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# Food safety and vegetables: contamination of baby-leaf salads by human pathogens

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## Abstract

Baby leaves are leafy vegetables harvested at an early stage of growth (up to the eighth true leaf) and consumed as salads. They can be marketed as unprocessed products or, more commonly, subjected to minimal processing and sold in a convenient ready-to-eat form, which offers the advantage of ease of consumption. It is well known that vegetables may host human pathogens like *Salmonella enterica* and *Escherichia coli*, possibly leading to the occurrence of foodborne diseases. From this point of view, the consumption of salads may raise particular concerns as they are eaten raw and often without prior proper washing. This thesis presents the results of three experiments on the issue of foodborne diseases in baby-leaf salads, with the final objective to provide information useful for increasing the safety of these products. The first and the second experiments aimed to highlight possible differences in the susceptibility to human pathogens contamination between 30 accessions of baby leaves belonging to different species and/or varieties and to understand the relationship between the susceptibility and leaf traits. In the first experiment the 30 accessions were surface inoculated with a suspension of *E. coli* ATCC 35218 containing $1 \times 10^7$ cells/mL and the attachment was measured 1.5 h after inoculation. Significant differences in attachment were detected between the accessions. The three most susceptible accessions (romaine lettuce 'Bionda degli Ortolani', Swiss chard, and rocket) and the three least susceptible (wild rocket 'Yeti', wild lettuce, and lamb's lettuce 'Trophy F1') were selected and characterized for leaf micro-morphological traits (stomata density and size, surface roughness) and water content. Scanning electron microscopy was used to analyse the stomatal parameters. Roughness was measured by an innovative portable 3D digital microscope. No significant correlation between the attachment of *E. coli* on the leaves and stomatal parameters was detected, while the attachment was positively correlated with roughness and water content. *E. coli* population in surface-inoculated leaves was also measured after a UV treatment, which was found to be less effective in reducing bacterial contamination in the rougher leaves. This result suggested that roughness offers UV protection to *E. coli* cells, further highlighting its impact on the microbiological safety of baby-leaf greens. In the second experiment the attachment of *S. enterica* on the leaves of the 30 accessions was evaluated after 5 min of inoculation, revealing differences in susceptibility to contamination by *S. enterica* between the baby-leaf salads. Wild lettuce (*Lactuca serriola* L.) and lamb's lettuce 'Trophy F1' (*Valerianella locusta* [L.] Laterr.) showed the lowest level of contamination, while sorrel was the most contaminated. The differences in attachment found between the salads were then related to the following leaf traits: hydrophobicity, roughness, and epicuticular waxes. Attachment was correlated to hydrophobicity (measured as contact angle) and epicuticular waxes, but not to roughness, presumably due to the short incubation time. The most important wax components for attachment were alcohols and, in particular, the 3-D wax crystals of C26 alcohol, but fatty acids probably also had a role. Both these compounds increased hydrophobicity. The presence of thymol, whose antimicrobial properties are well known, was found in lamb's lettuce. Finally, the third experiment was conducted to preliminary evaluate fluorescence microscopy as a mean to directly track *E. coli* proliferation in baby leaves. Cultivated lettuce (romaine lettuce 'Bionda degli Ortolani') and wild lettuce, which had previously shown a different susceptibility to *E. coli* contamination, were used for the experiment. Fluorescence microscope observations were performed at time 0 and 24 h after inoculation. The fluorescence area increased over the 24-hour incubation interval. Wild lettuce showed a smaller area of fluorescence than romaine (-56.1%),

and a fluorescence intensity approximately 19.1% lower, confirming to be less susceptible to *E. coli* contamination in comparison with cultivated lettuce. The results obtained in this thesis can turn into practical implications and give rise to some recommendations for growers. The screening of a large number of baby leaves for the susceptibility to contamination by *E. coli* and *S. enterica* highlighted the most and the least risky in terms of food safety. This information can guide growers' choice and be of interest for the ready-to-eat industry. Besides, the thesis provided findings on leaf traits associated with a decrease in proliferation of human pathogens in salads. Such traits could be considered in breeding programs with the goal of obtaining cultivars less prone to human pathogens. Finally, although preliminary, the results of the third experiment indicated a new approach for real-time observation and monitoring of bacterial proliferation on leaf surfaces, providing valuable information in the field of plant-bacteria interactions. In conclusion, this work offers insights for enhancing the food safety of leafy vegetables.

**Keywords:** baby-leaf vegetables, human pathogens, leaf roughness, contact angle, epicuticular waxes, 3-D wax crystals, bacterial internalization, cutting technique, fluorescence microscopy

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# 1. General introduction

## 1.1 Foodborne diseases and vegetables

Foodborne diseases represent a widespread issue throughout the world. The World Health Organization estimates that approximately 600 million people a year, nearly one in 10 worldwide, fall ill due to the consumption of contaminated food, with 420,000 deaths recorded among them[1]. It should be emphasized that the impact of foodborne disease extends beyond the affected individuals, having significant economic repercussions. On one hand, there are costs related to the individual, including medical expenses, care, and absenteeism from work/school. On the other hand, there are societal costs, which encompass decreased worker productivity, expenses for epidemic research, income loss due to the closure of food businesses, legal costs for disease-related litigations, and expenses for public health services[2].

Various agents such as toxic chemicals, pathogens, and parasites can contaminate food at different stages of the production and preparation process. Prevention strategies and protocols have been devised and developed by numerous researchers, regulatory bodies, and governments. However, despite considerable scientific advancements, foodborne illnesses persist, remaining a significant cause of morbidity and mortality globally. Although foodborne illnesses are more common in developing countries, particularly in Africa and Southeast Asia, with specific vulnerable groups such as children, immunocompromised individuals, pregnant women, and the elderly, these illnesses are not confined solely to these regions or demographic groups. Typically, such diseases are associated with foods like meats and eggs; however, a significant increase in outbreaks has also been documented due to the consumption of fresh vegetables[3].

The microflora of fresh vegetables is usually dominated by molds, yest and bacteria not dengerous to humans, nevertheless, the occasional presence of pathogenic bacteria, parasites and viruses capable of causing human infections has been extensively documented[4]. In recent years, the vegetable market has significantly increased. This popularity can be mainly attributed to the growing awareness of the relationship between health and the consumption of fresh vegetables. In fact, it has been demonstrated that a diet rich in fresh plant products prevents some chronic diseases such as diabetes, cardiovascular diseases, hypertension, obesity, and even cancer[4]. Currently, the fastest-growing sector in the vegetable industry, whose market globally valued at USD 10.78 billion in 2020 and expected to further expand in the next years is that of ready-to eat salads[5]. Many are the leafy vegetables that can be processed, packaged in air or modified atmosphere, refrigerated, and consumed within a short period after harvesting[6]. These products can be marketed worldwide and throughout the year. Since they are consumed raw, they are recognized as potential carriers of diseases and, thus, pose a threat to global food safety[6].

Most industrialized nations, particularly the United States, have efficient data collection systems and therefore possess extensive and comprehensive sets of information indicating the extent and severity of outbreaks, and the pathogen linked to the consumed product. Based on such information, preventive protocols have been developed with the aim of avoiding further outbreaks. Unfortunately, this level of data is not as much as available for developing countries, especially in Africa, where resources are lacking to effectively track incidents caused by foodborne illnesses. Anyway, also in developed countries, despite the advances in epidemiological investigation

approaches and techniques that allow to identify the associations between products and pathogens, the extent of foodborne diseases linked with fresh vegetables consumption is probably largely underestimated[3]. One of the main difficulties is the relatively short shelf life of fresh salads: when an outbreak is identified, these products have often already been disposed of as waste. Hence, frequently, determining the actual source of contamination proves challenging, prompting investigators to speculate when attempting to identify a particular source. Additionally, smaller and less significant outbreaks are not studied. Moreover, of greater significance is the fact that outbreaks of foodborne diseases occur sporadically within populations, making them elusive to detection through routine epidemiological surveillance. It is important to note that if an outbreak is severe, unusual, or sudden, it typically has a significant impact on public health. Therefore, if the causative agent and the implicated product are not quickly identified, there is a significant risk of the epidemic spreading to multiple countries, with inevitable consequences also for international trade.

Of greater concern for food safety are bacteria like *Shigella* spp., *Salmonella enterica* spp., *Escherichia coli*, *Campylobacter* spp., *Listeria monocytogenes*, *Yersinia enterocolitica*, *Bacillus cereus*, *Clostridium botulinum*, as well as viruses and parasites like *Giardia lamblia*, *Cyclospora cayetanensis*, and *Cryptosporidium parvum*[7]. *E. coli* and *S. enterica*, which are among the most frequent, will be discussed in the following paragraphs, being the pathogens studied in this thesis.

### *1.1.1 Escherichia coli*

*E. coli*, a member of the Enterobacteriaceae family, is the most widespread commensal bacterium in the gastrointestinal tracts of humans and warm-blooded animals, normally engaging in mutually beneficial associations with its hosts. However, certain strains of *E. coli* are human pathogens responsible for a broad spectrum of diseases that can be transmitted through contaminated food. Prior to the identification of specific virulence factors in pathogenic strains, *E. coli* was primarily classified based on serological identification of "O" (lipopolysaccharides, LPS) and "H" (flagellar) antigens. Today, *E. coli* strains are classified into pathotypes based on the type of virulence factor present and the clinical symptoms exhibited by the host. The pathotype most commonly mentioned in news related to outbreaks of foodborne origin is Enterohemorrhagic *E. coli* (EHEC), sometimes also referred to as Verocytotoxin-producing *E. coli* (VTEC) or Shiga toxin-producing *E. coli* (STEC). *E. coli* O157, a STEC, is one of the leading causes of gastrointestinal illnesses worldwide. Signs and symptoms of infection caused by this human pathogen may include diarrhea, severe stomach cramps, and vomiting; the infection can progress to hemolytic-uremic syndrome (HUS) and death. The bacterium can be transmitted to humans through contaminated food and water, direct person-to-person transmission, and contact with animals or their environment[8].

Among Western countries, the United States is one of the areas where outbreaks of foodborne illnesses caused by *E. coli* have been frequently reported. According to the Centers for Disease Control and Prevention (CDC), in collaboration with public health officials in various states, the United States Department of Agriculture's Food Safety and Inspection Service (FSIS), and the U.S. Food and Drug Administration (FDA), a total of 390 outbreaks of *E. coli* O157 were reported in the epidemic data collection from 2003 to 2012.

The implicated transmission modes were primarily foodborne (255 outbreaks, 65%), followed by contact with animals (39, 10%), person-to-person contact (39, 10%), waterborne transmission (15, 4%), and other/unknown sources (42, 11%). Consequently, foodborne illness outbreaks accounted for the majority of illnesses (3,667 cases,

74% of the total involved cases), hospitalizations (1,035 cases, 81%), physician-diagnosed cases of hemolytic-uremic syndrome (HUS) (209 cases, 70%), and deaths (25 cases, 70%). Out of the 255 reported outbreaks of foodborne illnesses, 29 were attributed to the consumption of leafy vegetables. The majority of these incidents involved lettuce (22 outbreaks, 76%), including romaine (3), iceberg (1), and mixed leafy greens[9]. Other vegetables in this category included spinach (4, 13%) and unspecified types of leafy salads (3, 10%). The investigation revealed that hospitalization rates were higher in outbreaks attributed to foods generally consumed raw compared to cooked foods, and deaths more commonly occurred in outbreaks attributed to leafy vegetables (7 deaths, 0.8% of illnesses in leafy vegetable outbreaks), fruit (6, 11%), and beef (5, 0.4%)[9].

Regarding Europe, it is important to mention the outbreak caused by the *E. coli* O104:H4 (STEC) pathogen that occurred in Central Europe during late spring 2011. Nearly 4000 people were infected, mainly in Germany, with over 900 cases of hemolytic-uremic syndrome (HUS), resulting in 54 deaths. Tracing the origin of the epidemic by competent authorities was a challenging and still partially unresolved task. Epidemiological investigations pointed to lettuce sprouts as the possible contaminated source. Fenugreek sprouts were also considered a potential source, and the contamination's origin could have been a common import of seeds from Egypt arriving in Rotterdam, distributed in Germany, and partially redistributed in the United Kingdom and France. As a consequence of this severe outbreak, most EU Member States significantly increased the number of checks aimed at detecting the presence of STEC in food placed on the market[10]. However, it is noteworthy that, as reported in the latest edition of the European Union summary report on trends and sources of zoonoses, zoonotic agents, and foodborne outbreaks for the period 2012-2017, most foodborne outbreaks caused by *E. coli* STEC reported in Europe were associated with the consumption of animal-origin foods.

During the period 2012-2017, 52 strong pieces of evidence for foodborne outbreaks caused by STEC were reported, involving 987 cases, 214 hospitalizations, and 4 deaths[11]. The strength of evidence for reporting an outbreak at the EU level is based on an assessment of all available categories of evidence (descriptive, epidemiological, or microbiological). Source attribution analysis suggested that beef and beef products, milk and dairy products, tap water including well water, and vegetables, fruits, and their products were the main food vehicles for STEC infection in the EU. Specifically, the category vegetables, fruits, and their products was responsible for seven reported outbreaks with strong evidence, causing 575 cases, 73 hospitalizations, and 2 deaths[11]. The food category beef and beef products accounted for 15 of these outbreaks, causing 143 cases and 76 hospitalizations. These data confirm the importance of the vegetables, fruits, and their products category as a source of STEC infections.

### *1.1.2 Salmonella enterica*

*Salmonella enterica* is a species of bacterium belonging to the genus *Salmonella*. The *Salmonella* genus is part of the Enterobacteriaceae family, which includes many other bacteria commonly found in the intestines of humans and animals. *Salmonella* is a gram-negative, rod-shaped, and facultative anaerobe, meaning it can survive in both the presence and absence of oxygen. The species *Salmonella enterica* is further divided into numerous serovars (or serotypes), each with distinct surface antigens. These serovars are classified based on variations in two major surface structures: the O antigen (lipopolysaccharide) and the H antigen (flagellar protein). The combination of

these antigens leads to the identification of specific *Salmonella* serovars. Several strains of *Salmonella enterica* do not cause any disease in humans, and some are naturally present in the intestines of animals without causing illness[12]. On the contrary, certain serovars are wellknown for their pathogenicity, and can result in significant public health concerns being associated with a variety of illnesses collectively known as salmonellosis. Salmonellosis can manifest as gastroenteritis (inflammation of the stomach and intestines), bacteremia (bacterial infection in the blood), and typhoid fever (caused by specific serovars such as *Salmonella* Typhi). *Salmonella* infections are typically contracted through the consumption of contaminated food or water. Foods such as undercooked poultry, raw eggs, unpasteurized milk, and contaminated fruits and vegetables are common sources of *Salmonella.* Additionally, contact with infected animals or their environment can also lead to transmission.

Foodborne illnesses caused by pathogens represent a significant global public health concern, prompting countries to allocate substantial resources to address this issue. Bacterial infections from contaminated food are a cause for alarm in both developed and developing nations. In Europe, *Salmonella*, together with *Campylobacter*, stand out as the primary culprits behind foodborne illnesses[13,14]. According to the European Centre for Disease Prevention and Control (ECDC), *Salmonella*, responsible for the highest number of human infections, afflicted 91,857 individuals in the EU in 2018, accounting for 33% of total foodborne oubreaks reported in Member States that year.

Although traditionally plants were not considered hosts for human pathogens like *Salmonella,* over the last few decades they have demonstrated to be possible ecological niches for these organisms[15].

In the United States, there is a decline in food poisoning outbreaks related to raw eggs and seafood, whereas outbreaks attributed to fruits and vegetables are on the rise[16,17]. This trend persists despite field surveys in the United States indicating low *Salmonella* contamination during pre-harvest production. Fruits and vegetables have been linked to 130 outbreaks since 1996[16,17]. Bennett et al. (2015)[18] observed that tomatoes, in particular, were implicated in 15 multi-state salmonellosis outbreaks between 1990 and 2010. Traceback analysis suggested that contamination occurred during the production or processing stages.

Devleesschauwer et al. (2017)[19] observed that although outbreaks of salmonellosis linked to fruits and vegetables have been well-documented, their occurrence remains sporadic. Furthermore, the same authors stated that multiple factors must converge for outbreaks involving fruits and vegetables to occur. These factors encompass the presence of vectors, crop maturity level, physiological defects, the presence of native biota that may hinder or promote human pathogens, irrigation practices, and more. The role of environmental conditions and farm practices is crucial in determining the factors that render plants susceptible to *Salmonella* proliferation, both pre and post-harvest. The study conducted by Devleesschauwer et al. (2017)[19] confirmed that harvesting tomatoes while still green significantly reduces *Salmonella* infestation, as does harvesting after a period of high humidity. Pre-harvest application of copper, iron, potassium, nitrogen, or foliar sprays did not have an impact on post-harvest contamination.

## 1.2 Baby-leaf salads

### *1.2.1 Description*

In recent years, there has been a notable increase in people's consumption of fruits and vegetables, known for their rich content of bioactive substances. These are compounds recognized for their dual benefit: they not only provide

essential nutrients for the human body, but also contribute positively to human health[20]. Sprouts, microgreens and "baby leaf" vegetables represent a rapidly expanding market segment in the sector of vegetable products. In many countries around the world, both chefs and consumers are increasingly attracted by these products, appreciating them for their sensorial appeal and nutritional properties.

Baby leaves are leafy vegetables harvested at an early stage of growth (up to the eighth true leaf) and consumed as salads[21]. Baby leaf salads are usually processed as ready-to eat produce, often in mixed salads[22]. Ready-to-eat salads are increasingly appreciated by consumers not only because they constitute a rich source of minerals, vitamins and phytochemicals with significant antioxidant potential[23], but also for the ease of use[24]. As compared to mature leaves, baby leaves have the advantage that they can undergo processing without additional preparation since the entire leaves are used. Furthermore, due to the small stem diameter, there is a reduced wound response, resulting in less bruising and minimal oxidation[21]. All this implies an extended storage potential for ready-to-eat salads, showcasing improved color retention, nutritional quality, and microbiological attributes[25]. On the contrary, mature leaves, which are cut during the processing, undergo physical damage, leading to heightened respiration rates, biochemical changes, and microbial spoilage[26]. These factors contribute to the deterioration of color, texture, and flavor of fresh-cut produce, reducing shelf-life[27].

### *1.2.2 Utilized species*

Lettuce (*Lactuca sativa* L.) stands out as one of the most significant species employed as a baby leaf vegetable. Various types of lettuce, distinguished by different, attractive, colors and shapes, have been selected to create salad mixes that combine the finest quality characteristics from different varieties. Other numerous species belonging to different botanical families can also be used as baby-leaf crops, suitable for both raw and cooked consumption. For instance, spinach (*Spinacia oleracea* L.), Swiss chard (*Beta vulgaris* L.), mustard (*Brassica juncea* L.), and kale (*B. oleracea* L.) are primarily cooked vegetables[28]; nevertheless, they are also utilized in their raw form, especially in mixed salads. Orach (*Atriplex hortensis* L.), of the Chenopodiaceae family, can serve as a spinach substitute, offering a similar flavor but without the puckering effect caused by oxalic acid[28]. Species primarily utilized as raw "baby leaf" include endive (*Cichorium endivia* L.), chicory (*C. intybus* L.), lamb's lettuce (*Valerianella locusta* L.), common purslane (*Portulaca oleracea* L.), chervil (*Anthriscus cerefolium* Hoffm.), perilla (*Perilla frutescens* [L.] Britt.), and several Brassicaceae, such as rocket (*Eruca vesicaria* L.), wild rocket (*Diplotaxis tenuifolia* L.), mizuna (*Brassica rapa* L. Mizuna group), tatsoi (*B. rapa* Tatsoi group)[28], and watercress (*Nasturtium officinale* R. Br.). Chicory and endive, while less commonly used than lettuce, still hold a notable presence as some of the most recognized and popular horticultural products globally; despite significant variations in cultural practices and utilization, they are widespread in nearly every country as an important component of the dietary habits of both Western and Eastern populations[29]. Mizuna, tatsoi, and perilla are Oriental vegetables, while corn salad enjoys popularity in Western Europe[28]. Several of the above-mentioned species are common wild plants that have undergone a selection process bringing to the current cultivated form. One of the most successful cases involves wild rocket that in Italy, since the initial attempts in the early 1990s, is now cultivated on over 1000 hectares[21]. The consumption of wild rocket has seen an increase since 1990, partly driven by the initiatives of renowned chefs who have incorporated it into various recipes. Many others are the wild leafy plants still uncultivated or underutilized, that could be profitably used to produce baby leaves

(Baldi et al., 2022). Some examples are dandelion (*Taraxacum campylodes* G.E. Haglund), small burnet (*Poterium sanguisorba* L.), wild mustard (*Sinapis arvensis* L.), sorrel (*Rumex acetosa* L.), and wild lettuce (*Lactuca serriola* L.)[30,31].

