Biointerface Research in Applied Chemistry
Platinum Open Access Journal (ISSN: 2069-5837)

Short Communication
Volume 11, Issue 2, 2021, 8716 - 8724
https://doi.org/10.33263/BRIAC112.87168724

Pseudomonas aeruginosa as a Potential Contaminant of Packed Fresh-Cut Lettuce in a Controlled Atmosphere. The Role of Phenotypes muc$^+$/muc$^-$

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Received: 3.07.2020; Revised: 4.08.2020; Accepted: 6.08.2020; Published: 8.08.2020

Abstract: In order to shed light on contamination risks along the ready-to-eat chain of fresh commodities by emerging foodborne pathogens, we investigated the biofilm development *in vitro* of two *Pseudomonas aeruginosa* strains on fresh-cut lettuce (*Lactuca sativa* L. var. Iceberg). The experiment was performed employing a floating bioreactor system where modified atmosphere package conditions were mimicked, and fresh-cut lettuce disks of 2 cm^2 were put into contact with a 10^6 CFU/mL of a phenotypic mucoid *P. aeruginosa* phenotype (*muc*$^+$) or a non-mucoid one (*muc*$^-$). Following a simulated 2-day refrigerated-shelf quantitative Real-Time PCR, designed on a target gene region of the 16S rRNA gene, defined the different *muc* phenotypes behavior on biofilm in lettuce phyllo-plane. Between the two strains, a development difference of nearly 1.0 log CFU/cm^2 occurred, with the *muc*$^+$ phenotype being the most settled and adherent. This result clearly showed a distinct contamination risk according to *P. aeruginosa* phenotype and the need to develop real-time, specific, fast, and easy to use detection protocols along with specific sanitation systems for modified atmosphere package ready-to-eat commodities.

Keywords: ready-to-eat produce; emerging foodborne agents; biofilms; *mucA* gene; exopolysaccharide alginate.

1. Introduction

Today's lifestyles and health concerns have led to a significant rise in fruit and vegetable consumption balanced by a remarkable growth of ready-to-eat (RTE) products on the market [1]. Within this context, fresh-cut produce is acquiring a crucial role thanks to its fresh-like appearance, taste, flavor, and handiness. Still, the primary and secondary phase of the RTE production faces unsolved criticisms related to the product's short shelf-life and safety issues [2]. Indeed, following harvest senescence hasten in most crops and processing ensuing steps (grading, washing, peeling, cutting, and packaging) further speed up quality spoilage. In addition, the extracellular release of cytoplasm nutrients, following cell-wall ruptures by cutting, provides a favorable milieu for the fast growth of foodborne pathogens, especially bacteria and yeast [3]. Since fresh-cut commodities, such as leafy greens (e.g., lettuce, parsley, etc.) and fruit salads, are consumed raw, they represent a potential transmission vehicle for human chronic infections [4]. To withstand those threats, fresh-cut produce is sanitized, packed

