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Review

Incidence and microbiology of salad-borne disease

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Abstract

Because consumers have developed a better understanding of the health benefits associated with eating fresh produce, salads are increasingly being served in the home, in restaurants and as ready-to-eat pre-packaged foods. The associated increase in food-borne diseases must be seen in view of these benefits. Bacterial pathogens can survive on growing plants over prolonged periods of time under field conditions. In addition, the ability of human pathogens to infiltrate plant tissues has been recognized recently. Prepackaged, precut lettuce (i.e. damaged plant tissue) may pose a risk as an emerging source of food-borne illness. Surprisingly, there are no clear differences in pathogen contamination reported between organically grown produce and conventionally grown produce. The present intervention strategies are inadequate to ensure by 100% that salads are not contaminated with food-borne bacterial pathogens. The relative infrequency of outbreaks associated with preharvest contamination with *Shigella*, an organism with humans as its major reservoir, and the relative frequency of those associated with *Salmonella* or Shiga toxin-producing *Escherichia coli*, organisms with animals as their major reservoirs, underline the role of domestic and wild animals as dominant sources of preharvest contamination of vegetable salads. In the overwhelming majority of reported outbreaks, the contamination of salads did not occur before harvest nor did it originate at the processing plants, but rather the source could be traced to symptomatic or asymptomatic food handlers. Thorough epidemiological and microbiological investigation of all food-borne outbreaks is essential to finally assess the importance of preharvest contamination of salads.

Keywords: Salad, Infection, Outbreak, Food-borne

Review Methodology: The authors searched the following databases: CAB Abstracts and Medline (keyword search terms used: salad, infection, outbreak, food-borne). In addition the authors used references from articles obtained by this method to check for additional relevant material. The authors also spoke to colleagues and checked for any upcoming studies not yet published.

Introduction

Vegetables are often eaten raw, and hence are potential vehicles to transmit human pathogens. Vegetables can become contaminated throughout the food chain from production to consumption, via, for example, treatment with water for fertilization, irrigation or pest control, contact with animal or human excreta, food handlers or by cross-contamination in domestic environments. The quantity of produce eaten per capita has been increasing steadily over the past two decades, creating a heightened potential for produce-related food-borne disease. The obesity epidemic in Europe and in North America has prompted consumers to eat healthier diets that include more fresh vegetables [1]. Salads constitute one of the most popular diet foods. The rise in food-borne illness associated with salad vegetables can be attributed partly to this higher consumption level [2, 3]. The rise in food-borne illness associated with salad vegetables is also connected with the increase in availability of ready-to-eat vegetables [4]. Here, the increased handling of produce can augment cross-contamination, and cutting or shredding operations release nutrients that can sustain bacterial growth [4]. Recent research has even demonstrated the ability of enteric pathogens to become internalized within growing salad vegetables [4]. For countries increasingly using organic farming, such as Austria, this has special consequences. It is not clear whether organically grown produce poses a higher risk as compared with conventionally cultivated vegetables. Because more organic fertilizers are used, pathogenic bacteria from farm animals are more likely to reach the organic vegetable fields either directly through manure or indirectly through contaminated water. Human pathogens in raw produce took on national prominence in the USA in 2006 when 205 illnesses (including 31 haemolytic uraemic syndrome (HUS) cases and 3 deaths) in 26 states were reported as a consequence of the consumption of fresh, bagged, baby spinach contaminated with *Escherichia coli* O157:H7 [5]. In 2007, the same pathogen was identified in salad greens as the source of disease outbreaks in the Netherlands and in Iceland [6]. There are many specific aetiological agents linked with produce-associated outbreaks. Bacteria (*Salmonella* and *Shigella* spp., Shiga toxin-producing *E. coli* (STEC), *Listeria monocytogenes* and *Campylobacter jejuni*), viruses (norovirus and hepatitis A virus) and parasites (*Cyclospora cayetanensis*, *Cryptosporidium parvum* and *Giardia lamblia*) have been associated with cases of human illness following consumption of contaminated produce [7]. In this review, the authors try to give an overview of the incidence and microbiology of salad-borne disease.

Incidence

The data available on the incidence of salad-borne diseases must be scrutinized in view of the poor quality of available data on food-borne illness in general. In Austria (total population: 8 million), there were 3587 statutorily reported cases of salmonellosis in 2007 but 4050 human primary isolates were sent to the national reference centre for *Salmonella* [8].

Furthermore, microbiologically confirmed food-borne cases are considered to be just the tip of the iceberg. In estimating the burden of disease within a population it is important to recognize that many people do not consult a physician when they become ill with gastrointestinal symptoms or complaints, and that not all physicians send in stool samples for microbiological verification. Microbiologically confirmed salmonella cases account for only 2.6–6.9% of the estimated total number of *Salmonella* infections [9, 10] and thus it may be calculated that actual cases of *Salmonella* infection in Austria in 2007 reached approximately 60 000–160 000.

