Organic and conventional fruits and vegetables contain equivalent counts of Gram-negative bacteria expressing resistance to antibacterial agents

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Summary

Resistance to antibiotics is a major public health problem which might culminate in outbreaks caused by pathogenic bacteria untreatable by known antibiotics. Most of the genes conferring resistance are acquired horizontally from already resistant commensal or environmental bacteria. Food contamination by resistant bacteria might be a significant source of resistance genes for human bacteria but has never been precisely assessed, nor is it known whether organic products differ in this respect from conventionally produced products. We showed here, on a large year-long constructed sample set containing 399 products that, irrespective of their mode of production, raw fruits and vegetables are heavily contaminated by Gram-negative bacteria (GNB) resistant to multiple antibiotics. Most of these bacteria originate in the soil and environment. We focused on non-oxidative GNB resistant to third-generation cephalosporins, because of their potential impact on human health. Among them, species potentially pathogenic for immunocompetent hosts were rare. Of the products tested, 13% carried bacteria producing extended – spectrum beta-lactamases, all identified as Rahnella sp. which grouped into two phylotypes and all carrying the blA¹ gene. Thus, both organic and conventional fruits and vegetables may constitute significant sources of resistant bacteria and of resistance genes.

Introduction

The European Food Safety Authority (EFSA) recently attempted to ascertain to what extent food constitutes a vehicle for the acquisition by humans of antimicrobial-resistant bacteria or resistance genes, to grade the risks of such acquisition, and to identify control options (EFSA, 2008). These problems have arisen at a time when many pathogenic Gram-negative bacteria (GNB), such as members of the family Enterobacteriaceae, have become resistant to most antibiotics (Levy and Marshall, 2004), resulting in difficult-to-treat infections that make it necessary to avoid further increases in resistance, because very few new antibiotics are becoming available (Shlaes, 2003). In this situation, better understanding of the paths via which resistance to antibiotics spreads might be crucial in helping to control such dissemination.

Intestinal commensal GNB are good recipients for the horizontal transfer of genes from environmentally resistant by transient inhabitants (Salyers et al., 2004) and can further disseminate resistance in pathogenic species (Alekshun and Levy, 2006). This resistance was found to decrease in subjects fed sterilized food (Corpet, 1988) but was frequent in vegetarians (Guinee et al., 1970; van den Braak et al., 1997). Exposure to resistant GNB has been traced to contaminated vegetables (Kapperud et al., 1995) as well as fruits, when either are eaten raw, with environmental bacteria acting as potential sources of resistance (Rossolini et al., 2008). Contamination and
subsequent infections are indeed feared in patients with leukaemia (Remington and Schimpff, 1981) and cystic fibrosis (Moore et al., 2001).

In France, most fruits and vegetables are conventionally produced, but organic products are also available and are attractive to consumers (Bio Agence, 2008). Manure from farms using antibiotics increases resistance in soil bacteria (Binh et al., 2007; Ghosh and LaPara, 2007; Heuer and Smalla, 2007), and this resistance may contaminate agricultural products. However, producers of organic fruit and vegetables must adhere to European rules (Anonymous, 2000): these forbid fertilization by chemical agents, but allow the use of manure from organic farming and sewage. The overall result of these conditions as regards the exposure of fruit and vegetable consumers to resistant bacteria is not known.

Because the EFSA stressed that the role of food in the transfer of resistance was insufficiently documented (EFSA, 2008), we conducted the present study, which demonstrated that irrespective of their mode of production (conventional or organic), many raw fruits and vegetables carry GNB which are essentially of environmental origin and express resistance to antibiotics.

**Results**

We tested 399 products (15.4 ± 0.5/week) including 292 (73.2%) vegetables and 107 (26.8%) fruits. Of these, 92 (23.1%), 139 (34.8%) and 168 (42.1%) were grown in, on and above the soil respectively. Details concerning product samples are given in Table S1. Due to differences in availability, slightly more products were conventional (218, 54.6%) than organic (181, 45.4%). There were also some seasonal variations in the number of samples tested (Table S2).

