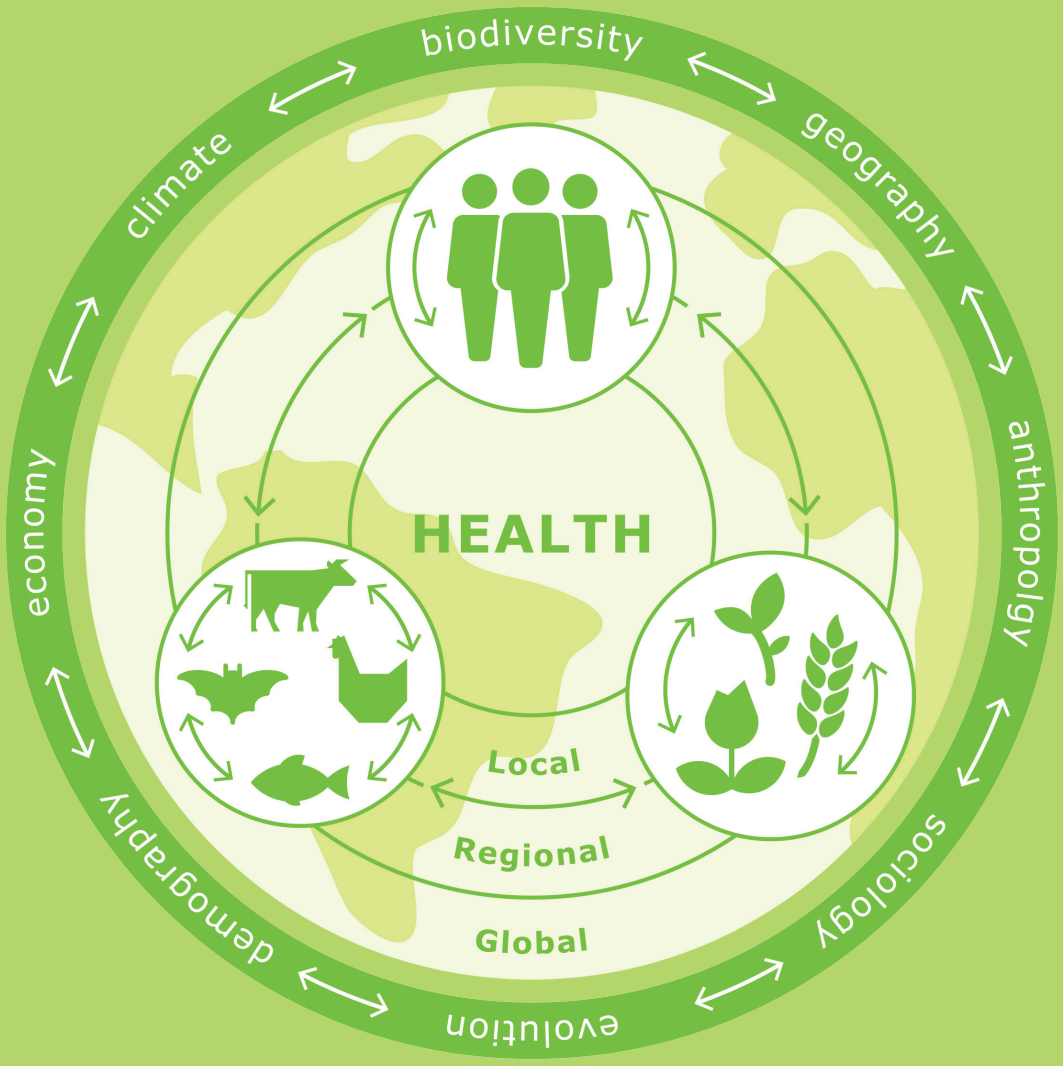
Towards microbial safety of fresh vegetables in Rwanda



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Towards microbial safety of fresh vegetables in Rwanda

James Noah Ssemanda

Thesis

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1

General introduction and outline of the thesis

Foodborne illness

Foodborne illnesses originate from ingesting contaminated food. Acute symptoms of foodborne illnesses include mild and self-limiting watery diarrhea, vomiting, headache, nausea, abdominal pain and severe symptoms like bloody diarrhea, paralysis and abortion (1). In long term, foodborne illnesses can also include reactive arthritis, Guillain Barré Syndrome and hemolytic uremic syndrome (HUS) (2, 3) and in some cases, cancer (4). Foodborne illnesses are caused by bacteria, parasites, viruses, toxins, metals, and prions (5). This study focused on bacterial agents and Figure 1.1. shows examples of the pathways through which these agents can cause foodborne illnesses.

Historically, foodborne illnesses can be traced back to ancient time (6). In their study, doctors at Maryland University (US) in 1998 postulated that "Alexander the Great" may have died of typhoid fever at Babylon around 323 B.C., though by that time it was considered as poisoning due to rivalry (6, 7). Other notable figures in history that are reported to have succumbed to foodborne illnesses include King Henry I of England year 1135 and US President Zachary Taylor, year 1850 (6). While people continued to suffer from these foodborne illnesses, knowledge about particular pathogens was still limited until the late 19th century (8). Indeed most major foodborne pathogens were only discovered during the last two centuries *i.e.*. *Trichinella spiralis* in 1835, *Salmonella* in 1885, *Staphylococcus aureus* 1914, *Clostridium perfringens* 1945, *Campylobacter jejuni*, *Yersinia enterocolitica*, *Escherichia coli* O157:H7 and *Vibrio cholerae* in period between 1975 to 1985 (8).

From historical to present times, foodborne pathogens continue to cause substantial morbidity and mortality in the world, and do hamper social-economic development (9). Factors highlighted to contribute to this burden of illness include: the lack of implementation of known preventive and control measures, the emerging and re-emerging of foodborne pathogens such as the emergence of microbial antibiotic resistant strains, the increasing potential of spread due to fast tracked globalization, and the surging number of susceptible population (3, 10-14).

While a lot of progress has been made in studying food borne pathogens, known pathogens continue to re-emerge by occupying different niches and or acquire virulence genes while in some cases completely new pathogens emerge (11). On new emerging pathogens, a good example is pathogenic *E. coli.* In the early 1980s focus was on *E. coli* O157: H7 (15) but other serotypes have now also caused severe outbreaks like the *E. coli* O104: H4 in Germany in 2011 (16). Furthermore, the continued use of antibiotic agents such as amoxicillin and tetracycline in human medicine to treat infectious diseases (12, 15) and antibiotics massively used to treat farm animals, has led to the emergence and spread of antimicrobial resistant strains. Although most foodborne illnesses are self-limiting, the use of antibiotics against antibiotic resistant strains has in some cases led to ineffective treatment, and prolonged duration of illness

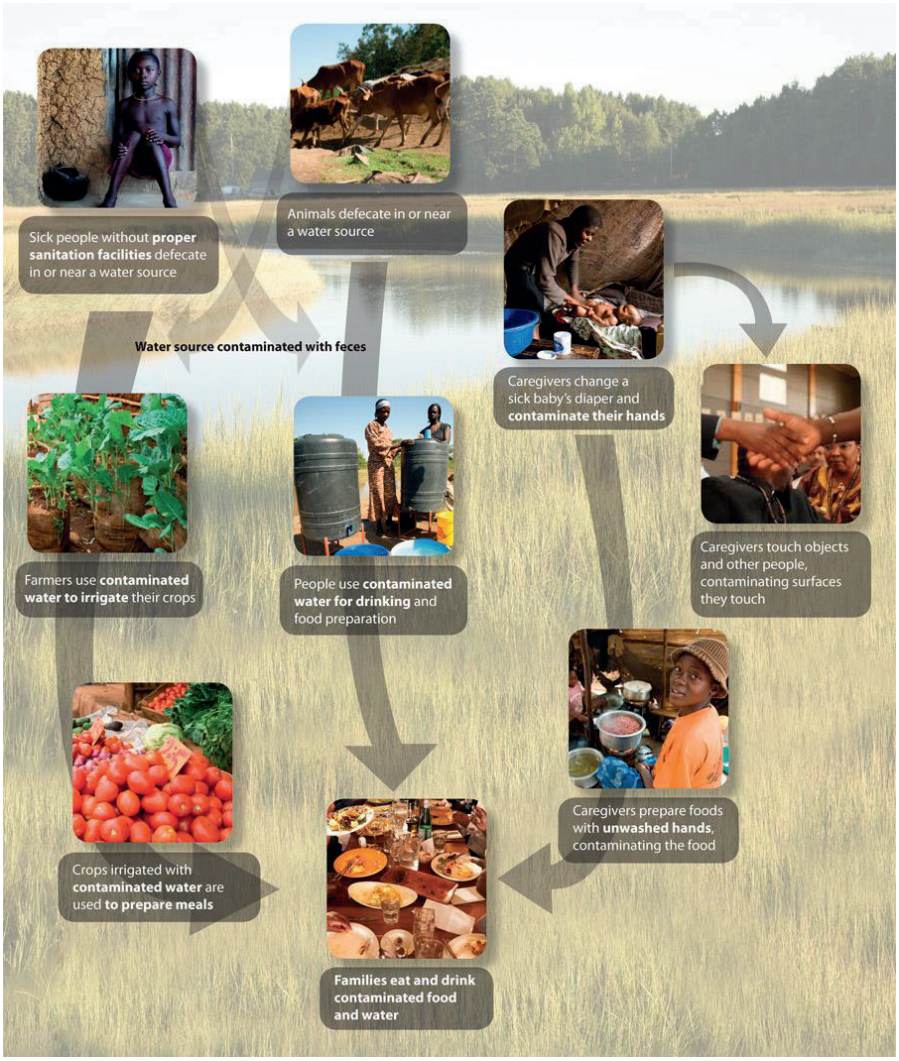

Figure 1.1: Example of pathways to foodborne diseases [source: CDC, 2012 (17)].

and deaths. Researchers from different countries have continued to isolate antibiotic resistant strains and genes in food, water and environment. For example, in the US, *Salmonella* Newport isolates from surface waters were reported to be multidrug resistant (18). In China, *L. monocytogenes* (19), *Salmonella* spp. (20), enterotoxigenic *E. coli* (ETEC) (21) were isolated from ready to eat foods and were also to found to be antibiotic resistant to a number of antibiotic agents. In Africa, antibiotic resistant strains of pathogenic *E. coli* (22, 23), *Salmonella* spp. (24), *Enterococcus* spp. (25) have also been isolated.

Globalization has led to increased chances of spread of human pathogens (11). Food supply chains have been internationalized *ie.* a "Rwandan", "Dutch" or 'French" salad can be prepared from vegetables imported from another country with different levels of implementation of food safety standards. In some cases, outbreaks have spread from one country to another (26-28) or

in many USA states (29-31) due to imported foods and movement of people. It is anticipated that globalization will even be more intensified in future and addressing the associated foodborne illness will require a global approach.

Food illnesses especially from opportunist pathogens like *L. monocytogenes* may in future become very important due to increased size of the population at risk. The recent successes in medicine have led to a rise in the population of immuno-compromised individuals. HIV/AIDS and cancer patients now live longer than before and these individuals are very susceptible to foodborne pathogens. Another predisposing factor is the increasing use of antacid drugs (13) that have been reported to have a protective role to some foodborne pathogens against gastric acids and enhance susceptibility to infection (13, 14).

Consumption of meals away from homes is on the rise and food handlers in food service establishments have continued to be implicated in foodborne outbreaks (32-36) and associated with food borne pathogens (36-38). Although people can also get ill due to contaminated food prepared in households, the contamination of food at food service is more likely to affect a larger number of consumers and to be recognized as a food related outbreak. Moreover it has been argued that in most food service establishments, food handlers are low income earners who may work even when they are ill (12) thereby increasing the chances of food contamination.

Disease surveillance systems

Public health authorities in various countries have developed disease surveillance systems to get a view of the magnitude of the health burden, and eventually to prioritize control measures and interventions. The level of development of these surveillance systems is greatly influenced by availability of economic resources. Surveillance systems are systematically and continuously operated to collect, consolidate and analyze disease epidemiological data so as to generate information for public health action (1, 39). Figure 1.2 summarizes components of an effective surveillance system. The major role of a disease surveillance system is to monitor trends of the target disease or illness over time to identify high-risk groups, locations, seasons, outbreaks, route(s) of transmission and risk factors (40).

The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) of the United Nations are at the fore front of coordinating international efforts to detect, control and prevent foodborne illnesses. Globally the WHO takes a pivotal role in coordinating a “network of networks” which links together existing formal and informal local, regional, national and international networks of laboratories and medical centres (41). In Rwanda, disease surveillance and response duties are mandated to Rwanda biomedical center (RBC) which in turn coordinates with WHO through the Ministry of Health (42). Other examples of these networks are the European centre for disease prevention and control (ECDC) (43), the US Centres for Disease Prevention and Control (CDC, Fig. 1.3) (44), the newly launched Africa Centres for Disease Prevention and Control (Africa CDC) (45). Figure 1.3 shows an example of data sources and data flow through a national notifiable disease surveillance system to WHO (46).

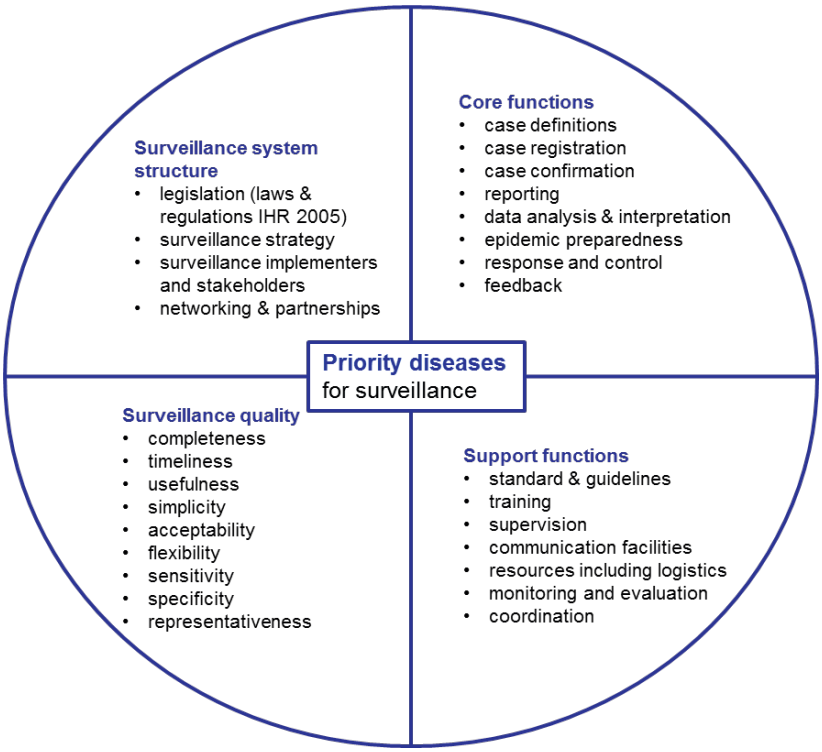

Figure 1.2: Components of surveillance and response systems [source: WHO, 2006 (47)]. IHR= International Health Regulations.

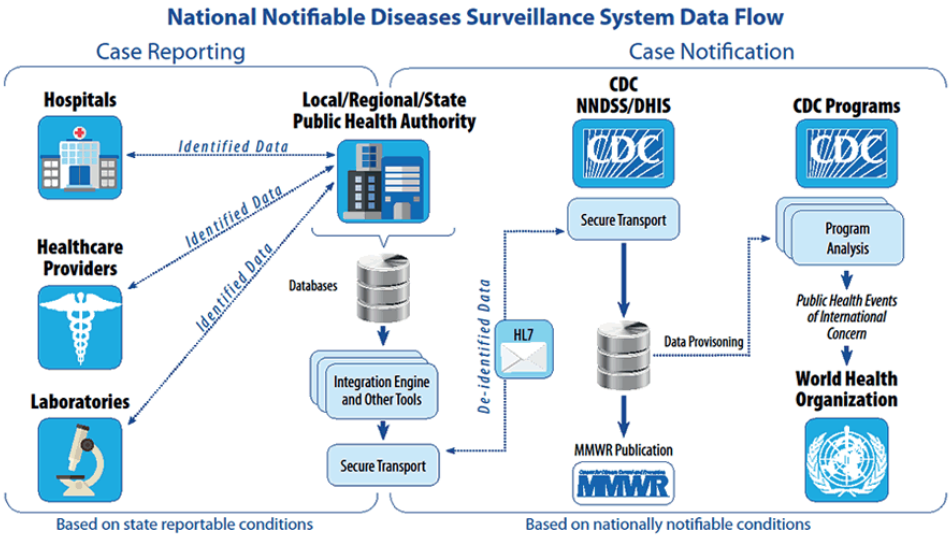

Figure 1.3: Data flow in the United States national notifiable disease surveillance system (NNDSS), [source: CDC, 2016 (46)]: CDC= centres for disease prevention and control, MMWR= Morbidity and mortality weekly report, DHIS= Division of health informatics and surveillance.

Data collection in a disease surveillance system can be classified as active, sentinel or passive surveillance (48). In active surveillance, designated active surveillance staff regularly visit health facilities in person to search for suspected cases, talking to health-care providers and reviewing medical records to identify suspected cases of disease under surveillance. When a case is found, the active surveillance staff then investigate, document clinical and epidemiological data, arrange to send appropriate laboratory specimens and report the information rapidly, in accordance with the national policy. Passive surveillance relies on the cooperation of health-care providers, laboratories, hospitals, health facilities and private practitioners to report the occurrence of a vaccine-preventable disease to a higher administrative level where data once received, are compiled and analyzed to monitor disease patterns and possible outbreaks. Sentinel surveillance involves the deliberate collection of high-quality data from a limited network of carefully selected reporting sites with a high probability of seeing cases of the disease in question, employing good laboratory facilities and experienced well-qualified staff.

In the African region, the WHO has classified surveillance systems into four categories: i) no formal surveillance, ii) syndromic, iii) laboratory based and iv) integrated food chain disease surveillance systems (1). No formal surveillance as a system is typical of countries where there is political instability, recent history of war or extreme poverty to an extent that a public health system is generally not a priority or inexistent. Certain aspects of disease surveillance are often undertaken by external agencies such as nongovernment organizations (NGOs). Syndromic surveillance makes use of data from work and school absenteeism, emergency calls, hospitals, over-the-counter drug sale records, internet searches, and other data sources to detect unusual disease patterns. Laboratory based surveillance systems is made up of various country wide clinical laboratories that test, report or collect specimens (stool or blood) to identify disease causing agents in a location, season or risk population. The generated data or collected specimens are sent to the national reference laboratory. Lastly, in the integrated food chain disease surveillance system, epidemiologic data from animals, food and humans is collected, analyzed and interpreted to inform public health action. In this system, it is possible to attribute the burden of foodborne illnesses to a particular etiological agent and food source. Recently in Rwanda a surveillance system for infectious diseases has been established and Chapter 2 of this thesis explores to what extent the burden of food related illnesses can be estimated.

