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Older Leaves of Lettuce (*Lactuca* spp.) Support Higher Levels of *Salmonella enterica* ser. Senftenberg Attachment and Show Greater Variation Between Plant Accessions than do Younger Leaves.

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Abstract

Salmonella can bind to the leaves of salad crops including lettuce and survive for commercially relevant periods. Previously studies have shown that younger leaves are more susceptible to colonization than older leaves and that colonization levels are dependent on both the bacterial serovar and the lettuce cultivar. In this study, we investigated the ability of two *Lactuca sativa* cultivars (Saladin and Iceberg) and an accession of wild lettuce (*L. serriola*) to support attachment of *Salmonella enterica* serovar Senftenberg, to the 1st and 5-6th true-leaves and the associations between cultivar-dependent variation in plant leaf surface characteristics and bacterial attachment. Attachment levels were higher on older leaves than on the younger ones and these differences were associated with leaf vein and stomatal densities, leaf surface hydrophobicity and leaf surface soluble protein concentrations. Vein density and leaf surface hydrophobicity were also associated with cultivar-specific differences in *Salmonella* attachment, although the latter was only observed in the older leaves and was also associated with level of epicuticular wax.

Introduction

Between 1992 and 2006, 23% "food poisoning" outbreaks in England and Wales were linked to prepared salad crops (Little and Gillespie 2008), with lettuce being one of the salads most frequently associated with illness caused by food-borne pathogens (Warriner *et al.* 2003). Over a similar timeframe, *Salmonella enterica* was associated with 30% of such outbreaks in the USA (second only to *E. coli*) (Brandl 2006). Despite recent implementation of assured production protocols, improvements in quality assurance testing, and the advent of statutory monitoring through non-governmental agencies (FSA, EFSA etc), which have reduced the incidence of food-poisoning outbreaks (Monaghan 2006), incidents related to consumption of fresh produce still occur, indicating continuing issues with contamination by human pathogens.

Human pathogenic bacteria, including *Salmonella* can bind to the leaves of various salad crops including lettuce and can survive in the phyllosphere (the aerial part of plants) for periods in excess of 30 weeks (Islam *et al.* 2004; Stine *et al.* 2005). Standard post-harvest decontamination procedures typically involve short immersion of salad crops in solutions containing approximately 20-200 μg/ml of active chlorine. These treatments reduce bacterial numbers but do not completely eliminate either the natural microbial population or human pathogens (Beuchat and Brackett 1990; Seo and Frank 1999; Lang *et al.* 2004). This is a particular issue with "minimally processed" salad crops, which are normally eaten uncooked, as there is a limit to the stringency of decontamination that can be applied without ruining the saleability of the produce.

Previous work has identified the younger leaves of plants as being able to support higher levels of enteric human pathogens than older leaves (Brandl and Amundson 2008), however, cultivar-specific variation in the level of contamination of lettuce has also been

demonstrated for a number of food poisoning-associated *Salmonella* strains (Barak *et al.* 2008) and *Salmonella enterica* serovars have also been shown to differ in their ability to colonise lettuce plants (Klerks *et al*. 2007) suggesting both plant cultivar and pathogen serovar as potential sources of variation. In previous work, we have shown that the composition of the naturally occuring bacterial population which developed on field-grown lettuce differed significantly with cultivar, particularly in respect of the *Enterobacteriaceae* (the bacterial family to which *Salmonella* belongs) (Hunter *et al.* 2010). This variation was found to be associated with plant morphotype (a composite of leaf and whole-plant morphological traits) and levels of soluble carbohydrate and phenolic compounds. In this work, we examined whether plant leaf age and / or cultivar had an effect on the ability of lettuce leaf tissue to support attachment of the same *Salmonella* isolate and if any such differences could be correlated with differences in leaf surface characteristics.

Materials and Methodology

Plant accessions and experimental design

Lettuce (*Lactuca sativa*) cultivars Saladin (iceberg type) and Iceberg (batavian type) and the wild lettuce relative *Lactuca serriola* (US96UC23) were raised under glass (18-22 ^{o}C) in modular arrays (P40; Plantpak, Maldon, United Kingdom) of Levington F2s compost (Levington Horticulture Limited, Ipswich, UK). The oldest or newest fully expanded true leaves (corresponding to the 1st and approximately 5th or 6th true leaves) were inoculated with *S.enterica* ser. Senftenberg strain 070885 or used for leaf characteristic measurements at 6 weeks post-sowing. Separate sets of 40 plants of each accession randomly distributed within the modular trays were raised consecutively and plants from each set tested for both bacterial adhesion and plant leaf characteristics. Tests comparing characteristics of younger and older leaves were made on leaves from the same plants.