Evidence-based measures for the prevention of food-borne disease require information on the sources and transmission routes of infection [11]. Outbreaks frequently offer the only arena for gaining such knowledge. Improved knowledge, in turn, provides a basis for improved prevention recommendations. Outbreak identification and the subsequent microbiological and epidemiological investigations therefore play a central role in prevention and control of infections from foodstuffs [11]. Whereas the incidence of sporadic notifiable disease can be seen as an 'unavoidable event', the occurrence of a food-borne outbreak is often an indication of inadequate standards of hygiene [12]. If the sources of an outbreak can be identified and successfully contained, then the occurrence of further outbreaks and subsequent sporadic cases can be prevented. Outbreak investigations are thus an instrument for evaluation and improvement of existing preventive measures [12]. Public health investigators would benefit by having legal authorization to investigate outbreaks, including the collection of data and the interviewing of ill and well persons, without needing the institutional clearances usually necessary in non-emergency settings.

Only if food-borne outbreaks are investigated can the causative pathogens be identified within food products and preventative measures be implemented during food production or food processing. Although data may be missing on the true scale of food-borne outbreaks, interpretation and evaluation of the available reported outbreaks remain important.

A European Union (EU) average of 1.2 outbreaks per 100 000 people was reported among 22 EU member states in 2006 [13]. The highest number of outbreaks per 100 000 was reported in Latvia, with 13.5 outbreaks per 100 000; Austria reported 7.4 outbreaks per 100 000 and Germany 1.7 outbreaks per 100 000 [13]. Portugal reported 0.1 outbreaks per 100 000 in 2006, Spain 0.8 outbreaks per 100 000 and Poland 1.5 outbreaks per 100 000 inhabitants. Using the Swedish Tourist Database, de Jong and Ekdahl have calculated the estimated burden of *Salmonella* disease in EU member states [14]. Numbers of returning travellers who had fallen ill with *Salmonella* were used to estimate an annual incidence of the disease within each country. Based on these calculations, the risk of contracting *Salmonella* in Portugal was 79.9 per 100 000 travellers, the risk in Poland was 76.5 per 100 000 travellers and in Spain 72 per 100 000 [14]. Although in 2006, Portugal, Poland and Spain reported a lower number of outbreaks per 100 000 people in comparison with Austria, there was a higher risk of *Salmonella* infection among the travellers visiting these countries. This may indicate that the level of reporting in these countries is not as detailed as the level of reporting in Austria and not that there is a higher rate of outbreaks in Austria.

In 2007, the three most common sources of domestically acquired food-borne infection in Austria were eggs (18.2%), meat (especially poultry) (16.4%) and fish (0.8%); these data correspond fairly well with those given for the EU in general in the Community Summary Report of Zoonoses for 2006 (eggs: 17.8%; meat: 14.5%; fish: 4.6%). In Austria, the source of infection was known in only 37% of the outbreaks; no source of infection was given by reporting member states in 42.6% of outbreaks [81]. In produce, because of the short shelf life of the product, illnesses are hard to trace, especially in sporadic cases [15]. In 2007 in Austria, vegetables were blamed for less than 1% of domestically acquired food-borne infections; in the European Community Summary Report of Zoonoses for 2006 vegetables and vegetable juices accounted for 1.9% of the individually reported outbreaks of all member states [13].

However, in countries where surveillance of food-borne illness is extensive, a significant proportion of outbreaks have been attributed to fresh produce [16]. For example, in England and Wales, 5.5% of food-borne outbreaks documented during the period from 1992 to 2000 were associated with the consumption of salad vegetables or fruit [17]. Nearly 60% of these illnesses were associated with *Salmonella* and norovirus. In England and Wales, salad, vegetables and fruit caused 6.4 and 10.1% of all outbreaks with a known food vehicle in the respective periods of 1993–1998 and 1999–2000. [16]. Also, in the USA, produce-associated outbreaks accounted for a growing proportion of all food-borne outbreaks documented from 1973 to 1997, increasing from 0.7% of all outbreaks in the early 1970s to 6% in the 1990s [2, 7]. According to Centers for Disease Control and Prevention (CDC) data, from 1998 to 2002, an average of 25 674 food-borne illnesses and 18 deaths in the USA are caused by 1329 outbreaks annually [18]. The number of outbreaks traced to plant consumption (vegetables, fruits and nuts) during that same period averaged 56 and caused 2109 illnesses each year. The proportion of outbreaks attributed to plant consumption was 4.2% [18].

Worldwide, bacterial food-borne zoonotic infections are the most common cause of reported cases and outbreaks of human infectious intestinal disease, with *Salmonella* and *Campylobacter* accounting for over 90% of all reported cases of bacteria-related food poisonings [19]. Salads only play a minor role as the source of disease. Nevertheless, examples of food-borne outbreaks traced to salad vegetables can be found worldwide.

Campylobacteriosis

Campylobacteriosis is the most common bacterial cause of diarrhoea in developed countries [13]. Contrarily, from 1990 to 2004, only three outbreaks of *Campylobacter* linked to contaminated leafy greens were reported in the USA [16]. In Austria, food-borne outbreaks of campylobacteriosis were traced to contaminated poultry meat and to raw cows' milk, but so far never to vegetables [20–22].

