Food and Bioproducts Processing

A Comparison of Vegetable Leaves and Replicated Biomimetic Surfaces on the Binding of Escherichia coli and Listeria monocytogenes

--Manuscript Draft--

Manuscript Number:	
Article Type:	VSI: FCFP2022
Keywords:	Biomimetic surfaces; leaves; food industry; biofouling; Escherichia coli; Listeria monocytogenes.
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Abstract:	Biofouling in the food industry is a huge issue, and one way to reduce the amount of cleaning is to design naturally cleaning surfaces based on biomimetic designs. Four self-cleaning leaves (Tenderheart cabbage, Cauliflower, White cabbage and Leek) were analysed for their surface properties and artificial replicates were produced. The leaves and surfaces were subjected to attachment, adhesion and retention assays using Escherichia coli and Listeria monocytogenes . For the attachment assays, the lowest cell numbers occurred on the least hydrophobic, smooth surfaces. Following the adhesion assays, use of surfaces with an intermediate S q and demonstrated the lowest bacterial adhesion. However, following the retention assays, the chemistry of the surface may have affected the results since opposite surface effects were demonstrated to reduce cell retention on the leaf which was least hydrophobic and on the biomimetic replicate surfaces which were rougher and hydrophobic. Although the surfaces were promising in reducing bacterial binding, the results suggest that different experimental assays exerted different influences on the conclusions. This work demonstrates that, in addition to surface attributes such as hydrophobicity and roughness, biological factors, environment, and the type of methodologies used need to be taken into consideration.
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Highlights

- Four self-cleaning leaves were analysed, and replicates were produced.
- Lowest cell numbers were attached to the least hydrophobic, smooth surfaces.
- Cells adhered to surfaces with intermediate S_q and ΔG_{iwi} surface properties.
- The surfaces were promising in reducing bacterial binding.
- Different experimental assays exerted different influences on the conclusions.

A Comparison of Vegetable Leaves and Replicated Biomimetic Surfaces on the Binding of *Escherichia coli* and *Listeria monocytogenes*

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Abstract

Biofouling in the food industry is a huge issue, and one way to reduce the amount of cleaning is to design naturally cleaning surfaces based on biomimetic designs. Four self-cleaning leaves (Tenderheart cabbage, Cauliflower, White cabbage and Leek) were analysed for their surface properties and artificial replicates were produced. The leaves and surfaces were subjected to attachment, adhesion and retention assays using *Escherichia coli* and *Listeria monocytogenes*. For the attachment assays, the lowest cell numbers occurred on the least hydrophobic, smooth surfaces. Following the adhesion assays, use of surfaces with an intermediate S_q and ΔG_{iwi} demonstrated the lowest bacterial adhesion. However, following the retention assays, the chemistry of the surface may have affected the results since opposite surface effects were demonstrated to reduce cell retention on the leaf which was least hydrophobic and on the biomimetic replicate surfaces which were rougher and hydrophobic. Although the surfaces were promising in reducing bacterial binding, the results suggest that different experimental assays exerted different influences on the conclusions. This work demonstrates that, in addition to surface attributes such as hydrophobicity and roughness, biological factors, environment, and the type of methodologies used need to be taken into consideration.

Keywords: Biomimetic surfaces; leaves; food industry; biofouling; *Escherichia coli*; *Listeria monocytogenes*.

1. Introduction

Biofilms formed by foodborne pathogens that occur in and on food industry equipment are a major problem since they are a frequent source of product contamination, resulting in economic losses for processors and posing serious health concerns for consumers (Chmielewski and Frank 2003). Safer food production may entail high cleaning costs and severe environmental impacts (such as water and energy consumption, wastewater production, and increasing bacterial resistance to antimicrobial agents) to reduce contamination (Moreira et al. 2016). Therefore, the development of new antifouling strategies focused on preventing bacterial colonization and biofilm formation instead of their elimination is very promising for the industrial sector.

Surface modification to prevent contamination is a key topic of research and several different approaches have been developed (Rajab et al. 2017, Vorobii et al. 2022, Silva et al. 2021, Matinha-Cardoso et al. 2021). One solution has been in the development of biomimetic, superhydrophobic surfaces and these have shown great potential applications in many fields (Hu et al. 2018). One of the most well-known biomimetic surfaces that has been replicated using a number of different engineering approaches is the superhydrophobic lotus-like surface, which presents self-cleaning abilities due to its particular wetting regime (Moerman and Frank 2014). Although most leaves appear smooth to the naked eye, under a microscope, from a microbiological perspective, their surfaces contain a huge number of macro- (> 5 μm), micro- (≤ 5 μm - 0.5 μm), and nano-scale (≤ 0.5 μm) papillae and structures that are coated in a hydrophobic wax. This hierarchical structure in which the macro- and micro-scale surface features have nano-scale roughness contributes to the hydrophobic properties of the surface, reducing the area on which water, debris and microorganisms can attach (Moerman and Frank 2014). A wide range of engineering approaches have been used to try to replicate such surfaces and these include some more complicated methods such as using a soft lithography technique

on stainless steel plates to reproduce the surface properties of leaves from *Colocasia esculenta*, *Crocosmia aurea* and *Salvinia molesta* (Arango-Santander et al. 2021), and using nanosecond laser technology on the surface of titanium alloy, functionalized with organic polysilazane to produce titania nano petals or nanorod layers (Li et al. 2013). However, there has been an increasing interest in reproducing the surface properties of biomimetic surfaces using simpler methodologies. These have included reproducing the leaves of the lotus and rice leaf topography on gold surfaces using polydimethylsiloxane (PDMS), and then chemically modifying with alkanethiol (Zhao et al. 2010), recreating two bamboo varieties and *Ginkgo biloba* using a PDMS replicating protocol (Legrand et al. 2021), reproducing the morphology and wettability of water bamboo leaves using PDMS (Guan et al. 2015), and replicating the surface of the *Gladiolus hybridus* (Gladioli) leaf using silicone material to create a negative mould of the leaf surface, followed by using dental wax to produce a biomimetic surface (McClements et al., 2021).