Leaf-adhesion assays followed the method of Shaw *et al* (2008), as modified by Berger *et al* (2009): 10mm wide strips were trimmed from 6 replicate freshly excised leaves of each age from each plant accession into 30mm diameter petri dishes and immersed in 4mL of a *Salmonella* Senftenberg culture grown to an OD_{600} of 1.00 (6 - 7 $x10^8$ CFU mL^{-1}) at room temperature in Luria Bertani (LB) broth. Samples were incubated statically at room temperature for 1hr to allow bacterial adhesion, but minimize time for subsequent proliferation (thus minimizing the influence of confounding factors). This corresponds to Cevallos-Cevalos *et al.* (2012) who found that 100% of leaf adhedsion in tomato had occurred by 1hr. Non-adherent bacteria were removed by three 5min washes in 3mL of sterile distilled water (SDW) at 80 rpm in an orbital shaker. A 6mm diameter (approx 28 mm^2) leaf disk was excised from each sample and macerated in SDW. The macerates were serially diluted in SDW and incubated on MacConkey agar overnight at 37^{o}C to recover leaf-associated bacteria. An additional disk was excised from each sample and fixed in 3% glutaradehyde in100mM sodium phosphate buffer pH 7.3 and processed for scanning electron microscopy (SEM) (Knutton 1995) to confirm bacterial attachment.

Leaf characteristics

Leaf characteristics were determined from five replicate samples of each leaf age from individual plants from each accession, grown contemporaneously, under the same conditions as those used for bacterial inoculation. Data for physical characteristics and leaf surface hydrophobicity were extracted from captured images using the Image Tool software (freeware available from University of Texas Health Science Center San Antonio at http://ddsdx.uthscsa.edu/dig/download.html.). Five detached leaves for each plant accession and leaf age category were weighed and full-leaf images of all samples used to determine leaf area and to measure distances between veins, along the mid-rib of each leaf. These latter

measurements were used to calculate inter-vein distance (an inverse estimator of vein density) following the method of Uhl and Mossberger (1999). To measure water droplet contact angle (an estimator of surface hydrophobicity), leaf strips from the broadest portion of leaves from each physiological age from 5 replicate plants of each accession were cut and mounted onto microscope slides. Two strips were cut from each leaf, one mounted adaxial side uppermost; the other abaxial side uppermost and five 2µl droplets of sterile, ultra-pure water were place on the surface of the central portion of each leaf strip. Edge-on images of the droplets were captured for contact angle measurement. Leaf strips were only handled at the ends and the regions handled not used for subsequent testing. Values for both upper and lower leaf surfaces were acquired for all plant accessions, and the data for both surfaces combined.

Stomatal density and cell perimeter density were estimated from SEM images Tissue was washed with 100mM sodium phosphate buffer, pH 7.3, fixed for 1hr in 250mM glutaradehyde, then 1hr in 40mM OsO_4, ethanol dried and sputter coated with Platinum (Knutton 1995). Three random fields of view from each leaf surface (upper and lower) of a single leaf of each age from 5 replicate plants of each accession were examined. To estimate stomatal density, stomatal counts in each field of view were made from images taken at approximately 150x magnification. Estimates of cell perimeter density required higher magnification images (approximately 500x). The number of cells in each field of view (n) was determined, and the combined linear perimeter distance (d) of five individual whole cells from each field of view also measured. The cell perimeter density for each field for view (area = a) was then estimated as: $[n \times (d/5)]/a$. The areas of the fields of view for both magnifications were calculated from scale bars incorporated into the digital image output from the SEM.