Methods for estimating burden of foodborne illnesses

Data generated from foodborne surveillance systems have to be computed and reorganized in a way usable by public health policy makers to compare the health of different populations, inform on priorities for health service delivery and planning, and to analyze the cost-effectiveness of health interventions, among others (49, 50). To obtain estimates of the burden of foodborne illnesses from surveillance data, summary measures of population health (SMPH) have been employed (5, 9, 51-54). SMPH are classified into two broad families: health expectancies and health gaps (49, 55). Examples of health expectancies SMPH include the HLE (healthy life expectancy), DFLE (disability-free life expectancy), QALE (quality adjusted life expectancy) (49, 55) while health gaps summary measures include the QALY (quality adjusted life years) and the DALY (disability adjusted life years) (56). Among these SMPH, the DALY

metric has gained wide recognition internationally (57) and is for instance used in the global and national studies of the burden of disease (5, 9, 51-54). DALYs are healthy life years lost, calculated by adding the adjusted number of years lived with disability (YLDs) and the number of years of life lost due to premature mortality (YLLs) (58) where:

YLD =Number of cases × duration till remission or death × disability weight

YLL= Number of deaths × life expectancy at the age of death

DALY = YLD +YLL

Global estimates from the Foodborne Disease Burden Epidemiology Reference Group (FERG) of the WHO in 2010 indicate that 33 million DALYs were due to the studied 31 foodborne hazards (9). Diarrheal disease agents caused nearly 70% of this burden and 31% of the DALYs were from sub-Saharan Africa. According to Havelaar et al. (9), the global burden of foodborne diseases is comparable to major infectious diseases of "the big three"; HIV/AIDS, malaria and tuberculosis. Major etiological agents with individual DALY losses of 1 to 10 million DALYs in the 2010 FERG study were non-typhoidal *Salmonella enterica*, *Salmonella* Typhi, enteropathogenic *E. coli*, *Taenia solium,* Norovirus*, Campylobacter* spp., enterotoxigenic *E. coli, Vibrio cholerae,* Hepatitis A Virus *and Shigella* spp.

Vegetables and foodborne illnesses

Risk managers require information on the relationship between food and foodborne pathogens from farm to consumption in order to develop evidence based food safety policies. In developing these policies, risk managers should be able to attribute cases of foodborne illnesses to the food vehicle(s) or other sources responsible for illness (59) and this activity is referred to as food attribution when food items are involved. Depending on the availability of resources and data, different food attribution approaches can be employed, *i.e.* analysis of outbreak data, case-control studies, microbial subtyping and source tracking methods, expert elicitation, risk assessments (59). Due to the resource demanding nature of food attribution, most studies so far have only been conducted in high income countries *viz.* Netherlands (60), United states (61-63), UK (64), New Zealand (65), Scandinavian countries (66-68). Before conducting a food attribution exercise, it is important that food items are put in categories agreeable to the stakeholders (risk managers, researchers and consumers) (59). In line with the topic of this study, discussions on food attribution focused on the illness due to consumption of vegetables.

In the United States, Painter et al. (63) conducted a food attribution study from foodborne outbreak data from 1998 to 2008 for 17 food commodities (Fig.1.4) and reported that leafy vegetables, dairy products and poultry products caused the highest number of foodborne illnesses, hospitalizations and deaths respectively. In the Netherlands, vegetables were ranked 4th among 11 food items by expert elicitation in 2008 for transmission of 17 common foodborne pathogens (69) while globally, vegetables were estimated to be responsible for 60 to 80 % of illness from foodborne parasites in 2015 (70). From these food attribution studies, it is evident that vegetables are reported among the major food items associated with foodborne illnesses

and indeed studies on reported foodborne outbreaks and on the prevalence of foodborne pathogens associated with vegetables, support this ranking.

From studies on foodborne outbreaks, *viz.* in the United States (63) and the European Union (71), the number of reported foodborne illnesses linked to fresh vegetables has been increasing. In Table 1.1, examples of selected outbreaks linked to fresh vegetables for the last decade are presented. Most notably is the German incident in 2011 in which a total of 4075 reported cases, including 54 deaths were registered due to enterohemorrhagic *E. coli* O104:H4 with sprouts being implicated as the food vehicle (72). Occurrence and pathogenic significance of these pathogens in vegetable/produce-associated outbreaks seem to vary with time and location. Sivapalasingam et al. (73) reported that in 2004 in the US, 60% of the produce-associated outbreaks were caused by bacterial pathogens, of which 48% were caused by *Salmonella* spp. Callejòn et al. in 2015 (71), highlighted that norovirus was the main pathogen responsible for 59% and 53% of these outbreaks in the United States and the European Union respectively, followed by *Salmonella* (18% in the United States and 20% in European Union). It should be noted that most of the outbreaks reported in Table 1.1. are from developed countries. Likely reasons for this overrepresentation are the developed systems and availability of resources to detect and investigate outbreaks up to the level of identifying the food source.

There is an increasing number of studies in which foodborne pathogens have been isolated from fresh vegetables (74-76). In Table. 1.2, the prevalence of foodborne pathogens in fresh vegetables from selected studies around the globe is summarised. Generally, the range of pathogenic microorganisms associated with fresh produce can be classified into three categories: bacteria such as *Salmonella* spp., pathogenic *E. coli*, *L. monocytogenes*), viruses like *Hepatitis A*, norovirus, and parasites *viz. Entamoeba histolytica, Cryptosporidium parvum*, *Cyclospora cayatenensis*.

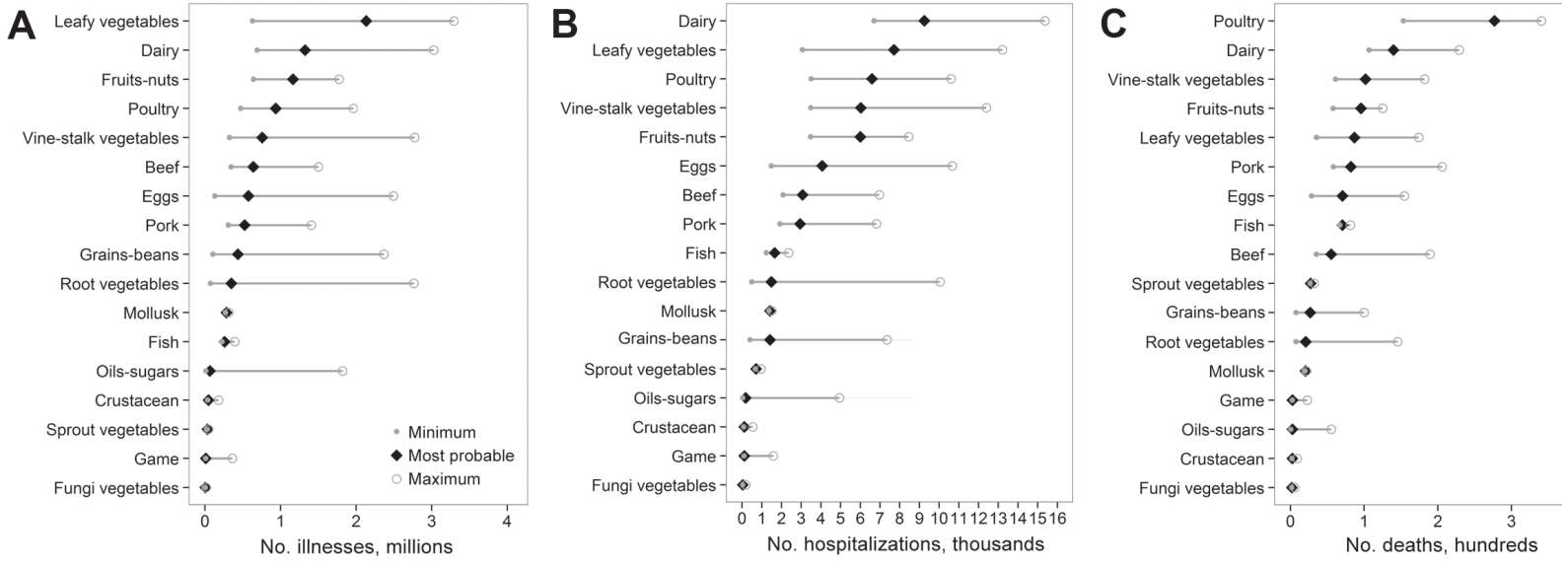

Figure 1.4: Minimum, most probable, and maximum estimates of the annual number of foodborne illnesses (A), hospitalizations (B), and deaths (C) from all etiologies attributed to food commodities, United States, 1998–2008 [source: Painter et al., 2013 (63)].

Table 1.1: Examples of reported foodborne outbreaks associated with fresh vegetables for the last decade in different countries

Year of outbreak	Pathogen	Reported human cases	Vegetable implicated	Country (ies) of outbreak	Reference
2016	*Salmonella* Reading, *Salmonella* Abony	36	Alfalfa sprouts	United States	(77)
	Listeria monocytogenes	19	Packaged salads	United States	(78)
2015	*Salmonella* Poona	907	Cucumber	United States	(79)
2014	*Salmonella* Bovismorbificans	34	Salads and Sprouts	Germany, Switzerland	(80)
	Norovirus	19	Lettuce, Tomato	Australia	(81)
2013	*Cyclospora cayetanensis*	631	Mixed salads, Cilantro	United States	(82)
	Salmonella Saintpaul	84	Cucumber	United States	(83)
2012	*Escherichia coli* O157:H7	33	Spinach, Spring Mix	United States	(84)
	Escherichia coli O26	29	Sprouts	United States	(85)
2011	*Escherichia coli* O104:H4	4,075	Sprouts	Germany	(16, 72)
	Shigella sonnei	46	Fresh basil	Norway	(86)
2010	Hepatitis A	13	Semi-dried tomatoes	Netherlands	(87)
	Escherichia coli O157:H7	26	Shredded lettuce	United States	(88)
2009	*Salmonella* Cubana	20	Onion, Sprouts	Canada	(89)
	Salmonella Saintpaul	235	Alfalfa sprouts	United States	(90)
2008	*Salmonella* Saintpaul	1442	Fresh peppers	United States	(91)
	Escherichia coli O157:H7	3	Iceberg lettuce	Canada	(89)
2007	*Salmonella* Weltevreden	45	Alfalfa sprouts	Norway, Denmark,...	(91)
	Shigella sonnei	175	Raw baby corn	Australia, Denmark	(91)

Table 1.2: Prevalence of foodborne pathogens in fresh vegetables at retail in selected studies around the Globe

Study location	Vegetable type	Pathogens investigated and their % prevalence	Overall prevalence	Reference
Europe				
Italy	Fresh leafy vegetables, fresh-cut" or "ready-to-eat" vegetables	*Salmonella* spp. (0.7%), *Listeria monocytogenes* (0.8%), *Escherichia coli* O157:H7 (0.1%), thermotolerant *Campylobacter* (0.7%), *Yersinia enterocolitica* (0.5%), norovirus (0.04%).	3% (72/2532)	(92)
Germany	Cucumber, carrots, herbs, leaf lettuce, and ready-to-eat mixed salad leaves	*Listeria monocytogenes* (1%), *Salmonella* spp. (0.5%), pathogenic *E. coli* (14%), Shiga toxin–producing *Escherichia coli* (0.5%)	16% (32/200)	(93)
Norway	Lettuce, pre-cut salad, herbs, parsley,	*Escherichia coli* O157: H7 (0%), *Salmonella* spp. (0%), *Listeria monocytogenes* (0.5%), *Yersinia enterocolitica* (3.0%).	4% (21/530)	(94)
Spain	Whole fresh vegetables, sprouts, ready to-eat salads	*Listeria monocytogenes* (0.7%), *Salmonella* spp. (1.3%), *E. coli* O157:H7 (0%), *Yersinia enterocolitica* (0%), thermotolerant *Campylobacter* (0%)	2% (6/279)	(95)
Czech Republic	Whole, fresh cut and frozen vegetables, sprouts	*Listeria monocytogenes* (0.8%), *Salmonella* spp. (0.4 %)	1.2% (3/249)	(96)
The Americas				
Canada	Green onions, tomatoes, leafy herbs, leafy vegetables	*Salmonella* spp. (0.1%), *Escherichia coli* O157: H7 (0%), *Shigella* spp. (0%), *Campylobacter* spp. (0%), *Listeria monocytogenes* (0.3%).	0.4% (80/26323)	(97)
California, USA	basil, yard long beans, bitter squash, okra, squash stems and leaves, cilantro	*Salmonella* spp. (6.6%), *Escherichia coli* O157: H7 (0%)	6.6% (16/242)	(98)
Mexico	Jalapeno and Serrano peppers	Diarrheagenic *E. coli* pathotypes: enterotoxigenic *E. coli* (7%), Shiga toxin-producing *E. coli* (25%)	32% (64/200)	(99)
Brazil	Minimally processed vegetables (lettuce watercress, chard, spinach, cabbage, carrot, arugula, chicory, escarole and tomatoes)	*Salmonella* spp. (0.8%)	0.8 % (4/512)	(100)

Table 1.2 Continues…

Asia				
Iran	Unwashed (Parsley, spearmint, scallion, basil, coriander, dill, cress, leek, tarragon, radish and purslane)	*Ascaris lumbricoides* eggs (14%), *Taeniid* spp. eggs (9%), *Toxocara* spp. eggs (3%), *Trichostrongylus* spp. eggs (4%), *Giardia* spp. cysts (8%).	38% (116/304)	(101)
India	Cabbage, carrot, chili, coriander, cucumber, fenugreek leaves, lettuce, mint leaves, radish, tomatoes, turnip	*Cryptosporidium* oocysts (6%), *Giardia* cysts (5%)	11% (31/284)	(102)
Malaysia	Carrot, potatoes, pennywort, cabbage, parsley, winged beans, yard long beans, Tomato, cucumber	*Listeria monocytogenes* (23%)	23% (69/306)	(103)
China	Mixed vegetables	*Listeria monocytogenes* (2%)	2% (1/58)	(104)
Singapore	Tomatoes, carrots, lettuces, fresh-cut salads and sprouts	*Salmonella* spp. (0%), *Escherichia coli* O157: H7 (0%)	0% (0/83)	(105)
Oceania				
Australia & New Zealand	Lettuce, Salad leaves, tomatoes, cucumber	*Salmonella* spp. (0.1%), *Listeria monocytogenes* (0.1%)	0.2% (7/4483)	(106)
New Zealand	Lettuce, cabbage, carrot, cucumber	*Listeria monocytogenes* (0%)	0% (0/96)	(107)
Africa				
South Africa	Basil	*Salmonella* Typhimurium (0.9%), *Escherichia coli* O157: H7 (0%)	0.9% (4/463)	(108)
Burkina Faso	Lettuce	*Salmonella* spp. (50%)	50% (10/20)	(109)
Egypt	Leafy green vegetables (14 types)	*Escherichia coli* O157: H7 (5.3%)	5.3% (26/483)	(110)
Nigeria	Lettuce, cabbage, garden egg, carrot, cucumber, green peppers	Eggs of *Ascaris* spp. (0.5%), hookworm (1.2%), *Trichuris* spp. (0.5%), *Taenia/Echinococcus* spp. (0.5%), and *Strongyloides stercoralis* (0.6%).	3.5% (40/1130)	(111)
Zambia	Fresh-cut organic mixed vegetables and green beans	*Listeria monocytogenes* (20%), *Salmonella* spp.(23%), *C. perfringens* (0%).	43% (69/160)	(112)

Consumption trends for fresh vegetables

A diet rich in vegetables has been associated with health benefits like reduced risk to cancers and cardiovascular diseases (113). Consumption of vegetables is increasing year by year (114-116) and reports indicate that a large portion of these vegetables are consumed raw (117). Internationally, the WHO has recommended consumption of 400g of fruits and vegetables/ day (118) while nationally in Rwanda, the Ministerial Order (No. 002/2008) of the Rwandan Ministry of Agriculture and Animal Resources stipulates that every family in Rwanda must have a backyard garden of vegetables, termed as "*akarima k'igikoni*". Remarkable growth in trade of fresh vegetables in globalized market settings has been registered (119) and in most countries, agricultural practices to increase the production levels of vegetables have been adopted (120, 121).

Pathways for microbial contamination of fresh vegetables along the supply chain

The prevalence of foodborne pathogens in fresh vegetables varies from one locality to another depending on the conditions and handling practices along the supply chain. Figure 1.5. illustrates the mechanisms and conditions by which fresh vegetables and other foods can become contaminated with pathogenic microorganisms and serve as vehicles of human disease. Food handlers, regulators public health officials and other stake holders have a challenging task to prevent and control microbial contamination of agricultural commodities like fruits and vegetables that are eaten raw (122). This is because pathogens such as *L. monocytogenes* are naturally present in soil, and their presence on fresh vegetables is not rare (123). Other pathogens like *Salmonella* spp., *C. jejuni*, parasites like *E. histolytica*, and viruses (norovirus) can contaminate fresh vegetables through vehicles such as raw or improperly composted manure, irrigation water containing untreated sewage, or contaminated wash water (123, 124). Other probable sources of contamination include contact with domestic and wild animals. Unpasteurized products of animal origin offers another avenue through which pathogens can access vegetables due to cross contamination during food preparation (123). Surfaces, including human hands, which come in contact with whole or cut vegetables, may also become potential points of contamination throughout the "farm to fork" continuum (123).

Generally, to effectively control contamination of vegetables at farm level and along the entire supply chain, it is important to understand the complex interactions between human pathogens and plant structures. Figure 1.6 shows how bacteria can attach and hide in vegetable leaf structures. Studies have highlighted that pathogens can not only attach on the outer surface of plants but can also intrude the inside of fresh vegetables (125-127). Pathogens on the surface of the fresh vegetables can be reduced or eliminated if washed with safe water but on the other hand, transfer of these pathogens in the whole batch can also be aided (128, 129). Occurrence of pathogens in the internal parts of vegetables may also render the washing process ineffective (127). Overall, the adhesion of pathogens to surfaces and the internalization of pathogens limits

the effect of conventional washing and chemical sanitizing methods in preventing transmission from contaminated fresh vegetables (130).