The recreation of the properties of biomimetic surfaces is complex. A superhydrophobic surface typically has an apparent water contact angle (CA) greater than 150° and small CA hysteresis (Ramachandran et al. 2014). It has been suggested that the superhydrophobic properties of the surface can be influenced by the surface structure and material composition (Peng et al. 2013). However, it has also been shown that surfaces can exhibit a high contact angle coupled with either low or high adhesion by virtue of surface topography alone (Peng et al., 2013). Some superhydrophobic surfaces have been shown to have a high CA and, at the same time, strong adhesion with water and, therefore, large CA hysteresis, a phenomenon that was called ‘rose petal effect’ (Ramachandran et al., 2014). Both types of surfaces may be replicated and adapted to understand the interactions between the surfaces, biofouling and interfacial phenomena. One key area where superhydrophobic surfaces that repel water could be extremely useful is in the food industry to reduce bacterial binding to surfaces. Two of the

most important pathogens that occur in the food industry are *Listeria monocytogenes* and *Escherichia coli*. Both are opportunistic foodborne pathogens:. *L. monocytogenes* is the causative agent of listeriosis, whilst *E. coli* is found in water and food, and can cause foodborne disease (de Grandi et al. 2018; Klayman et al. 2009). Bacterial attachment, adhesion, and retention are a prerequisite for biofilm formation, and such issues can lead to poor hygienic conditions in food processing environments (Røder et al. 2015).

In this work, surfaces were replicated to utilise the antifouling properties that occur naturally on the surfaces of plant leaves. The aim of this study was to replicate the self-cleaning surfaces of cabbages - *Brassica oleracea* (Tenderheart), *Brassica oleracea capitate* (White cabbage), *Brassica oleracea var. botrytis* (Cauliflower), and *Allium ampeloprasu* (Leek) - using a casting technique. Negative silicone moulds of the leaves surfaces were manufactured and dental wax was used to create the biomimetic surfaces because it is a low-cost, easily mouldable material and mimics the crystalline hydrocarbons found on several hydrophobic leaves (McClements et al. 2021). The biomimetic wax surfaces were then compared with the original leaves (control) to determine the effectiveness of plant-based surfaces in counteracting bacterial attachment, adhesion and retention. In this instance, bacterial attachment was defined as the initial stage of interaction between bacterial cells and the surface, and is followed by adhesion (stronger chemical bonds between surface-bacteria), and finally retention on a surface (final step before biofilm formation) (Rajab et al. 2018). These results help to understand how mimicking the topography of a self-cleaning leaf and testing using a range of bacterial binding methodologies can impact the antifouling behaviour of a replicated biomimetic surface.

2. Materials and Methods

2.1 Production of biomimetic surfaces

To fabricate biomimetic replicates, several biological samples of the same leaf type were mounted with double-sided tape on a smooth surface and an addition-cured silicone duplicating system (Shera Duo-Sil H, Shera GmbH & Co. KG, Germany) was poured on the adaxial surfaces of the leaves in order to produce a negative mould. Dental wax (Kemdent Eco dental wax, UK) was then poured onto the negative mould, creating a positive wax surface for each leaf (McClements et al. 2021). A 15 mm diameter steel hole punch was used to create equally sized coupons.

2.2 Surface characterization

The brassica leaves and leek, together with the biomimetic wax surfaces, were characterized regarding the surface hydrophobicity, roughness (by Optical Profilometry, OP) and morphology (using Scanning Electron Microscopy, SEM).

2.2.1 Scanning Electron Microscopy (SEM)

The original leaves and biomimetic wax surfaces were soaked for 24 h at 4 °C in 4% (v/v) glutaraldehyde (Agar Scientific, UK), washed with sterile water, dried overnight, and finally stored in a desiccator until visualisation to remove any trace of water from almost-dry samples. The samples were then fixed (adaxial side up) to SEM stubs using carbon pads (Agar Scientific, UK) and sputter-coated with gold in an SEM coating system (Polaron, UK). The sputter coating conditions were: 5 mA (plasma current), pressure < 0.1 mbar, 800 V, and argon gas for 30 s. The secondary electron detector of a Supra 40VP scanning electron microscope (Carl Zeiss Ltd., UK) was used to obtain the images at an accelerating voltage of 2 kV and a magnification of 5000×.

2.2.2 Optical Profilometry (OP)

The surface roughness of the leaves and wax replicates were evaluated using a MicroXAM (phase-shift) surface mapping microscope (ADE Corporation, XYZ model 4400 mL system, USA) with an AD phase-shift controller (Omniscan, UK). Each analysis was carried out using extended range vertical scanning interferometry, and the MAPVIEW AE 2.17 (Omniscan, UK) image analysis system was utilized to extract the root-mean-square roughness (Sq) (n = 9) (Skovager et al. 2013).

2.2.3 Surface hydrophobicity

The surface-energy components of the leaves and replicates were calculated according to the work by van Oss and colleagues (Van Oss et al. 1986; van Oss 1995; van Oss and Giese 1995), which considers the contact angles of three test liquids including water to estimate the interfacial free energy (ΔG_{iwi}). The contact angles of each surface were determined using a drop goniometer (GH11 model, Krüss, France) and a PC-based data analysis system as described in McClements et al. (2021). The interfacial free energy was used as a measure of the hydrophobicity of a surface where greater (negative) ΔG_{iwi} values correspond to more hydrophobic surfaces.

2.3 Microbial adhesion to hydrocarbons (MATH) assay

Bacterial cell surface affinity to hydrocarbons was measured according to the MATH assay described by Whitehead et al. (2005). *Escherichia coli* and *Listeria monocytogenes* overnight cultures were centrifuged at 567 g for 10 min, washed three times in PUM buffer pH 7.1 (PUM buffer: $K_2HPO_4.3H_2O$ 22.2, KH_2PO_4 7.26, urea 1.8, $MgSO_4.7H_2O$ 0.2 g L^{-1}) and resuspended to an optical density (OD) of 1.0 at 400 nm. A volume of 5 mL of washed cells suspended in PUM buffer was added to round bottom glass test tubes of 15 mm diameter and 1 mL n-hexadecane (Sigma-Aldrich, USA) was added to the test suspension. The suspensions were

mixed by vortexing for 2 min and then incubated for 30 min at 37 °C. The lower aqueous phase was transferred to a cuvette and the OD was determined at 400 nm. The calculation used to determine the percentage affinity to hydrocarbons was (Equation 1):

$$\% \, affinity = 1 - \frac{A}{A_0} \times 100 \qquad [1]$$

where A_0 is the optical density of the microbial suspension measured at 400 nm before mixing, and A is the optical density following mixing with hydrocarbon and extraction of the aqueous phase measured at 400 nm.

2.4 Attachment, adhesion and retention assays

E. coli NCIB 9484, a common laboratory strain (Gill and Penney 1977), or *L. monocytogenes* Scott A, an isolate from a foodborne outbreak (Briers et al. 2011), was inoculated into tryptone soy broth (TSB; Oxoid, UK) and incubated overnight at 37 °C with shaking (New Brunswick Scientific, USA). Appropriate dilutions in sterile distilled water were performed to obtain an OD of 0.5 at 540 nm, corresponding to 5.5×10^8 *E. coli* or *L. monocytogenes* colony forming units (CFU)/mL.