into a modified atmosphere, and kept refrigerated [5]. Ongoing researches to withstand the outbreak of resistant strains is constantly reporting on new treatments, more effective, and differently acting [6, 7]. However, outbreaks of foodborne diseases start frequently by an over-reliance on the food safety management system [8]. Along the food chain, all steps have a strict interdependence, and contamination risk assessment must start from the field to warrant quality and safety at all subsequent stages [9]. In addition, the increasing need to use wastewater in agriculture and the occurrence of groundwater contamination introduce new critical issues [10]. The latter influence the microbial load introduced by fresh produce into the processing line affecting the cross-contamination risk significantly. Holvoet *et al*. [11] evidenced that above-average microbial loads at harvest, increase cross-contamination, and total psychrotrophic aerobic bacterial count resulted in useless as a food safety indicator. In addition, planktonic and biofilm bacterial cells have different susceptibility to disinfectants as well as attachment characteristics to food or processing equipment surfaces [12, 13]. This complex microbe-produce interaction and the development of disinfectant-resistant strains hurry-up the need for easy, efficient, and fast methods to detect and map early microbial settlements along the chain. In this regard, medical research has developed several methods; however, most are invasive, costly, time-consuming, need specialized labs, and trained personnel. In addition, yet they have not been applied along the farm-to-fork chain to warn on over-all microbial composition [14, 15]. According to recent statistics on foodborne illnesses in the USA and the European Union, among the over 250-reported foodborne diseases, leafy vegetables are implicated in several outbreaks, and most common agents are *Norovirus*, *Salmonella* spp., and *Escherichia coli* [16]. Still, other opportunistic ubiquitous pathogens with high adaptation capacity and resistance towards sanitation treatments are increasingly involved in acute and chronic infections related to food contamination [17, 18]. Emerging foodborne outbreaks, like those involving Pseudomonades, are strictly related to cropping environment, processing protocols, as well as diet changes, all factors influencing the background microflora balance [19, 20]. In this respect, the World Health Organization ranks *Pseudomonas aeruginosa* as critical due to its high antibiotic resistance and a broad range of adaptive mechanisms [21]. Among adaptations occurring in *P. aeruginosa* subjected to biotic/abiotic stresses, quorum sensing is pivotal to switch from the planktonic form to the more resistant and adherent biofilm one [22]. Little literature on *P. aeruginosa* behavior under abiotic stresses such as the ones occurring along the fresh-cut chain (sanitation, nutrient/oxygen variation, low temperature) is available [23]. For this reason, the effect of mimicked fresh-cut lettuce package conditions on the development of two *P. aeruginosa* phenotypes was investigated by a feasible detection methodology.

2. Materials and Methods

2.1. Lettuce leaf sample preparation and bioreactor structure.

Freshly harvested lettuce heads (*Lactuca sativa* cv 'Iceberg') were attained from a local farm producing leafy greens addressed to the processing industry (Fresco & Pronto S.r.l., Monserrato (CA), Italy). In order to simulate the local commercial chain for fresh-cut produce, lettuce heads were immediately moved from the field to the laboratory, soiled external leaves discarded and then, recovered leaves were dumped into a cold-water-sodium hypochlorite solution (4°C; 50 mg L^{-1} total chlorine). The disinfection process lasted for 15 min by gently shacking the bath. After removal, leaves were rinsed with sterile water and drained by means of a vegetable-centrifuge. Finally, the middle area of unbruised lettuce leaves was used to

prepare tissue disks employing a cork-borer (ø 1.5 cm) under sterile conditions. Disks were gently rinsed with deionized water and kept cold (4 °C) until use (max one h).

In order to obtain comparable and replicable results, a specific growth-monitoring system was developed, owning a temperature (0 - 25 ± 1 °C) and oxygen level (1 - 0.5 kPa) control. In addition, the contact area between *P. aeruginosa* cells and the lettuce phylloplane was standardized in order to monitor bacterial growth according to a specific leaf area. In this work, bacterial growth occurred in a floating bioreactor system (Fig. 1), where the contact area between *P. aeruginosa* cell suspension and the leaf surfaces was set at 2 cm^2. The floating-bioreactor system was developed with the aim to study microbe-host adhesion at the laboratory level, applying comparable settings to those occurring during standard shelf-life conditions of Modified Atmosphere Packed (MAP) fresh-cut produce [24].

2.2. Pseudomonas aeruginosa phenotypes, storage, and preparation.

Experiments were performed with two reference *P. aeruginosa* strains: a mucoid phenotype, alginate producer (ATCC 15442) highly biocide-resistant (muc^+), and a non-mucoid one (ATCC 2783) highly susceptible (muc^-). Until used, both isolates were stored in stock-vails (50 µL) in a tryptic soy agar (TSA) medium with 15% glycerol at -80 °C (Microbiol Uta, Italy). Before each experiment, a stock of the frozen bacterial culture was slowly thawed and plated on TSA medium. Following 24 h incubation at 37 °C, a single colony was removed and inoculated into a 500 mL flask with sterilized Mueller Hinton Broth (Microbiol, Cagliari, Italy) and incubated at 37°C for eight h, until the growth middle logarithmic phase was reached [25,26]. Then, for each *P. aeruginosa* strain, a final concentration of 10^6 CFU/mL bacterial cell was prepared and employed as the starting inoculum for the 2 cm^2 lettuce leaf specimen placed in the floating bioreactor system.