The prevalence of samples with GNB growing on antibiotics ranged from 70.4% for kanamycin to 21.3% for ciprofloxacin, with no significant differences between conventional and organic products (Fig. 1A). However, significant differences were observed between products grown

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**Fig. 1.** Prevalence of resistance (% rate, A and B) and bacterial densities (log CFU g⁻¹, C and D) of Gram-negative bacteria growing on antibiotic selective agar in 399 samples of the 10 fruits and vegetables most often eaten raw in France. (A and C: conventional products ▲ versus organic products ●. B and D: products grown above the soil ▲ versus on the soil ▲ or in the soil □).
in and on the soil, and those grown above it (Fig. 1B, \(P < 0.001\) for all markers).

The densities of resistant bacteria (Fig. 1C and D) varied among resistance markers, and was as high as \(10^4\) cfu g\(^{-1}\) of product for tetracycline, chloramphenicol and nalidixic acid. However, here again, densities were not significantly different in organic and conventional products, but were significantly lower in products grown above ground (\(P < 0.001\) for all markers).

The overall median resistance score was 80% (interquartile range: 10–90%), again with no significant difference between organic and conventional products, but with a median score of 40% for products grown above the soil, and as high as 90% for those grown on or in it (Fig. 2).

In all, 321 different non-oxidative GNB resistant to third-generation cephalosporins were isolated from 199 (49.9%) products (1.6 ± 0.7-positive sample). Ninety-five per cent belonged to three genera only (70.8% to \textit{Acinetobacter}, 6.8% to \textit{Stenotrophomonas} and 17% to \textit{Rahnella}) whereas the remaining 5% were distributed among nine genera (\textit{Proteus}, \textit{Pantoea}, \textit{Klebsiella}, \textit{Ewingella}, \textit{Escherichia}, \textit{Erwinia}: one strain each, and \textit{Hafnia}, \textit{Serratia} and \textit{Enterobacter}: two, three and four strains respectively). Species distribution did not differ significantly according to type of production or mode of culture, except that \textit{Rahnella} strains were significantly more strongly associated with products grown in or on the soil than with those grown above it (\(P < 0.001\), data not shown). However, species were evenly distributed between organic and conventional products (data not shown).

Resistance phenotypes (resistant to amoxicillin, ticarcillin, cephalothin, intermediate to cefotaxime and ceftriaxone, susceptible ceftazidime and imipenem, and to combinations such as amoxillin-calvulanic acid, and piperacillin-tazobactam) suggested the presence of class A extended-spectrum \(\beta\)-lactamase (ESBL) in 51 isolates. Strikingly, all these isolates were identified as \textit{Rahnella} sp. These results suggested that these isolates carried the \textit{bla\textsubscript{RAHN-1}} gene as described (Bellais et al., 2001). The \textit{bla\textsubscript{RAHN-1}} gene was detected in all of 51 isolates. The \textit{bla\textsubscript{RAHN-1}} gene was detected in all of 51 isolates. The phylogenetic analyses performed on the 16S rRNA/\textit{rpoB} concatenated sequences showed that \textit{Rahnella} strains were indeed divided into two branches separated by high bootstrap values (Fig. 3). The first comprised 37 isolates (including 36 isolates from this study and \textit{Rahnella aquatilis} genomospecies 1 CIP105589), over 98.6% of whose nucleotides were identical to those of the \textit{Rahnella} reference strain CIP7865T. The second branch included 17 isolates (including 15 isolates from this study and \textit{R. aquatilis} CIP103904 and \textit{R. aquatilis} genomospecies 2 CIP105588) fewer than 98.2% of whose nucleotides were identical to those of the \textit{Rahnella} reference strain CIP7865T. The second branch included 17 isolates (including 15 isolates from this study and \textit{R. aquatilis} CIP103904 and \textit{R. aquatilis} genomospecies 2 CIP105588) fewer than 98.2% of whose nucleotides were identical to those of the reference strain. There was no significant difference between the distribution of the two phylogenetic branches of \textit{Rahnella} in conventional and organic products, or in products grown in contact with the soil or above it (data not shown). The antibiotic resistance pattern to \(\beta\)-lactams of \textit{Acinetobacter} spp. isolates which were frequently isolated are shown in Table 1.

