Artificial Phylloplanes Resembling Physicochemical Characteristics of Selected Fresh Produce and Their Use in Bacteria Attachment/Removal Studies

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Abstract

The recurrence of food-borne illness outbreaks caused by consumption of fresh produce highlights the importance of developing a good understanding of the bacteria-leaf-surfaces interactions. In this study, we proposed and developed a new method to fabricate artificial phylloplanes that mimic the topographical and epicuticular characteristics of fresh produce, to be used as a platform for the development of food safety interventions for fresh produce. Romaine lettuce and spinach were selected to create phylloplane replicas using a double-cast procedure. The surface hydrophobicity of the artificial phylloplanes made from polydimethylsiloxane (PDMS) was modified by adding a non-ionic surfactant with different hydrophilic-lipophilic balance (HLB) values to match the hydrophobicity of produce leaves. Key epicuticular wax compounds identified from the natural spinach and lettuce leaves were coated on the leaf replica to mimic the chemical composition of natural leaf surfaces. These surrogate surfaces were used to study the attachment *Escherichia coli* O157:H7 and *Listeria innocua*. In addition, these surfaces are reusable, and have surface hydrophobicity, surface roughness values and epicuticular wax compositions similar to fresh produce. The artificial phylloplanes of fresh produce can be used as a platform for studying the interactions between human pathogens with produce surfaces and for developing new sanitation strategies.

1. Introduction

Fresh and fresh-cut produce continue to be associated with outbreaks of foodborne illness. This has promoted researchers to develop understanding of the interactions between human pathogens with produce phylloplanes, with the purpose of providing insight into pathogens colonization and persistence on produce surfaces, route of contamination, and means to remove pathogens more effectively or decontaminate produce surfaces[1-2]. Adaxial and abaxial surface of plant leaves provide habitats for a diverse assemblage of microorganisms including fungi, yeast, viruses and bacteria[3-4]. Leafy greens can become contaminated with microorganisms at multiple stages in the farm-to-fork continuum. For instance, during growth, produce surfaces can be contaminated with soil, improperly processed manure, or contaminated irrigation water[5-7]. During processing of fresh produce, such as cutting, microorganisms present on outer leaves may come into contact with inner leaves causing cross-contamination of the final product[8-10]. In most cases, bacteria must come into contact with produce surfaces to initiate adhesion and colonization.

The variation in chemistry and topography of plant surfaces oftentimes results in inconsistence in attachment studies[11]. Research regarding interaction between food-borne pathogens and fresh produce leaves is limited compared to that on phytopathogens[12-13]. Most previous studies regarding bacteria-fresh produce interaction were focused on the effect of bacterial species, inoculation method, and produce surface characteristics on the attachment of bacteria to produce[14]. whereas most studies with fresh produce are aim at determining the best combination of sanitizer concentration and processing time to remove bacteria[15-18].

Studies have shown that the properties of fresh produce phyllosphere such as produce surface roughness, surface hydrophobicity, and epicuticular composition play an important role in bacteria attachment and removal[19-21]. Noticeably, these properties undergo constant changes during produce pre-harvest growth and post-harvest transportation and storage, as fresh produce are living plant tissues with active metabolic activities. Produce variety and growth conditions (weather, soil, water, fertilization, etc.) also play a key role determining the produce surface properties. Consequently, the produce surface conditions in sanitation experiments performed by different research groups would not be the same for the same produce type and same sanitizer. As a result, most previous sanitation tests were performed, in a sense, under uncontrolled conditions with regard to produce surface properties. This may be the reason for often observed inconsistent sanitation data reported by different research groups. There is a need to develop a platform for studying bacteria and fresh produce interactions, as well as produce sanitation with controllable and constant surface property phylloplanes that resemble produce surface physical, biochemical, and biological properties.

The requirements for an artificial phylloplane that can be considered as a replica of a natural produce leaf surface should thus include 1) resembling the 3D topological features of natural produce leaf surfaces, 2) having a similar surface hydrophobicity, 3) having a similar epicuticular chemical composition, mainly epicuticular wax composition, 4) producing a similar bacterial attachment pattern, and 5) reproducible and reusable, including autoclave-able and compatible with stomacher.

In the past decade, some research groups have attempted to develop man-made microstructures that could be used as a replica of natural produce surfaces, with varying degrees of success. The early work of University of California, Davis had developed plant surface structure analogs with a microfabrication method (photolithography)[11, 22-25]. To study the effects of plant surface microstructure on attachment of *Escherichia coli* O137:H41, they fabricated uniformly patterned vertical micro-pillars, pyramids, or grooves on polydimethylsiloxane (PDMS) pieces to mimic trichomes, stomata, and ridges between plant cells on produce leaf surfaces, respectively. They used the silanization method to produce hydrophobic surfaces on the silicon, resulting in hydrophobic microstructures similar to those on natural plant surfaces. As the first documented effort, their goal was to use analogs of trichomes, stomata, and intercellular grooves “with controlled shapes, sizes, and distributions to avoid uncontrolled variables that occur on natural plant surfaces.” Therefore, their method only produced man-made arrays of vertical trichomes, stomata, and grooves with uniform shape and size, not the 3D topology of the produce surfaces. The initial work at The University of Illinois was evolved from making simple patterns on PDMS films[26] to fabrication of PDMS surfaces with 2D patterns of natural produce leaves produced from the SEM image of spinach leaf surface[18]. They also developed a method to modify surface hydrophobicity of PDMS by mixing it with different ratios of surfactant with different hydrophile-lipophile balance (HLB) values and pouring it onto a silicon wafer mold with features resembling the surface of spinach leaves. Their method provided a means to mimic hydrophobicity of any leaf surfaces. But it cannot reproduce a 3D surface topology from produce leaves. A simple two-stage double-casting method to transfer of 3D natural patterns on Trembling Aspen leaf surfaces was developed by McDonald et al.[27]. They used PDMS to produce

negative mold of leaf surface. Then a self-assembled monolayer of 2H-perfluorodecyltrichlorosilane (FDTS) was used as an anti-adhesion agent to facilitate transfer of micro-patterns to PDMS positive replica. Slightly later, a USDA ARS group reported a method to reproduce 3D fresh produce surface topology on a PDMS film[28]. After obtaining the negative 3D image of the produce surface on PDMS, they utilized a relatively complex chemical surface modification method to coat the negative PDMS surface with a layer of Pd nanoparticles to make a PDMS negative mold. Then the positive PDMS leaf surface analog was produced using a thermal molding method (120 °C, 20 minutes) from the negative mold. The leaf surface replica after the thermal molding process happened to have a water contact angle (WCA) similar to that of spinach. Recently, Doan et al.[3] reported a two-step casting process to generate topomimetic “replicasts” in PDMS that resembled leaf surface topography at submicrometer scale and used it to study microbial colonization of the phyllosphere with *P. agglomerans*, a plant pathogen to study the removal of food-borne pathogen *E. coli* from spinach leaves.

Noticeably, almost all previous studies exploring biological surface replica have focused on topological or physical reproduction of the surfaces. Much less efforts have been placed on developing artificial plant surfaces with similar chemical and biological properties with natural leaves. To fill in this gap, we conducted a comprehensive investigation to develop a PDMS-based artificial phylloplane surface to resemble the topographical, chemical, and epicuticular characteristics of romaine lettuce and ‘Carmel’ spinach to high fidelity. This method enables us to modify and control produce leaf surface properties such as surface roughness, surface hydrophobicity, and epicuticular composition so that to provide an insight into the role played by leaf surface property on sanitation treatment. The PDMS leaf replica was used as a substrate to evaluate attachment and removal of *E. coli* O157:H7 EDL933 and *Listeria innocua*. In addition, we examined the reusability of these phylloplane surfaces by exposing them to commonly used disinfection practices in laboratory settings.

