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Article

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Contamination of microgreens by *Salmonella enterica* and *Escherichia coli* is influenced by selection breeding in chicory (*Cichorium intybus* L.)

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Abstract

The aim of this study was to assess whether selection breeding in chicory (*Cichorium intybus* L.) led changes in the susceptibility to *Salmonella enterica* and *Escherichia coli* contamination and whether the anatomical traits of the leaves are involved in the possible changes. Five chicory genotypes subjected to different intensities of selection were compared at the microgreen stage. Bacterial retention was evaluated after leaf incubation for 1.5 h on the surface of the bacterial suspension, followed by rinsing, grinding, plating on selective media, and colony forming unit (CFU) counting. The density of stomata and trichomes, total stomatal length and width, stomatal pit width, surface roughness and sharpness were evaluated. The intensively selected genotype (Witloof) was significantly more prone to contamination (2.9±0.3 lg CFU/cm^2) as the average of the two bacterial types than the wild accession (Wild; 2.3±0.4 lg CFU/cm^2) and the moderately selected genotypes (two leaf chicories, Catalogna type, and root chicory 'Magdeburg'; on average, 1.9±0.3 lg CFU/cm^2). Witloof microgreens also showed larger stomata (on average +34% for stoma width and +44% for pit width), which could justify, at least in part, the higher susceptibility to enterobacterial contamination. In fact, when contamination was performed in the dark (closed stomata), the bacterial retention in Witloof was significantly reduced in comparison with the opened stomata (–44%) and in Wild (–26%). Differences in retention between Witloof and Wild were still observed after UV treatment. The hierarchical clustering performed by grouping the leaf anatomical features was consistent with the chicory genetic groups. Our results suggest that the domestication process can affect the safety of produce and that the micromorphological traits of the leaves may be involved.

Keywords: chicory genotypes; enterobacteria; contamination; raw-consumed vegetables; micro vegetables; selective breeding; food safety; stoma.

Introduction

Selective breeding has steadily contributed to the genetic enhancement of crop plants during recent centuries. The artificial selection process is addressed to strengthen specific traits, such as yield, tolerance/resistance to phytopathogens and abiotic stress conditions, organoleptic properties, chemical composition, etc. (Heckenroth *et al.*, 2016). However, during the selection process, some favourable or unfavourable traits may be unintentionally lost or selected (Tanksley and McCouch, 1997), including traits possibly involved in the contamination of produce by human pathogens.

It is known that produce can host several human pathogens (e.g. *Salmonella*, *Shigella*, *Listeria* or pathogenic *Escherichia coli*) without exhibiting any sign of spoilage (Barak and Schroeder, 2012), which raises concerns about food safety (Aiyedun *et al.*, 2020). The main risks originate from raw-consumed products such as fruits and vegetables, whose contamination with foodborne pathogens has often resulted in international outbreaks causing multimillion-dollar damage to the fresh produce industry (Heaton and Jones, 2008; Marshall *et al.*, 2020). Among vegetables, leafy greens were often involved in those outbreaks in association with both *E. coli* (Herman *et al.*, 2015; Kintz *et al.*, 2019) and *Salmonella enterica* (Hanning *et al.*, 2009; Herman *et al.*, 2015). Microgreens are an emerging salad crop consisting of tender immature greens harvested within 10–20 days from seedling emergence when the first pair of true leaves are more or less developed (Bulgari *et al.*, 2017). Microgreens are thought to be a potential vector for foodborne pathogens (Riggio *et al.*, 2019). To date, no outbreaks associated with them have been reported; however, the occurrence of recalls due to their contamination by *Salmonella* and *Listeria* (Misra and Gibson, 2020) recommends studies on this topic.

Some differences in susceptibility to the attachment and retention of enterobacteria have been noticed between different crop species, including at the microgreen stage (Wright and Holden, 2018) and different cultivars of the same species (Lenzi *et al.*, 2021). Nevertheless, specific traits involved in the observed variability have seldom been investigated (Devleesschauwer *et al.*, 2017; Henriquez *et al.*, 2020; Lenzi *et*

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al., 2021). In spinach, different colonization efficiencies in different cultivars were ascribed to different leaf surface morphologies, including roughness and stomatal density (Macarisin *et al.*, 2013; Mitra *et al.*, 2009). Jacob and Melotto (2020) found significant variations among 11 lettuce genotypes in bacterial leaf attachment and internalization and a significant correlation of the internalization rate with the stomatal pore width and area.

