Aquavalens Project

"Protecting the health of Europeans by improving methods for the detection of pathogens in drinking water and water used in food preparation."

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List of risk factors associated with each of the industries examined

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Implications of the results of Deliverable Report 12.1

Implications of the results for the Work Package (WP 12)

This deliverable clearly identifies the food products most related to outbreaks of sickness related to microbial contamination. The matrix also identifies the top 10 microorganisms associated with food related outbreaks. The source of contamination of food stuffs from outbreaks is also highlighted. This will form the basis of the sampling plan D12.2.

Implications of the results for this Cluster 3

The matrix highlights the ready to eat food stuffs and contamination sources that should be targeted for site selection. The matrix highlights the pathogens to be targeted for cluster 1 and 2. The matrix will be used for the site selection and development of the sampling plan (D12.2)

Implications of the results for the whole project

Disease outbreaks are more associated with microbial contamination of ready to eat foods than bottled waters. The pathogens most closely associated with these food related outbreaks are clearly identified and the tools for most of these have been developed in cluster 1.

Indicate key external stakeholders interested in the results of Deliverable Report 12.1

The results will be informative for producers, shops and consumers of ready to eat foods. The European and national food safety authorities will also be interested in the matrix from a risk perspective.

Which internal partners should your deliverable be sent to?

All the participants in Cluster 3.

Risk Matrix for Pathogen Occurrence on Ready-to-Eat Foods and Bottled Water

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Introduction

Fresh fruits and vegetables are an important component of a healthy and balanced diet. Their consumption is increasing worldwide for several decades [1], as a result of the promotion of healthier lifestyles. However, the shift towards a healthier diet increases the risk of disease related to these Ready-to-Eat (RTE) foods. Indeed, these foodstuffs do not undergo any processing step prior to human consumption to ensure the effective removal of contaminants such as chemical residues or pathogenic microorganisms. Furthermore, their increased consumption, allied to the globalization and large scale of production of RTE foodstuffs [2], implicates longer distribution times and greater distribution distances, which entails more complexities in terms of food safety management [3]. Some RTE crops are washed prior to consumption and this also represents a potential contamination route of RTE foods. Consequently, the number of RTE food related disease outbreaks is rising in recent years [2, 4-6]. In Europe, foodstuffs of non-animal origin were responsible for 5% of the total foodborne disease outbreaks in 2007 and this has increased to 30% in 2011 [7]. Bottled water is an important water supply for humans. Between 1997 and 2004 the consumption of bottled water increased by 60% in north America and Europe and by 200% in South America and Asia [8, 9].

Therefore, investigating the source of microbial contamination of RTE foods and bottled water and the evaluation of reliable techniques for detecting contamination are important considerations for food safety. This risk matrix aims to look at important factors involved in the microbial contamination of foodstuffs, focusing on identifying the most relevant RTE foodstuffs linked to microbial disease outbreaks, the most relevant pathogenic microorganisms involved in said outbreaks, and also the pathways in which the selected microorganisms contaminate foodstuffs (with a particular focus on the role of water in the contamination process). The gathered data will be used to identify the foods most susceptible to contamination and the most frequent microorganisms associated with contamination globally.

1. Contamination Sources

Microbial contamination of fresh produce and fruit has been widely reviewed in the literature [10, 11]. When considering the farm-to-fork chain, microbial contamination of fresh produce can occur at multiple steps of the process [12]. Contamination can take place during the cultivation of fresh produce, at harvest, during preparation/washing, transport to shops, and even at the final step in the consumers' kitchen (Fig.1).

Contamination during cultivation can be caused by soil, untreated animal manure/human biosolids, either directly applied as a fertilizer [13, 14] or due to runoff events caused by extreme weather events (heavy rains) [15], utilization of contaminated water or untreated wastewater for irrigation, preparation of pesticides for application on RTE crops and washing of tools and equipment [16-18]. Contaminated seeds are also worth noting, as they pose a serious issue concerning the consumption of sprouts [19-21].

