml, and 0.1 ml of the water samples were used to inoculate tubes containing 10 ml of lactose broth. The 10-ml volume was mixed with double-concentrated lactose broth to maintain equal ingredient concentrations across dilutions. The formation of gas bubbles after 48 h at 35°C was interpreted as a positive presumptive test. Total and fecal coliforms were discriminated by inserting a sterile wooden applicator into each positive test and plunging it to the bottom of new fermentation tubes containing 10 ml of brilliant green lactose bile broth or *Escherichia coli* broth. These tubes were incubated for 24 h at 35°C (brilliant green lactose bile broth) or 45°C (*E. coli* broth) for enumeration of total and fecal coliforms, respectively. The number of gas-positive tubes was converted to MPN values/100 ml by means of a standard statistical table.

RESULTS

Microbiological quality of the lettuce and water samples collected. Crops contained between 10^4 and 10^7 CFU of resistant bacteria/g fresh weight (Fig. 1A). Viable counts on selective medium supplemented with oxytetracycline consistently equaled or exceeded those obtained with gentamicin by 1 or 2 orders of magnitude (Fig. 1A). The lowest number of oxytetracycline-resistant bacteria was isolated from a conventional farm where gentamicin-resistant bacteria were not detected (Fig. 1A). The organic farm, in contrast, showed a high abundance of gentamicin-resistant bacteria (Fig. 1A). Antimicrobial-resistant bacteria reached proportions of 10% or even 100% (Fig. 1B). In accordance with the viable counts, resistance coefficients were equal or higher for oxytetracycline than for gentamicin (Fig. 1B).

Total coliforms were ubiquitously found (Fig. 2). Fecal co-

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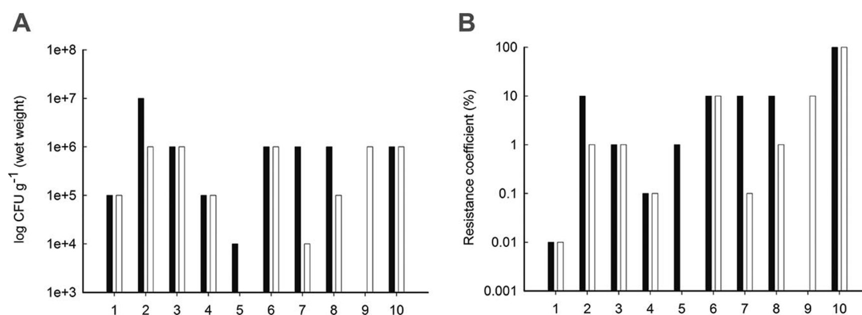

FIG. 1. Total (A) and proportional (B) abundance of culturable oxytetracycline-resistant (black bars) and gentamicin-resistant (white bars) bacteria on the lettuce phyllosphere. Collection sites are presented on the *x* axis. Data for the oxytetracycline-resistant community from farm 9 were not available.

liforms were detected in seven samples, but their abundance was approximately half of that of total coliforms (Fig. 2). The level of contamination of irrigation water samples with enteric bacteria did not correspond to the abundance of antimicrobial-resistant bacteria on the lettuce (Fig. 1 and 2).

FAME analyses. Lipid sets indicating the presence of the genera *Pseudomonas* (10:0 3OH, 12:0 2OH, and 12:0 3OH) and *Xanthomonas* (11:0 iso 3OH and 13:0 iso 3OH) and members of the *Enterobacteriaceae* (14:0 3OH) were detected in a number of FAME profiles from oxytetracycline-resistant communities (Table 1). Gentamicin-resistant communities mainly included lipid markers of the *Cytophaga-Flavobacterium-Bacteroides* branch, such as 15:0 iso 3OH, 17:0 iso 3OH, and 17:0 iso cis9, and elevated amounts of branched fatty acids (Table 1).

The results of two multivariate statistical procedures used on these data confirmed the expected discrimination between sites and between bacterial communities grown on different antimicrobials (data not shown).

Richness and composition of the resistant communities according to t-RFLP data. The profiles of oxytetracycline-resistant communities showed between 16 and 34 t-RF (Table 2). Similarly, the numbers obtained for gentamicin-resistant communities ranged from 14 to 26 t-RF (Table 2). This richness corresponded to Shannon's diversity indices of between 2.0 and 3.0. Moreover, differences in diversity were generally not significant (Table 2). The anticipated dissimilarity between t-RFLP profiles was verified by calculation of the Morisita index and two additional multivariate statistical procedures (data not shown).