2. Results And Discussion

2.1 Bactericidal effect of PDMS utilized for the development of artificial phylloplanes.

As seen in Figure 1, the process to develop artificial phylloplanes involved a double casting procedure utilizing PDMS as the base polymer. The PDMS-double-casting technique has been widely utilized in other applications such as replication of “high-aspect-ratio microstructures”, development of components of nanophotonic devices, as well as fabrication of microfluidic devices[29-32]., One of the advantages of using PDMS as a base for double casting process is the low cost, low labor and time involved with the procedure. However, one of the obstacles of utilizing PDMS is the high hydrophobicity of the material (WCA=120°), which is different from most fresh plant phylloplanes[33]. Studies have shown that mixing non-ionic surfactants directly with PDMS can lower the hydrophobicity of PDMS[34-35] and thus in our case improve the wettability of the artificial phylloplanes. Nonetheless, in the food industry a common practice is to utilize surfactant as a component of chemical sanitizers for wash of fresh produce[8, 36-37]. It is therefore necessary to understand if the PDMS surfaces with surface hydrophobicity modified by a

surfactant is toxic to bacteria. As seen in Figure 2, after up to 24 hours of growth in the non-ionic surfactant solutions with HLB of 7 and 11, there is no significant change (or reduction) in *E. coli* O157:H7 population at each sampling time (0, 2, 12, 24 hrs.) for each of the surfactants tested. Thus, the artificial phylloplanes with addition of surfactants do not possess a bactericidal to *E. coli* cells.

2.2 Physiochemical properties of master mold and artificial phylloplanes

A qualitative comparison of the surface microstructures of the fresh produce leaves and the artificial phylloplanes, or PDMS replica is shown by CLSM and SEM micrographs. The CLSM and SEM images of the fresh leaves (A, D), master mold (B, E) and artificial phylloplanes (C, F) are presented side-by-side with the 'Romaine' lettuce (Figure 3) and 'Carmel' spinach (Figure 4 and Figure 6). As seen in Figures 3B, 3E, 4B, and 4E, with the replication method, the created master molds (B, E) exhibit negative impressions of the features of fresh leaves (Figures 3A, 3D, 4A, 4D). By comparing Figures 3D, 3F and Figures 4D, 4F, one can see that the artificial phylloplanes are true replicas of fresh leaves. Features such as guard cells, open stomas, and vertical variations (peaks and valleys) of different sizes and scales were replicated.

To further examine the fidelity of the artificial phylloplanes to the fresh leaves, the surface hydrophobicity and surface roughness of the natural leaves and PDMS replicas were measured. As shown in Table 1, no significant differences ($P > 0.05$) were observed in the hydrophobicity (water contact angle) values between the fresh leaf of 'Romaine' lettuce and the lettuce artificial phylloplane made with 10% surfactant. Similarly, no significant differences ($P > 0.05$) in the hydrophobicity values of the 'Carmel' spinach fresh leaf and the spinach artificial phylloplane made with 10% surfactant (values). Also, we were able to determine that a slightly hydrophobic sample could be achieved by mixing PDMS and surfactants at concentrations <5%. Moreover, as seen in Table 1 no significant differences were observed between the surface roughness (mm) of the fresh 'Romaine' lettuce and its PDMS replica, as well as between the fresh leaf of 'Carmel' spinach and the spinach artificial phylloplane ($P > 0.05$).

2.3 Epicuticular composition of developed artificial phylloplanes

One important component of fresh leaves is their epicuticular wax, which acts as a barrier that prevents loss of water from the surface of the plant and as a barrier from abiotic stresses[38-39]. Epicuticular wax composition varies depending on the species, cultivar, age and environmental factors. On leaf surfaces, the wax usually exists in the form of a mixture of smooth amorphous layer and hierarchical structures (crystals)[33, 40-41].

To faithfully replicate produce leaves, besides mimicking the leave topological properties and regulating the PDMS surface hydrophobicity to match that of natural leaves, the artificial phylloplanes should also represent the leave surface chemical composition, mainly the wax composition. For that purpose, a chemical solution of different long-chain hydrocarbons mixed with chloroform (Table 1) was used to coat the artificial phylloplanes using a spin coating process. The compounds in the chemical solution were chosen to represent the key compounds of the epicuticular wax according to the work of Lu et al.[33]. After

coating, to exam if the PMDS replica surface has been coated with the compounds, a FTIR analysis was performed.

Figure 5 shows the infrared spectra for PDMS without wax coating, and for the artificial phylloplanes of the 'Romaine' lettuce and 'Carmel' spinach coated with wax. Each of the infra-red (IR) active functional groups is highlighted with a band depending on the functional group region. As seen on Figure 5, the alkene bands are at 3090 cm^{-1}, ketone bands are at 1750 cm^{-1}, and PDMS silicone groups at 1020-1074 cm^{-1}. The appearance of the new alkene and ketone bands on the IR spectra of PDMS replica confirms that the wax compounds are deposited on PDMS surfaces. The epicuticular wax in the form of a mixture of small crystals and amorphous layer can also observed on the natural and artificial spinach leaves in the SEM images in Figure 6 E-F.

2.4 Attachment of *E. coli* O157:H7 and *L. innocua* to natural and artificial spinach leaves.

A comparison of the attachment of *E. coli* O157:H7 and *L. innocua* to natural surface and artificial phylloplanes of the 2 produce types is shown in Table 2. No significant difference between the attachment of *E. coli* O157:H7 and *L. innocua* to surfaces of the natural and the hydrophobic artificial phylloplanes, respectively, was found. This finding suggests that bacterial cells may have a similar interaction with the PDMS leaf replica developed in this study and that of a natural biological leaf surface, at least regarding attachment of the bacterial cells to two kinds of surfaces.

The surface hydrophobicity is shown to affect bacterial attachment. Between the 2 artificial produce surfaces, significantly more ($P < 0.05$) cells were attached to hydrophilic (WCA = 70) surfaces than on the hydrophobic surfaces (WCA = 110). Similarly, and between the natural leaf surface and the hydrophobic artificial surfaces, significantly more ($P < 0.05$) cells were found on the fresh produce surfaces (WCA = 74 for spinach and WCA =71 for lettuce) than on the hydrophobic PDMS replica (WCA = 110) of them. These findings are in agreement with Crick et al.[42] who evaluated the effect of hydrophobicity of various surfaces on the attachment of *E. coli* and *S. aureus* and found that the hydrophobicity of PDMS reduced the attachment of both types of bacteria compared to hydrophilic surfaces such as glass. The attachment of *E. coli* O157:H7 onto fresh and artificial spinach leaves is shown in Figures 6A and 6D. Some attached *E. coli* cells can be identified in Figures 6A and 6D. No significant differences in the attachment patterns between fresh and artificial phylloplanes can be found.

2.5 Reusability of artificial phylloplanes

In order to prove that the artificial phylloplanes of lettuce and spinach will work as an effective low-cost platform to study factors that promote bacterial attachment, we evaluated the reusability of artificial phylloplanes after exposing them to two rounds of disinfection with ethanol and two rounds of heat sterilization using an autoclave.

As seen in Figure 7, no significant changes in surface hydrophobicity (water contact angle) were observed when the hydrophilic and hydrophobic spinach artificial phylloplanes were disinfected with 70% (v/v)

ethanol or disinfected by two rounds of sterilization at 121°C for 30 min ($P > 0.05$). Although changes in surface hydrophobicity of up to 7° ± 2° were observed for lettuce artificial phylloplane disinfected with 70% ethanol and sterilization at 121°C for 30 min ($P < 0.05$), these changes did not cause the sample to become hydrophobic. Lastly, no significant differences in surface hydrophobicity were observed when lettuce hydrophobic artificial phylloplanes were disinfected with ethanol or sterilization ($P > 0.05$)

Furthermore, as seen in Figure 8, the FTIR spectra shows that after exposing the artificial phylloplanes of lettuce and spinach to two rounds of sterilization at 121°C for 30 min, no changes in epicuticular wax composition were observed. The signal of IR-active functional groups from the surfaces compared was still identifiable. Thus, the artificial phylloplanes can be used for at least three times for experiments of bacterial attachment.