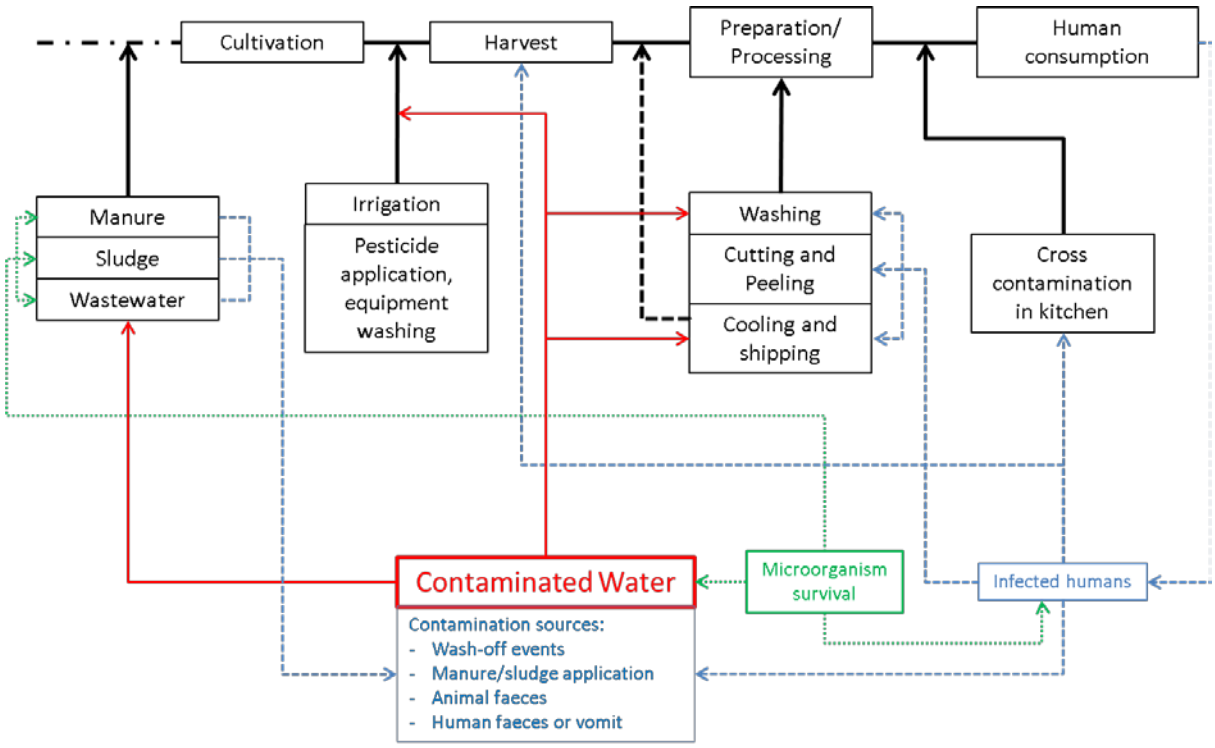

Figure 1: Fresh produce - contamination pathways in the Farm-to-fork chain

Focusing on the post-crop phase of the farm-to-fork chain, manipulation of foodstuffs by infected pickers during harvest [22, 23], as well as poor hygienic conditions such as absence of toilets and hand-washing facilities, or manipulation of foodstuffs without gloves [24] are potential risk factors regarding RTE food related outbreaks. Contact of foodstuffs with soil contaminated with manure, namely dropped fruits, such as apples and oranges [25-27], can also be a source of concern. Washing