C. jejuni, a microaerophilic food-borne pathogen, survives poorly on plants, perhaps because of a lack of microsites with sufficiently low oxygen concentrations in that habitat [23]. The pathogen has higher survival rates in the rhizosphere than in the phyllosphere, as well as on leaves that are wounded or contaminated with soil than on clean healthy leaves [23]. These observations may partly explain the occasional presence of *C. jejuni* on fresh produce and the (although rare) occurrence of produce-associated outbreaks of campylobacteriosis, despite its generally weak epiphytic fitness [16]. In a study of 3200 uncooked ready-to-eat organic vegetables from retail markets in the UK no *Campylobacter* was detected [24]. McMahon and Wilson did not detect *Campylobacter* in 86 organic vegetable samples from large supermarket chains in Northern Ireland [25]. Low isolation rates of 2 (3.6%) of 56 fresh vegetables and 2 (0.5%) of 400 ready-to-use vegetables were reported by Kumar *et al.* and Federighi *et al.*, respectively [26, 27]. Of 200 products of precut ready-to-eat salads sampled at retail in Spain in 2006, none yielded *Campylobacter* spp. [13]. Exceptionally high rates were presented by Chai *et al.*, with average prevalence from 29.4 to 67.7% of *Campylobacter* in 309 samples of raw vegetables used in ulam, a popular Malaysian salad dish, obtained from three different supermarkets [28].

Non-typhoidal Salmonellosis

Salmonellosis associated with the consumption of salad items is relatively uncommon, although its occurrence is well accepted. In a widespread outbreak of multiresistant *Salmonella enterica* serotype Typhimurium DT104 infection in England, affecting 351 people, illness was epidemiologically linked to the consumption of lettuce away from home in the three days prior to the onset of illness [29]. Investigations in Iceland into a Europe-wide outbreak of multiresistant *Salmonella* Typhimurium DT204b (affecting 125 people in England and Wales) revealed an association between illness and the consumption of imported iceberg lettuce [30]. Fisher and O'Brien reported an outbreak of *Salmonella* Newport infection in England associated with the consumption of prepacked salad that was identified following the isolation of *Salmonella* Newport from a salad item as part of a routine survey of retail prepared ready-to-eat salad vegetables [31]. In 2008, an outbreak of *Salmonella* Saintpaul infections associated with multiple raw produce items affected at least 1442 persons in the USA [32]. Epidemiological and microbiological results pointed toward jalapeno peppers as the major vehicle by which the pathogen was transmitted; tomatoes possibly were a vehicle early in the outbreak. The outbreak strain was cultured from a jalapeno pepper sample and from a sample of water from a holding pond used for irrigation.

During the period from 1990 to 2004, the number of tomato-associated *Salmonella* outbreaks increased in frequency and magnitude (1616 cases; nine outbreaks) [7]. In 2004, three outbreaks of *Salmonella* infection associated with eating Roma tomatoes were detected in the USA and

Canada [33]. No clear source of contamination was identified following environmental investigation of four packers and associated farms. In 2004, consumption of rocket lettuce (also known as arugula) produced in Italy as precut mixed salad was associated with 21 cases of *Salmonella* Thompson infection in Norway [34]. Sweden and England also reported an increase of cases of illness. Swedish authorities reported the finding of *Salmonella* Thompson in the incriminated rocket lettuce. In a large outbreak of *Salmonella* Thompson caused by contaminated cilantro (coriander) used in restaurant-made salsa, inoculation studies were done to investigate the growth characteristics of *Salmonella* in fresh leaf vegetables. The investigation of this outbreak of *Salmonella* Thompson, linked to cilantro suggested that it resulted from pre-harvest contamination of the produce [35]. It was shown that chopping the leaves facilitated growth, and that *Salmonella* Thompson after 1 day incubation at 26°C could grow from 10^4 to 10^7 colony-forming units per gram when incubated at 26°C [35, 36]. In 2008, Sweden reported 10 cases of *Salmonella* Napoli (RASFF alert number 2008.1399); patient isolates were indistinguishable by pulsed field gel electrophoresis (PFGE) from a strain isolated from a batch of rocket salad and from the 2004 isolates [34]. In Austria, no single outbreak has been traced to consumption of salad contaminated with non-typhoidal *Salmonella* so far.

In 2006, 18 EU member states investigated 7571 plant products of 'vegetables, fruits and herbs' for *Salmonella*, which may reflect the increased political attention in this area following several international *Salmonella* outbreaks caused by lettuce and basil [13]. Islam *et al*. also investigated the survival of *Salmonella* Typhimurim on lettuce and parsley grown in fields treated with contaminated composts or irrigation water [37]. Results indicated that the organisms persisted for 63 days on lettuce and for 231 days on parsley. Of 1870 samples of precut lettuce, two samples (0.1%) tested in the Netherlands yielded *Salmonella* in low numbers. However, Austria did isolate *Salmonella* Mbandaka (n=8) and *Salmonella* Bere (n=4) from sprouted seeds in a small investigation including only 20 samples [13]. In the USA, the Food and Drug Administration (FDA) conducted a survey of imported fresh produce and found 4% of the products to be contaminated [38]. The presence of *Salmonella* spp. in tested produce may have been the result of workers' handling of the product, animal manure used as fertilizers, or harvesting equipment [38]. Considering the sheer volume of salads imported each year, even a low level of contamination poses a significant human health threat.