Chicory (*Cichorium intybus* L.) is a herbaceous perennial plant both wild and cultivated for different uses (Tesi, 2010), including microgreens (Di Gioia *et al.*, 2017; Teng *et al.*, 2021). Native to Eurasia, it has a cosmopolitan distribution, and its use as a wild vegetable is widespread, especially in the Mediterranean region (Tardío *et al.*, 2016). According to the classification of Kiers *et al.* (2000), the cultivated forms of *C. intybus* can be divided into three groups: (1) leaf chicory group (leafy vegetable consumed fresh or cooked); (2) root chicory group, characterized by a large taproot (originally used to obtain a coffee substitute, currently mainly grown for inulin production but, especially in Italy, also consumed as a vegetable); and (3) Witloof group (a vegetable formed by a tight head of etiolate leaves obtained by forcing the root under artificial conditions). The Witloof group is thought to have originated in approximately 1850 from 'Magdeburg', an old root chicory cultivar (Raulier *et al.*, 2016). Cultivated forms are all genetically different compared to the wild form, with groups 1 and 2 being subjected to less intensive selection than Witloof chicory (Raulier *et al.*, 2016).

In this study, we hypothesized that chicory genotypes may vary in susceptibility to *Salmonella enterica* and *E. coli* contamination due to selection breeding and that the surface topography and micromorphological traits of the leaf may play a crucial role in the differences in retention. We tested these hypotheses at the microgreen stage, compared the retention of *S. enterica* and *E. coli* in artificially contaminated microgreen leaves of wild chicory and domesticated genotypes, and analysed leaf micromorphology by scanning electron microscopy (SEM).

Materials and Methods

Plant materials and bacterial strains

As starting plant material, seeds of five chicory genotypes, including one wild accession and four domesticated genotypes subjected to more or less intensive selection, were used: (a) local wild chicory (Wild), whose seeds were collected in the wild on the banks of Arno River in the Florence area (Italy; nonselected genotype); (b) leaf chicory Catalogna type accession 1, from Fratelli Ingegnoli, Milan, Italy (Leafy accession 1); (c) leaf chicory Catalogna type accession 2, from B&T World Seed, Aigues-Vives, France (Leafy accession 2); (d) root chicory 'Magdeburg', from Fratelli Ingegnoli, Milan, Italy (Magdeburg; moderately selected genotypes); and (e) Witloof type (Witloof), from Fratelli Ingegnoli, Milan, Italy (intensively selected genotype).

After sowing, seeds were kept in the dark at 20 °C for 48 h to promote germination. Seedlings were hydroponically grown in a floating system in a growth chamber at 25±2 °C (day) and 17±2 °C (night) with a photoperiod of 16 h under fluorescent lighting units OSRAM L36 W/77 (Osram, Munich, Germany; 36 W, 120 cm in length, 26 mm in diameter) up to the microgreen stage (16 days after sowing). A half-strength Hoagland's nutrient solution (macroelements expressed in mmol/L and microelements in µmol/L: N 7.5, P 0.5, K 3.0, Ca 2.5, Mg 1.0, Fe 25.0, B 23.1, Mn 4.6, Zn 0.39, Cu 0.16, Mo 0.06; pH: 5.56; CE: 1.12 mS/cm) was used. Microgreen leaves were cut at the base of the petiole and immediately used for contamination. The average fresh weight, dry weight, and leaf area of the leaves are reported in Table 1. Fresh weight and dry weight were measured before and after oven desiccation at 80 °C for 48 h or until a constant weight. Leaf area was determined by a LI-3000 planimeter (LI-COR, Lincoln, NE, USA).

Table 1. Fresh weight (FW), dry weight (DW) and leaf area (LA) of the chicory leaves exposed to enterobacterial contamination.

Chicory genotype	FW (mg)	DW (mg)	LA (cm^2)
Wild	25.37±0.99	1.88±0.08	1.86±0.07
Leafy accession 1	28.37±1.12	1.94±0.10	2.03±0.07
Leafy accession 2	26.68±0.98	2.05±0.11	2.12±0.06
Magdeburg	27.64±0.90	1.88±0.12	2.51±0.10
Witloof	23.12±0.90	1.33±0.10	1.56±0.08

Data are means of 18–31 replicates±SE.

Salmonella enterica serovar Typhimurium ATCC 14028 and *Escherichia coli* ATCC 35218 were used for contamination. *S. enterica* is a pathogen; *E. coli* 35218, although classified as biosafety level 1, has specific virulence genes associated with different *E. coli* pathotypes related to human and animal infections, such as *fimH*, *papA*, *papC*, *papG*, *papE*, *sfaS*, *hlyA* (gene for α-haemolysin), *kpsM*, *fyuA*, and *ompT* (Chapman *et al.*, 2006).