### *1.2.3 Nutritional properties*

Leafy vegetables are widely recognized for their abundance of vitamins, fiber, minerals, and other beneficial phytochemicals[32]. Nevertheless, it is important to note that the biosynthesis, composition, and concentration of health-promoting compounds can vary significantly among different leafy vegetables. First of all this variability is influenced by genetic factors, bringing to differences between species and among cultivars and populations within the same species. For instance, lettuce serves as a notable source of ascorbic acid, folate, and, consistent with its green nature, chlorophyll. Additionally, the majority of lettuce cultivars are abundant in carotenoids (such as β-carotene, lutein, and zeaxanthin) and various flavonoids, including flavanols, mainly quercetin, and flavones like apigenin and luteolin[33]. Furthermore, red cultivars contain anthocyanins[34]. Consequently, the antioxidant activity of red baby leaf lettuce cultivars could potentially be up to 11 times higher than that of green baby leaf lettuce[35]. Rocket, while containing similar core nutrients as lettuce and sharing many of the same phytochemicals, lacks anthocyanins[36]. Environmental conditions, growing practices, harvesting methods, postharvest handling conditions, and the stage of maturity are other factors influencing the nutrient and phytochemical content of leafy vegetables[37].

## 1.3 Sources of pre-harvest contamination

The prevention of contamination of vegetables by human pathogens begins in the field through the identification and and elimination of the possible sources of contamination. The principles of Good Agricultural Practices (GAP) and Good Hygiene Practices (GHP) remain fundamental prerequisites in the pre-harvest product safety management strategy. As a first preventive measure, cultivation should never be conducted in areas where the known or suspected presence of pathogens could pose an unacceptable likelihood of pathogen transfer to the crops. Furthermore, regarding hygiene measures, equipment and tools used during cultivation and harvesting should be maintained in good hygenic condition at all times through various sanitation practices[38]. In the cultivation phase the use of immature manure or other contaminated organic fertilizers, the use of irrigation water contaminated with fecal material, and the access of livestock or wildlife to the field are the main causes leading to pre-harvest microbial contamination of the product[6].

The use of organic fertilizers such as animal manure, slurry, slaughterhouse waste, and sewage sludge is widespread[3]. These organic materials serve as an economically advantageous nutrient source for agricultural purposes, but numerous studies demonstrate that they also pose a significant risk of pathogenic contamination of products directly in the field[3]. Even the treatments they are usually subjected to before their distribution in the soil, like composting, anaerobic digestion, pelletization, drying, etc., do not guarantee their safety. Although many of these treatments involve a thermal process, concerns have been raised about their ability to satisfactorily eliminate enteric bacteria[3]. The application of organic fertilizers to the soil can lead to direct contamination of crops, especially during heavy rainfall or irrigation with water jets that cause splashes on the leaves[3]. This application also allows the incorporation of pathogens into the soil matrix. Piles of manure stored near cultivation areas can also

pose risk of contamination due to runoff[39]. Therefore, pathogens present in contaminated manure can be swiftly transported within the soil system, where their movement (both vertically and horizontally) is influenced by the soil type and the amount of water present in the matrix, and reach plants.

The survival of pathogens in manure and other organic fertilizers is a significant risk factor, and depends on various factors, including the source of origin, chemical-physical characteristics, the applied treatment technique, and the degree of incorporation into the soil. Franz et al. (2005)[40] observed that the diet of cattle can influence the incidence of *E. coli* O157:H7 and *S. enterica* in manure[41,42]. The bacteria persist longer in manure obtained from cattle fed high-energy but low-fiber diets, such as maize silage, compared to animals that have received low-energy diets with higher fiber content, such as straw. The method of fertilizer application can also impact the survival time of pathogens. Indeed, bacteria tend to decrease more rapidly when the fertilizer is surface-applied compared to when it is incorporated into the soil. This is likely due to faster drying and exposure to UV rays on the soil surface[39].

The pathogen's ability to survive in the environment (and on products) is also an essential factor for the risk of human infection. Actual risks depend on numerous variables, including environmental conditions, bacterial load, the ability to multiply efficiently outside a mammalian host, the infectious dose for humans, and the response of the host plant[3]. As reported in some studies, the survival of pathogens in the soil depends on various factors, including soil type, mainly based on based on organic matter content, particle size, moisture, and water retention capacity, moisture, pH, temperature, nutrient availability, agronomic practices, and biological interactions in the soil[3]. Clayey soils, for example, promote the absorption of microorganisms into soil particles. Clays protect bacterial cells, creating a barrier against microbial predators and parasites. *E. coli*, e.g., can persist for up to 25 weeks in clayey soils, but for much less time (8 weeks) in sandy soils[3]. Therefore, pathogen survival rates are lower in sandy soils, which also have a low water retention capacity. Soil water potential is determined by soil properties and water supplies from precipitation and/or irrigation, and it has been shown to be one of the factors that significantly influence microbial transport and survival in the soil[3]. Generally, cool and humid environments are conducive to bacterial survival. Regarding *E. coli*, some studies have demonstrated that its survival is higher in organic soils and under flooding conditions[43]. Population peaks have been recorded following the rise of the water table caused by significant rainy events[3]. Concerning pH and temperature, soils with a neutral pH and lower temperatures ensure better survival of enterobacteria[3].

Fertilizers not only can be a source of contamination, but they can also influence the survival of pathogens in the soil. In fact, they are an important source of nitrogen, phosphorus, and secondary elements such as calcium, manganese, and sulfur, which promote the growth of enterobacteria[44]. Additionally, organic matter improves nutrient retention in the soil, serves as a carbon source, and enhances moisture retention as well. These factors have an effect on human pathogens directly, but also indirectly, by influencing the soil microflora. Some bacteriophages, protozoa, nematodes, and other soil organisms can parasitize non-indigenous pathogens, thereby limiting their survival[3]. Furthermore, an increase in pathogen survival (in some cases, regrowth has been observed) has been documented in sterile soils or those with relatively low biological activity[3]. The study conducted by the same authors also indicates that non-indigenous enteric pathogens compete poorly for nutrients and are susceptible to inhibition by indigenous soil bacteria. Therefore, the impact of the physical, chemical, and biological conditions of the soil on the persistence of human pathogens in it, and consequently on product contamination should not

be underestimated. Due to the wide range of variables associated with product contamination by fertilizers, regulatory bodies have established a minimum time interval between fertilization and harvest. This is necessary to ensure microbiological safety, and the timing varies depending on different organisms/states. As an example, in Good Agricultural Practices (GAP) guidelines, a commonly accepted interval between application and harvest is 120 days[3].

Irrigation water represents a possible source for contamination of plant products both directly and through soil contamination. In increasing order of microbiological contamination risk, the water sources used for irrigation can be classified as follows: potable or rainwater, groundwater, surface water, and wastewater[45]. The microbiological quality of rainwater is good. Groundwater is generally microbiologically safe unless it has been contaminated with surface runoff or leaching of pathogens or other sources of contamination near the water table. Surface waters, which are the primary source of irrigation in many countries, including open canals, ponds, lakes, rivers, and streams, are much more susceptible to pathogenic contamination compared to groundwater. Sewage discharges, defecation by wild and domestic animals, runoff from contaminated fields, industrial and municipal effluents are all potential causes of surface waters contamination. Finally, wastewater is generally of poor microbiological quality and therefore would requires treatment involving coagulation, flocculation, and filtration of suspended solid material, followed by disinfection before it can be used for irrigating crops.

In general, water sources other than rainwater used for irrigation are often minimally treated or left untreated because the treatment process to bring the water up to drinking water standards, which would be the ideal condition, can be costly and time-consuming[45]. Crops irrigated with wastewater show a higher incidence of enteropathogens. Epidemiological studies on foodborne outbreaks associated with the consumption of fruits and vegetables reveal a greater occurrence of enterobacterial diseases in areas where wastewater irrigation is practiced, especially in cases of minimal or no water treatment[3]. The transportation of irrigation water through channels may involve interaction with microbial reservoirs located in bottom sediments, just as transportation through pipes can involve interactions with biofilms on pipe surfaces, compromising the microbiological quality of water. The method of storing irrigation water can also have an effect on the transmission of pathogens. Alegbeleye et al. (2018)[3] found that water quality is rapidly degraded in ponds and storage tanks due to microbial loads from the feces of avian species or other wildlife. One of the pathogens most frequently implicated in outbreaks related to irrigation water is *E. coli* O157:H7. Under certain temperature, pH, and UV light conditions, this bacterium is capable of surviving for an extended period in water, consequently being able to contaminate the soil and directly the plants. It is known that the bacterium can survive for longer periods in groundwater compared to surface water, as the former tends to be cooler, provides protection from sunlight, and has lower biological activity. Regarding irrigation systems, sprinkler systems pose a higher risk of microbiological contamination compared to drip systems, as the latter minimize contact between the crops and irrigation water[46]. The greatest risk associated with contaminated water is when crops are irrigated close to harvest, as this increases the likelihood that the products, soon to be introduced to the market, may contain the pathogen at the time of consumption. Therefore, a proper time interval between irrigation and harvest should be conscientiously followed[3].

## 1.4 Sources of post-harvest contamination

The processing of vegetables to obtain fresh-cut products involves numerous operations carried out between the initial stage of raw material harvesting and the final packaging phase. Throughout these operations, efforts are made to preserve the nutritional, sensory, and aesthetic qualities, namely the freshness of the product[47]. In general, ready-to-eat fresh products have a shorter shelf life than the fresh starting material due to cutting and preparation operations that cause mechanical damage to tissues, leading to oxidative phenomena, faster loss of texture, and increased susceptibility to microorganisms. To maintain the freshness of the product as much as possible, thereby increasing its commercial value and safety of use, it is essential to carry out all stages of the processing using appropriate techniques and exercising utmost care[48].

Due to the heterogeneity of raw materials and final products, technological processes for minimally-processed fruit and vegetable products are quite diverse and can vary in terms of automation levels. Not all operations are strictly necessary for different types of vegetables, and some may be optional for certain products[48].

During processing, the most important factors influencing the microbiological safety of ready-to-eat salads are:

- *Temperature.* Temperature is a crucial factor in all stages from harvesting, transportation, processing, storage, to retail sale. Maintaining the cold chain is a necessary strategy to prevent/reduce bacterial growth of human pathogens, and it has been highlighted that high temperature is a significant factor contributing to the increase in foodborne outbreaks[38]. Therefore, even though it is challenging, the use of low temperatures (between 0 and 4°C) should begin as early as possible and be maintained from the field, to the processing center, through shipping, and to the point of sale[47].
- *Equipment.* Processing equipment surfaces have been recognized as critical points for microbial contamination. In the inspection phase, conveyor belts or mechanical systems that allow gently shaking or floating the salads using air currents to separate cotyledons and other foreign bodies of various kinds from the product of interest can be sources of contamination. Similarly, conveyor belts used for moving waste away can pose a contamination risk. For instance, the presence of long conveyor belts could create hazardous dripping points on the product, leading to hygiene issues if these belts are not adequately cleaned[49]. Cutting machinery can also be a source of contamination; therefore, it is essential for the blades to be disinfected regularly to prevent the accumulation of organic residues responsible for microbial proliferation. A study aimed at assessing cross-contamination of *E. coli* O157:H7 during the processing of ready-to-eat fresh vegetables found that the conveyor belt and shredder are critical points for microbial contamination[50]. Castro-Ibàñez et al. (2017)[38] observed that the cellular juices from shredded lettuce were visible on the cutting blades and on the discharge chute of the shredder, as well as on all contact areas of the product with the conveyor belt. Similarly, machinery used for washing must undergo continuous sanitization; the lack of which may promote the formation of microbial biofilms on the surfaces of the system. These biofilms can be challenging to remove even with the cleaning practices commonly employed in the food industry[49].
- *Washing.* This operation represents a crucial phase in the processing of ready-to-eat products as it allows for the removal of dirt, foreign materials, exudates from cut surfaces, and microorganisms. Since fresh,

ready-to-eat vegetables do not undergo intensive microbiological inactivation or preservation treatments during processing, washing is the sole processing stage that helps reduce their microbial load[3]. However, the use of water during processing has been identified as a potential source of cross-contamination by human pathogens. In this regard, a study reported that during washing, a small percentage of microorganisms, including pathogenic *E. coli*, were transferred from the aqueous phase to the lettuce, highlighting the vulnerability of fresh vegetables to cross-contamination during washing[51]. However, it is important to note that this experiment was conducted without the use of disinfectants in the water. In this context, some studies have emphasized the importance of maintaining water quality during washing by employing appropriate disinfectants despite these disinfectants have a limited direct antimicrobial effect on products, meaning they do not strongly impact bacterial biofilms and do not inhibit the internalization of microorganisms through stomata and wounds[38]. Today, a wide variety of antimicrobial washing solutions can be applied to reduce microbial populations on minimally-processed products. Several factors can influence disinfection, such as the initial bacterial load and type of microorganisms, the type of surface being washed, the type of disinfectant used, the possible internalization of pathogens, temperature, and the exposure time to the disinfectant[38]. Chlorine is one of the most widely used disinfectants because it is easy to apply, highly effective for deactivating microorganisms, persistent, and relatively cost-effective[38]. It is crucial to use the correct concentration, as too low concentrations have minimal effects on microorganisms, while too high concentrations may lead to chemical contamination of the product[52]. Recently, there has been significant research focused on the pros and cons of using chlorine to disinfect wash water. These studies have raised growing concerns about public health associated with the use of chlorine derivatives during washing, primarily due to the formation of disinfection by-products (halogenated compounds) harmful to humans[38,53]. This is why the use of chlorine-based disinfectants for washing minimally-processed products has been prohibited in some European Union countries, including Germany, the Netherlands, Denmark, Belgium, and Switzerland. Due to these bans, research has developed alternative disinfection methods such as the use of hydrogen peroxide, ozone, peracetic acid, electrolyzed water, ultraviolet rays, ultrasonics, etc.[54]. However, the predominant use of chlorine, its effectiveness, and low cost have not yet been seriously challenged by the use of any other sanitizing agent[38].

- *Drying.* After washing, the raw material is dried using centrifugal drying machines with adjustable speeds based on the product's characteristics. Centrifugal dryers, at times, can damage plant tissues if the centrifugal force exceeds a certain limit. Therefore, ready-to-eat products are often left with excessive moisture inside the packaging (bags or trays), significantly increasing the risk of microbial proliferation[47]. In the case of delicate vegetables, such as baby leaves, continuous airflow drying tunnels can be employed as an effective alternative to the centrifugation process to prevent product damage and subsequent microbial growth[49].
- *Packaging.* The final operation of the processing for ready-to-eat salads occurs in the assembly and packaging room. In case of mixed salads, packaging is carried out using an automated system with a machine for portion control of different vegetable species based on weight[38]. Packaging in controlled hygienic conditions immediately after drying plays a fundamental role in microbiological protection. One

of the most widely used methods is Modified Atmosphere Packaging (MAP), which involves modifying the atmospheric composition inside the packaging. This technology is aimed not only at extending the shelf life of leafy vegetables, but also at inhibiting or delaying the growth of pathogenic microorganisms, primarily due to the low concentration of oxygen[55]. In addition, there is a growing interest in the development of packaging materials containing natural antimicrobial agents used as an additional barrier to prevent the growth of foodborne pathogens. This approach avoids the direct application of antimicrobials on the food, which can enhance consumer appeal. Another emerging alternative is the use of edible films containing antimicrobials. This technique has proven to be a useful tool in protecting food against microbial spoilage and reducing the risk of pathogen growth[38].

- *Workers.* Non-compliance by workers with Good Manufacturing Practices (GMP) and Good Hygienic Practices (GHP) represents a contamination risk during the processing of minimally-processed products. In this regard, some researchers have demonstrated that lettuce leaves can be easily contaminated by the hands of workers in inadequate hygienic conditions[38].

### 1.5 Attachment, internalization and persistence of human pathogens in plants

The contact of human pathogens with the plant is a fundamental prerequisite for contamination and subsequent transmission of foodborne diseases to humans. Once in contact with the plant, these microorganisms are capable of adhering and proliferating or at least surviving on plant surfaces[3]. Adhesion to leaf surfaces is a complex but relatively rapid process involving multiple physical, chemical, and biological factors[56]. In the case of enterobacteria such as *E. coli*, this process is initially facilitated by chemotaxis and bacterial motility. Specifically, once contact with plant surfaces occurs, two phases can take place:

- Initial adhesion phase (occurring during the first few seconds after plant-bacteria contact), involving the formation of a weak, reversible, and non-specific bond (usually dependent on physical factors such as hydrophobicity and the charge of the bacterial cell surface).
- Binding phase: This second phase involves a strong and irreversible bond between the bacterium and the plant. This process requires the presence of bacterial attachment or virulence factors, such as fimbriae, flagella, and polysaccharides. Some laboratory experiments with *E. coli* and lettuce have demonstrated the establishment of this phase after a few hours[57].

The ability of human pathogens to adhere, survive, and proliferate on plant surfaces depends not only on bacterial properties but also significantly on plant species, the stage of plant and leaf development, environmental conditions, and leaf traits[56,58-61].

In general, the majority of above-ground plant surfaces are covered by the cuticle, on the outermost part of which lie the cuticular waxes. Cuticular waxes are primarily composed of long-chain fatty acids (more than 20 carbon atoms), alcohols, aldehydes, alkanes, esters, etc. Due to their chemical composition, these surface waxes provide a hydrophobic coating on most above-ground plant organs, protecting them from various environmental stresses[61]. Additionally, the crystalline form of epicuticular wax can take various shapes (such as films, plates, rods, etc.), which are specific to each genotype and can be altered by changes in environmental conditions. Since an increase

in the size and number of crystalline facets of the wax reduces the contact area with water, the three-dimensional morphology of the wax crystal influences the hydrophobicity of the leaf surface[61]. Therefore, the epicuticular wax layer is likely to affect the adhesion process of human pathogens to plants[61]. Leaf topography is also an important factor influencing the adhesion process of pathogens on plants. The presence of stomata, lenticels and trichomes, and leaf surface roughness promotes adhesion by providing protective niches for bacteria[60,62,63]. Several studies have demonstrated that stomata are involved in the retention of *Salmonella spp.* and *E. coli* in plant leaves[58,60,64,65]. Both the number and size of stomata play a role. For example, in spinach, it has been observed that cultivars with lower stomatal density were less susceptible to contamination with *E. coli*[58]. Among 11 lettuce genotypes, the persistence of *S. Typhimurium* 14028s and *E. coli* O157:H7 was lower in those with smaller stomata[60]. The plant species and leaf age are closely related to different roughness levels, leading to a highly heterogeneous leaf surface and spatial variation in nutrients, water, and the resident microbiota[60]. Such heterogeneity can impact the fate of human pathogens when they come into contact with the leaf surface. In this regard, some studies have highlighted the significant role of leaf age in the adhesion, persistence, and survival process of human pathogens in plants[59,66]. Greater survival on the surface of young leaves compared to old ones (as well as on the abaxial leaf surfaces compared to adaxial ones) can be explained by differences in nutrient availability, different environmental and biological conditions, and physical factors such as roughness[56]. For instance, different persistence of *E. coli* O157:H7 on leaves was observed among various spinach cultivars with different leaf lamina roughness[58]. Damaged plant tissues are much more susceptible to colonization than undamaged tissues of the same type. Additionally, enteric pathogens preferentially attach to cut surfaces where more nutrients may be available for their growth and survival. In this regard, some studies have highlighted increased adhesion of *E. coli* O157:H7 and *Listeria monocytogenes* on cut lettuce leaves compared to whole leaf surfaces[57,58]. Furthermore, damage to lettuce plant stems has led to the release of sugar-containing latex, supporting the growth and rapid increase of *E. coli* O157:H7 populations[57]. Once adhesion occurs on the surfaces of products, bacterial pathogens can become trapped in complex structures called biofilms. The formation of such structures by epiphytic bacteria or plant pathogens has been known for a long time; however, the discovery that enteric pathogens themselves could form biofilms on plant surfaces is more recent[57]. As a result, in recent years, the number of studies on the formation of biofilms by enteric bacteria on the surfaces of vegetable products has significantly increased[39,57]. Biofilms are defined as a collection of microorganisms enclosed within an exopolymeric matrix, capable of providing protective action against various stresses[67]. These structures likely represent a survival strategy for the plant surface to withstand the hostile environment in the field as well as during harvesting, transportation, and product storage. Bacteria within biofilms are more challenging to remove from plant surfaces and are more resistant to inactivation, thus exhibiting a higher survival rate[39]. Internalization can occur through natural openings on the plant surface (stomata, lenticels, etc.) or through artificially caused wounds, natural lesions, or at sites of physical damage caused by plant pathogens. Indeed, stomata not only can provide a protective niche for these bacteria but also represent a preferential route for penetration into the plant[62]. As for wounds in plant tissue or injuries caused by plant pathogens, they can serve as entry points, creating a microenvironment more favorable for the internalization and survival of human pathogens such as *Salmonella spp.* and *E. coli* O157:H7. Some investigations on commercial products have indicated a higher likelihood of finding high levels of *Salmonella* inside products when they have been damaged by plant

pathogens such as those causing soft rot, compared to their healthy counterparts[68,69]. Other studies suggest that rubbing or cutting lettuce leaves results in increased internalization of *E. coli* O157:H7 compared to leaves that remain intact[57,70]. The entry of human pathogens into the plant can also occur through absorption by the roots, carried by contaminated water. In this case, internalization and movement within the plant may not require any active biological process by the bacteria. Similarly, the movement of water through natural leaf openings, caused, for example, by a temperature differential between the product and wash water, can be a cause of internalization[69]. The plant species and developmental stage influence the potential for internalization, with an effect that is not always straightforward. For instance, in Matthews et al. (2009)[57], younger lettuce leaves and roots were associated with a higher risk of internalization by *Salmonella* and *E. coli* O157:H7. However, in other studies, older plants were found to be more susceptible to internalization compared to younger plants[59](Hunter et al., 2015). Additionally, while in young lettuce plants *E. coli* survived better in older leaves rather than in young and middle-aged leaves, in mature plants, the pathogen population size was significantly higher on both older and younger leaves compared to middle-aged leaves[57,59]. The presence of endogenous bacteria in the soil or on the plant can also influence the internalization and colonization of enteric pathogens. Positive interactions may arise from the presence of other bacteria or fungi that can provide carbon sources (through the degradation of cell wall polymers or the induced secretion of sugars) that would otherwise be inaccessible to human pathogens; alternatively, these microorganisms may suppress the plant's defense response[69]. On the other hand, negative interactions may occur because human pathogens, while attempting to colonize the surface or interior of a host plant, must compete with the natural microflora[69]. In this regard, studies have compared the internalization of *Salmonella* Newport and *E. coli* O157:H7 in *Arabidopsis thaliana* in autoclaved and non-autoclaved soil. Internalization was higher in autoclaved soil, likely due to the suppression of antagonistic bacteria such as *Enterobacter asburiae*, which colonize the root surface[57]. However, these effects do not seem to be universal, as it has been demonstrated that co-inoculation of human pathogens with some epiphytic microorganisms (*Wausteria paucula*, *Erwinia carotovora*) leads to an increase in the growth level of human pathogens[69].