Although few incidents or threats of intentional contamination of food with hazardous agents on a massive scale have been documented, it is prudent always to consider the possibility of deliberate action. In 1984, members of a religious commune contaminated salad bars in ten restaurants in the USA with *Salmonella* Typhimurium, causing 751 cases of salmonellosis. The possibility that intentional contamination caused the outbreak was initially rejected, as no one claimed responsibility for the incident. In fact, the attack was planned as a covert tactical strike, a trial run for a more extensive attack intended to disrupt local elections [39]. Deliberate contamination cannot be prevented with 100% certainty [40]. However, preparedness for naturally occurring outbreaks or the accidental release of agents equals consolidating preparedness for intentional biothreats [40].

Infection with STEC

Infections with STEC are associated with a wide spectrum of diseases in humans, including diarrhoea, haemorrhagic colitis and HUS, the most common cause of acute renal failure in childhood. Infections with STEC are rare when compared with the incidence of campylobacteriosis or salmonellosis, but can be the third most common bacterial cause of diarrhoea [41]. The most important virulence characteristic of an STEC strain, also called enterohaemorrhagic *E. coli* (EHEC), is its ability to produce and release cytotoxins known as verotoxins because of their cytopathic effect on Vero cells or as Shiga toxins because of relatedness to the toxin of *Shigella dysenteriae*. EHEC O157:H7 is the most prevalent serotype associated with human disease worldwide [42]. However, several non-O157:H7 Shiga toxin-producing serotypes including O26:H11/H$^-$, O103:H2, O111:H8/H$^-$ and O145:H28/H$^-$ have been increasingly reported, especially in Europe. These non-O157 serotypes ferment sorbitol and are thus indistinguishable from normal intestinal coliforms by classic microbiological diagnostic procedures based on screening with sorbitol MacConkey agar.

Fresh produce is a prominent food vehicle of *E. coli* O157:H7 outbreaks in the USA [43]. During the period from 1982 to 2002, produce was associated with 38 of 138 food-borne outbreaks and 34% of 5269 cases of food-borne *E. coli* O157:H7 illness. Approximately 18% of produce-related outbreaks were linked to lettuce, and the outbreaks were not associated with kitchen-level cross-contamination [43]. In 1996, about 8000 children in Japan became ill with *E. coli* O157:H7 infection from contaminated radish sprouts served in school lunches [44]. Contaminated seeds shipped internationally were the most likely source of the Sakai City outbreak as well as previous outbreaks of sprout-related *Salmonella* infections [45, 46]. In 2007, an outbreak of STEC simultaneously occurred in the Netherlands and Iceland, affecting at least 48 people [6]. Of more than 100 samples of the ready-to-eat lettuce mixtures prepackaged in the Netherlands, none tested positive for *E. coli* O157. In one outbreak caused by lettuce, the rinsing water used by the grower was the most likely source of contamination, as it contained *E. coli* O157:H7 [47]. Contaminated water was also suspected as the source in an STEC O157 outbreak related to iceberg lettuce in Sweden, although no microbiological evidence was found [48]. The trace-back investigation in another lettuce outbreak in the USA implicated two possible sources: one at a local farm and another in six farms shipping under the same label [49]. Microbiological evidence could not be established, so the transmission route remained unclear. In Austria, food-borne outbreaks of STEC infections were traced to contaminated meat and to raw milk, but so far never to vegetables [50–52].

The association between *E. coli* O157:H7 and lettuce has been investigated by a number of laboratories both because of the plant's commercial significance and because of repeated outbreaks linked to lettuce consumption [6, 47, 49]. Laboratory and greenhouse level studies demonstrated transmission of *E. coli* O157:H7 from contaminated soil and irrigation water [53, 54]. In a field-scale investigation conducted by Islam *et al*., *E. coli* O157:H7 was found to persist on lettuce (>77 days) and parsley (>177 days) after being planted in fields treated with contaminated manure composts or irrigation water [55]. Although it has been shown that lettuce can become infiltrated by *E. coli* O157, making it impossible to wash off, in most cases the bacteria stay on the surface of the leaves [56]. Of 280 vegetable products (unspecified) sampled in Germany, Slovenia and Spain in 2006, none yielded STEC [13].

Shigellosis

Shigellosis is an acute bacterial disease of the large and small intestine caused by one of four *Shigella* species. Shigellosis caused by *Shigella sonnei* is more frequent in industrialized countries, whereas *S. dysenteriae*, *Shigella boydii* and *Shigella flexneri* account for most isolates from developing countries. Similar to STEC, the low infectious dose (10–100 bacteria) favours high transmissibility with a secondary attack rate estimated at 40% [57]. A 1986 outbreak of *S. sonnei* in Texas affected 347 customers who had eaten at three separate restaurants; commercially distributed shredded lettuce was epidemiologically and microbiologically implicated [58]. In Austria, salad was the most probable source of infection in a youth hostel outbreak caused by *S. sonnei* affecting 53 people in 2008; the hostel warden, who tested stool-culture-positive for *S. sonnei* but did not fall sick with shigellosis, was assumed to be the source of the salad contamination [57]. Two *S. sonnei* outbreaks in several European countries, including Norway, Sweden and the UK in 1994 were linked to contaminated iceberg lettuce from Spain [59, 60]. In Norway, 110 culture-confirmed cases of infection were recorded [60]. The presence of *Shigella* in the suspected food source could not be documented retrospectively, despite comprehensive bacteriological examinations.

