Chapter

Pathogenic *Escherichia coli*: An Overview on Pre-Harvest Factors that Impact the Microbial Safety of Leafy Greens

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Abstract

Consumption of fresh leafy greens has been repeatedly reported and linked to pathogenic *Escherichia coli*-associated foodborne illnesses outbreaks. Leafy greens are mostly eaten raw, based on the increased consumers' preferences for natural, nutritious diets. Recent studies indicate the incidence of infections caused by pathogenic *Escherichia coli* remained almost unchanged or even increased. In this context, fresh produces increased the awareness about their primary contamination level, namely the pre-harvest phase. Fully eliminating pathogenic *Escherichia coli* from pre-harvest environment proved to be impossible. Emphasis must be placed on the pre-harvest factors that affect the food safety and, subsequently, on the identification of possible mitigation strategies that can be used on-farm for reducing the risk of leafy greens contamination with pathogenic *Escherichia coli*.

Keywords: pathogenic *Escherichia coli*, leafy greens, foodborne illnesses outbreaks, pre-harvest on-farm contamination factors, pre-harvest microbial safety mitigation strategies

1. Introduction

Leafy greens are mostly eaten raw, based on the increased consumers' preferences for natural, nutritious diets. The consumption of leafy greens is recommended to reduce the risk of malnutrition, diet-related chronic diseases such as cardiovascular diseases, cancer, metabolic disorders, and may help to slow down the cognitive decline with aging [1–4]. For preserving their bioactive compounds, leafy greens are commonly consumed raw, and the lack of a kill-step to inactivate the potentially present pathogens leads to greater risk to the health of consumers. Among other fresh produce, leafy greens are more exposed to pathogen contamination because they grow low to the ground and can be easily contaminated in open fields. The increased consumption of fresh, ready-to-eat leafy greens has been repeatedly, reported worldwide and linked to pathogenic *Escherichia coli* (herein *E. coli or E. coli* O157:H7) associated foodborne illnesses outbreaks.

Pathogenic *Escherichia coli* group is comprised of six pathotypes out of which Shiga toxin-producing *E. coli* (STEC)—STEC (also be referred to as Verocytotoxin-producing *E. coli* (VTEC) or enterohemorrhagic *E. coli* (EHEC)) is the one most associated with foodborne outbreaks [5]. Some other STEC *E. coli* strains, namely *E. coli* O145, *E. coli* O26, and *E. coli* O104:H4 were involved in rare foodborne outbreaks due to consumption of shredded lettuce, raw clover sprouts, and raw sprouted seeds [6–8].

According to World Health Organization (WHO) pre-harvest food safety is an important element in creating sustainable food safety policies and must be considered in the context of *farm-to-fork* for the protection of human health [9]. Despite the existing food safety regulations and the undertaken on-farm food safety measures, according to recent studies performed by the United States Center of Diseases Control (CDC), European Centre for Disease Prevention and Control (ECDC) in collaboration with the European Food Safety Authority (EFSA) the incidence of infections caused by pathogenic *Escherichia coli*, between 2016 and 2019, remained almost unchanged or even increased (**Table 1**) [10–14]. In this context, in the past years, these fresh produces emerged as a food safety concern that, ultimately, increased the awareness about their primary contamination stage, namely pre-harvest. It has been established that once contaminated leafy greens leave the farm's site it will be difficult to prevent further transmission of *E. coli* to consumers. Usually, large quantities of contaminated leafy greens are recalled from the markets, a fact which pose a great economic burden on leafy greens growers but also on public health [15]. The on-farm contamination with pathogenic *Escherichia coli* largely depends on agricultural and environmental factors, unsafe on-site agronomic practices including the harvesting stage, and ineffective or missing post-harvest decontamination steps. However, eliminating completely the presence of the *E. coli* from the on-farm, the natural growing environment of leafy greens, during pre-harvest stage, proves to be impossible due to the high number of factors which are involved in the harboring and transmission of this pathogen. Based on the vast number of on-field and experimental results it was unanimously agreed that it is more feasible to first understand the main agricultural factors affecting the

Year	Type of vehicle	Total reported cases (hospitalizations/deaths)	Pathogen involved in the outbreak	Sources of contamination
2020	Spinach; romaine lettuce	40 (20/0)	*E. coli* O157:H7	• Cattle feces
2019	Romaine lettuce	167 (85/0)	*E. coli* O157:H7/ strains of Shiga toxin-producing *E.coli* (STEC)	• Farm in proximity to cattle grazing land; • On-farm water drainage basins
2018	Red leaf lettuce; green leaf lettuce	62 (25/0)	*E. coli* O157:H7	• Agricultural water reservoir
2017	Romaine lettuce and other leafy greens	25 (9/1)	*E. coli* O157:H7	• Source of contamination not identified
2016	Alfalfa sprouts	11 (2/0)	*E. coli* O157 (STEC O157)	• Farming practices
2014	Raw clover sprouts	19 (44%/0)	*E. coli* O121 (STEC O121)	• Farming practices

[a] *Reported by the CDC, between 2014 and 2020.*
[b] *Source: https://www.cdc.gov/ecoli/outbreaks.html.*

Table 1.
Selected pathogenic E. coli outbreaks associated with fresh leafy greens[a,b].

prevalence, incidence, and survival of this pathogen, as well as pathogen contamination of leafy greens, which ultimately negatively impacts the microbial safety of the produce. In turn, this will assist leafy greens producers to improve their on-farm pre-harvest agronomic practices for reducing the pathogen contamination to levels that will be a lesser hazard to public health.

Subsequent to the reported pathogenic *Escherichia coli*-related foodborne outbreaks, epidemiological trace-back studies identified the following as main contamination factors: (a) the use of manure, as a soil organic fertilizer; (b) irrigation water; (c) the domestic and wild animals which either can be found in the proximity of the growing sites or as free-roaming animals; and (d) on-farm human activity [15–17].

Similarly, in European countries, over the years, the consumption of fresh leafy greens led to multiple foodborne outbreaks. For example, in Germany (2011) the consumption of sprouts led to 3816 total illnesses (810 hospitalizations and 54 deaths) due to *E.coli* O104:H4. Between 2010 and 2011, in England, Wales and Scotland, 252 fell ill and one died following the consumption of raw leeks; the identified pathogen being *E.coli* O157 PT8. In Denmark (2010), the consumption of lettuce resulted in 264 illnesses due to *E. coli* ETEC O6:K15:H16. The consumption of fresh basil, provoked in Denmark (2006) about 200 illnesses due to *E. coli* ETEC O92:H- and O153:H2 [18].