Surface contamination of microgreens

All the bacterial inocula started from –20 °C glycerol stocks. One millilitre from an overnight inoculum in lysogeny broth (Oxoid, Hampshire, UK) was washed three times in sterile physiological solution (PS; NaCl 0.85% (mass concentration) in H_2O) to clean the cells. Washed cells were diluted in PS to obtain the working bacterial suspension [containing approximately 10^7 cells/mL, which allowed easy counting of colony-forming units (CFU) on Petri plates]. For the contamination protocol, we updated the method previously adopted by Barak *et al.* (2007). Thirty millilitres of the working bacterial suspension was transferred into empty Petri plates (Fisher Scientific, Rodano, Italy). For each genotype, 7 leaves per Petri plate were placed onto the surface of the suspension on the adaxial side, keeping the petioles out from the plates to avoid contamination through the cut tissue. Leaves were incubated for 1.5 h at 25 °C under static conditions. After incubation, the leaves were washed to remove unattached bacterial cells. Three Petri plates for each set of 7 leaves were filled with 30 mL of sterile PS. The leaves were therefore placed on the adaxial side on the first Petri plate and gently washed by 30 rotations; then, the same procedure was repeated on the second and third plates. Rinsed leaves were ground with a mini-pestle (into 1.5-mL tubes) in 0.5 mL of PS. After grinding, 20 µL was plated onto selective and differential media xylose lysine desoxycholate agar (XLD; Oxoid, Hampshire, UK) for *Salmonella* and MacConkey agar (Oxoid, Hampshire, UK) for *E. coli* and incubated at 37 °C overnight. CFU were counted and log base 10 (lg) transformed. At least 5 biological replicates (different

seeding sets) and 7 technical replicates (leaves from the same seeding set) were used. To account for different leaf sizes of the tested genotypes (Table 1), CFU were normalized per cm^2 of leaf area.

Contamination of leaves with closed stomata was performed by using *E. coli*. Witloof and Wild microgreens were kept in the dark for 24 h before the contamination experiments. The contamination was performed as described above in a dark room with a red light to avoid stomatal opening. Aperture and closure of stomata were confirmed by using a Leica optical microscope at 1×200 magnification. Three biological replicates and 6 technical replicates were performed. To account for the different leaf sizes of the two genotypes (Table 1), CFU were normalized per cm^2 of leaf area.

Witloof and Wild microgreens contaminated as described above were also UV-treated to decontaminate them on the surface. Five leaves per accession were exposed for 5 min under a UV lamp (UVC≥90% with 108.4 μW/cm^2 from 0.5 m) placed at a distance of approximately 10 cm. Unexposed leaves were used as negative controls, while as positive controls, three separate drops of 30 μL of 10^8 *E. coli* cell/mL inoculum were placed onto plastic and exposed to the same UV treatment. After exposure, cells were harvested, and CFU were counted, normalized per cm^2 of leaf area and lg converted as mentioned above.

SEM analysis

Scanning electron microscopy was used to analyse the following micromorphological traits of the adaxial leaf page of the microgreens of the different chicory genotypes: density of stomata, density of trichomes, and stomata size (length, width, and stoma pit width). Before SEM observations, fresh leaf samples were coated with a thin layer of gold (about 10 nm) to reduce shrinkage while ensuring the preservation of cell structures as close to the natural state as possible (Pathan *et al.*, 2010). Nine observations (3 leaves and 3 sections per leaf) were performed in each genotype. Photographs at two different magnifications were taken: 1×300 for counting stomata and trichomes and 1×600 for measuring stomata sizes. Stomata and trichomes were counted on the images (view field ranging from 920 to 923 μm^2), and then the number per surface unit (1 mm^2) was calculated by a simple proportion. A millimetre ruler was used to measure stomata size; the measure was expressed in μm by means of a proportion considering the bar of known length present in each image.

SEM image analysis to test the roughness and sharpness of the leaf surface

Roughness (Roughness index, Ra) and sharpness (Kurtosis index, Rku) were measured by using the plugin SurfCharJ 1q (www.gcsca.net) installed in the ImageJ package (NIH, Bethesda, CA, USA; Chinga *et al.*, 2007). Fifteen 1×300 magnification SEM images for each genotype were processed through background subtraction and conversion to 32 bits for each image. Then, SurfCharJ 1q was used, choosing the options 'level surface' and 'local roughness analysis' (=2.54 cm).