S. sonnei has been shown to multiply in shredded lettuce and lettuce extract under certain conditions [58]. It is therefore likely that *Shigella* can survive on iceberg lettuce in numbers sufficient to cause disease but insufficient to allow detection by the laboratory methods used. The most relevant reservoir of *Shigella* spp. is humans. As total *E. coli* counts are a much more sensitive parameter for faecal contamination, salad samples are not routinely tested for *Shigella* in the EU.

Listeriosis

L. monocytogenes, the causative agent of listeriosis, may be isolated from soil, animals, irrigation water, surfaces of processing plants and numerous food products [61]. Cross-contamination in processing plants, retail outlets, food service facilities and homes may contribute to the transmission of the pathogen. The ubiquitous nature and extraordinary ability of this pathogen to adapt to multiple environments hinder conclusive outbreak investigations and control. Unlike other food-borne pathogens, *L. monocytogenes* naturally occurs in the soil. In fact, most produce contaminated with *L. monocytogenes* can be traced back to the soil [61]. While most food-borne pathogens may survive, but do not grow on lettuce under storage conditions, *L. monocytogenes* has been shown to multiply on lettuce under storage conditions [62, 63]. An outbreak of listeriosis in Canada in 1981 has been linked to cabbage used by a regional producer to prepare coleslaw [64]. Given the ability of *L. monocytogenes* to grow on lettuce in storage conditions and the prevalence in lettuce, the fact that no listeriosis cases have been reported in connection with consuming contaminated salad is paradoxical [1]. Also in Austria outbreaks of listeriosis were traced back to cheese and cold cut meat, but so far not to vegetables [65, 66].

Of 101 products of precut ready-to-eat salads sampled at retail in Belgium in 2006, none yielded *L. monocytogenes* (81). In Europe, *L. monocytogenes* must not be present in levels above 100 cfu/ml during the shelf life in order to comply with microbiological criteria [13]. Surveys to determine the prevalence of *Listeria* in various foods in the USA indicated that 0.74% of bagged salad yielded *L. monocytogenes* [67]. Another microbiological examination of nearly 3000 samples of salad vegetables from food service areas and self-service salad bars revealed that 97% were of acceptable microbiological quality [68]. *Salmonella* spp., STEC, and *Campylobacter* spp. were not detected in any of the samples examined; one sample was positive for *L. monocytogenes*.

Viral Infections

In the USA from 1998 to 2000, 17% (13 of 76) of norovirus-associated outbreaks were linked to fruits or vegetables [69]. Food handlers are implicated more often (48 of 94) in outbreaks linked to viruses as opposed to outbreaks (20 of 102 outbreaks) involving bacteria [7]. An analysis of norovirus outbreaks from 2002 through 2006 recorded in a European norovirus surveillance database revealed that the 224 food-borne outbreaks were associated with thirty different food categories, including shellfish, fruit, fancy cakes, buffets, sandwiches and salads [70]. Numerous outbreaks of hepatitis A were associated with lettuce [71–73]. In Austria, salad vegetables were the main reservoirs for food-borne norovirus infections affecting 182 workers in an outbreak in 2006; the most likely source of food contamination was a kitchen assistant, who prepared the salad manually [74]. Food handlers working despite manifest diarrhoea or vomiting (often in fear of losing their jobs) are a common cause of food-borne norovirus outbreaks. In 2003 an outbreak of hepatitis A affected >700 people in the USA; contamination of the causative green onions was not associated with a food service worker but occurred at some point during growth, harvest, processing or distribution to the consumer [75]. In Austria, three food-borne outbreaks of hepatitis A were documented in recent years, none of them associated with salads [76].

Irrigation water and manure fertilizer can serve as vehicles for dispersing viruses, like other microbes, onto crops in the field. Viruses inoculated onto vegetables, lettuce, carrots, radishes, cucumbers and tomatoes could be recovered for up to 8 days postchallenge. The persistence of enteric viruses on vegetables has been found to be plant-dependent. For example, hepatitis A virus persists longer on lettuce, compared with carrots or fennel [77]. Hepatitis A virus survived for 4, 7 and 9 days postchallenge on carrots, fennel and lettuce, respectively [77]. Enhanced persistence on lettuce was speculatively linked to the wrinkled surface of the leaves, which provided greater protection for the virus. The possibility that enteric viruses could interact with growing plants in the field has not been considered to any great extent, due to the inability of viruses to multiply in the environment. So far, the internalization of enteric viruses into the inner tissue of plants has not been shown [4]. However, by gaining access to protected sites, such as roots or closed foliage, the virus can achieve a survival time of up to 60 days [78].