The biomimetic coupons and the fresh leaves were analysed for attachment (by spray plus wash), adhesion (by spray), and retention (by 1-h static incubation) assays with monocultures of the selected bacteria (Rajab et al. 2018, McClements et al. 2021). Before being used, the leaves were also cut into 15 mm diameter circles, washed with sterile distilled water and air-dried in a class 2 flow hood for 1 h. For attachment and adhesion assays, replicates of biomimetic surfaces and original leaves were attached to a vertical stainless steel tray and the bacterial suspension was sprayed (Spraycraft Universal Air Propellant, Shesto, UK) over the surfaces for 10 s. Immediately after spraying, the surfaces were divided into two sets (n = 3

each), one was laid horizontally and left to dry (adhesion assay) and the other was rinsed using a water spray bottle (attachment assay). For retention assays, surfaces were submerged in 25 mL of cell suspension for 1 h at 37 °C (n = 3). Then, the cell suspension was poured off and the coupons or leaves were rinsed with sterile distilled water. All surfaces from the three microbiological assays were then prepared for CFU enumeration by being added to 2 mL of phosphate-buffered saline (PBS; Oxoid, UK), vortexed for 1 min to ensure the removal of most adhered cells and plated out onto tryptone soy agar (TSA; Oxoid, UK). The agar plates were incubated for 18 h at 37 °C and the colony enumeration was performed in three independent experiments (n = 9).

2.5 Statistical analysis

Statistical analysis was carried out using non-parametric Mann-Whitney testing in SPSS® Statistics 26 software (IBM, USA). The error bars shown in the graphs correspond to the standard deviation (SD) or standard error (SE). Differences between samples were considered statistically significant for p values < 0.05.

3. Results and Discussion

The leaves selected for replication in this study demonstrated slippery, superhydrophobic surfaces with sliding angles less than 10° such as have been described by the Cassie–Baxter model. In this model, the water droplet contacts the tips of the largest surface protrusions, resulting in a large air fraction which is trapped at the bottom of the surface, thus generating a non-wetting phenomenon, allowing water droplets to easily roll off the surfaces (Peng et al. 2013). The leaf surfaces were analysed for their surface properties, and the replicated surfaces were analysed in the same way so that the degree of replication of the surfaces could be

checked, and to determine the effect of the surface properties on the attachment, adhesion and retention of *E. coli* and *L. monocytogenes.*

3.1 Surface characterization

SEM of the real and wax replica surfaces revealed that the macro-topographies of all the surfaces demonstrated some variations in roughness when compared to the original leaf surface (Figure 1). The most obvious differences were seen between the original (Figure 1b) and the replicated biomimetic Cauliflower leaf surfaces (Figure 1f). Although the macro- and micro-topographies of the surfaces were well reproduced, the nano-topographies on the biomimetic replicated surfaces were less evident. Work by others using moulding methods has demonstrated that the surface features of two bamboo varieties and *Ginkgo biloba* replicated using PDMS resulted in the loss of the nanometric features during the replication process (Legrand et al. 2021). In addition, when the hierarchical patterns of water bamboo leaves (with features from sub-millimeter to micron-scale range) were well reproduced, it was found that there was an absence of nanostructures on the replicated surface, and it was suggested that this was due to the melting of plant epidermal wax during the curing process (Guan et al. 2015).

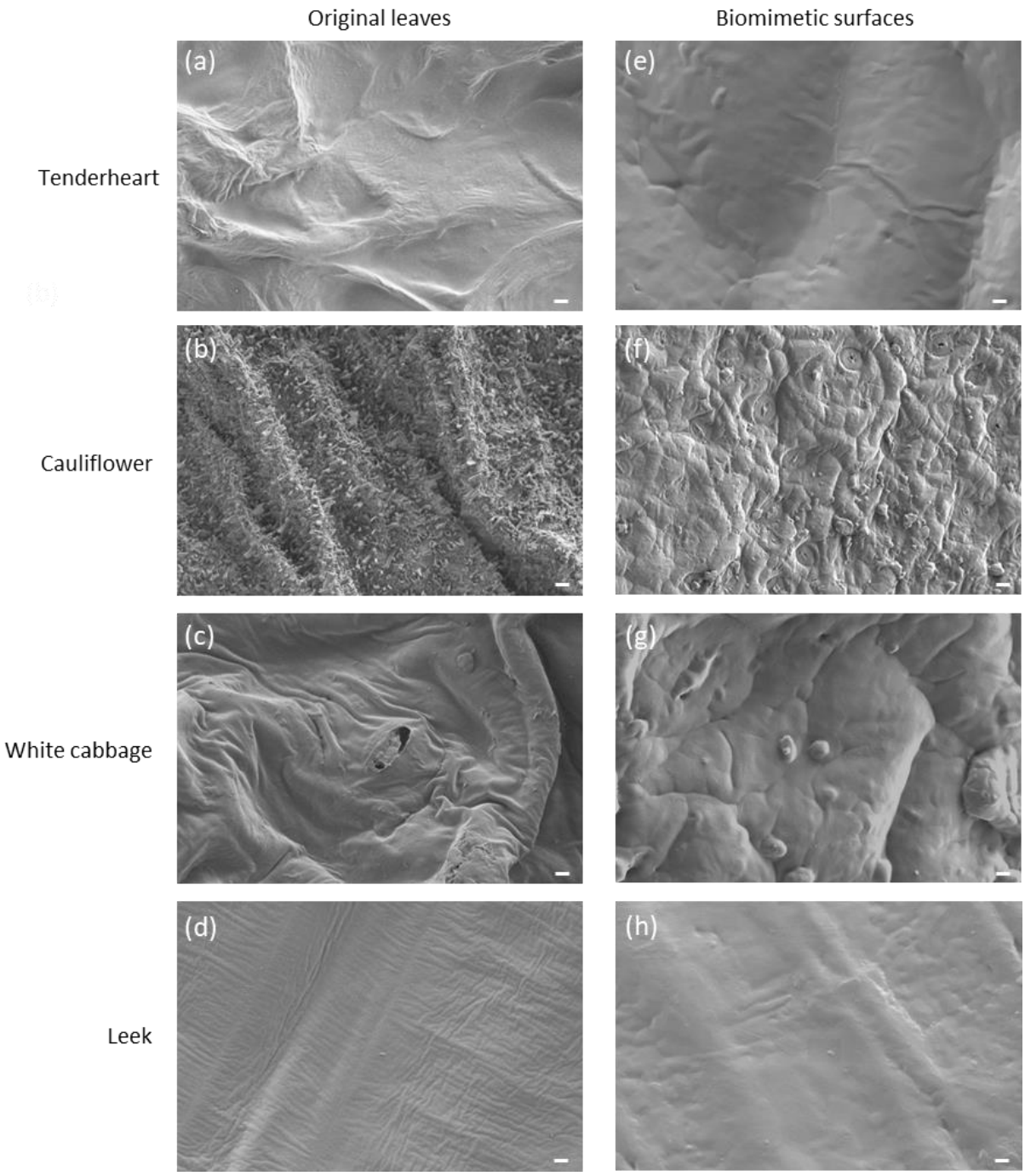


Figure 1. SEM micrographs of the (a-d) original leaves and (e-h) biomimetic wax surfaces of Tenderheart (a and e), Cauliflower (b and f), White cabbage (c and g), and Leek (d and h). Magnification of 5000×, Scale bar of 2 µm.