2.3. DNA extraction.

Following 48 h of the simulated MAP conditions, the leave specimens were removed from the floating bioreactor, and a 1 cm^2 circle, from the center of each leaf-specimen, was removed by a cork-borer (8 mm Ø) and employed to obtain *P. aeruginosa* DNA. The extraction of microbial DNA was achieved by the hexadecyltrimethylammonium bromide (CTAB) protocol. In short, leave tissue was chopped (with a stainless blade), suspended in 400 µL of ultrapure water DNAse free (Gibco, Invitrogen Paisley, Scotland UK) and ground thoroughly using an Ultra TURRAX® - Tube Drive (IKA, Germany) with sterile glass balls for 3 min at shaking power 7. Subsequently, 70 µL of a 10% sodium dodecyl sulfate solution (SDS) and five µL of proteinase K (10 mg/mL) (Sigma-Aldrich, ST. Louis, Missouri, USA) were added and vortexed (2 min high speed), then the resulting mixture was incubated for 10 min at 65 °C. Following incubation, 100 µL of NaCl [5 M] and 100 µL of CTAB/NaCl (0.274 M CTAB and 0.877 M NaCl, Sigma-Aldrich) were added to the vial, vortexed and incubated at 65 °C for 10 min. After incubation, 750 µL of SEVAG (Chloroform: Isoamyl alcohol-v:v; 24:1, Sigma-Aldrich) was added, and the mixture was medium speed vortexed for 10 sec. Then, the mixture was centrifuged (5 min at 5600 *RCF*), and 0.6 volumes of isopropanol (Sigma-Aldrich) were added to the supernatant, which was stored at -20 °C for 30 min. The cooled mixture was centrifuged (30 min. at 5600 *RCF*), the pellet recovered and left to dry at room temperature for 20 min. Afterward, the dried pellet was suspended in 20 µL of molecular biology-grade distilled

water (Gibco, Invitrogen Paisley), vortexed, and two µL of it was used as DNA suspension for the real-time PCR reaction.

2.4. Real-time PCR conditions and quantitation curve.

The total mass of the two investigated *P. aeruginosa* phenotypes (muc- or muc+) developed within 48 h on the lettuce leave surface under MAP conditions at 4°C was determined through the method reported by Denotti *et al.*[27]. Briefly, a real-time PCR (RT-PCR) protocol was performed by using the Light Cycler instrument with the Light Cycler DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany). PCR reaction involved a region of the 16S rRNA gene. The primers for the PCR OG644 (5'-GGGAGGAAGGGCAGTAAGTT - 3') and OG645 (5'-ACCACCCTCTACCGTACTCT-3') were designed to a flanking sequence of 228 bp (GenBank accession AJ549293) by using Primer3web program .version 4.1.0. The PCR profile was as follows: (i) denaturation at 95 °C for 30 s and (iii) 40 cycles of 1 s at 95°C, 10 s at 50°C, 3 s at 72°C and 3 s at 81 °C. (iv) The melting curve was performed for 1 s at 95, 45, and 95 °C. Transition rates were: 5 °C/s in 72 °C segment, 0.1 °C/s in 45 °C segment and 20 °C/s for another step. Fluorescence was detected at the end of the 81 °C segment (avoiding aspecific fluorescence due to primer-dimers, in the PCR step (single mode) and at 45 °C segment in the melting step (continuous mode) in the F1 channel. (Figure 2). *P. aeruginosa* cells amount was evaluated in the lettuce leaves by a standard curve made on DNA extracts obtained from different *P. aeruginosa* cell suspensions with a concentration ranges from 10^6 to 10^2 genomes/ µl. The amount of bacterial DNA concentration in the leaf extracts was calculated by an interpolated threshold cycle with a standard curve (Figure 3). The prediction *in silico* of melting temperatures was evaluated by using m To evaluate *P. aeruginosa* adhesion index, we have used the subsequent equation [27]:

$$AI° = ([DNA]*V°/2)/S°$$

AI° is the bacterial adhesion coefficient measured in *P. aeruginosa* genomes /cm^2 on a lettuce leaf surface.
[DNA]= bacterial genomes in 2 µl, calculated by PCR real-time standard curve interpolation.
V°= volume of DNA extract suspension (50 µl during our experiments). S°= leaf surface used for DNA
extraction (1 cm^2).