Parasitic Infections

Food-borne outbreaks of cyclosporiasis and cryptosporidiosis have been associated with the consumption of lettuce, basil, snow peas and a variety of other vegetables and fresh fruits [7]. In 2005, fresh basil was implicated as the source of illness in 293 laboratory-confirmed cases of cyclosporiasis in Florida [7]. In 2005, also in the USA, approximately 50 cases of cyclosporiasis were associated with consuming contaminated snow peas imported from Guatemala [79]. The raw snow peas were washed in municipal water and added to a salad. The modes of contamination of the fresh produce have not been determined but may include using contaminated water for irrigation, applying pesticides and cleaning the product immediately following harvest [79]. Döller *et al.* reported on a food-borne cyclosporiasis outbreak affecting 34 people who attended lunches at a German restaurant [80]. The only foods associated with significant disease risk were two salad side dishes prepared from lettuce imported from southern Europe and spiced with fresh green leafy herbs. In 1997, fresh mesclun, a mixture of various types of baby lettuce, and fresh basil were implicated in several clusters of *Cyclospora*-related food poisoning in the USA [64, 81–83]. Ethelberg *et al.* reported on a cryptosporidiosis outbreak that affected at least 99 people in Denmark in 2005, associated with consumption of whole carrots [84]; the carrots were kept in a large bowl of water. One hypothesis was that individuals may have taken carrots from the bowl using their hands, and this action could have contaminated the carrots if any of these people were human carriers of the parasite. Chicken salad (including chopped celery) was the cause of a cryptosporidiosis outbreak affecting 15 persons in Minnesota in 1995 [85]. The food preparer in this outbreak may have contaminated the implicated salad after changing the diaper of an asymptomatically infected child. Although salad-borne transmission of *C. parvum* has been suspected previously, evidence supporting this mode is limited, as it is for *G. lamblia* [86]. For 21 people who fell ill after attending a dinner at a church in New Mexico, lettuce, onions, and tea or coffee were most strongly associated with giardiasis by logistic regression analysis [87]. Washing lettuce in contaminated water was the most probable source; because the lettuce and onions were all cut on the same board, cross-contamination could have occurred. Stuart *et al.* studied risk factors for sporadic giardiasis in England in 1999, and found an increased risk for giardiasis associated with eating lettuce [88]. Outbreaks of giardiasis in the USA have been attributed to eating salad contaminated by food handlers [89].

Robertson and Gjerde analyzed 475 samples of various fruits and vegetables for parasite contamination and found 6% to be contaminated with either *Cryptosporidium* oocysts or *Giardia* cysts [90]. Moore *et al.* reviewed the available literature on detecting *C. parvum* in lettuce and reported prevalence of up to 14.5% [91]. Discovery of contamination usually occurs following a human food-borne outbreak, but in this case random sampling revealed low levels of parasitic contamination. *Cyclospora* oocysts have been isolated from lettuce in Peru [92], and Egypt [93] and from green leafy vegetables in Nepal [94]. Ripabelli *et al.*, working in Italy on experimentally contaminated lettuce, were unable to detect *Cryptosporidium* in un-inoculated lettuce leaves using filtration, immunomagnetic separation, light microscopy and PCR [95]. Irrigation and fertilization practices may also lead to contamination with *G. lamblia* [96].

Internalization of Human Pathogens in Plants

The early beliefs that most illnesses linked to fresh produce are the result of postharvest microbial contamination rather than contamination in the field is to be challenged by recognizing potential preharvest crop contamination. For most food-borne pathogens associated with produce, the major source of contamination is human or animal faeces [97]. Some pathogenic microorganisms, such as hepatitis A virus and *Shigella* spp., have only higher primates as reservoir, so when outbreaks caused by these organisms occur, they are almost certainly related to poor personal hygiene or improper waste disposal. The most probable routes of contaminating vegetables are fertilization with human waste or faecally contaminated water used to irrigate crops, prepare pesticides, or to freshen and clean produce at their origin. In addition to contaminating field crops through the water route, it must be considered that seasonal field workers often do not have access to appropriate sanitary facilities. *L. monocytogenes* naturally inhabits the soil environment. *E. coli* O157:H7 and *Salmonella* also have been demonstrated to survive in soil over prolonged periods of time (>80 days) [98, 99]. Therefore, contamination of salads with soil can be another source for infecting humans.

Bacterial human pathogens have also been shown to survive for long periods on growing plant tissue [100]. Recently, investigators were able to demonstrate that human pathogens can also colonize the tissues of growing plants [36, 100–102]. The presence of non-phytopathogenic micro-organisms within healthy tissue of vegetables was described in the 1960s, but only recently it has been accepted that bacteria can reside in the internal structures of undamaged plants [103–106]. Previously, most of the studies on non-pathogenic plant–microbe interactions addressed the symbiosis between legumes and rhizobia, which induce nodule formation and nitrogen fixation on legume roots. Furthermore, it has been realized that many other plant-colonizing bacteria show beneficial effects on plant growth and health [106]. Very recently, it has been recognized that plants may also act as a reservoir of human pathogens. STEC and *Salmonella*, both previously regarded as pathogens linked to foods of animal origin, have emerged as the two most common causative agents of produce-related outbreaks. Several studies suggest even a true pathogenic response in the plant when it gets infected by a human pathogen. Pathogenicity-related genes were expressed in lettuce plants upon colonization by *Salmonella* bacteria [107]. Moreover, in *Arabidopsis* a complex response pattern similar to that caused by a typical plant pathogen has been observed upon attack by *Salmonella*, demonstrating that the plant represents a true host system for the bacterium [108]. However, a certain plant cultivar-bacterial serovar specificity has been observed implying that some bacterial strains/serovars colonize plants more efficiently than others [109].