3. Conclusions

In this study, a double-casting method to fabricate artificial phylloplanes that mimic with high fidelity the physical, chemical, and biological characteristics of fresh leaves of lettuce and spinach was developed with a soft polymer (PDMS). The surface hydrophobicity of the PDMS fresh produce leaf replica was manipulated with addition of non-ionic surfactant with different HLB values to match the hydrophobicity of produce leaves. A method was developed to coat the PDMS leaf replica with the main epicuticular wax compounds extracted and identified from the natural spinach and lettuce leaves to replicate the chemical composition of the natural leaf surfaces. Similarities in bacterial attachment patterns between the fresh produce leaves and artificial phylloplanes were observed. The PDMS leaf replicas are reusable, economical, and recyclable. The artificial produce leaf phylloplanes can be used as platform to investigate the interactions between bacteria and produce phylloplanes, and to develop new or enhanced fresh produce decontamination strategies.

4. Materials And Methods

4.1 Greenhouse production of leafy vegetables

“Romaine” lettuce (*Lactuca sativa* L.) and “Carmel” spinach (*Spinacia oleracea* L.) were used in this study. They were grown in a greenhouse as previously described[37]. Briefly, lettuce and spinach cultivar seeds purchased from Johnny’s Selected Seeds (Winslow, ME) were germinated in 32-cell plant plug trays filled with Sunshine LC1 (Sun Gro Horticulture, Vancouver, British Columbia, Canada) professional soil mix. Seedlings were grown in a greenhouse at University of Illinois under a 25°C/17°C and 14 h/10 h day/night temperature regimen with supplemental lighting. Twenty days post-germination, the seedlings were transferred to 4-liter pots. Leaf tissues from the “Carmel” spinach plants were harvested 40-45 days after sowing seeds and that from the “Romaine” lettuce plants were harvested 50-65 days after sowing seeds. For this study, leaves were harvested at market maturity. Since commercial crop seeds were purchased from a seed company, it is not applicable to the IUCN policy state as it does not involve any risk of extinction.

4.2 Bacterial strain preparation

Escherichia coli O157:H7 and *Listeria innocua* obtained from the food microbiology culture collection at the University of Illinois at Urbana-Champaign were used in this experiment. Bacterial inoculums were prepared by repeated sub-culturing on Tryptic Soy Agar (TSA) plates for *L. innocua* and TSA plates containing 50 mg/L of nalidixic acid (Sigma Aldrich, St. Louis, MO) for *E. coli*. Cultures of *L. innocua* were grown in Tryptic Soy Broth (TSB) and that of *E. coli* in TSB with 50 mg/L of nalidixic acid (Sigma Aldrich, St. Louis, MO) for 22 hours at 37 °C. Cells were harvested by centrifugation at 4 °C and 2,455 *g* for 10 min. and washed twice in sterile 0.1% peptone water. The recovered bacterial precipitates were diluted in 10 mL of 0.1% peptone water; the initial inoculation level was 2.1 x 10^8 *CFU/ml* for *E. coli* O157:H7 and $2.5 \text{ x } 10^8$ *CFU/ml* for *L. innocua.*

4.3 Development of a reproducible artificial phylloplane

4.3.1 Preparation of polydimethylsiloxane (PDMS) elastomer

Polydimethylsiloxane (PDMS) Sylgard® 184 Silicone Elastomer Kit (Dow Corning Corporation, MI, USA) was prepared according to manufacturer instructions. Briefly, a mixture of base/curing agent at ratio of 10:1 was prepared and thoroughly mixed for 5 min. The base/curing agent mixture was degassed under low vacuum until no oxygen bubbles were visibly present in the mixture. In addition, a PDMS-surfactant solution was prepared by mixing degassed PDMS and 1 to 10% (v/v) Caprol-PGE860® surfactant (Abitec, OH, USA) and thoroughly mixed for 5 minutes. The PDMS-surfactant solution was degassed under low vacuum until no oxygen bubbles were visibly present in the mixture.

4.3.2 Master mold for reproducing artificial phylloplanes using a double casting method

A negative impression (master mold) was developed by collecting leaf samples of lettuce and spinach grown in a greenhouse until commercial maturity. Leaf samples were rinsed with distilled water to remove soil and debris from the surface. The leaf samples were taped (3M, Minnesota, USA) to the bottom of a 4-inch glass Petri Dish (Fisher scientific, NH, USA), and degassed PDMS was poured on top of the taped leaf until the petri dish was full to 75% capacity. Solidification took place by placing the sample for 8 hours under refrigeration (4 °C ± 1 °C), followed by 22 hours solidification under controlled temperature (25 °C ± 1 °C) and relative humidity (70% ± 3%). The obtained inverted PDMS master molds were treated for 10 min with a solution of 1% hydroxypollymethylcellulose (HPMC) (Sigma Aldrich, MO, USA) diluted in 1M phosphate-buffered saline (pH 3.0) and stored at 25 °C ± 1°C.

4.3.3 Reproducible artificial phylloplanes with topography and hydrophobicity similar to “Romaine” lettuce and “Carmel” spinach

The PDMS-surfactant solution was poured onto HPMC-treated PDMS master molds, and the molds were cured in a hot plate for 5 min at 100 °C ± 5 °C. Hardened PDMS surface was removed from the PDMS master mold and stored at 25 °C until further use or was spin-coated with 200 mL of epicuticular

chemical solution containing the main epicuticular wax compounds of “Carmel” spinach leaves and “Romaine” lettuce. The spin-coated samples were placed in a glass petri dish and cured under low vacuum for 15 min and stored in airtight sampling bags until further use.

4.3.4 Evaluation of the bactericidal effect of surfactants on *E. coli* O157:H7

E. coli O157:H7 87-23 was utilized to evaluate if the addition of the surfactant onto the PDMS solution would cause toxicity to microorganisms. Two different surfactants (Caprol-3GO and Caprol PGE-860) were selected and prepared at two different concentrations. They are both “non-ionic, non-alkoxylated emulsifier” with hydrophile-lipophile balance (HLB) values of 7 and 11, respectively (Abitec Corp., 2014). Each surfactant was diluted in 0.1% peptone water to a final concentration of 7% or 10% (v/v) and stirred for 10 min in a biological cabinet until dissolved. A tenfold dilution of *E. coli* and surfactant solution was prepared in glass test tubes and placed in an incubator equipped with a shaker. The samples were shaken at 120 RPM and a survival growth curve was determined by sampling the solution at 0, 2, 12, 24 hours. The *E. coli* cells surviving on surfactant solutions were enumerated via spread plating with Tryptic Soy Agar (TSA) plates containing 50 mg/L of nalidixic acid and incubated for 24 hours at 37°C.

4.4 Preparation of epicuticular chemical solution.

Based on the information presented by Lu et al. (2015), a wax solution containing the key epicuticular wax compounds of “Carmel” spinach leaves were prepared by mixing chloroform with 22% (w/v) of the alkane octadecenol, 54% (w/v) of the fatty alcohol 1-hexacosanol, and 24% (w/v) of the fatty acid myristic acid. The mixture was placed in airtight containers and stirred for 1 hour in a water bath (70 °C ± 2°C). Similarly, a wax solution containing the key epicuticular wax compounds of “Romaine” lettuce leaves was prepared by mixing chloroform with 41% (w/v) of the alkane heneicosane, 20% (w/v) of the fatty alcohol 1-hexacosanol, and 39% (w/v) of the fatty acid myristic acid. The mixture was placed in airtight containers and stirred for 1 hour in a water bath (70 °C ± 2°C). To prevent evaporation and precipitation of epicuticular wax, the wax solutions were kept in airtight containers and placed in a darkroom until further use.

4.4.1 Confirmation of deposition of epicuticular chemical solution on surfaces

Using a Pasteur pipette, approximately 200 mL of epicuticular chemical solution was placed in direct contact with attenuated total reflectance (ATR) crystal on a multibounce plate at controlled ambient temperature (25 °C). An FTIR spectrometer (Thermo Nicolet Nexus 670) connected to the software SPECTRUM ® was used during FTIR data collection. FTIR spectra were recorded from 8 scans at a resolution of 4 cm^{-1} at 4000–400 cm^{-1}. These spectra were subtracted against background air spectrum. After every scan, a new reference air background spectrum was taken. The ATR plate was carefully cleaned in situ by cleaning the sample holder with ethanol twice and dried with soft tissue paper before placing the next sample. Cleanliness was verified by collecting a background spectrum and compare to the previous one. These spectra were recorded as absorbance values at each data point in triplicate.