Optical profilometry was used to quantify the surface roughness of the leaves (Figure 2). Regarding the topography of the original leaf surfaces, the White cabbage demonstrated the

lowest S_q value (3.5 µm), thus being the smoothest surface, whilst the roughest original leaf surface was the Leek (S_q = 5.4 µm). The least rough biomimetic replicated surface was the Leek surface (S_q = 2.0 µm), whereas the roughest biomimetic replicated surface was the White cabbage (S_q = 5.3 µm). There was only a significant difference demonstrated between the original and the biomimetic replicated surface for the Leek ($p < 0.05$). Thus, in agreement with the work of others, although the moulding techniques used were simpler than other production methodologies, there may be a loss in the resolution of the surface features. However, it has also been demonstrated that plants without the presence of macro- and micro- features can show superhydrophobicity (McClements et al. 2021), hence the relationship between the surface properties and the superhydrophobicity of a surface is still unclear.

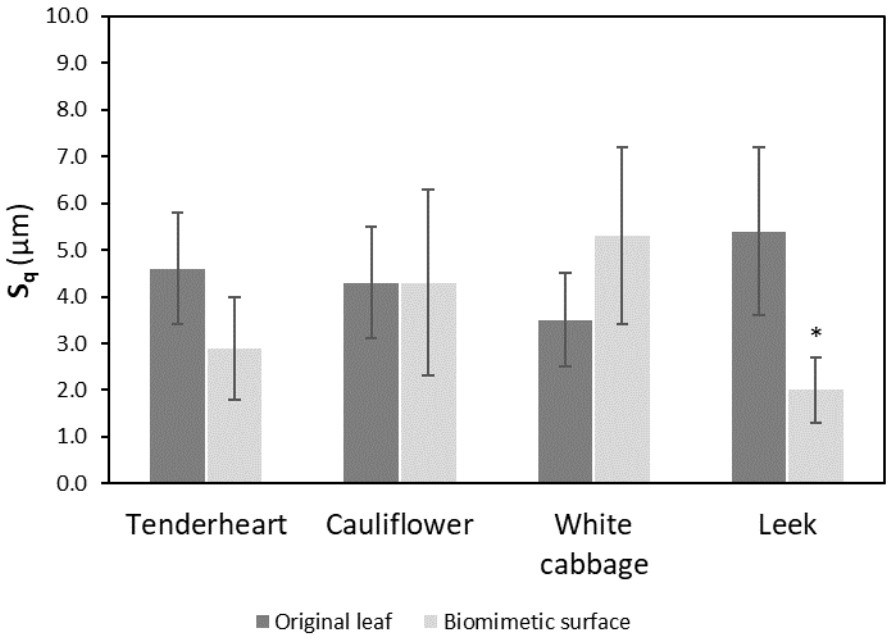


Figure 2. Root-mean-square roughness (S_q) of the original leaf (Tenderheart, Cauliflower, White cabbage and Leek) and the corresponding biomimetic surface obtained by OP. Values are means ± SEs. Asterisk denotes a significant difference between the original and replicates of the same leaf (* $p < 0.05$).

The hydrophobicity (free energy of transfer, ΔG_{iwi}, Figure 3) revealed that the White cabbage leaf had the most hydrophobic character (ΔG_{iwi} = -88.7 mJ/m^2), followed by Cauliflower, whilst Leek had the least hydrophobic surface tested (ΔG_{iwi} = -30.4 mJ/m^2). On the replica biomimetic surfaces, the White cabbage was again the most hydrophobic (ΔG_{iwi} = -87.4 mJ/m), whilst the Tenderheart cabbage replica was the least hydrophobic surface (ΔG_{iwi} = -3.6 mJ/m). The topography of a surface affects its wetting state (Timonen et al. 2013; Xu et al. 2013). This may be one reason why the surface properties demonstrated inconsistencies between the original leaf and the biomimetic replicate surfaces. In agreement with our work, when replicated biomimetic surfaces have been produced by others, it has been found that in some cases, the contact angle measurements showed that natural leaves were highly hydrophobic, but such hydrophobicity could not be transferred to the metallic plates (Arango-Santander et al. 2021). In addition, it was found that the water contact angle values on artificial Water Bamboo leaf replicates were lower than on the original surfaces (Guan et al. 2015). In another study, although the biomimetic wax surface and Gladioli leaves had extremely similar surface roughness parameters, the water contact angle of the Gladioli leaf was found to be significantly higher than the replicated biomimetic surfaces (McClements et al., 2021). Hence, these studies demonstrate the challenges in using a simplified method to produce biomimetic surfaces.