Under laboratory conditions, adhesion of *Salmonella*, STEC, and *L. monocytogenes* to roots follows location patterns similar to those reported earlier for various plant-associated bacteria [110]. In a study in which lettuce seedlings grown in soil were irrigated with a suspension of *E. coli* O157:H7, the pathogen was detected on various parts of the root [53]. Enteric bacteria penetrate also through natural openings. The leaf surface has numerous stomata that can provide entry into the substomal cavity. Stomata have been identified as natural openings that permit the entry of bacterial cells into lettuce [49, 111]. *E. coli* O157:H7 inoculated onto cut lettuce pieces was found entrapped within stomata approximately 20 μm beneath the leaf surface [111]. Inoculation of cilantro plants with a green fluorescent protein (gfp)-labelled outbreak strain of *Salmonella* Thompson and incubation under warm and humid conditions revealed that this pathogen formed microcolonies preferentially in the vein area of the leaf [36]. Higher bacterial prevalence in the vein area was reported also for *E. coli* O157:H7 on lettuce leaves [104]. The enhanced wetting ability of this part of the leaf may allow for easier contact of inoculum cells with the plant or enhanced attachment. Also, it is possible that specific factors on the surfaces of leaf veins, where nutrients are more abundant, facilitate the attachment of bacteria [100]. Lettuce has been used as a model vegetable to demonstrate the internalization of human pathogens primarily because of its commercial importance. Watchel *et al*. cultivated lettuce within soil microcosms irrigated with water containing different inoculation levels (10^2, 10^4, 10^6, 10^8 cfu/ml) of *E. coli* O157:H7 [53]. At the highest dose level, but not at lower cell densities, *E. coli* was associated with the roots of plants by day 3. *E. coli* numbers at the lower doses progressively increased over the cultivation period to reach levels of 10^3–10^4 on roots. Although the majority of *E. coli* was recovered from the roots, lower numbers (approximately 10^2 cfu/g) were associated with hypocotyls and cotyledons of 10-day-old lettuce plants. Using confocal laser microscopy, *E. coli* was observed within the vascular system of the hypocotyls. Soloman *et al*. propagated lettuce seedlings in soil inoculated with gfp-labelled *E. coli* O157:H7 (at cell densities of 10^4, 10^6 or 10^8 cfu/g) [54]. The authors reported that *E. coli* O157:H7 could be recovered from the internal tissue of seedlings inoculated with the highest cell density, but not at the lower inoculation levels applied.

Shredded Lettuce

Prepackaged precut salad mixes are attractive to consumers because of the ease of preparation and to producers because the modified atmosphere of the packaging dramatically increases product shelf life [1]. In an *in vitro* study, cut lettuce leaves immersed in a suspension of *E. coli* O157:H7 were exposed to fluorescent antibody specific to this pathogen and observed by confocal microscopy. *E. coli* O157:H7 attached predominantly to the cut edges of leaves; fewer cells attached to the intact cuticle region of leaves but were observed near stomata, on trichomes, and on veins [111]. The preferential attachment of *L. monocytogenes* and *E. coli* O15:H7 to the cut edges of lettuce leaves may be related to the interactions of lectins on the surfaces of these pathogens with specific carbohydrates that leak from the damaged plant tissue [112]. The fact that salad vegetables are usually eaten raw is compounded by the increase in popularity of prepackaged salad products that are unlikely to be washed by the consumer [6]. Singh *et al*. have shown that washing with water or using different sanitizers is not effective in eliminating *E. coli* O157:H7 contamination of shredded lettuce leaves [113].

Organic Versus Conventionally Grown Produce

Organic produce has become increasingly popular because of the belief that vegetables grown without the application of chemical pesticides and synthetic fertilizers are of higher quality and healthier than those grown with modern agronomic practices. Organic crops may be fertilized with natural fertilizer such as manure. Because organic crops may be in contact with animal faeces, one might think organics have a higher likelihood of being contaminated with pathogens [1]. Himathongkham *et al.* studied the survival of *E. coli* O157:H7 and *Salmonella* Typhimurium in cow manure and cow manure slurry [114]. An exponential linear destruction was observed for *E. coli* O157:H7 and *Salmonella* Typhimurium in cattle manure and manure slurry stored at 42 or 37°C. To achieve a $\geq 10^5$-fold reduction of *E. coli* O157:H7 and *Salmonella* Typhimurium, the manure should be stored for 105 days at 4°C or 45 days at 37°C [114]. It remains uncertain what the inactivation mechanism is in manure. There is no evidence to suggest that organic produce carries a higher risk of being contaminated with human pathogens than does conventionally grown produce [4]. However, the presence of a larger variety of natural microbes in organically cultivated soil can lead to a better defence capacity against pathogens [115]. When colonizing plants, human pathogens come into contact with native bacteria and must successfully compete with a highly diverse microflora to establish themselves [116]. Van Elsas *et al.* demonstrated that the complexity of the soil microflora clearly influences the survival and persistence of a pathogen in soil [117]. Furthermore, it has been shown that competition already occurs in manure, where cattle diet influences the persistence of human pathogens [118]. In a recent study, the prevalence of STEC and distribution of Shiga toxin encoding genes was investigated in manures collected from 16 organic and in 9 low-input conventional Dutch dairy farms [119]. The abundance of pathogenic *E. coli* was higher in organic farms, although differences were not statistically significant. However, more conventional farms were positive for all Shiga toxin-producing virulence genes, which form a potentially highly virulent combination. Semenov *et al*. suggested that microbial communities in organically managed soil have a more predictable behaviour and are more stable because of a relatively high microbial diversity and oligotrophic conditions with respect to easily available substrate [120]. Consequently, organic soils in that study showed a higher resilience to incremental rates of manure infected with *E. coli* O157:H7. The ecology of the pathogens *E. coli* O157:H7 and *Salmonella* in the soil–plant environment has been reviewed by Franz and van Bruggen [121].