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# 2. Objectives

The Ph.D. project was aimed to characterize different vegetable species at the baby leaf stage for susceptibility to enterobacteria contamination, and to identify leaf phenotypic traits possibly related to susceptibility, with the final objective to provide information useful for the food safety of these products.

The Ph.D. project specific objectives were:

- to screen thirty different baby-leaf accessions (crops of different species and/or varieties, some wild relatives, plus other wild greens) for the degree of susceptibility to *E. coli* and *S. enterica* contamination;
- to characterize the baby-leaf accessions for leaf phenotypic traits possibly involved in the observed susceptibility: roughness, density and size of stomata, amount and morphology of epicuticular waxes, hydrophobicity;
- to find a correlation between the contamination degree and the observed phenotypic traits;
- to evaluate fluorescence microscopy as a possible mean to track the proliferation of *E. coli* on baby leaves and to highlight differences in susceptibility between species.

## 3. List of papers

The thesis is composed by three papers (one published, one submitted, and one in preparation):

**First paper - Foliar roughness and water content impact on *Escherichia coli* attachment in baby leafy greens.** *Stefania Truschi*, Ada Baldi, Piero Bruschi, Ilaria Cacciari, Massimiliano Marvasi, Anna Lenzi.* **Published in Biology** 2023, 12(1), 102; https://doi.org/10.3390/biology12010102.
(*corresponding author)

**Second paper - Relationship between *Salmonella enterica* attachment and leaf hydrophobicity, roughness, and epicuticular waxes: a focus on 30 baby-leaf salads.** *Stefania Truschi, Lorenzo Marini, Ilaria Cacciari, Ada Baldi, Piero Bruschi, Anna Lenzi, Johanna Baales, Viktoria V. Zeisler-Diehl, Lukas Schreiber, Massimiliano Marvasi.* **Submitted to Journal of the Science of Food and Agriculture.**

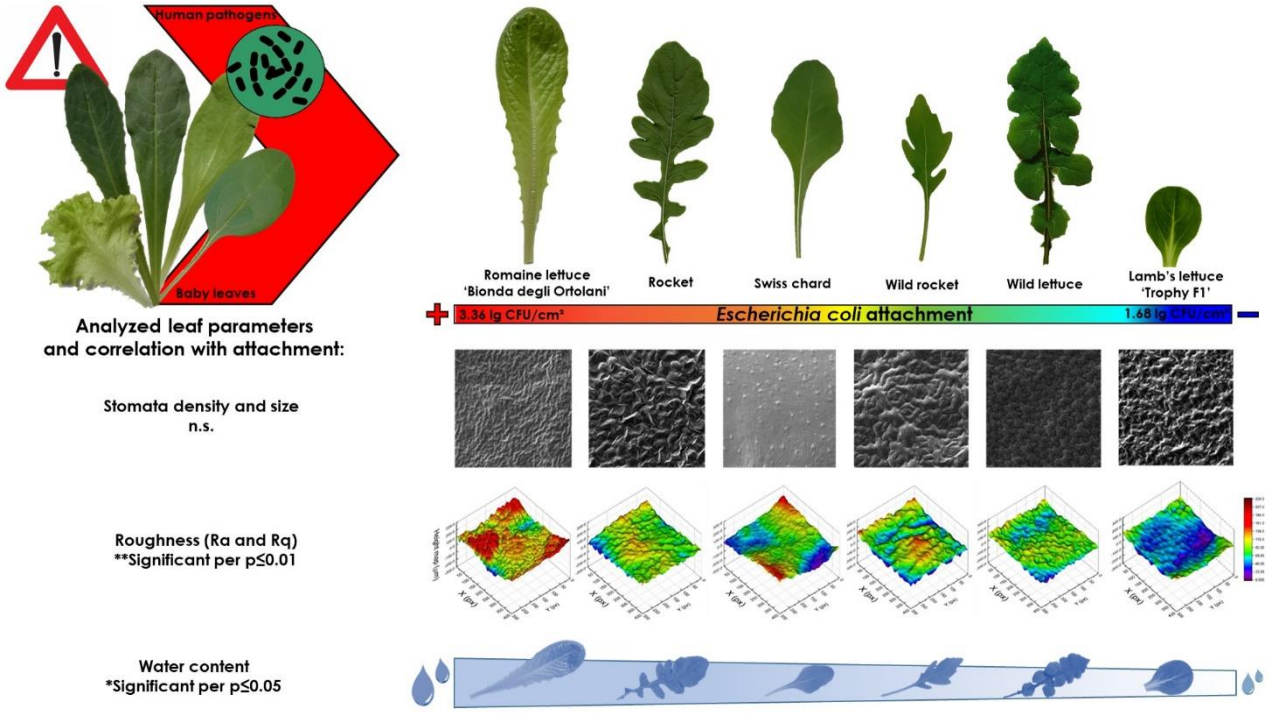
**Third paper - Fluorescence microscopy for directly tracking the proliferation of *Escherichia coli* in baby leaves of cultivated and wild lettuce.** *Stefania Truschi, Marianna Arvaniti, Massimiliano Marvasi, Anna Lenzi, Marco Napoli, Panagiotis Skandamis.* **In preparation**.

The experiments were carried out in the period between October 2020 and November 2023 at: the Department of Agriculture, Food, Environment and Forestry (DAGRI), University of Florence; The Department of Biology, University of Florence; the Institute of Cellular and Molecular Botanics (IZMB) University of Bonn, Germany (quantification/characterization of waxes and wettability analysis shown in paper 2); the Department of Food Science and Human Nutrition, Agricultural University of Athens, Greece (AUA), Laboratory of Food Quality Control and Hygiene (inoculations and observations under the fluorescence microscope, discussed in paper 3).

# 4. First paper

## Foliar roughness and water content impact on Escherichia coli attachment in baby leafy greens

**Graphical abstract**




### 4.1 Introduction

Baby leaves are leafy vegetables harvested at an early stage of growth (up to the eighth true leaf) and consumed as salads[1]. They can be commercialized as unprocessed produce but are often minimally processed and sold in a ready-to-eat form, which offers the advantage of ease of consumption. With the increased consumer awareness of the relationship between health and the presence of fresh vegetables in the diet, the demand for minimally processed fruits and vegetables is constantly growing[2]. The global size of the ready-to-eat salad market was valued at USD 10.78 billion in 2020 and is expected to further expand in the next years[3].

A high number of species are grown as baby leaf salads. Lettuce, with many types of different colours and shapes, is the most important, but also a lot of other vegetable crops are used, including endive (*Cichorium endivia* L.), chicory (*Cichorium intybus* L.), spinach (*Spinacia oleracea* L.), Swiss chard (*Beta vulgaris* L.), mustard (*Brassica juncea* L.), kale (*Brassica oleracea* L.), rocket (*Eruca sativa* Miller), etc.[1]. Furthermore, wild greens (leafy plants gathered in the wild and used as food) have also been demonstrated to be suitable for producing baby leaves[4]. The different leaves can be marketed individually or as salad mixes.

It is well known that vegetables are prone to host human pathogens, including Enterobacteriaceae[5], with contaminations that may occur through various routes from farm to fork[6]. Salads entail great risks since they are

consumed raw, and usually without prior proper washing. For fresh-cut salads, a raising trend in foodborne disease has been observed following the increase in consumption. Fresh produce was reported to be a major vehicle of foodborne diseases in both U.S. and Europe, in most cases with the coliforms *Salmonella enterica* and *Escherichia coli* serotype O157:H7 as causative agents[7]. One of the last food safety alerts reported by the Centers for Disease Control and Prevention (CDC) for the U.S. in 2021 involved a multistate *E. coli* outbreak linked to a packaged salad containing spinach, mizuna, kale and chard baby leaves[8].

Attachment to the plant phyllosphere is the first step in product contamination and can be followed by internalization in the absence of timely decontamination treatments[9,10]. The involvement of leaf traits (e.g., veins, stomata, trichomes, roughness and wettability) in bacterial attachment and further persistence has been demonstrated. These features mainly depend on plant species and cultivars and on leaf age[11-15]. Stomata are one of the preferred sites of bacteria for attachment[16] because they can provide protection, moisture and nutrients for their survival[17] and represent a route of internalization into the plant foliar tissue[18,19]. The presence of bulges and hollows on leaf surface influences bacteria distribution and movement, at the same time increasing the area for the attachment[20,21]. *E. coli* attachment and persistence was found to be positively correlated with stomatal density, stomata size and surface roughness by many authors[11,22,23,24]. It can be hypothesized that also the leaf water content can play a role in the contamination by human pathogens. In fact, Yadav et al.[25] demonstrated that bacterial colonization of the phyllosphere in eight Mediterranean plant species depends on leaf hydration, where leaves with a higher water content are more highly colonized[25].

In this study, *E. coli* ATCC 35218 was used as a model microorganism to evaluate the susceptibility to enterobacteria contamination in 30 accessions of baby leaves belonging to 13 different species. Then, a selected number of accessions (those with the highest and the lowest susceptibility) were characterized for leaf micro-morphological traits (stomata density and size and surface roughness) and water content to identify the leaf traits possibly involved in the bacterial adherence. Information about the susceptibility of different baby leaf accessions to *E. coli* contamination and the related traits can contribute to increasing the food safety of the fresh vegetable industry.

## 4.2 Materials and Methods

### *4.2.1 Baby leaves cultivation and characterization*

Plants of 30 different accessions belonging to 13 species were tested (Table 4.1). After sowing, seeds were kept for 48 h in the dark at 20 °C for promoting germination. Seedlings were hydroponically grown in a floating system in a growth chamber at 21 ± 2 °C (day) and 14 ± 2 °C (night) with a photoperiod of 16 h under fluorescent lighting units OSRAM L36W/77 (36 W, 1400 lm, 120 cm in length, 26 mm in diameter) up to the baby-leaf stage (five/six weeks after sowing, depending on the species). A full-strength Hoagland's nutrient solution N 15.0 mM, *p* 0.10 mM, K 6.0 mM, Ca 5.0 mM, Mg 2.0 mM, Fe 50.0 µM, B 46.2 µM, Mn 9.2 µM, Zn 0.78 µM, Cu 0.32 µM, Mo 0.12 µM) was used. Baby leaves were cut at the base of the petiole, and immediately used for the bacterial attachment assay. The average fresh weight, dry weight, leaf number, and leaf area of the baby leaf plants are reported in Table S1. Fresh weight (FW) and dry weight (DW) were measured before and after oven desiccation at 80 °C for 48 h or until a constant weight, and water content was calculated as [(FW-DW)/FW]*100. Leaf area was determined by

a planimeter LI-3000 (LI-COR, Lincoln, NE, USA). Colour was measured with the Handy Colorimeter NR-3000 (Nippon Denshoku Kogyo C., LTD.) and expressed as a*, b* and L* values (Table S2).

**Table 4.1** Detailed list of the 30 baby leaf accessions used in the study.

| | Family | Specie | Variety/Type/Cultivar | Accession Name |
|---|---|---|---|---|
| 1 | Asteraceae | *Cichorium intybus* L. | var. *sativus* Witloof type | Witloof chicory |
| 2 | | | var. *foliosum* cv. 'Magdeburgo' | Chicory 'Magdeburgo' |
| 3 | | | - | Wild chicory Ingegnoli |
| 4 | | | - | Wild chicory B&T |
| 5 | | | - | Wild chicory local |
| 6 | | | var. *foliosum* cv. 'Biondissima di Trieste' | Chicory 'Biondissima di Trieste' |
| 7 | | | var. *foliosum* cv. 'Spadona da taglio' | Chicory 'Spadona da taglio' |
| 8 | | *Cichorium endivia* L. | var. *crispum* | Endive |
| 9 | | *Lactuca sativa* L. | var. *crispa* Lollo verde type | Lollo verde lettuce |
| 10 | | | var. *crispa* Lollo rossa type | Lollo rossa lettuce |
| 11 | | | var. *crispa* blonde type | Blonde lettuce |
| 12 | | | var. *crispa* cv. 'Pamela' | Lettuce 'Pamela' |
| 13 | | | var. *longifolia* cv. 'Bionda degli ortolani' | Romaine lettuce 'Bionda degli Ortolani' |
| 14 | | | var. *longifolia* cv. 'Maraichere' | Romaine lettuce 'Maraichere' |
| 15 | | *Lactuca serriola* L. | - | Wild lettuce |
| 16 | | *Taraxacum campylodes* G.E.Haglund | - | Dandelion local |
| 17 | | | - | Dandelion Ingegnoli |
| 18 | Brassicaceae | *Eruca sativa* Miller | - | Rocket |
| 19 | | *Diplotaxis tenuifolia* L. | - | Wild rocket Ingegnoli |
| 20 | | | cv. 'Yeti' | Wild rocket 'Yeti' |
| 21 | | *Brassica rapa* L. | subsp. *chinensis* | Pak choi |
| 22 | | | subsp. *nipposinica* | Mizuna |
| 23 | | *Brassica juncea* L. | - | Wasabina leaf mustard |
| 24 | | | - | Red Giant leaf mustard |
| 25 | | | - | Red leaf mustard |
| 26 | Chenopodiaceae | *Beta vulgaris* L. | subsp. *cycla* | Swiss chard |
| 27 | | | subsp. *cycla* cv.'Bull's Blood Artica' | Red chard 'Bull's Blood Artica' |
| 28 | | *Spinacia oleracea* L. | cv. 'Cugoe RZ F1' | Spinach 'Cugoe RZ F1' |
| 29 | Valerianeceae | *Valerianella locusta* L. | cv. 'Trophy F1' | Lamb's lettuce 'Trophy F1' |
| 30 | Polygonaceae | *Rumex acetosa* L. | - | Sorrel |

Seed source: Fratelli Ingegnoli, Milan, Italy (Accessions 1, 2, 3, 6, 7, 8, 13, 17, 19, 21, 29, 30); B&T World Seed, Aigues-Vives, France (Accession 4); Locally gathered in the wild in Florence, Italy (Accession 5); Sativa Bio, Rheinau, Switzerland (Accessions 9, 10, 22, 24); Maraldi Sementi, Cesena, Italy (Accessions 11, 12, 20, 26, 27, 28); Vivosem, Macerata, Italy (Accession 14); Provencemonamour, Paris, France (Accession 15); locally gathered in the wild in Lucca, Italy (Accession 16); Gargini sementi, Lucca, Italy (Accession 18); Tutto-Semi, Wilts, England (Accessions 23, 25).

### *4.2.2 Bacterial strain*

*Escherichia coli* ATCC 35218 was used for the inoculation assays. This strain (classified as biosafety level 1) has specific virulence genes associated with different *E. coli* pathotypes related to human and animal infections[26].

*4.2.3 Surface inoculation of the baby leaves*

The bacterial inocula was started from a −20 °C glycerol stock. Briefly, one mL from an overnight inoculum in Lysogeny Broth (LB, Oxoid, UK) was washed and diluted in sterile Physiological Solution (PS, NaCl 0.85% w/v in $H_2O$) to clean the cells from LB medium[27]. Disks 1.5 cm in diameter were cut from the leaves of the 30 accessions used in this study and put in Petri plates. Fifty µL of the working bacterial suspension (containing approximately $1 \times 10^7$ cells/mL) were placed onto the centre of the leaf disk on the adaxial side. Leaves were incubated for 1.5 h at 25 °C in static. After incubation, the disks were washed three times in 30 mL of sterile PS in Petri plates to remove unattached bacterial cells. The leaf disks were placed on the adaxial side on the first Petri plate and gently washed by manually rotating the plates clockwise 30 times; then, the same procedure was repeated on the second and the third plate. Rinsed disks were grinded with a mini-pestle (into 1.5 mL tubes) in 0.5 mL of PS. After grinding, 20 µL were plated onto selective and differential media MacConkey Agar (Oxoid, UK) and incubated at 37 °C overnight. Colony-forming units (CFU) were counted and log base 10 (log) transformed. Two different seeding sets (each consisting of about 84 plants) and the third true leaf of 5 plants for seeding set were used for each accession (10 leaves in total/accession). Among the 30 accessions, we selected the three most and three least contaminated.

*4.2.4 Analysis of leaf micro-morphological traits*

Stomatal parameters and roughness were determined in the six selected accessions.

*Stomata*

Scanning electron microscopy (SEM) was used to analyse the stomata density and size (length, width and stomatal rim area) of the adaxial leaf surface. Before SEM observations, fresh leaf samples were coated by a thin layer of gold (~10 nm) to reduce shrinkage while ensuring the preservation of cell structures as close to the natural state as possible[28]. Eight observations (the third true leaf of 4 plants at the baby leaf stage and 2 sections per leaf) were performed in each accession. Photographs at 1 × 300 and 1 × 600 were used for counting stomata and measuring stomata sizes, respectively. Stomata were counted on the images (view field ranging from 923 to 929 µm$^2$), and then the number per surface unit (mm$^2$) was calculated by a simple proportion. ImageJ software[29] 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.

*Roughness*

A portable prototype of 3D digital microscope was used to measure the leaf surface roughness[30]. Basically, its setup includes three elements: an optoelectronic imaging group (a CCD digital camera and a fixed focus optical element), a calibrated stage for translating this group along the optical axis, and a smart polymeric lightning system[31]. The 3D reconstruction of the surface under inspection was obtained by elaborating a sequence of defocused images (Video S1) and implementing a suitable algorithm based on shape from a focusing technique[32]. Surface roughness parameters were extracted from 3D reconstruction, according to ISO standards[33]. In particular, we focused the analysis of plant surface on the average roughness (Ra) and root mean square roughness (Rq). A suitable optical element was used in order to achieve a field of view of about 7 × 5.2 mm and a vertical resolution of about 10 µm. It is worth noting that these kinds of measurements are completely non-contact, as well as non-destructive; this

overcomes the disadvantages of using a contact stylus profilometer with soft biological samples. Five different analyses in two leaves (10 replicates) were performed for each accession.