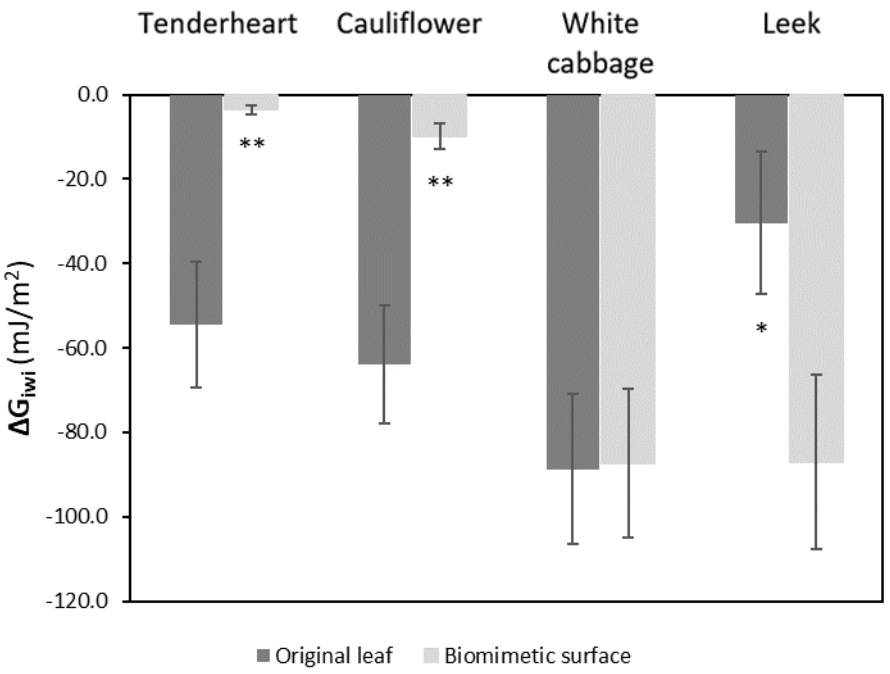


Figure 3. Hydrophobicity of the original leaf (Tenderheart, Cauliflower, White cabbage and Leek) and the corresponding biomimetic surface. Values are means ± SEs. Asterisks denote significant differences between the original and replicate of the same leaf (* $p < 0.05$ and ** $p < 0.01$).

The microbial adhesion to hydrocarbon (MATH) assay was carried out to determine the hydrophobicity between the bacterial strains used in this study, and it was found that *L. monocytogenes* was significantly more hydrophobic (95%) than *E. coli* (3%) (Figure 4). In agreement with these results, *E. coli* has been reported as being hydrophilic in nature (Rivas et al. 2005), although the hydrophobicity of *L. monocytogenes* can vary depending on a number of factors (Lee et al. 2017).

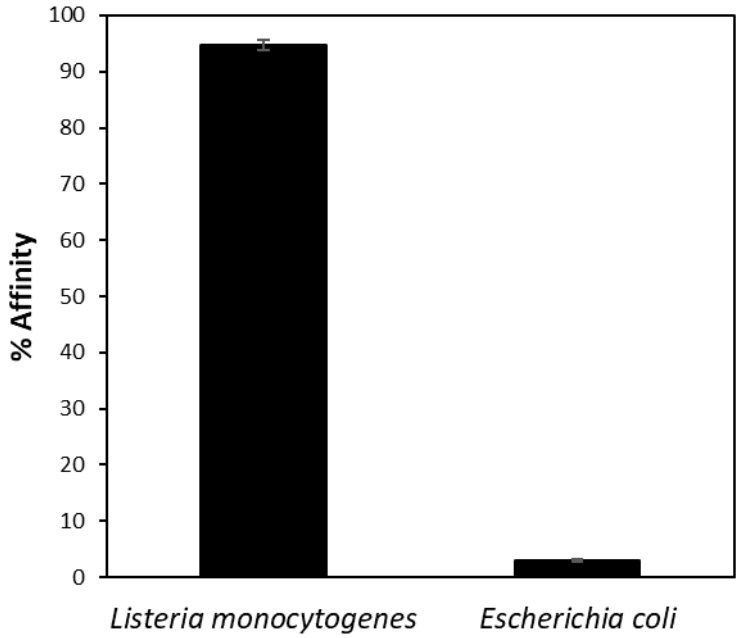


Figure 4. Percentage affinity of the bacterial strains *E. coli* and *L. monocytogenes* toward hydrocarbons. Values are means ± SDs.

3.2 Attachment, adhesion and retention assays

In general, *L. monocytogenes* (Gram-positive bacteria) were bound to both the original leaf and biomimetic replicated surfaces in lower numbers than the Gram-negative bacteria *E. coli* (Figure 5 to 7). This was most evident in attachment and retention assays where a difference of ~ 0.30 Log CFU/cm^2 existed between species, regardless of the surface type. This could be related to the surface hydrophobicity of the *L. monocytogenes* strain whereby it was found to be significantly more hydrophobic than *E. coli* (Figure 4). This is in contrast to work by McClements et al. (2021) who found that only following retention assays that *L. monocytogenes* bound in lower numbers to Gladioli leaf and biomimetic replica surfaces. Further, in work by others, on smoother surfaces, it was demonstrated that *L. monocytogenes* and *Staphylococcus aureus* retention to the surfaces were mostly affected by surface microtopography, whereas retention of *E. coli* to the coatings was mostly affected by the coating physicochemistry (Whitehead et al. 2015), and this may be a clear effect of topography.

Although there is conflicting evidence, it has been suggested that the hydrophobicity of a bacterial cell is largely influenced by the residues and structures on the surface of the cell (van der Mei et al. 1991). Positive relationships between physicochemical surface properties and bacterial attachment have been reported (Liu et al. 2004), however, others have found no evidence of such relationships (Bettelheim et al. 1995; Rivas et al., 2007).

Following the attachment assays, from the results of the original leaves, the Tenderheart cabbage leaves (which were the least hydrophobic) attached most *E. coli* and *L. monocytogenes* (6.85 log CFU/cm^2 and 6.54 log CFU/cm^2, respectively; Figure 4a and b). However, *L. monocytogenes* cells were also attached on the Leek surfaces in similar numbers (6.53 log CFU/cm^2), which was the roughest surface and the second most hydrophobic leaf surface. *E. coli* were least attached on the Leek leaves (6.52 log CFU/cm^2), which were the roughest surfaces with the second greatest hydrophobicity. On the other hand, *L. monocytogenes* were least attached to the White cabbage surface (5.68 log CFU/cm^2), which was the smoothest and most hydrophobic surface.

On the biomimetic replicate surfaces, *E. coli* was attached in the greatest numbers on the replica biomimetic Leek surface (6.40 log CFU/cm^2) (smoothest and least hydrophobic), and in the least numbers on the White cabbage biomimetic surface (5.49 log CFU/cm^2) (roughest and most hydrophobic). *L. monocytogenes* was attached in the greatest numbers on the Leek biomimetic surface (6.44 log CFU/cm^2) (smoothest and least hydrophobic), and in the least numbers on the White cabbage biomimetic surface (6.02 log CFU/cm^2) (roughest and most hydrophobic). Hence, for *E. coli,* attachment on both the leaves and biomimetic replicate surfaces, and *L. monocytogenes* on the biomimetic replicate surfaces, cell attachment was influenced by both surface hydrophobicity and roughness (i.e., lowest cell numbers on least hydrophobic, smooth surfaces). However, on the leaf surfaces, *L. monocytogenes* was most influenced by surface roughness.