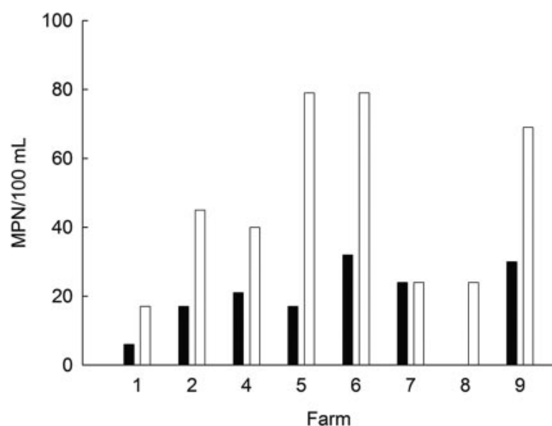

FIG. 2. MPN of fecal (black bars) and total (white bars) coliforms in irrigation water samples. This type of material was not available at farms 3 and 10.

Gammaproteobacteria and *Betaproteobacteria* were found to dominate the profiles of oxytetracycline-resistant communities (Table 3). For example, *Ralstonia* species (or a close relative) and either *Xanthomonas* or *Stenotrophomonas* species were detected in most of these profiles (Table 3). *Burkholderiales* and bacteria from the *Cytophaga-Flavobacterium-Bacteroides* branch dominated in gentamicin-resistant communities (Table 3).

Detection of resistance genes and broad-host-range plasmids in plate community DNA. The initial screening for resistance genes revealed strong hybridization signals by the detection of *tet* genes coding for efflux proteins. This type of resistance mechanism was omnipresent and could later be assigned to several genes classes (Table 4). In contrast, *tet* genes for ribosomal protection proteins were not found. The detection of gentamicin-modifying enzymes was limited to aminoglycoside acetyltransferases of the family *aac*(3)-II or *aac*(3)-VI. However, their signal intensities were low, and their occurrence could be corroborated only in pooled samples containing DNA from farms 1 to 4 and farms 9 and 10. Plate community DNA samples from two farms included four different *tet* genes, but most other samples rarely had more than two different *tet* genes (Table 4). The genes *tet*(B) and *tet*(C) occurred in all samples. This pair was commonly accompanied by *tet*(A) and, to a lesser extent, by genes coding for additional tetracycline efflux proteins (Table 4).

Weaker hybridization signals were associated with the detection of BHR plasmids (Table 5). IncP-1 plasmids were detected in plate community DNA from both oxytetracycline- and gentamicin-resistant communities, including the DNA samples from those farms where up to four different *tet* genes were found (Tables 4 and 5). Elements from this incompatibility group were concomitantly found three times in both types of resistant communities (Table 5).

On the other hand, plasmids of incompatibility group Q were restricted to the oxytetracycline-resistant community of a single farm (Table 5). In this instance, IncP-1 plasmids were also detected (Table 5).

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TABLE 1. Relative abundance of selected FAMEs detected in biomass from oxytetracycline- and gentamicin-resistant communities isolated from the lettuce phyllosphere

FAME	Relative abundance (%) in biomass from farm[a]:																	
	1		2		3[b]	4		5[c]	6		7		8		9		10	
	O	G	O	G	G	O	G	O	O	G	O	G	O	G	O	G	O	G
10:0 3OH	8.7	1.7		0.8	0.8	1.8	0.8	1.1	6.8	0.5	1.0	1.6	0.5	1.4			1.7	0.7
12:0 2OH	4.2		3.4			1.5			1.4		1.0							
12:0 3OH	6.0		4.8		0.6	3.4		1.2	4.3		1.8		0.9		0.5		4.1	
14:0 3OH	0.3							2.0					1.0		1.8		0.5	
16:0 3OH		1.8		1.0			1.2											
11:0 iso 3OH					0.4	0.4												
13:0 iso 3OH			0.4		0.9	0.6											0.4	
15:0 iso 3OH		2.1		1.7			1.2											
17:0 iso 3OH		5.7		1.9	0.6		2.3								0.6			
15:0 iso	1.0	19.9	8.5	15.8	37.5	16.0	14.7		0.9	2.7	3.1	0.4	10.8		19.7	39.3	17.1	11.0
15:0 anteiso	0.6	1.0	3.2	1.9	4.8	5.7	0.2				0.9	0.2	3.8	4.2	5.2	1	5.1	3.8
17:0 iso		0.3	0.4	0.4	2.1	0.8	0.4						0.3	0.5	0.7	1.0	0.8	0.3
17:0 iso cis9	0.3	1.1		0.4	0.6	0.7									1.3	3.3		
16:1 cis9	11.6	27.5	16.3	25.2	12.6		30.3	24.0	3.0	20.4	13.2	17.9	4.6	16.7	6.3	11.8	12.8	20.8
18:1 cis11	7.4	9.0	10.6	8.6	5.3	5.4	7.4	9.4	5.0	8.0	12.1	4.4	5.1	6.7	7.5	2.4	8.7	6.5
17:0 cyclo 9-10	7.6	0.9	8.2	4.7	4.0	9.1	2.6		18.7	15.4	9.9	21.9	18.7	11.8	12.1	2.6	9.3	9.4
19:0 cyclo 11-12			0.3				0.6		2.0	0.7	0.6		4.5	0.7	1.9			

