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Detection of *Arcobacter* and *Helicobacter pylori* Contamination in Organic Vegetables by Cultural and PCR Methods

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***Abstract*—**The most demanded organic foods worldwide are those that are consumed fresh, such as fruits and vegetables. However, there is a knowledge gap about some aspects of organic food microbiological quality and safety. Organic fruits and vegetables are more exposed to pathogenic microorganisms due to surface contact with natural fertilizers such as animal manure, wastes and vermicompost used during farming. Therefore, the objective of this work was to study the contamination of organic fresh green leafy vegetables by two emergent pathogens, *Arcobacter* spp. and *Helicobacter pylori*. For this purpose, a total of 24 vegetable samples, 13 lettuce and 11 spinach were acquired from 10 different ecological supermarkets and greengroceries and analyzed by culture and PCR. *Arcobacter* spp. was detected in five samples (20%) by PCR, four spinach and one lettuce. One spinach sample was found to be also positive by culture. For *H. pylori*, the *H. pylori Vac*A gene-specific band was detected in 12 vegetable samples (50%), 10 lettuces and two spinach. Isolation in the selective medium did not yield any positive result, possibly because of low contamination levels together with the presence of the organism in its viable but non-culturable form. Results showed significant levels of *H. pylori* and *Arcobacter* contamination in organic vegetables that are generally consumed raw, which seems to confirm that these foods can act as transmission vehicles to humans.

Keywords*—Arcobacter* spp., *Helicobacter pylori*, organic vegetables, Polymerase Chain Reaction, PCR.

I. Introduction

ORGANIC production is an important instrument to agri-food sustainable production. The term "organic (ecological) agriculture" refers to agricultural methods using natural substances and processes, comprising the use of organic fertilisers, a predominant reliance on ecosystem services and non-chemical measures for pest prevention and control [1]. Despite the great interest of consumers in this type of product, at present, little is known about organic food microbiological quality and safety compared to conventional one. The main problem for organic food is bacterial contamination; because it seems that consumers have not assumed that organic food actually has similar microbiological risks to those commonly consumed, and therefore, must be handled under appropriate conditions to prevent the occurrence of foodborne diseases.

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The most abundant organic foods worldwide are those consumed without prior transformation. Among them, fresh fruits and vegetables stand out [1]. Fresh leafy vegetables, sprouts, fruits and other ready-to-eat vegetables can pose a serious health problem if their production, harvesting, storage and distribution are not closely controlled.

Some of the established microbiological contamination sources of fresh vegetables include contaminated manure, irrigation water, soil, livestock and wildlife, being fertilizer and irrigation the most crucial risk factors [2]-[4]. Organic fruits and vegetables are exposed to pathogenic microorganisms during farming, due to the use of natural fertilizers such as animal manure, wastes and vermi-compost.

Paradoxically, there are very few scientific works about the microbiological quality of organic vegetables and, although it seems that common practices in the organic sector (in particular, allowing sufficient time for transformation of manure and crop rotation) leads to food with similar or even lower levels of microbiological hazards [1], [3], [5], most authors remark that further efforts are needed to understand and control the risks associated with organic produce from harvest to consumption [6].

Enterobacteriaceae, such as *Salmonella* or Shiga toxin-producing *Escherichia coli* (STEC), *Listeria* spp., and norovirus, are the main agents causing foodborne disease outbreaks related to fresh produce. However, there are many other emergent pathogens which can contaminate raw or minimally processed vegetables, such as *Helicobacter pylori* or *Arcobacter* spp. [7]-[9].

H. pylori is one of the most prevalent pathogens worldwide, being the unique microorganisms classified up to date as carcinogenic agent level I by the World Health Organization [10]. It is the causative agent of upper gastrointestinal tract diseases, mainly gastritis, peptic and duodenal ulcers. This bacterium is also closely related with gastric cancer, mucosal-associated-lymphoid-type (MALT) lymphoma and non-Hodgkin's gastric lymphoma [11].