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Unrooted neighbour-joining tree of concatenated 16S rDNA/rpoB sequences of 51 Rahnella sp. strains isolated from fruits and vegetables, and of four Rahnella reference strains from the Collection Institut Pasteur. Members of the Yersinia genus were used as the outgroup. Values above the lines are bootstrap values expressed in per cent (only values greater than 90% are shown). Numbers in parentheses are nucleotide sequence accession numbers: the first for 16S rDNA, and the second for rpoB. Scale bar = accumulated changes per nucleotide.

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Table 1. Prevalence of antibiotic resistance to \(\beta\)-lactamin for *Acinetobacter* sp. isolates.

<table>
<thead>
<tr>
<th>Identification</th>
<th>No. of isolates</th>
<th>TIC</th>
<th>TCC</th>
<th>PIP</th>
<th>TZP</th>
<th>CTX</th>
<th>CAZ</th>
<th>IPM</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acinetobacter calcoaceticus</em></td>
<td>179</td>
<td>29</td>
<td>17.3</td>
<td>67</td>
<td>8.9</td>
<td>57</td>
<td>42.5</td>
<td>0.6</td>
</tr>
<tr>
<td><em>Acinetobacter sp.</em></td>
<td>32</td>
<td>12.5</td>
<td>2.2</td>
<td>62.5</td>
<td>9.4</td>
<td>87.5</td>
<td>71.9</td>
<td>3.1</td>
</tr>
<tr>
<td><em>Acinetobacter baumanii</em></td>
<td>9</td>
<td>33.3</td>
<td>0.6</td>
<td>77.8</td>
<td>0</td>
<td>66.7</td>
<td>22.2</td>
<td>0</td>
</tr>
<tr>
<td><em>Acinetobacter johnsonii</em></td>
<td>2</td>
<td>50</td>
<td>1.1</td>
<td>50</td>
<td>100</td>
<td>100</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td><em>Acinetobacter junii</em></td>
<td>2</td>
<td>50</td>
<td>0.6</td>
<td>50</td>
<td>100</td>
<td>50</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td><em>Acinetobacter lowii, Acinetobacter haemolyticus, Acinetobacter rhizosphaerae</em></td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

AMC, amoxicillin + clavulanic acid; AMX, amoxicillin; CAZ, Ceftazidime, IPM, imipenem.; CTX, cefotaxime; PIP, piperacillin; TCC, ticarcillin; TZP, piperacillin + clavulanic acid; TIC, ticarcillin; T2P, piperacillin + tazobactam.

**Discussion**

This study led to three main findings. First, raw fruits and vegetables were frequently associated with the presence of resistant GNB; second, organic and conventional products were similar in this respect, and third, certain organic and conventional products carried genes encoding ESBL.

Indeed, resistant GNB were not only present in many products, but high densities were found for most of the classes of antibiotics used in human medicine. However, the fact that the products were not washed before testing clearly shows that our results express the maximum likelihood of colonization. It is not possible to say whether or not this is unusual, because the present work apparently constitutes the first attempt to evaluate bacterial resistance in raw fruits and vegetables in a comprehensive and quantitative manner. It would be interesting to evaluate the impact of washing fruits and vegetables on the amount of resistant bacteria. However, to our knowledge, no standard protocol of washing has been proposed and it is not known which would decrease bacterial counts without altering the organoleptic properties of the products. That the products tested were grown in different production areas does, however, suggests that some general conclusions can be drawn from our results. Note that we do not wish to imply that fruit and vegetable consumption is associated with an increased risk of being colonized by resistant bacteria, because other foods are known to be contaminated by resistant bacteria (White et al., 2004; Gyles, 2008) and it has been shown that dietary habits do not significantly predict resistance in faecal enterobacteria (Sannes et al., 2008). Here, we found no difference between the resistance to bacteria of organic and conventional products, when the incidence of antibiotic-resistant bacteria was previously found to be lower in faeces from organic pigs and broiler chickens than from conventionally bred animals (Hoogenboom et al., 2008), and although conventional dairy cattle were reported to harbour a higher prevalence of antimicrobial-resistant enterobacteria than organic dairy cattle (Call et al., 2008). Altogether, this suggests that there is no direct link between the bacterial resistance of animal products and that of fruits and vegetables.