2. Leafy greens are an easy target for contamination with pathogens: mechanisms of microbial contamination

2.1 General considerations

Leafy greens are known as an important vector for microbial hazards responsible for foodborne outbreak illnesses and almost 20% of leafy greens contamination with pathogens takes place on-farm [19, 20]. In leafy greens, *E. coli* O157:H7 is found to be more frequent than other pathogens due to its ability to contaminate mostly via biofilm formation on the produce surface which could explain the large number *E. coli* O157:H7 related outbreaks [21]. The on-farm fate of enteric pathogens on leafy greens depends on multiple conditions that the pathogenic bacteria are facing in the soil-produce environment, and on the pathogen's ability and strategies to survive and contaminate the fresh produce, such as biofilm formation or internalization. In the preharvest stage, due to the pathogen-produce interaction pathways and mechanisms, some of the pathogens could become endopathogenic in leafy greens—a stage which raises serious food safety concerns since the post-harvest decontamination treatments have almost a null chance to reduce the numbers of viable cells to a harmless level [22]. The "*points-of-entry*" used by pathogens to contaminate the leafy greens are the plant's rhizosphere and/or phyllosphere. Due to its richness in nutrients (root exudates including compounds released as a consequence of root cell metabolism or after lysis of plant cells), the root zone (rhizosphere) is an excellent environment for pathogens and it could support the presence of 10^6 to 10^9 bacteria per gram of roots [23]. On the phylloshere, *E. coli* O157:H7 is capable of attaching on these plant's parts, and can remain viable for weeks to months, and even multiply if the environmental conditions are favorable (i.e., warm temperatures, high humidity, adequate nutrients, plant's leaves' characteristics and integrity) [21]. Compared to the rhizosphere, the leaves surface nutrients are scarce. The nutrients found on leaves, probably originating from mesophyll and epidermal cell exudates that leak onto the leaves surface from wounds and broken trichomes, are not distributed homogenously. Since the phyllosphere is subjected to many stress factors which can have rapid fluctuations will affect the bacterial survival: temperature, solar radiation and humidity, phyllospheres typically could support fewer than 10^3 to 10^7 pathogen per gram of leaf [19]. Therefore, understanding the pathogen contamination pathways and mechanisms will provide important information to fresh produce growers for either adopting preventive actions or protecting their produce during the pre-harvest stage.

2.2 Biofilm formation

Leafy greens, as pathogenic biofilm carriers, pose a great threat to produce microbial safety since the biofilms poses a great resiliency towards decontamination methods applied during post-harvest processing (i.e., chemical washing solutions) [24, 25]. The general mechanisms of leafy greens contamination by pathogens' colonization takes place in phases: (a) attachment to phylloshere and/or to rhizoplane, and (b) pathogens' adaptation to environmental factors followed by survival and multiplication on the plant parts. The whole general bacterial attachment-colonization mechanism takes pace in a similar manner for human enteric pathogens that are either environmentally shed by domestic animals and/or wildlife: cattle, sheep, pigs, goats, wild birds, deer, mice, insects, or can originate from other sources: soil, manure, irrigation waters, etc. [26]. The leafy greens' structure (leaves' roughness, leaves' surface degree of porousness, crests etc.), influences the pathogen's attachment phenomenon that results in biofilm formation. When leaves are damaged (i.e., cuts, wounds) the pathogen may further become internalized due to pathogen's multiplication in these areas where damaged plant tissue exudes inner nutrients [27]. In addition, the amount of contaminating bacteria is a factor which can affect the degree of pathogen's attachment to the leafy greens. The colonization of leafy greens, as the first stage of biofilm formation, could take place through multiple routes, such as: contaminated soil (i.e., via dust or splashes), roots, seeds, or by wetting of produce leaves (i.e., via irrigation waters) and depends on the pathogens' ability to adapt to the new environment following the attachment phase. Once colonization takes place, biofilm formation is initiated. According to Ximenes and Tarver biofilm formation on leafy greens (i.e., lettuce, spinach, basil, cilantro, green onions, and parsley) by enteric pathogens involves in several stages: (a) initial contact of *E. coli* with the leafy greens and pathogen's subsequent attachment to the produce; (b) *E.coli* cells' proliferation and cells' aggregation by the excretion of the extracellular polymeric substances – which helps the formation of the initial "matrix" where the pathogen will grow and multiply; (c) *E. coli* biofilm maturation, and (d) sporadic *E. coli* cells' dispersion or detachment into the environment and contaminate other produce from the vicinity of the "infected" produce [26, 28]. According to Beattie and Lindow, bacteria found on leaves possess two major strategies which they can apply for their attachment, growth and survival, and biofilm formation on the plant surface: (a) "*tolerance strategy*" that requires the bacteria's ability to resist exposure to environmental stresses on leaf surfaces; or (b) "*avoidance strategy*", in this case bacteria seek plant sites that are protected from those stresses. Using these bacterial strategies, a general step-by step-model of leaf colonization and biofilm formation was developed: 1. the landed bacteria on the leaf surface are randomly distributed; 2. some of bacteria will enter into the leaf via openings such as stomata while some will stay on the surface of the plant leaves and modify the local environment to fit their needs; 3. surface adhered bacteria start to multiply and to form aggregates or micro-colonies, which subsequently will develop into biofilms [29]. Subsequent to the tight adhesion on favorable sites found on plants (niches), the biofilm formation process is facing environmental factors, plant properties, and the innate plant microbiota [20]. Nevertheless, once the biofilm is formed it has the capability to protects the rest of attached bacteria against environmental stressors (i.e., desiccation, UV radiation etc.), from the plant immune response, and from endogenous (plant-origin) or exogenous (indigenous microorganisms-origin) antimicrobial compounds. Studies on the attachment of human enteric *E. coli* indicate that it can rapidly adhere to a variety of growing plant tissues such leaves and roots. Surface attachment is possible due to the presence of the plant's cuticles and the plant's surface characteristics. The cuticle present on the plant surfaces favors attachment of hydrophobic molecules and any breaks in the cuticle may expose the hydrophilic structures for further attachment [30]. The characteristics of the plant's surface is also important in the microbial adhesion process. For example, the surface roughness of the plant parts depends on the nature and age of the plant, and it is important not only for adherence but also for the pathogen's survival on the produce as demonstrated for *E. coli* O157:H7 adhesion on leaves of different spinach cultivars [31]. The microbiota found on the plants is not homogenously distributed on the leaf surface, bacterial cells predominantly attaching and colonizing on specific sites of leaf surfaces such as epidermal cell wall junctions, in grooves along veins and depressions, or beneath in the cuticle [29]. Under certain factors (on-field circumstances, bacterial unspecific binding based on hydrophobic and electrostatic interactions), attachment phenomenon could be reversible. However, when the pathogen cells form the exopolymeric material, are able to fix themselves more strongly on the leafy greens, the attachment is irreversible and the pathogen cannot be removed by washing treatments [20, 32, 33].