*4.2.5 UV treatment*

The leaves of the six selected accessions were surface-inoculated as described above (5 leaf disks per accession) and then exposed for 5 min under a UV lamp (UVC ≥ 90% with 108.4 μW/cm² from 0.5 m) placed at a distance of 10 cm. After exposure, CFU were counted and log transformed as mentioned above. As a control, three separate drops of 50 μL of $10^7$ *E. coli* cell/mL inoculum were put onto a smooth plastic surface and exposed to the above described UV treatment. The effectiveness of the treatment on the control was demonstrated by complete bacterial inactivation after plating onto MacConkey Agar.

*4.2.6 Statistical analysis*

Data were subjected to the Shapiro–Wilk test for normality and Levene's test for homogeneity of variances. Analysis of variance (ANOVA) was performed using the CoStat software (version 6.45, Monterey, CA, USA). Significance was set at $p \leq 0.05$ (Tukey test). The Pearson's correlation test ($p \leq 0.05$) was used to determine the relationship between the *E. coli* ATCC 35218 attachment and all the considered leaf traits.

## 4.3 Results

*4.3.1 E. coli attachment in the baby leaves*

Variability in *E. coli* ATCC 35218 attachment was observed among the 30 studied baby leaf accessions, even within the same species (Table 4.2). The romaine lettuce 'Bionda degli Ortolani' showed the highest contamination, with significant differences compared to wild chicory (local accession), romaine lettuce 'Maraichere', lettuce 'Pamela', sorrel, wild rocket 'Yeti', wild lettuce and lamb's lettuce 'Trophy F1' ($p < 0.001$). The latter resulted in being less susceptible than all the other accessions. Considering the data by species, differences were found not only in *Lactuca sativa,* but also in *Diplotaxis tenuifolia*; conversely, no differences were observed within *Taraxacum campylodes*, *Brassica juncea, Brassica rapa*, *Cichorium intybus* and *Beta vulgaris*. Among the lettuces, 'Bionda degli Ortolani' showed a contamination level of 3.36 ± 0.36 log CFU/cm², significantly different from 'Maraichere' (2.65 ± 0.34 log CFU/cm²) and 'Pamela' (2.45 ± 0.20 log CFU/cm²). In *Diplotaxis tenuifolia*, 'Yeti' was less susceptible than the Ingegnoli accession. Based on the results of the attachment assays, we selected six accessions (romaine lettuce 'Bionda degli Ortolani', Swiss chard, and rocket as the most susceptible, and wild rocket 'Yeti', wild lettuce, and lamb's lettuce 'Trophy F1' as the least susceptible; Figure 4.1). Further analyses aimed to identify traits possibly involved in the bacterial retention.

**Table 4.2** *E. coli* ATCC 35218 attachment in 30 accessions of baby leaves.

| Accession | Attachment log CFU/cm² | Accession | Attachment log CFU/cm² |
|---|---|---|---|
| Romaine lettuce 'Bionda degli Ortolani' | 3.36 ± 0.36a | Dandelion (local) | 2.94 ± 0.32abcd |
| Rocket | 3.15 ± 0.14ab | Chicory 'Biondissima di Trieste' | 2.93 ± 0.31abcd |
| Swiss chard | 3.11 ± 0.20ab | Pak-choi | 2.93 ± 0.24abcd |
| Endive | 3.11 ± 0.26ab | Lollo verde lettuce | 2.90 ± 0.30abcd |
| Spinach 'Cugoe RZ F1' | 3.10 ± 0.23ab | Blonde lettuce | 2.87 ± 0.21abcde |
| Mizuna | 3.10 ± 0.20ab | Red leaf mustard | 2.83 ± 0.27abcde |
| Wild chicory (B&T) | 3.03 ± 0.43abc | Wild chicory (Ingegnoli) | 2.81 ± 0.36abcde |
| Dandelion (Ingegnoli) | 3.02 ± 0.46abc | Red chard 'Bull's Blood Artica' | 2.79 ± 0.27abcde |
| Red Giant leaf mustard | 3.01 ± 0.25abc | Wild chicory (local) | 2.68 ± 0.55bcde |
| Wasabina leaf mustard | 3.00 ± 0.48abc | Romaine lettuce 'Maraichere' | 2.65 ± 0.34bcde |
| Wild rocket (Ingegnoli) | 2.98 ± 0.32abcd | Lettuce 'Pamela' | 2.45 ± 0.20cde |
| Chicory 'Magdeburgo' | 2.97 ± 0.46abcd | Sorrel | 2.40 ± 0.69de |
| Lollo rossa lettuce | 2.96 ± 0.50abcd | Wild rocket 'Yeti' | 2.36 ± 0.52e |
| Chicory 'Spadona da taglio' | 2.96 ± 0.21abcd | Wild lettuce | 2.34 ± 0.53e |
| Witloof Chicory | 2.95 ± 0.46abcd | Lamb's lettuce 'Trophy F1' | 1.68 ± 0.39f |

Different letters show significant differences for $p \leq 0.05$, Tukey test. Data are means ± SD (n = 10).

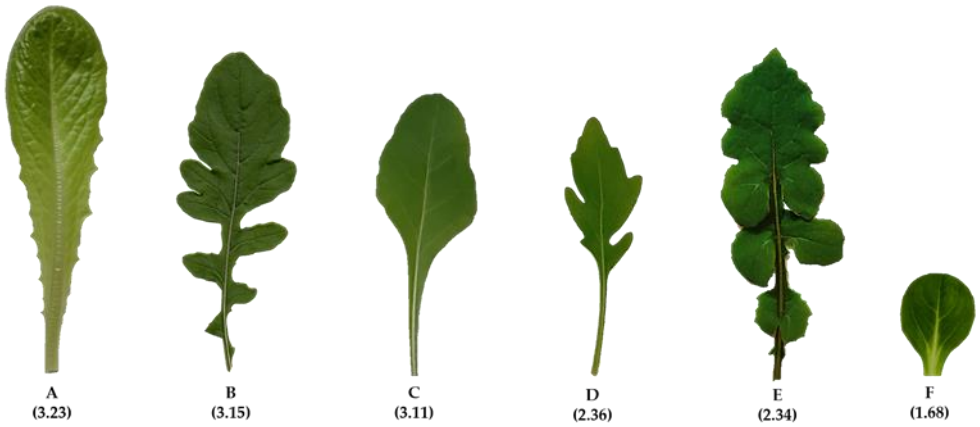



**Figure 4.1** The six selected accessions: romaine lettuce 'Bionda degli Ortolani' (**A**), Swiss chard (**B**), and rocket (**C**) as the most susceptible to contamination, and wild rocket 'Yeti' (**D**), wild lettuce (**E**), and lamb's lettuce 'Trophy F1' (**F**) as the least susceptible. Attachment values in brackets (log CFU/cm²).

*4.3.2 Leaf micro-morphological traits and water content*

The SEM images (Figure 4.2) highlighted significant differences in stomata density and size (length, width and rim area) between the six selected baby leaf accessions (Table 4.3). In wild lettuce, these parameters were not measured as this species did not have stomata on the adaxial leaf surface (Figure 4.2 E). Some of the observed differences were consistent with the contamination data: e.g., the absence of stomata in wild lettuce, the highest and lowest stomata density in rocket (92.20 ± 28.40) and lamb's lettuce 'Trophy F1' (57.14 ± 5.47), respectively, and the highest stomatal rim area in Swiss chard (45.58 ± 6.78 μm²). However, no significant correlation of *E. coli* attachment ATCC 35218 versus any of the stomatal parameters was detected (data not shown).

Three-dimensional digital microscopy revealed significant differences ($p < 0.001$) between the accessions for both the roughness parameters, with romaine lettuce 'Bionda degli Ortolani' and Swiss chard (more susceptible

accessions) showing higher values (Ra = 58.81 ± 11.52 and 54.70 ± 15.84, respectively; Rq = 71.56 ± 13.71 and 66.92 ± 18.35, respectively) than less susceptible accessions, i.e., wild rocket 'Yeti' (Ra = 41.33 ± 4.01; Rq = 50.23 ± 5.01), wild lettuce (Ra = 39.43 ± 12.42; Rq = 51.14 ± 15.49) and lamb's lettuce 'Trophy F1' (Ra = 24.74 ± 10.92; Rq = 31.38 ± 14.24) (Table 4.3). From a visual point of view, the differences in leaf roughness can be observed in the 3D reconstructions shown in Figure 4.3. A rough estimate of the Ra parameter can be obtained considering the differences between the maximum and minimum height values, which are higher in 'Bionda degli Ortolani' (Figure 4.3 A) and lower in lamb's lettuce 'Trophy F1' (Figure 4.3 F). The Pearson's test revealed a significant correlation between contamination level and both Ra and Rq values (Figure 4.4 A, B).

Moreover, significant differences ($p < 0.001$) between the selected accessions were observed in leaf water content (Table 4.3). The accession most susceptible to contamination ('Bionda degli Ortolani') showed the highest leaf water content (95.36 ± 0.84%) and differed from all the others, but especially from the least susceptible accessions, i.e., wild lettuce (89.79 ± 1.13%) and lamb's lettuce 'Trophy F1' (90.22 ± 2.30%). The correlation between *E. coli* ATCC 35218 attachment and water content was significant (Figure 4.4 C).

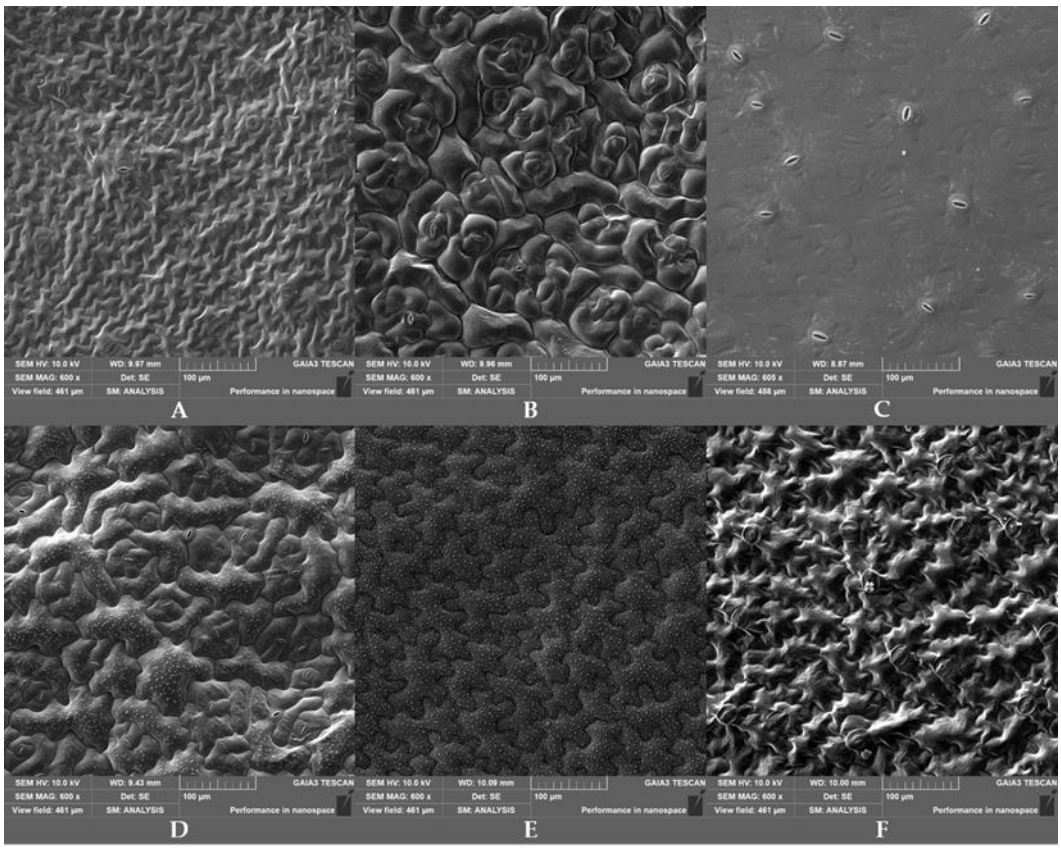



**Figure 4.2** SEM image (600×) of adaxial leaf surface of the six selected accessions: romaine lettuce 'Bionda degli Ortolani' (**A**), Swiss chard (**B**), rocket (**C**), wild rocket 'Yeti' (**D**), wild lettuce (**E**), and lamb's lettuce 'Trophy F1' (**F**). The absence of stomata can be noticed in wild lettuce.

**Table 4.3** Stomatal parameters (density, length, width, rim area), roughness (Ra and Rq) of adaxial leaf surface and water content (%) in six selected accessions of baby leaves (+ = most susceptible to contamination; — = less susceptible).

| Stomatal Density (n/mm²) | Stomata Length (µm) | Stomata Width (µm) | Stomatal Rim Area (µm²) | Ra | Rq | Water Content (%) |
|---|---|---|---|---|---|---|
| Romaine lettuce 'Bionda degli Ortolani' (+) | | | | | | |
| 71.96 ± 6.11ab | 25.93 ± 1.96b | 20.56 ± 3.28c | 23.03 ± 5.52bc | 58.81 ± 11.52a | 71.56 ± 13.71a | 95.36 ± 0.84a |
| Rocket (+) | | | | | | |
| 92.20 ± 28.40a | 23.46 ± 1.39c | 15.66 ± 1.92d | 11.70 ± 3.55c | 45.81 ± 7.74bc | 56.58 ± 9.79bc | 93.66 ± 1.59b |
| Swiss chard (+) | | | | | | |
| 61.65 ± 10.80b | 30.10 ± 1.98a | 21.85±3.38b | 45.58±6.78a | 54.70 ± 15.84ab | 66.92 ± 18.35ab | 92.66 ± 1.62b |
| Wild rocket 'Yeti' (−) | | | | | | |
| 80.29 ± 25.67ab | 21.85 ± 1.64d | 14.38±2.23e | 24.35±7.63b | 41.33 ± 4.01c | 50.23 ± 5.01c | 92.08 ± 1.03bc |
| Wild lettuce (−) | | | | | | |
| - | - | - | - | 39.43 ± 12.42c | 51.14 ± 15.49c | 89.79 ± 1.13c |
| Lamb's lettuce 'Trophy F1' (−) | | | | | | |
| 57.14 ± 5.47b | 30.19 ± 1.33a | 23.83 ± 2.05a | 19.84 ± 1.52bc | 24.74 ± 10.92d | 31.38 ± 14.24d | 90.22 ± 2.30c |

Different letters show significant differences for $p \leq 0.05$, Tukey test. Data are means ± SD (n = from 3 for roughness to 152 for stomata length and width).

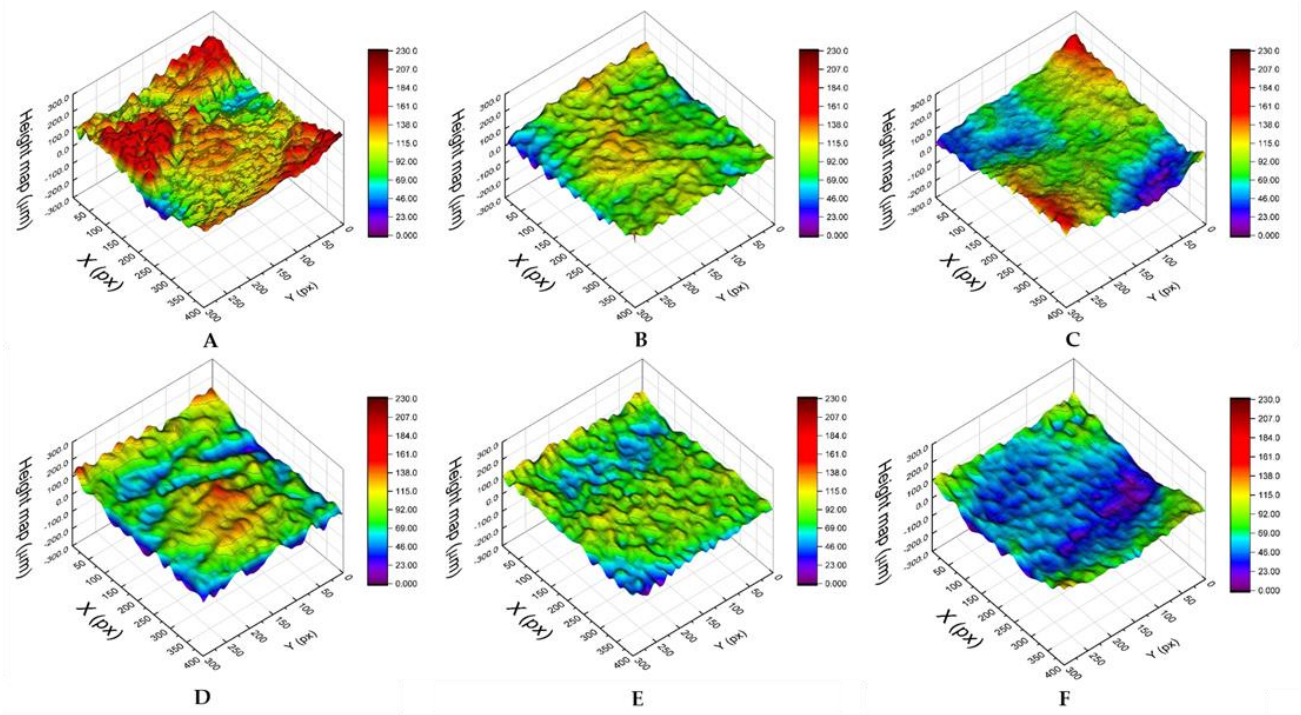



**Figure 4.3** Examples of leaf surface 3D reconstructions of the six selected accessions: romaine lettuce 'Bionda degli Ortolani' (**A**), Swiss chard (**B**), rocket (**C**), wild rocket 'Yeti' (**D**), wild lettuce (**E**) and lamb's lettuce 'Trophy F1' (**F**). X and Y axes are expressed in pixels (1 pixel = 17.5 µm), with heights reported on the vertical axis (µm). The corresponding color ramp is shown on the right of each reconstruction.

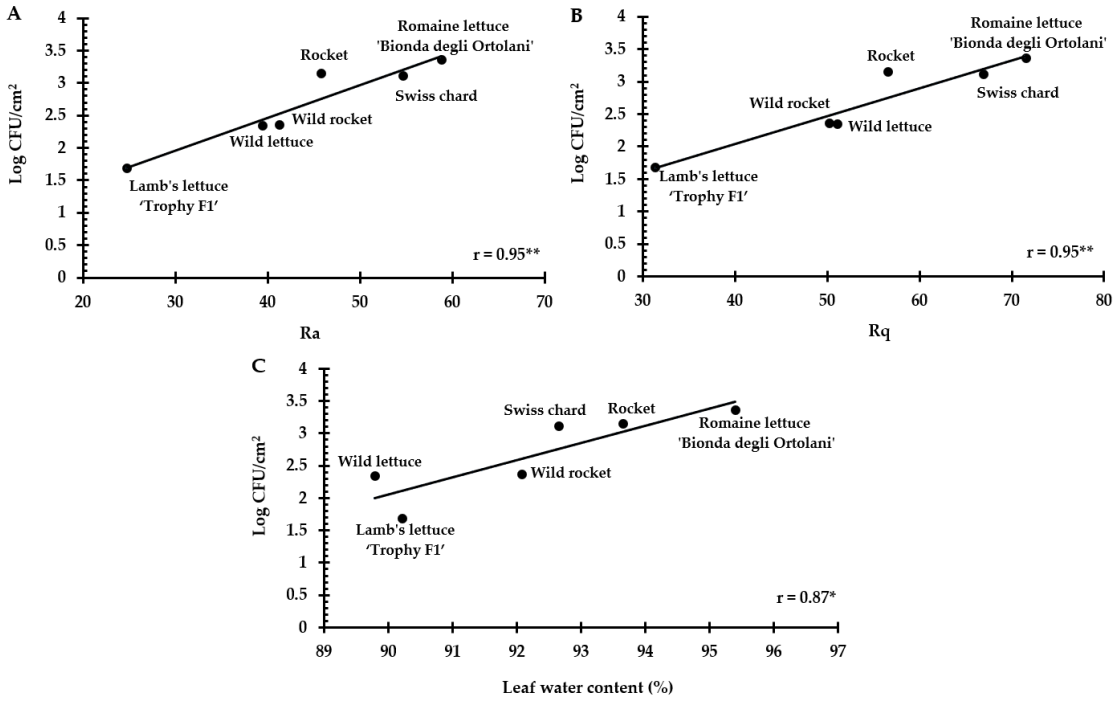



**Figure 4.4** Correlation between *E. coli* ATCC 35218 attachment and (**A**, **B**) roughness (Ra and Rq) and (**C**) leaf water content % in the six selected accessions. *Significant per $p < 0.05$; **significant per $p < 0.01$.