of fresh produce may prove to be non-effective to remove microorganisms, as they may remain attached to plant surfaces [28] or become internalized in the edible parts of the plant [29], and thus, not accessible for efficient removal. In addition, the quality of water used to wash fresh produce can be a source of microbial contamination [30, 31], as washing water is often reutilized, which may promote the spread of pathogenic microorganisms between clean and contaminated plants [32-35]. Also, water used for washing typically contains high content of organic matter, resulting from the accumulation of soil, debris and cut-produce tissue fluids from RTE crops [35, 36], and this may increase pathogen survival [36]. Some soft fruits, such as raspberries and blueberries, are not suitable for efficient washing, due to loss of commercially important properties, namely shape and visual aspect. Cutting and peeling of foodstuffs are potentially hazardous due to microorganisms present in the tools and surfaces in contact with the food being prepared [37, 38]. The quality of water used for spraying (cooling) or making ice (shipping) is also a risk to consider, namely when the water source is non-chlorinated, which enables pathogenic microorganisms to contaminate foodstuffs and cause disease [39, 40]. Finally, manipulation of RTE foodstuffs in the kitchen of the final consumer should be considered as a risk, due to possible cross contamination with other pathogenic microorganism sources, such as raw meats and eggs [41-43].

2. Microbial Contamination Risk Matrix

The number of fresh produce related outbreaks has been increasing over the years [2, 4-6]. In order to prepare a risk matrix on microbial contamination of RTE foods, reports of RTE food related outbreaks were assessed, focusing on the causative microorganisms, the foodstuff(s) involved, and the contamination pathways, when available. In total 453 outbreaks were considered, from 1922 to 2013. These 453 outbreaks resulted in 48,131 reported disease cases, 117 reported deaths, and implicated 13 different microorganisms, 48 foodstuffs and 10 main contamination pathways.

2.1. Foodstuffs susceptible to microbial contamination

RTE foodstuffs are of particular importance as a source of microbial disease outbreaks, as they do not undergo any preparation step that effectively removes pathogenic microorganisms prior to human consumption. In addition, the notable increase in their consumption favours the occurrence of microbial disease outbreaks.

From the data collected for this study, leafy-green vegetables - lettuce, salad, basil, parsley, spinach, clover, cilantro, cress, watercress, and celery - are responsible for the most reported cases (27.9%) and outbreaks (57.5%), while sprouted plants – alfalfa, fenugreek, mung beans, anise seeds, bean

sprouts and fennel seeds - account for the highest fraction of reported deaths (39.4%). Table 1 presents the 10 most relevant foodstuffs implicated in reported outbreaks, cases and deaths, and there is considerable consistency between these rankings. Thus these crops should be targeted for sampling and microbial quality testing across the EU.

Table 1: Most relevant foodstuffs implicated in RTE food related outbreaks

	No. of cases	**No. of deaths**	**No. of outbreaks**
1	Radish (10126)	Fenugreek (50)	Salad (182)
2	Salad (7780)	Cantaloupe (32)	Lettuce (74)
3	Raspberries (4584)	Coleslaw (17)	Fruit[1] (35)
4	Fenugreek (4018)	Tomatoes (8)	Alfalfa (33)
5	Lettuce (3713)	Lettuce (5)	Raspberries (21)
6	Alfalfa (3116)	Celery (5)	Apple juice (12)
7	Fruit[1] (2030)	Onions (3)	Mung beans (12)
8	Celery (1481)	Orange juice (2)	Tomatoes (10)
9	Berries[1] (1409)	Apple juice (2)	Pepper (8)
10	Orange juice (1235)	Pepper (2)	Orange juice (7)
Total	39492	126	394

Radish is the foodstuff responsible for the highest case count. It is a root vegetable, commonly consumed raw in salads. As the edible part of this vegetable is in contact with the soil, irrigation water, as well as the presence of contaminated manure or animal faeces, may play a role in possible contamination with pathogenic microorganisms [44-46]. The sprouting of radish seeds should be also considered a critical step, as it is normally done in conditions that favour microbial growth [47].