Water-soluble leaf surface components (protein, sugars and phenolic compounds) were extracted following a modification of the method of Mercier and Lindow (2000). Individual leaves were detached from plants, imaged to enable determination of leaf area then immersed in 10mL of sterile ultra-pure water overnight at 4^{o}C with care taken to avoid immersion of the cut ends of the leaf bases. Each of 5 replicate samples for each leaf age from each plant accession comprised combined extracts from 5 leaves from separate plants, filter sterilised though a 0.2µm syringe filter (Nalgene , VWR International (UK), Lutterworth, UK) and lyophilized. The dried sample was re-suspended in 500µL of ultra-pure water. Separate extractions were made for each component. Protein concentrations were determined by A_{595} meaurements against a standard curve of BSA using the Coomassie (Bradford) protein assay kit (Fisher Scientific UK, Loughborough, UK). Sugar concentrations were determined using a modification of the 3,5-dinitrosalycilic acid (DNS) procedure of Bernfeld (1955): 250µl of sample was boiled for 10min with 750µl of [44mM DNS; 500mM NaOH; 700mM potassium sodium tartrate], then cooled to room temperature. A_{540} measurements were compared to a glucose standard curve. Soluble phenolic compounds were measured using a variation of the method of Zhang *et al* (2006): 50µL of sample was diluted 1:10 with SDW and incubated for 5min at room temperature with 100µL Folin Ciocalteu reagent (Sigma-Aldrich Company Ltd, Dorset, UK). 2mL of 600mM Na_2CO_3, was then added and the supernatant cleared by centrifugation for 1min at 13,000rpm after 2hr incubation at room temperature. A_{765} measurements of the supernatant were made against an SDW blank and compared to a gallic acid standard curve to estimate phenolic compound concentrations.

Leaf wax content was estimated by a modification of the process of Bakker *et al,* (1998) from 5 replicate samples of each leaf age from each plant accession: For each replicate sample five detached leaves were sequentially extracted by gentle agitation for

15min at room temperature in the same 10mL volume of chloroform. The chloroform extracts were filtered into pre-weighed vials and evaporated to constant weight at room temperature to determine the weight of extracted wax.

Statistical analyses

The significances of differences in the various characteristics measured were tested by ANOVA. Comparisons were made both within and between leaf age categories. Characteristics showing significant differences were tested for correlation with Salmonella levels using Spearman's rank correlation test. The significance of any identified correlations was then determined using a permutation test that allowed for tied ranks and differences in sample size

Results and Discussion

We investigated attachment of *Salmonella* Senftenberg to leaves of three lettuce accessions (Table 1). This revealed that overall levels of attachment were significantly higher ($P < 0.0001$) on older leaves than younger ones (Table 1) and that significant differences existed between accessions (P 0.0463). Post-hoc tests revealed no significant differences between *Salmonella* attachment to younger leaves. In older leaves, significantly greater attachment ($P < 0.05$) to *L. serriola* US96UC23 than to the other two accessions was observed. While Brandl and Amundson (2008), showed that *Salmonella* colonize younger plant leaves more heavily than older leaves, in this study, plants were tested at a much younger age (1-6 true leaves) than those in the previous work (10-12 true leaves and mature heads). Brandl and Amundson also measured combined attachment and persistence/proliferation over 72 h at 38^{o}C, whilst the assay timeframe in this study (1h) is too short for bacterial proliferation to be a major factor, suggesting that different parameters may influence initial bacterial attachment

and subsequent growth.

Significant differences were observed in all tested leaf characteristics between leaf ages (Table 1). Nutrition levels, particularly soluble sugar concentrations, are reportedly important in determining bacterial load of leaves. Mercier and Lindow (2000) reported significant correlations between initial leaf sugar concentrations and bacterial (*Pseudomonas fluorescens*) populations developing over 48 h, whilst Brandl and Amundson suggested higher levels of available nutrition in the younger leaves as a potential driver for the higher levels of colonization they noted. This study showed significantly increased concentrations of soluble leaf-surface sugars in young leaves of all cultivars compared to the older leaves but no significant correlation (P 0.070) with *Salmonella* attachment levels indicating that soluble sugar levels do not significantly influence *Salmonella* attachment. Sugar levels may, however, result in a more favourable environment for bacterial growth on the surface of younger leaves, which could become the determining factor in the overall colonization level over time.

A significant positive correlation was observed between *Salmonella* attachment and inter-vein distance. Significant negative correlations were also identified with water soluble protein concentration, water droplet contact angle (hydrophobicity) and stomatal density (Table 1). Plant cultivar may also be a factor: Brandl and Amundson used a romaine type lettuce (cv. Paris Island), whilst iceberg and batavian types (cvs. Saladin and Iceberg respectively) and the wild relative *L. serriola*, were used in this study. These plants have considerably different leaf morphologies compared to the romaine type and morphotype has been shown to influence bacterial colonization of lettuce, particularly by members the *Enterobacteriaceae* (which includes the genus *Salmonella*) (Cevallos-Cevallos *et al.* 2012, Hunter *et al.* 2010). Differences in *Salmonella* attachment levels between the cultivars used in this work, were identified, although these were only significant in older leaves (P 0.0122),

with cv. Saladin supporting significantly ($P > 0.05$) higher levels of attachment than either cv. Iceberg or *L. serriola*. Cultivar-level differences in leaf surface soluble protein, sugar and wax concentrations, water droplet contact angle and inter-vein distance were also detected in the corresponding older leaf samples. Of these, inter-vein distance and surface wax were positively correlated with *Salmonella* attachment, whilst hydrophobicity was negatively correlated (Table 2).