*4.3.3 UV experiment*

In the six selected accessions, *E. coli* ATCC 35218 survival was measured in the surface-inoculated leaves after UV treatment. UV rays significantly reduced *E. coli* ATCC 35218 contamination in all the accessions (Figure 4.5). Nevertheless, a lower reduction in the attachment was achieved in romaine lettuce 'Bionda degli Ortolani' (−8%) and rocket (−17%) compared to the accessions showing lower roughness (wild rocket 'Yeti', wild lettuce and lamb's lettuce 'Trophy F1'; −47% on average) (Figure 4.5). Swiss chard differed from 'Bionda degli Ortolani' and wild rocket 'Yeti'.

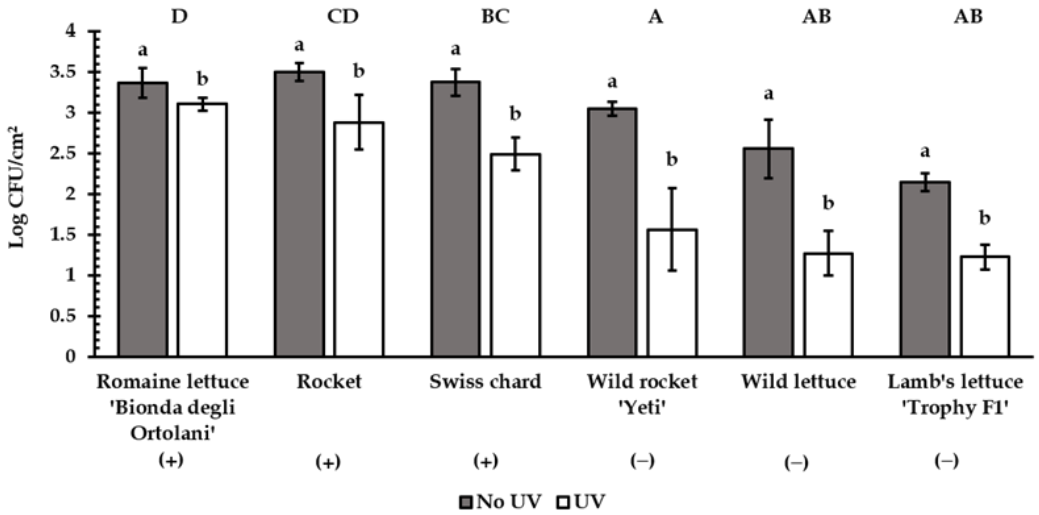



**Figure 4.5** Reduction effect of UV treatment on *E. coli* ATCC 35218 attachment in the six selected accessions (+ = rougher; − = less rough). Different lowercase letters show significant differences ($p < 0.05$) for each accession; different uppercase letters show significant differences ($p < 0.001$) in the net bacterial population reduction between accessions (Tukey test, n = 5). Bars represent ± SD.

## 4.4 Discussion

Intraspecific variability in the susceptibility to human pathogen contamination has been observed by many authors in several vegetable crops, among which lettuce was one of the most studied[34]. In particular, Jacob and Melotto[23] found differences in the attachment and persistence of *S. enterica* and *E. coli* between nine cultivars of this species. Our study confirmed differences in *E. coli* attachment within lettuces, even between accessions belonging to the same type (Table 4.2). Interestingly, 'Bionda degli Ortolani' and 'Maraichere', whose susceptibility resulted to be different, are both romaine lettuces. Romaine lettuce has been involved in many food disease outbreaks in the U.S. and, in particular, in *E. coli* infections[35-38]. These alerts are generically referred to this lettuce type, considered as one of the riskiest leafy vegetable in terms of foodborne diseases. However, since our data showed that romaine lettuce contamination varies by cultivar, a reduction in the risks associated with this lettuce could be achieved through varietal choice. Also, for *Diplotaxis tenuifolia*, we observed intraspecific variability (Table 4.2) which, to our knowledge, had not yet been found in this species. No differences in *E. coli* ATCC 35218 attachment were detected within the *Taraxacum campylodes*, *Brassica juncea, Brassica rapa*, *Cichorium intybus* and *Beta vulgaris* accessions. Conversely, the same accessions of *C. intybus* used in this study but compared at the microgreen stage (immature greens harvested when cotyledons the first pair of true leaves are more or less developed) showed a different susceptibility to *S. enterica* and *E. coli* contamination[39]. Actually, the stage of the leaf may affect the contamination, as noticed by Hunter[40].

It is known that the leaf micro-morphological traits influence the attachment and persistence of human pathogens on vegetables. Many studies have shown that stomata are involved in the contamination of leafy greens with *S. enterica* and *E. coli* [11,23,41,42]. Bacteria were found on guard cells, within and underneath the stomata cavity, and on the crevices in proximity to them[19]. In our study, the six selected baby leaf accessions differed in stomata (Table 4.3). Nevertheless, the Pearson's test revealed that the bacterial retention (1.5 h after inoculation) and stomatal parameters were not correlated. This finding agrees with the results reported by Jacob and Melotto[23], who did not find a correlation between stomata aperture width, pore area, and density and *E. coli* or *S.* Typhimurium attachment onto leaves of different lettuce genotypes. On the other hand, the same authors found that the bacterial persistence 10 days after surface inoculation was correlated with the stomatal aperture width and pore area[23]. Also, Macarisin[11] observed that stomata density affected the persistence of *E. coli* O157:H7 in different spinach cultivars. Therefore, as suggested by Jacob and Melotto[23], for lettuce, stomata seem to have a role in bacterial penetration into the leaves, while the attachment might be influenced by other properties of the leaf surface. Traits such as trichomes, hydrophobicity, vein areas, cuticular waxes, surface irregularities and surface proteins and sugars can be associated with different attachment levels[40,43,44]. Palma-Salgado et al.[24] found that leaf surface roughness was positively correlated to *E. coli* adhesion in different leafy greens. Also, in spinach, roughness was an important factor determining the differential attachment and persistence of *E. coli* on leaves[11]. Our results support these previous findings. In fact, a significant correlation between the contamination level and roughness parameters (Ra and Rq values) was observed (Figure 4.4).

Leaf roughness can also reduce the effectiveness of sanitizing treatments for vegetables. Doan et al.[14] and Yi et al.[45] reported that the variation in leaf topography accounted for differences in survival of *E. coli* in chlorine-treated leafy greens of different species and cultivars. Surface roughness revealed to be a factor protecting *E. coli* cells from

treatment with chlorine also in fruits and seeds[46,47]. UV radiation can be used as an alternative to chemicals for the sanitization of food products[48]. Different studies demonstrated the effectiveness of UV rays to control foodborne pathogens in fresh-cut vegetables, including lettuce[49,50]. In general, the role of surface topography on the effectiveness of UV treatment for bacterial inactivation was demonstrated by Woodling and Moraro in stainless-steel surfaces[51]. In our study, UV treatment resulted in less *E. coli* ATCC 35218 attachment reduction in rougher accessions than in those showing less roughness (Figure 4.5). Therefore, our results suggest that leaf roughness may offer protective niches to bacteria from UV rays.

Finally, the positive correlation between *E. coli* ATCC 35218 attachment and leaf water content that we found could suggest that the internal water status may impact the bacterial contamination of the leaves. Interestingly, in Yadav et al.[25], leaf water content was positively correlated with bacterial colonization of the phyllosphere in eight Mediterranean plant species. *S. enterica* colonization was enhanced by water-congestion in green tomato fruits[52]. The mechanism by which tissue water amount influences bacterial contamination in plants could be related to physical and/or chemical factors associated to tissue turgidity.

## 4.5 Conclusions

The screening of 30 baby leaf accessions for the susceptibility to *E. coli* ATCC 35218 contamination confirmed that variability exists among different leafy crop species, types of the same species and cultivars. The differences within the same species suggest that the choice of the cultivar can be a means to reduce the risk of foodborne diseases linked to the consumption of salads. Even within var. *longifolia* of lettuce (romaine type), which is the most involved in food disease outbreaks in the U.S., and for this reason is considered one of the riskiest salads, it seems possible to find genotypes (e.g. cv. 'Maraichere') less susceptible to contamination than others (*e.g.*, cv. 'Bionda degli Ortolani'). These findings could find a practical application in orienting growers and the vegetable industry towards safer crops.

Our study also showed that leaf roughness and water content impact on *E. coli* ATCC 35218 attachment in baby greens. The use of an innovative portable 3D digital microscope highlighted the differences in the leaf roughness of six selected accessions. Among the measured leaf micro-morphological traits, roughness was the only one to be positively correlated with the *E. coli* ATCC 35218 contamination level. Roughness also somehow offered UV protection to bacteria. In fact, when *E. coli* ATCC 35218 retention in surface-inoculated leaves was measured after UV treatment, bacterial contamination was reduced to a minor extent in the rougher leaves. This finding further underlies the impact of leaf roughness on the safety of vegetables, suggesting that sanitization treatment through UV rays should be modulated accounting for this parameter. The correlation between *E. coli* ATCC 35218 attachment and leaf water content was also significant, suggesting that the internal water status can impact the bacterial contamination of the leaves.

Looking forward, new findings on leaf characteristics associated with lower susceptibility to attachment and proliferation of human pathogens could be used in selection breeding in order to obtain safer cultivars and thus increase food safety in the vegetable industry. In particular, our results identified roughness as a trait to be considered in breeding programs.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biology12010102/s1, Table S1: Number of leaves (NL), leaf fresh weight (FW) and dry weight (DW), leaf area (LA); and leaf water content (LWC) of the 30 baby leaf accessions (average ± SD); Table S2: Colour (a*, b*, L*) of the 30 baby leaf accessions (average ± SD); Video S1: 3D reconstruction of the surface under inspection obtained elaborating a sequence of defocused images implementing a suitable algorithm based on shape from the focusing technique.

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# 5. Second paper

## Relationship between *Salmonella enterica* attachment and leaf hydrophobicity, roughness, and epicuticular waxes: a focus on 30 baby-leaf salads

### 5.1 Introduction

Human pathogens have conventionally been associated with foods originating from animal sources. Nevertheless, during the last decades, several outbreaks associated with fruits and vegetables occurred, showing that plants are an important vector of food-borne diseases[1]. Contamination frequently takes place when crop plants encounter biologically polluted irrigation, wastewater, contaminated fertilizers, small carrier animals, and poor hygiene practices, before or after the harvest[2,3]. According to the EFSA and ECDC 2021 zoonoses report[1], the frequency distribution of strong-evidence foodborne outbreaks, by food vehicle, showed that vegetables and juices (and similar products) caused a higher number of outbreaks when compared to the traditional high-risk broiler meat. The same report listed the *Salmonella*/vegetables and juices association at the 7th position in the ranking of the top-ten pathogen/food pairs causing strong-evidence outbreaks in the EU in 2021, with 11 outbreaks[1]. Baby-leaf vegetables are particularly susceptible to contamination because of the tender leaves (high water content) associated with the early growth stage, and their use in salads, which involves eating them raw[4]. The first step in the contamination of salads with bacteria is the attachment on the leaf surface, which, in the absence of timely decontamination treatments, can be followed by internalization[5,6].

The interaction between bacteria and leaves is a complex process influenced by various factors, including the physical and chemical characteristics of the leaf surface[7]. Previous research has shown that the leaf surface plays a critical role in providing attachment sites for human pathogens such as *Salmonella enterica* and *Escherichia coli*[9] and influences the establishment of bacterial communities on the leaves[10,11]. Surface roughness, in particular, can provide numerous microhabitats for bacteria to attach and hide, offering protection from external factors such as wind, rain, and sunlight, including protection from UV radiation[9,11,12,13]. In spinach, leaf blade roughness and stomata density influenced the persistence of *E. coli* O157:H7[13]. Trichomes, stomata and leaf veins also contribute to the overall roughness. Doan et al. (2020)[9] showed that leaf venation prevented the recovery of *E. coli* from the surface of spinach leaves using water washing and rinsing, even in the presence of a detergent, and increased the ability of the bacterium to survive chlorine washing.

Another factor involved in bacterial attachment is the hydrophobic or hydrophilic nature of the leaf surface. In general, bacteria are more likely to attach to hydrophilic surfaces due to favorable interactions with water molecules and surface charges[14]. The presence of epicuticular waxes (waxes of the outermost layer of the leaf surface) makes leaf hydrophobic to an extent dependent on their amount and chemical composition, thus influencing the attachment of human pathogens[15,16].

The aims of this study were: i) to evaluate the attachment of *S. enterica* on the leaves of 30 green salads contaminated at the baby-leaf stage; ii) to test whether the differences in susceptibility to *Salmonella* contamination among the salads were related to the following leaf traits: hydrophobicity, roughness, and epicuticular waxes; leaf water status

was also considered. It is important to know these relations, not only for understanding plant-bacteria interactions but also for providing information possibly useful to improve baby leaves safety in a farm-to-fork scenario.

## 5.2 Materials and Methods

### *5.2.1 Production of the baby leaves*

Baby leaves of 30 different accessions belonging to 13 species were tested. Seeds of the 30 accessions were used as starting material. The detailed list of the accessions and the seed source are reported in Truschi et al. (2023)[11]. Seeds were sown in polystyrene alveolate trays filled with vermiculite at a density of 3000 seeds $m^{-2}$. After sowing, trays were kept for 48 h in the dark at 20 °C for promoting germination, and were then transferred in a floating system in a growth chamber at 21 ± 2 °C (day) and 14 ± 2 °C (night) with a photoperiod of 16 h under fluorescent lighting units OSRAM L36W/77 (36 W, 1400 lm, 120 cm in length, 26 mm in diameter). A full-strength Hoagland's nutrient solution (macroelements expressed in mM and microelements in μM: N 15.0, P 0.10, K 6.0, Ca 5.0, Mg 2.0, Fe 50.0, B 46.2, Mn 9.2, Zn 0.78, Cu 0.32, Mo 0.12) was used. At the baby-leaf stage (five/six weeks after sowing depending on the species), plants were cut at the base of the petiole and immediately inoculated. Besides, the following leaf parameters were analysed: hydrophobicity, roughness, wax and water content. The water content was measured in 8-12 plants per accession using the formula [(FW-DW)/FW]*100, where FW is fresh weight and DW is dry weight measured after oven drying at 80 °C until constant weight.

### *5.2.2 S. enterica surface inoculation of the baby leaves*

*S. enterica* subsp. *enterica* serovar Typhimurium ATCC 19585 was used for the surface inoculation.

The inocula started from a -20 °C glycerol stock. Briefly, 1 mL from an overnight inoculum in Lysogeny Broth (LB, Oxoid, UK) was washed three times and diluted in a sterile Physiological Solution (PS, NaCl 0.85% w/v in $H_2O$) to clean the cells from LB medium. Leaf disks 1.3 cm in diameter were cut from 5 leaves of different plants for each accession. Twenty μL of the working bacterial suspension (containing approximately $5x10^7$ cells/mL) were placed onto the centre of the leaf disks on the adaxial side and incubated for 5 min at 25 °C in static. After incubation, the leaf disks were gently picked up with sterile tweezers and washed four times in 15 mL of clean PS in glass tubes to remove un-attached bacterial cells. Rinsed disks were subsequently grinded with a mini-pestle in 0.5 mL of PS into 1.5 mL tubes. After grinding, 20 μL of the suspension was plated onto selective and differential media XLD Agar (Oxoid, UK) and incubated at 37 °C overnight. Colony-forming units (CFU) were counted and log base 10 (lg) transformed.

### *5.2.3 Contact angle measurement*

The hydrophobicity of the leaf surface of the 30 accessions was quantified by measuring the contact angle using a drop shape analyzer equipped with a video camera and connected to a computer (DSA 25E; Krüss, Hamburg, Germany). Leaf samples were carefully placed on clean microscopic slides using a double-sided adhesive tape. Droplets of pure water (10 μl) were carefully placed on the adaxial side of the leaf surfaces. Each droplet equilibrated 10 s on the surface before measurement. Contact angles were measured using the sessile drop method.

This method determines the contact angle from the shadow image of a sessile drop and is based on an ellipse algorithm (tangent-1). Five different measurements were performed for each accession. Each measurement was done with a freshwater droplet.

*5.2.4 Leaf surface roughness*

A portable 3D digital microscope used for imaging the leaf surface and measuring local roughness was based on the "deep focus" technique[17]. The operating principle is based on the simple observation that, due to the limited depth of focus of each optical system, only objects placed at a suitable distance from the sensor form in-focus images, while those located at different distances appear out of focus (blurred). Thus, in order to reconstruct the surface of an object, a series of images of the same scene are acquired, corresponding to different positions of the optical group (including imaging optics, digital camera, and smart lighting system)[18,19,20], translated along the optical axis (scan). Each image will contain in-focus and out-of-focus parts of the surface under examination. A dedicated software processes the sequence of images. From each image, the focused areas are extracted, and, in order to reconstruct the 3D surface, composed together to obtain the depth scale once the translation is provided. In this work, the minimum step of the calibrated translation stage was of the order of magnitude of the microns, the field of view of about 7x5.2 mm, thus determining a vertical resolution of about 10 μm. Surface roughness parameters were extracted from 3D reconstruction, according to ISO standards[21], focusing the analysis of plant surface on the average roughness (Ra). These measurements are completely non-contact as well as non-destructive; this overcomes the disadvantages of using a contact stylus profilometer with soft biological samples. For each accession, up to 12 different analyses (replicates) were performed.

*5.2.5 Quantification and qualification of epicuticular waxes*

Glass vials with broad rims and a central opening with a defined area were filled with chloroform (1.5 ml). Intact leaves of each accession (with 3 replications) were carefully placed on a clean Teflon disk. Due to the different size of the leaves, two defined areas were used (0.384 and 1.25 $cm^2$).

The leaf side of interest was gently pressed on the opening of the glass vial and turned upside down for 10 s to allow wax extraction by chloroform in the vial. Subsequently, the wax extract was directly spiked with the $C_{24}$ alkane (internal standard) and the volume was reduced to 200 μl under a gentle stream of nitrogen at 60 °C. Prior to gas chromatography, samples were derivatized using BSTFA [N, O-bis-(trimethylsilyl)-trifluoroacetamide, Merck] at 70 °C for 45 min. For derivatization, 20 μl BSTFA and 20 μl pyridine as a catalyst were added to the samples dissolved in 200 μl of chloroform. Quantification was performed by on-column injection analyzing 1 μl of each sample in a gas chromatograph connected to a flame ionization detector (GC-FID: Agilent 5980; column: 30 m DB-1 with an inner diameter of 0.32 mm and film 0.1 μm, Agilent). Identification of wax was achieved by mass spectrometry (GC: Agilent 6890 N; MS: Agilent 5973 N mass selective detector; column: 30 m DB-1MS with an inner diameter of 0.32 mm and film 0.1 μm). Identification of the individual peaks was based on fragmentation patterns of the peaks and by comparing obtained mass spectra with stored mass spectra in the NIST 2011 library.

Moreover, images of the epicuticular wax crystals were taken using a Scanning Electron Microscope (SEM). The images were captured under low vacuum at 10 kV and 800× and 8000× resolution from at least three different samples per accession.

*5.2.6 Statistical analysis*

All the statistical analyses were performed using the Rstudio software[22] (version 4.3.1). Data were subjected to the Shapiro–Wilk test for normality and Levene's test for homogeneity of variances to verify the ANOVA's assumptions, using the *car* package[23]. Since the assumptions were not respected, a Linear Mixed Model (LMM) was applied to all the parameters, considering the accessions as fixed factor and the repeated measures as random factor, using the *lme4* package[24]. Then, the Tukey test was applied ($p \leq 0.05$) using the *multcompView* package[25]. Moreover, the Pearson's correlation test ($p \leq 0.05$) was used to determine the relationship between *S. enterica* ATCC 19585 attachment and the considered leaf traits, using the *bruce*R package[26]. Wax components that were detected in only a small number (1-7) of accessions (aldehydes, alkanes and esters) were excluded from the correlation test. A Principal Component Analysis (PCA) was carried out using R Studio with *FactorMine*R and *Factorextra* packages for all recorded data[27,28]. The highly correlated variables ($r > 0.90$) were excluded from the PCA. Finally, a partial least squares (PLS) model was established to predict the amount of *S. enterica* attachment (Y variable) by specifying six variables (X variables: contact angle, surface roughness, C26 alcohol, alcohols, fatty acids, and water content) using the *mdatools* package[29]. Leave-One-Out Cross-Validation (LOOCV), using the coefficient of determination ($R^2$), and the root mean square error of prediction (RMSE) were applied to verify the PLS model. Parameters that had variable importance for projection (VIP) <0.8 were considered not to make a major contribution to dimensionality reduction in PLS.