Leafy-green vegetables are responsible for a high number of reported cases, particularly salad [23, 48], lettuce [13, 43, 49], and celery [50-52]. Leafy-green vegetables are susceptible to contamination by contaminated irrigation water [16, 17], as the edible part of the plants is exposed to pathogenic microorganisms present in water. The plant morphology may also be important – as an example, water can be retained on lettuce leaves, which increases the potential for contamination. Crop fertilization with contaminated manure [13] is reported as a cause of microbial contamination of leafy-green vegetables. The preparation of packaged salads is also a point of concern, as

[1] The foodstuffs designated as “fruit” and “berries” refer to all the cases in which there is no available information on the specific fruit or berry, respectively, involved in the outbreak.

contamination can occur during the preparation and packaging (infected handlers) [53], as well as during washing (contaminated water, or cross contamination between plants) [30].

Fruits and fruit juices are shown as relevant foodstuffs for RTE food related disease outbreaks. Soft fruits particularly, namely raspberries or other berries [24, 54-57], constitute a contamination risk since they are not suitable for intensive washing due to loss of important properties and commercial value. They are normally handpicked and packaged on site, being later exported to consumer countries. Hygiene in picking and packaging processes [24], as well as manipulation by infected handlers [22, 58], are reported causes of microbial contamination. Other fruits and fruit juices are linked with microbial contamination mainly caused by contaminated food handlers [59-61], and usage of unwashed dropped fruits on manure contaminated soil [27, 62-64].

Edible sprouts, such as alfalfa [65-67], fenugreek [68] and mung beans [69-71], are also a serious concern in RTE food safety. Sprout seeds are soaked in water, and sprouted in warm and moist conditions, highly favourable for microbial growth [47]. The water used for sprouting may act as a contamination source, and also facilitate cross contamination between clean and contaminated sprouts [47]. Most importantly, contaminated seeds represent the main contamination factor for sprouts [66, 67, 71-73], as microorganisms present on the seeds may become internalized during the germination process [74]. Seeds may become contaminated due to fertilization with contaminated manure, or irrigation with contaminated water [19, 75].

To test the efficacy of the microbial detection methods should target root vegetables (radish), salad (lettuce), and soft fruit (raspberries), both with and without irrigation across a range of EU countries. In addition sprouting beans should also be targeted.

2.2. Microorganisms

Bacteria, virus and parasites all play a relevant part in RTE food related outbreaks. Table 2 presents the most relevant pathogenic microorganisms, concerning the number of reported cases, deaths, and outbreaks considered in the preparation of this risk matrix.

Table 2: Most relevant microorganisms implicated in RTE food related outbreaks

	No. of cases	**No. of deaths**	**No. of outbreaks**
1	*Escherichia coli* (15298)	*Escherichia coli* (52)	Norovirus (243)
2	Norovirus (13069)	*Listeria* (52)	*Salmonella* (95)
3	*Salmonella* (8689)	*Salmonella* (10)	*Escherichia coli* (31)
4	Hepatitis A (5030)	Hepatitis A (3)	Hepatitis A (30)
5	*Cyclospora* (3566)		*Cyclospora* (16)
6	*Shigella* (1037)		*Shigella* (10)
7	*Campylobacter* (344)		*Bacillus* (6)
8	*Cryptosporidium* (338)		*Listeria* (5)
9	*Bacillus* (266)		*Giardia* (5)
10	*Listeria* (239)		*Cryptosporidium* (4)
Total	47876	117	445

Escherichia coli is responsible for the highest number of reported cases. Non-pathogenic strains of *E. coli* are common inhabitants of the gastrointestinal tract of humans and animals. However, some strains are virulent and capable of causing disease to the gastrointestinal, urinary and nervous systems. Two specific strains were responsible for the outbreaks recorded in this study: *E. coli* O157:H7 and *E. coli* O104. Both strains are Shiga toxin producers, causing gastrointestinal illness, and also capable of causing hemolityc uremic syndrome (HUS). Studies on *E. coli* O157:H7 report survival times ranging from 1 day to 21 months in manure in different experimental designs, such as laboratory settings either at different constant temperatures [76-79] or variable conditions [80], as well as field studies [79, 81-84]. Contamination of crops may occur via uptake by the root system, splash dispersal from the soil surface, or by irrigation water [29]. Microbial internalization into edible portions of RTE foodstuffs, such as tomato, radish sprouts, bean sprouts and lettuce, reported in the literature [29]. Reported cases implicated manure [13], or sprouts whose seeds were contaminated [68, 85].