Stomata and grooves along the length of leaf veins are known to be preferential bacterial attachment sites on leaf surfaces (Mariano and McCarter 1993). In this study, negative correlations were shown with stomatal density and leaf vein density. SEM images both from this work (Fig1) and previous studies of *Salmonella* Senftenberg (Berger *et al.* 2009) revealed diffuse attachment patterns rather than an association with the typical preferential bacterial attachment sites (i.e. veins and stomata), although Cevallos-Cevallos *et al.* (2012) implicated trichome density as a factor in *Salmonella* attachment to tomato leaves (we were unable to obtain sufficiently robust trichome density measurements to permit analysis). It is interesting to note however, that in contrast to the distribution of *Salmonella* Senftenberg on lettuce, *Salmonella* serovar Thompson has been shown to be associated with vein structures on leaves of corriander (*Corriandrum sativum*) (Brandl and Mandrell 2002), suggesting a stain-specific component to attachment in *Salmonella*. This may reflect differences in attachment mechanisms: Thin aggregative fimbriae (known as tafi or curli), have been shown to play a role in *S*. Newport attachment to alfalfa sprouts (Barak *et al.* 2005) and *Salmonella* attachment to tomato leaves (Cevallos-Cevallos *et al.* 2012), whilst flagellae have been shown to be strongly involved in attachment of *S*. Senftenberg to a range of salad leaves (Berger *et al*. 2009).

Furthermore, expansion of the leaf surface as the leaves matured, resulting in reduced densities of these characteristics in older leaves (Van Volkenburgh 1999) may have

contributed to the observed negative correlations. Although leaf expansion rates were not measured in this study, a similar level of reduction in the mean density of both veins and stomata (2-3 fold) between younger and older leaves was noted in all accessions.

The negative correlation between *Salmonella* attachments and protein levels suggested the presence of proteins on the leaf surface that either favoured growth of antagonistic micro-organisms or reduced either general bacterial adhesion or survival or that of *Salmonella* specifically. SEM of inoculated samples revealed no indication of non-target micro-organisms and the timescale of the experiment (1h) would have provided only limited opportunity for microbial growth and/or interaction, consequently microbial antagonism seems unlikely. Proteins and peptides with anti-microbial properties have been reported in leaf washes and guttation fluid from a number of species however (Young *et al.* 1995; Grunwald *et al.* 2003; Shepherd *et al*. 2005). The deposition of such peptides on the leaf surface is likely to be influenced by localized water distribution and flow and could result in channelling of leaf surface water along the areas of lower hydrophobicity e.g. vein structures (Leben C. 1988) conceivably leading to increased localised concentrations of antimicrobial peptides in these regions potentially contributing to the observed negative correlation of *Salmonella* attachment with vein density.

Leaf wax levels have been shown to directly influence adherence of microorganisms to leaf surfaces (Beatie 2002). A negative correlation (P 0.027) was observed between *Salmonella* levels and hydrophobicity. There was, however, no significant correlation between overall levels of surface wax and hydrophobicity (*P* 0.479) as might be expected, suggesting that total wax content was not the factor determining the hydrophobicity of the leaf surface.

Successional differences in epicuticular leaf wax composition during leaf development have been shown in cherry laurel (*Prunus laurocerasus*) (Jetter and Schäffer

2001). These changes, detectable in leaves as young as 10 days old, could potentially result in different levels of surface hydrophobicity without significant impact on total epicuticular wax levels. Leaf surface SEM images of *L. serriola* and the two *L. sativa* cultivars appeared to show physically different wax micro-structures (Fig 2). Although certain wax components have been associated with specific wax crystalloid micro-structures (Barthlott *et al.* 1998), the observed differences did not correspond with *Salmonella* attachment from older leaves: Counts were significantly higher from cv. Saladin (*L.sativa*) than from cv. Iceberg, whilst counts from cv. Iceberg and *L. serriola* (showing different wax morphology) were not significantly different. Differences in wax composition between closely related species such as *Thellungiella halophila* and *T. Parvula* have been reported (Teusnik *et al.* 2002), which did not result in any observable differences in leaf waxiness however: It is possible that such differences could result in leaf surface environments with different hydrophobicity. Furthermore, the epicuticular wax layer of leaves erodes over time (Beatie 2002). Such erosion could produce different micro-environments on the leaf surface and eroded surfaces may also provide additional physical niches for bacterial attachment.