[a] O, oxytetracycline resistant; G, gentamicin resistant.
[b] Data were not available for the oxytetracycline-resistant community from this farm.
[c] Gentamicin-resistant bacteria were not detected in this sample.

DISCUSSION

Lettuce was selected as a subject of study because it is one of the most important leafy vegetables consumed worldwide. Furthermore, it is eaten raw, and it comes into direct contact with soil during a great part of its production cycle. The decision to restrict the analysis to gram-negative bacteria was based on their frequent association with human infections. This choice also had to do with the fact that bacteria from this group abundantly colonize the phyllosphere (17) and with the possibility that enteric bacteria reach crops through irrigation with contaminated water or as a result of fertilization with noninactivated manure (13).

TABLE 2. t-RF richness, Shannon's entropy index, and t tests on diversity of antimicrobial-resistant communities isolated from the lettuce phyllosphere

Farm	Oxytetracycline		Gentamicin		P^c
	No. of t-RF	H′[d]	No. of t-RF	H′	
1	19	2.1	24	2.4	0.08
2	17	1.9	16	2.1	0.2
3	18	2.3	15	2.2	0.6
4[a]			14	2.1	
5[b]	24	2.4			
6	24	2.6	26	2.7	0.6
7	29	2.9	19	2.3	<0.05
8	34	3.0	19	2.4	<0.05
9	16	2.3	20	2.3	0.7
10	32	2.8	15	1.9	<0.05

[a] Data were not available for the oxytetracycline-resistant community from this farm.
[b] Gentamicin-resistant bacteria were not detected in this sample.
[c] A probability value of <0.05 indicates a significant difference in diversity between two samples. Shannon indexes of oxytetracycline-resistant and gentamicin-resistant communities from a single sample did not differ significantly.
[d] H′, Shannon's entropy index.

This study delivers evidence for the accumulation, survival, and viability of oxytetracycline- and gentamicin-resistant bacteria on the lettuce phyllosphere. In this regard, it supports a previous report that implicated lettuce in the dissemination of antimicrobial-resistant bacteria (38). However, our plate counts of total and resistant bacteria were 1 or 2 orders of magnitude above those previously reported for lettuce (4) and other fresh vegetables (26) by European investigations.

We are aware of the fact that the inclusion of a cultivation step reduced the richness and complexity of the analyzed microbial communities. For this reason, our results for the diversity and abundance of antimicrobial-resistant bacteria represent an underestimation of the real picture in the 10 farms studied and possibly elsewhere in the country (due to the general acceptance of antimicrobials by Costa Rican farmers).

The analysis of microbial biomass or DNA obtained directly from lettuce would not have provided unambiguous phenotypic evidence of antimicrobial resistance and would have implied the need to pool biomass from an excessive amount of plants for satisfactory sensitivity. Irrespective of the methodology used, our results are in accord with data from previous investigations that showed an extent of oxytetracycline resistance that was broader than gentamicin resistance in the environment (25). This observation agrees with the more intensive use of the former antimicrobial in plant agriculture (40).

Antimicrobial-resistant populations were isolated on the same medium but at different times for the determination of viable counts and for community analyses (3 versus 5 days). However, the distortion caused by colonies that grew during these two additional days can be expected to be negligible because of their small size. Degradation of antibiotics during incubation was minimized by preparing plates freshly and by keeping them in the dark. Furthermore, aqueous solutions of tetracyclines do not exhibit more than 10% decomposition

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TABLE 3. Length, relative abundance, and preliminary assignment of selected t-RF to bacterial taxa