2.5. Statistical analysis.

The absolute quantification of total bacteria with RT-PCR was performed by *Escherichia coli* standard curve following a previously published protocol [27]. In these experiments, the standard curve linear correlation R^2 ranged from 0.97 to 0.99. For each analysis, three distinct biological replicas were made, and quantitative data were expressed as values mean ± SD. For each sample, the threshold cycle variation comprises ± 0.8 was considered significant. The growth and adhesion were compared in *muc-* and *muc+* experimental groups by using Fisher's exact test. Data were considered statistically significant for $P \leq 0.05$.

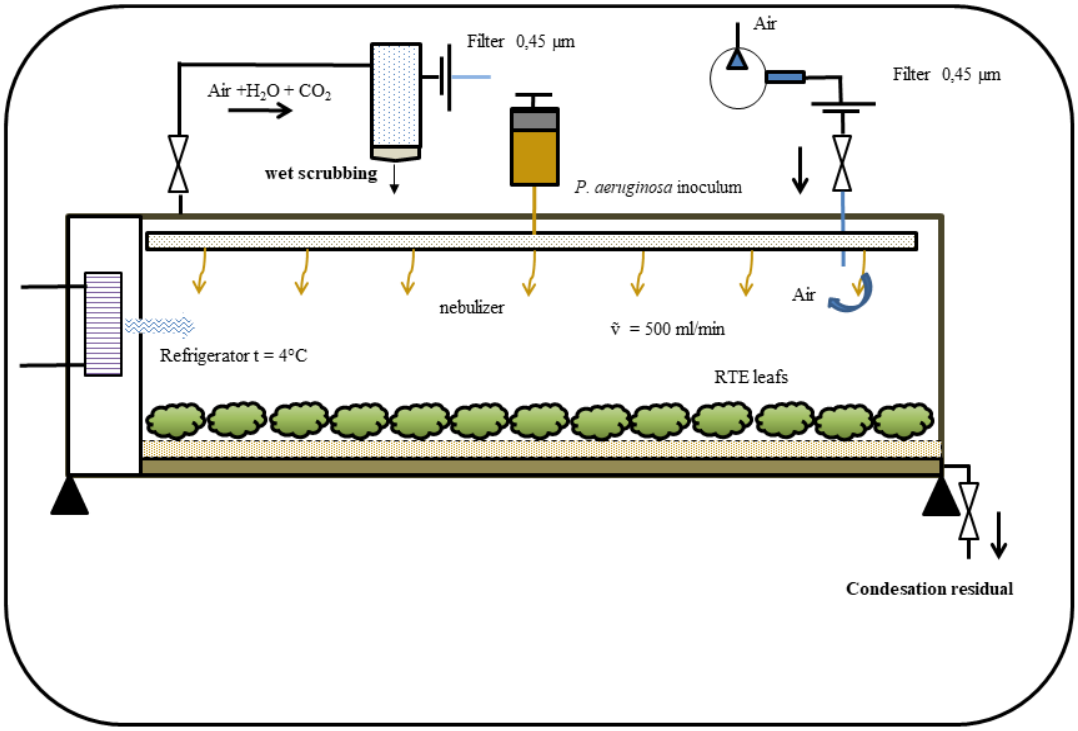

Figure 1. Schematic diagram of the floating bioreactor system used to simulate the contamination of fresh-cut ready to eat (RTE) lettuce by *P. aeruginosa* (*muc*+/*muc*-) when kept under MAP conditions. Leaf contact-surface was of 2 $cm^{2,}$ and the sterile water volume 100 mL.