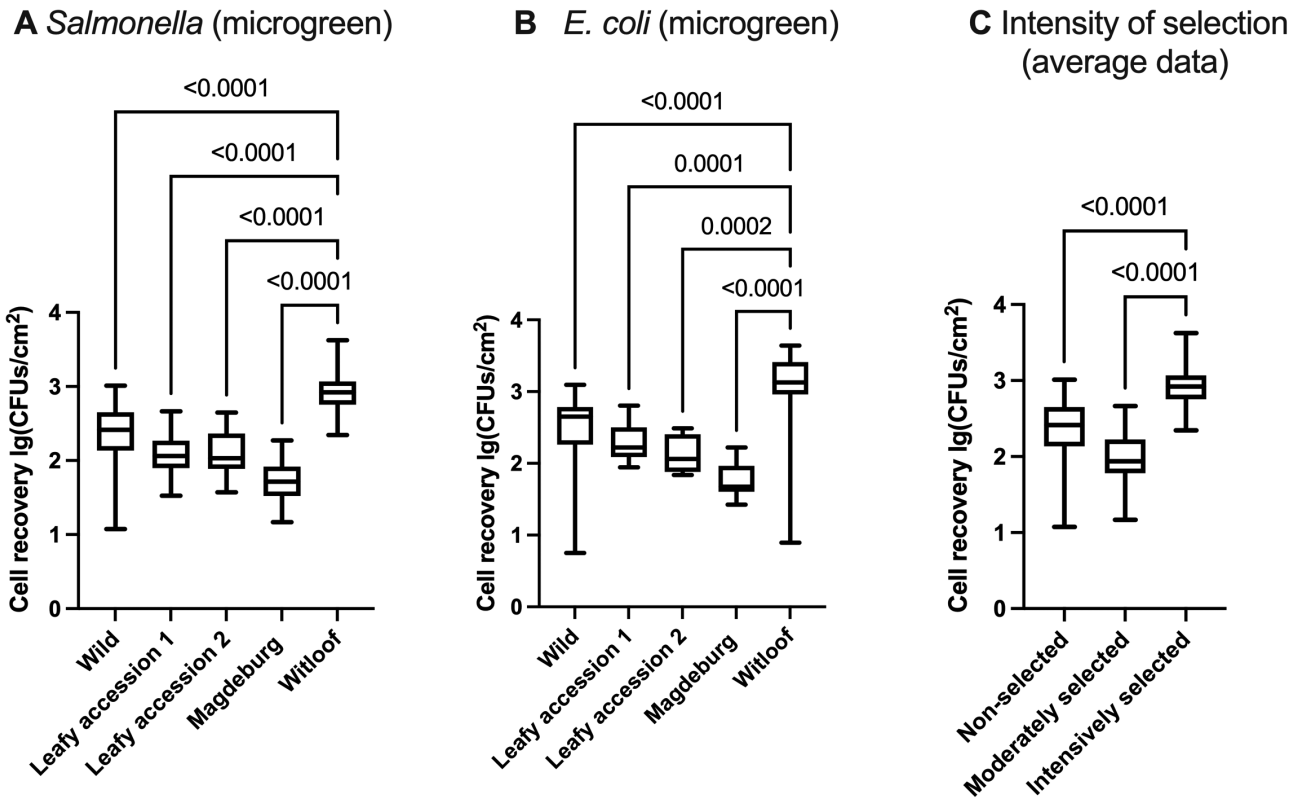

Figure 1. Recovery of ***Salmonella*** and ***E. coli*** cells from microgreen leaves of different chicory genotypes. In (C) data are averaged for the three moderately selected genotypes (Leafy accession 1, Leafy accession 2, and Magdeburg) and for *Salmonella* and *E. coli*. In box plots, the boundaries of boxes include the lower and upper quartiles, the thick line within the box is the median, and whiskers indicate the degree of dispersion of the data. Horizontal lines represent pairwise statistically significant differences, and values are the *P* values ($P<0.05$); n.s.: not significant. Number of replicates=18.

As a negative control, a grey flat image was used, resulting in no roughness or sharpness.

Statistical analyses

PRISM 9.0 (GraphPad Software Inc., San Diego, CA, USA) was used to carry out analysis of variance (ANOVA) and post-hoc Tukey's test (data in Figures 1–4) for $P<0.05$. IBM SPSS v.27 (IBM, Armonk, NY, USA) was used for hierarchical clustering based on micromorphological features (density of stomata and trichomes, stomata total length and width, stoma pit width, Ra and Rku) for the data presented in Figure 4.