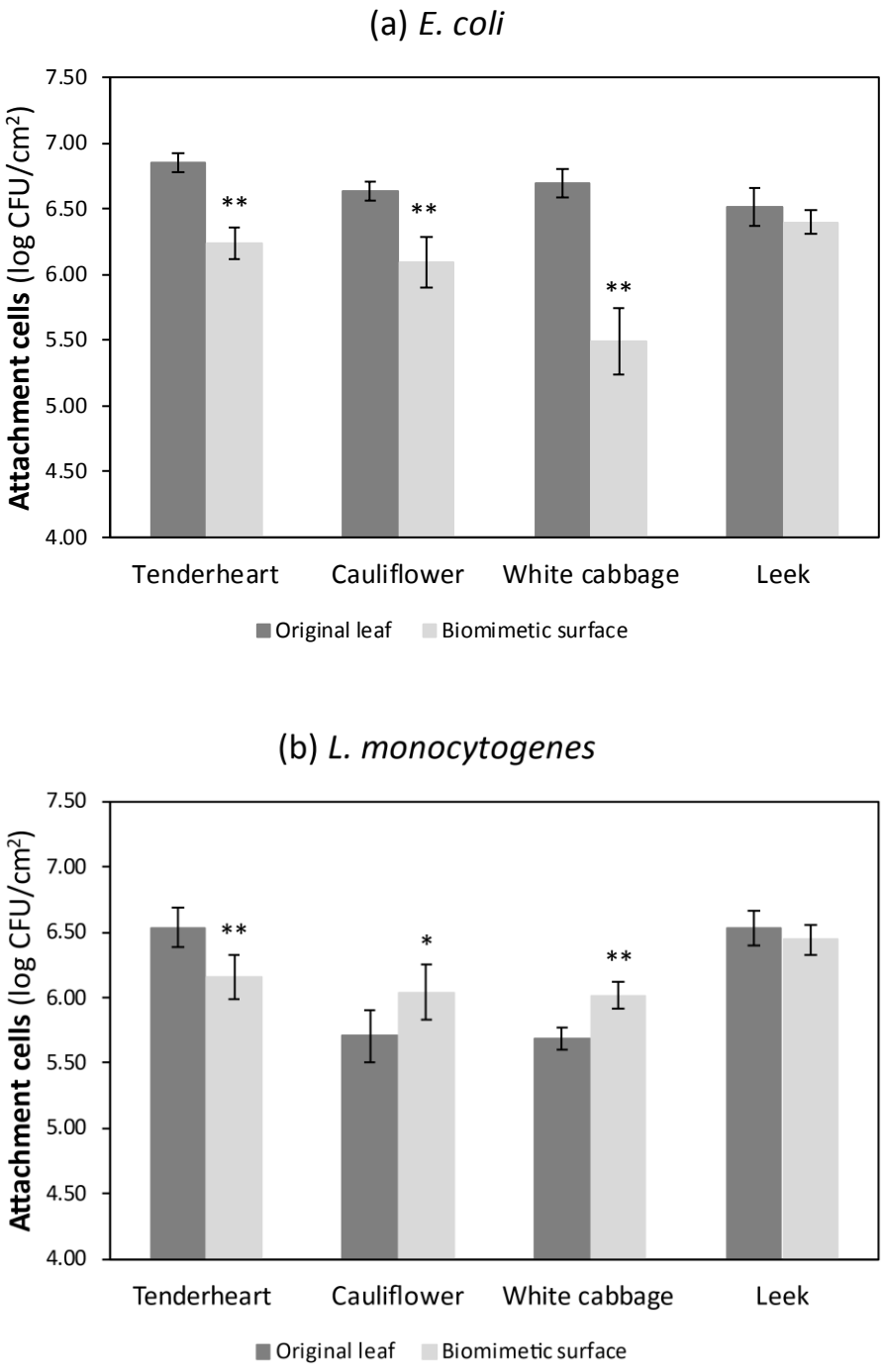


Figure 5. Number of (a) *E. coli* and (b) *L. monocytogenes* culturable cells following the attachment assay on the original leaf (Tenderheart, Cauliflower, White cabbage and Leek) and the corresponding biomimetic surface. The means ± SDs for three independent experiments are presented. Asterisks denote significant differences between the original and replicate of the same leaf (* $p < 0.05$ and ** $p < 0.01$).

For the adhesion assays (Figure 6), *E. coli* and *L. monocytogenes* adhered on the leaf surfaces in the greatest numbers to the Tenderheart cabbage (7.00 log CFU/cm^2) and Cauliflower leaves (6.56 log CFU/cm^2). *E. coli* and *L. monocytogenes* adhered on the leaf surfaces in the lowest numbers to the White cabbage (6.50 log CFU/cm^2 and 5.51 log CFU/cm^2, respectively). For the adhesion assays on the biomimetic surfaces, *E. coli* (Figure 6a) and *L. monocytogenes* (Figure 6b) adhered on the surfaces in the greatest numbers to the biomimetic Leek (6.61 log CFU/cm^2) and the White cabbage surfaces (6.78 log CFU/cm^2). Both bacterial strains adhered on the biomimetic surfaces in the lowest numbers to the Tenderheart cabbage (6.27 log CFU/cm^2) and the Cauliflower surfaces (6.12 log CFU/cm^2). In summary, following the use of the adhesion assay, it was difficult to elucidate the surface properties that reduced microbial adhesion. However, it could be speculated that the use of surfaces with a S_q value between 2.9 and 4.3 µm, and a ΔG_{iwi} value between -54.5 and -63.9 mJ/m^2 resulted in the least bacterial retention on the surfaces.