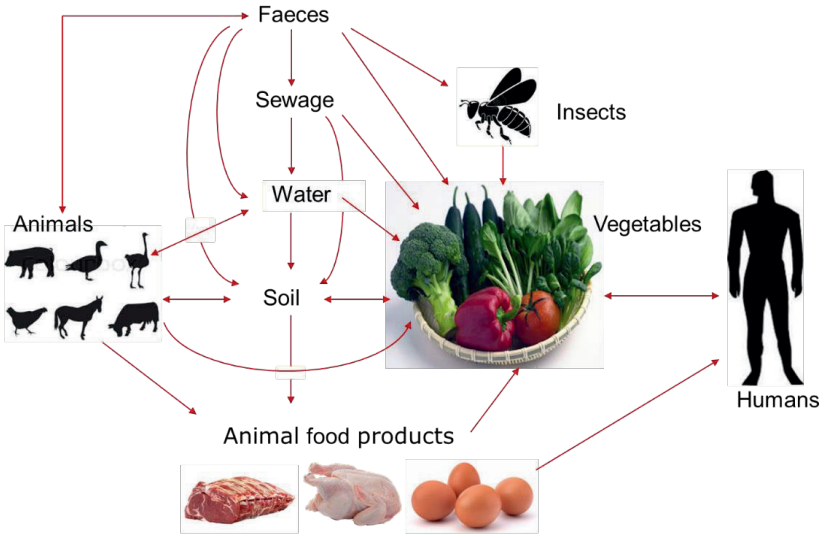

Figure 1.5: Mechanisms by which fresh vegetables and other foods can become contaminated with pathogenic microorganisms and serve as vehicles of human disease. [modified from Beuchat 1996 (131)].

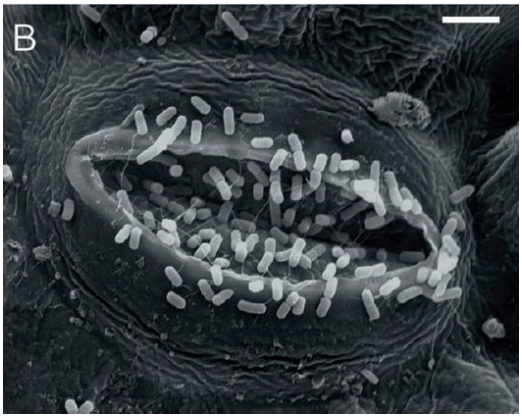

Figure 1.6: Scanning electron micrograph showing binding of pathogenic *E. coli* to exterior and interior of vegetable leaf structure (stomatae).[source: Berger et al. 2010 (132)].

Overall, it should be noted that if human pathogens are introduced to fresh vegetables along the supply chain, they have the potential to survive and they are also difficult to eliminate prior to consumption. The presence of foodborne pathogens in fresh vegetables is of great public health concern if these vegetables are to be consumed raw. Fortunately, the microbial safety of fresh vegetables is today not a “terra incognita”, a lot is now known thanks to the indicative studies that have been conducted in different countries like those in the EU (Veg-I-Trade project) (133-135), the USA (123, 136-139), Canada (27, 76, 89, 97, 140), and the Netherlands (74, 141-143),

to mention a few. Indicatives studies in these countries have been conducted to: (i) identify microbial populations in different farming systems in order to detect emerging and re-emerging pathogens; (ii) better understand the interactions between host, pathogen and environment that contribute to inter-species jumps and adaptation in a new host; (iii) generate more precise information on the infection and transmission behaviour of pathogens under different farming systems and human populations to estimate the burden of disease; (iv) understand the conditions, motives and priorities of farmers and other stakeholders so as to develop interventions that can lead to community based disease control (144). In Rwanda so far, no microbial safety indicative study has been conducted along the vegetable supply chain.

Study objective and outline

In this study we aim to conduct an indicative study by estimating the burden of foodborne infectious diseases, investigating the current microbial safety status and performing a quantitative microbial risk assessment for fresh vegetables in Rwanda, identifying critical activities and opportunities for improvement, identifying specific food safety roles of the different multidisciplinary stakeholders in the context of the supply chain in Rwanda. Figure 1.7. shows the conceptual approach of this study.

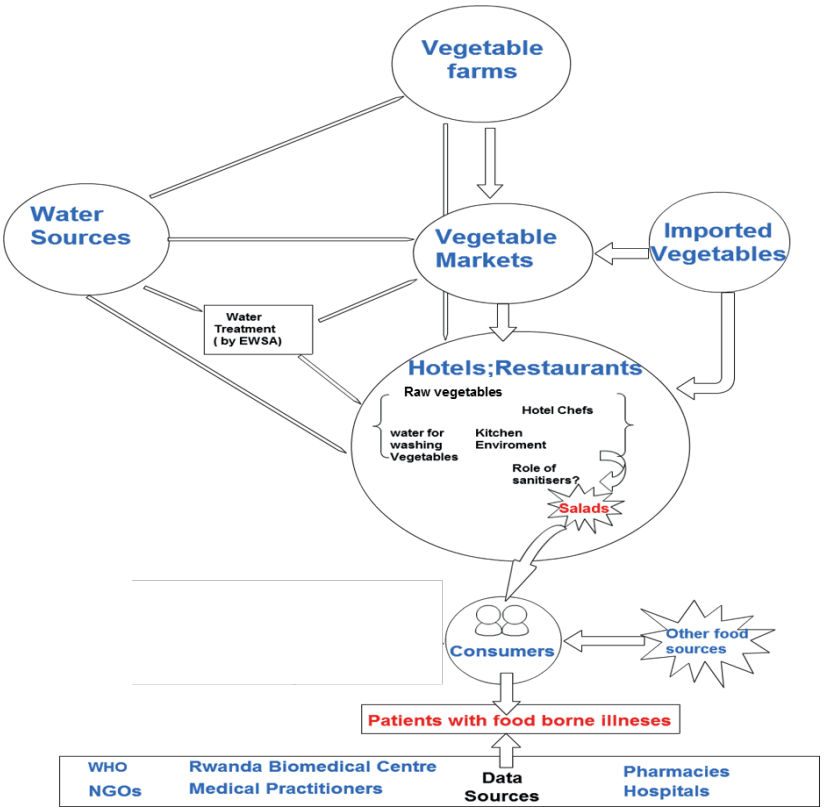

Figure 1.7: Schematic representation of the conceptual approach used in this study.

This thesis is made up of six chapters. In this chapter (**Chapter 1)**, the background and rationale for the study are presented. **Chapter 2** provides the estimates of the burden of food related illnesses in DALYs and also discusses a methodology that can be adopted in resource scarce settings. In **chapter 3**, the levels of indicator microorganisms in fresh vegetable along the supply chain are studied from farms, markets to food service establishments (hotels, restaurants and bars) to obtain an insight into the effect of the current handling practices on the microbial levels and identify areas of priority for interventions. In **chapter 4**, the prevalence of foodborne pathogens in farms vegetables and agricultural water is presented in conjunction with their risk exposure factors. **Chapter 5** discusses the reduction of microbial counts during kitchen scale washing and sanitization of salad vegetables from the food service establishments in Rwanda and laboratory trials to screen the applied vegetable chemical sanitizers and also identify the most effective washing technique(s). Finally in **Chapter 6**, we present results of a "farm to fork" microbial risk assessment based on the WHO/FAO CODEX alimentarius approach, discuss the study findings in general, make conclusions and recommendations, and present future perspectives. Overall, the findings and discussions in this thesis provide more insight for risk managers in countries especially those at an infant stage of operating an integrated food chain system, a system that is recommended by the WHO (1) and FAO (144) as indispensable in order to detect, control and prevent foodborne illness.

References

1. **The World Health Organization of the United Nations** (WHO). 2012. Manual for integrated foodborne disease surveillence in the WHO Africa Region. Available at: http://apps.who.int/iris/bitstream/10665/170262/1/foodborne-disease-manual.pdf. Accessed 02nd September 2016.

2. **Food and Drug Administration (FDA) of the United States**. 2012. Bad bug book, foodborne pathogenic microorganisms and natural toxins. second edition. Available at: https://www.fda.gov/downloads/food/foodsafety/foodborneillness/foodborneillnessfoodbornepathogensnaturaltoxins/badbugbook/ucm297627.pdf. Accessed 19th May 2017.

3. **Lasky T.** 2002. Foodborne illness - Old problem, new relevance. *Epidemiology* 13:593-598.

4. **Mughini-Gras L, Schaapveld M, Kramers J, Mooij S, Neefjes-Borst EA, van Pelt W, Neefjes J.** 2018. Increased colon cancer risk after severe *Salmonella* infection. *PloS one* 13:e0189721.

5. **Thomas MK, Murray R, Flockhart L, Pintar K, Pollari F, Fazil A, Nesbitt A, Marshall B.** 2013. Estimates of the burden of foodborne illness in Canada for 30 specified pathogens and unspecified agents, Circa 2006. *Foodborne Pathogens & Disease* 10:639-648.

6. **Michigan State University Extension**. 2014. History of food safety in the U.S. – part 1. Available at: http://msue.anr.msu.edu/news/history_of_food_safety_in_the_us_part_1. Accessed 02nd June 2017.

7. **Food Safety News.** 2011. Bugs through the ages: the foodborne illness fight. Available at: http://www.foodsafetynews.com/2011/01/fbi-through-the-ages/#.VBrpSxaa8tE. Accessed 08th July 2017.

8. **Roberts CA.** 2001. The food safety information handbook. Greenwood Publishing Group, Westport, CT, United States, pp 25-28.

9. **Havelaar** et al. 2015. World Health Organization global estimates and regional comparisons of the burden of foodborne disease in 2010. *PLoS Medicine* 12:e1001923.

10. **Scott E.** 2003. Food safety and foodborne disease in the 21st Century. *Canadian Journal of Infectious Diseases & Medical Microbiology* 14:277-280.

11. **Tauxe RV, Doyle MP, Kuchenmüller T, Schlundt J, Stein CE.** 2010. Evolving public health approaches to the global challenge of foodborne infections. *International Journal of Food Microbiology* 139:S16-S28.

12. **Kasowski EJ, Gackstetter GD, Sharp TW**. 2002. Foodborne illness: new developments concerning an old problem. *Current Gastroenterology Reports* 4:308-318.

13. **Bavishi C, Dupont H.** 2011. Systematic review: the use of proton pump inhibitors and increased susceptibility to enteric infection. *Alimentary Pharmacology & Therapeutics* 34:1269-1281.

14. **Cobb CA, Curtis GD, Bansi DS, Slade E, Mehal W, Mitchell RG, Chapman RW.** 1996. Increased prevalence of *Listeria monocytogenes* in the faeces of patients receiving long-term H2-antagonists. *European Journal of Gastroenterology & Hepatology* 8:1071-1074.

15. **Newell DG, Koopmans M, Verhoef L, Duizer E, Aidara-Kane A, Sprong H, Opsteegh M, Langelaar M, Threfall J, Scheutz F, der Giessen JV, Kruse H.** 2010. Food-borne diseases - The

challenges of 20years ago still persist while new ones continue to emerge. *International Journal of Food Microbiology* 139:S3-S15.

16. **Rasko DA, Webster DR, Sahl JW, Bashir A, Boisen N, Scheutz F, Paxinos EE, Sebra R, Chin C-S, Iliopoulos D, Klammer A, Peluso P, Lee L, Kislyuk AO, Bullard J, Kasarskis A, Wang S, Eid J, Rank D, Redman JC, Steyert SR, Frimodt-Møller J, Struve C, Petersen AM, Krogfelt KA, Nataro JP, Schadt EE, Waldor MK.** 2011. Origins of the *E. coli* strain causing an outbreak of hemolytic–uremic syndrome in Germany. *New England Journal of Medicine* 365:709-717.

17. **Centers for Disease Control and Prevention (CDC). 2012.** Global water, sanitation, & hygiene (WASH). Pathways to diarrhea. Available at: http://www.cdc.gov/healthywater/global/diarrhea-pathways.html, Accessed 17th September 2016.

18. **Li B, Vellidis G, Liu H, Jay-Russell M, Zhao S, Hu Z, Wright A, Elkins CA.** 2014. Diversity and antimicrobial resistance of *Salmonella enterica* isolates from surface water in southeastern United States. *Applied & Environmental Microbiology* 80:6355-6365.

19. **Wang G, Qian W, Zhang X, Wang H, Ye K, Bai Y, Zhou G.** 2015. Prevalence, genetic diversity and antimicrobial resistance of *Listeria monocytogenes* isolated from ready-to-eat meat products in Nanjing, China. *Food Control* 50:202-208.

20. **Yang X, Huang J, Wu Q, Zhang J, Liu S, Guo W, Cai S, Yu S.** 2015. Prevalence, antimicrobial resistance and genetic diversity of *Salmonella* isolated from retail ready-to-eat foods in China. *Food Control* 60:50-56.

21. **Zhang S, Wu Q, Zhang J, Lai Z, Zhu X.** 2016. Prevalence, genetic diversity, and antibiotic resistance of enterotoxigenic *Escherichia coli* in retail ready-to-eat foods in China. *Food Control* 68:236-243.

22. **Lamprecht C, Romanis M, Huisamen N, Carinus A, Schoeman N, Sigge GO, Britz TJ.** 2014. *Escherichia coli* with virulence factors and multidrug resistance in the Plankenburg River. *South African Journal of Science*, 110: 01-06.

23. **Olaniran AO, Naicker K, Pillay B.** 2009. Antibiotic resistance profiles of *Escherichia coli* isolates from river sources in Durban, South Africa. *World Journal of Microbiology & Biotechnology* 25:1743-1749.

24. **Akinyemi KO, Iwalokun BA, Foli F, Oshodi K, Coker AO.** 2011. Prevalence of multiple drug resistance and screening of enterotoxin (stn) gene in *Salmonella enterica* serovars from water sources in Lagos, Nigeria. *Public Health* 125: 65-71.

25. **Ben Said L, Klibi N, Dziri R, Borgo F, Boudabous A, Ben Slama K, Torres C.** 2016. Prevalence, antimicrobial resistance and genetic lineages of *Enterococcus* spp. from vegetable food, soil and irrigation water in farm environments in Tunisia *Journal of the Science of Food & Agriculture* 96: 1627-1633.

26. **Jernberg C, Hjertqvist M, Sundborger C, Castro E, Löfdahl M, Pääjärvi A, Sundqvist L, Löf E.** 2015. Outbreak of *Salmonella* Enteritidis phage type 13a infection in Sweden linked to imported dried-vegetable spice mixes, December 2014 to July 2015. *Eurosurveillance* 20: 21194.

27. **Allen KJ, Kovacevic J, Cancarevic A, Wood J, Xu J, Gill B, Allen JK, Mesak LR.** 2013. Microbiological survey of imported produce available at retail across Canada. *International Journal of Food Microbiology* 162:135-142.

28. **Raguenaud ME, Le Hello S, Salah S, Weill FX, Brisabois A, Delmas G, Germonneau P.** 2012. Epidemiological and microbiological investigation of a large outbreak of monophasic *Salmonella* Typhimurium 4,5,12: i: - in schools associated with imported beef in Poitiers, France, October 2010. *Euro surveillance* 17:20289.

29. **Sharapov UM, Wendel AM, Davis JP, Keene WE, Farrar J, Sodha S, Hyytia-Trees E, Leeper M, Gerner-Smidt P, Griffin PM, Braden C.** 2016. Multistate outbreak of *Escherichia coli* O157:H7 infections associated with consumption of fresh spinach: United States, 2006. *Journal of Food Protection* 79:2024-2030.

30. **McCollum JT, Cronquist AB, Silk BJ, Jackson KA, O'Connor KA, Cosgrove S, Gossack JP, Parachini SS, Jain NS, Ettestad P, Ibraheem M, Cantu V, Joshi M, DuVernoy T, Fogg Jr NW, Gorny JR, Mogen KM, Spires C, Teitell P, Joseph LA, Tarr CL, Imanishi M, Neil KP, Tauxe RV, Mahon BE.** 2013. Multistate outbreak of listeriosis associated with cantaloupe. *New England Journal of Medicine* 369:944-953.

31. **Neil KP, Biggerstaff G, MacDonald JK, Trees E, Medus C, Musser KA, Stroika SG, Zink D, Sotir MJ.** 2012. A novel vehicle for transmission of *Escherichia coli* O157:H7 to humans: Multistate outbreak of *E. coli* O157:H7 infections associated with consumption of ready-to-bake commercial prepackaged cookie dough-United States, 2009. *Clinical Infectious Diseases* 54:511-518.

32. **Maritschnik S, Kanitz EE, Simons E, Höhne M, Neumann H, Allerberger F, Schmid D, Lederer I.** 2013. A food handler-associated, foodborne norovirus GII.4 Sydney 2012-outbreak following a wedding dinner, Austria, October 2012. *Food & Environmental Virology* 5:220-225.

33. **Nicolay N, McDermott R, Kelly M, Gorby M, Prendergast T, Tuite G, Coughlan S, McKeown P, Sayers G.** 2011. Potential role of asymptomatic kitchen food handlers during a food-borne outbreak of norovirus infection, Dublin, Ireland, March 2009. *Eurosurveillance* 16:3.

34. **Angelillo IF, Viggiani NMA, Rizzo L, Bianco A.** 2000. Food handlers and foodborne diseases: Knowledge, attitudes, and reported behavior in Italy. *Journal of Food Protection* 63:381-385.

35. **Lo SV, Connolly AM, Palmer SR, Wright D, Thomas PD, Joynson D. 1994.** The role of the pre-symptomatic food handler in a common source outbreak of food-borne SRSV gastroenteritis in a group of hospitals. *Epidemiology & Infection* 113:513-521.

36. **Figgatt M, Mergen K, Kimelstein D, Mahoney DM, Newman A, Nicholas D, Ricupero K, Cafiero T, Corry D, Ade J, Kurpiel P, Madison-Antenucci S, Anand M.** 2017. Giardiasis outbreak associated with asymptomatic food handlers in New York State, 2015. *Journal of Food Protection* 80:837-841.

37. **Abdel-Dayem M, Al Zou'bi R, Hani RB, Amr ZS.** 2013. Microbiological and parasitological investigation among food handlers in hotels in the Dead Sea area, Jordan *Journal of Microbiology, Immunology & Infection* 47: 377-380.

38. **Gebreyesus A, Adane K, Negash L, Asmelash T, Belay S, Alemu M, Saravanan M.** 2014. Prevalence of *Salmonella* Typhi and intestinal parasites among food handlers in Mekelle University student cafeteria, Mekelle, Ethiopia. *Food Control* 44:45-48.

39. **Nsubuga P, White ME, Thacker SB, Anderson MA, Blount SB, Broome CV, Chiller TM, Espitia V, Imtiaz R, Sosin D.** 2006. Public health surveillance: a tool for targeting and monitoring interventions. *Disease Control Priorities in Developing Countries* 2:997-1018.