## 5.3 Results

*5.3.1 S. enterica attachment in baby leaves*

Differences in *S. enterica* attachment were observed among the 30 baby-leaf accessions (Table 5.1). Sorrel, Red Giant leaf mustard, pak-choi, rocket, endive, Swiss chard, and mizuna all showed a contamination level higher than 3.7 log CFU/cm² and were found to be significantly more susceptible to *Salmonella* contamination than wild lettuce, lamb's lettuce, wild rocket 'Yeti', lettuce 'Pamela', Lollo Rossa lettuce, dandelion Ingegnoli, wild rocket Ingegnoli, and blonde lettuce (from 1.63 to 3.29 log CFU/cm²). Intermediate values (from 3.39 to 3.66 log CFU/cm²) were measured in wild chicory Ingegnoli, romaine lettuce 'Maraichere', chicories 'Magdeburgo' and 'Spadona da Taglio', wild chicories (B&T and local), spinach 'Cugoe RZ F1', romaine 'Bionda degli Ortolani', red leaf mustard, local dandelion, Lollo Verde lettuce, red chard 'Bull's Blood Artica', chicories Witloof and 'Biondissima di Trieste', and mizuna. Lamb's lettuce 'Trophy F1' and wild lettuce were significantly different from all the other accessions, with the lowest level of contamination ($1.79 \pm 0.54$ and 1.63 log CFU/cm², respectively).

**Table 5.1** *S. enterica* attachment [log(CFU/cm²)], contact angle (°), leaf roughness (Ra), and leaf water content (%), in 30 baby-leaf accessions.

| ID† | Botanical family | Species | Accession | Attachment Log(CFU/cm²) | Contact angle (°) | Leaf roughness (Ra) | Leaf water content (%) |
|---|---|---|---|---|---|---|---|
| 7 | Asteraceae | *Cichorium endivia* L. | Endive | 3.72 ± 0.01 a | 46.76 ± 5.78 gh | 13.88 ± 0.97 ef | 91.40 ± 3.31 ab |
| 6 | | *Cichorium intybus* L. | Chicory 'Biondissima di Trieste' | 3.59 ± 0.02 abc | 40.38 ± 3.80 hi | 18.09 ± 3.55 cdef | 93.65 ± 0.94 a |
| 2 | | | Chicory 'Magdeburgo' | 3.42 ± 0.01 abcde | 55.76 ± 1.07 fgh | 13.89 ± 3.93 ef | 92.77 ± 1.36 a |
| 22 | | | Chicory 'Spadona da Taglio' | 3.44 ± 0.02 abcde | 28.57 ± 7.04 i | 31.34 ± 9.39 abcdef | 92.18 ± 0.55 ab |
| 5 | | | Wild chicory (B&T) | 3.42 ± 0.01 abcde | 45.12 ± 8.70 gh | 43.83 ± 11.16 ab | 91.64 ± 0.82 ab |
| 1 | | | Wild chicory (Ingegnoli) | 3.39 ± 0.01 abcdef | 52.14 ± 5.41 gh | 26.50 ± 3.85 abcdef | 93.56 ± 2.79 a |
| 4 | | | Wild chicory (local) | 3.44 ± 0.07 abcde | 45.72 ± 6.82 gh | 15.86 ± 2.47 def | 90.68 ± 3.54 ab |
| 3 | | | Witloof chicory | 3.62 ± 0.1 abc | 39.62 ± 2.15 hi | 14.84 ± 2.19 ef | 92.46 ± 1.54 a |
| 29 | | *Lactuca sativa* L. | Blonde lettuce | 3.29 ± 0.03 bcdef | 51.09 ± 3.87 gh | 23.91 ± 2.95 abcdef | 96.11 ± 0.83 a |
| 28 | | | Lettuce 'Pamela' | 3.11 ± 0.02 ef | 48.68 ± 2.10 gh | 41.88 ± 12.51 abc | 93.30 ± 1.31 a |
| 9 | | | Lollo Rossa lettuce | 3.17 ± 0.03 def | 44.35 ± 2.38 ghi | 27.77 ± 3.19 abcdef | 94.78 ± 0.97 a |
| 8 | | | Lollo Verde lettuce | 3.49 ± 0.03 abcde | 58.12 ± 8.81 fg | 38.13 ± 8.29 abcde | 95.75 ± 0.20 a |
| 23 | | | Romaine lettuce 'Bionda degli Ortolani' | 3.46 ± 0.08 abcde | 48.62 ± 4.54 gh | 24.00 ± 2.65 abcdef | 95.10 ± 0.65 a |
| 30 | | | Romaine lettuce 'Maraichere' | 3.40 ± 0.03 abcde | 92.28 ± 1.09 cd | 19.04 ± 5.11 bcdef | 92.89 ± 1.56 a |
| 33 | | *Lactuca serriola* L. | Wild lettuce | 1.63 ± 0.39 g | 135.54 ± 5.52 a | 20.73 ± 5.98 bcdef | 90.31 ± 0.55 ab |
| 15 | | *Taraxacum officinale* L. | Dandelion (Ingegnoli) | 3.22 ± 0.02 cdef | 55.34 ± 1.74 fgh | 12.18 ± 1.30 f | 89.59 ± 0.49 ab |
| 14 | | | Dandelion (local) | 3.49 ± 0.02 abcde | 50.27 ± 1.40 gh | 13.95 ± 1.79 ef | 85.19 ± 9.61 b |
| 20 | Brassicaceae | *Brassica juncea* [L.] Czern. | Red Giant leaf mustard | 3.77 ± 0.01 a | 71.34 ± 1.85 ef | 46.80 ± 15.43 a | 94.04 ± 0.52 a |
| 16 | | | Red leaf mustard | 3.49 ± 0.03 abcde | 85.77 ± 6.80 de | 19.68 ± 2.54 bcdef | 93.08 ± 0.56 a |
| 25 | | | Wasabina leaf mustard | 3.76 ± 0.01 a | 70.94 ± 7.10 ef | 20.69 ± 4.54 bcdef | 93.72 ± 0.46 a |
| 19 | | *Brassica rapa* L. | Mizuna | 3.66 ± 0.09 ab | 104.76 ± 2.52 bc | 19.72 ± 9.71 bcdef | 93.41 ± 0.83 a |
| 17 | | | Pak-choi | 3.75 ± 0.01 a | 85.37 ± 5.36 de | 40.29 ± 14.21 abcd | 95.49 ± 0.29 a |
| 32 | | *Diplotaxis tenuifolia* [L.] D.C. | Wild rocket 'Yeti' | 2.98 ± 0.09 f | 109.72 ± 1.83 b | 18.38 ± 6.34 cdef | 92.66 ± 0.55 a |
| 24 | | | Wild rocket (Ingegnoli) | 3.25 ± 0.06 bcdef | 108.92 ± 2.75 b | 28.19 ± 11.09 abcdef | 93.95 ± 1.04 a |
| 10 | | *Eruca vesicaria* [L.] Cav. subsp. *sativa* [Mill.] Thell | Rocket | 3.73 ± 0.01 a | 92.27 ± 3.55 cd | 28.08 ± 9.23 abcdef | 93.33 ± 1.53 a |
| 31 | Chenopodiaceae | *Beta vulgaris* L. | Red chard 'Bull's Blood Artica' | 3.56 ± 0.02 abcd | 97.78 ± 7.49 bcd | 21.07 ± 2.35 bcdef | 93.38 ± 1.51 a |
| 27 | | | Swiss chard | 3.72 ± 0.03 a | 90.27 ± 2.22 cd | 25.89 ± 10.52 abcdef | 93.19 ± 2.12 a |
| 26 | | *S. oleracea* L. | Spinach 'Cugoe RZ F1' | 3.45 ± 0.01 abcde | 88.46 ± 7.24 d | 29.24 ± 17.84 abcdef | 93.29 ± 0.28 a |
| 21 | Polygonaceae | *R. acetosa* L. | Sorrel | 3.79 ± 0.04 a | 46.19 ± 5.53 gh | 21.98 ± 1.50 abcdef | 93.04 ± 1.23 a |
| 12 | Valerianaceae | *V. locusta* [L.] Laterr. | Lamb's lettuce 'Trophy F1' | 1.79 ± 0.54 g | 82.39 ± 7.25 de | 12.87 ± 3.07 f | 89.92 ± 1.76 ab |
| | | | *p-value (LMM)* | *** | *** | *** | *** |

†Accession Identification Number. Data are means ± SD. In the same column, different letters show statistically significant differences for $p < 0.001$ (Tukey test).

*5.3.2 Contact angle*

Significant differences in hydrophobicity were found between the 30 accessions. Wild lettuce leaves showed the highest contact angle (136.5 ± 6.97°), different from all the other accessions, thus resulting in the worst wettability. On the contrary, chicory 'Spadona da Taglio' had the smallest contact angle (28.49 ± 5.75°), but without significant differences compared to other chicories ('Biondissima di Trieste' and witloof) and to Lollo Rossa lettuce (Table 5.1).

*5.3.3 Roughness and water content*

Three-dimensional digital microscopy revealed significant differences ($p < 0.001$) for the Ra parameter among the 30 accessions. As shown in Table 5.1, the smoothest leaf surfaces (lowest Ra values) were found in lamb's lettuce 'Trophy F1' and dandelion Ingegnoli, with values (12.87 ± 3.07 µm and 12.18 ± 1.30 µm, respectively) different compared to wild chicory B&T, the lettuces 'Pamela' and Lollo Verde, pak-choi, and Red Giant leaf mustard. The latter accession had the roughest leaves (46.80 ± 15.43 µm). From a purely visual point of view, the difference between lamb's lettuce and Red Giant leaf mustard can be observed in the 3D reconstructions of the leaf surface shown in Figure 5.1. A rough estimate of the different degrees of roughness can be obtained by observing the height difference between the bulges and cavities (expressed by the scale of colors).

All the baby-leaf salads but the two dandelion accessions had a water content higher than 90%. In some lettuces (blonde, Lollo Verde, and romaine 'Bionda degli Ortolani') and in pak-choi it was even higher than 95% (Table 5.1). Local dandelion showed the lowest value (85.19%).

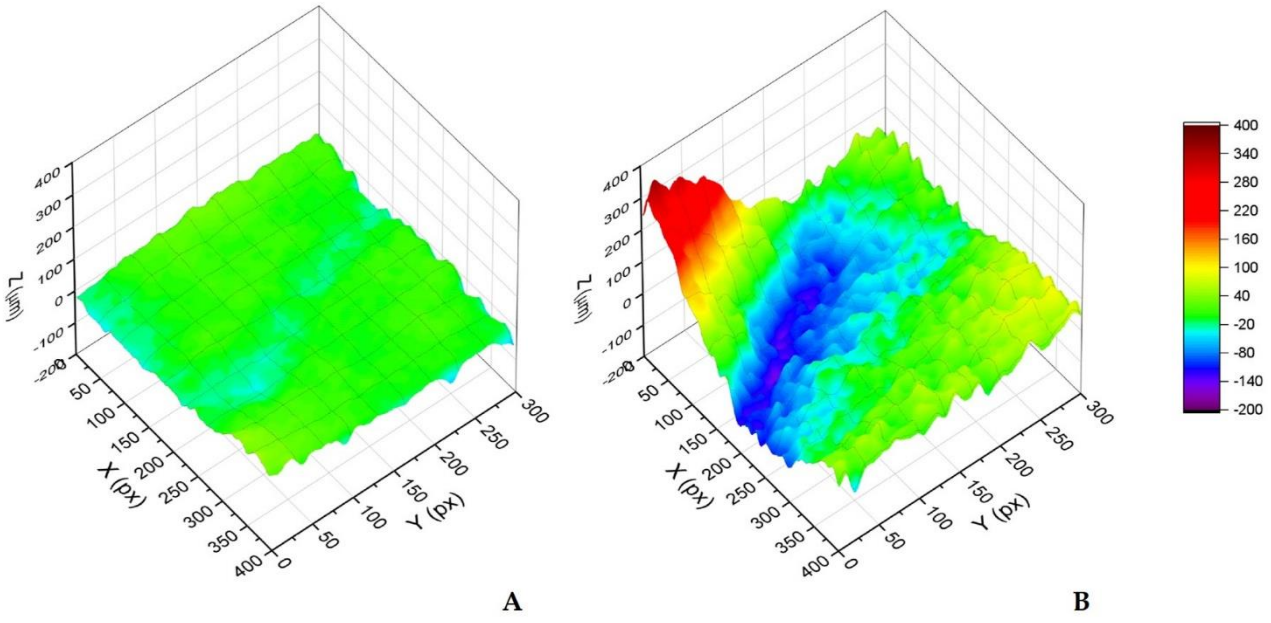


**Figure 5.1** Examples of leaf surface 3D reconstructions: lamb's lettuce 'Trophy F1' (A) and Red Giant leaf mustard (B). X and Y axes are expressed in pixels (1 pixel = 17.5 µm), with heights reported on the vertical axis (µm). Observing the height differences between bulges and cavities (expressed by the scale of colors) it is possible to obtain a rough estimate of the different degrees of roughness. The color ramp is shown on the right side of the figure.

*5.3.4 Wax quantification and characterization*

In all baby-leaf salads the epicuticular waxes included fatty acids and alcohols, while aldehydes, alkanes, and esters, were only found in a limited number of accessions (Table 5.2). Only wild lettuce showed aldehydes (0.82 ± 0.08 μg/cm²); alkanes were detected in spinach 'Cugoe RZ F1' (0.69 ± 0.16 μg/cm²), wild lettuce (0.20 ± 0.04 μg/cm²), and sorrel (0.07 ± 0.01 μg/cm²); whereas esters in the lettuce group (*L. sativa*) and wild lettuce (*L. serriola*), with values ranging from 0.07 to 0.79 ± 0.02 μg/cm². Alcohols were the main component of waxes (about 80% of the total waxes as an average of the 30 accessions). Only in rocket and wild rocket did the fatty acid content exceed that of alcohol (Table 5.2). Both the wild rockets had significantly higher fatty acid amounts (2.66 ± 0.45 μg/cm² and 2.05 ± 0.89 μg/cm², respectively) than all the other accessions. Low fatty acids values were found in chicories, especially in local wild chicory (0.04 ± 0.02 μg/cm²), 'Biondissima di Trieste' (0.05 ± 0.02 μg/cm²), witloof (0.05 ± 0.02 μg/cm²), and 'Magdeburgo' (0.06 ± 0.01 μg/cm²), as well as in local dandelion (0.07 ± 0.02 μg/cm²) and Wasabina leaf mustard (0.09 ± 0.01 μg/cm²) (Table 5.2). Wild lettuce was by far the accession with the highest content of alcohols (12.32 ± 1.35 μg/cm²), of which even 84.3% was C26 alcohol. Wild lettuce was followed by accessions with about 3 μg/cm² alcohols: lettuce 'Pamela' (3.34 ± 1.08 μg/cm²), Lollo Rossa lettuce (3.02 ± 0.70 μg/cm²), lamb's lettuce 'Trophy F1' (2.87 ± 0.88 μg/cm²), and romaine lettuce 'Maraichere' (2.76 ± 0.39 μg/cm²). In these accessions, the C26 alcohol accounted for 8% to 22% of the total alcohols. Higher percentages of this component were observed in other accessions (e.g. 55% in Wasabina leaf mustard), but with low absolute values. The lowest alcohol concentration was found in sorrel (0.34 ± 0.07 μg/cm²). Alcohols were strongly correlated with total wax content (Figure 5.2). Consistently, wild lettuce and sorrel were the accessions with the highest (14.23 ± 3.60 μg/cm²) and the lowest (0.51 ± 0.09 μg/cm²) total wax amount, respectively, as can be seen in SEM images (Figure 5.3). In lamb's lettuce, the gas chromatographic analysis revealed also the presence of thymol, a monoterpenoid phenol, in the amount of 0.26 ± 0.07 μg/cm² (data not shown).

**Table 5.2** Chemical composition of epicuticular waxes in 30 baby-leaf accessions.

| ID† | Accession | Fatty acid (µg/cm²) | Alcohol (µg/cm²) | Aldehyde (µg/cm²) | Alkane (µg/cm²) | Ester (µg/cm²) | Tot wax (µg/cm²) |
|---|---|---|---|---|---|---|---|
| 7 | Endive | 0.10 ± 0.05 cd | 0.77 ± 0.20 hijk | - | - | - | 0.87 ± 0.16 cde |
| 6 | Chicory 'Biondissima di Trieste' | 0.05 ± 0.02 d | 1.30 ± 0.20 fghijk | - | - | - | 1.34 ± 0.18 bcde |
| 2 | Chicory 'Magdeburgo' | 0.06 ± 0.01 d | 1.77 ± 0.03 cdefghij | - | - | - | 1.83 ± 0.11 bcde |
| 22 | Chicory 'Spadona da Taglio' | 0.19 ± 0.1 bcd | 1.81 ± 0.21 cdefghij | - | - | - | 1.99 ± 0.30 bcde |
| 5 | Wild chicory (B&T) | 0.06 ± 0.00 d | 2.17 ± 0.23 bcdefgh | - | - | - | 2.23 ± 0.23 bcde |
| 1 | Wild chicory (Ingegnoli) | 0.23 ± 0.03 bcd | 2.11 ± 0.44 bcdefghi | - | - | - | 2.34 ± 0.41 bcde |
| 4 | Wild chicory (local) | 0.04 ± 0.02 d | 1.59 ± 0.22 cdefghijk | - | - | - | 1.63 ± 0.22 bcde |
| 3 | Witloof chicory | 0.05 ± 0.02 d | 1.43 ± 0.03 efghijk | - | - | - | 1.48 ± 0.05 bcde |
| 29 | Blonde lettuce | 0.17 ± 0.07 bcd | 2.39 ± 0.16 bcdef | - | - | 0.09 ± 0.03 c | 2.65 ± 0.14 bcde |
| 28 | Lettuce 'Pamela' | 0.18 ± 0.03 bcd | 3.34 ± 1.08 b | - | - | 0.07 ± 0.00 c | 3.59 ± 1.05 b |
| 9 | Lollo Rossa lettuce | 0.17 ± 0.02 bcd | 3.02 ± 0.70 bc | - | - | 0.16 ± 0.02 b | 3.34 ± 0.72 bc |
| 8 | Lollo Verde lettuce | 0.16 ± 0.03 bcd | 1.52 ± 0.29 defghijk | - | - | 0.14 ± 0.01 b | 1.82 ± 0.02 bcde |
| 23 | Romaine lettuce 'Bionda degli Ortolani' | 0.26 ± 0.03 bcd | 1.70 ± 0.07 cdefghijk | - | - | 0.16 ± 0.04 b | 2.12 ± 0.20 bcde |
| 30 | Romaine lettuce 'Maraichere' | 0.14 ± 0.05 bcd | 2.76 ± 0.39 bcde | - | - | 0.09 ± 0.01 c | 2.99 ± 0.43 bcde |
| 33 | Wild lettuce | 0.10 ± 0.05 cd | 12.32 ± 1.35 a | 0.82 ± 0.08 | 0.20 ± 0.04 b | 0.79 ± 0.02 a | 14.23 ± 3.60 a |
| 15 | Dandelion (Ingegnoli) | 0.12 ± 0.07 cd | 2.36 ± 0.31 bcdefg | - | - | - | 2.47 ± 0.38 bcde |
| 14 | Dandelion (local) | 0.07 ± 0.02 d | 1.70 ± 0.21 cdefghijk | - | - | - | 1.77 ± 0.20 bcde |
| 20 | Red Giant leaf mustard | 0.50 ± 0.01 bcd | 0.50 ± 0.14 jk | - | - | - | 0.55 ± 0.14 de |
| 16 | Red leaf mustard | 0.77 ± 0.24 b | 0.75 ± 0.19 hijk | - | - | - | 1.52 ± 0.61 bcde |
| 25 | Wasabina leaf mustard | 0.09 ± 0.01 d | 0.44 ± 0.11 jk | - | - | - | 0.53 ± 0.11 de |
| 19 | Mizuna | 0.37 ± 0.19 bcd | 0.61 ± 0.32 jk | - | - | - | 0.98 ± 0.5 bcde |
| 17 | Pak-choi | 0.28 ± 0.12 bcd | 0.59 ± 0.32 jk | - | - | - | 0.87 ± 0.44 cde |
| 32 | Wild rocket 'Yeti' | 2.66 ± 0.45 a | 0.71 ± 0.09 ijk | - | - | - | 3.37 ± 0.77 bc |
| 24 | Wild rocket (Ingegnoli) | 2.05 ± 0.89 a | 0.93 ± 0.66 ghijk | - | - | - | 2.98 ± 1.34 bcde |
| 10 | Rocket | 0.75 ± 0.25 bc | 0.50 ± 0.13 jk | - | - | - | 1.25 ± 0.35 bcde |
| 31 | Red chard 'Bull's Blood Artica' | 0.17 ± 0.04 bcd | 1.64 ± 0.20 cdefghijk | - | - | - | 1.81 ± 0.24 bcde |
| 27 | Swiss chard | 0.14 ± 0.05 bcd | 1.61 ± 0.23 cdefghijk | - | - | - | 1.75 ± 0.23 bcde |
| 26 | Spinach 'Cugoe RZ F1' | 0.46 ± 0.14 bcd | 0.92 ± 0.19 hijk | - | 0.69 ± 0.16 a | - | 2.06 ± 0.46 bcde |
| 21 | Sorrel | 0.11 ± 0.01 cd | 0.34 ± 0.07 k | - | 0.07 ± 0.01 c | - | 0.51 ± 0.09 e |
| 12 | Lamb's lettuce 'Trophy F1' | 0.27 ± 0.05 bcd | 2.87 ± 0.88 bcd | - | - | - | 3.14 ± 0.93 bcd |
| | *p-value (LMM)* | *** | *** | *** | *** | *** | *** |

†Accession Identification Number.