The contamination of vegetables with *H. pylori* and the subsequent introduction of these raw vegetables into the food chain have been suggested to be one of the transmission pathways of the bacterium to humans, and different authors have found rates of contaminated leafy vegetables and salads samples between 10-35% [12], [13]. The use of faecal irrigation water seems to be the main factor leading to the contamination of fruits and vegetables with *H. pylori* [12].

The genus *Arcobacter* is a member of the family

Campylobacteraceae. Arcobacters are foodborne pathogens causing diarrheal illness in humans [14]. It has been suggested that water may play an important role in the transmission of these organisms and it has been previously found in water contaminated with heavy metals and in fresh vegetables [15], [16]. Moreover, the number of foodborne diseases caused by these organisms is increasing, which represents a challenge for food safety [17].

The knowledge of contamination levels in foods is essential for the prevention of microbiological hazards for consumers. Conventional culture methods are considered the gold-standard for determining the microbiological status of samples. Culture also allows for isolating the pathogens, and thus characterizes them for epidemiological purposes. However, *Arcobacter* and *Helicobacter* are very fastidious organisms, difficult to culture from environmental samples, and long times are needed to obtain results. Moreover, standardized detection methods have yet to be established. Molecular methods, such as PCR based methods, have already proved to be valuable tools for rapid *Arcobacter* and *Helicobacter* detection in environmental and food samples, being more rapid, sensitive and specific than culture [18], [19].

We have not found any published study focused specifically on the contamination of organic vegetables with these two pathogens. Therefore, the objective of this research was to study the presence of *Arcobacter* spp. and *Helicobacter pylori* in organic fresh green leafy vegetables, for human consumption, by using cultural and molecular methods.

II. Material and Methods

A total of 24 vegetable samples, 13 lettuce and 11 spinach, were acquired from 10 different Spanish specialized ecological supermarkets and greengroceries, from June to December 2020. Each sample batch was composed for five lettuce units or five spinach bunches taken from the same commercial point. All vegetables were fresh, unpacked, not washed and without previous disinfection.

Samples were maintained under refrigeration and processed in less than 4 hours since sampling. Lettuce and spinach leaves were sliced in pieces and mixed.

A. Isolation of H. pylori

For *H. pylori* detection, 25 g from each batch was aseptically disposed into sterile stomacher bags with 225 ml of Wilkins-Chalgren anaerobe broth (Oxoid CM0643, UK) supplemented with 5% horse serum (Oxoid SR0035, UK) and *Helicobacter pylori* selective supplement (Dent) (Oxoid SR147E, UK). The samples were homogenized in a stomacher machine for 5 min and incubated at 37 °C under microaerophilic conditions (5% oxygen, 10% carbon dioxide, and 85% nitrogen) (CampyGen®, Oxoid CN0035A, UK) for 48 h. After this time, 100 µl of the enriched samples were dropped by duplicate on a 0.45 µm cellulose membrane filter laid on the surface of Wilkins-Chalgren Agar with Dent supplement and 5% of defibrinated horse blood (Oxoid SR0050C, UK). After 30 min, membranes were removed and plates were incubated for other 48 h under microaerophilic atmosphere. Then, characteristic colonies were selected, and identification was performed by means of Gram stain and specific PCR, as described below.

B. Isolation of Arcobacter spp.

A sample of 25 g from each batch were homogenized with 225 ml Arcobacter Broth (AB) (Oxoid CM0965, UK) with Cephoperazone-Amphotericin B-Teicoplanin (CAT) selective supplement (Oxoid SR174E, UK) and incubated at 37 °C under microaerophilic conditions for 48 h. Then, 100 µl were transferred by duplicate onto the surface of Arcobacter agar plates with 5% defibrinated horse blood supplemented with CAT, using a 0.45 µm cellulose membrane filter [20]. After one hour incubation at 30 °C in aerobic atmosphere, the filters were removed and the plates were incubated for 48 h at 37 °C under microaerophilic conditions. One to four presumptive *Arcobacter* colonies were selected from each plate, checked by Gram stain and identified by PCR.

C. PCR Detection of H. pylori and Arcobacter spp.

For both, Arcobacter-CAT and Wilkins-Chalgren-Dent broths, 1.5 ml aliquots were taken directly and after the 48 h enrichment period.