Results

Escherichia coli and *Salmonella enterica* retention was assessed in different chicory genotypes (subjected to different intensities of selection) after the surface contamination of microgreens. The intensively selected Witloof significantly retained more *S. enterica* cells (2.9±0.3 lg CFU/cm^2) and *E. coli* cells (3.0±0.5 lg CFU/cm^2) than the other genotypes (Figures 1A and 1B). The lowest values were found in Magdeburg (1.7±0.3 lg CFU/cm^2 and 1.7±0.2 lg CFU/cm^2 for *Salmonella* and *E. coli*, respectively). When data were averaged for the three moderately selected genotypes and the two bacteria, Witloof was confirmed to be significantly more

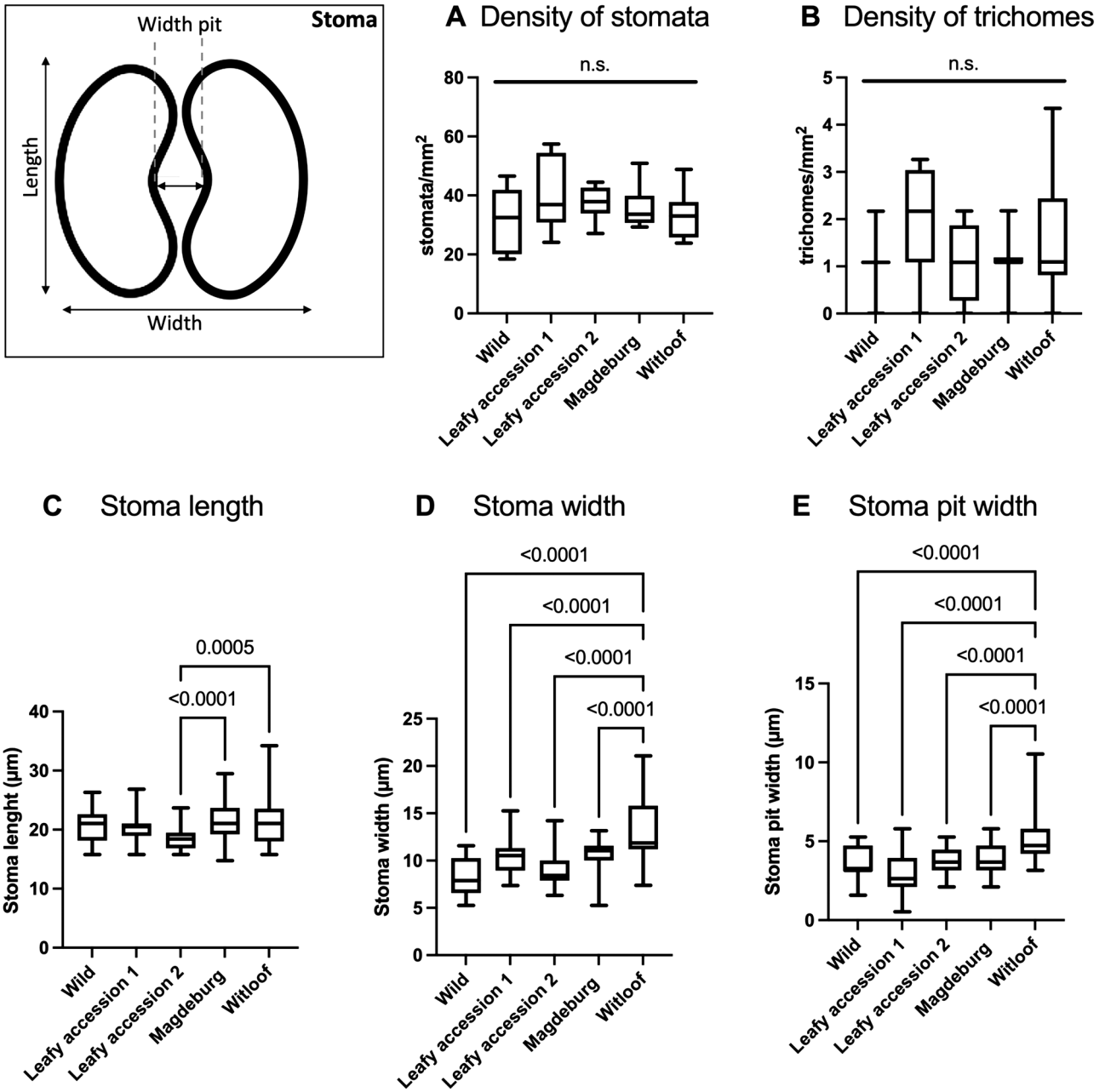

Figure 2. Stomata density, trichome density, and stomatal size (length, width, and stoma pit width, as shown in the picture on the top left) in microgreen leaves of different chicory genotypes. In box plots, the boundaries of boxes include the lower and upper quartiles, the thick line within the box is the median, and whiskers indicate the degree of dispersion of the data. Horizontal lines represent pairwise statistically significant differences, and values are the *P* values ($P<0.05$); n.s.: not significant. For panels A and B, the number of replicates=9; panels C to D, the number of replicates from 25 to 48.