Studies showed that both, produce and bacterial properties, are factors involved in attachment of pathogenic *E. coli*. Leafy greens surface properties (i.e., cuticles, roughness) is favoring the pathogen attachment and colonization at specific sites of leaves: base of trichomes, stomata, epidermal cell wall junctions, or in grooves existing along the produce veins and depressions [29]. The study by Takeuchi *et al.* indicated specific attachment and colonization sites the cut surfaces of lettuce are rich in water and nutrients and offer *E. coli* O157:H7 stress-protection [34]. *E. coli* strains possess an attachment-adhesion system due to its ability to produce a diversity of pili and fimbriae and non-fimbrial adhesins, that function as 'professional' adhesion systems, and flagella; these compounds could play alternative functions in attachment and adhesion stages [35, 36]. An earlier study led by Torres *et al.* showed that *E. coli* O157:H7 possesses several redundant protein adhesins and the overexpression of each adhesin alone is sufficient to promote binding to alfalfa sprouts [37]. Ximenes *et al.* indicated the importance of some bacterial hydrolytic enzymes, such as: pectinases, cellulases, proteinases, and amylases which can further enhance the ability of pathogens to invade and spread on plant tissues [26]. Several experimental studies showed that *E. coli* ability to adhere and attach varies in time and some influence factors could be the initial number of viable pathogenic cells contaminating the plant and the type of leafy green. For arugula leaves, 2 $\log_{10}$ CFU/g of pathogen attached after 60 min, for lettuce leaves attachment time varied between 25 and 120 min (final level of pathogenic viable cells being 1–2.5 $\log_{10}$ CFU/cm^2) and for spinach approximately 3 $\log_{10}$/spinach leaf attached in less than 60 min [31, 38, 39].

2.3 Internalization

Experimental studies indicated that there are many mechanisms used by *E. coli* O157:H7 to contaminate and internalize both the leafy greens root and leaves tissues [40–42]. From the roots, the pathogen can pass to the leaves by using the produce's vascular system or can penetrate the produce internal tissues using the existing wounds or other natural "openings" of the leaf system [42–44]. While due to the difficulty to study the pathogen internalization in the natural growing plant environment, internalization has been extensively studied in systems that mimic the natural environment, many factors which can promote produce contamination are yet to be clarified. Although the leafy greens possess physical and chemical defense mechanisms to restrict the internalization of pathogens under certain circumstances the produce defense can be disrupted either by biological or mechanical means and *E. coli* O157:H7 access to produce inner tissues is favored [45–47]. Once the pathogen penetrates the produce inner tissues it can potentially evade the produce defense systems by adapting to the plant environment and becomes internalized [48].

Generally, it has been accepted that the internalization of pathogens depends on several factors, such as: (a) plant type, age, and exposure time to pathogen; (b) produce growing system (i.e., soil, hydroponic, aquaponic), (c) the level of contamination of produce with the pathogen, (d) the type and the degree of roots or leaves injury, (e) length of time given to the pathogen to spread from injured roots to the mature leaves etc. (**Table 2**) [40].

For soil-grown plants, internalization was observed as a sporadic phenomenon and with low incidence. Usually, the contaminated soil, could have a little to no influence on the noted internalization, soil presenting a relatively low risk of internalization as compared to other produce growing systems (i.e., hydroponic or aquaponic systems). Generally, the soil-grown produces are protected by environmental stressful conditions which are not favoring the pathogen internalization [40, 49]. The pathogen internalization in soil-grown leafy greens remains controversial: while several studies on leafy greens (lettuce or spinach) grown on contaminated soil have shown that internalization of *E. coli* O157:H7 could occur [50, 51] other researchers found little to no pathogen internalization in soil-grown produce [52]. When pathogen internalization in leafy greens grown in soil was observed, the incriminated factors were either the root damage during growth or soil's microbial profile lacking the microorganisms that may compete with the pathogen [53]. Despite the extensive experimental studies, there are many possible factors which can interact together in promoting the pathogen internalization, and it remains controversial whether *E. coli* O157:H7, when introduced through soil or irrigation water, could internalize the edible parts of the mature produce. For example, the specific role of produce type in bacterial internalization is very difficult to assess in detail given the multiple existing interfering variables. In this regard, it was found that *E. coli* O157:H7 was able to internalize into inoculated seeds of cress, spinach, and lettuce [54]. In spinach plants, internalization was observed in the root tissue or seedlings but not in mature leaves [55]. Plant roots appear to be preferred by the pathogen as attachment and entrance site, and the roots contamination was reported to be dependent on roots health status (healthy, non-damaged roots versus damaged roots) and on the degree of pathogen contamination level [40]. While produce roots are getting mature, the differences in the produce developmental stages may also influence the ability of *E. coli* O157:H7 to interact with the produce, the pathogen could be enabled to enter the produce leaves by traveling through the root system [56]. Hora *et al.* [55] found that the degree of *E. coli* O157:H7 internalization of the spinach roots depends on the type of roots damage and produce age but it does not favor the internalization of leaves (**Table 3**).

When the produce contamination occurs, produce age, produce exposure to pathogen, and contact length of time with the pathogen can result in possible internalization of the pathogen [40]. Produce leaf's age has been shown to influence the growth and survival of *E. coli* O157:H7; young lettuce leaves were found to be associated with a greater risk of pathogen contamination and internalization [21].

Type of produce	Pathogen	Growth system	Inoculation method and level	Plant age	Internalization status
Spinach lettuce, parsley	*E.coli* O517:H7 (Shiga toxin negative)	Field-grown	Drip irrigation or compost (2, 4, or 6 log_{10} CFU/mL)	Transplanted to field 8 weeks, inoculated 1, 8, and 10 weeks after transplant	No internalization was detected in any leaf tissues; detection in root occurred at one sampling time
Whole romaine lettuce and iceberg lettuce	*E. coli* O157:H7	Soil	4 $log_{10}$and 6 log_{10} CFU/g	Seedlings with 4–5 leaves were transplanted and inoculated 30 days after transplant	Heat and drought stress applied individually or in combination did not promote internalization
Leaves of romaine lettuce and iceberg lettuce	*E. coli* O157:H7 (5 strains cocktail)	Soil	Inoculated 3 and 6 log_{10} CFU/mL by manure, soil, and water	Inoculated when 3–4 leaves present, analyzed on days 26 and 60 post-inoculation	All samples were negative for bacterial at all inoculums, routes of inoculation, and times post-inoculation
Green ice lettuce	*E. coli* O157:H7	Manure amended soil	Inoculated manure with 8, 6, and 4 log_{10} CFU/g and added to lettuce flats	Days 3, 6, and 9 post-planting seedlings were cut 1 cm above the soil surface	*E. coli* O157:H7 was visualized at depths of up to 45 μm below the tissue surface; edible portions can become contaminated through transport by the root system
Spinach	*E. coli* O157:H7	Soil (drench)	6 log_{10} CFU/mL	Inoculated at 4-leaf stage, analysis on days 0, 7, and 14	No internalization into leaf observed by direct plating and qPCR; bacterial presence on roots observed by confocal laser microscopy on day 7 and 14
Spinach	*E. coli* O157:H7	Hydro-ponic system	Low (5 logs) or high (8.5 logs)	Plants (12-day) inoculated, allowed to grow for 21 days	*E. coli* O157:H7 was recovered from shoot tissue from 3 replicates on days 14 and 21
Cress, spinach, lettuce	*E. coli* O157:H7	Hydro-ponic system	Seeds were soaked in bacterial cell suspension (2 log_{10} CFU/mL)	Plants surface sterilized, seedlings analyzed on day 9	*E. coli* O157:H7 was recovered from external and internal tissues of all plants