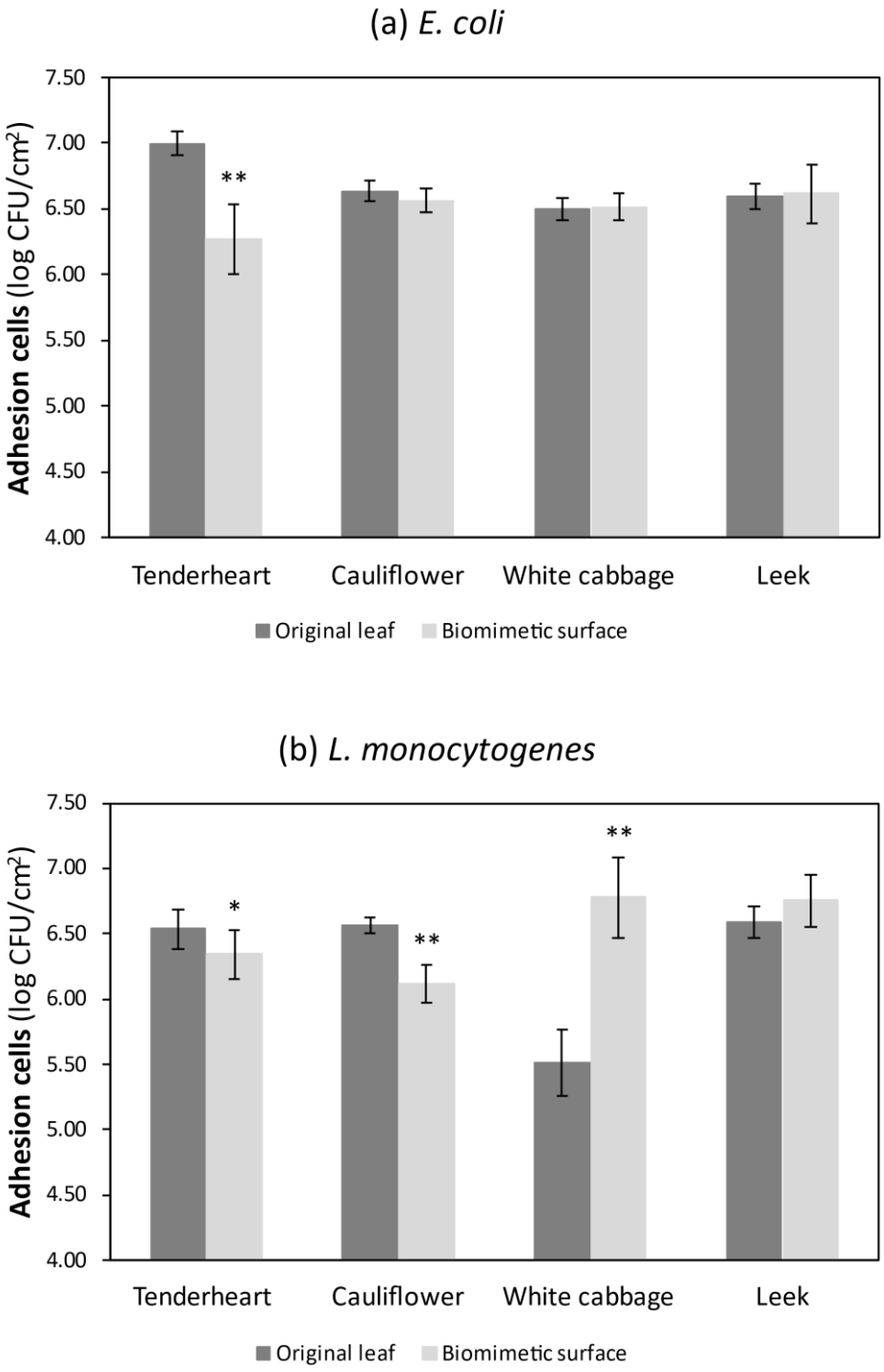
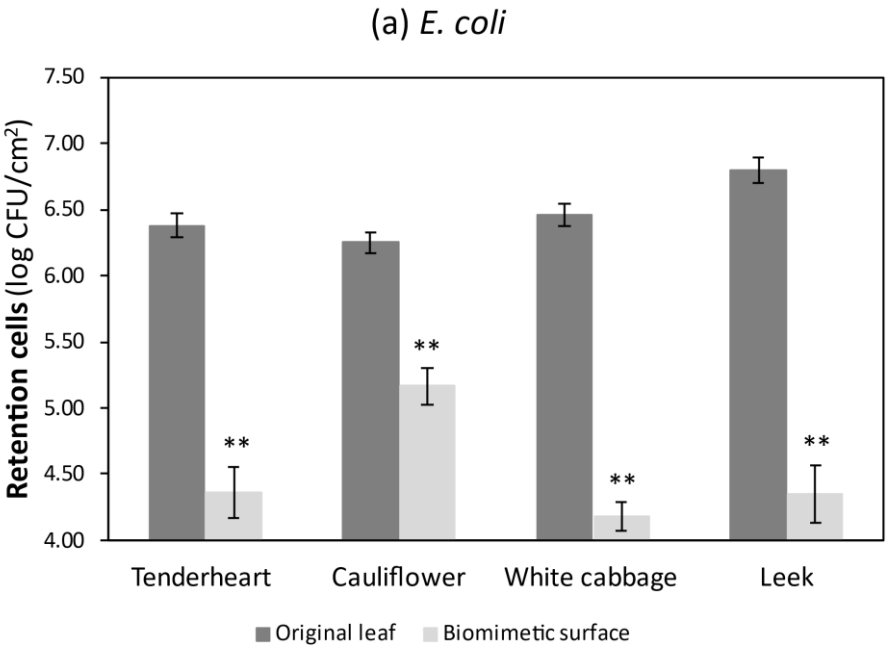


Figure 6. Number of (a) *E. coli* and (b) *L. monocytogenes* culturable cells following the adhesion assay on the original leaf and the corresponding biomimetic surface. The means ± SDs for three independent experiments are presented. Asterisks denote significant differences between the original and replicate of the same leaf (* $p < 0.05$ and ** $p < 0.01$).

For the retention assays on the original leaf surfaces, *E. coli* (Figure 7a) and *L. monocytogenes* (Figure 7b) were retained on the leaf surfaces in the greatest numbers to the Leek leaf surface (6.80 log CFU/cm^2 and 6.28 log CFU/cm^2, respectively). Nevertheless, *E. coli* and *L. monocytogenes* retained on the leaf surfaces in the lowest numbers to the Cauliflower leaf (6.25 log CFU/cm^2 and 5.18 log CFU/cm^2, respectively). Following retention assays on the biomimetic surfaces, *E. coli* and *L. monocytogenes* were retained on the replicate surfaces in the greatest numbers to the Cauliflower (5.17 log CFU/cm^2) and Leek leaves (4.95 log CFU/cm^2), and in the lowest numbers to the White cabbage replicate surface (4.18 log CFU/cm^2 and 4.44 log CFU/cm^2, respectively). Therefore, on the plant leaves, the rough, hydrophobic surfaces increased the retention of bacterial cells, whilst surfaces with S_q values around 4.3 µm and which were least hydrophobic reduced bacterial retention. On the replicated biomimetic surfaces, the rougher, hydrophobic surfaces decreased bacterial retention.