Norovirus (NoV) and Hepatitis A (HAV) are responsible for a high amount of reported cases [24, 57, 86, 87]. NoV is linked to the majority of reported outbreaks (53.6%). These viruses are excreted in

high number in human faeces, and are transmitted via the faecal-oral route. They are present in several groundwater resources [88-90], which can be explained by their small particle size, ranging from 27 to 35 nm [91, 92], allowing them to bypass the attenuation effect (filtration) of soil layers. NoV is reported to survive in different water sources (groundwater, surface water and tap water) for as much as 5 weeks [93, 94], as well as in different fruits and vegetables (berries and leafy green vegetables) for as much as 90 days in refrigerated conditions [95], or 10 days in non-refrigerated conditions [96, 97]. Also, Murine Norovirus (MNV), a surrogate for NoV, is reported to remain infective in soil after 60 days [98]. HAV is reported to survive for over 90 days in leafy green herbs and berries [95], and survive for 21 days in oysters in a depuration tank [99]. HAV is also described to maintain infectivity in soil after 30 days [98]. Studies with MNV demonstrated internalization of viral particles in lettuce in hydroponic culture, and also viral particle retention in the soil [100]. It has also been demonstrated that MNV and HAV can be internalized by spinach and green onions, both in static hydroponic culture and nutrient film technique culture [98]. Leafy green vegetables [23, 101] and soft fruits [57, 87, 102] are the main foodstuffs implicated in the transmission of NoV, as shown by this risk matrix. HAV is mainly transmitted by soft fruits [54, 58, 103].

Salmonella genus comprises over 2700 serotypes, from which 200 are commonly associated with human illness, the most common being *S. typhimurium* and *S. enteriditis*. However, and as stated in the literature [29], data compiled in the present study revealed the contamination of RTE foodstuffs by other, less common serotypes of *Salmonella*: 42 different serotypes were reported as responsible for RTE food related disease outbreaks, with *S. javiana* [31, 104, 105], *S. saintpaul* [70, 106], *S. rubislaw* [106] and *S. montevideo* [31, 107, 108] accounting for the majority of the outbreaks considered in this study. *Salmonella* is widespread in the environment and normally found in farm effluents and any material subjected to faecal contamination [109, 110]. It is reported to survive up to 405 days in manure amended soil and/or manure, under different conditions, either in laboratory environment experimental designs [77, 111, 112], or field settings [81, 82, 113-115]. RTE foodstuff contamination and *Salmonella* internalization patterns are similar to those reported for *E. coli* [29]. The majority of reported *Salmonella* cases are caused by RTE sprouts whose seeds were contaminated [67, 70, 116].

Cyclospora and *Crysptosporidium* are the two human protozoan pathogens relevant in the preparation of this risk matrix, as they are responsible for a notable amount of reported cases, with 3566 and 338 reported cases, respectively (Table 2). These protozoa require a suitable host for replication, but they can survive for significant amounts of time outside a host, as they produce, in their lifecycle, environmentally resistant cysts, or oocysts. These environmentally resistant forms

are, in fact, responsible for the transmission of the parasites between hosts. They are excreted in the faeces of an infected host (either human or animal), and then are ingested by other individuals. Contamination mainly occurs via contact of contaminated faeces with plants directly [64, 117], or by water that has been contaminated with infected faeces [18, 118].