Potential interactions between pathogens and antagonists vary according to which pathogens and host plants are being studied and which bacteria are used as antagonists. In most cases, the co-inoculation of seeds with naturally occurring rhizobacteria does not reduce the persistence or number of enteric pathogens on plants, with the exception of *Enterobacter cloacae*, which reduced the numbers of *E. coli* O157:H7 and *L. monocytogenes* on lettuce [122]. Another *Enterobacter*, *E. asburiae*, was also observed to be antagonistic toward *E. coli* O157:H7 on plants, lowering colonization on lettuce 20- to 30-fold [123]. This effect is most likely to be due to competition for carbon and nitrogen sources, since *E. asburiae* and *E. coli* both utilize over 20 of the carbon sources present in plant exudates [123]. The level of indigenous microflora in foods is generally expected to be higher than that of a pathogen on a food. However, a study of organically and conventionally grown spring mixes (which may contain radicchio, beet tops, baby red romaine and arugula) showed no statistical differences in populations of microorganisms [124]. The growth and metabolic activity of the indigenous flora have a large influence on the intrinsic environment of the food [125]. Lactic acid bacteria that are naturally occurring on vegetables can inhibit pathogen growth by lowering pH, producing antimicrobial compounds, competing for nutrients and producing H_2O_2 [126]. Mukherjee *et al*. analysed vegetables produced by organic and conventional farmers to determine the coliform count and the prevalence of *E. coli* O157:H7 and *Salmonella* [127]. The percentages of *E. coli*-positive samples in conventional and organic produce were 1.6 and 9.7%, respectively. However, the *E. coli* prevalence in certified organic produce was 4.3%, a level not statistically different from that in conventional samples. Organic lettuce had the largest prevalence of *E. coli* (22.4%). Organic samples from farms that used manure or compost aged less than 12 months had a prevalence of *E. coli* 19 times greater than that of farms that used older materials. Serotype O157:H7 was not detected in any produce samples, but *Salmonella* was isolated from one organic head of lettuce and one organic green pepper. In Europe, the objective of the European PathOrganic research project is to detect and minimize potential risks due to bacterial load in organic vegetable products (PathOrganic in the CORE Organic Program: http://pathorganic.coreportal.org/). In Austria, Switzerland, Germany, Sweden and Denmark, vegetable farms are currently screened systematically for possible pathogen (*E. coli, Salmonella*, *L. monocytogenes*, *Campylobacter* and *Staphylococcus aureus*) load of their vegetable produce. Thereby any pathogenic agents in vegetable plants, the soil, the compost and fertilizers will be detected. The aim of this strategy is to define the so-called 'critical checkpoints' in the chain of production. These identify which essential controls and measures can be implemented to eliminate pathogen load on vegetable produce as much as possible.

Despite its relative infrequency, food-borne outbreaks associated with vegetable salads have become a food safety issue [1]. Besides increased utilization of prepackaged precut salad products and increased propagation of organic farming, multiple other factors may be involved in the increase of food-borne illness associated with fresh vegetables: increased sales and consumption of minimally processed vegetables, increased use of automated equipment for processing, wider distribution of products resulting in increased storage times, increased popularity of fresh salad consumption, large volumes of vegetables with centralized preparation, an increased number of meals eaten outside the home, the emergence of pathogens with lower infection doses and demographic changes causing a increased size of at-risk population [16].

Conclusion

Because consumers have developed a better understanding of the health benefits associated with eating fresh produce, salads are increasingly being served in the home, in restaurants, and as ready-to-eat prepackaged foods. The associated increase in food-borne diseases associated with salads must be seen in view of these benefits. Bacterial pathogens can survive on growing plants over prolonged periods of time under field conditions. In addition, the ability of human pathogens to infiltrate plant tissues has recently been recognized. Prepackaged, precut lettuce (i.e. damaged plant tissue) may pose an emerging source of food-borne illness. Surprisingly, there are no clear differences in pathogen contamination reported between organically grown produce and conventionally grown produce. Present intervention strategies are inadequate to ensure by 100% that salads are not contaminated with food-borne bacterial pathogens. The relative infrequency of outbreaks associated with preharvest contamination with *Shigella*, an organism with humans as its major reservoir, and the relative frequency of those due to *Salmonella* or STEC organisms with animals as their major reservoirs, underlines the role of domestic and wild animals as dominant sources of preharvest contamination of vegetable salads. In the overwhelming majority of reported outbreaks the contamination of salads has neither been preharvest nor originated at the processing plants, but rather the source could be traced to symptomatic or asymptomatic food handlers. Food handling and food preparation are crucial processes that require appropriate hygienic measures, especially washing hands with soap after defecation and preventing food handlers with gastroenteritis from working. Finally, to assess the importance of salads contaminated before harvest, thorough epidemiological and microbiological investigation of all food-borne outbreaks is essential.

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