Farm	t-RF of oxytetracycline-resistant communities			t-RF of gentamicin-resistant communities		
	Length (bp)	Relative abundance (%)	Possible taxa	Length (bp)	Relative abundance (%)	Possible taxa
1	205	16	*Burkholderiales*	101	25	*Bacteroides/Flavobacterium*
	207	28	*Ralstonia* or close relative	225	10	*Chryseobacterium*
	215	27	*Xanthomonas/Stenotrophomonas*	564	24	*Bacillus/Comamonas*
2	205	21	*Burkholderiales*	101	20	*Bacteroides/Flavobacterium*
	207	25	*Ralstonia* or close relative	110	15	Unidentified
	215	25	*Xanthomonas/Stenotrophomonas*	205	22	Burkholderiales
3	205	19	*Burkholderiales*	213	23	*Xanthomonas/Stenotrophomonas*
	213	20	*Xanthomonas/Stenotrophomonas*	225	23	*Chryseobacterium*
	225	17	*Chryseobacterium*	564	18	*Bacillus/Comamonas*
4[a]				100	20	*Sphingobacterium/Cytophaga*
				101	16	*Bacteroides/Flavobacterium*
				205	23	*Burkholderiales*
5[b]	82	18	*Alphaproteobacteria/Sphingomonas*			
	84	17	*Sphingomonas*			
	207	25	*Ralstonia* or close relative			
6	207	14	*Ralstonia* or close relative	89	19	*Capnocytophaga/Flavobacterium*
	215	13	*Xanthomonas/Stenotrophomonas*	226	19	*Ancylobacter/Sulfobacillus*
	225	13	*Chryseobacterium*	368	8	*Actinomycetales*
7	112	11	Unidentified	107	14	Unidentified
	205	11	*Burkholderiales*	205	22	*Burkholderiales*
	207	12	*Ralstonia* or close relative	213	14	*Xanthomonas/Stenotrophomonas*
8	84	11	*Sphingomonas*	92	14	*Flavobacteriaceae*
	102	11	*Prevotella*	205	18	*Burkholderiales*
	215	11	*Xanthomonas/Stenotrophomonas*	216	19	*Xanthomonas/Shewanella*
9	215	22	*Xanthomonas/Stenotrophomonas*	205	11	*Burkholderiales*
	225	21	*Chryseobacterium*	213	24	*Xanthomonas/Stenotrophomonas*
	372	14	*Enterobacteriaceae/Actinobacteria*	225	24	*Chryseobacterium*
10	84	12	*Sphingomonas*	92	21	*Flavobacteriaceae*
	207	18	*Ralstonia* or close relative	215	27	*Xanthomonas/Stenotrophomonas*
	215	18	*Xanthomonas/Stenotrophomonas*	225	22	*Chryseobacterium*

[a] Data were not available for the oxytetracycline-resistant community from this farm.
[b] Gentamicin-resistant bacteria were not detected in this sample.

when maintained at 37°C for 3 days (11), and several of their degradation products have the same potency as their parental compounds (7).

Costa Rican legislation bans the use of water with more than 100 fecal coliforms/100 ml for irrigation of leafy vegetables (24). Therefore, the level of contamination with fecal coliforms was in compliance with national and international legislation on water quality (3). We did not address the persistence of enteric bacteria on the lettuce phyllosphere, but we provide molecular and chemotaxonomical evidence of their presence in the resistant communities isolated. Additional research is required to reveal further sources of resistant bacteria, or resistance genes, at the beginning of a new growing cycle.

TABLE 4. Detection of *tet* genes in plate community DNA of oxytetracycline-resistant populations isolated from the lettuce phyllosphere

Farm[b]	Signal intensity[a]							
	tet(A)	*tet*(B)	*tet*(C)	*tet*(D)	*tet*(G)	*tet*(H)	*tet*(L)	*tet*(M)
1	+	++	++	−	−	−	−	−
2	−	++	+++	−	−	−	−	−
3	−	++	++	−	−	−	−	−
5	−	++	+	−	−	−	−	−
6	+	++	+	−	+	−	−	−
7	++	++	+	−	−	−	++	−
8	++	++	++	−	−	−	−	−
9	+	+++	+	−	−	−	−	−
10	+	++	+	−	−	−	−	−

[a] Estimates of signal intensity range from positive (+) to strongly positive (+++). Negative samples are indicated with a −.
[b] Plate community DNA was not available for farm 4.