Shigella, Campylobacter, Bacillus and *Listeria* are the other bacterial genera considered relevant to RTE food related outbreaks. *Shigella* is a pathogenic bacteria closely related to *E. coli* and *Salmonella* [119]. *Shigella* has been shown to survive in water at 4°C with no decline in population after 26 days [120], and survive in different fruits and vegetables for as much as 14 days in refrigerated conditions [120-122]. It has also been demonstrated that *Shigella* is capable of growth in different fruits and vegetables, when kept at room temperature [120, 122, 123]. Finally, survival under vacuum or modified atmosphere packaging was also demonstrated [124]. These characteristics make *Shigella* a relevant pathogen in RTE food related disease. Cases reported in this study include contamination via infected handlers [53] and water used for irrigation and refrigeration [16, 17, 40]. *Campylobacter* is a causative agent of acute bacterial gastroenteritis. Transmission of the microorganism mainly occurs via handling of livestock (particularly poultry) [125]. Events of cross contamination of RTE foodstuffs with contaminated livestock are reported in the preparation of this matrix [43, 48]. *Bacillus* is reported in this study only in association with spices [126, 127], which have potential to cause outbreaks as their application is not limited to a particular dish or meal, and takes place normally after the food is cooked. Reported cases were caused by *B. cereus*, *B. pumilus* and *B. subtilis* [126, 127]. This genus is widely distributed in soil and plant material. It is also characterized by forming heat, radiation, dehydration and disinfection resistant endospores that may attach to equipment and processing surfaces [128, 129]. *Listeria* is the pathogenic microorganism with the highest mortality per case (21.7%) reported in this study [51, 52, 130-132]. All the reported cases were caused by *L. monocytogenes*. This species is naturally occurring in terrestrial environment, fresh and salt water, livestock manure, decaying plant materials and many raw foods associated with these environments [133, 134]. *L. monocytogenes* is able to grow and multiply during refrigeration, as well as survive at relatively low water activity [133], which makes it a pathogen of special concern to RTE foodstuffs. Reported cases in this study include contamination with manure [51, 52], and at the manufacturing of foodstuffs [131, 132].

2.3. Contamination Pathways

For the majority (76%) of the reported outbreaks included in this study, the specific contamination source was not determined [135-139]. Table 3 details the known contamination sources on the reported cases.

Table 3: Contamination sources of RTE food related outbreaks

Contamination source	No. of outbreaks
Unknown source	333
Contaminated seed	34
Contaminated water	23
Contaminated food handler	16
Contaminated manure	13
Handling environment/surface contamination	6
Contaminated processing facility	4
Cross contamination with raw meets	3
Unrefrigerated food item	3
Damaged/spoiled food item	2
Weather event causing sewage overflow	1

From the outbreaks whose contamination source is known, special relevance should be given to the contamination of seeds [71, 116] and water, to the manipulation of foodstuffs by infected handlers [53, 59, 140], and to the application of contaminated manure [13, 14, 141], as they are responsible for the majority of outbreaks. Focusing specifically on water as the cause for RTE food related disease outbreaks, reported events include contamination of irrigation water by faecal matter [142], and specifically manure [13], as well as contamination of oysters' culture water due to sewage overflow [143]. Usage of non-potable water for irrigation [75] was also a source of concern. Washing foodstuffs with non-potable [144] or non-chlorinated [40] water were also responsible for outbreaks reported in this study. Events of cross-contamination during washing were also reported [30].

Considering specific pathogenic microorganisms, *E. coli* contamination is mainly reported through the application of contaminated manure [13], Hepatitis A is mainly spread by infected handlers [22, 58], while *Salmonella* is mainly transmitted by contaminated seeds [66, 70], and to a lesser extent, contaminated water [31, 104, 108].

3. Bottled Water

By definition of the European Union Law, a natural mineral water is “microbiologically wholesome water, originating from an underground water table or deposit and emerging from a spring tapped at one or more natural or borehole sources”. Natural mineral water must not be disinfected and, therefore, contains an autochthonous microbiota that originates from its source [145]. These microorganisms are of little concern for healthy consumers [146]. Despite this, the presence of human pathogens can occur, indicating a possible contamination at the source, or during the bottling process.