40. **Khera A.** 2016. Surveillance systems for foodborne illnesses. Food Safety in the 21st Century: Public Health Perspective. Elsevier Inc. Chapter 4, pp 41-51.

41. **The World Health Organization (WHO) of the United Nations**. 2017. Global infectious disease surveillance. Available at: http://www.who.int/mediacentre/factsheets/fs200/en/. Accessed 25th July 2017.

42. **Rwanda Biomedical Center.** 2016. Epidemic surveillance and response (ESR) division. Available at: http://www.rbc.gov.rw/index.php?id=348. Accessed 5th August 2017.

43. **European Centre for Disease Prevention and Control (ECDC).** 2017. Surveillance and disease data. Available at: https://ecdc.europa.eu/en/surveillance-and-disease-data. Accessed 5th August 2017.

44. **Centres for Disease Control and Prevention of the United States** (CDC). 2017. Surveillance and disease data. Available at: https://www.cdc.gov/. Accessed 5th August 2017.

45. **Nkengasong JN, Maiyegun O, Moeti M**. 2017. Establishing the Africa Centres for Disease Control and Prevention: responding to Africa's health threats. *The Lancet Global Health* 5:e246-e247.

46. **Centres for Disease Control and Prevention of the United States** (CDC). 2016. Modernizing our public health surveillance systems. Available at: https://www.cdc.gov/ophss/csels/dhis/blogs/yoon-201611.html. Accessed 5th August 2017.

47. **The World Health Organization (WHO) of the United Nations**. 2006. Communicable disease surveillance and response systems: Guide to monitoring and evaluating. Available at: http://www.who.int/csr/resources/publications/surveillance/WHO_CDS_EPR_LYO_2006_2.pdf. Accessed 28th July 2017.

48. **The World Health Organization (WHO) of the United Nations**. 2017. Types of Surveillance. Available at: http://www.who.int/immunization/monitoring_surveillance/burden/vpd/surveillance_type/en/. Accessed 5th August 2017.

49. **Murray C, Salomon J, Mathers C.** 2002. A critical examination of summary measures of population health. In Murray C, Salomon J, Mathers C, Lopez A (ed.), Summary Measures of Population Health. WHO Press, Geneva.

50. **Council NR.** 2011. Accounting for health and health care: approaches to measuring the sources and costs of their improvement. National Academies Press.

51. **Torgerson PR, Devleesschauwer B, Praet N, Speybroeck N, Willingham AL, Kasuga F, Rokni MB, Zhou XN, Fèvre EM, Sripa B, Gargouri N, Fürst T, Budke CM, Carabin H, Kirk MD, Angulo FJ, Havelaar A, de Silva N.** 2015. World Health Organization estimates of the global and regional disease burden of 11 foodborne parasitic diseases, 2010: A Data synthesis. *PLoS Medicine* 12:e1001920.

52. **Gkogka E, Reij MW, Havelaar AH, Zwietering MH, Gorris LGM. 2011.** Risk-based estimate of effect of foodborne diseases on public health, Greece. *Emerging Infectious Diseases* 17:1581-1590.

53. **Havelaar AH, Kemmeren JM, Kortbeek LM.** 2007. Disease burden of congenital toxoplasmosis. *Clinical Infectious Diseases : an Official Publication of the Infectious Diseases Society of America* 44:1467-1474.

54. **Murray** et al. 2012. Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990–2010: a systematic analysis for the global burden of disease study 2010. *The Lancet* 380:2197-2223.

55. **Molla M, Madans JH, Wagener DK, Crimmins EM.** 2003. Summary measures of population health: Report of findings on methodologic and data issues. National Center for Health Services, Hyattsville, Maryland.

56. **Gold MR, Stevenson D, Fryback DG.** 2002. HALYs and QALYs and DALYs, Oh My: similarities and differences in summary measures of population Health. *Annual Review of Public Health* 23:115-134.

57. **Devleesschauwer B, Haagsma JA, Angulo FJ, Bellinger DC, Cole D, Döpfer D, Fazil A, Fèvre EM, Gibb HJ, Hald T.** 2015. Methodological framework for World Health Organization estimates of the global burden of foodborne disease. *PLoS One* 10:e0142498.

58. **Devleesschauwer B, Havelaar AH, Maertens de Noordhout C, Haagsma JA, Praet N, Dorny P, Duchateau L, Torgerson PR, Van Oyen H, Speybroeck N.** 2014. DALY calculation in practice: a stepwise approach. *International Journal of Public Health* 59: 571-574.

59. **Batz MB, Doyle MP, Morris Jr JG, Painter J, Singh R, Tauxe RV, Taylor MR, Wong DMALF**. 2005. Attributing illness to food. *Emerging Infectious Diseases* 11: 993-999.

60. **Mangen M-JJ, Bouwknegt M, Friesema IHM, Haagsma JA, Kortbeek LM, Tariq L, Wilson M, van Pelt W, Havelaar AH.** 2015. Cost-of-illness and disease burden of food-related pathogens in the Netherlands, 2011. *International Journal of Food Microbiology* 196:84-93.

61. **Herman KM, Hall AJ, Gould LH**. 2015. Outbreaks attributed to fresh leafy vegetables, United States, 1973-2012. *Epidemiology & Infection* 143:3011-3021.

62. **Beatty ME, Shevick G, Shupe-Ricksecker K, Bannister E, Tulu A, Lancaster K, Alexander N, Zellner DE, Lyszkowicz E, Braden CR.** 2009. Large *Salmonella Enteritidis* outbreak with prolonged transmission attributed to an infected food handler, Texas, 2002. *Epidemiology & Infection* 137:417-427.

63. **Painter JA, Hoekstra RM, Ayers T, Tauxe RV, Braden CR, Angulo FJ, Griffin PM**. 2013. Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998-2008. *Emerging Infectious Diseases* 19:407-415.

64. **Little CL, Pires SM, Gillespie IA, Grant K, Nichols GL.** 2010. Attribution of human *Listeria monocytogenes* infections in England and Wales to ready-to-eat food sources placed on the market: Adaptation of the Hald *Salmonella* source attribution model. *Foodborne Pathogens & Disease* 7:749-756.

65. **King N, Lake R, Campbell D.** 2011. Source attribution of nontyphoid salmonellosis in New Zealand using outbreak surveillance data. *Journal of Food Protection* 74:438-445.

66. **Ranta J, Matjushin D, Virtanen T, Kuusi M, Viljugrein H, Hofshagen M, Hakkinen M.** 2011. Bayesian temporal source attribution of foodborne zoonoses: *Campylobacter* in Finland and Norway. *Risk Analysis* 31:1156-1171.

67. **Wahlström H, Andersson Y, Plym-Forshell L, Pires SM.** 2011. Source attribution of human *Salmonella* cases in Sweden. *Epidemiology & Infection* 139:1246-1253.

68. **Boysen L, Rosenquist H, Larsson JT, Nielsen EM, Sorensen G, Nordentoft S, Hald T. 2014.** Source attribution of human campylobacteriosis in Denmark. *Epidemiology & Infection* 142:1599-1608.

69. **Havelaar AH, Galindo AV, Kurowicka D, Cooke RM.** 2008. Attribution of foodborne pathogens using structured expert elicitation. *Foodborne Pathogens & Disease* 5:649-659.

70. **Hoffmann S, Devleesschauwer B, Aspinall W, Cooke R, Corrigan T, Havelaar A, Angulo F, Gibb H, Kirk M, Lake R.** 2017. Attribution of global foodborne disease to specific foods: Findings from a World Health Organization structured expert elicitation. *PLoS One* 12:e0183641.

71. **Callejon RM, Rodriguez-Naranjo MI, Ubeda C, Hornedo-Ortega R, Garcia-Parrilla MC, Troncoso AM.** 2015. Reported foodborne outbreaks due to fresh produce in the United States and European Union: trends and causes. *Foodborne Pathogens & Disease* 12:32-38.

72. **Frank C, Werber D, Cramer JP, Askar M, Faber M, an der Heiden M, Bernard H, Fruth A, Prager R, Spode A.** 2011. Epidemic profile of shiga-toxin–producing *Escherichia coli* O104:H4 outbreak in Germany. *New England Journal of Medicine* 365:1771-1780.

73. **Sivapalasingam S, Friedman CR, Cohen L, Tauxe RV.** 2004. Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. *Journal of Food Protection* 67:2342-2353.

74. **Wijnands LM, Delfgou-van Asch EHM, Beerepoot-Mensink ME, van der Meij-Florijn A, Fitz-James I, van Leusden FM, Pielaat A.** 2014. Prevalence and concentration of bacterial pathogens in raw produce and minimally processed packaged salads produced in and for The Netherlands. *Journal of Food Protection* 77:388-394.

75. **Loutreul J, Cazeaux C, Levert D, Nicolas A, Vautier S, Le Sauvage AL, Perelle S, Morin T.** 2014. Prevalence of human noroviruses in frozen marketed shellfish, red fruits and fresh vegetables. *Food & Environmental Virology* 6:157-168.

76. **Bohaychuk V, Bradbury R, Dimock R, Fehr M, Gensler G, King R, Rieve R, Barrios PR.** 2009. A microbiological survey of selected Alberta-grown fresh produce from farmers' markets in Alberta, Canada. *Journal of Food Protection* 72:415-420.

77. **Centres for Disease Control and Prevention of the United States** (CDC). 2016. Multistate outbreak of *Salmonella* Reading and *Salmonella* Abony infections linked to alfalfa sprouts (Final Update). Available at: https://www.cdc.gov/salmonella/reading-08-16/index.html. Accessed 27th November 2017.

78. **Centres for Disease Control and Prevention of the United States** (CDC). 2016. Multistate outbreak of listeriosis linked to packaged salads produced at Springfield, Ohio Dole processing facility (Final Update). Available at: https://www.cdc.gov/listeria/outbreaks/bagged-salads-01-16/index.html. Accessed 27th November 2017.

79. **Centres for Disease Control and Prevention of the United States** (CDC). 2015. Multistate outbreak of *Salmonella* Poona infections linked to imported cucumbers (Final Update). Available at: https://www.cdc.gov/salmonella/poona-09-15/index.html. Accessed 27th November 2017.

80. **Knoblauch AM, Bratschi MW, Zuske MK, Althaus D, Stephan R, Hächler H, Baumgartner A, Prager R, Rabsch W, Altpeter E, Jost M, Mäusezahl M, Hatz C, Kiefer S.** 2015. Cross-border outbreak of *Salmonella* Bovismorbificans: Multiple approaches for an outbreak investigation in Germany and Switzerland. *Swiss Medical Weekly* 145.

81. **The Australian Government Department of Health Network for Surveillance of Foodborne Diseases (OZFoodNet).** 2017. Quarterly report, 1 October to 31 December 2014. Available at: http://health.gov.au/internet/main/publishing.nsf/Content/cda-cdi4101k.htm. Accessed 29th November 2017.

82. **Food and Drug Administration (FDA) of the United States**. 2016. FDA Investigates 2013 multistate outbreak of Cyclosporiasis. Available at: https://www.fda.gov/Food/RecallsOutbreaksEmergencies/Outbreaks/ucm361637.htm#updates. Accessed 29th November 2017.

83. **Centres for Disease Control and Prevention of the United States** (CDC). 2013. Multistate outbreak of *Salmonella* Saintpaul infections linked to imported cucumbers (Final Update). Available at: https://www.cdc.gov/salmonella/saintpaul-04-13/index.html. Accessed 27th November 2017.

84. **Centres for Disease Control and Prevention of the United States** (CDC). 2012. Multistate outbreak of shiga toxin-producing *Escherichia coli* O157:H7 infections linked to organic spinach and spring mix blend (Final Update). Available at: https://www.cdc.gov/ecoli/2012/O157H7-11-12/index.html. Accessed 27th November 2017.

85. **Centres for Disease Control and Prevention of the United States** (CDC). 2012. Multistate outbreak of shiga toxin-producing *Escherichia coli* O26 infections linked to raw clover sprouts at Jimmy John's restaurants (Final Update). Available at: https://www.cdc.gov/ecoli/2012/o26-02-12/index.html. Accessed 27th November 2017.

86. **Guzman-Herrador B, Vold L, Comelli H, MacDonald E, Heier B, Wester A, Stavnes T, Jensvoll L, Aanstad AL, Severinsen G**. 2011. Outbreak of *Shigella sonnei* infection in Norway linked to consumption of fresh basil, October 2011. *Eurosurveillance* 16:20007.

87. **Petrignani M, Harms M, Verhoef L, Van Hunen R, Swaan C, Van Steenbergen J, Boxman I, i Sala RP, Ober H, Vennema H.** 2010. Update: a food-borne outbreak of hepatitis A in the Netherlands related to semi-dried tomatoes in oil, January-February 2010. *Eurosurveillance* 15:19572.

88. **Centres for Disease Control and Prevention of the United States** (CDC). 2010. Multistate outbreak of human *E. coli* O145 infections linked to shredded romaine lettuce from a single processing facility (Final Update). Available at: https://www.cdc.gov/ecoli/2010/shredded-romaine-5-21-10.html. Accessed 27th November 2017.

89. **Kozak GK, Macdonald D, Landry L, Farber JM**. 2013. Foodborne outbreaks in Canada linked to produce: 2001 through 2009. *Journal of Food Protection* 76:173-183.

90. **Centres for Disease Control and Prevention of the United States** (CDC). 2009. Multistate outbreak of *Salmonella* Saintpaul infections linked to raw alfalfa sprouts (Final Update). Available at: https://www.cdc.gov/salmonella/2009/raw-alfalfa-sprouts-5-8-2009.html. Accessed 27th November 2017.

91. **Lynch MF, Tauxe RV, Hedberg CW.** 2009. The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities. *Epidemiology & Infection* 137(3), 307-315.

92. **Losio MN, Pavoni E, Bilei S, Bertasi B, Bove D, Capuano F, Farneti S, Blasi G, Comin D, Cardamone C, Decastelli L, Delibato E, De Santis P, Di Pasquale S, Gattuso A, Goffredo E, Fadda A, Pisanu M, De Medici D.** 2015. Microbiological survey of raw and ready-to-eat leafy green vegetables marketed in Italy. *International Journal of Food Microbiology* 210:88-91.

93. **Fiedler G, Kabisch J, Böhnlein C, Huch M, Becker B, Cho GS, Franz CMAP.** 2017. Presence of human pathogens in produce from retail markets in northern Germany. *Foodborne Pathogens & Disease* 14:502-509.

94. **Johannessen GS, Loncarevic S, Kruse H.** 2002. Bacteriological analysis of fresh produce in Norway. *International Journal of Food Microbiology* 77:199-204.

95. **Abadias M, Usall J, Anguera M, Solsona C, Vinas I.** 2008. Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. *International Journal of Food Microbiology* 123:121-129.

96. **Vojkovská H, Myšková P, Gelbíčová T, Skočková A, Koláčková I, Karpíšková R.** 2017. Occurrence and characterization of food-borne pathogens isolated from fruit, vegetables and sprouts retailed in the Czech Republic. *Food Microbiology* 63:147-152.

97. **Denis N, Zhang H, Leroux A, Trudel R, Bietlot H.** 2016. Prevalence and trends of bacterial contamination in fresh fruits and vegetables sold at retail in Canada. *Food Control* 67:225-234.

98. **Pan F, Li X, Carabez J, Ragosta G, Fernandez KL, Wang E, Thiptara A, Antaki E, Atwill ER.** 2015. Cross-sectional survey of indicator and pathogenic bacteria on vegetables sold from Asian vendors at farmers' markets in Northern California. *Journal of Food Protection* 78:602-608.

99. **Cerna-Cortes JF, Gómez-Aldapa CA, Rangel-Vargas E, Torres-Vitela MR, Villarruel-López A, Castro-Rosas J.** 2012. Presence of some indicator bacteria and diarrheagenic *E. coli* pathotypes on jalapeño and serrano peppers from popular markets in Pachuca City, Mexico. *Food Microbiology* 32:444-447.

100. **Sant'Ana AS, Landgraf M, Destro MT, Franco BDGM.** 2011. Prevalence and counts of *Salmonella* spp. in minimally processed vegetables in São Paulo, Brazil. *Food Microbiology* 28:1235-1237.

101. **Fallah AA, Pirali-Kheirabadi K, Shirvani F, Saei-Dehkordi SS.** 2012. Prevalence of parasitic contamination in vegetables used for raw consumption in Shahrekord, Iran: Influence of season and washing procedure. *Food Control* 25:617-620.

102. **Utaaker KS, Kumar A, Joshi H, Chaudhary S, Robertson LJ**. 2017. Checking the detail in retail: Occurrence of *Cryptosporidium* and *Giardia* on vegetables sold across different counters in Chandigarh, India. *International Journal of Food Microbiology* 263:1-8.

103. **Ponniah J, Robin T, Paie MS, Radu S, Ghazali FM, Kqueen CY, Nishibuchi M, Nakaguchi Y, Malakar PK.** 2010. *Listeria monocytogenes* in raw salad vegetables sold at retail level in Malaysia. *Food Control* 21:774-778.

104. **Yu T, Jiang X.** 2014. Prevalence and characterization of *Listeria monocytogenes* isolated from retail food in Henan, China. *Food Control* 37:228-231.

105. **Seow J, Ágoston R, Phua L, Yuk H-G**. 2012. Microbiological quality of fresh vegetables and fruits sold in Singapore. *Food Control* 25:39-44.

106. **Food Standards Australia and New Zealand**. 2011. Supporting Document 2. Review of foodborne illness associated with selected ready-to-eat fresh produce. Proposal P1015. Primary production & processing requirements for horticulture. Available at: https://www.foodstandards.gov.au/code/proposals/documents/P1015%20Horticulture%20PPPS%201CFS%20SD2%20Illness%20review.pdf. Accessed 29th November 2017.

107. **Zhu Q, Gooneratne SR, Hussain M.** 2016. Detection of *Listeria* species in fresh produces samples from different retail shops in Canterbury, New Zealand. *Advances in Food Technology & Nutritional Sciences* 2:96-102.

108. **De Bruin W, Otto D, Korsten L.** 2016. Microbiological status and food safety compliance of commercial basil production systems. *Journal of Food Protection* 79:43-50.

109. **Traoré O, Nyholm O, Siitonen A, Bonkoungou IJO, Traoré AS, Barro N, Haukka K.** 2015. Prevalence and diversity of *Salmonella enterica* in water, fish and lettuce in Ouagadougou, Burkina Faso. *BMC Microbiology* 15:1-7.

110. **Khalil RKS, Gomaa MAE, Khalil MIM.** 2015. Detection of shiga-toxin producing *E. coli* (STEC) in leafy greens sold at local retail markets in Alexandria, Egypt. *International Journal of Food Microbiology* 197:58-64.