4.5 Comparison of physical, chemical and biological characteristics of the artificial phylloplanes vs leafy greens surfaces

4.5.1 Determination of surface hydrophobicity

Surface hydrophobicity was measured as previously described by Lu et al., 2015. Briefly, nine disks (approximate area of each disk 2.01 cm^2) from each vegetable cultivar were excised and used for determination of surface hydrophobicity. The disks were rinsed with distilled water to remove soil and debris and dried using Kimwipes® (Kimberly-Clark, Irving TX. USA) with gentle patting motions. The dried disks were then taped (3M, Minnesota, USA) to a microscope glass slide exposing the adaxial surface of leaves. The glass slides containing the leaves disks were covered with moistened paper towel and aluminum foil to prevent dehydration. Similarly, nine disks (approximate area of each disk 2.01 cm^2) from each artificial leaves with and without epicuticular chemical solution were excised and taped (3M, Minnesota, USA) to a microscope glass slide exposing the adaxial surface of leaves and covered with aluminum foil to prevent contamination with debris and dust particles. Water contact angle of all surfaces was obtained using a goniometer (KSV Instruments, Stockholm, Sweden) model CAM 200. Using a calibrated pipette, 5 mL of deionized water was placed at the center of each disk and within 20 seconds five contact angle readings were measured.

4.5.2 Determination of surface roughness

Produce leaf surface samples and artificial leaves were prepared following the same procedure used for contact angle measurement and surface roughness was measured as previously described by Lu et al. [33]. A confocal microscope (NanoFocus, µSurf explorer) was used to determine 3-dimensional surface parameters. Area-average root mean square roughness (-S_q bar) was obtained from the average of a number of linear root mean square roughness S_q measured from the 3-D image reconstructed from 2-D laser confocal images over an area of . Image analysis was done using software Mountains (Digital Surf, France)

4.5.3 Attachment of *Escherichia coli* O157:H7 and *Listeria innocua* to artificial phylloplanes vs leafy greens surfaces

Prior to inoculation, romaine lettuce and spinach leaves were cleaned by rinse step with sterile Milli-Q water to remove debris and patted dried using Kim wipes® (Kimberly-Clark, TX). Each artificial phylloplane was sterilized using 10 min of UV light. A diluted bacteria solution was prepared by diluting 1 mL of *E. coli* O157:H7 and *L. innocua* inoculum in 9 mL of 1*X* PBS buffer (Initial Inoculum *E. coli* = 7.0 Log_{10} PFU/ml and *L. innocua* = 8.4 Log_{10} PFU/mL). Using sterile tweezers each piece was transferred to an empty sterile petri dish and 100 µL of each bacteria solution in PBS buffer was spot inoculated at 10 different spots on adaxial surface. The petri dish was loosely capped and incubated for 2 hours at 25 °C ± 1 °C in a biological cabinet. After the incubation, the samples were transferred to a sterile container with 1*X* PBS buffer at ratios of 1:10 (surface: buffer solution) and agitated for 1 min to remove loosely

attached bacteria. Afterwards, each sample was transferred to a sterile sampling bag containing 1*X* PBS buffer and pummeled for 1-minute to remove all bacteria attached to the surface. The remaining supernatant was collected, spread in selective media incubated for 24 hours at 37°C.

4.6 Reusability of the artificial phylloplanes

To determine PDMS-based artificial phylloplane surfaces endurance to commonly used disinfection practices, they were exposed to two different disinfection procedures. Changes in surface hydrophobicity and epicuticular composition were evaluated. The PDMS-based surface samples were immersed twice in 70% ethanol (v/v) for 36 hours, air dried inside a safety cabinet for 2 hours and stored at 25 °C for 24 hours prior analysis. In addition, PDMS-based surface samples were placed inside an autoclave at 121°C for 30 min and were stored at 25 °C for 24 hours prior analysis. Surface hydrophobicity was determined following the previously described procedure, while epicuticular composition was determined following procedure in 2.4.1.

4.7 Scanning electron microscopy

Surface characterization was carried using a scanning electron microscope. Microimages of the epicuticular surfaces were taken using a FEI Quanta FEG 450 ESEM (Hillsboro, OR, USA). The images were captured under low vacuum at 20 kV and at 400´, 800´, and 1200´ resolution from at least three different samples.

4.8 Statistical analysis.

The experiments were performed with a complete randomized design (CRD) with each treatment conducted three times. Bacterial counts were subjected to log transformation before statistical analysis. Data were analyzed using a general linear model available in SAS version 9.1 (SAS Institute, Raleigh, NC, USA), and with Origin-Pro 2016 (OriginLab Corporation, MA, USA). Mean separation was determined using Tukey's test with α= 0.05. Relationships were considered significant when the *P* value was < 0.05.

Declarations

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Tables

Table 1. Physical and chemical properties of fresh leaves, negative impression, and artificial leaves.

Samples	Contact angle (Degrees) Mean ± STD DEV	Surface roughness (mm) Mean ± STD DEV	Epicuticular wax composition (mg/cm^2)			
			Fatty alcohol (mg / cm^2)	Alkane (mg / cm^2)	Fatty acid (mg / cm^2)	Total wax content (mg / cm^2)
'Carmel' spinach						
Fresh sample	74.0 ± 6.0 (c)	8.0 ± 1.2 (b)	NA§	NA	NA	10.0 ± 0.7[1]
PDMS mold	87.0 ± 3.0 (b)	18.0 ± 6.0 (a)	NA	NA	NA	NA
Artificial surface made with 1% surfactant	110.0 ± 2.0 (a)	11.0 ± 3.0 (b)	2.1	4.2	4.2	10.4 ± 0.4
Artificial surface made with 10% surfactant	68.0 ± 2.0 (c)	12.0 ± 3.1 (b)	2.1	4.2	4.2	10.4 ± 0.4
'Outredgeous' romaine lettuce						
Fresh sample	71.0 ± 7.0 (c)	9.0 ± 3.0 (a)	NA	NA	NA	19.9 ± 8.2[1]
PDMS mold	92.0 ± 2.0 (b)	13.0 ± 5.0 (a)	NA	NA	NA	NA
Artificial surface made with 1% surfactant	109.0 ± 2.0 (a)	8.0 ± 1.0 (a)	10.7	4.4	4.8	19.9 ± 0.9
Artificial surface made with 10% surfactant	76.0 ± 6.0 (c)	8.0 ± 1.1 (a)	10.7	4.4	4.8	19.9 ± 0.9

[1] Reference value taken from Lu and Ku et al 2015

§ No wax added

a-c: sample means for contact angle within each cultivar tested (column #2) with different letters are significantly different (a=0.05)

a-b: sample means for surface roughness within each cultivar tested (column #3) with different letters are significantly different (a=0.05)

Table 2 Attachment of *Escherichia coli* O157:H7 and *Listeria innocua* to artificial phylloplanes.

Cultivar	condition tested	Contact angle (Degrees)	*Escherichia coli* O157:H7 EDL 933 Mean ± Std Dev	*Listeria innocua* Mean ± Std Dev
'Carmel' spinach	Fresh sample	74	5.0 ± 0.2 [(a)]	5.9 ± 0.1 [(b)]
	Artificial surface, 10% surfactant	70	4.7 ± 0.7 [(a)]	6.8 ± 0.2 [(a)]
	Artificial surface, 1% surfactant	110	4.5 ± 0.4 [(a)]	5.7 ± 0.2 [(b)]
'Outredgeous' Romaine lettuce	Fresh sample	71	5.2 ± 0.06 [(a)]	5.7 ± 0.1[(b)]
	Artificial surface, 10% surfactant	70	5.0 ± 0.06 [(b)]	6.3 ± 0.3[(a)]
	Artificial surface, 1% surfactant	110	4.3 ± 0.1 [(c)]	4.7 ± 0.3 [(c)]

a-c: sample means for each bacteria strain within each cultivar tested (column) with different letters are significantly different (a=0.05)