In this study, bottled water was reported to be the origin of 5 outbreaks implicating *E. coli*, *Salmonella*, *Shigella* and *Pseudomonas aeruginosa*. Only one death, caused by *P. aeruginosa* was reported. The *Salmonella* outbreak was caused at a local bottled water producer, due to the existence of a strain-carrier pigeon loft near the production facility [147]. Both *P. aeruginosa* outbreaks were linked to intensive care units, namely bottled water used to prepare milk for baby feeding in a neonatal intensive care unit [148], as well as bottled water used to prepare orally administered medications and oral fluids replacement in an intensive care unit [149].

4. Conclusion

The elaboration of this risk matrix for RTE food related disease outbreaks allowed the formulation of several conclusions regarding key aspects of the subject.

Firstly, regarding the foodstuffs involved in the recorded outbreaks, one can consider three major groups of concern: leafy green vegetables, sprouted plants, and fruits (particularly soft fruits and fruit juices). This study identified radish and tomatoes, salad, lettuce and celery (leafy green vegetables), fenugreek, alfalfa and mung beans (sprouted plants), raspberries and other berries, orange juice and other fruits (fruits and fruit juices) as the foodstuffs linked to the higher risk of microbial disease outbreaks. On the other hand, bottled water as a RTE foodstuff does not represent an important source of microbial contamination, as it is not linked to a relevant number of outbreaks. This selection was based on the number of reported cases, as a measure of social and economic impact on the consumers, as well as the number of reported outbreaks, which highlight the susceptibility of a given foodstuff to microbial contamination. Special attention should be given to sprouted plants, as the sprouting of contaminated seeds is a major issue in terms of health risk. Leafy green vegetables are also a point of concern, due to exposition of the edible part of the plant to irrigation waters, which increases the potential for contamination. Soft fruits, as raspberries and strawberries, are also considered to be foodstuffs of high risk of microbial disease outbreaks, as they are not suitable for efficient washing, and are subjected to manual handling, which increases the risk of microbial contamination.

Focusing on the microorganisms implicated in the RTE food related disease outbreaks, this risk matrix identified the bacteria, viruses and protozoan pathogens linked to the higher risk of disease. *Escherichia coli* strains O157 and O104, several serovars of *Salmonella*, *Shigella sonnei* and *S. flexneri*, *Campylobacter jejuni* and *Listeria monocytogenes* were the pathogenic bacteria flagged as greatest risk of disease outbreaks. Norovirus and Hepatitis A were the viruses associated with higher risk of contamination. Concerning the pathogenic protozoa, *Cyclospora* and *Cryptosporidium* were chosen as the most relevant microorganisms in terms of disease risk. This selection is based on the number of reported cases, deaths and outbreaks caused by each microbial pathogen.

Finally, setting the focus on the contamination pathways involved in the microbial contamination of RTE foodstuffs, the majority of the reported cases has no associated contamination source. Considering only the reported outbreaks whose source of contamination is known, the contamination of seeds, the usage of contaminated water (for irrigation, application of pesticides and as an enabler of cross contamination during washing of foodstuffs), and the manipulation of foodstuffs by previously infected handlers play the greatest role in the transmission of

microorganisms. Special focus should be given to the role of water as a vehicle for transmission of microorganisms, specifically as a vehicle of cross contamination during sprouting of seeds of sprouted plants, such as alfalfa, fenugreek or mung beans, as well as cross contamination between clean and contaminated plants during washing of fresh produce.

Table 4: Risk matrix identifying a range of RTE foods/water/juice to target under a range of farm practices, processing methods, and transport/storage, and a list of the target microorganisms to test across the risk matrix.

Food Stuff	**Farm practice**	**Processing**	**Transport/storage**	**Target microorganisms**
Radish	± irrigation	± washing	shop shelf	*Escherichia coli*
Lettuce	± irrigation	± washing	shop shelf	Norovirus
Raspberries	± irrigation		shop shelf	*Salmonella*
Bean sprouts	± seeds	± washing?? ± sprouting	shop shelf	Hepatitis A
Bottled water			shop shelf	*Cyclospora*
Orange juice		± washing??	shop shelf	*Shigella*
				Campylobacter
				Cryptosporidium
				Bacillus
				Listeria

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