**Adapted from [40].*

Table 2.
Examples of Escherichia coli O157:H7 internalization status in leafy greens grown in different environments.*

As an example, in spinach grown under greenhouse conditions, the internalization of *E. coli* O157:H7 in the leaves is rare and mostly is taking place from outside of the produce to the inside if the plant surface is exposed to a heavy contamination with the pathogen [57]. Some studies show that hydroponic systems favor a greater

Root treatment (damage) type	Number of sampled spinach plants	Number of *E. coli* O157:H7 positive samples	
		Roots	Leaves
Control[a]	9	9	0
Cutting of seminal root[b]	8	8	0
Removal of root hairs[c]	8	8	0
Biological damage[d]	8	8	0

*Source: [55].
[a]Roots without damage; spinach plant was not removed from soil.
[b]Seminal root was severed from 5-week-old spinach plants; plants were repotted.
[c]After the removal of root hairs transferred to soil.
[d]Roots inoculated with nematodes (Meloidogyne hapla); plant age-14 days.

Table 3.
The degree of E. coli O157:H7 internalization of spinach roots and leaves following different types of root damages.*

internalization of leafy greens compared to soil-growing system [58–60] and the water, as a growing environment, is indicated as the main source of produce contamination via pathogen uptake by roots [61, 62]. In aquaponic growing systems, under certain circumstances, STEC *E. coli* can internalize both roots and the plant leaves. STEC *E. coli* first internalizes the roots which are mechanically injured due to manipulation during transplanting. Subsequently internalization into the leaves occurs when the pathogen is given sufficient time to spread into the plant shoots and into mature leaves. Internalization of STEC *E. coli* into the whole plant grown in aquaponic system seems to be dependent on the plant age at the time of root injury: if the infection takes place during the early stage of plant development the STEC *E. coli* internalization in the whole young plant is favored [63].

Although the variability of the published experimental results is great, several conclusions can be reached in relationship with leafy greens pathogen internalization: (a) the produce growth environment plays an important role in pathogen internalization; (b) internalization is a plant-pathogen specific interaction; (c) health status of the roots does not enable the uptake of pathogen into produce, and (d) the presence of internalized pathogens into roots of plants is cannot be used as an indicator for pathogen internalization in leaves and does not directly correlate with internalized pathogens in the produce leaves.

3. Leafy greens pre-harvest pathogen contamination: risk factors and management strategies

3.1 General considerations

For the leafy greens grown in open fields, during pre-harvest stage, there is a constant and concomitant exposure to factors which favor the produce contamination with pathogens. While manure (i.e., improperly stored raw manure, improperly treated or composted manure) deposited nearby plating fields or without using any protective barriers, agricultural soil (manure amended or non-amended), and irrigation water, are considered main risks for the microbial safety of the leafy greens. Other factors such as the presence of domestic or wild animals, which are usually shedding the enteric pathogens via feces, and field workers are involved in leafy greens pathogen contamination [64, 65]. Proper identification and management of these factors are paramount for reducing the contamination of leafy greens in the pre-harvest stage [16, 66, 67].

3.2 Non-amended agricultural soil: the soil-substrate management

The non-amended agricultural soil ("soil" herein) represents a habitat for pathogenic E. *coli*, as well as for other microorganisms (pathogenic or not), and it is recognized as a potential environmental factor which could contribute to pre-harvest leafy greens contamination [68, 69]. In soil, the fate of *E. coli* (i.e., survival or die-off rates) depends on a myriad of soil properties, such as: abiotic (physico-chemical composition) and biotic properties (inherent existing microbiota), the growing soil localization (i.e., nearby unprotected animal farming operations, sewage etc.), and soil type (i.e., sandy, clay etc.) [68, 70, 71]. From an experimental standpoint, due to the difficulty to study and predict the effect of a combination of factors of influence, as well as their importance against the pathogenic *E. coli*, focus has usually been placed upon a single factor or soil component [71, 72]. Based on the experimental results, the soil-related factors which can influence the pathogenic *E. coli* survival have been divided into soil's biotic and abiotic characteristics [68, 70, 73]. Soil's biotic profile is very complex and experimental targeted studies indicated a high die-off of *E. coli* O157:H7 rates in soils containing rich microbial communities (i.e., bacteria and fungi), especially those characterized by a high metabolic diversity, and an increased *E. coli* O157:H7 concentration in sterile soils due the absence of competitive and/or predatory interactions [74]. Competition for existing nutrients, the release of secondary metabolites, such bacteriocins [75], by the microbial community, or direct antagonism could determine the fate of *E. coli* O157:H7 in soil [72, 75]. Zhang *et al.* and Majeed *et al.* confirm that the Gram-negative bacteria exhibit a greater antagonism against *E. coli* O157:H7 than the Gram-positive bacteria and are known to out-compete Gram-positive bacteria for nutrients in soil [70, 76]. Soil temperature can affect the activity of microbial communities against *E. coli* O157:H7. At 18°C the decrease of the pathogen was likely caused by enhanced antagonistic activity among soil microorganisms [74]. Also, Vidovic *et al*. confirms that *E. coli* O157:H7 declined more rapidly at 22°C compared to 4°C in autoclaved soil [77].

Since the survivability of *E. coli* O157:H7 is considered a huge risk for contaminating the leafy greens or other fresh produce, the determination of essential nutrients availability including carbon, nitrogen, trace elements, salinity, soil's pH and temperature are paramount prior to planting [78]. Zhang *et al.* found that the soil's pH influences the survival of *E. coli* O157:H7. While low pH soil values could shorten the *E. coli* O157:H7 survival to 6–7 days, in a more neutral pH *E. coli* O157:H7 could survive between 32 and 33 days. In addition, the association of an acidic soil with the richness in organic carbon could result in a prolonged survival of *E. coli* O157:H7. This experimental study indicates the fact that the soil pH influences the adsorption and desorption of soil minerals by the pathogen, nutritional availability of soil components, and heavy metal toxicity [70]. Similarly, Li and Stevens showed that the soil with low pH reduces the risk of contamination regardless the virulence of *E. coli* O157:H7 strains [79]; however, it was noted that the virulent *E. coli* O157:H7 strains survived less than the nonvirulent ones [68]. Cools *et al*. indicated that the soil's content in organic matter can be more influential on the pathogen survival than soil type [80, 81]. In this context, Brennan *et al*. found that clay loam soil has a greater nutrient availability and a fine texture which is favoring the long-term survivability of pathogenic *E. coli* [82]. In addition, the clay soil has more available micropores that favors the nutrient adsorption by the pathogen [83, 84]. For example, Fenlon *et al.* were able to isolate inoculated pathogenic *E. coli* over 4 months from clay and loam soils, and for 8 weeks from sandy soils [85]. For minimizing the long-term persistence of pathogenic *E. coli* in soil before planting, regulators and researchers are proposing several mitigation strategies (**Table 4**).