111. **Adamu NB, Adamu JY, Mohammed D.** 2012. Prevalence of helminth parasites found on vegetables sold in Maiduguri, Northeastern Nigeria. *Food Control* 25:23-26.

112. **Nguz K, Shindano J, Samapundo S, Huyghebaert A.** 2005. Microbiological evaluation of fresh-cut organic vegetables produced in Zambia. *Food Control* 16:623-628.

113. **He FJ, Nowson CA, Lucas M, MacGregor GA.** 2007. Increased consumption of fruit and vegetables is related to a reduced risk of coronary heart disease: meta-analysis of cohort studies. *Journal of Human Hypertension* 21:717-728.

114. **Jacxsens L, Ibañez IC, Gómez-López VM, Fernandes JA, Allende A, Uyttendaele M, Huybrechts I.** 2015. Belgian and Spanish consumption data and consumer handling practices for fresh fruits and vegetables useful for further microbiological and chemical exposure assessment. *Journal of Food Protection* 78:784-795.

115. **Miller V, Mente A, Dehghan M, Rangarajan S, Zhang X, Swaminathan S, Dagenais G, Gupta R, Mohan V, Lear S.** 2017. Fruit, vegetable, and legume intake, and cardiovascular disease and deaths in 18 countries (PURE): a prospective cohort study. *The Lancet* 390:2037-2049.

116. **FAO STAT (Food and Agriculture Organization of the United Nations, statistics division).** 2015. http://faostat3.fao.org/compare/E. Accessed on 03rd May 2016.

117. **Cook R.** 2011. Tracking demographics and US fruit and vegetable consumption patterns. *Department of Agricultural and Resource Economics*, University of California, Davis. Available at: https://arefiles.ucdavis.edu//uploads/filer_public/2014/05/19/blueprintseoeconsumptioncookfinaljan2012figures.pdf. Accessed 30th May 2017.

118. **The World Health Organization (WHO) of the United Nations.** 2004. Fruit and vegetables for health. Report of a joint FAO/WHO workshop, 1–3 September 2004, Kobe, Japan. Available at: http://www.who.int/dietphysicalactivity/publications/fruit_vegetables_report.pdf. Accessed 02th April 2014.

119. **Johnson R.** 2016. US Trade Situation for fruit and vegetable Products. Report prepared for members and committees of US Congress. Available at: https://fas.org/sgp/crs/misc/RL34468.pdf. Accessed 25th June 2017.

120. **De la Peña R, Hughes J.** 2007. Improving vegetable productivity in a variable and changing climate. *Journal of SAT Agricultural Research* 4:1-22.

121. **Hiroyuki K, Holmer R, Linwattana G, Nath P, Keatinge J.** 2013. Growing role of vegetables in food security and nutrition in Asia. SEAVEG 2012 high value vegetables in southeast Asia: production, supply and demand. AVRDC-World Vegetable Center. pp 27.

122. **Hedberg C, Angulo F, White K, Langkop C, Schell W, Stobierski M, Schuchat A, Besser J, Dietrich S, Helsel L.** 1999. Outbreaks of salmonellosis associated with eating uncooked tomatoes: implications for public health. *Epidemiology & Infection* 122:385-393.

123. **Beuchat LR, Ryu JH.** 1997. Produce handling and processing practices. *Emerging Infectious Diseases* 3:459-465.

124. **Heaton JC, Jones K.** 2008. Microbial contamination of fruit and vegetables and the behaviour of enteropathogens in the phyllosphere: A review. *Journal of Applied Microbiology* 104:613-626.

125. **Erickson MC.** 2012. Internalization of fresh produce by foodborne pathogens. *Annual Review of Food Science and Technology* 3:283-310.

126. **Ongeng D, Muyanja C, Geeraerd AH, Springael D, Ryckeboer J.** 2011. Survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in manure and manure-amended soil under tropical climatic conditions in Sub-Saharan Africa. *Journal of Applied Microbiology* 110:1007-1022.

127. **Donkor ES, Lanyo R, Kayang BB, Quaye J, Edoh DA.** 2010. Internalization of microbes in vegetables: Microbial load of Ghanaian vegetables and the relationship with different water sources of irrigation. *Pakistan Journal of Biological Sciences* 13:857-861.

128. **Palma-Salgado S, Pearlstein AJ, Luo Y, Park HK, Feng H.** 2014. Whole-head washing, prior to cutting, provides sanitization advantages for fresh-cut Iceberg lettuce (*Latuca sativa* L.*). International Journal of Food Microbiology* 179:18-23.

129. **Gil MI, Selma MV, López-Gálvez F, Allende A.** 2009. Fresh-cut product sanitation and wash water disinfection: Problems and solutions. *International Journal of Food Microbiology* 134:37-45.

130. **Lynch MF, Tauxe RV, Hedberg CW.** 2009. The growing burden of foodborne outbreaks due to contaminated fresh produce: Risks and opportunities. *Epidemiology & Infection* 137:307-315.

131. **Beuchat LR.** 1996. Pathogenic microorganisms associated with fresh produce. *Journal of Food Protection* 59:204-216.

132. **Berger CN, Sodha SV, Shaw RK, Griffin PM, Pink D, Hand P, Frankel G.** 2010. Fresh fruit and vegetables as vehicles for the transmission of human pathogens. *Environmental Microbiology* 12(9), 2385-2397.

133. **Holvoet K, Sampers I, Seynnaeve M, Uyttendaele M.** Relationships among hygiene indicators and enteric pathogens in irrigation water, soil and lettuce and the impact of climatic conditions on

contamination in the lettuce primary production. *International Journal of Food Microbiology* 171: 21-31.

134. **Kirezieva K, Luning PA, Jacxsens L, Allende A, Johannessen GS, Tondo EC, Rajkovic A, Uyttendaele M, van Boekel MAJS**. 2015. Factors affecting the status of food safety management systems in the global fresh produce chain. *Food Control* 52:85-97.

135. **Allende A, Castro-Ibáñez I, Lindqvist R, Gil MI, Uyttendaele M, Jacxsens L.** 2017. Quantitative contamination assessment of *Escherichia coli* in baby spinach primary production in Spain: Effects of weather conditions and agricultural practices. *International Journal of Food Microbiology* 257**:**238-246.

136. **Buck J, Walcott R, Beuchat L.** 2003. Recent trends in microbiological safety of fruits and vegetables. *Plant Health Progress* **10:**1094.

137. **Nightingale K, Schukken Y, Nightingale C, Fortes E, Ho A, Her Z, Grohn Y, McDonough P, Wiedmann M.** 2004. Ecology and transmission of *Listeria monocytogenes* infecting ruminants and in the farm environment. *Applied & Environmental Microbiology* 70:4458-4467.

138. **Strawn LK, Fortes ED, Bihn EA, Nightingale KK, Gröhn YT, Worobo RW, Wiedmann M, Bergholz PW.** 2013. Landscape and meteorological factors affecting prevalence of three food-borne pathogens in fruit and vegetable farms. *Applied & Environmental Microbiology* 79:588-600.

139. **Chapin TK, Nightingale KK, Worobo RW, Wiedmann M, Strawn LK.** 2014. Geographical and meteorological factors associated with isolation of *Listeria* species in New York state produce production and natural environments. *Journal of Food Protection* **77:**1919-1928.

140. **Falardeau J, Johnson RP, Pagotto F, Wang S.** 2017. Occurrence, characterization, and potential predictors of verotoxigenic *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* in surface water used for produce irrigation in the Lower Mainland of British Columbia, Canada. *PLoS One* 12: e0185437.

141. **Franz E, Semenov AV, Van Bruggen AHC.** 2008. Modelling the contamination of lettuce with *Escherichia coli* O157:H7 from manure-amended soil and the effect of intervention strategies. *Journal of Applied Microbiology* 105:1569-1584.

142. **Franz E, Van Bruggen AHC.** 2008. Ecology of *E. coli* O157:H7 and *Salmonella enterica* in the primary vegetable production chain. *Critical Reviews in Microbiology* 34:143-161.

143. **Liu C, Hofstra N, Franz E.** 2013. Impacts of climate change on the microbial safety of pre-harvest leafy green vegetables as indicated by *Escherichia coli* O157 and *Salmonella* spp. *International Journal of Food Microbiology* 163:119-128.

144. **The Food and Agriculture Organisation of the United Nations (FAO)**. 2008. Contributing to "One World, One Health". A strategic framework for reducing risks of infectious diseases at the animal–human–ecosystems Interface. Available at ftp://ftp.fao.org/docrep/fao/011/aj137e/aj137e00.pdf. Accessed 01st October 2013.

2

Estimates of the burden of illnesses related to foodborne pathogens as from the syndromic surveillance data of 2013 in Rwanda

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Abstract

Food related illnesses contribute significantly to the global burden of disease and the estimates of these illnesses are important to develop evidence based food safety policies. However estimating the burden of these illnesses is complex. There is paucity of input data, and developing and sustaining disease surveillance systems that provide the input data is resource-intensive. In most developing countries with relative peace, the initial, faster and cheaper kind of health data is generated through syndromic surveillance. In this study, we estimated the burden of food related clinical features and illnesses (watery diarrhea, bloody diarrhea, suspected cases of cholera and typhoid fever) by making use of various syndromic surveillance data sources in Rwanda. Data sources were the reported cases as by the notifiable surveillance system, an opinion survey with health care providers about the prevalence of clinical features related to foodborne pathogens and over the counter prescription of drugs associated with foodborne illnesses. Study findings indicate that for the year 2013, watery diarrhea occurred all year round as by the surveillance system data, resulting to an estimated 672 (95% credible interval [CrI] 424 — 932) DALY per million inhabitants, bloody diarrhea was seasonal coinciding with the rainy months and caused an estimated 213 (95% CrI 50 — 475) DALY per million, typhoid and cholera cases were sporadic with an estimated 73 (95% CrI 57 — 91) and 1 (95% CrI 0 — 2) DALY per million respectively. Our DALY estimates from the different data sources were in the same range for combined cases of watery diarrhea, bloody diarrhea and cholera, but significantly different for typhoid fever. The methodology applied in this study can be adopted in resource-scarce settings where most data is from syndromic surveillance (a common phenomenon in most developing countries) other than the desired integrated food chain and laboratory-based surveillance systems, to pave way for future improved estimates of the burden of foodborne illnesses.

Introduction

Worldwide, foodborne illnesses are a threat to public health and social-economic development. Causes of food borne illnesses include bacteria, parasites, viruses, toxins, metals, and prions (1, 2) but the risk due to microbiological agents is higher due to their ability to emerge, re-emerge and adapt to various niches (3). Foodborne illnesses are usually characterised by acute conditions like gastroenteritis and in some cases by long term sequelae such as hemolytic uremic syndrome (due to shiga toxin-producing *Escherichia coli*), Guillain-Barré syndrome (*Campylobacter* spp.), or central nervous system (CNS) abnormalities (*Listeria monocytogenes*) (2). Other associated long-term sequelae also include chronic arthritis (*Campylobacter* spp., *Shigella* spp. and *Salmonella* spp. can be involved); mental retardation, seizures, paralysis, blindness, or deafness (*L. monocytogenes*); the incurable irritable bowel syndrome (IBS) (bacterial pathogens) and mental retardation and crossed eyes in newborns (*Toxoplasma gondii*) (4). Recently colon cancer has been associated with severe *Salmonella* spp. infections (5). In addition, foodborne illness may also exacerbate other underlying medical conditions (6).

While infectious diseases like malaria, HIV/AIDS and tuberculosis ("the big three") continue to dominate the agenda of health programmes in most African countries (7), it has been estimated that foodborne illnesses comparably and significantly contribute to the overall burden of disease (2). Estimates from the World Health Organisation (WHO) in 2010 indicate that 31% of the 33 million Disability Adjusted Life Years (DALYs) lost globally due to 31 foodborne hazards was from sub-Saharan Africa with diarrheal disease agents causing nearly 70% of this burden (2). Studies on costs of foodborne illnesses from the USA (8-10) reported economic losses estimated at 0.1 to 0.2 % the national gross domestic product(GDP) in 2015 due to productivity and medical care costs (11). Estimated costs due to diarrhea in Rwanda in the year 2000 were in the range of 2.5 to 5% of the GDP (12).

To implement effective control measures, risk managers need information on the magnitude of the burden of foodborne illnesses in order to prioritise, develop and implement risk based food safety policies (13). However, assessing the burden of foodborne illness is complex. First, disease surveillance systems that provide the data used in these assessments are resource-intensive and to date all countries are still developing their systems (14). Second, a number of acute foodborne illnesses are self-limiting, implying that some patients do not seek medical care and as a consequence, the real burden is underreported (15-17). Third, many pathogens that are usually associated with food can also be transmitted from the environment or from direct contact with animals or infected persons (18, 19). Cognizant of this complexity, the WHO through the Foodborne Disease Burden Epidemiology Reference Group (FERG) has encouraged national and international studies to assess the burden of foodborne illness. The FERG and volunteer researchers have since adopted the DALY (disability adjusted life year) concept to summarise and rank diseases.

Foot note: Abbreviations
OTC= Over the counter drug sales, HCPs=Health care providers, RBC=Rwanda biomedical centre, RSSB=Rwanda social security board, NISR=National institute of statistics Rwanda, ORS=Oral rehydration salts, MoH=Ministry of Health, DW=Disability weight

The DALY is a measure that combines the Years of Life Lost due to premature death (YLL) and the Years Lost due to Disability (YLD) from a disease or condition, for varying degrees of severity, making time itself the common metric for death and disability (20). Studies on food borne illnesses at a global level (2, 21), provide world estimates but with many assumptions due to paucity of data. At country level, most studies come from the developed countries with elaborate disease surveillance systems *i.e.*. England (22), USA (23), Netherlands (19), Greece (18) and Canada (1). More studies on the burden foodborne illnesses from developing countries are required, however, availability of disease surveillance data to use in these studies is still among the major challenges. Syndromic surveillance data that are collected by using standard case definitions of clinical features like watery diarrhea and suspected cases of illnesses (typhoid fever) without laboratory diagnosis or from surrogate data sources (*viz.* over-the-counter prescription sales, opinion of health care providers) can provide a cheaper and fast data option (24, 25).

In this study, we provided insight into- and estimated DALYs for foodborne illnesses in Rwanda using syndromic surveillance data of 2013 as a reference year, presented a methodology and also identified data gaps to contribute to future improved estimates in Rwanda and other developing countries. Because of the kind of data used this study *i.e.*. non-laboratory confirmed cases and surrogate health data, the term “foodborne illnesses” should be used with caution and hereafter replaced with “food related illnesses” (further definitions in the glossary, Appendix A).

Methods

Study Approach

Figure 2.1 shows the approach used to investigate the burden of food related illnesses in Rwanda. Our study was based on the syndromic surveillance data aspects explained by the WHO manual for integrated foodborne disease surveillance in the WHO Africa Region (24). Data from the opinion survey with health care providers (HCPs), the national notifiable surveillance system, and over the counter (OTC) drug prescriptions were used to show trends and estimates of YLL, YLD and DALY for food related- clinical features and illnesses.

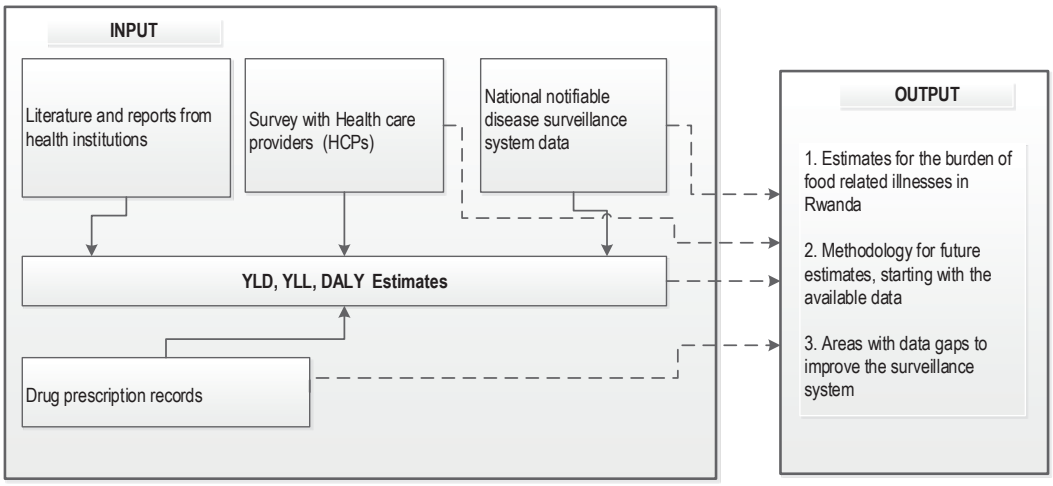


Figure 2.1: Study approach and data sources (inputs) used in this study. YLD, years lived with disability for prevalent cases of illness; YLL, years of life lost due to premature death caused by illness in the population; DALY= (YLD +YLL), disability-adjusted life years.

Clinical features (watery diarrhea and bloody diarrhea), and illnesses (cholera and typhoid fever) related to foodborne pathogens were investigated. For cholera and typhoid fever illnesses, this study was based on suspected cases. Standard case definitions of watery diarrhea, bloody diarrhea and suspected cases of cholera and typhoid fever as used in this study are presented in the glossary (see Technical Appendix A). We acknowledge that diarrheal diseases are caused by infectious and non-infections agents but because in most developing countries, these diseases are mainly caused by infectious agents (26), we assume in this study that diarrheal cases were all infectious in nature.

Ethical clearance

This study was approved by both the Rwanda National Health Research Committee (ref: NHRC/2014/PROT/0148) and the Rwanda National Ethics Committee (No. 130/RNEC/2014).

Data collection

Opinion survey with Health care providers

Over a period of three months (January to March, 2014), a face to face questionnaire (see online Technical Appendix A, section 6) was administered to 128 HCPs at their respective health facilities (private and public hospitals, clinics and health centres) in the City of Kigali and the other four provinces of Rwanda to investigate about the perceived prevalence of selected clinical features (watery diarrhea and bloody diarrhea) and illness (typhoid fever), commonly related to foodborne pathogens (27). The perceived prevalences were in form of percentage rank categories *ie*. 0% (not seen any case in year 2013), between—: 0-1%, 1-20%, 20-40%, 40-60%, 60-80% and 80-100%. We explained the importance of the study to the HCPs and thereafter asked the HCPs to allocate percentage prevalence ranks to the clinical features and typhoid fever illness based on the consultation with patients in the year 2013. HCPs that

participated in the study were general practising doctors and grade 1 nurses (most likely to handle patients with clinical features in the questionnaire). Percentage (%) rankings for any clinical feature or illness with less than 10 respondents (HCPs) were considered as outliers and excluded in the incidence calculations for the DALY estimates (Tab. 2A 4, Technical appendix A) because these rank results led to unrealistically large uncertainty.