The bacterial DNA was extracted using a commercial kit (GenElute Bacterial Genomic DNA Kit NA2110, Sigma-Aldrich, USA), following the manufacturer's instructions.

H. pylori specific VacA primers were used to amplify a 394 bp fragment from vacuolating cytotoxin A (*VacA)* gene [21], [22]. A final reaction volume of 50 µl was made by addition of 5 µl of each DNA template, 0.5 µM of each primer, 0.2 mM of each deoxynucleotide, 1.5 mM $MgCl_2$ and 5 U of *Taq* polymerase (Meridian Bioscience BIO-21060, USA). The amplification consisted of an initial DNA denaturing step at 95 °C for 5 min, followed by 33-cycle reaction (94 °C for 1 min; 57 °C, 1 min; 72 °C, 1 min) and a final extension step at 72 °C for 5 min. Reference strain *H. pylori* 11637 NCTC was included in all assays.

Specific *Arcobacter* spp. PCR was performed by using ARCO1 (5′-GTCGTGCCAAGAAAAGCCA-3′) and ARCO2 (5′-TTCGCTTGCGCTGACAT-3′) primers, for amplifying a 331-bp fragment of 23S rDNA gene [18]. *A. butzleri* DSM 8739 was used as a positive control for all the assays.

PCR products (5 mL) were detected by electrophoresis on 1.2% (wt/vol) agarose gel prepared in 1 X Tris-Acetate EDTA (TAE) buffer and stained with Red Safe (iNtRON Biotechnology 21141, South Korea) at 95 V for 90 min. The amplicons were visualized in a UV transilluminator. A 100-bp DNA ladder (Thermo Fisher Scientific SM0241, Germany) was used as a molecular weight marker.

III. Results and Discussion

A. Arcobacter spp. Detection

One spinach sample (4% out of the 24 analyzed organic vegetable batches) was found to be positive by culture. *Arcobacter* sp. was detected in five samples (20%) by PCR (see Fig. 1). The highest detection levels (4/5) were obtained

in spinaches (see Table I). Most of the positive results (4/5) were obtained after the enrichment step. It has been reported that the combination of PCR with an enrichment step increases the level of viable cells, while dead cells and inhibitors are diluted [23]. Only in one of the five positive samples (one spinach batch) was detection possible without enrichment, suggesting high contamination levels in this sample.

There are not many studies about the occurrence of arcobacters on vegetables. Our results showed contamination levels similar to those reported in Spain in 2011 [24] and 2017 [18], which found 14% and 20% PCR positive rates, respectively, in two different studies performed on non-organic conventional vegetables. In another work developed in a spinach-processing plant in Germany, *Arcobacter* sp. was detected in 35% of the analyzed samples [25]. *Arcobacter* has been also found in 27.5% out of 160 pre-cut vegetables in Italy, including lettuce spinach, rocket and valerian [26].

TABLE I
HELICOBACTER PYLORI AND *ARCOBACTER* SPP. DETECTION IN VEGETABLE SAMPLES

Type of Sample	*H. pylori* 0h	*H. pylori* 48h	*Arcobacter* 0h	*Arcobacter* 48h	Culture Detection
Lettuce	+	-	-	-	
Spinach	-	-	-	-	
Lettuce	+	-	-	-	
Spinach	-	-	-	+	
Lettuce	+	-	-	-	
Spinach	-	-	-	+	*Arcobacter* sp
Lettuce	+	+	-	-	
Spinach	-	-	-	-	
Spinach	-	-	-	-	
Lettuce	+	-	-	-	
Lettuce	+	-	-	+	
Spinach	-	-	-	-	
Lettuce	+	-	-	-	
Spinach	-	-	-	-	
Lettuce	-	-	-	-	
Lettuce	+	-	-	-	
Spinach	-	-	-	-	
Lettuce	-	-	-	-	
Lettuce	-	-	-	-	
Spinach	-	-	-	-	
Lettuce	-	+	-	-	
Spinach	+	+	+	+	
Lettuce	+	+	-	-	
Spinach	+	-	-	+	

+: positive; -: negative.