Recommendation	References
In agricultural areas where the risk of pathogen presence is high and the pathogen could be transferred to fresh produce crops without having in place a validated kill step process, planting should not be carried out.	[86]
Stoppage of soil amendment for a period of time prior to harvesting of fresh produce. After the use of manure, the "*90 to 120 days rule*" of not harvesting farm produce 90 days (for farm produce whose edible parts touch the soil) or 120 days (for farm produce whose edible parts do not touch the soil) must be applied.	[87, 88]
For either reducing the level of pathogens in the soil or applying the time rule to reduce pathogen to acceptable levels, the assessment of the planting land history, and of the adjacent land activities is required. Reducing the human (anthropogenic) activities which could disturb the nutrient resources and modifying the competition between native microbial communities and invasive species.	[79, 89, 90]
Similar hazards raise concern for proximity to waste stockpiling and management, composting operations, and run-off from areas of concentrated wildlife populations and urban environments.	[91]
Topographical features of the growing fields and adjacent land should also be considered in a hazard analysis to identify potential contamination sources.	[18]
Implementation of Good Agricultural Practices (GAP) and intervention strategies focused on the construction of ditches, establishment of buffer areas/physical barriers, setting up of fences around the farms to prevent animal intrusion, to re-direct or reduce runoff from animal production or other waste management operations.	[87, 92–94]
Before planting, the soil's acidity must be tested.	[95]
Encouraging growers to apply HACCP system in their primary production stage.	[24, 96, 97]

Table 4.
Examples of mitigation strategies recommended to be applied to growing soils and adjacent lands.

3.3 Manure and manure amended soils

In the fresh produce pre-planting and pre-harvest stages, amending the soil with organic fertilizers, such as manure, or biosolid fertilizers is a cost-effective alternative to chemical fertilizers, the later posing a great threat to humans and environment due to their potential toxicity. In farming, the use of manure is of paramount importance to enhancing the soil's fertility by primarily increasing its content of nutrients and other organic compounds required for improved production yields and agricultural sustainability. From a practical standpoint, manure is the solid part resulted after the segregation of the solid and liquid portions of the organic residual compounds from different origins (i.e., cattle, poultry, pigs etc.). Since manure has been used as an old, traditional farming practice, the advantages of using manure for soil replenishing with nutrients is well known. The studies performed over the last decades are scientifically validating the additional, multiple benefits of amending soils with manure: improving the soil's microbial diversity along with soils' agricultural properties such as soil density and structure (i.e., loosening up/breaking down the heavy soils), increment of water holding capacity [98], soil erosion, and to maintain the quality of "exhausted" soils due to the repeated use of agricultural lands—by application at the beginning of each growing season [99].

The addition of manure is performed before planting the soil and at different time periods during the fresh produce growth stages but not immediately before the harvesting stage. Manure can be applied as: solid manure (i.e., aged manure, compost, manure slurries, or manure tea). Among the identified pitfalls of soil manuring, the most important aspect is that the manure contains high levels of pathogens which can contaminate the leafy greens due to its ability to harbor and spread both animal- and human-origin pathogens including the *E. coli* O157:H7 in the farming environment. For minimizing to reduce this microbial risk, as a thumb rule, the manure must be generally added into the soil after being processed (aging or composting) and not at a stage near the produce harvesting time.

As a route of produce contamination, internalization of *E. coli* O157:H7 has been found highly prevalent on leafy greens, including lettuce leaves, when the soil has been fertilized with contaminated manure possibly due to the intake of the pathogen up through the leaves via the produce's root system [50]. Ekman *et al.* found that the *E. coli* can survive in manure amended soils and the viable *E. coli* O157:H7 numbers were declining by at least 3 logs after 50 days of manure application to the soil, regardless of the season of application [100]. Maximizing the time between manure application and harvest stage of the leafy greens is one avenue to allow the natural reductions of the target pathogen into the soil. Additionally, in manure amended soils, existing pathogens can colonize the seedlings during germination, or transfer from the manure amended soil to the leafy greens through water splashing (during irrigation or rain) or through soil dust [101, 102]. Islam *et al.* found more than 10 CFU/g *E. coli* O157:H7 on parsley and lettuce even when these produces were harvested after 160 and 70 days, respectively, when soil was amended with manure containing $\log_{10}$ 7 CFU/g *E. coli* [49]. In an experimental transfer "soil-to-crops" of *E.coli* O157:H7-inoculated manure, Suslow predicted that, once the contaminated manure was incorporated into the soil, a 99% reduction of *E. coli* O157:H7 viable population could take place after 60–120 days depending on soil type but also on other factors yet to be determined [103]. Later, other several other factors responsible for leafy greens contamination with manure pathogens were indicated by Baker and were based on the high variation of farming practices, from site to site: the use of untreated manure; the differences in manure storage methods, type of manure applied treatment including the time of manure piles resting undisturbed; the manure-handling equipment cleaning, sanitation, and segregation practices; lack of protection against wild animals of the manure sitting piles' [104]. The type of manure, aged (dried and compact) or manure slurry, and temperature could also influence the survivability of *E. coli* O157:H7. Under experimental conditions, Himathongkham found that the *E. coli* O157:H7 survival in aged cattle manure was higher at 20°C, while in fresh cattle manure slurry (1-part aged manure and 2-parts water) survivability was higher at 4°C and slightly reduced at 20°C [105]. Jiang *et al.* observed a more rapid decline of *E. coli* O157 in manure-amended unautoclaved soil at 21°C than at 5°C. This was attributed to an increase in microbial activity with temperature and consequently, greater competition for nutrients. These findings are important for elucidating the influence of temperature on *E. coli* O157:H7 survival in different types of manures used for soil fertilization [106].

It is established that the contaminated, untreated, or improperly treated manure has been implicated, worldwide, as a major source of pathogenic *E. coli* O157:H7-related foodborne outbreaks due to the consumption of leafy greens and fresh produce. Therefore, efficient manure management strategies and policies are required to be established and used on-farm. The public health is the ultimate, main objective of the manure management strategies which, for being successful, require multi-pronged approaches. Once adopted, these management strategies and policies should efficiently mitigate the negative impact of manure on the environment and on the leafy greens. Epidemiological and experimental studies conducted by CDC and FDA indicated manure as a major factor in the outbreaks due to *E. coli* O157:H7 and Shiga toxin-producing *E. coli* (STEC) [107–109].

To reduce the target pathogen and minimize the risk of leafy greens contamination via use of manure, FDA established a set of Good Agricultural Practices (GAPs),

Manure treatment status	Type of requirement for the application of manure.	Minimum application interval
Untreated	• Manure does not have contact with the leafy greens during application or the potential for contact with the leafy greens is minimized during manure application.	"Reserved"[a]
Treated[b,c]	• Manure does not have contact with the leafy greens during or after application.	0 days
	• Manure is applied in a manner that minimizes any potential contact with the leafy greens during or after application.	0 days

**Adapted from [110].*
[a]FDA is conducting additional research, working with other researchers, and working to conduct a formal risk assessment [111].
[b]A scientifically valid controlled physical, chemical, or biological process, or a combination of scientifically valid controlled physical, chemical, and/or biological processes to meet the requirements of microbial standard for E. coli O157:H7.
[c]Relevant national standard or E. coli O157:H7 is not detected using a method that has a detection limit of 0.3 MPN (Most Probable Numbers) per 1 gram or per 1 mL if liquid (i.e., agricultural manure tea) is being sampled as analytical portion.

