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Does organically produced lettuce harbor higher abundance of antibiotic resistance genes than conventionally produced?

Bokai Zhu [a], Qinglin Chen [b], Songcan Chen [c], Yong-Guan Zhu [b,c,*]

[a] Department of Biological Science, College of Agriculture and Life science, Cornell University, Ithaca 14853, United States
[b] Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China
[c] State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

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ABSTRACT

The demand for organic food products, especially for organic vegetables has been growing rapidly in the last few decades. However, the risk of introducing more antibiotic resistant genes (ARGs) and antibiotic resistant bacteria (ARB) to the vegetables by organic production procedures has long been overlooked. In our study, by using high-throughput quantitative PCR and 16sRNA Illumina sequencing technology, we investigated the abundance and diversity of ARGs and the microbial communities in conventionally (CPL) and organically produced lettuce (OPL). A total of 134 ARGs were detected in the phyllosphere and leaf endophyte of the samples. Absolute copy numbers of ARGs in phyllosphere were 8-fold higher in the OPL than in CPL. We also observed a significant difference in the microbial communities between OPL and CPL, and a lower diversity of both phyllosphere and leaf endophytic bacteria in OPL than in CPL. The Mantel test and variation partitioning analysis (VPA) suggested that the profile of ARGs is strongly affected by bacterial community compositions. Network analysis between ARGs and bacterial taxa indicated that eight bacterial families were implicated to be the potential hosts of ARGs. These results provide insights into the impacts of organic farming on the profiles of bacterial and ARG compositions in vegetables.

1. Introduction

The market of organic food has been growing rapidly in the last few decades, and the primary reason people pay more for organic food is the personal health concerns (USDA Economic Research Service, 2016). Fresh fruits and vegetables have been the top selling produce categories since the beginning of the modern organic food industry (Laux, 2013). During the production of certificated organic vegetables, chemical fertilizers and pesticides are strictly forbidden due to their potential harm they may cause to individuals and the environment. There is then a general perception that organic vegetables are safer and healthier than the conventionally (non-organic) produced vegetables.

To replace chemical fertilizers, animal manures are often used as organic fertilizers in the production of certificated organic foods. However, it has been recommended that good agricultural practice should be implemented to minimize potential contamination of pathogens in organic production due to the use of animal manure (de Quadros Rodrigues et al., 2014). Furthermore, animal manures are frequently sourced from confined animal feeding operations (CAFOs) where antibiotics are used to treat animal diseases and promote growth (Ji et al., 2012). Only a small proportion of the antibiotics applied are absorbed by the animals, and between 30 and 90% of the antibiotics were excreted in the manure (Heuer et al., 2011a; Heuer et al., 2011b; Ji et al., 2012; Negreanu et al., 2012). Additional to this biohazard contamination, manures have been shown to be a major carrier of antibiotic resistant bacteria (ARB), and a rich reservoir of antibiotic resistant genes (ARGs) (Zhu et al., 2013; Fang et al., 2015; Lester et al., 2006).

Long-term applications of manure have been shown to increase the abundance of ARGs in soil (Chen et al., 2016). When manure is applied as a fertilizer, residual antibiotics and ARGs can disperse into the soil and increase the selection pressure on ARB in the soil (Wang et al., 2015b). Moreover, the residual ARGs in the manure can transfer to bacteria in the soil via horizontal gene transfer (HGT) (Gaze et al., 2011; Sentchilo et al., 2013; Zhang et al., 2011). The introduction of manure as a rich source of antibiotics and ARGs to soil increases the risk of introducing more ARB to the soil bacterial community. Additionally, soil microbiomes contribute profoundly to the development of below- and above-ground plant bacterial communities, and genomic study has shown that there is a large functional overlap between leaf- and root-derived bacteria (Bai et al., 2015). As a consequence, the bacterial community of plants growing on manure-amended soil can be influenced by

* Corresponding author at: Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, No.1799 Jimei Road, Xiamen 361021, China.
E-mail address: ygzhu@rcees.ac.cn (Y.-G. Zhu).

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variations in the soil bacterial communities. Several studies have reported the presence of ARGs on the surface of vegetables and fruits (Abriouel et al., 2008; Boehme et al., 2004; Durso et al., 2012; Rodríguez et al., 2006). For instance, our previous study detected ARGs on different parts of lettuce (including root endophytes, leaf endophytes, and the phyllosphere) that were grown in a manure-amended soil (Wang et al., 2015a). However, until now only a few studies have investigated specifically the differences in ARGs and bacterial communities between conventionally produced and certificated organic vegetables. Ruimy et al. (2010) found that fruits and vegetables from organic and conventional production contained similar counts of antibiotic resistant Gram-negative bacteria (Ruimy et al., 2010). Using standard culture methods, Tango et al. (2014) surveyed indicator bacteria and pathogens on various leafy vegetables in Korea. They did not observe significant differences in pathogenic bacteria between samples from organic and conventional production (Tango et al., 2014). Using culture-based method, Raphael et al. (2011) reported that Gram-negative Saprophytes isolated from retail organic and nonorganic spinach harbored extended-spectrum beta-lactamase (ESBL) genes (Raphael et al., 2011). The above-mentioned studies relied on culture-based methods and focused on limited number of antibiotics. For a more comprehensive investigation, high throughput profiling of the microbial communities and resistome from organic and nonorganic vegetables is necessary.