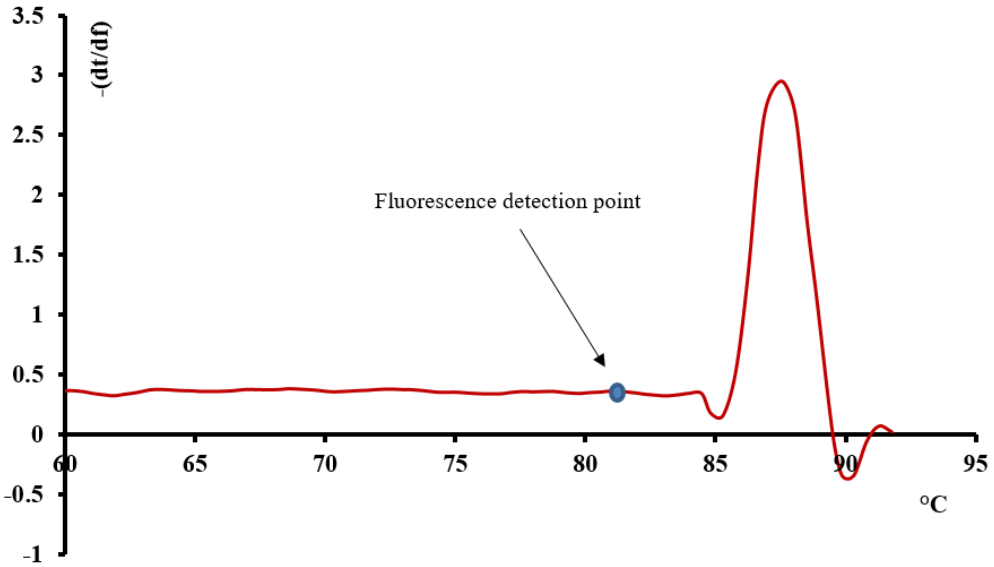

Figure 2. Melting curve profile obtained by light Cycle real-time PCR by using a *P. aeruginosa* ATCC 15442 DNA extract, positive sample showed a unique melting peak at 87.5 °C, in accordance with the values calculated in silico by the DNAmelt program.

3. Results and Discussion

According to the RT-PCR analysis, the development of *P. aeruginosa muc*+ and *muc*- on lettuce surface kept under MAP conditions was quite different following 48 h of storage (Figure 4). Colonization degree between the two phenotypes reached a difference of approximately 1 log ($P< 0.05$), with *muc*+ attaining over 1 x 10^9 *P. aeruginosa* genomes/cm^2 (Figure 4). Thus, results achieved by this research display a notable difference between the colonization and adhesion behavior of the two reference phenotypes used in this simulated

contamination experiment of fresh-cut produce. In particular, *muc*+ (ATCC 15442) increased the phylloplane-biofilm by 3 logs within 48 h. This result agrees with Holvoet *et al*. [11], evidencing how, under standard processing and shelf-life conditions, applied to fresh-cut ready-to-eat vegetables, the initial microbial load is critical, especially for *muc*+ phenotypes. Thus, the increased occurrence of contaminated irrigation/processing water with *muc*+ strains jeopardizes the sanitation system and increases the need to establish critical control points for hazard analysis along the processing line [8]. In addition, most water-sanitation protocols are based on the total coliforms count, which should be revised, taking into account emerging pathogens resistant to most used disinfectants [26, 28].

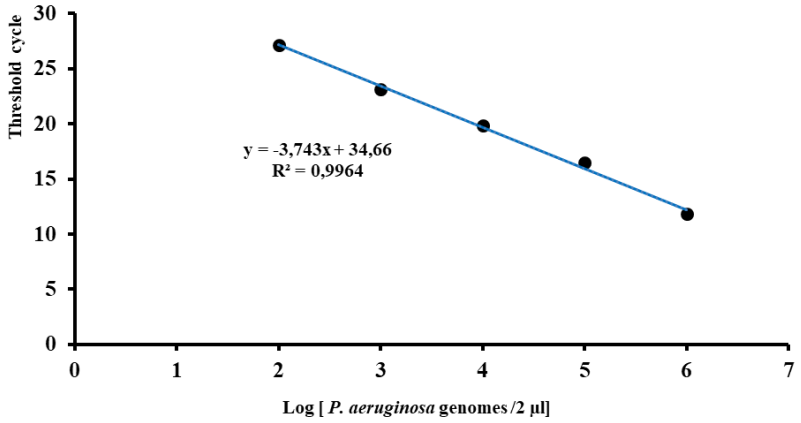

Figure 3. Real-time PCR standard curve related to serially diluted suspensions of *P. aeruginosa* cells. In these conditions, the linear dynamic range of quantification was observed in the range of 10^2–10^6 bacterial genomes /2 µl, corresponding to 5 * 10^3 – 5*10^8 CFU/mL.