Figures

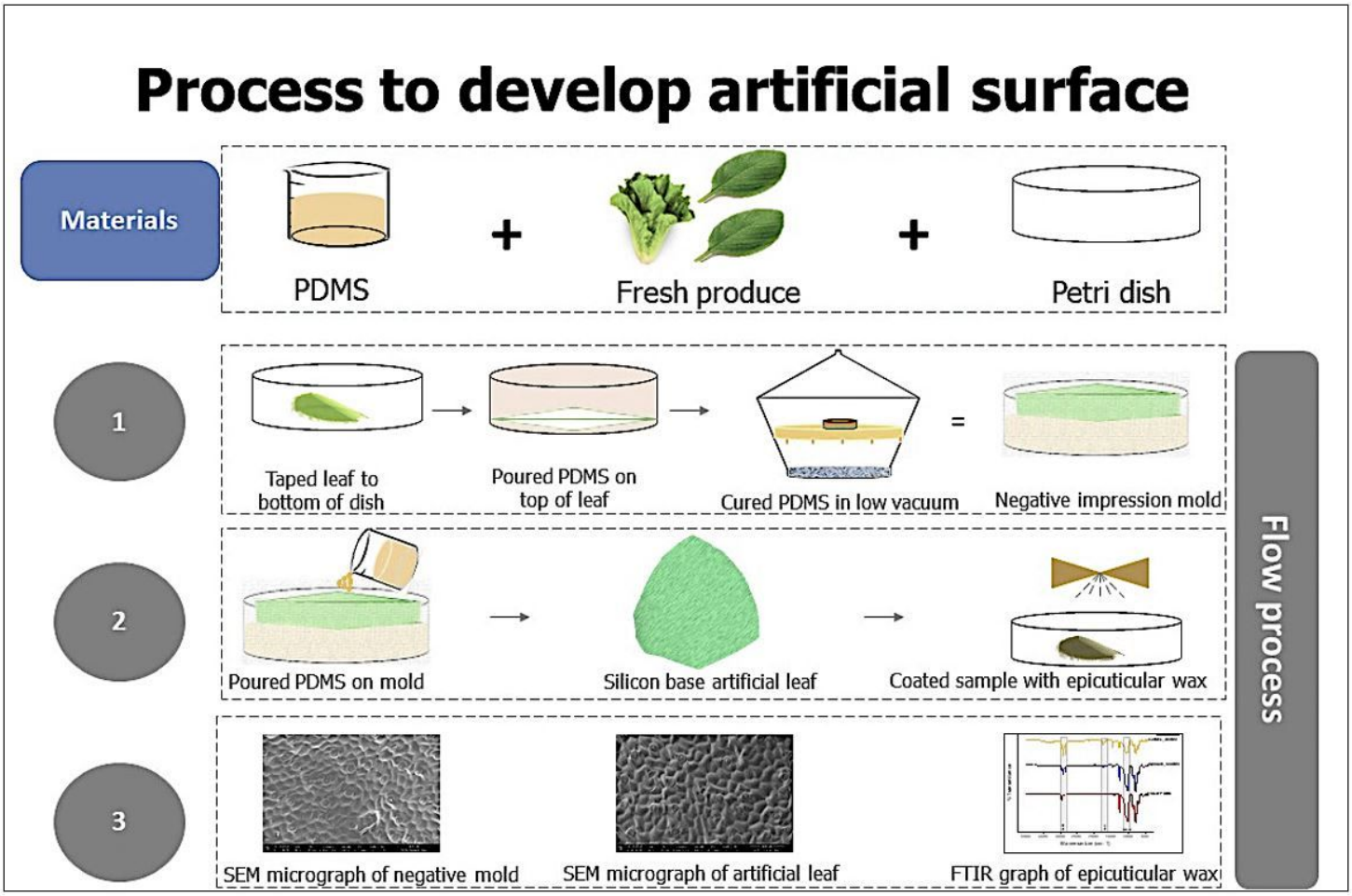

Figure 1

Flow process for the development of artificial surface.

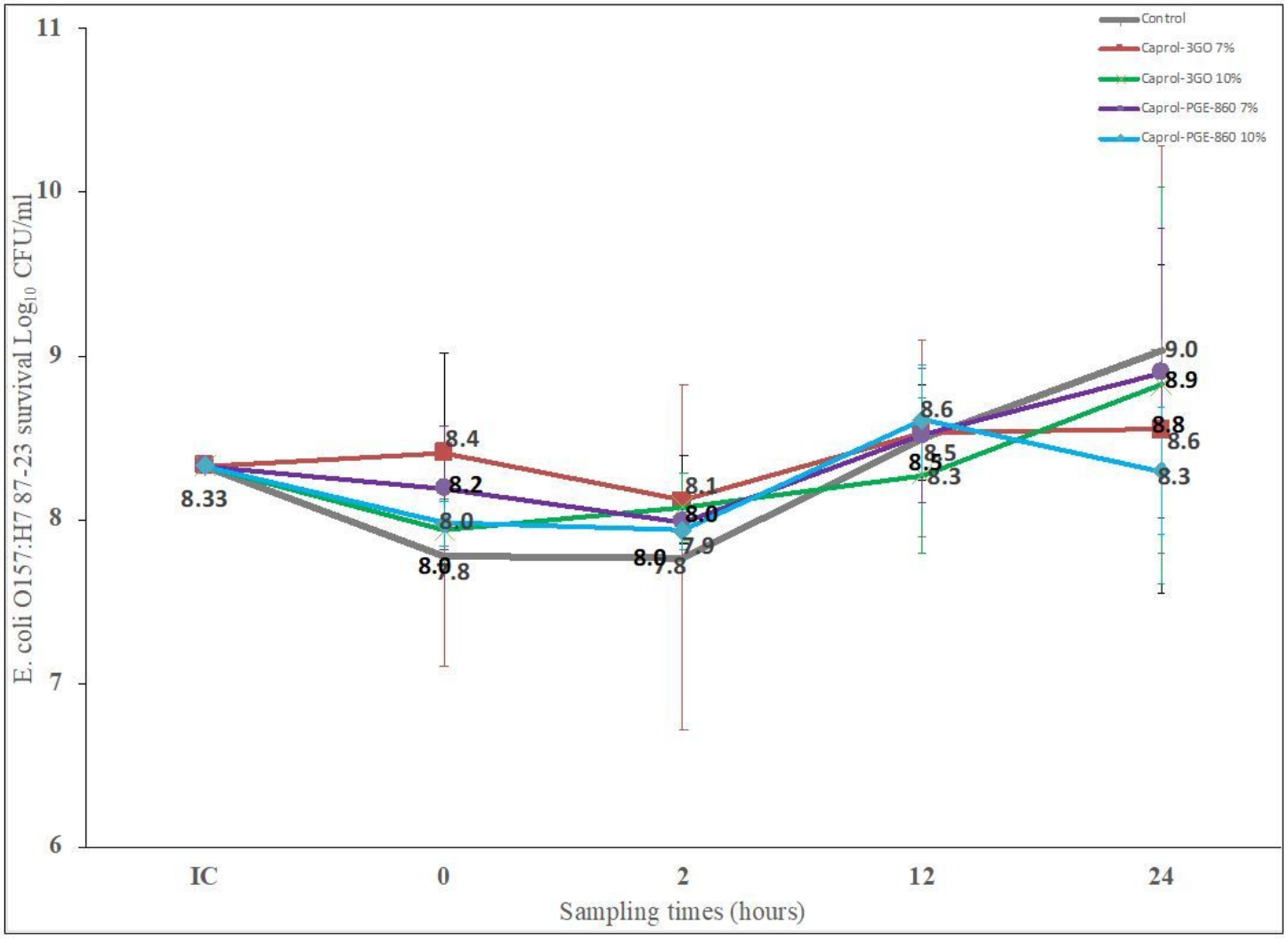

Figure 2

Growth rate of Escherichia coli O157:H7 in the presence of surfactant solutions at different incubation times. There were no significant differences in the survival counts of E. coli O157:H7 grown in different surfactant solutions, no significant differences within the different percentages (%) used, and no significant differences at the different sampling times by Tukey's test (□=0.05).