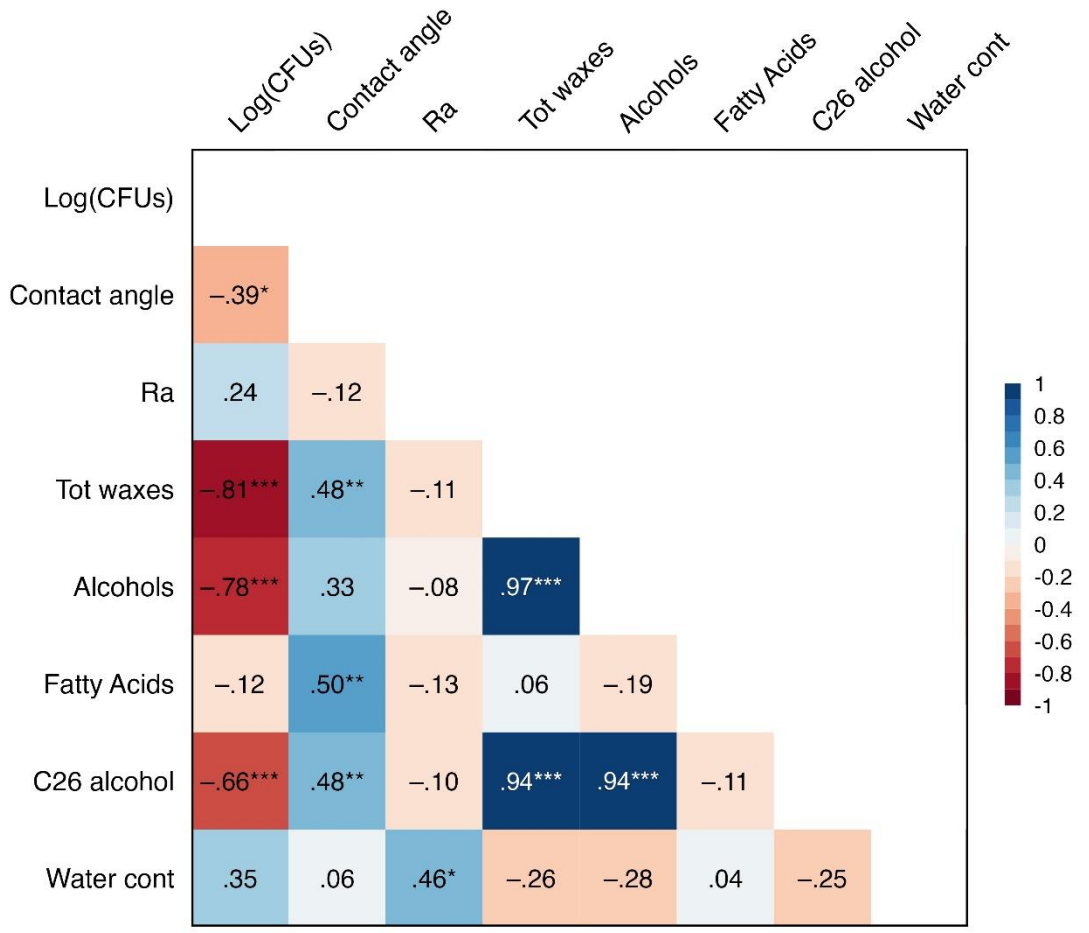


**Figure 5.2** Pearson's correlation test (p≤0.05) showing the relationship between *S. enterica* ATCC 19585 attachment and different leaf characteristics in 30 baby-leaf accessions. Heatmap represents the positive (blue) or negative (red) correlation. Significancy codes: p < 0.001 ***; ≤ 0.01 **; p≤ 0.05 *; p > 0.05 is not significant.

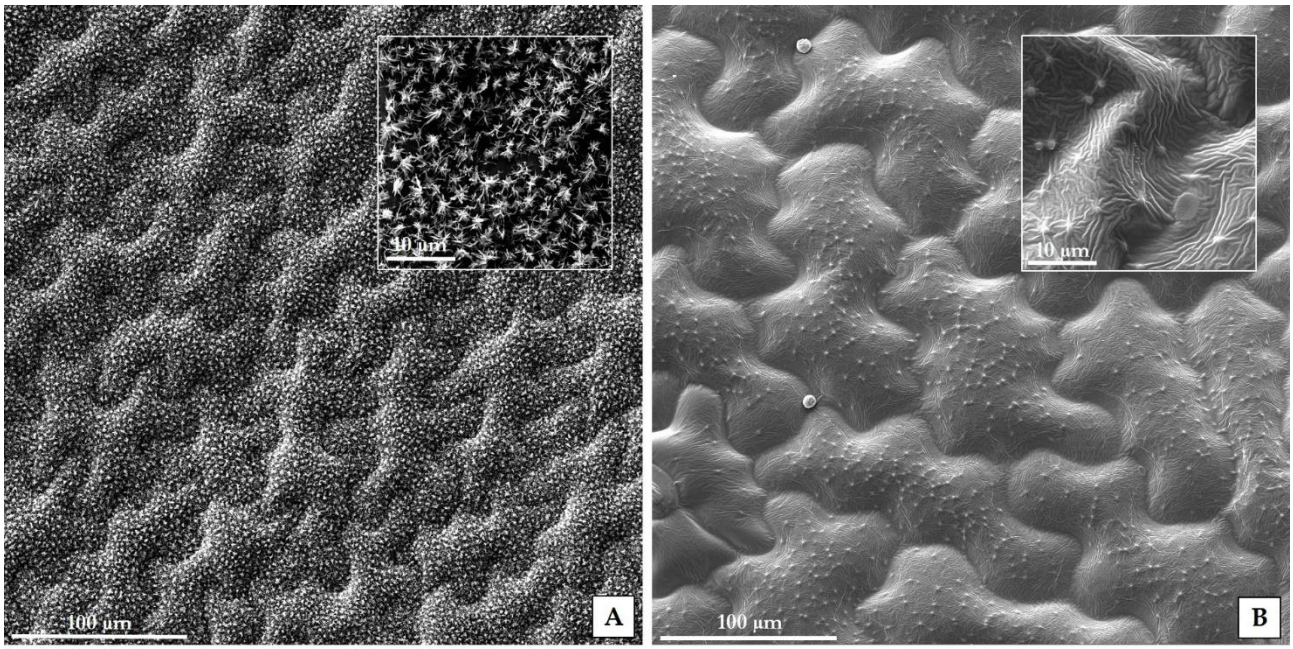


**Figure 5.3** SEM image (800×, and 8000× resolution) of adaxial leaf surface of wild lettuce (A), and sorrel (B).

*5.3.5 Correlation between S. enterica attachment and leaf hydrophobicity, roughness, and wax content*

The results of the Pearson's correlation test are shown in Figure 5.2. A significant negative correlation was observed between the *S. enterica* attachment and total waxes ($r = -0.81$; $p < 0.001$), alcohols ($r = -0.78$; $p < 0.001$), C26 alcohol ($r = -0.66$; $p < 0.001$), and contact angle ($r = -0.39$; $p < 0.05$). The latter parameter resulted to be positively correlated with total waxes ($r = 0.48$; $p < 0.01$), fatty acids ($r = 0.50$; $p < 0.01$), and C26 alcohol ($r = 0.48$; $p < 0.01$). As shown in Figure 5.2, roughness and water content were slightly correlated ($r = 0.50$; $p < 0.05$). The total wax amount was positively correlated with both alcohols ($r = 0.97$; $p < 0.001$) and C26 alcohol ($r = 0.94$; $p < 0.001$), which were correlated with each other ($r = 0.94$; $p < 0.001$).

*5.3.6 PCA results*

The first principal component (Dim1) of the PCA analysis explained 44.7% of the total variation among the accessions (Figure 5.4). The major parameters contributing to Dim1 were total waxes, *S. enterica* attachment, and alcohols (0.89, 0.81, and 0.80, respectively). The second component (Dim2) explained 21% of the total variation, with fatty acids providing the major contribution, followed by contact angle (0.71 and 0.48, respectively) (Figure 5.4 A). When grouped according to their botanical family, wild lettuce (accession 33) stood out within the Asteraceae for its high alcohol and wax content and its low contamination level (Figures 5.4 A and 5.4 B). Lamb's lettuce 'Trophy F1' (Valerianaceae, accession 12) was close to wild lettuce, while sorrel (Polygonaceae, accession 21), showing low waxes amount and high contamination level, had an opposite position. Brassicaceae accessions were divided into two clusters: the wild rocket accessions (24 and 32) were characterized by high fatty acid content, high hydrophobicity, and low *S. enterica* attachment, while all other accessions were less susceptible to *Salmonella* contamination and showed greater roughness. Chenopodiaceae formed a uniform cluster between Brassicaceae and Asteraceae (Figure 5.4 B).

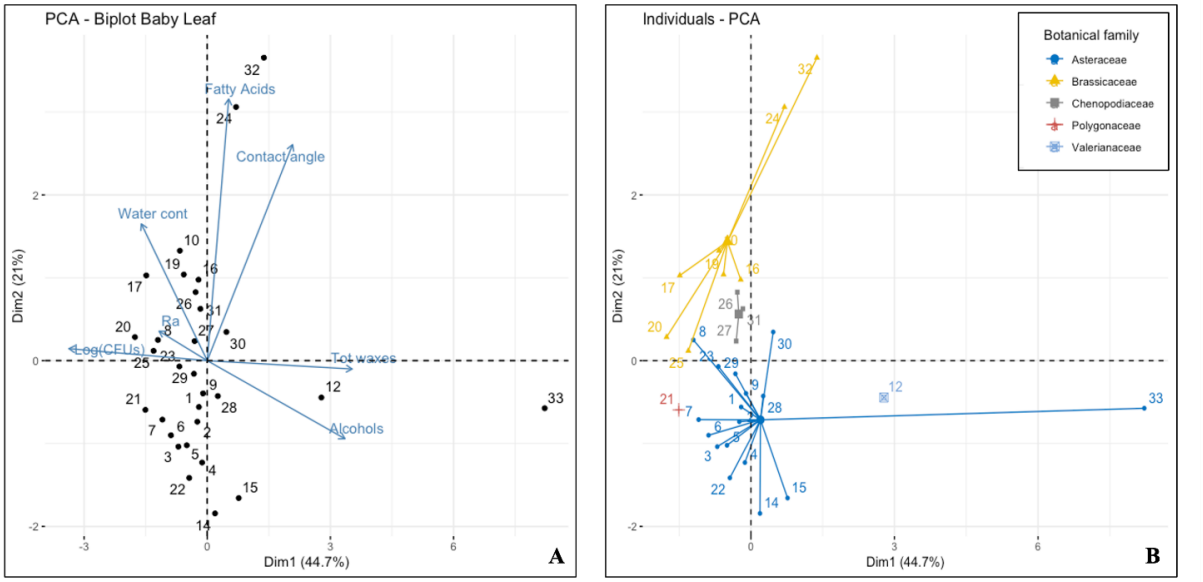


**Figure 5.4** Principal Component Analysis (PCA) considering leaves characteristics (panel A) and individuals grouped in botanical families (panel B).

*5.3.7 PLS results*

The PLS model highlighted three components sufficient to describe the six variables considered (Figure 5.5). The variance explained by the model was 78.3%, and $R^2$ was 0.78 (values between 0 and 1, with 1 indicating a perfectly fit model) with a low value of RSME (0.26) (this means there is a good measure of how well the model predicts the response, lower values of RMSE indicate a better fit). The variables that showed a high importance for projection score (VIP) were: alcohols (1.53), C26 alcohol (1.33), contact angle (0.87) and water content (0.87). The same variables reported a coefficient of determination lower than 32% on the *S. enterica* attachment. In particular, the highest values were reported in alcohol ($R^2$ = 0.31) and C26 alcohols ($R^2$ = 0.26).

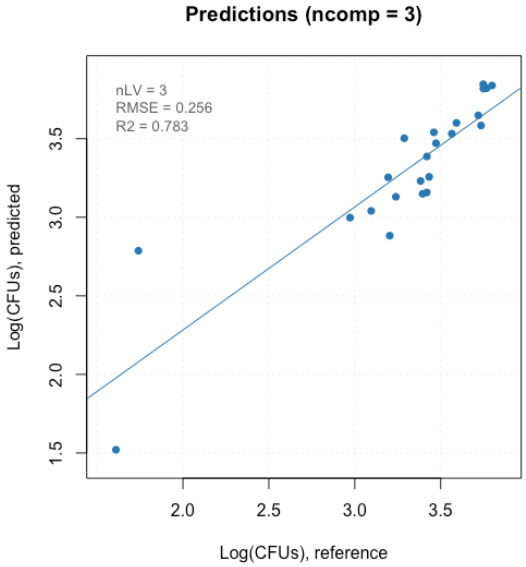


**Figure 5.5** Partial least squares (PLS) prediction model for the amount of *S. enterica* attachment using six variables (contact angle, surface roughness, C26 alcohol, alcohols, fatty acids, and water content).

## 5.4 Discussion

Differences in susceptibility to contamination by human pathogens have been reported by many authors in different vegetables[8,10,11,13]. Jacob and Melotto (2020)[10] found significant variations in the attachment and persistence of *S. enterica* and *E. coli* among 11 lettuce genotypes belonging to both *Lactuca sativa* (cultivated lettuce) and *L. serriola* (wild lettuce), and the latter species resulted to be less susceptible to contamination compared to the *sativa* group. In our study, differences in the attachment of *S. enterica* among 30 accessions of baby-leaf salads were observed and, in particular, wild lettuce showed the lowest level of contamination (Table 5.1). In a previous study[11], these 30 accessions were analyzed for their susceptibility to *E. coli* attachment with similar results, i.e., cultivated lettuce was more prone to *E. coli* attachment compared to their wild counterparts.

It is known that leaf traits can influence the behavior of human pathogens on vegetable crops. In this study, hydrophobicity, leaf roughness, and epicuticular wax composition of the 30 baby leaves were investigated to identify the surface properties possibly associated with the susceptibility to *S. enterica* attachment.

Leaf surface hydrophobicity can be best quantified by contact angle measurements. This is an indicator of the wettability of solid surfaces and ranges from 0° to 180°[30]. When it is 0°, the surface is completely wet, while, on

the contrary, 180° correspond to a completely non-wetting status; surfaces with contact angles greater than 90° are considered hydrophobic, while hydrophilic surfaces have contact angles less than 90°[31]. Among the 30 baby leaves, 76% resulted to be hydrophilic on the adaxial leaf surface (contact angle < 90°), while seven of them (wild lettuce, wild rocket 'Yeti' and Ingegnoli, mizuna, rocket, Red chard 'Bull's Blood Artica', and Swiss chard) had hydrophobic surfaces (contact angle > 90°) (Table 5.1). In particular, wild lettuce, the accession least susceptible to contamination, showed also the greatest contact angle (135.54 ± 5.52°). Sorrel, the most susceptible, had a hydrophilic surface (contact angle = 46.76 ± 5.78°). Considering all the accessions, a significant negative correlation between attachment and the contact angle was observed (Figure 5.2), supporting the findings of Hunter et al. (2015)[8] in lettuce.

The baby leaves also differed in roughness (Ra values) (Table 5.1). Consistently with Figure 5.1, Red Giant leaf mustard showed the roughest leaf surface (Figure 5.1 A) while lamb's lettuce 'Trophy F1' had a smooth surface (Figure 5.1 B). Although the latter species resulted also one of the least contaminated accessions, the Pearson's test revealed that *S. enterica* attachment was not correlated to roughness. Considering that we adopted an incubation time of 5 min, this result suggests that roughness was not a decisive factor in the early phases of the contamination process. This short inoculation time was adopted in agreement with other authors[32,33,34] to ensure that *Salmonella* attachment resulted solely from the surface wetting and to minimize the water absorption by the leaves. On the contrary, several authors[8,11,35] found that bacterial attachment was positively correlated with leaf roughness after longer surface inoculation (1, 1.5, and 2 h, respectively). In our study, roughness showed a slightly positive correlation with water content (Figure 5.2). To the best of our knowledge, no author found this relationship before. We hypothesized that turgidity given by the high water content can increase the difference in height between bulges and hollows on the leaf surface, in turn also increasing the roughness.

Epicuticular waxes form the outermost layer of the leaf surface that directly come in contact with human pathogens during plant contamination. Previous studies demonstrated that waxes can hinder *E. coli* and *S. enterica* attachment in different vegetable crops[8,32,36]. Similarly, rotavirus adsorption in 21 leafy greens was negatively correlated with total wax concentration, fatty acids, and alkanes[37]. In our study, a negative correlation between *S. enterica* attachment and the total waxes was observed (Figure 5.2). According to Pearson's correlation test, the wax components most correlated with attachment were alcohols, the main constituent of the waxes in the 30 baby leaves, and in particular C26 alcohol, the most abundant alcohol found (r = -0.78, and -0.66, respectively). The surface images taken by SEM revealed that the epicuticular layers of wild lettuce leaves (the accession with the highest concentration of waxes) showed the crystalline structures typical of C26 alcohol, while sorrel (the accession with the lowest concentration of waxes) had smooth layers (Figure 5.3). Interestingly, the two accessions were also the least and the most susceptible to *S. enterica* attachment. This result suggests that the visible crystalline wax structures on wild lettuce leaves contributed to the low *S. enterica* attachment we observed in this species. These findings agree with those reported by Ku et al. (2020)[32], who found a lower *S. enterica* attachment associated with more abundant epicuticular waxes and C26 alcohol on adaxial leaves of lettuce 'Two Star'. Ensikat et al. (2011)[16] suggested that the 3-D epicuticular wax crystal morphology influences the hydrophobicity of the leaf surface, as the size and the number of the crystalline facets are believed to reduce the contact area with water. In agreement with the study of Ensikat et al. (2011)[16], we observed a positive correlation between the contact angle and the C26 alcohol (Figure

5.2). The hydrophobicity was also positively correlated with fatty acids (Figure 5.2), similarly to what was found by Lu et al. (2015)[37]. That was not surprising considering that fatty acids are lipids containing long-chain hydrocarbons that end in a carboxylic acid functional group, thus resulting hydrophobic. It is known that fatty acids play a crucial role in defense against pathogens in plants[38]. Not only they form physical and chemical barriers, but also activate defence signaling pathways when they come in contact with phytopathogens. In particular, C16 and C18 fatty acids contribute to defence regulating basal, effector-triggered, and systemic immunity of plants[38]. Both of these fatty acids were detected in the *Diplotaxis tenuifolia* accessions (wild rocket 'Yeti' and wild rocket Ingegnoli) (data not shown), which among the 30 baby leaves had the highest fatty acid content as shown in Table 5.2 and Figure 5.4 A (accessions 24 and 32). Wild rockets were also among the less contaminated accessions (Table 5.1). Therefore, we hypothesize that in *Diplotaxis tenuifolia* fatty acids were crucial in limiting *S. enterica* attachment. However, due to the short inoculation time, they probably exert their role by increasing leaf hydrophobicity more than by more complex mechanisms. Indeed, wild rockets also showed a large contact angle (Table 5.1), resulting among the hydrophobic accessions. Considering all the 30 accessions, the incidence of fatty acids was less decisive; in fact, the Pearson's test revealed a non-significant correlation between their content and bacterial attachment (Figure 5.2). Thus, based on our results, fatty acids, as well as other factors, may have a predominant role in the susceptibility to *S. enterica* contamination depending on the species. In lamb's lettuce, which showed a level of contamination similar to wild lettuce (Table 5.1), the low attachment by *S. enterica* could perhaps be related to the presence of thymol, whose antibacterial effects are well known[39]. Such hypothesis is supported by Xu et al. (2008)[40], who found that Thymol had a detrimental effect on *E. coli* thanks to the ability to permeabilize and depolarize the cytoplasmic membrane.