Table 5.
Application requirements and the minimum application intervals of the manure depending on their treatment status and the potential on-field contact with leafy greens.*

as mitigation strategies to reduce the pathogen hazard. These strategies are related to the application and use of animal-origin manure and to the minimum application intervals for leafy greens according to manure treatment types (**Tables 5** and **6**) [110, 111]. In addition, minimizing direct or indirect contact between manure and the leafy greens especially at a stage closer to the harvesting time could be used as a method to reduce the contamination with *E. coli* O157:H7 [111].

Accordingly, there are other treatments on which fresh produce growers can rely on for minimizing the pathogens hazards, such as: allowing enough passage of time in conjunction with the action of other environmental factors (i.e., environmental temperature, moisture fluctuations, and solar ultraviolet irradiation) to ensure the manure is properly aged and decomposed before first application to fields. These type of manure treatments, are known as "*passive treatments*". Its disadvantage is that the treatments are time consuming compared to the "*active treatments*" because they depend on the type and source of manure, and on the climatic factors (regional and/or seasonal). When manure aging is used as a passive treatment U.S. FDA cautioned on not confusing this process with composting process, the latter being solely applied as an active treatment [111]. In addition, produce contamination with pathogens occurs if the manure is not treated before use, or if the untreated manure does not respect the recommended application method during produce growing (**Table 5**) [110].

The accepted manure active treatments consist in the application of a scientifically controlled processes such as: physical (i.e., thermal treatment), chemical (i.e., highly alkaline digestion), biological (i.e., composting), or a combination of those so that *E. coli* O157:H7 levels satisfy the microbial accepted standard levels (**Table 6**).

Similarly, in 2017, the European Union Commission in collaboration with the European Food Safety Agency (EFSA) established Good Agricultural Practices for the application of animal manures and the minimum pre-harvest intervals that should be followed when growers use organic fertilizers for leafy greens based on manure treatment types and manure microbial quality [112].

Examples of good agricultural practices (GAPs) for reducing the pathogen levels	
1. Manure treatments	
	1. Passive treatments: • Relying primarily on the passage of time, in conjunction with the influence of environmental factors: temperature, moisture fluctuations and natural ultraviolet (UV) irradiation; holding time for passive treatments will vary depending on regional and seasonal climatic factors and on the type and source of manure. • Growers should ensure the passive treated manure is well aged and decomposed before applying to fields.
	2. Active treatments: • Pasteurization, heat drying, anaerobic digestion, alkali stabilization, aerobic digestion, or combinations of these.
2. Manure handling and application	
	1. General: • Manure storage and treatment sites should be situated as far as practicable from fresh produce production and handling areas. • Consider barriers or physical containment to secure manure storage or treatment areas where contamination from runoff, leaching, or wind spread is a concern. • Consider good agricultural practices to minimize leachate resulting from manure storage or treatment areas contaminating produce. • Consider practices to minimize the recontamination potential of the treated manure.
	2. Untreated manure: • Consider incorporating manure into the soil prior to planting. • Applying raw manure, or leachate from raw manure, to produce fields during the growing season prior to harvest is not recommended. • Maximize the time between application of manure to produce production areas and harvest. • Where it is not possible to maximize the time between application and harvest, such as for fresh produce crops which are harvested throughout most of the year, raw manure should not be used.
	3. Treated manure[a]: • Avoid contamination of fresh produce from manure that is in the process of being composted or otherwise treated. • Apply good agricultural practices that ensure that all materials receive an adequate treatment.

**Source: [146].*
[a]If the manure is not treated on-farm then: (i) Growers purchasing manure should obtain a specification sheet from the manure supplier for each shipment of manure containing information about the method of treatment, (ii) Growers should contact state or local manure handling experts for advice specific to their individual operations and regions.

Table 6.
Control measures for minimizing E. coli O157:H7 and other microbial hazards.*

3.4 Use of irrigation waters

When grown in open fields, leafy greens can become contaminated inside roots and leaves with *E. coli* O157:H7 when irrigation is performed with contaminated water and by the irrigation method [49, 113] and could become the source of many outbreaks [114, 115]. The transmission of the pathogens from contaminated irrigation waters has been elucidated [50, 116], and secondary vehicles by which *E. coli* O157:H7 may contaminate the leafy greens were identified: flood irrigation with water contaminated either with animal feces or by contact with surface runoff [117, 118]. Experimental and on-field studies indicated the ability of the pathogen to survive for extended periods in water [119, 120].

Many different sources of water and methods are used for irrigation of fresh produce [121]. As water sources are identified two main groups: (a) surface water or treated wastewater (more prone to contamination and presents variables in water quality parameters); and (b) ground water reserves or collected rainfall water (which is less prone to contamination and more controlled from microbial quality standpoint if stored properly). Using drip or subsurface irrigation limits direct contact between edible plant tissue and irrigation water (splashes) and thus is less likely to introduce pathogens than furrow or sprinkler/overhead irrigation. Drip irrigation (subsurface irrigation) has less impact on leafy greens' contact with the pathogen and pathogen survival compared to other irrigation methods such as spraying, surface irrigation, and furrow which favor the subsequent survival of the pathogen up to 56 days [49, 122–124].

On the farm, to ensure the leafy greens protection from pathogen contamination, checking the water source history, application of preventive control measures to prevent contamination or to eliminate the pathogen (i.e., frequent sanitary surveys of water reservoirs and distribution systems, identification, and surveillance of drainages at the confluence points of water sources) are aspects of importance [14, 93, 125]. The preventive control measures are usually combined with different water treatments: filtration, disinfection, or solar irradiation (UV natural treatment) [89, 126, 127]. Similarly, FDA issued a set of GAPs for produce growers which includes: (a) identification of the source and distribution of water used and check its relative potential for being a source of pathogens; (b) maintain water wells in good working condition; (c) revision of existing practices and conditions to identify potential sources of contamination (direct or indirect contamination, contamination from human or animal waste); (d) check the current and historical use of land since agricultural water is frequently a shared resource with other operations or affected by human activity); and (e) test the irrigation water microbial quality [128]. Regarding the GAPs implementation, the Canadian Ministry of Agriculture, Food and Rural Affairs, proposed to farmers additional management practices to avoid or reduce the risk of contamination: (a) choose a different irrigation method (i.e., use drip or trickle irrigation systems rather than overhead sprinklers); (b) choose a different water source, or (c) for some irrigation systems and applications, water treatment is required to improve its quality (**Table 7**) [129].