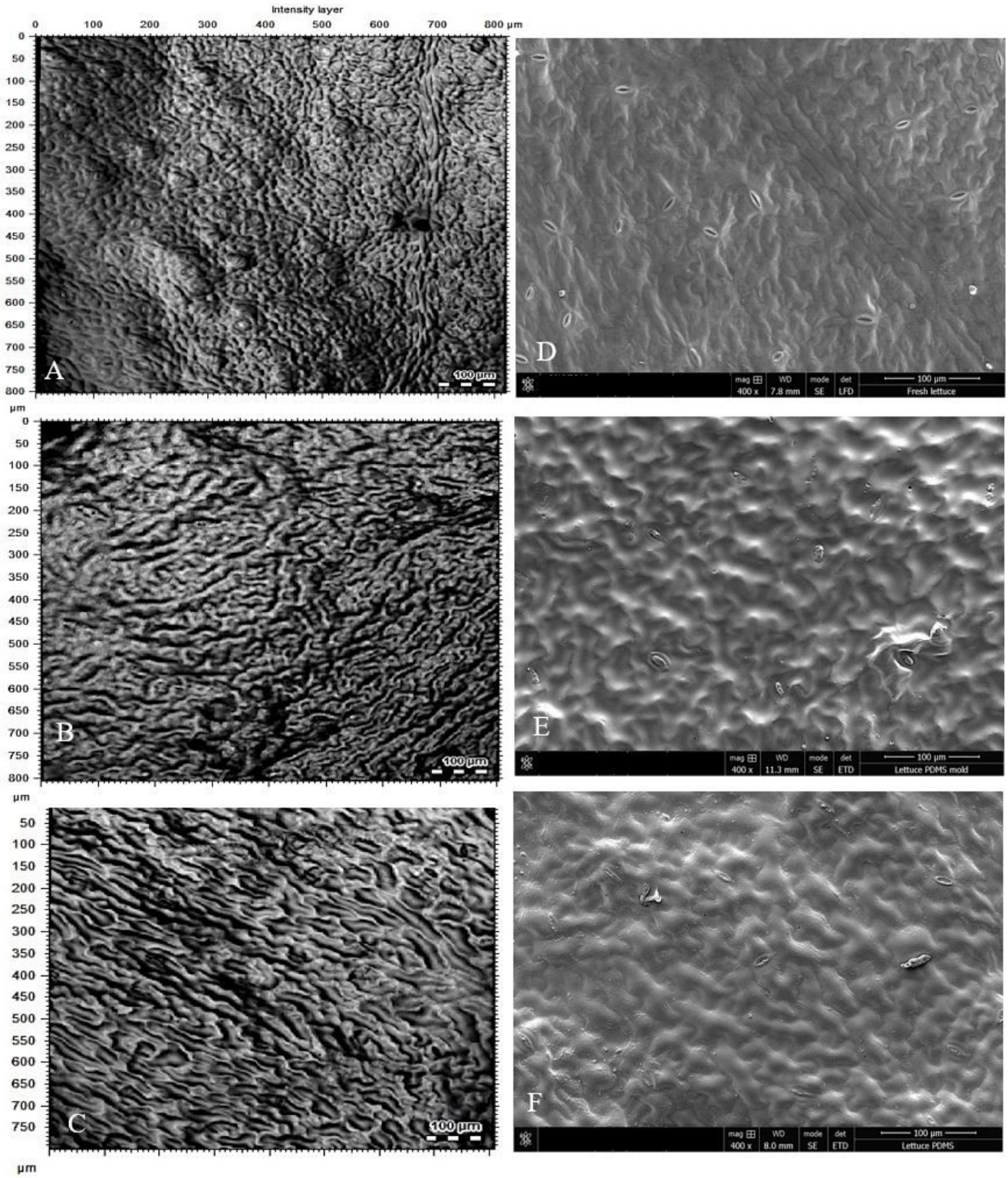

Figure 3

Selected lettuce micrographs using Confocal Microscope (CM) and Scanning Electron Microscope (SEM). A) CM plant leaf surface, B) CM PDMS mold C) CM artificial surface, D) SEM plant leaf surfaces, E) SEM PDMS mold, and F) SEM artificial surface.

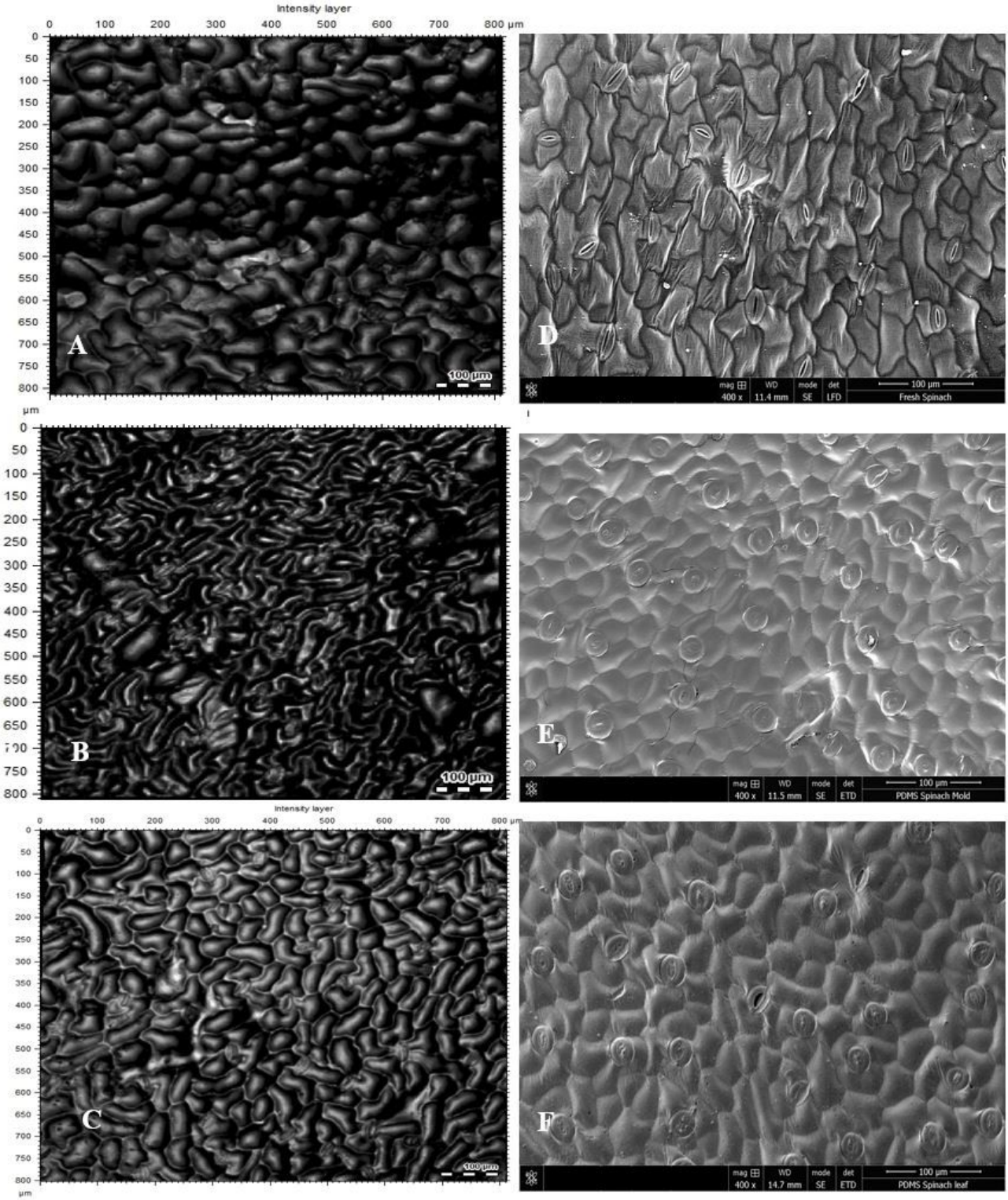

Figure 4

Selected Spinach micrographs using Confocal Microscope (CM) and Scanning Electron Microscope (SEM). A) CM plant leaf surface, B) CM PDMS mold C) CM artificial surface, D) SEM plant leaf surface, E) SEM PDMS mold, and F) SEM artificial surface.