Exploration of all the resistance genes and species present in the products examined was beyond the scope of this study. We chose here to focus on the resistance of non-oxidative GNB to third-generation cephalosporins, because this resistance is a major concern in human medicine (Souli et al., 2008). Recently, specific types of genes which confer extended resistance on beta-lactams, such as the CTX-M gene family, have emerged worldwide in enterobacteria, causing infections in humans (Pitout and Laupland, 2008), and their sources have been traced to environmental bacterial species (Canton and Coque, 2006). Here too, our results for resistance to third-generation cephalosporins indicated that environmental species were responsible for most of the colonization of fruits and vegetables, first, because most of the species isolated were indeed environmental, and second, because resistance was more frequent in the products grown in contact with the soil, and many soil bacteria indeed carry resistance genes (Dantas et al., 2008; Demaneche et al., 2008).

*Escherichia coli* and *Klebsiella* spp., which are both intestinal commensals of many mammals and are the GNB most frequently isolated from cases of infection in immunocompetent hosts, were very rare. This suggests that fruits and vegetables, whether conventional or organic, do not constitute a direct threat of infection of the general population. No pathogens such as *Salmonella* were isolated, but the study was not designed to isolate them if they were not resistant. *Stenotrophomonas* and *Acinetobacter* sp. were frequently found, but are only pathogenic for immunocompromised hosts.

The case of *Rahnella* was of particular interest, because it was the only species found to express ESBL. *Rahnella* strains are widely distributed in nature, and may be present in foods (Hamze et al., 1991; Lindberg et al., 1998; Hamilton-Miller and Shah, 2001; Ercolini et al., 2006). However, although the species can cause severe
opportunist infections (Maraki et al., 1994; Matsukura et al., 1996; Caroff et al., 1998; Tash, 2005), these have been rarely reported in immunocompetent hosts (Chang et al., 1999). The \textit{Rahnella} strains that we isolated were heterogeneous from a phylogenetic standpoint, but they all carried the \textit{bla}_{\text{RAHN}} gene, which has so far only been described as chromosomal, and has apparently never transferred to other species. Therefore, at least in theory, ingestion of \textit{Rahnella} strains should not be a major concern as regards the dissemination of bacterial resistance. However, the recent worldwide dissemination of the \textit{bla}_{\text{CTX-M}} genes family originated from \textit{Klyuyvera} sp., which are environmental enterobacteria, to human pathogens (Bellais et al., 2001; Humeniuk et al., 2002; Poirel et al., 2002; Rodriguez et al., 2004; Olson et al., 2005) suggests that such transfer of \textit{bla}_{\text{RAHN}} may not be impossible.

Taken together, our results fully support the EFSA concern about the presence of resistance genes in foodstuffs, and suggest that further investigations should be undertaken in this field.

**Experimental procedures**

**Sampling**

Between March 2003 and March 2004, one conventionally produced batch and one organically produced batch of four fruits (apple, peach, pear and strawberry) and six vegetables (carrot, celery, cucumber, lettuce, radish and tomato) produced in France were purchased at the National Market (Rungis, France) every two weeks, as available. Each batch weighed from 1 to 5 kg, and all were grown in the various national areas of production. However, no effort was made to obtain representative samples from producers. Organic products were differentiated from conventional ones by the label ‘Bio’ which indicates that the producer adheres to specific rules (Ministère de l’Agriculture et de la Pêche, 2009). All products were transported to the laboratory of one of the investigators (A.B.). There, 50 g of each product was immediately selected at random without washing or peeling, diluted 1:5, homogenized (Stomacher 80 Biomaster®, Seward, UK), coded, and frozen at \(-80°C\).