3.5 Other factors which can contribute to pathogen contamination of leafy greens

3.5.1 Domestic and wild animals

An extensive number of post-foodborne outbreaks epidemiological surveys recognize the interconnection between animal activity on or in the proximity of growing fields and, leafy greens contamination with pathogenic *E. coli* [49, 130]. Regardless the leafy greens production phase, animals, domesticated (i.e., nearby livestock and on-farm working animals) and wildlife, can shed and transfer *E. coli* O:157:H7 to the produce, even the animals do not display any signs of illness. Among animals themselves, a zoonotic vicious cycle can take place. In many instances, cross contamination via fecal matter between domestic and wild animals have been identified, and approximately 77% of the pathogens

Irrigation water source	Best management practices
Streams	a. Use an off stream settling pond-allows large particles that may contain pathogens to settle out of the water and reduce the potential contaminant load. b. Work with neighbors (animal farms, industrial parks, households etc.) to reduce livestock access to water sources. c. Establish vegetative buffer zones to filter water and slow down run-off.
Ponds	a. Fence pond to prevent animals, both wildlife and domestic, from defecating in or near water. b. Re-direct runoff so that it flows around the pond and avoids contaminants entering pond through runoff. c. Establish grassed waterways or vegetative buffer strips to filter water before it enters the pond. d. Install steep sides or rocky berms to discourage geese from nesting.
Stream-fed ponds	a. Avoid harvesting water during the peak flows after a rainfall—this water carries most of the sediment (and possibly pathogens) washed by the rainfall. b. Establish vegetative buffer zones to filter water and slow down run-off.
Wells	a. Mound up the ground around the outside of the well or well pit with clean earth to provide drainage for surface water so that runoff flows away from the well. b. Maintain well casing above grade. c. Ensure that well casing is intact and there are no cracks or openings. d. Don't allow any space between the well casing and the surrounding soil (this could act as a pathway for surface water to contaminate the well).

**Source: [129].*

Table 7.
Best management practices for different irrigation water sources.*

that infect livestock can also infect wildlife (deer, geese, rodents, foxes etc.) which, in turn, can re-infect the livestock populations [131, 132]. For the past 10 years, FDA investigation findings on previous foodborne illness outbreak indicate the proximity of cattle operations as a main contributing factor for pathogenic *E. coli* contamination of leafy greens, cattle being repeatedly demonstrated to be a persistent source of E. coli O157:H7 [133]. In addition, leafy greens can become contaminated with antibiotic-resistant *E. coli* which can represent a real danger for public health. This fact was discovered when the *E. coli* isolates from lettuce production sites were compared with the animal-derived *E. coli* strains, and it was determined that these antimicrobial-resistant strains was prevalent in cattle [134]. Due to this high risk, a relatively recent report was issued by the European Food Safety Authority (EFSA) which attempted to ascertain to what extent fresh produce represents a vehicle for the acquisition by humans of antimicrobial-resistant bacteria and to identify possible control options [135–137]. Since food safety is a shared responsibility among all sectors ample animal management guidelines and mitigation strategies were proposed for protecting leafy greens but also fresh produces from being contaminated with pathogens at any stage of production [96, 138]. The on-field protection of the produce against pathogen cross-contamination from the existing multiple sources, regardless if the contamination sources are placed on the farm's premises or outside the farm, several practical protection strategies can used without disturbing the production chain (**Table 8**).

Location and type of pathogen contamination sources	Examples of management strategies
On-farm:	
a. Working animals	When working animals are needed to be used during harvest, minimizing animal contact with the produce must be reduced by: • Establishing "*no harvest*" buffer zones since working animals defecate in the field. • For animal and manure handling, development of standard operating procedure (SOPs) regarding hand washing, cleaning and sanitizing tools, and other sanitary practices to be completed after animal handling.
b. Mixed farming (i.e., animal farming and leafy greens production)	When both livestock and fresh produce production facilities are located on the same farm, implement farm policies, such as: • Require workers from animal holding areas to change their shoes or boots and clothing before entering fresh produce fields to prevent cross-contamination. • Proceed to train the employees to identify contaminants and determine when to not harvest produce that is likely to be contaminated with a known or reasonably foreseeable hazard. • Train the workers to washing hands after touching animals or any waste of animal origin before handling the produce.
Outside of the farm/produce fields:	
a. Dairy, livestock, or poultry nearby production facilities	Avoid locating produce fields and packing areas adjacent to dairy, livestock, or poultry production facilities unless adequate physical barriers are put in place, such as: ditches, mounds, grass/sod waterways, diversion berms, and vegetative buffer areas. The physical barriers will help to re-direct or reduce runoff from animal production or waste management operations, and to exclude free-roaming livestock from fresh produce fields.
b. Other animals (such as livestock from nearby farms)	Monitor for any signs of animal entry such as the presence of feces or damage to the crop and consider adding barriers to prevent animal waste from adjacent fields from contaminating produce fields during heavy rains, especially if fresh produce is grown in low-lying fields or orchards. If any animal holding areas are nearby, assure that the produce fields are uphill and the manure or urine runoffs are away, downhill, from produce fields.
c. Wild animals, pests, birds	Addition of distress machines (i.e., sonic fences), scarecrows, reflective strips, or gunshots to ward off birds and pests from crops, and repellent substances.

**Adapted from [138].*

Table 8.
Examples of using on-farm management strategies to avoiding leafy greens pathogen contamination.*

3.5.2 Workers, on-farm activities, and farming equipment and tools

3.5.2.1 Farm workers

Authorized or unauthorized human activity, regardless the status, farm worker or trespassers, could take place on the farm premises and on growing fields and could result in the produce contamination with pathogens. However, due to the daily, continuous type of work, the farmers and farm workers are playing an important role in maintaining uncompromised the microbial safety of the fresh produce, leafy greens, respectively, while performing their duties. A survey of Midwestern United States farms brought up an important aspect: while the most farmers were familiar with GAPs, the GAPs were not fully implemented on farms because they did not believe that the fresh produce contamination with pathogens were the direct result of their on-farm practices and there are several factors which farmers view as obstacles in GAPs implementation (**Table 9**) [139, 140].