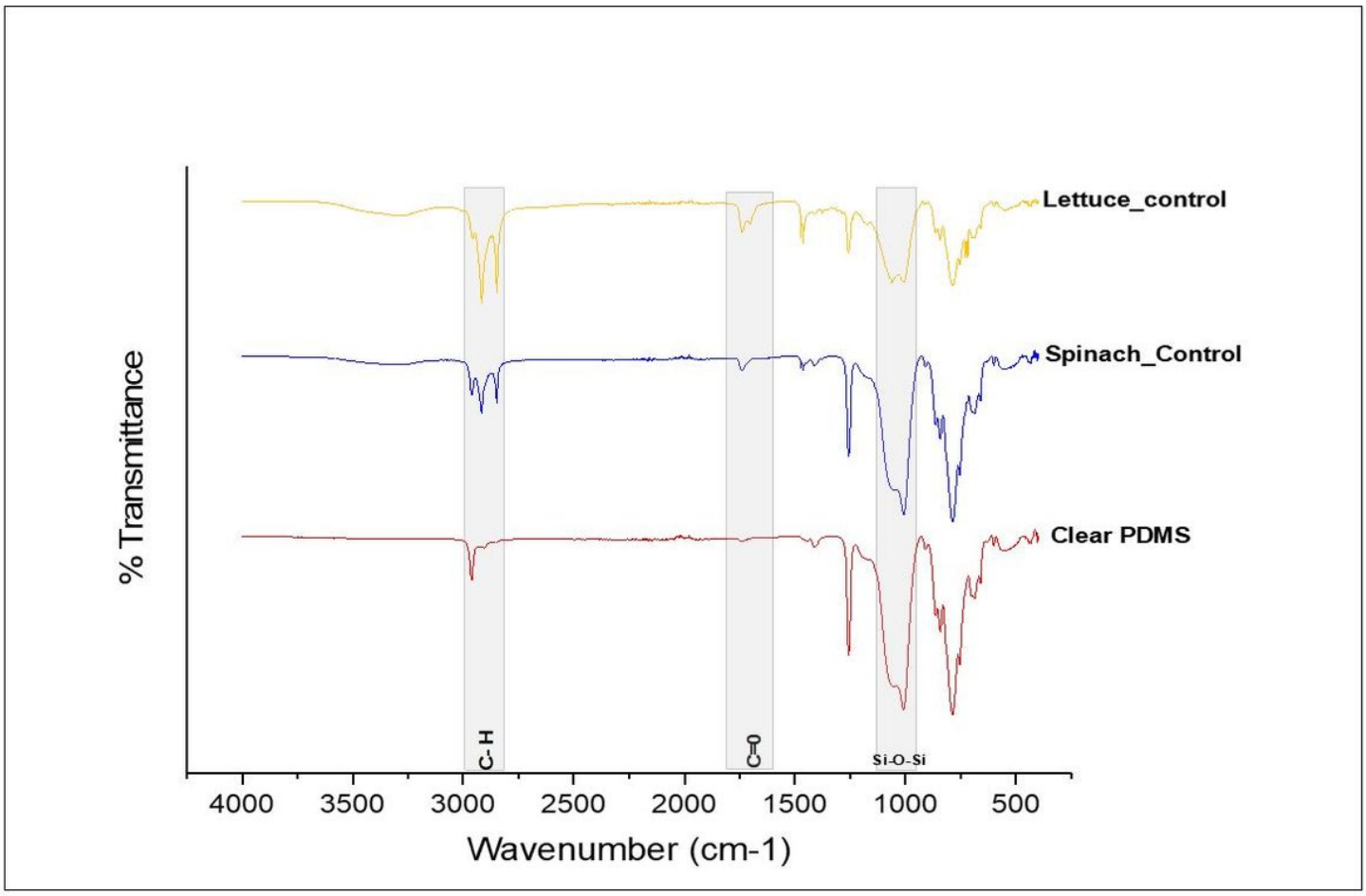

Figure 5

Confirmation of deposition of epicuticular wax on artificial phylloplane using FTIR *Highlighted zones indicate the presence of IR-active functional groups. The alkene band is at 3090 cm-1, the ketones band is at 1750 cm-1, PDMS silicone groups at 1020-1074 cm-1.

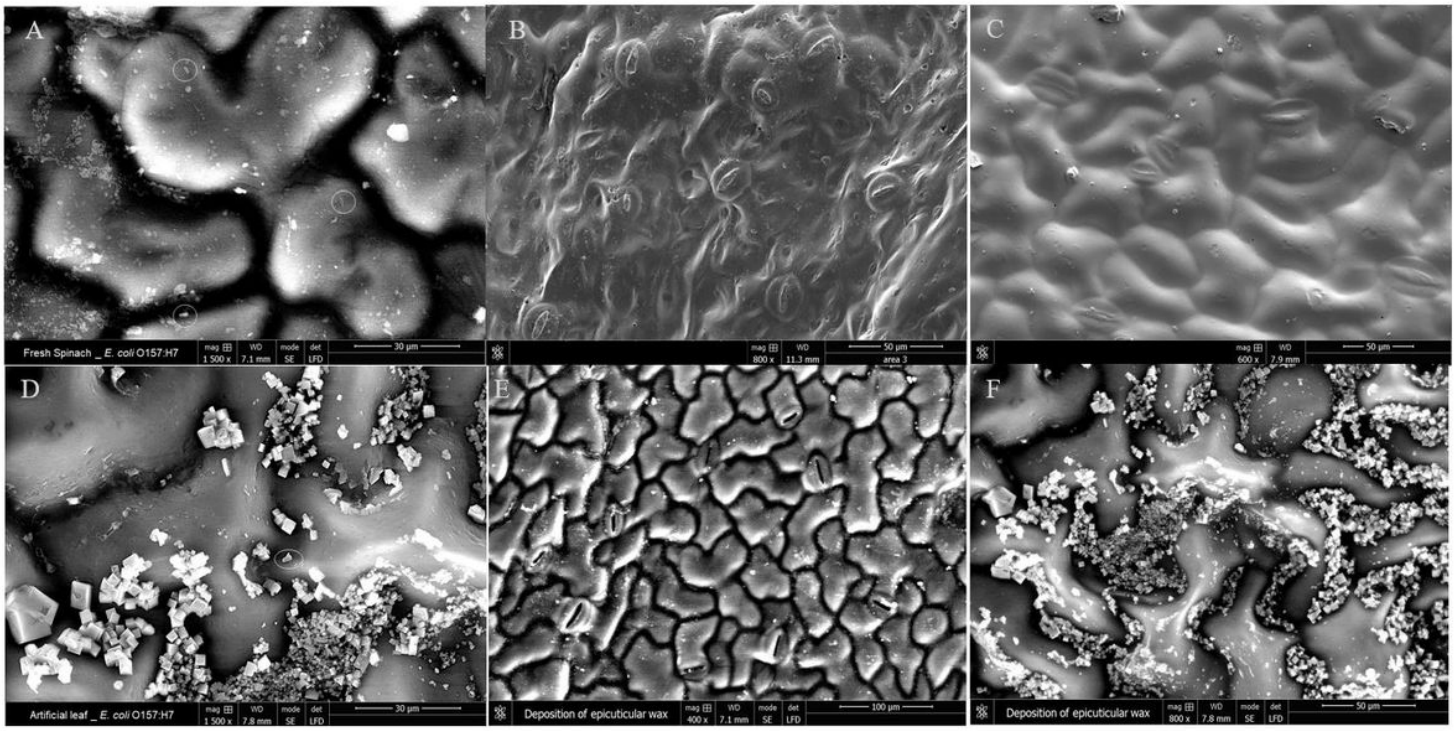

Figure 6

SEM micrographs for confirmation of bacterial attachment to produce surface and deposition of epicuticular wax on artificial phylloplane. (A) Fresh spinach leaf inoculated with E. coli O157:H7, (B) Negative impression mold, (C) Artificial leaf without wax, (D) Artificial leaf with wax inoculated with E. coli O157:H7, and (E) Artificial leaf with wax 400×, and (F) Artificial leaf with wax 800×. Presence of bacteria is highlighted with circle.