**Bacteriological methods**

Samples were defrosted in batches and GNB were counted on Drigalski agar (bioMérieux, Charbonnières-les-Bains, France) with one of the following antibiotic: 100 mg l\(^{-1}\) ampicillin, 2 mg l\(^{-1}\) ceftazidime, 2 mg l\(^{-1}\) cefotaxime, 50 mg l\(^{-1}\) nalidixic acid, 4 mg l\(^{-1}\) ciprofloxacin, 2 mg l\(^{-1}\) gentamicin, 50 mg l\(^{-1}\) spectinomycin, 20 mg l\(^{-1}\) kanamycin, 10 mg l\(^{-1}\) tetracycline, or 20 mg l\(^{-1}\) chloramphenicol, in order to isolate non-susceptible (i.e. resistant) strains only. Antibiotic concentrations were chosen as previously recommended (Société Française de Microbiologie, 2008).

All morphologically distinct oxidase-negative GNB from Drigalski agar containing cefotaxime or ceftazidime, chosen as examples of resistant bacteria with a potential impact on human health, were further studied. First, an internal fragment of 1357 bp of the 16S rRNA gene was amplified and sequenced for species identification, as described (Ruimy et al., 1994). Next, production of ESBL was detected by the disc-diffusion method, also as described (Société Française de Microbiologie, 2008). Because the detection test was only positive in strains identified as \textit{Rahnella} sp. (see below), positive isolates were further studied in more detail: first, they were more precisely identified by screening an internal fragment of the \textit{rpoB} gene, as described (Maraki et al., 1994). Then, their phylogenetic relationships were determined using the neighbour-joining (ortho USA) algorithm (Kimura two-parameter distance estimation) as implemented in MEGA 4.0 (Tamura et al., 2007), on the concatenated sequences of their 16S rRNA and \textit{rpoB} genes. The \textit{R. aquatilis} reference strains CIP7865T, CIP103904, CIP105588 and CIP105589 were used as controls. The accession numbers for the \textit{rpoB} and 16S rDNA sequences of \textit{Rahnella} sp. are GQ148917 to GQ149026. In addition, \(\beta\)-lactam susceptibility pattern of \textit{Acinetobacter} sp. isolates was determined as recommended (Société Française de Microbiologie, 2008) because they were the most frequent oxidase-negative GNB isolated from Drigalski agar containing cefotaxime or ceftazidime (Table 1).

The \textit{Rahnella} strains were then screened for the \textit{bla}_{\text{RAHN-1}} gene, previously described in \textit{R. aquatilis} (Bellais et al., 2001). For this purpose, two primers (Rahn-up, CTG-GAAATAGAAAGCGCG and Rahn-down, TCAATAACCT-GCGTCACA) were designed, to amplify an internal 721 bp fragment of the \textit{bla}_{\text{RAHN-1}} gene. Fragments were amplified in 50 \(\mu\)l final volume mixtures containing 100 ng of bacterial DNA, 400 nM each of the Rahn-up and Rahn-down primers, 250 \(\mu\)M of each deoxynucleoside triphosphate (Boehringer GmbH, Mannheim, Germany), 1 \(\times\) reaction buffer supplied by the manufacturer with 1.5 mM MgCl\(_2\), and 1 U of AmpliTaq DNA polymerase (Applera, Courtaboeuf, France), as follows: 94°C for 4.5 min, then 30 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, and 72°C for 10 min. PCR products were visualized under UV irradiation after agarose gel (2%, w/v) electrophoresis. The \textit{R. aquatilis} strains cited above were used as controls.

**Statistical analysis**

The prevalence of resistance was compared by the \(\chi^2\) or Fisher’s exact test, according to mode of production (conventional versus organic), and place of culture (above ground for apple, cucumber, pear, peach and tomato; on the ground for lettuce, celery and strawberry, or in the soil for carrot and radish). Geometric means of bacterial counts were compared between groups by the Kruskal–Wallis or Mann–Whitney test. The resistance score of each product was calculated as described (Murray et al., 1990). Statistical analyses were performed with Stata software, version 9.0 (Stata). \(P\) values of < 0.05 were considered significant.

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References

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Antibacterial resistance in fruits and vegetables


**Supporting information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Product, type of culture and mode of production. **Table S2.** Number of samples and type of product per date of sampling.

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