TABLE 5. Detection of plasmids from the IncP-1$_{\alpha\beta}$ and IncQ incompatibility groups in plate community DNA of gentamicin- and oxytetracycline-resistant communities isolated from the lettuce phyllosphere

Farm	Signal intensity[a]			
	Gentamicin		Oxytetracycline	
	IncP-1	IncQ	IncP-1	IncQ
1	−	−	−	−
2	+	−	−	−
3	+	−	W	−
4[b]	−	−		
5			W	+
6	−	−	W	−
7	+	−	+	−
8	−	−	W	−
9	W	−	W	−
10	−	−	−	−

[a] Estimates of signal intensity range from weakly positive (W) to strongly positive (+++). Negative samples are indicated with a −.
[b] Plate community DNA was not available for farm 4.

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The bacterial species carrying the pool of resistance genes are not known yet. Nevertheless, we believe this information to be of low relevance in practice, because iceberg lettuce together with bacteria on their surfaces are eaten as a whole, and consumers cannot eliminate specific populations prior to their ingestion. Iceberg salads have previously been shown by culture-dependent and culture-independent methods to include *Gammaproteobacteria* (*Pseudomonas*, *Xanthomonas*, *Erwinia*, *Enterobacter*, and *Serratia* species), *Betaproteobacteria* (*Janthinobacterium*, *Alcaligenes*, and *Oxalobacter* species), as well as some members of the *Cytophaga*-*Flavobacterium*-*Chryseobacterium* branch (20, 31). Our results are consistent with their occurrence on the lettuce phyllosphere. The identification of bacterial taxa based on the size of their t-RF could be ambiguous, especially if only one restriction enzyme is used (14). Consequently, our in silico predictions were complemented and substantiated with chemotaxonomical data obtained from a well-established technique (33). This combination does not provide the precision of a sequencing approach, but it is sufficient for a preliminary taxonomical characterization of the isolates and, at the same time, for rapid appraisal of differences in community structure. On the other hand, evenness estimators were not calculated because organisms that formed large colonies could have appeared to be dominant in spite of low colony numbers on the plates.

In comparison with conventional farming practices, it seems that the organic status of farm 9 had little, if any, effect on the attributes of antimicrobial-resistant communities inhabiting the lettuce phyllosphere. This resemblance indicates that the appearance of antimicrobial-resistant bacteria on the lettuce phyllosphere is a naturally occurring process. However, the possibility that this farm was used for conventional agriculture before changing to an organic production regimen cannot be excluded.

The screening for resistance genes revealed the presence of several tetracycline efflux proteins and one family of aminoglycoside acetyltransferases. The discovery of multiple *tet* genes in a plate community DNA sample could have resulted from the contribution of numerous individuals with single genes to the pool or from the carriage of multiple *tet* genes by a few of them.

We do not discard the possibility that additional acquired genes, known or novel ones, caused the resistant phenotypes of the isolates. Alternatively, mutations in the 16S rRNA gene (10) or multidrug transporters (1, 22, 28) could have been responsible for the attenuated sensitivity of these populations to oxytetracycline and gentamicin.

The widespread distribution of *tet*(A), *tet*(B), and *tet*(C) in the microbial world is a persuasive explanation for their high prevalence among the samples analyzed (30). The gene for the otherwise frequent efflux pump Tet(L) was sporadically detected, but this can be explained by the discrimination of gram-positive bacteria in this survey due to the inclusion of crystal violet in the selective medium. The same is true for *tet* genes coding for ribosomal protection proteins.

More than 50 aminoglycoside-modifying enzymes have been documented to date (39). Of these enzymes, mainly members from the families *aac*(6′) and *aac*(3) have been detected in environmental gram-negative bacteria (29, 35, 39). In a pioneering work dealing with gentamicin-resistant bacteria of environmental origin, isolates that hybridized to the *aac*(3)II-VI probe used here were identified as *Stenotrophomonas*, *Xanthomonas*, *Empedobacter*, and *Sphingobacterium* species (9). Lipid markers and t-RF compatible with these bacterial groups were also found in our analyses (Tables 1 and 3).

The resistant microbial populations cultivated included self-transmissible and mobilizable BHR plasmids of the incompatibility groups P and Q, respectively. We do not immediately implicate these factors in the horizontal transfer of the resistant genes detected, but we do consider that their overlapped presence opens a door for their potential dissemination on the plant surface or within the digestive tract of consumers. In this context, recent studies have demonstrated the transferability of a vancomycin resistance gene between enterococci in the digestive tract of human volunteers (16) and the conjugative transfer of aminoglycoside and macrolide resistance genes in mice treated with streptomycin (15).

This work is intended to encourage a discussion on whether high levels of resistant bacteria on crops equal low safety for consumers. On the other hand, the data presented can be used to advise respective authorities on the importance of surveillance activities and strict regulations on the appropriate use of these drugs.

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