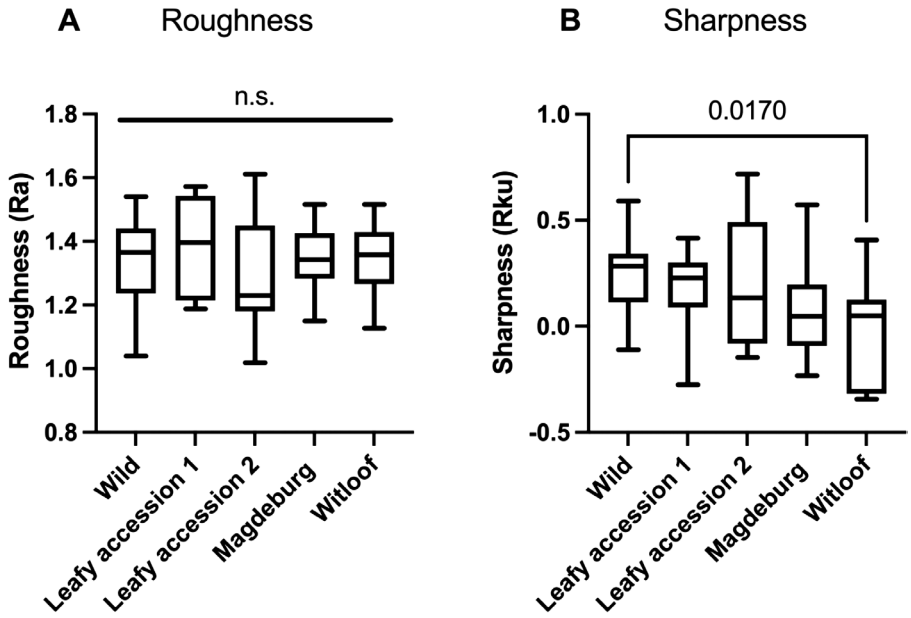

Figure 3. Leaf surface roughness (Roughness index, Ra) and sharpness (Kurtosis index, Rku) in microgreens of different chicory genotypes. Indexes were calculated based on SEM images. Higher Ra values indicate higher roughness; lower Rku values indicate higher sharpness. In box plots, the boundaries of boxes include the lower and upper quartiles, the thick line within the box is the median, and whiskers indicate the degree of dispersion of the data. Horizontal lines represent pairwise statistically significant differences, and values are the *P* values ($P<0.05$); =15. n.s.: not significant.

prone to contamination (2.9±0.3 lg CFU/cm^2) versus both the wild genotype and the moderately selected group ($P<0.001$; 2.3±0.4 and 1.9±0.3 lg CFU/cm^2, respectively; Figure 1C).

The micromorphological traits of the microgreen leaves (density of stomata and trichomes, stomata total length and width, stoma pit width, Ra and Rku) were then evaluated as possible factors responsible for the observed differences in retention. No significant differences between genotypes were found in the density of stomata and trichomes of the leaves (Figures 2A and 2B). In contrast, the stomata of the different genotypes showed significant differences in size. Witloof and Magdeburg showed longer stomata (21.4±4.2 µm and 21.8±3.3 µm in length, respectively) than Leafy accession 2 (18.5±1.9 µm; Figure 2C). Witloof had larger stomata in terms of width (13.1±2.9 µm) and stoma pit (5.1±1.4 µm) than all the other genotypes (Figures 2D and 2E). The assessment of the level of roughness (Ra) and sharpness (Rku) by the image analyses on SEM micrographs revealed that all the genotypes were equally rough (Figure 3A), while in terms of sharpness, Magdeburg and Witloof were significantly steeper when compared to the Wild accession (Figure 3B). The considered leaf traits were also used to construct a hierarchical clustering, and the clusters matched chicory genotypes. In fact, the cultivated accessions were separated according to the respective genetic group (leaf chicory, root chicory, witloof). The wild accessions clustered with Leafy accession 2 (Figure 4).

Finally, to assess the role of stomata in bacterial retention, two further experiments were conducted with *E. coli* in Witloof and Wild accessions: contamination was performed in the dark (closed stomata), and a second experiment was the UV treatments. When *E. coli* contamination was performed in leaves with closed stomata, the bacterial retention in Witloof was significantly reduced (1.7±0.2 lg CFU/cm^2) in comparison with the opened-stomata condition (3.0±0.1 lg CFU/cm^2) and resulted similar to the retention in the Wild

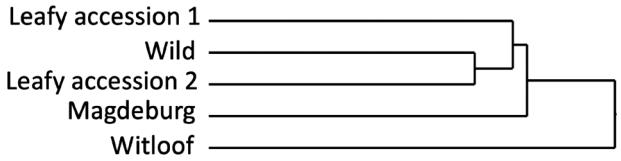

Figure 4. Hierarchical clustering groups of different chicory genotypes. The following traits measured in microgreen leaves were used for clusterization: density of stomata and trichomes, stomata total length and width, stoma pit width, Ra and Rku.

accession (1.7±0.1 and 2.3±0.1 lg CFU/cm^2 in the dark and in the light condition, respectively; Figure 5A). After UV treatment, the surface of Witloof retained significantly more cells than the wild accession (1.5±0.2 and 0.8±0.1 lg CFU/cm^2, respectively; Figure 5B).