In summary, different factors appear to influence attachment of *Salmonella* to lettuce leaves and subsequent proliferation, although some factors (e.g. venation) may be common to both phases of colonization. There may also be considerable serovar-specific differences in attachment mechanisms and it may not be possible to generalise from one serovar to others. In respect of *Salmonella* Senftenberg and lettuce leaves, the hydrophobic properties of the epicuticular wax layer appears to be significant, although wax layer composition or the level of wax erosion (or a combination of both) may be more important than the total amount of wax on the leaf surface.

All of these features will have some level of underlying genetic control. The fact that the lines used in this study are the parents of genetic mapping populations will facilitate

detailed investigations of the underlying genetics of epicuticular wax and other leaf surface characteristics. Determination of quantitative trait loci (QTL) for these characteristics and the use of transcriptomics technology to identify up- or down-regulated genes should allow specific targets to be identified. In parallel, QTL for attachment of *Salmonella* (or other pathogens of interest such as *E. coli*) may also be beneficial in targeting future genetic investigations. Since both the genetic mapping populations and a lettuce genome sequence are in the public domain, candidate genes underlying any QTL could be identified and the associated plant biochemical pathways investigated.

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Table 1. Bacterial counts and leaf surface characteristics for 1st and 5-6th true leaves from three lettuce accessions, showing results of ANOVA tests between leaf age categories with subsequent Spearman rank correlation coefficients and associated probabilities for association of characteristics with *Salmonella* counts.

	Salmonella Senftenberg (cfu ml^{-1})	Phenolic content (mg g^{-1})	Surface Protein (ng cm^{-2})	Surface Wax (µg cm^{-2})	Surface Sugars (µg cm^{-2})	Contact Angle (o)	Inter-vein distance (mm)	Stomatal density ($N^{o.}$ mm^{-2})	Cell perimeter density (mm mm^{-2})
Saladin (1st leaf)	21333[a]	81.8[a]	3.59[b]	409.1[d]	6.67[d]	54.8[c]	4.2[b]	165.2[c]	240.2[b]
Saladin (5-6th leaf)	54333[c]	97.1[a]	0.52[a]	28.6[a]	2.23[b]	55.4[d]	9.7[d]	79.8[a]	135.2[a]
Iceberg (1st leaf)	13166[a]	81.8[a]	1.43[a]	123.8[b]	2.19[b]	43.9[a]	6.6[c]	115.1[b]	199.4a[b]
Iceberg (5-6th leaf)	34166[b]	95.7[a]	0.18[a]	8.1[a]	0.87[a]	50.2[b]	12.5[e]	72.4[a]	162.2[a]
Serriola (1st leaf)	10816[a]	88.7[a]	7.18[c]	190.6[c]	8.13[e]	134.6[f]	2.9[a]	293.1[d]	153.9[a]
Serriola (5-6th leaf)	32666[b]	118.3[b]	1.56[a]	8.1[a]	2.75[c]	129.0[e]	6.8[c]	89.0[a]	132.7[a]
No. of plants	6	5	5	5	5	5	5	5	5
ANOVA (age)	<0.0001***	0.0031**	<0.0001***	<0.0001***	<0.0001***	<0.0001***	<0.0001***	<0.0001***	0.0090**
l.s.d. (P 0.05)	4821	20.2	2.68	21.8	0.08	0.2	0.2	23.1	68.0
Spearman correlation R		0.328	-0.780	-0.597	-0.506	-0.731	0.739	-0.795	-0.322
Spearman correlation P		0.116	0.044*	0.056	0.070	0.027*	0.035*	0.042*	0.172

*, **, *** - Results significant at 0.05, 0.01 and 0.001 level respectively (no asterisks indicate non-significant results), l.s.d. (P 0.05) - least significant difference between means representing a significant difference at the 0.05 level, R – Spearman rank correlation coefficient (vs. *Salmonella* Senftenberg counts), P- probability (significance of correlation).