Based on the PLS model results, the variables alcohols, C26 alcohol, contact angle and water content had the largest impact on the attachment of *S. enterica* in the 30 baby leaves we studied. Together, these variables can explain 78% of the variation in the bacterial attachment among the accessions. The highest coefficient of determination was observed between bacterial attachment and the concentration of alcohols ($R^2 = 0.31$) and C26 alcohol ($R^2 = 0.26$).

## 5.5 Conclusions

This study confirmed that different leafy vegetables have different susceptibility to contamination by *S. enterica*. Among the 30 baby-leaf salads investigated, the lowest attachment was found in wild lettuce (*Lactuca serriola*) and lamb's lettuce 'Trophy F1' (*Valerianella locusta*). The study also demonstrated that leaf surface properties influence attachment by this human pathogen. In all set of baby leaves the main was leaf surface hydrophobicity (measured as contact angle) and epicuticular waxes. Conversely, attachment was not correlated to roughness, but we hypothesise that this result might be due to the short incubation time used here. The most important wax components for susceptibility to *S. enterica* contamination were alcohols and, in particular, the 3-D wax crystals of C26 alcohol, which significantly increased hydrophobicity. These components were crucial in wild lettuce, while in lamb's lettuce 'Trophy F1' the reason for the low susceptibility was less evident. Perhaps a predominant role was due to thymol, whose antibacterial properties are well-known. Finally, in wild rocket accessions *S. enterica* attachment was probably hindered by fatty acids due to their hydrophobic nature.

These findings can help predict and control the attachment and contamination of leafy salads by enterobacteria. Furthermore, they provide useful information for breeding programs aimed at developing cultivars less susceptible to human pathogens, and therefore safer.

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# 6. Third paper

## Fluorescence microscopy for directly tracking the proliferation of *Escherichia coli* in baby leaves of cultivated and wild lettuce

### 6.1 Introduction

Baby leaves, being prepared as ready-to-eat salads, may undergo contamination during processing[1]. The production of fresh-cut leafy vegetables entails the application of several unit operations, including trimming, cutting, and washing. These operations are recognized to impact both the quality and microbiology of the final product[2]. Exposure to disinfectants, low temperatures commonly used in wash water, and mechanical forces encountered in channels, cutting equipment, or centrifuges are stressors that play a crucial role in determining the fate of contaminating microorganisms[3]. Lettuces, due to their typical tender leaves, are particularly vulnerable to mechanical damages that create conditions favorable to microbial invasion of tissues.

Over the past decade, the development of microscopic techniques has spurred a renaissance in its application of to the study of plant-bacteria interactions. Microscopic techniques, notably confocal scanning laser microscopy, have been used to examine the interaction between *E. coli* O157:H7 and cut lettuce surfaces[4,5]. Brandl et al. (2009)[6] report the use of fluorescence microscopy combined with the green fluorescence protein (GFP) to locate *S. enterica* and *E. coli* O157:H7 on fresh fruits and vegetables.

To date, there is a dearth of studies using fluorescence microscopy for the real-time monitoring of human pathogens on plant leaf surfaces. This preliminary study introduces the use of microscopic techniques as a novel approach for directly tracking *E. coli* replication on leaf surfaces of romaine lettuce 'Bionda degli Ortolani' and wild lettuce, which had previously shown a different susceptibility to *E. coli* contamination[7]. The methodological approach outlined here aims to explore and understand the complex interaction between bacteria and plant tissues.

### 6.2 Materials and Methods

#### *6.2.1 Plant material and bacterial strain*

Seeds of romaine lettuce 'Bionda degli Ortolani' (*Lactuca sativa* L.) from Fratelli Ingegnoli, Milan, Italy; and wild lettuce (*Lactuca serriola* L.) from Provencemonamour, Paris, France were sown in plant pots (15 cm in diameter, H 13 cm, volume 1.5 L) filled with soil at a density of 3000 seeds $m^{-2}$. After sowing, plants were transferred in a growth chamber at 22 ± 2 °C with a photoperiod of 16 h under fluorescent lighting units CULTILITE HPS Lamp 400W, and grown to the baby-leaf stage. *Escherichia coli* S-17-1 pHC60 with green fluorescence was used for the inoculation.

#### *6.2.2 Leaf inoculation, fluorescence microscope observations and images processing*

Adaxial leaf epidermis of romaine 'Bionda degli Ortolani' and wild lettuce was peeled off with special tweezers and put on a medium prepared with plant juice obtained from the two species, respectively, and solidified with agar. Epidermis was then inoculated with green fluorescent *E. coli*. Bacterial suspension for inoculation was prepared

from an overnight inoculum of *E. coli* in Lysogeny Broth with Tetracycline-HCl. The inoculum was washed and diluted two times in a sterile Ringers solution (RS). A mix of RS and plant juice was used for a third washing. Then, 10 µl of bacterial suspension was placed on top of the epidermis. An inverted fluorescence microscope (Leica, DMi8) equipped with an oil immersion 63× phase contrast objective and a DFC 7000T camera (Leica), was used for tracking *E. coli* proliferation in romaine lettuce 'Bionda degli Ortolani' and wild lettuce. Fluorescence microscope observations (view field 197.52 µm x 148.11 µm) were performed at time 0 and after incubation of inoculated epidermis at 37 °C for 24 h. Fluorescence area ($mm^2$) and intensity (arbitrary units, AU) were determined using the ImageJ software. Five images per species and per incubation time were considered.

### *6.2.3 Statistical analysis*

The analysis of area and intensity data was performed using RStudio (R 4.1.1). The normality of data was investigated using the Shapiro-Wilk's test. As both variables did not exhibit a normal distribution, a box-cox transformation was applied. A two-way analysis of variance (ANOVA) was conducted to assess the main effects of species and incubation time, as well as their interactions, on the variables area and intensity. Significance levels were indicated as follows: * = 0.05, ** = 0.01, *** = 0.001, and n.s. for not significant.

## 6.3 Results

From a visual standpoint, the differences in fluorescence area and intensity can be observed both at different incubation times and between the two species (Figure 6.1). At both 0 h and 24 h, romaine lettuce (Figure 6.1 A and 6.1 B, respectively) exhibited a larger fluorescence area compared to that of wild lettuce (Figure 6.1 C and 6.1 D, respectively). Furthermore, both romaine and wild lettuce show a noticeable increase in fluorescence area from 0 h to 24 h.

The previous visual observations were further confirmed by statistical analyses. Based on the ANOVA results, both factors (incubation time and species) significantly influenced fluorescence area (Table 6.1). The F-statistics revealed that incubation time was the most important factor affecting the fluorescence area, explaining approximately 83.3% of the total variance. The species was identified as the sole factor significantly impacting fluorescence intensity, accounting for about 61.7% of the total variance, and the interaction species x incubation time was not significant for both fluorescence area and intensity (Table 6.1). The fluorescence area and intensity of wild lettuce were approximately 56.1% and 19.1% smaller, respectively, compared to romaine lettuce (Table 6.2). The fluorescence area exhibited a 7.8-fold increase from incubation time 0 h to 24 h, whereas the fluorescence intensity decreased by approximately 11.8% over the 24-hour incubation interval.

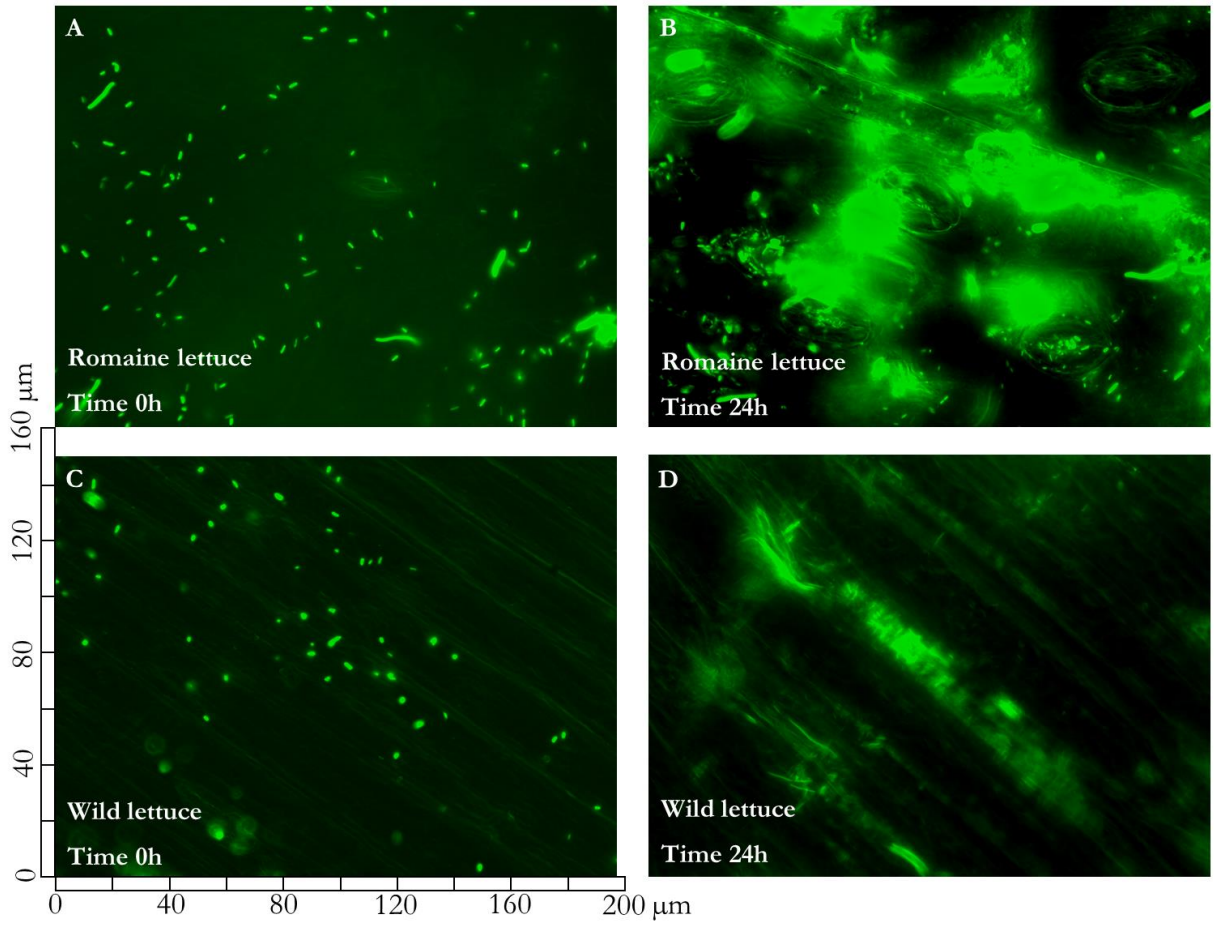


**Figure 6.1** Images of leaf epidermis captured with an inverted fluorescence microscope after 0 h and 24 h from the inoculation with fluorescent *E. coli*. The viewing field dimensions are 197.52 µm x 148.11 µm.

**Table 6.1** Results of the ANOVA for fluorescence area and intensity. The table columns report the degree of freedom (Df), the Fisher F (F) and the significance (p): * = 0.05, ** = 0.01, *** = 0.001, ns = not significant.

| Source of variability | Df | Area | | Intensity | |
|---|---|---|---|---|---|
| | | F | p | F | p |
| Species | 1 | 11.576 | ** | 13.826 | ** |
| Incubation time | 1 | 58.973 | *** | 3.789 | ns |
| Species x incubation time | 1 | 0.239 | ns | 4.81 | ns |
| Residual | 16 | | | | |

**Table 6.2** Effect of species and incubation time on fluorescence area ($mm^2$) and fluorescence intensity (AU). Data are averages (Avg) of five values. SE = standard error. Lowercase letters indicate significant differences according to the ANOVA; significance levels (sig): *** = $p \leq 0.001$, ** = $p \leq 0.01$, ns = not significant.

| | Area ($mm^2$) | | | Intensity (AU) | | |
|---|---|---|---|---|---|---|
| | Avg | SE | sig | Avg | SE | sig |
| Species | | | ** | | | ** |
| Romaine lettuce | 107.7 | 27.76 | a | 57.21 | 1.47 | a |
| Wild lettuce | 47.28 | 14.56 | b | 46.29 | 3 | b |
| Incubation time | | | *** | | | ns |
| 0 h | 15.86 | 3.96 | b | 54.99 | 1.33 | |
| 24 h | 139.11 | 19.75 | a | 48.51 | 3.64 | |

## 6.4 Discussion

The results of this preliminary study indicate that the fluorescence microscopy can be a mean for tracking bacterial proliferation in baby leaves. Bacterial growth can be monitored by means of fluorescence area. In fact, microscope observations after 24 h from the inoculation of leaf epidermis with fluorescent *E. coli* revealed an increase in this parameter. On the contrary, fluorescence intensity decreased meanwhile, although not significantly. This suggests that fluorescence intensity is not a suitable parameter to track bacterial proliferation, at least 24 h from inoculation. Nevertheless, shorter inoculation times should be investigated for a better understanding of the dynamic of fluorescence intensity. Fluorescence microscopy also highlighted greater *E. coli* proliferation in romaine lettuce than in wild lettuce. Romaine lettuce has been implicated in numerous foodborne illness outbreaks in the United States, particularly *E. coli* infections[8,9,10]. This lettuce type is considered one of the riskiest leafy vegetable in terms of foodborne diseases. Interestingly, Truschi et al. (2023)[7] found that, romaine lettuce 'Bionda degli Ortolani' showed the highest contamination by *E. coli* among 30 baby-leaf accessions, with a significant difference compared to wild lettuce. The results of this study support these previous findings.

## 6.5 Conclusions

To the best of our knowledge, no studies have been conducted for real-time monitoring of human pathogens on the leaf surface of vegetables using the fluorescence microscopy. Our preliminary results demonstrated that this technique can be an advanced tool for real-time observation and monitoring of bacterial replication at the cellular level. This methodology can facilitate a comprehensive understanding of bacterial behaviour within plant tissues, bring to light the spatial and temporal aspects of *E. coli* proliferation. Subsequent investigations will be conducted with additional incubation interval times to improve accuracy in tracking *E. coli* proliferation in baby leaves.

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# 7. General conclusions

The thesis work demonstrated that baby leaves of different species, and even of different varieties of the same species, may vary in the susceptibility to surface contamination by *E. coli* and *S. enterica*. Particularly noticeable was the low susceptibility of lamb's lettuce 'Trophy F1', wild lettuce, and wild rocket 'Yeti'. Interestingly, we found that var. *longifolia* of lettuce (romaine type), the lettuce most involved in food disease outbreaks in the U.S. and thus considered one of the riskiest salads, includes cultivars (i.e., 'Maraichere') less susceptible to contamination than others (i.e., 'Bionda degli Ortolani').

The different responses to contamination detected among the baby-leaf accessions studied in the thesis appeared to be associated with leaf micro-morphological traits. This result is in agreement with previous studies reporting that the characteristics of the leaf surface can influence the attachment of human pathogens in plants. Specifically, leaf roughness was found to be positively correlated with *E. coli* contamination level in baby leaves after 1.5 h of incubation. Furthermore, roughness seemed to offer UV protection to bacteria. Indeed, when *E. coli* retention in surface-inoculated leaves was measured after a UV treatment, bacterial contamination was reduced to a minor extent in the rougher leaves. This finding highlights the possible impact of leaf roughness on the effectiveness of sanitization treatments.

Leaf surface hydrophobicity (measured as contact angle) and epicuticular waxes resulted to be the crucial traits for the attachment of *S. enterica* on baby leaves after an incubation of 5 min. The most important wax components influencing the susceptibility to *S. enterica* contamination were alcohols, with a main role of the three-dimensional wax crystals of the C26 alcohol, which significantly increased hydrophobicity. The effect of these components was particularly evident in wild lettuce, whereas in lamb's lettuce the low susceptibility was more likely associated with the presence of thymol, known for its antibacterial properties. Finally, in wild rocket 'Yeti', the attachment of *S. enterica* was probably hindered by fatty acids, owing to their hydrophobic nature.

Fluorescence microscopy represents an advanced technique for real-time observation and monitoring of biological processes at the cellular level. In the context of this thesis, fluorescence microscopy was evaluated as a mean to directly monitor the proliferation of *E. coli* on the surface of baby leaves. The obtained results, although preliminary, suggested that this methodology can facilitate a comprehensive understanding of bacterial behaviour within plant tissues, bringing to light the spatial and temporal aspects of *E. coli* proliferation. Further investigations will be conducted with additional incubation interval times to improve accuracy in tracking *E. coli* proliferation in baby leaves.

In conclusion, the results obtained in this thesis can turn into practical applications and give rise to some recommendations for growers, offering insights for enhancing the food safety of leafy vegetables. The screening of a large number of baby leaves for the susceptibility to contamination by *E. coli* and *S. enterica* highlighted the most and the least risky from this point of view. This information could be used for orienting growers and the

vegetable industry towards safer crops. For example, the choice of the cultivar can be a means to reduce the risk of foodborne diseases linked to the consumption of salads. Besides, the thesis provided findings on leaf traits associated with the attachment of human pathogens in salads. Such traits could be considered in breeding programs with the goal of obtaining cultivars less prone to human pathogens, but also be taken into account in modulating the sanitation treatments during the processing of ready-to-eat salads. Finally, the preliminary results of the fluorescence microscopy study indicated a new approach for real-time observation and monitoring of bacterial proliferation on leaf surfaces that could provide valuable information in the field of plant-bacteria interactions.

## Appendix

During the three-year doctoral period, I also participated in laboratory experiments, data processing, and the writing of the following articles close to the topic of my thesis.

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**Article**

OXFORD

*Article*

# 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.

*horticulturae*

MDPI

*Article*

# Preliminary Assessment of Four Wild Leafy Species to Be Used as Baby Salads

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## Abstract

Wild edible leafy plants, thanks to their organoleptic characteristics and nutritional value that can make them be appreciated as salads by consumers, represent a good opportunity for growers and the fresh-cut industry, which are always looking for new crops to expand the number of products they offer. In this study, four wild species (dandelion, sorrel, wild chicory, and wild lettuce) were cultivated hydroponically up to the baby leaf stage in order to evaluate them as potential crops. At harvest, yield and antioxidant compounds, minerals, and nitrates content were assessed. The contribution to human mineral intake and the possible health risk associated with heavy metals were investigated. A characterization of the sensory profile was also carried out. Yield and chlorophylls and carotenoids content of the investigated species were comparable to those of common leafy vegetables. Variability in nitrate content was observed, with the lowest value in sorrel and the highest in dandelion. All species could contribute in Cr, Mg, and Se intake, and health risks due to heavy metals were excluded. Each species was well characterized by distinctive and peculiar sensory notes. In conclusion, the results of this preliminary study suggest that the four wild investigated species may be promising for baby leaf production.

**Keywords:** *Taraxacum campylodes* G.E. Haglund; *Rumex acetosa* L.; *Cichorium intybus* L.; *Lactuca serriola* L.; leafy vegetables; yield and quality; nitrate; dietary intake; health risk; sensory profile

Research Article

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# Pre-heated blades for harvesting baby-leaves reduce the risk of Escherichia coli internalization in leaves

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## Abstract

BACKGROUND: Pathogenic enterobacteria can travel through the plant vascular bundles by penetrating from cuts and persisting into ready-to-eat leafy greens. Because the cutting site is the main point of entrance and uptake, we tested how different cutting strategies can reduce bacterial internalization in leaves. Horizontal cuts at the base of the leaves were performed with two different types of tools: the first with a scalpel (by pulling the blade) and the second with a scissor-action that has blades that cuts by gliding against a thicker blade. Scissor-action generally makes closer border cuts. Blades of both types of tools have worked at 25 °C and 200 °C. The present study aimed to determine how these different types of cuts and temperatures affected bacterial uptake in leaves. Experiments were repeated on different plant genotypes and at different wilting stages.

RESULTS: Our findings showed that cutting baby-leaves with a scissor action at 200 °C significantly reduced the bacterial uptake compared to the not heated (which simulates a mechanized lettuce harvester). The most effective cutting treatments for reducing bacterial uptake were in the order: scissor 200 °C > scissor 25 °C > scalpel 200 °C > scalpel 25 °C. The scissor heated at 200 °C also prevented bacterial uptake on wilted baby-leaves.

CONCLUSION: The findings of the present study could provide a further contribution in terms of safety during harvest and suggest that a pre-heated blade supports safety during harvest of leafy greens. © 2022 Society of Chemical Industry.

Supporting information may be found in the online version of this article.

Keywords: baby leaves; bacterial internalization; bacterial uptake; produce safety; ready-to-eat produce; produce harvest