Discussion

During the past years, several enterobacterial outbreaks with vegetables as the source, including sprouts, salads, and leafy greens, have occurred (Herman *et al.*, 2015; Kintz *et al.*, 2019; Marshall *et al.*, 2020). Sources for produce contamination include water, wild animals, contaminated seeds, and inappropriate agricultural or handling practices, posing risks to food safety at different steps from farm to fork (Xiao *et al.*, 2014; Jay-Russell and Doyle, 2016; Lenzi *et al.*, 2021). Cultivation, harvest, processing, transporting, retail and food service establishments, and even handling in the home kitchen are all sensitive stages (Delibato *et al.*, 2018). In soilless cultivation systems, which are usually used to produce microgreens (Di Gioia *et al.*, 2017), human pathogens can be internalized within plant tissues via the uptake of contaminated nutrient solution through the root system or reach the aerial parts through substrates, seeds, ventilation systems (in hydroponics

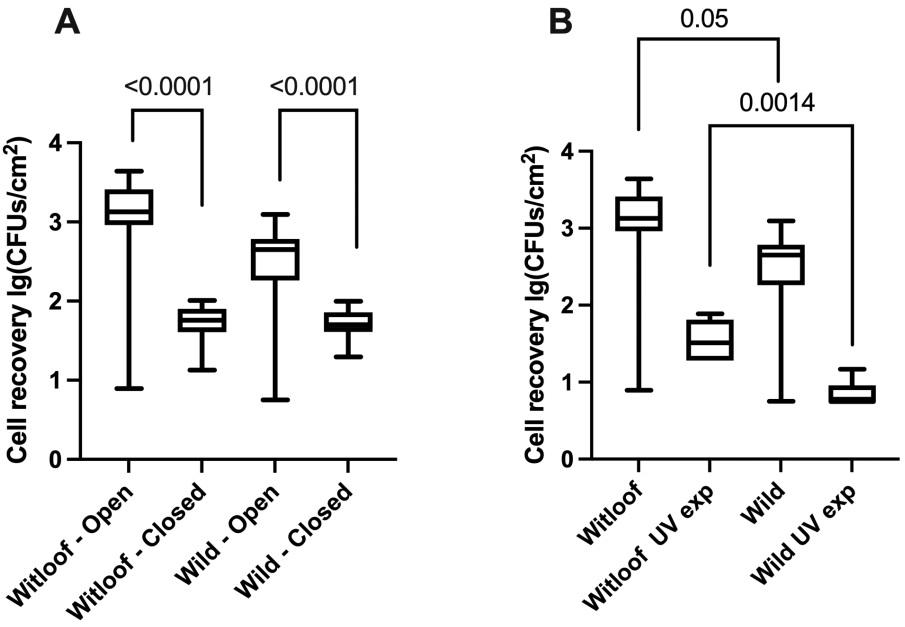

Figure 5. Contribution of stomata to leaf contamination. (A) Recovery of *E. coli* cells in microgreens of wild and witloof chicory after incubation of leaves with open versus closed stomata. (B) Surviving populations of *E. coli* on microgreen of wild and witloof chicory following UV treatment with single-sided exposure at a 10-cm distance between the sample and the lamp. In box plots, the boundaries of boxes include the lower and upper quartiles, the thick line within the box is the median, and whiskers indicate the degree of dispersion of the data. Horizontal lines represent pairwise statistically significant differences, and values are the *P* values ($P<0.05$). Number of replicates=5.

housed in built environments), insects and small reptiles accidentally accessing the system, which are all possible routes of contamination (Lenzi *et al.*, 2021).

Modern vegetable cultivars are selected for high yield, tolerance to biotic and abiotic stress, and specific quality traits, while the issue of preventing contamination by human pathogens has not yet been explored. Recently, the importance of including food safety among the goals of breeding has been proposed (Henriquez *et al.*, 2020; Jacob and Melotto, 2020).