Table 2. Bacterial counts and leaf surface characteristics for 5-6th true leaves from three lettuce accessions, showing results of ANOVA tests between accessions with subsequent Spearman rank correlation coefficients and associated probabilities for association of characteristics with *Salmonella* counts.

	Salmonella Senftenberg (cfu ml^{-1})	Phenolic content (mg g^{-1})	Surface Protein (ng cm^{-2})	Surface Wax (µg cm^{-2})	Surface Sugars (µg cm^{-2})	Contact Angle (o)	Inter-vein distance (mm)	Stomatal density (N$^{o.}$ mm^{-2})	Cell perimeter density (mm mm^{-2})
Saladin (5-6th leaf)	54333^{b}	97.1	0.52^{a}	28.6^{b}	2.23^{b}	55.4^{b}	9.7^{b}	79.8	135.2
Iceberg (5-6th leaf)	34166^{a}	95.7	0.18^{a}	8.1^{a}	0.87^{a}	50.2^{a}	12.5^{c}	72.4	162.2
Serriola (5-6th leaf)	32666^{a}	118.3	1.56^{b}	8.1^{a}	2.75^{c}	129.0^{c}	6.8^{a}	89.0	132.7
No. of plants	6	5	5	5	5	5	5	5	5
ANOVA (cv)	0.0122*	0.6000	0.0165*	<0.0001***	<0.0001***	<0.0001***	<0.0001***	0.3022	0.3000
l.s.d. (P 0.05)	8962	nt	0.53	5.3	0.40	3.1	1.1	nt	nt
Spearman correlation R		nt	-0.357	0.668	-0.151	-0.747	0.420	nt	nt
Spearman correlation P			0.063	<0.0001***	0.187	<0.0001***	0.0009***		

*, **, *** - results significant at 0.05, 0.01 and 0.001 level respectively (no asterisks indicate non-significant results), l.s.d. (P 0.05) - least significant difference between means representing a significant difference at the 0.05 level, nt – not tested further since ANOVA revealed no significant differences in characteristic between accessions. R – Spearman rank correlation coefficient (vs. *Salmonella* Senftenberg counts). P-probability (significance of correlation).