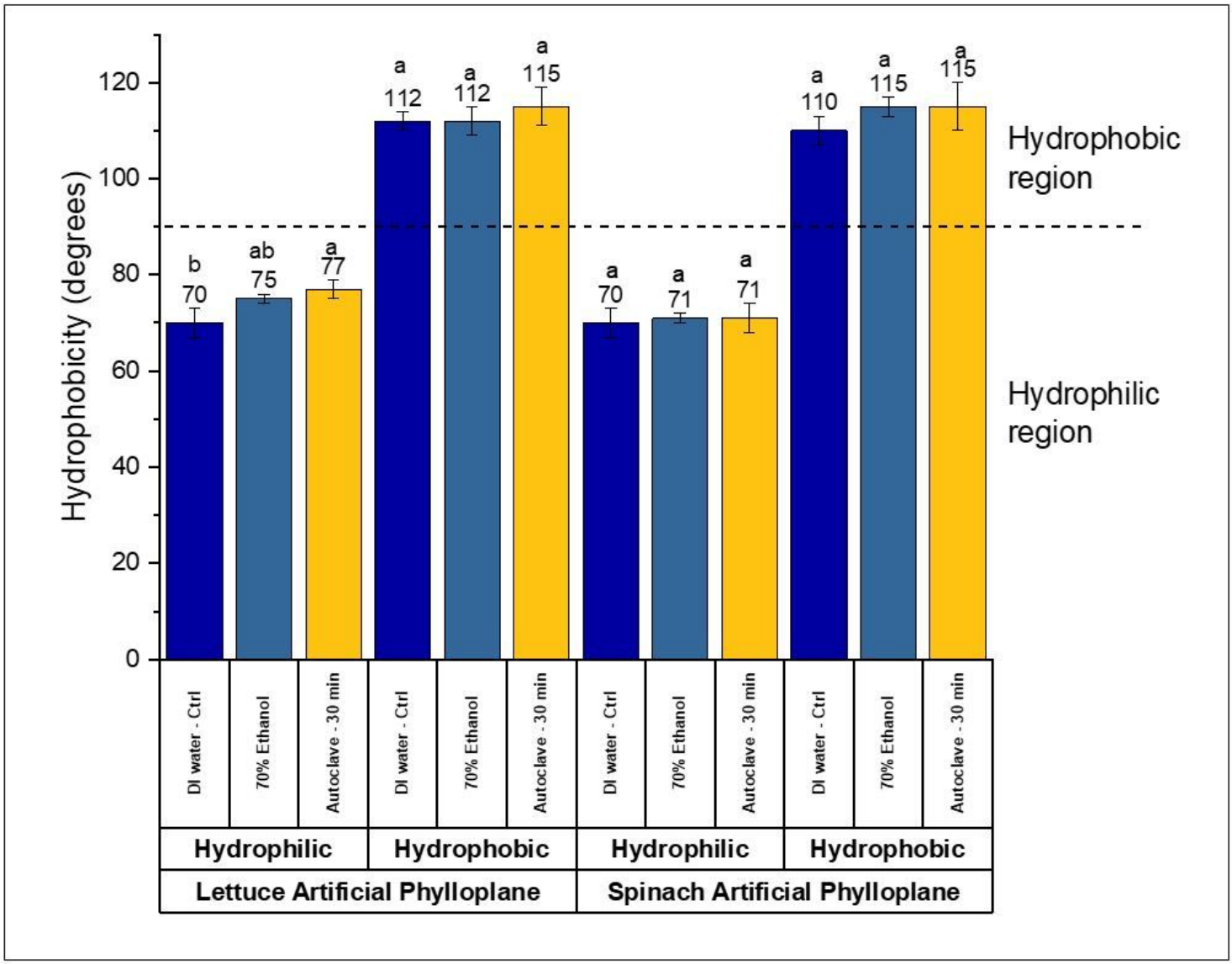

Figure 7

Changes in physical properties of artificial phylloplane after disinfection procedure a-b: Means (columns) with different within each condition tested (control, ethanol, autoclave) with different letter are significant different (= 0.05)

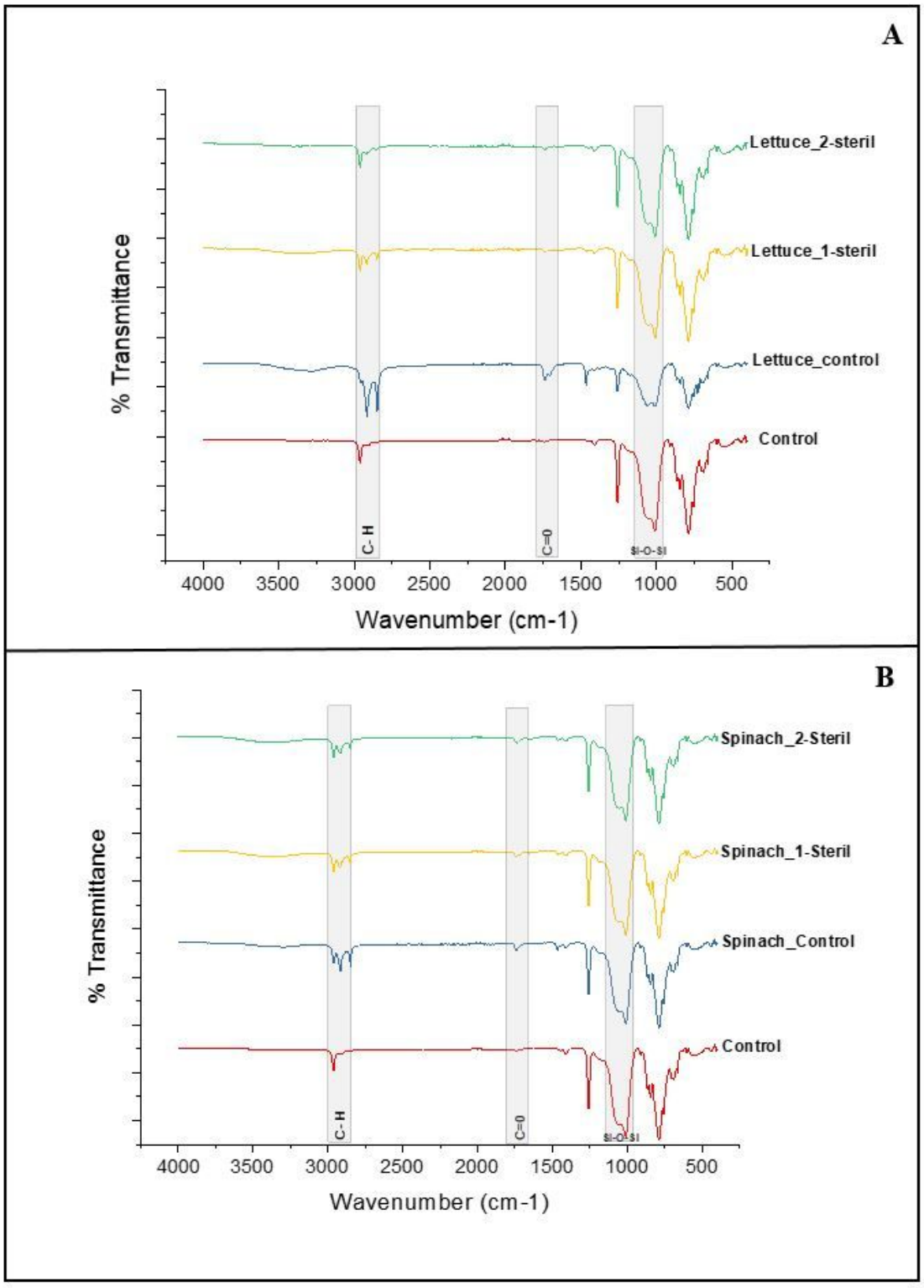

Figure 8

FTIR micrographs confirming the presence of epicuticular wax after sterilization with autoclave. (A) Lettuce (B) Spinach. *Highlighted zones indicate the presence of IR-active functional groups. The alkene band is at 3090 cm-1, The ketones band is at 1750 cm-1, PDMS silicone groups at 1020-1074 cm-1

Supplementary Files

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