National notifiable surveillance system data

The epidemic surveillance and response division of Rwanda Biomedical Centre (RBC) receives and compiles weekly suspected cases of illnesses like cholera, typhoid fever and cases of clinical features; watery and bloody diarrhea from health facilities all over Rwanda based on case definitions as presented in the glossary (Technical Appendix A). In March 2014, with permission from RBC, we retrieved data of reported cases of watery diarrhea, bloody diarrhea, cholera and typhoid fever for the year 2013. In the Rwandan health system, further actions after this syndromic surveillance can involve confirmation of these cases in the National Reference Laboratory but this study focussed on the reported cases of clinical features and suspected cases of the illnesses under investigation.

Over the Counter drugs (OTC) sales

In two months period (April to May) of year 2014, we collected data on drug prescription from 37 major pharmacies in Rwanda. Rwanda Social Security Board (RSSB), pharmacy division was identified as a one stop centre for drug sales records coupled with prescriptions by medical practitioners. In 2010, the RSSB insurance scheme was estimated to cover 3.7 % (28) of the population in Rwanda of about 10.5 million people (29). Drug sales records as prescribed by the medical practitioners were used to minimise the unrealistic drug sale trends that result from the practice of patients who repetitively buy drugs from individual pharmacies without seeking medical care. With permission from the RSSB management, we accessed the RSSB archive of drug insurance claim forms for RSSB affiliates and their dependants. Data collected covered prescription per pharmacy and age of patient for antibiotics, anthelmintics, antiprotozoal and oral rehydration salts (ORS) for the year 2013.

Estimation of YLD, YLL and DALY for food related illnesses

In summary, we applied a step wise approach in computing YLD, YLL and DALY as was explained by Devleesschauwer et al. (30). DALY estimates were computed from the following expression (31):

DALY= YLD + YLL

Where

YLD ($n \times DW \times L$) are computed by multiplying the number of incident cases (n) of a given illness in a population with the disability weight (DW) and the average duration of the case until remission or death (L in years).

YLL ($n_d \times LE$) are computed by multiplying the number of deaths (n_d) at a given age with the year lost due premature death as compared to the standard life expectancy at that age (LE in years).

We computed YLD, YLL and DALY for water diarrhea, bloody diarrhea, cholera and typhoid fever. Data sources were the epidemic surveillance and response division of Rwanda Biomedical Centre (RBC), an opinion survey with health care providers (HCPs), prescription records for oral rehydration salts (ORS), Rwanda Ministry of Health reports (MoH), Rwanda National Institute of statistics reports (28, 29), the WHO and data in other published literature (Fig. 2.1). Details of the methodology used, are provided in Technical Appendix A of this chapter.

Data analyses

A bean plot from "R" statistical software (version 3.3.2) was used to visualise data from the HCPs survey (32). DALY estimates were computed in "R" software environment for statistical computing and graphics using a DALY- calculator (30) set at 20,000 iterations. Data from epidemiology (RBC), survey with HCPs, ORS sales records, health reports and literature were reorganised to suit the inputs parameters of the DALY calculator. Mortality rates and the associated uncertainty were computed using @risk 7.5 software (Palisade corporation, USA) at 20,000 iterations (see Technical Appendix A, section 4.8, this Chapter). In IBM SPSS Statistics 22, one way ANOVA followed by Tukey's post hoc tests, was used to determine statistical significance between the number prescriptions/1000 persons/year of drugs among the age groups and drug categories. Statistical significance was set at 0.05.

Results

Opinion survey with HCPs

Figure 2.2, shows the % prevalence of watery diarrhea, bloody diarrhea, and typhoid fever as perceived by HCPs in the year 2013. Results show perceived prevalence of watery diarrhea with mean perceived prevalence around 40% and estimates of HCPs divided among all the prevalence categories except for the 0% category (not seen any case). For bloody diarrhea and typhoid fever the mean perceived prevalence was around 10% and estimates of most HCPs concentrated around the 0-1 % category.

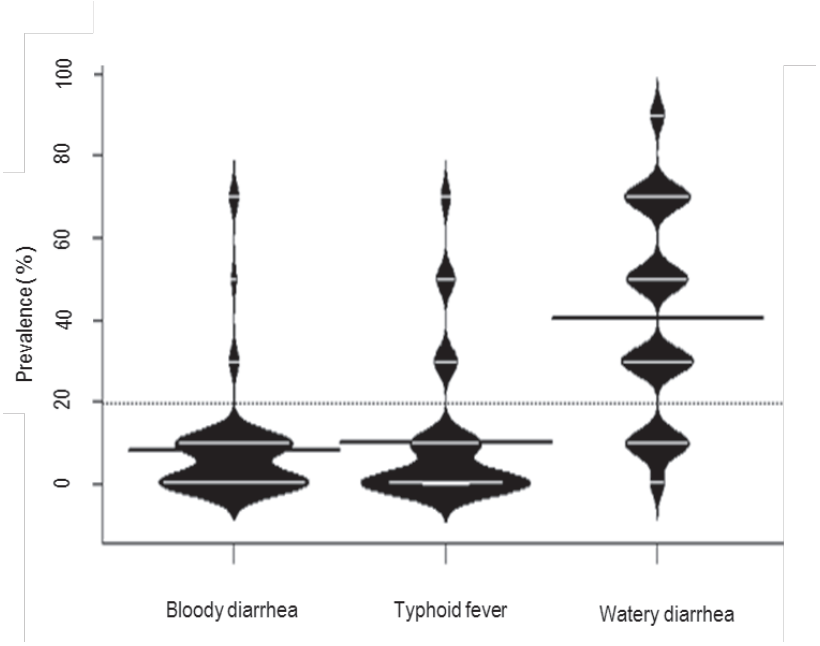


Figure 2.2: Prevalence (%) of clinical features related to foodborne pathogens as from an opinion survey with health care providers (HCPs, n=128) in health facilities across Rwanda for the year 2013. The number of "bean pods" show the difference in opinion of HCPs on the % prevalence of each clinical feature, while the size and shape of the "bean pods" shows the number of HCPs who chose a particular % prevalence category. White bands in each "bean pods" show the median. Black line crossing "bean pods" is the overall mean % prevalence for each clinical feature. The dotted line horizontal line, is the overall mean % prevalence of all the clinical features. Prevalence = estimated number of patients with clinical feature out of the total patients received in 2013 per HCP.

Reported cases of food related illnesses, year 2013

Figure 2.3 shows the cases for watery diarrhea, bloody diarrhea, cholera and typhoid fever reported in the year 2013 to the national notifiable surveillance system of the epidemic surveillance and response division of RBC from country wide health facilities.

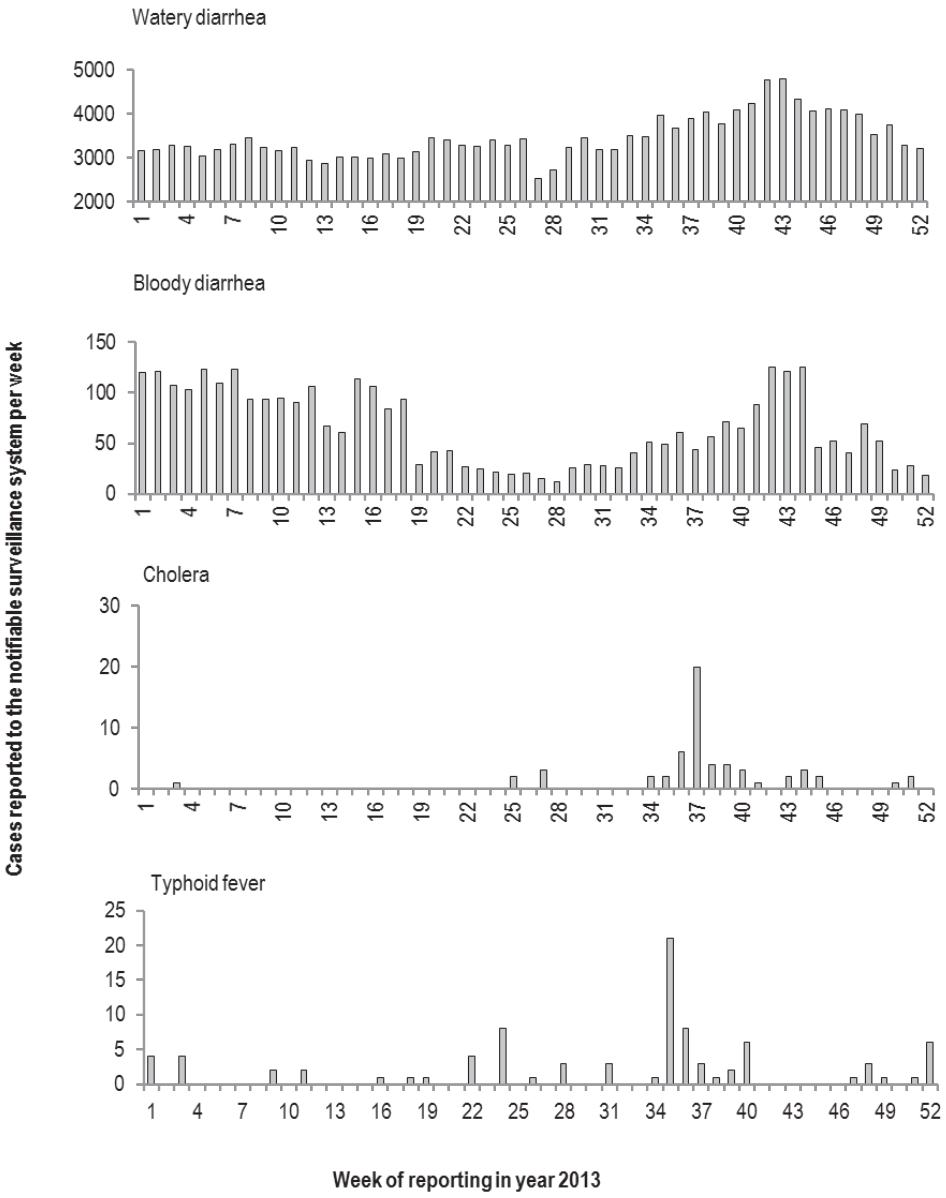


Figure 2.3: Cases of watery diarrhea, bloody diarrhea, cholera and typhoid fever reported weekly by country wide health facilities to the Epidemic Surveillance and Response Division of Rwanda Biomedical Centre (RBC) in the year 2013.

The reported cases of these illnesses were reported out of the whole population which was estimated to be around 10.5 million inhabitants (29). The annual reported cases for watery diarrhea were 180,121 cases with a weekly reported average of 3464 cases. For bloody diarrhea, annual reported cases were 3395, with a weekly reported average of 65 cases. Cholera and typhoid fever occurred sporadically with an average of 1 case and 2 cases per week respectively, with peaks at the second half of the year.

Trends of drugs prescribed for food related illnesses

Figure 2.4 shows that antibiotics had the highest monthly prescriptions/pharmacy that peaked in the second half of the year compared to anthelmintic and antiprotozoal drugs that remained relatively stable throughout the year. In Figure 2.5, the number prescriptions/1000persons/year were also significantly higher ($p < 0.05$) for antibiotics compared to antiprotozoal and anthelmintics across all age groups. Although not statistically significant ($p>0.05$), the age groups of 30 to 39 years registered the highest number of prescriptions/1000persons/year, when all the three drug categories (antibiotics, antiprotozoal and anthelmintics) were combined.

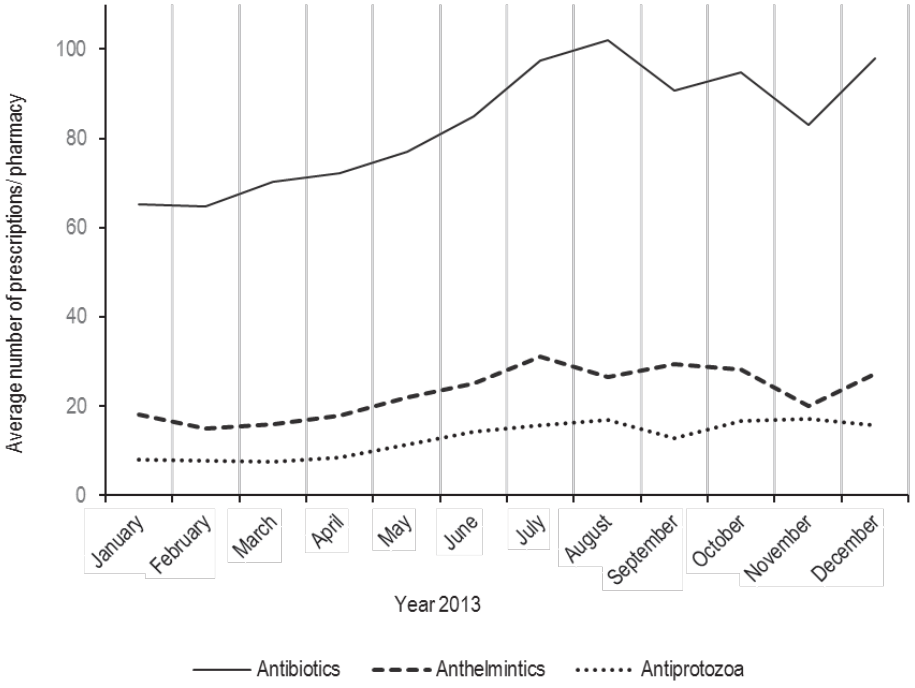


Figure 2.4: Monthly prescription of antibiotics, anthelmintic and antiprotozoal as by insurance claim forms received at Rwanda Social Security Board (RSSB) for the year 2013 from 37 major pharmacies country wide. The RSSB insurance scheme was estimated to cover 3.7 % of the population in Rwanda, year 2010 (28).

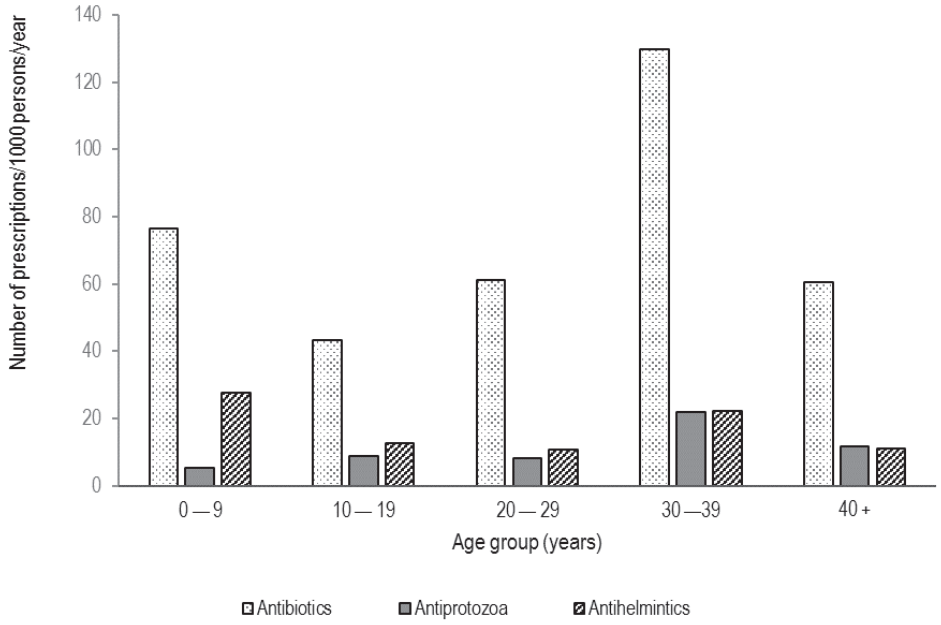


Figure 2.5: Number prescriptions for antibiotics, anthelmintic and antiprotozoal per age group of patients as by insurance claim forms received at Rwanda Social Security Board (RSSB) for the year 2013.

Estimates for YLD, YLL and DALY of food related illnesses

Table 2.1 shows the details of our estimates for YLD, YLL, DALY and deaths due to watery diarrhea, bloody diarrhea and cholera from RBC, HCPs and ORS prescription data and typhoid fever from RBC, HCPs data. DALY estimates per million inhabitants from RBC data on average were highest for watery diarrhea (672) followed by bloody diarrhea (213), typhoid fever (73) and cholera (1) with the YLL component contributing to over 90% except for cholera. Table 2.1 also shows that with HCPs data, the YLD component is more pronounced in the DALY estimates compared to RBC (surveillance) data. Cases from ORS prescription data resulted to DALY estimates in the same range (approximately 1.2: 1) with the DALY estimates from combined cases of watery diarrhea, bloody diarrhea and cholera from RBC data (Tab. 2.1). Figure 2.6 provides a graphical comparison of the DALY estimates, from RBC, HCPs data for watery diarrhea, bloody diarrhea, typhoid fever. DALY estimates from HCPs survey were in the same range with the DALY from RBC data, while for typhoid fever, HCPs survey DALY estimates were 15 times higher than the DALY from RBC data.

Table 2.1. Estimates of YLD, YLL, DALY and deaths per million inhabitants⊠, caused by food related illnesses in Rwanda circa 2013

	Output estimates							
	YLD		YLL		DALY		Deaths	
Illness	mean	95% CrI ᵠ	mean	95% CrI ᵠ	mean	95% CrI ᵠ	mean	95% CrI ᵠ
Watery diarrhea								
RBC data*	74	21 — 163	598	355 — 842	672	424 — 932	9	6 — 12
HCPs opinion survey ǂ	427	90— 1,128	601	358 — 840	1,028	573 — 1,759	,,	,,
Bloody diarrhea								
RBC data*	1	0 — 2	213	49 — 474	213	50 — 475	3	1 — 7
HCPs opinion survey ǂ	21	1 — 88	213	48 — 474	234	63 — 499	,,	,,
Typhoid fever								
RBC data*	3	1 — 10	70	54 — 86	73	57— 91	1	1 — 1
HCPs opinion survey ǂ	1,018	7 — 4,273	70	54 — 86	1,088	78— 4,340	,,	,,
Choleraᵞ								
RBC data	1	0 —1	0	0 — 0	1	0 — 2	0	0 — 0
Diarrheal diseases Ω								
RBC data*	76	23 — 165	811	499 — 1,160	887	566 — 1,242	12	8 — 17
ORS prescription ⏥	35	8 — 78	1,010	751 — 1,327	1,045	783 — 1,364	16	13 — 20

* RBC data: Estimates generated from surveillance data submitted weekly to Rwanda Biomedical Centre in 2013.
ǂ HCPs opinion survey: Estimates generated from data obtained from opinion survey with Health care providers in Rwanda first three months of 2014
ᵞ Cholera: Opinion survey with HCPs did not cover Cholera
ᵠ 95% CrI: 95% Credible interval representing the 2.5 and 97.5 percentiles.
,, Opinion survey with HCPs did not cover estimates of number of death, so same mortality rates were used as for RBC data.
⊠ The population of Rwanda in 2012 was reported to be around 10,515,973 inhabitants by the National institute of statistics of Rwanda (NSIR)
Ω Estimates for diarrheal diseases were computed from cases with clinical features (watery and bloody diarrhea) and suspected cases of cholera illness
⏥ ORS prescription: Estimates generated from oral rehydration salts pharmacy prescription records.