B. H. pylori Detection

The *H. pylori Vac*A gene specific band was detected in 12 vegetables samples (50%), 10 lettuce and two spinach (see Table I, Fig. 2). These results are higher than those reported by other authors; in a survey developed in Iran, they found that close to 20% of unwashed lettuce were *H. pylori* positive [12]. Similar qPCR contamination levels were reported in another study, which analyzed salads and fresh vegetables and found around 14% of samples contaminated with *H. pylori* [13].

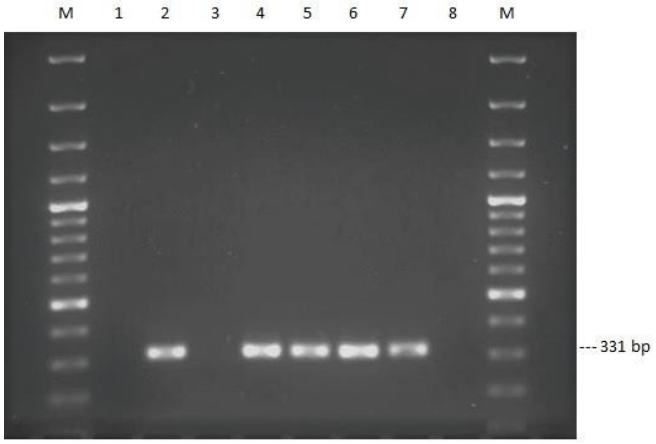

Fig. 1 Gel electrophoresis showing the 23S rDNA amplified fragment of *Arcobacter* spp. in vegetable samples and isolates. Lanes M: 100-bp ladder; Lane 1: spinach sample before enrichment; Lane 2: spinach sample after enrichment; Lane 3: lettuce sample before enrichment; Lane 4: lettuce sample after enrichment; Lanes 5-6: isolates by culture; Lane 7: *A. butzleri* DSM 8739; Lane 8: negative control

Detection rates were higher before enrichment (11 *vs.* four samples), probably due to the overgrowth of competitive microbiota during this step. This result is not surprising, and has been previously reported by other authors [27].

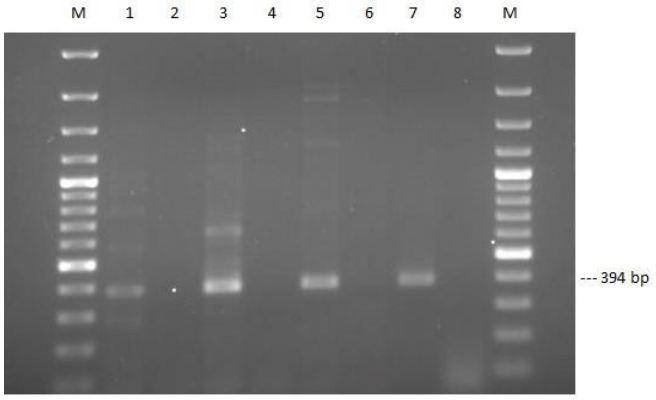

Fig. 2 Gel electrophoresis showing the specific VacA amplified fragment of *Helicobacter pylori* in vegetable sample. Lanes M: 100-bp ladder; Lane 1: lettuce sample before enrichment; Lanes 2: lettuce sample after enrichment; Lane 3: lettuce sample before enrichment; Lanes 4: lettuce sample after enrichment; Lane 5: lettuce sample before enrichment; Lanes 6: lettuce sample after enrichment; Lane 7: *H. pylori* 11637 NCTC; Lane 8: negative control

Isolation in selective medium did not yield any positive result, being this fact possibly related to the presence of *H. pylori*, not only at low levels, but also into a viable but non-culturable (VBNC) form. Main differences between culture and molecular techniques to recover *H. pylori* from foods are due to the fact that a proportion of *H. pylori* cells are in VBNC form in the environment [19], [28].

IV. Conclusion

Our results show significant levels of *H. pylori* and *Arcobacter* contamination in organic vegetables. As these food products are generally consumed raw, they could pose a potential public health risk. However, as the size of the sample is too small, more exhaustive studies are necessary before reaching definitive conclusions.

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