Also, multiple studies indicated that the workers' clothing, hands, feet, and training are involved in fresh produce contamination with pathogens [141–143]. The survey data obtained by Antwi-Agyei *et al.* supports the fact that on-farm workers' hygiene practices could favor the on-field produce contamination with pathogen via hands- and feet-to-soil contact: (a) 73% of workers are practicing open field defecation, while only 25% use a public toilet, and 2.4% other toilets; (b) the percentage of farmers' prior contact to fecal contamination was 69 (as hand-to-soil contact) and 74 (as feet-to-soil contact) [144]. In this context, the data from **Table 7** correlates with other findings related to farm workers hygiene and on-field activities, and, more important with the willingness and the ability of farmers to provide proper conditions for avoiding fresh produce contamination by field workers. Practically, the hygiene interventions specifically designed for produce farm workers and workers' hygiene behavior is affected by farmers on-site policies and offered food safety training. Surveys performed by Bartz *et al.*, Antwi-Agyei *et al.*, and Fabiszewski *et al.* support the fact that the farm workers' hands are the main contamination vehicle of leafy greens during pre-harvest activities, such as: bed preparation, transplanting, soil tilling, weed removal, irrigation, due to the lack of field workers' accessibility of toilets, handwashing posts, eating and resting posts, training, and facilitative work policies to encourage workers to respect and practice the on-field hygiene [141, 142, 144, 145]. According to common guide issued in 1998 by FDA in collaboration with U.S. Department of Agriculture (USDA) and CDC, both farmers and workers must be reminded: (a) that anything that comes in contact with fresh produce has the potential of contaminating it and, for most foodborne pathogens associated with the fresh produce, the major source of contamination is human or animal feces, and (b) worker hygiene and sanitation practices during production, harvesting, sorting, packing, and transportation play a critical role in minimizing the potential for microbial contamination of fresh produce [146]. The multiple survey-based research on farm management and on farm workers indicate several mitigation strategies that could be implemented concomitantly (**Table 10**).

Type of factors	(%)
High costs of workers' training and GAPs implementation	67
Lack of time for implementing GAPs	68
Non-existent on-farm technical solutions (i.e., water and soil testing, testing the health status of workers etc.)	26
Lack of knowledge of GAPs	17
Lack of knowledge how to prioritize and implement the GAPs	27
Lack of personnel training opportunities	35
On-farm implementation of GAPs minimizes the growers' profit	40

[*]Adapted from: [140].
[a]Number of survey respondents, n = 143.

Table 9.
Examples of factors that farmers consider obstacles in implementing GAPs[a] on their farms[,a].*

Strategies to avoid on-field pathogen contamination by workers activities or via workers hands	References
Encourage workers to taking time away from their activities to use sanitary facilities (toilets, hand washing posts), especially if these facilities are not placed within a reasonable distance from the growing fields to avoiding the pathogen transfer from hands to leafy greens.	[147, 148]
Farmers should consider that workers, who are conditioned by the payment of hourly or daily pre-established quantity of harvested fresh produce and by the fragile nature of leafy greens, may be strongly discouraged from taking time away from their activities to use distant sanitary facilities and, as a consequence they will use the actual growing fields as a "*sanitary facility*". Therefore, the position and the number of handwashing posts and toilets must be well established prior to start growing the leafy greens.	[144, 147]
Farmers must establish labor policies and food safety training to encourage workers to adopt hygiene behavior even when the temporary on-farm workforce represents a unique challenge to farmers, and although these activities could affect financially the farmers.	[141, 142, 145]

Table 10.
Examples of mitigation strategies for being applied concomitantly for ensuring on-farm food safety during pre-harvest stage.

3.5.2.2 Farming equipment and tools

Farming is labor intensive and require a variety of working equipment and tools for land preparation (i.e., primary and secondar tilling, primary and secondary applications of manure/fertilizers or pesticides and insecticides, sowing the seeds or transplanting the seedlings, folding, irrigation etc.) and management, manure management, and workers protective equipment. Focusing on the pre-harvest phase of the farm-to-fork chain, the manipulation of leafy greens in the field is of particular concern due the risk of cross-contamination of the produce from unsanitary, soiled farm equipment. Little or no cleaning and sanitation between activities, lack of equipment and tools segregation, and lack of proper storage represent major causes of concern since these can become a direct source of produce contamination with pathogens [66, 93, 148]. In addition, processing of produce in the field such manual practices, and mechanical activities should be performed in ways that reduce the contamination of produce from soil, workers, or equipment surfaces [66]. Since on-farm surveys indicated that some farms do not clean and sanitize properly their equipment, or the equipment was most commonly cleaned by using only water without applying detergents and sanitizers. However, if water alone is used for cleaning the equipment and tools, famers should use only water with high microbiological quality (comparable with drinking water). Several management strategies (standard operation procedures and good hygiene practices, good agricultural practices) were designed to assist farmers and farm workers to reduce the microbial hazard and the microbial cross-contamination between equipment and tools and the fresh produce (**Table 11**) [149–151].

4. Conclusions

A better understanding of the pathogens' behavior in pre-harvest environments will support the developing of effective on-farm food safety management strategies (GAPs, HACCP) and interventions that will ensure the delivery of a safe produce to the consumer. Leafy greens should be given a high food safety priority since they are an important vehicle for pathogenic *E. coli* and are playing an important role in the emergence of new foodborne outbreaks. There are many possible sources of contamination of leafy greens due to their exposure to many different environmental factors, and multiple handling phases until reaching the consumers. Moreover,

What	How
• Equipment and tools that may contact raw produce should be sanitized and maintained clean to reduce the risk of cross-contamination. • Produce contact implements should be cleaned using adequate washing, sanitizing, and rinsing protocols, and the frequency of these operations should be determined, and the schedule maintained. • Cleaning of implements should be performed in a separate area and at appropriate times to prevent contamination of growing produce. • Storage of these implements should be in a clean area separate from that of manure/compost to avoid contact and cross-contamination.	*Step 1*: The surface should be rinsed so any obvious dirt and debris are removed. *Step 2*: Apply an appropriate detergent[a] and scrub the surface. *Step 3*: Rinse the surface with water that is the microbial equivalent of drinking water (potable). *Step 4*: Apply an appropriate sanitizer[a]. *Step 5*: If the sanitizer requires a final rinse, this will require an extra step, namely surface air dry.

[a]*Detergents and sanitizers must be food grade.*

Table 11.
Examples of on-farm cleaning and sanitation procedures for equipment and tools.

pathogenic *E. coli* could survive in leafy greens for commercially relevant periods even multiple disinfection procedures are applied. Therefore, pre-harvest stage must be viewed and approached as an important process which favors the contamination with pathogenic *E. coli*. The improvement of leafy greens microbial safety can be achieved by embracing the farming management strategies which will help growers to re-examine their own farming processes for reducing or eliminating the food safety risks. Comprehensive surveys, risk assessments, and scientific research on pre-harvest factors are needed to continue to identify risks, mitigation priorities, and the efficacy of different intervention strategies. Because of the frequent growers' failure to implement food safety rules and guidelines on their production premises, the existing mitigation strategies are not a "silver bullet" for minimizing the risk of leafy greens pathogen contamination. Therefore, both regulators and researchers should use the existing and the new incoming information for proposing and continuously designing potential mitigation strategies to be implemented by farmers for reducing the risk of leafy greens contamination with pathogenic *Escherichia coli* to harmless levels. These mitigation strategies have to undergo changes and be re-designed to address newly identified and reported on-farm deviations or violations of the food safety guidelines or of the Good Agricultural Practices (GAPs). Accordingly, the farmers and farm managers should be persuaded and helped to undergo more training sessions. National and international organizations and agencies, and researchers must support farmers to maximize their understanding and adherence to food safety guidelines for increasing their awareness on their role in the assurance of food safety throughout the leafy greens *farm-to-fork* continuum.

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