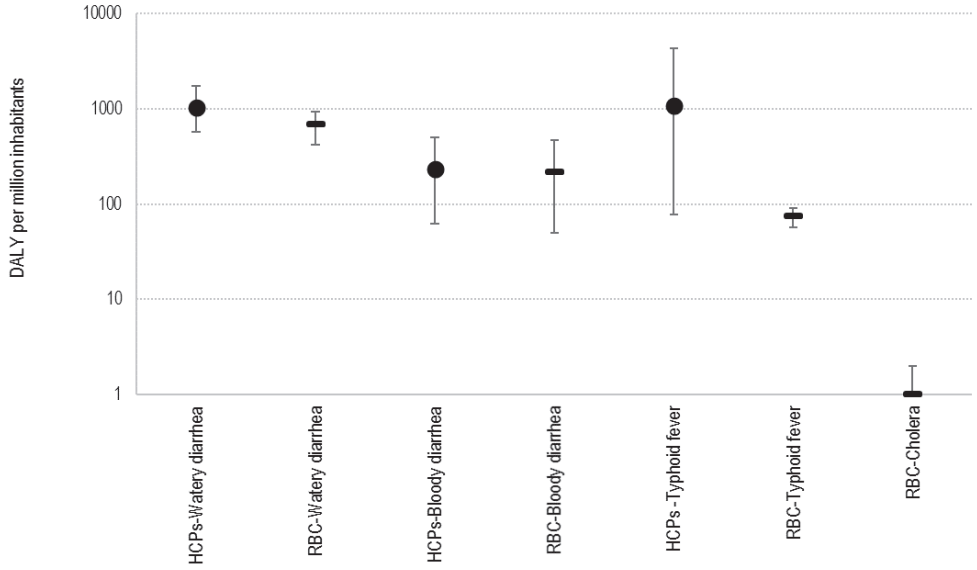


Figure 2.6: Estimates of DALY for clinical features (watery diarrhea and bloody diarrhea) and illnesses (typhoid fever and cholera) related to foodborne pathogens, generated by computing data from both the Epidemic Surveillance and Response Division of Rwanda Biomedical Centre (RBC) and the Opinion survey with health care providers (HCPs) in the year 2013. Estimates are presented on a logarithmic scale on the y-axis. Whiskers represent 95% credible intervals.

Discussion

Study findings

In this study we have presented the reported cases and the opinion of HCPs on the prevalence of selected food related illnesses and estimated YLD, YLL and DALY for watery diarrhea, bloody diarrhea and cholera, ORS prescription cases and typhoid fever for the year 2013. In addition, trends in drug prescription for antibiotics, antiprotozoal and anthelmintics in 2013 have also been presented.

Watery diarrhea had the highest number of cases and occurred all year round. Bloody diarrhea was seasonal coinciding with the rainy season in Rwanda, while cholera and typhoid fever occurred were sporadic. The results of the reported cases seem to be in agreement with the estimates of % prevalence from our HCPs survey and DALY estimates. Watery diarrhea had the highest mean prevalence (Fig. 2.2) from the HCP survey. The high number of cases and DALY estimates coupled with the year round occurrence of watery diarrhea compared to other studied illnesses may be attributed to the vast range of causative agents (26) with a high prevalence especially in developing countries. In Egypt, one of the most commonly aetiological agents for watery diarrhea, enterotoxigenic *E. coli* (heat labile toxin strains of ETEC), was reported to be prevalent in similar levels all year round among hospitalized children (33).

Rotavirus, one of the major causes of profuse watery diarrhea was detected in 39 – 52% of hospitalized children in Ghana, Kenya, Uganda and Zambia in 2008 (34). Studies from different countries have reported prevalence of 14.9% for ETEC in rectal swabs of hospitalized acute diarrhea patients in Indonesia (35) and 9.3% for non typhoidal *Salmonella* in faecal samples of diarrhea patients in Chad (36). For bloody diarrhea compared to watery diarrhea, few aetiological agents are commonly reported, *i.e.*. bacteria (*Shigella* spp., Enterohemorrhagic *E. coli*) (37, 38), parasites (*Entamoeba histolytica*) (39) and the seasonal trends may be explained by the reported connection of these aetiological to weather seasons (40). Cholera cases peaked in the last quarter of the year (September to December) during the rainy season in Rwanda. Similar trends were reported in Bangladesh (41) and Kenya (42) but differed from the trend reported in Ghana (40). Typhoid fever outbreaks were dispersed over the year but like cholera also peaked in the rainy season of the final quarter of the year similar to findings from India (43) and Zimbabwe (44). The occurrence of these sporadic cases of typhoid and cholera during the rainy season has been linked to the flooding of water sources and increased runoff water in situation were surface water is used for household sores, drinking and food preparation (45-47).

From previous studies, trends of local sales for weekly aggregate OTC drugs were comparable to the outbreak epidemic curves (48). The observed trends in prescription per pharmacy or per age group for antibiotics, antiprotozoal and anthelmintic can therefore also provide a hint about the manifestation of these aetiological agents, although it is not clear whether they are food related or not. OTC drug sales method has been identified as one way in which community health information on illnesses can be obtained even before medical care and notification (48). In this study the peak in prescriptions of antibiotics in the second half of the year coincided with the peak in reported cases for bloody diarrhea, typhoid and cholera. The age of 0-9 and 30-39 year had the highest number of prescriptions for antibiotics. These findings correlate with the incidence of invasive non typhoidal *Salmonella* spp. reported from other nearby Africa countries (Malawi and South Africa) for these age groups (49).

Reported cases of watery diarrhea were 50 times higher than the cases of bloody diarrhea, but in terms of DALY estimates, watery diarrhea was only greater than bloody diarrhea by a DALY factor of 3. This trend can explain why it is not enough to look at only the number of cases, prevalence or incidence without considering the other aspects of the disease envelope, especially the disease severity. The DALY estimates therefore provide a risk-based perspective of the impact of each illnesses on the population health of a country (18). In prioritizing interventions, policy makers can be advised to address the causes of the studied illnesses in the order of watery diarrhea — bloody diarrhea — typhoid fever — cholera. In most of our DALY estimates, the YLL component was greater than YLD by over 80%. This can be possible because the illnesses in this study were acute with a short duration to influence the YLD component or these illnesses mainly cause death in children resulting to large YLL estimates as it has been reported that children under 5 year old bear a greater burden of foodborne diseases in the Sub Saharan African regions (2).

Our DALY estimates from HCPs survey were in a close range of about 1 to 1.5 fold with the DALY estimates from reported cases (RBC) for watery diarrhea and bloody diarrhea. The same trend was observed for the DALY estimates to combined cases of watery diarrhea, bloody diarrhea and cholera combined) from RBC data compared to the DALY estimates from ORS prescription. This comparison suggests that HCP data can also be used. However, HCP opinion

survey should be used with caution for illnesses which occur sporadically where some HCPs can finish the whole year without handling a single case. In this study, the sporadic occurrence of typhoid fever (Fig. 2.3) can explain the large 95% credible interval and the significant difference observed between the DALY estimates for typhoid fever from HCPs and RBC data (Fig. 2.6). Our study has demonstrated that different syndromic surveillance data sources (epidemiology, opinion survey and drug prescriptions) can be used for DALY estimates. This approach can be effective in situations where syndromic data is readily available compared to the desired integrated food chain and laboratory based surveillance data (24).

In Table 2.2, we compared our DALY estimates from notifiable surveillance cases (RBC) with the DALY of comparable illnesses reported in previous studies. In this study, DALY estimates for combined cases of watery diarrhea, bloody diarrhea and cholera were 40 times lower compared to the DALY of diarrhea diseases reported by von Witzke et al. (12) in Rwanda, year 2000 (Tab. 2.2). This decline in DALY estimates for diarrhea over the years is in agreement with what was reported by the Institute for Health Metric and Evaluation (IHME) for the period between 1990 to 2013 (50). Rwanda was also classified among countries in Sub-Saharan Africa that have DALY estimates of diarrheal diseases significantly lower than the mean (50). In addition, our DALY estimates for diarrhea in Rwanda were 20 times lower than the DALY estimates from IHME in 2013 (51). This difference in DALY is justifiable because in the IHME study the deaths estimates used were 10 times more than our study and deaths numbers as observed from this study greatly influences the DALY outcome of short term illnesses. Furthermore, the DALY estimates for typhoid fever in this study were about 15% of the 2010 estimates from each of the two WHO Africa regions (Tab. 2.2). Considering that there are more than 20 countries in each of these WHO regions (2), these findings support what Havelaar et al. (2) acknowledged that the estimates in their study were conservative and more on the side of underestimation. Comparing DALY estimates across different studies at the moment, should also be done with caution due to the differences in methodology and approach. Nevertheless, continued comparison of DALY estimates can be a basis for setting public health objectives, contribute to the quest to harmonise study approaches and act as a reminder of the gap in estimating the real burden of foodborne illnesses.

Table 2.2. Comparison of incidence based DALY estimates for food related illnesses in Rwanda and other countries/regions

Illnesses	DALY per million Inhabitants	Reference year	Country/ Region	Reference
Diarrheal diseases				
Diarrheal diseases ǂ	8,890 [95 % CrI 1960 – 17,310]	2010	WHO-AFR D	(Havelaar et al., 2015)
Diarrheal diseases ǂ	8,240 [95 % CrI 4,470 – 13,260]	2010	WHO-AFR E	(Havelaar et al., 2015)
Diarrheal diseases ǂ	51,582	2012	WHO-African region	WHO, 2014
Diarrheal diseases ǂ	99,909	2000	WHO-African region	WHO, 2014
Diarrheal diseases ǂ	38,189	2000	Rwanda	(von Witzke et al., 2005)
Diarrheal diseases ǂ	20,281 [95 % CrI 11,536 – 31,265]	2013	Rwanda	(IHME, 2016 b)
Diarrheal diseases*	887 [95% CrI 566 – 1,242]	2013	Rwanda	This study
Typhoid fever				
Typhoid fever ǂ (as *Salmonella* typhi)	470 [95% CrI 0 – 1690]	2010	WHO-AFR D	(Havelaar et al., 2015)
Typhoid fever ǂ (as *Salmonella* typhi)	520 [95% CrI 0 – 1870]	2010	WHO-AFR E	(Havelaar et al., 2015)
Typhoid fever*	73 [95% CrI 57 – 91]	2013	Rwanda	This study

* Estimates generated from surveillance data submitted weekly to Rwanda Biomedical Centre in 2013. Estimates in this study for diarrheal diseases were computed from cases with clinical features (watery and bloody diarrhea) and suspected cases of cholera disease.
ǂ Data expressed per million inhabitants
DALY, disability-adjusted life years; CrI, credible interval; WHO-AFR D, World Health Organisation African Region D; WHO-AFR E, World Health Organisation African Region E

Data gaps and limitation of the study

For all the reported cases, we assumed that all diarrheal cases are mainly infectious in origin as in most developing countries (26). Even with the assumed infectious nature of these diarrheal cases, it was not possible to underpin and rank from this study the most important foodborne pathogens. Various foodborne pathogens can lead to diarrhea. For example, there is need to investigate the burden of non-typhoidal *Salmonella* in additional to the already existing surveillance efforts of typhoid fever (targeting *Salmonella* Typhi). We used population survey studies in USA for the multiplier for underreporting due to medical seeking behaviour and underdiagnosis. Discrepancies may come the fact that multipliers from a developed country were used in this study, this approach may result to underestimation of the burden. DALY estimates in this study would improve if population surveys in Rwanda cover medical seeking behaviours for different illnesses in future. We also assumed that all cases of illnesses registered at health facilities were reported to the notifiable surveillance system at RBC. During this study, the data from the notifiable surveillance system was not stratified by age and sex, so is our DALY estimates. Since diarrheal diseases can also originate from non-food sources such as unhygienic shared sanitation facilities and open defecation (52), attributing cases to sources will provide the clear burden estimates according to sources. For all illnesses, we assumed that all cases in this study were acute and therefore the duration of chronic cases and their related sequelae not considered. We used the WHO deaths estimates per age group (53) to distribute per age group the total deaths reported by the MoH for each studied illnesses. Registering deaths according to aetiological agents, vehicles and demographics will also lead to improved estimates in future.

For the over the counter (OTC) drug prescription/sales records, the data was paper based and collecting data from individual pharmacies was not possible. We were able to cover the year 2013 in 37 pharmacies for affiliates and dependants (about 3.7% of population of Rwanda) (28) of the public insurance scheme at Rwanda Social Security Board. Data collection would have been easier and more accurate if all pharmacies had electronic records of sales and prescriptions that are submitted to a one stop centre at a regulated time, but this study shows that even in the absence of an electronic system, pharmacy data can be used to estimate food related illnesses.

In this study we have used data from three syndromic surveillance sources, *ie.* data from the notifiable surveillance system at RBC, OTC drug prescription and from opinion survey with HCPs to estimates outcome based DALY. Each data source has its advantages and dis advantages. Notifiable surveillance data are easy to retrieve if the electronic reporting system is working (54). However the data from the surveillance system have to be supported by population surveys to determine medical seeking behaviour (55) and in some cases there are under notification and underdiagnosis from health facilities (15, 56). Data from drug sales can be more representative and may not require adjusting for medical seeking behaviour especially in situations where patients can access pharmacies with or without HCP's advice. The drawbacks for this data are that it is difficult to collect data from private and public pharmacies,

and some patients repetitively buy drugs without HCP consultation and this can hype the number of cases and at times misdiagnosis. Opinion survey data requires no pre-existing data collection infrastructure but the data generated is very subjective and greatly influenced by trends, seasons, location for the illnesses understudy. If a survey is conducted in areas where an outbreak has just occurred, the illness understudy may be accorded unrealistic high incidence. In Table A.4 of the Technical appendix A, high and fluctuating incidences of the studied illnesses can be observed.

Conclusion and recommendations

We have provided trends and estimates for the burden of food related illnesses in Rwanda for the year 2013 based on syndromic surveillance data. We envision that the data gaps identified, and the methodology used in this study will guide future studies on estimating the burden of foodborne illnesses in Rwanda and other developing countries. It is important to note that as developing countries aspire to acquire laboratory based- and integrated food chain disease surveillance systems, the data from the cheaper and available syndromic surveillance data sources can still be used to provide guiding estimates. As recommended by WHO (24), estimating the burden of disease is a step in the right direction towards introducing integrated food chain surveillance systems. Integrated food chain surveillance systems will prepare these countries to address the current and future challenges from foodborne illnesses. This preparedness will involve national multi-task teams (HCPs, food safety experts, academia, agriculturalists) to collect pathogen information from humans, animals and plants. This information will support improved studies to estimate the burden of foodborne diseases based on pathogen identification and ranking, source attribution, acute disease and sequelae ranking, all with aim of supporting evidence based public health policies.

Disclaimer. The findings and conclusions in this study are those of the authors and do not necessarily represent the official position of the institutions where input data were sourced.

Appendix

Technical Appendix A

Outcome based DALY estimates.

In this appendix, the methodology used to estimate outcome based DALY is presented for watery diarrhea, bloody diarrhea, cholera and typhoid fever in Rwanda, year 2013. Syndromic surveillance data was used in this study. Data sources were the epidemic surveillance and response division of Rwanda Biomedical Centre (RBC), an opinion survey with health care providers (HCPs), prescription records for oral rehydration salts (ORS), Rwanda Ministry of Health reports (MoH), Rwanda National Institute of statistics reports (28, 29), the World Health Organisation (WHO) and data in other published literature. DALY estimates were computed using the R-DALY calculator (57).

1. **Glossary**

   In the context of this study, the following terms and abbreviations were defined/applied as follows:

   Age weighting, refers to a factor which can be used in DALY calculations to carter for the relative value of a year of healthy life lived at different ages (58).

   Bloody diarrhea. Case refers to any person with diarrhoea and whose stool contains visible traces of blood.

   Case fatality rate (CFR). Proportion of reported cases of a specified disease or condition which are fatal within a specified time. CFR in this study are used in percentage proportions (%).

   Cholera. Suspected cases, a patient aged 5 years or more develops severe dehydration or dies from acute watery diarrhoea. A case of cholera is confirmed when *Vibrio cholerae* O1 or O139 is isolated from any patient with diarrhoea (59). DALY calculations in this study included the 0-4 years age group considering that all age groups can suffer from cholera.

   DALY (**Disability-Adjusted Life Years)**, summary metric of population health which measures how many years of healthy life are lost due to premature death and or life lived with the disease and or disability. DALY are the sum of two components: years of life lost due to premature mortality (YLL) and years lived with disability (YLD) (31).

   DALY calculator, a DALY package for calculating DALY and performing uncertainty and sensitivity analyses (57).

   Disability weight (DW) is a weighing factor that reflects the severity of the disease on a scale from 0 (perfect health) to 1 (equivalent to death). Disability weights are generated through large-scale empirical investigation in which judgments about health losses associated with many causes of disease and injury are elicited from the general public in diverse communities (60).

   Discounting for time, refers to the inclusion of discount rates in DALY calculations to carter for future health life years lost (61). Discounting for time in DALY calculations is borrowed from economic principles (58).

   Duration of disease, refers to the time interval from manifestation of the illness to the time of healing for nonfatal cases or death for fatal cases**.**

Food-related pathogens, refers to aetiological agents that can cause human illnesses/diseases through food and other sources.

Food related illnesses, refers to the illnesses that can be caused mainly through food and water notwithstanding other causes. Because non confirmed cases are used in this study, the term "food related illnesses" is used instead of foodborne illnesses.

GBD (Global Burden of Disease) a systematic, scientific effort to quantify the comparative magnitude of health loss due to diseases, injuries, and risk factors by age, sex, and geographies for specific points in time (31).

HCPs: Health care providers, including general practising doctors and grade 1 nurses

Incidence rate, new cases of disease occurring per unit of population, per unit time (number of new cases per 1,000 persons per year).

GBD 2010, life expectancy table. Represents the aspiration for healthy lifespan for all individuals, both female and male (31).