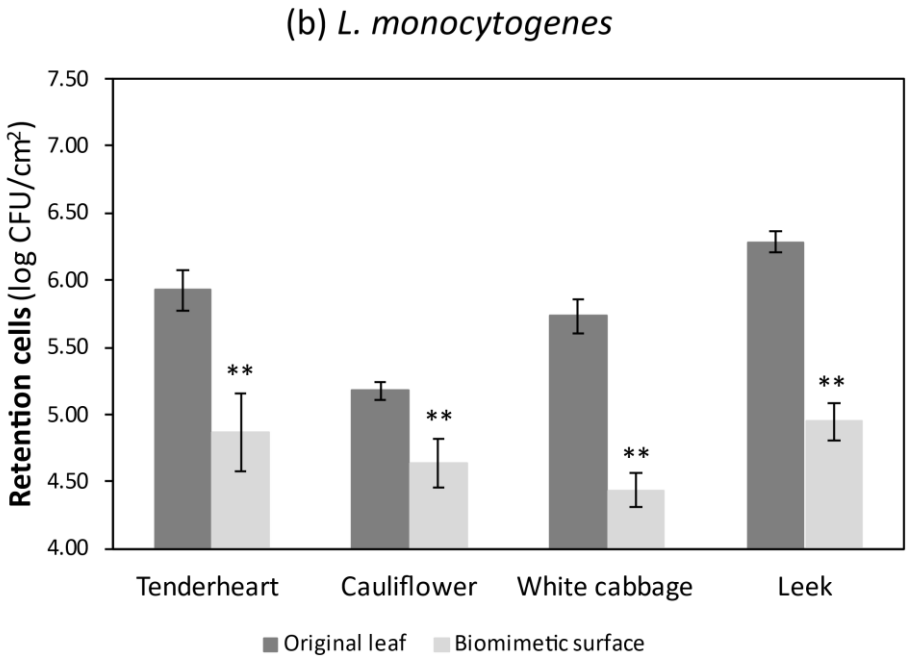


Figure 7. Number of (a) *E. coli* and (b) *L. monocytogenes* culturable cells following the retention assay on the original leaf and the corresponding biomimetic surface. The means ± SDs for three independent experiments are presented. Asterisks denote significant differences between the original and replicate of the same leaf (* $p < 0.05$ and ** $p < 0.01$).

When comparing biomimetic with the original leaf surface, it was observed that, in most cases, all types of replica biomimetic surfaces were more efficient at reducing the numbers of bacteria that bound to the surface than the natural leaves. These higher removal rates were particularly noticeable in the attachment assays with *E. coli* (Figure 5a), where the biomimetic surfaces showed on average less 0.62 log CFU/cm^2, as well as in the bacterial retention assays with both bacteria (Figure 7a and b). In this case, reductions of on average 1.92 and 1.05 log CFU/cm^2 were achieved for *E. coli* and *L. monocytogenes* ($p < 0.01$), respectively, with biomimetic surfaces of White cabbage and Leek showing to be the most promising surfaces. This is in agreement with work by McClements et al. (2021) who compared the self-cleaning properties

of biomimetic produced surfaces against *E. coli* and *L. monocytogenes,* where it was found that the biomimetic surfaces retained fewer bacteria than the control surfaces.

In general, for the attachment assays, the lowest cell numbers occurred on the least hydrophobic, smooth surfaces. Following the adhesion assays, use of surfaces with an intermediate S_q and ΔG_{iwi} demonstrated the lowest bacterial adhesion. However, following the retention assays, it seems that the chemistry of the surface may have affected the results since opposite surface effects were demonstrated to reduce cell retention on the leaf which was the least hydrophobic and on the biomimetic surfaces which were rougher and hydrophobic. In agreement with other previous work (Liauw et al. 2020), the overall results suggest that the different methods exerted different influences on the surface and bacterial binding. This is an important finding since this may be one of the reasons for the conflicting evidence regarding the effect of surface properties on bacterial binding. The attachment assays include a spraying step directly following cell application to the surface, and hence the bacteria only have a few seconds to bind. In this case, the surfaces that were the least hydrophobic and smooth retained the least bacteria, suggesting that the immediate inclusion of a washing step altered the hydration dynamics between the surface and bacteria. Such an assay may be representative of where unwanted fouling occurs on a surface and is immediately removed. The adhesion assay does not involve a wash step, so the bacteria that bind to the surface are able to adhere, and in this case, surfaces with intermediate S_q and ΔG_{iwi} demonstrated the least bacterial retention. Such a scenario may occur when fouling arises on a surface but is not immediately cleaned. In the retention assay, the bacteria could bind to the surface whilst in suspension for a longer time, showing different interactions that can only be assumed to be due in part to the chemistry of the surface, but this requires further investigation. Such an assay may be representative of foodstuffs that are stored in a vat for a longer period of time. In agreement with our work, bacterial binding on replicated biomimetic surfaces is not a straightforward phenomenon.

Biomimetic surfaces that were prepared using the soft lithography technique demonstrated that two of the surface models used showed positive results for reduction of *C. aurea* and *C. esculenta*, while the other showed an increase in bacterial adhesion (*S. molesta*) (Arango-Santander et al. 2021). However, other authors have demonstrated that biomimetic surfaces inhibited *E. coli* adhesion (Hu et al. 2018) and have a bacteriostatic effect on *S. aureus* (Li et al. 2013). On reproduced *Laminaria japonica* biomimetic surfaces, the antifouling effect against *E. coli* was also found to be effective (Zhao et al. 2020). Hence, the findings from this work show that, in addition to surface attributes such as hydrophobicity and roughness, the biological factors and environment, as well as the type of methodologies used, need to be taken into consideration when designing self-cleaning surfaces based on biomimetic principles, particularly if the surface is to be used in future scale up.

4. Conclusions

The replication of biological surfaces has great potential in applied surface technology. These preliminary results showed that via a casting approach, wax surfaces mimicking the structure of vegetable leaves could be prepared and that these surfaces seem to be promising in preventing bacterial binding. In general, for the attachment assays, the lowest cell numbers occurred on least hydrophobic, smooth surfaces. For the adhesion assays, surfaces with an intermediate S_q and ΔG_{iwi} revealed the lowest bacterial adhesion. However, following the retention assays, it seems that the chemistry of the surface may have had an effect on the results. In further experiments, we will concentrate on the choice of appropriate multispecies cultures and polymers to get closer to the conditions found in real scenarios where biofilms are established in the food industry.

Funding

This work was financially supported by: LA/P/0045/2020 (ALiCE), UIDB/00511/2020 and UIDP/00511/2020 (LEPABE), funded by national funds through FCT/MCTES (PIDDAC), and by the European Union's Horizon 2020 Research and Innovation Programme under grant agreement no. 952471 (SurfSAFE). L.C.G. Thanks to the Portuguese Foundation for Science and Technology (FCT) for the financial support through the Scientific Employment Stimulus - Individual Call - [CEECIND/01700/2017]. Thanks also to Manchester Metropolitan University for their financial support of this work.

Conflicts of Interest.

The authors have no competing or conflicts of interest.

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Conflicts of Interest. The authors declare no conflicts of interest.