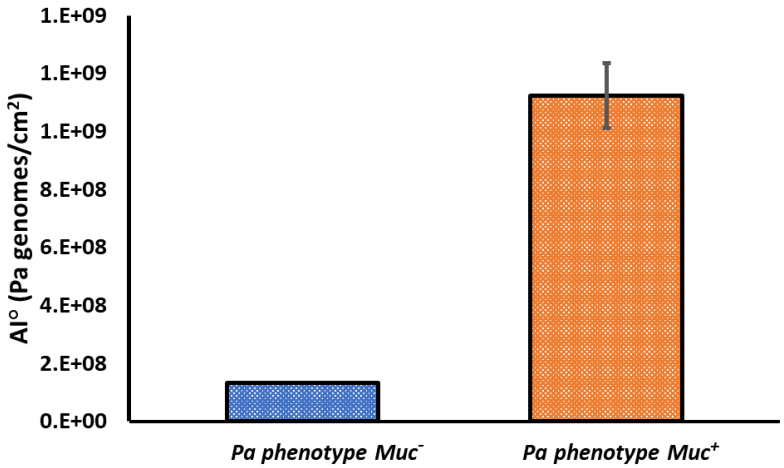

Figure 4. Mean of biofilm amount between *muc*- and *muc*+ phenotypes as determined by real-time PCR and expressed as adhesion index (AI), total genomes of *P. aeruginosa* per cm^2 of *L. sativa* leaf kept under modified atmosphere conditions at 4 °C for 48 hours.

4. Conclusions

Special considerations must be given to the ready-to-eat chain of fresh-cut produce in that sanitation is threatened by produce perishability and structure (surface anatomy, cuts, epicuticular waxes, etc.), as well as cross-contamination (lack of appropriate commodity hazard assessment) and increased recycling of water [10,13,17,18]. All of these criticisms are favorable to the outbreak of foodborne agents, especially when the cold-chain is not maintained

along with the post-processing phase. The spread of several foodborne diseases is halted by low temperatures, but short warm-ups may defeat microbial safety, as evidenced by E. coli acid-resistant isolates [29]. Still, the present work evidence that also under cold and subatmospheric oxygen partial pressure conditions, the muc+ phenotype of *P. aeruginosa* was able to grow and colonize lettuce phylloplane in a relatively short period (48 h), considering the commercial 5 to 7 d shelf-life for fresh-cut produce. This result is likely related to the mutation of the mucA gen, which is a negative regulator of the extracytoplasmic sigma-factor σ22, responsible for the overproduction of alginate [30]. The resistance role of alginate in *P. aeruginosa's* biofilm formation during the infection process has been explained by Lim J. et al. [31] in cystic fibrosis and evidence of how alginate significantly contributes to strengthen the biofilm structure and increase the stickiness to surfaces. Thus, this observation, along with the results reported by Worlitzsch et al. [32] on *P. aeruginosa* muc+ resistance to low oxygen levels, corroborates the results of the present research. The capacity of *P. aeruginosa* to mutate from a no-mucoid to a constitutively mucoid phenotype (missense GAC65GGC Asp-Gly) as a stress adaptation poses a serious problem for the fresh-cut chain where a broad range of different environmental conditions (stresses) occur between the field and the table. In conclusion, as reported for other foodborne pathogens by different authors [33-37], the evidence is provided that also for *P. aeruginosa* there is need to hurry-up appropriate food safety management systems and fast screening methods, such as PCR real-time analysis [35], able to identify mutated mucA phenotypes, characterized by alginate hyper-production.

Funding

This research was funded by the project "Real Time Check IV Gamma" - Por Fesr 2014/2020 – Asse Prioritario I "Ricerca Scientifica , Sviluppo Tecnologico e Innovazione".

Acknowledgments

This research has no acknowledgment.

Conflicts of Interest

The authors declare no conflict of interest.

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