In this study, we sampled vegetable products directly from a fresh food market. We sampled products from eight different producers and evaluated the effect of the organic production process on ARGs abundance and diversity of the phyllosphere and endophytes of vegetables at the point of retail. All the organic producers included in this study have national certification for organic food production and are located in different areas of China. We used high-throughput quantitative PCR (HT-qPCR) which included 296 validated primer sets targeting almost all major classes of ARGs. We also used Illumina sequencing in this study to provide a more comprehensive profile of bacterial communities in vegetables. In addition, the co-occurrence pattern between ARGs and bacterial taxa was revealed by network analysis and provides potential host information for the ARGs.

2. Materials and methods

2.1. Sampling

Lettuces were purchased directly from the supermarket, from eight different producers including three conventionally producers (CPL) and five organic producers (OPL) All five organic producers (companies) possess national certification for organic farming, and the organically production is based on the China national organic food standard-GB/T 19630.1-2011. According to the standard, chemical fertilizers, pesticides, growth regulating agents etc. are forbidden during the production. This standard recommends the use of bio-fertilizers from organic sources if possible, and in China most bio-fertilizers are derived from animal manures or manures mixed with crop residues. In addition, the bio-fertilizers applied to organic production systems must be fully composted. The national standards also recommend that pathogens should be minimized in bio-fertilizers used in organic production, but other bacteria (including antibiotic resistance bacteria) are not on the list. A total of 48 samples (48 heads of lettuces) were gathered. Only one producer's product was stored in a refrigerator when selling, all 7 other producers' products were stored at the room temperature when selling in the market. We took only fresh lettuce samples that were on sale for less than one day to avoid uncontrolled bacteria growth and other factors that might influence the results. For each producer, we randomly sampled three packaged products, with six replicate samples for each product and three were used for analysis of the phyllosphere (CPL-phyllo and OPL-phyllo) and the other three were used for analysis of the leaf endophyte (CPL-endo and OPL-endo). The samples for each replicate were all collected from the upper part of the lettuce leaves and cut into pieces around 2 cm × 2 cm with sterilized scissor.

2.2. DNA extraction

Before DNA extraction, the samples for leaf endophyte analysis were immersed in 30% hydrogen peroxide for 30 min to eliminate the phyllosphere bacterial community, and then washed with sterilized water three times. After that, the samples were treated with 70% ethanol for 1 min and washed in sterilized water again (Wang et al., 2015a). To confirm that the surface disinfection process was successful, the last wash solution for each sample was incubated in TSB culture, at 37 °C, 180 rpm, for 7 days, to test that there were no phyllosphere bacteria remaining. Then around 0.4–0.7 g of leaves were used for extraction with the FastDNA SPIN Kit (MP Biomedical, Santa Ana, California, USA). For the phyllosphere samples, around 2–4 g of leaves were transferred into a 50 ml centrifuge tube. The transfer procedure was conducted under sterile conditions in order to avoid contamination from the air. The centrifuge tubes were shaken at 200 rpm and 30 °C on a shaking incubator for 2 h after adding 45 ml autoclaved 1 × phosphate buffered saline supplemented with 0.02% Tween20. The washing solution was filtered with a sterilized nylon net and then centrifuged at 7500 rpm for 30 min. The pellets were preserved using the sodium phosphate buffer from the FastDNA SPIN Kit. The remaining procedure follows the FastDNA SPIN Kit protocol. The quality of DNA was checked by spectrophotometric analysis using NanoDrop ND-1000 (Nanodrop, USA). The concentration of DNA was determined using the QuantiFluor® dsDNA system (Promega,Madison, Wisconsin, USA) according to the manufacturer's instruction with a microplate reader (Spectramax M5, USA). The DNA was stored at −20 °C until use.

2.3. High-throughput quantitative PCR

To evaluate the abundance and diversity of ARGs in the samples, high-throughput quantitative PCR (HT-qPCR) reactions of ARGs were performed using the Wafergen SmartChip Real-time PCR system. A total of 296 primer sets were used in the PCR, targeting 285 ARGs, 8 transposases, 1 class I intergron, 1 clinic intergron and 1 16s rRNA (Table S1). The 285 ARGs primers covered almost all major antibiotic resistance genes (Su et al., 2015). The initial enzyme was activated at 95 °C for 10 min. The PCR cycling process was: denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s. This amplification process cycled 40 times. Wells with multiple melting peaks or an amplification efficiency that is beyond the range (90%–110%) were discarded. For each sample, 2 replicates were made. We utilized a detection limit where any replication with a threshold cycle (C_T) number larger than 31 is considered negative and only samples with where both replicates were positive were regarded as a positive detection of the specific ARGs. The relative copy number of ARGs and MGEs was calculated (using Eq. 1) and transformed to absolute copy numbers by normalizing to 16S rRNA gene copy numbers which were quantified separately from the Wafergen platform (Looft et al., 2012; Ouyang et al., 2015; Schmittgen & Livak, 2008). The average number of 16S rRNA encoding genes per bacterium is currently estimated at 4.1 (Klappenbach et al., 2001). Bacterial cell numbers were then estimated by dividing 16S rRNA gene copy numbers by this value, and the normalized copy number of ARGs per bacterial cell was calculated (Stalder et al., 2014; Su et al., 2015).

$$\text{Relative gene copy number} = 10^{(31-C_T)/(10/3)} \quad (1)$$

2.4. Illumination sequencing and analyses of 16S rRNA gene

To investigate bacterial community structure and composition, the V4-V5 region of bacterial 16 s rRNA was selected for amplification with primers F515 GTGCCAGCMGCCGCGG and R907 CCGTCAAT

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