In this work, we show that selection intensity exerts a role in the contamination potential of chicory microgreens by *Salmonella* and *E. coli*. Higher bacterial retention following artificial contamination was observed in the most intensively selected chicory genotype, namely, Witloof. In addition, stomata seemed to be involved in the differences in contamination observed between genotypes according to selection intensity. The chicory genotypes did not differ in stomatal densities, but the stomatal size was higher in Witloof microgreens than in the moderately selected genotypes and the wild accession. A similar shift in stomatal traits associated with plant domestication was observed by Milla *et al.* (2013). These authors studied stomatal density and size in 24 herbaceous species, including chicory, and observed that on the adaxial side of the leaf (which was the side subjected to contamination in our study), the number of stomata did not change, but their dimensions increased in cultivated plants compared to their wild ancestors (Milla *et al.*, 2013). We believe that the larger stomata we observed in the intensively selected chicory genotype could justify, at least in part, its higher susceptibility to *S. enterica* and *E. coli* contamination. Additionally, in lettuce, genotypes less susceptible to contamination by *S.* Typhimurium 14028 and *E. coli* O157:H7 showed lower stomatal sizes (Jacob and Melotto, 2020). Several studies have demonstrated that stomata are involved in *Salmonella* and *E. coli* leaf retention. Most of the studies focused on lettuce (Gomes *et al.*, 2009; Kroupitski *et al.*, 2009; Goldberg *et al.*, 2011; Jacob and Melotto, 2020), but other leafy vegetables, such as arugula, basil, parsley, and spinach, were also investigated (Golberg *et al.*, 2011; Saldaña *et al.*, 2011). In these studies, bacteria were found on stomata, within and underneath the stomata cavity, and on the crevices in proximity to them. In our research, when *E. coli* contamination was performed in the dark (closed stomata), retention in Witloof and Wild decreased significantly in comparison with retention in the light (open stomata), and no difference was observed between the two genotypes. When UV light was used to decontaminate the surface, allowing only the internalized cells to survive, Witloof (larger stomata pit width) retained more *E. coli* cells when compared with the wild. Internalization through stomatal pores has practical implications because in this case, bacteria can withstand washing and even survive disinfection treatments, posing serious problems to the ready-to-eat industry (Gomes *et al.*, 2009; Kroupitski *et al.*, 2009). Takeuki and Frank (2001) observed that the viability of *E. coli* O157:H7 on lettuce leaves after chlorine treatment was higher for bacteria that had entered stomata than for cells on the leaf surface.

The density and size of stomata are not the only risk factors when considering plant contamination by human pathogens. Indeed, trichomes, vein areas, surface irregularities, hydrophobicity, cuticular waxes, and surface proteins and sugars can be associated with different attachment rates (Brandl *et al.*, 2013; Hunter *et al.*, 2015; Ku *et al.*, 2020). In spinach cultivars, a study of leaf topography showed that both a high density of stomata and leaf blade roughness increased leaf contamination by *E. coli* O157:H7 (Macarisin *et al.*, 2013). In contrast, in our study, the roughness of the adaxial leaf

page was not significantly different among genotypes. The steepness of peaks measured as Rku were instead sharper for Magdeburg and Witloof than for Wild, but the role of this parameter was unclear.

Interestingly, the hierarchical clustering performed by clustering all the anatomical features considered in our study (density of stomata and trichomes, stomata total length and width, stomatal pit width, Ra and Rku) was consistent with the genetic differentiation and the constitution of genetic groups within the cultivated germplasm of *C. intybus* (leaf cultivars, root cultivars, and witloof) demonstrated by the study of Raulier *et al.* (2016).

Conclusions

The domestication process not only influences agronomic traits but can also accidentally affect the safety of produce. Breeders should not ignore the possibility that some traits promoting contamination may be unintentionally selected during the process. On the other end, traits possibly hindering contamination could be intentionally selected to make vegetable produce safer. From this point of view, stomata and leaf morphology are important traits that should be considered during the breeding of leafy vegetables. Bigger stomata as measured in the microgreens of the intensively selected Witloof may have practical implications allowing bacteria to find a protected niche where to persist during the washing/disinfection steps. Further research needs to be addressed to understand if and to what extent other changes in morphological traits due to selective breeding may affect the persistence and to find possible ways to counteract the contamination potential. In addition, knowledge of such selected traits can help to improve washing procedures and to develop new technologies (Marvasi, 2017) for the processing of ready-to-eat vegetables.

Author Contributions

Conceptualization and methodology: Anna Lenzi, Ada Baldi, Massimiliano Marvasi, Piero Bruschi; Performing the experiment: Letizia Lombardelli, Stefania Truschi, Massimiliano Marvasi; Data curation: Massimiliano Marvasi; Writing original draft preparation: Anna Lenzi, Massimiliano Marvasi, Piero Bruschi; Writing, review and editing: Ada Baldi, Stefania Truschi. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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