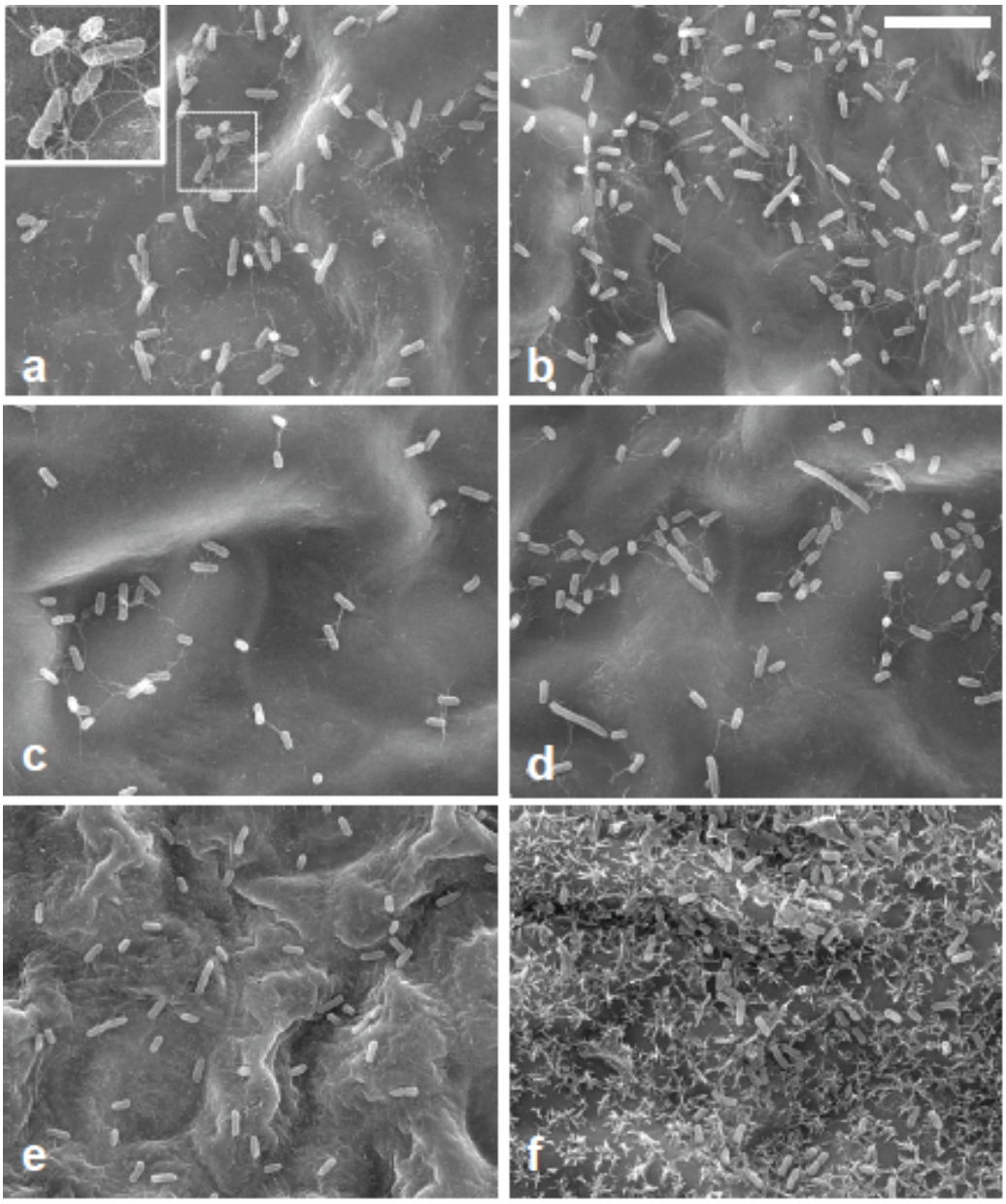

Figure 1. Scanning electron micrographs of *Salmonella enterica* serovar Senftenberg attachment to lettuce cultivars Saladin (a, b), Iceberg (c, d) and *L. serriola* US96UC23 (e, f) on 1st true leaves (right-hand panels) and 5th true leaves (left-hand panels). All lines supported a diffuse adhesion pattern at both growth stages, often associated with filamentous networks (panel a inset). Bar = 10µm. All samples were taken rom from the centre of the leaf lamella:

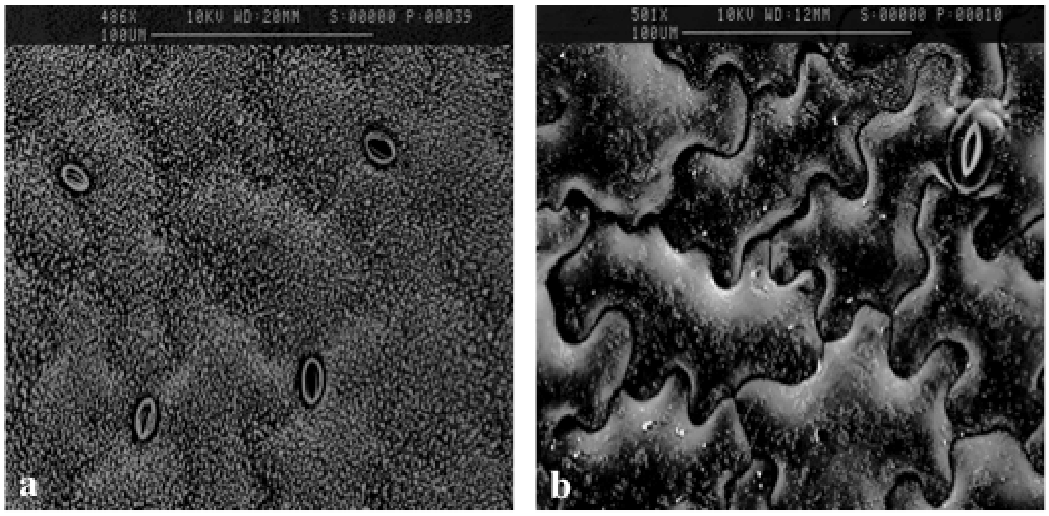

Figure 2. Scanning electron micrographs of the upper surfaces of the 5th true leaf of lettuce cultivar Iceberg (a) and accession *L. serriola* US96UC23 (b), showing different wax layer structures in *L. serriola* compared to the other two cultivars as typified by cv. Iceberg. The white arrows indicate stomata. The dark lines are the periphery of individual cells.