MoH: Ministry of Health of Rwanda (http://www.moh.gov.rw/index.php?id=2).

Mortality, number of deaths due to a specific disease in a population per unit time. (Units in this study were, deaths per 1,000 persons per year)

Multiplier, refers to the multiplication factor used to upscale the reported numbers to carter for the magnitude of underestimation due to medical seeking behaviour (15) and underdiagnosis.

NISR: National Institute of Statistics of Rwanda, a public institution in Rwanda mandated to collect, analyse and disseminate, among others duties, information on population housing census, demographic health survey and population conditions of living (http://www.statistics.gov.rw/).

Onset of disease, age of onset in years is the age at which an individual acquires, develops, or first experiences a condition or symptoms of a disease.

ORS (Oral rehydration salts), a glucose electrolyte solution given to patients by mouth to combat dehydration especially due to diarrhea (62).

Perceived prevalence, in this study perceived prevalence refers to the proportion of individuals in the population, suffering from the illnesses under study in a given period, based on the opinion or experience of health care providers.

PERT distribution (Program Evaluation and Review Technique). A probability distribution which is based on the β distributions, originally referring to a project management tool (63). The PERT distribution can be specified either using a minimum, maximum and modal value, or by three percentile points, such as a median value and 95% credible intervals. As from Kirk et al. (64) we used this distribution widely in our analysis, as it allows for asymmetric distributions.

RBC (Rwanda Biomedical Centre), a public institution in Rwanda under the Ministry of Health, mandated among others duties to conduct syndromic- and epidemic surveillance and response (http://www.rbc.gov.rw/).

Treatment proportion, the proportion of individuals in the whole population, suffering from the illnesses under study, that seek medical care.

Typhoid fever. Suspected case, any person with gradual onset of steadily increasing and then persistently high fever (38°C and above), chills, malaise, headache, sore throat, cough, and, sometimes, abdominal pain and constipation or diarrhoea. Confirmed case: Suspected case confirmed by isolation of *Salmonella* typhi from blood, bone marrow, bowel fluid or stool (65).

Watery diarrhea. Case refers to a person with three watery stools or more within 24 hours.

WHO, the World Health Organisation of the United Nations.

YLD (Years Lived with Disability) is a component of DALY estimates computed by multiplying the number of incident cases of a given illness in a population with the disability weight (DW) and the average duration of the case until remission or death (years) (31).
YLL (Years of Life Lost due to premature mortality), is a component of DALY estimates, computed by multiplying the number of deaths at a given age with the standard life expectancy at that age (31).

2. Adjusting raw data from the national notifiable surveillance system at Rwanda Biomedical Centre (RBC) to carter for under reporting.

In Rwanda it is mandatory for health care facilities to report all cases of watery diarrhea, bloody diarrhea and suspected cholera and typhoid fever to the epidemic surveillance and response division of Rwanda Biomedical Centre (RBC), but due to factors like medical seeking behaviour, it is possible that some cases of these illnesses within the population are not reported. In other studies, to carter for this data gap, researchers have used underreporting factors or multipliers obtained from population studies or assumptions (23, 64, 66). Cognizant of the possible underreporting in the RBC raw data and the paucity of population data on medical seeking behaviour for these illnesses in Rwanda , we multiplied the numbers of cases for every illness with a multiplier derived from previous studies in other countries as shown in Table A.1. but all working calculations were in Microsoft excel. It was assumed that all cases for patients who seek medical care for watery and bloody diarrhea are reported to RBC. For typhoid fever and cholera which are attributed to specific pathogens (*Salmonella* typhi and *Vibrio cholerae*), we took are more sensitive approach and adjusted the raw data to carter for underdiagnosis (Tab. A.1) even when this study is based on all suspected cases.

Table A.1. Number of cases of food-related illnesses reported to Rwanda Biomedical Center (RBC) in 2013 adjusted with multipliers

Illness	Numbers of cases Reported at RBC, 2013	Justification for multipliers used in this study and data source (s)	Derived multiplier	Total adjusted number of cases (rounded off)
Watery diarrhea	180,121	We derived the multipliers from FoodNet –USA population in surveys of year, 2000-2001, 2002-2003 and 2006-2007 for the proportion of survey respondents with **non-bloody diarrhea** who sought medical care. Proportions (95% credible interval) were 0.15, 0.18, and 0.20 for low, modal and high values respectively (23).	PERT distribution Low= 5.00 Modal= 5.56 High= 6.67	PERT distribution Low= 180,121×5.00 **=900, 605 cases** Modal= 180,121 ×5.56 **=1, 001, 476 cases** High= 180,121× 6.6667 **=1, 201, 407 cases**
Bloody diarrhea	3,395	We derived the multipliers from FoodNet –USA population in surveys of year, 2000-2001, 2002-2003 and 2006-2007 for the proportion of survey respondents with **bloody diarrhea** who sought medical care. Proportions (95%	PERT distribution Low= 1.96 Modal= 2.86 High= 5.26	PERT distribution Low= 3,395 ×1.96 **=6,654 cases**

		credible interval) were 0.19, 0.35 and 0.51 for low, modal and high values respectively (23).		Modal= 3,395 ×2.85 **=9710 cases** High= 3,395 × 5.26 **=17, 858 cases**
Cholera	58	Due to severity and outbreak nature of cholera, we assumed 100% medical seeking and hence multiplier of 1.	Constant 1.00	PERT distribution Low= 58 × 18.4 **=1067 cases** Modal= 58 ×33.1 **=1920 cases** High= 58 × 57.5 **=3335 cases**
		To carter for under-diagnosis factor, we used under diagnosis factor of 33.1 (18.4 -57.5) (23)	PERT Low= 18.4 Modal= 33.1 High= 57.5	
Typhoid fever	88	We derived multipliers from the approach of Scallan et al. (2011) where for *Salmonella enterica*, serotype Typhi, medical seeking proportion due to both bloody and non-bloody proportion were considered from FoodNet –USA population in surveys of year, 2000-2001, 2002-2003 and 2006-2007. Bloody diarrhea, 95% credible interval, (0.19, 0.35 and 0.51) and non-bloody diarrhea (0.15, 0.18, and 0.20). The modal of these values (0.15, 0.18, 0.19, 0.20 0.35, 0.51) was assumed to be equal to the mean and a PERT distribution was obtained as follows: 0.15, 0.26, 0.51 for low, modal and high values respectively	PERT distribution Low= 1.96 Modal= 3.85 High= 6.67	PERT distribution Low= 88 ×1.9608 × 7.6 **=1311 cases** Modal= 88 ×3.85 × 13.3 **=4506 cases** High= 88 × 6.6667 × 38.4 **=22539 cases**
		To carter for underdiagnosis factor, we used under diagnosis factor of 13.3 (7.6 - 38.4)(23)	PERT distribution Low= 7.6 Modal= 13.3 High= 38.4	

3. **Adjusting data from prescription of Oral rehydration Salts (ORS) in year 2013**

From the average annual prescription of ORS, we also derived possible number of cases due to diarrheal diseases, assuming all the ORS prescriptions in records of Rwanda Social Security Board (RSSB) was for diarrhea patients. Calculations for the numbers of cases (ORS-cases) shown in Table A. 2 were based on the approach in section 2 above.

Table A.2. Number of cases of diarrheal diseases derived from prescription of ORS in RSSB records in 2013, adjusted with multipliers

Illness	Numbers of cases	Justification for multipliers used in this study and data source (s)	Derived multiplier	Total adjusted number of cases (rounded off)
Diarrheal diseases	Monthly average prescription of ORS/ pharmacy (2013) = **3 cases/ pharmacy/month** Approximate number of pharmacies in Rwanda = **440 pharmacies** **ORS cases = 440 × 3 × 12 = 15,840 cases**	We derived the multipliers from FoodNet –USA population in surveys of year, 2000-2001, 2002-2003 and 2006-2007 for the proportion of survey respondents with both **non-bloody diarrhea and bloody diarrhea** who sought medical care. **Non bloody diarrhea**. Proportions (95% credible interval) were 0.15, 0.18, and 0.20 for low, modal and high values respectively (23). **Bloody diarrhea**. Proportions (95% credible interval) were 0.19, 0.35 and 0.51 for low, modal and high values respectively (23). We choose values: **Low = 0.15** **Modal** = Average (0.15, 0.18, 0.20, 0.19, 0.35, 0.51) = **0.26** **High = 0.51**	PERT distribution Low= 1.96 Modal= 3.87 High= 6.67	PERT distribution Low= 15,840 × 1.96 × 3.64 **=113,009 cases** Modal= 15,840 × 3.87 × 3.64 **=223,135 cases** High= 15,840 × 6.67 × 3.64 **= 384,576 cases**
		Multiplier for ORS use in Rwanda. From MoH in 2014-2015, ORS use was around 27.5% (67)	Constant 3.64	

4. General input parameters for the DALY calculator

4.1. Calculation of incidence from RBC data and ORS prescription (cases/1000persons/year)

We calculated the incidence as follows (Eq. A.1):

Equation A.1

$$Incidence = \frac{Number\ of\ cases}{Population\ under\ study} \div {}^{1}/_{1000\ persons} \div {}^{1}/_{year}$$

The RBC data was not age or gender specific, so in calculating incidence, we chose to consider the whole population of 10,515,973 inhabitants in 2012 (29) without age/gender stratification.

Table A.3. Incidence of food-related illnesses in 2013

Illness	Total adjusted number of cases(rounded off)	Incidence (cases/1000persons/year)
Watery diarrhea	PERT distribution Low =**900, 605 cases** Modal =**1, 001, 680 cases** High =**1, 200, 812 cases**	PERT distribution Low = 85.64 Modal = 95.25 High = 114.19
Bloody diarrhea	PERT distribution Low =**6,657 cases** Modal=**9670 cases** High=**17, 869 cases**	PERT distribution Low = 0.633 Modal = 0.922 High= 1.699
Cholera	PERT distribution Low = **1067 cases** Modal=**1920 cases** High==**3335 cases**	PERT distribution Low = 0.1015 Modal = 0.183 High= 0.317
ORS-diarrhea	PERT distribution Low =**112,948 cases** Modal =**221,543 cases** High = **384,361 cases**	PERT distribution Low = 10.74 Modal = 21.08 High = 36.6
Typhoid fever	PERT distribution Low =**1311 cases** Modal =**4502 cases** High =**22528 cases**	PERT distribution Low = 0.125 Modal = 0.428 High = 2.142

4.2. Incidence estimates from opinion survey data with health care providers (HCPs)

Perceived prevalence of watery diarrhea, bloody diarrhea and typhoid fever were obtained by administering a questionnaire (section 6 of this appendix) in early 2014 to randomly selected health care providers (HCPs) (n=128) who conduct consultancies with patients at their respective health facilities (private and public hospitals, clinics and health centres) in Kigali city and in the other four provinces of Rwanda. Highly specialised HCPs were excluded from the survey as they routinely deal with specialized illnesses. Table A.4 shows the frequency of choice for every prevalence percentage range by HCPs for watery and bloody diarrhea and typhoid fever. Cholera was not included in the study. The incidence (**cases/1000persons/year**) for watery diarrhea and bloody diarrhea and typhoid fever was determined from the obtained data as shown in Table A.4 (All working calculations were performed in Microsoft excel)

Table A.4. Results of opinion survey with health care providers and calculation of estimated cases for the different illnesses

Percentage (%) range as in questionnaire	**Average % value.**	**Number of health care providers who chose a percentage range as perceived prevalence for a particular illness**		
		Watery diarrhea	**Bloody diarrhea**	**Typhoid fever**
0	0 %	0	0	22
0-1	0.5%	6*	66	53
1-20	10%	28	52	30
20- 40	30%	32	4*	11
40-60	50%	27	2*	8*
60-80	70%	29	4*	4*
80-100	90%	6*	0	0
Total number health care providers interviewed		**128**	**128**	**128**
perceived % prevalence of illness in Rwanda in 2013.		PERT distribution Low= 10% Modal= 30% High= 70%	PERT distribution Low= 0.5% Modal= 0.5% High= 10%	PERT distribution Low= 0 % Modal= 0.5 % High= 30%

Number of Cases According to the MoH, the HCP (nurses) to patients ratio was around 1 to 1,291 by year 2012 (68, while the total number of HCPs who do patient consultancies was 2302 (509 general practitioners + 1793 grade A1 nurses) {, #3542): Therefore Number of cases = Mean perceived % prevalence × Total number of possible patients per HCP (1,291) × Total number of HCPs	PERT distribution Low= 297,188 cases Modal= 891,565 cases High= 2,080,317 cases	PERT distribution Low= 14,859 cases Modal= 14,859 cases High= 297,188 cases	PERT distribution Low= 0 cases Modal= 14,859 cases High= 891,565 cases
Number of cases considering under reporting due medical seeking behaviour **Same underreporting factors as in Table A.2**	PERT distribution Low = 297,188 × 5 **=1.49×10^6 cases** Modal= 891,565 ×5.55 **= 4.95×10^6 cases** High= 2,080,317 ×6.67 = **1.39×10^7 cases**	PERT distribution Low= 14,859 × 1.96 **= 2.91×10^4 cases** Modal= 14,859 ×2.86 **= 4.25×10^4 cases** High= 297,188 × 5.26 **= 1.56×10^6 cases**	PERT distribution Low = 0× 1.96 **= 0 cases** Modal= 14,859 × 3.85 **= 4.25×10^4 cases** High= 891,565 × 6.67 **= 1.19×10^7 cases**
Estimated incidence (**cases/1000persons/year**) From Equation A.1	PERT distribution Low= 141 Modal= 471 High= 1319	PERT distribution Low= 2.8 Modal= 4.0 High= 149	PERT distribution Low= 0 Modal= 11 High= 1130

*Percentage (%) rankings from less than 10 respondents (HCPs) were considered as outliers and excluded in the incidence calculations as they lead to unrealistically large uncertainty.

4.3. Population under study

Data about the population demographics was obtained from the Rwanda fourth population and housing census conducted by the National Institute of Statistics of Rwanda (29). The data was reorganised to suit the age categories programmed in the R-DALY calculator (57) as in Table A.5

Table A.5. Population distribution by age in Rwanda 2012

Age group.	No. of Males	No. of Females
0 - 4	768,340	774,057
5 - 14	1,208,114	1,406,385
15 -44	2,350,514	2,463,900
45 - 59	465,048	506,953
60 +	262,853	299,811

4.4. Life expectancy table

Life expectancy table used in the global burden of disease in 2010 (GBD 2010) for both males and females was adopted (31).

4.5. Social values

4.5.1. Discounting for time

In this study, a discount rate of 0 % was applied similar to the GBD of 2010 (31).

4.5.2. Age-weighting

No age weighting was used in this study.

4.6. On set age: (in years)

4.6.1. Watery diarrhea, bloody diarrhea and cholera

Even though children (under 5 years) and old people (above 59 years) are more susceptible, diarrheal diseases can lead to morbidity across all age groups (69). The default age groups in R-DALY calculator were arranged in beta PERT distribution and applied in this study, since the onset of acute diarrheal diseases can occur at any age. Average age for each age group was taken as the modal value (Tab A.6).

Table A.6. On set age for diarrhea diseases

	Age in years		
Age group	modal	Min	Maximum
0 - 4	2	0	4
5 - 14	9.5	5	14
15 - 44	29.5	15	44
45 - 59	52	45	59
60 +	77.5	60	95

4.6.2. Typhoid fever

Default age groups in R-DALY calculator we used in a beta PERT distribution since illness due to typhoid can occur at any age (70) as shown in Tab. A. 6.

4.7. Disability Weight (DW) of disease: (range [0-1])

4.7.1. Watery diarrhea, bloody diarrhea and cholera

The global burden of disease study for the year 2010 (GBD 2010) involved countries like Tanzania in the same region as Rwanda. In the GBD 2010, diarrheal diseases were classified as mild, moderate and severe and awarded disability weights (DW) (60). All reported cases of bloody diarrhea and cholera were assumed to be severe; the GBD 2010 DW for severe diarrhea were, modal= 0.281 (low=0.184 – high=0.399) in a beta PERT distribution(60). We also assumed that all reported cases of watery diarrhea had moderate and severe episodes (DW moderate + DW severe) with the modal as the average (0.133,0.202, 0.299, 0.18, 0.281, 0.399), leading to a PERT distribution of; modal = 0.250 (low=0.133 – high=0.399).

4.7.2. Typhoid fever

DW of 0.6 was applied as from a study by Gkogka et al. (18).

4.8. Mortality: deaths/1000 persons/ year

4.8.1. Watery diarrhea, bloody diarrhea and cholera

We used the deaths (335 deaths) due to diarrheal diseases reported by Ministry of Health of Rwanda for the year 2012 (71) and a pert distribution was obtained as shown in Box 1 to carter for uncertainty under reporting and recording.

Box 1 Deaths due to diarrheal diseases in Rwanda, 2013			
Symbol	**Variable**		**Source**
dD	Deaths due to diarreal diseases	335	MoH, 2013
unD	Deaths due to unknown causes	5,063	MoH, 2013
tD	Total deaths for all causes	12,172	MoH, 2013
kD	Deaths due to known causes = (tD-unD)	7,109	Calculated
pdD	Proportion of known deaths due to diarhea = (dD/kD)	0.0471	Calculated
xD	Deaths due to diarhea not registered = (pdD*unD)	239	Calculated
mD	Most likely deaths due to diarrhea = (dD+xD)	574	Calculated
hD	Highest possible deaths due to diarrhea = 2* mD	1147	Assumption
	Output: Risk pert distribution low = **335** deaths modal= **574** deaths High = **1147** deaths		

To estimate the number of deaths for patients with only watery diarrhea or bloody diarrhea as clinical features, and or cholera disease with respect to the total death burden diarrheal diseases, we used the case fatality rates (CFR) derived from the most suspected respective causative pathogens from literature as in Table A.7. Box 2 shows the methodology used to generate mortality rates and the associated uncertainty were computed using @risk 7.5 software (Palisade corporation, USA) at 20,000 iterations.

Table A. 7. Case fatality rates of common etiologic agents causing diarrheal diseases (in %).

Bloody diarrhea			
	Pathogen	CFR %	Reference
1	*Shigella* spp.	0.1,1, 4, 7, 15	(6) (72)
2	*Camplylobacter jejuni*	0.1, 3, 10, 0.05	(6, 18)
3	Non typhoidal *Salmonella*	0.5, 0.14, 19, 20, 25	(6),(73-75)
4	*Entamoeba histolytica*	0.1, 0.2, 0.3	(18)
5	STEC	0.8, 3, 5	(6)
6	EIEC	Like *Shigella* spp.	(76)
			Pert (0.1, 5.7, 25)
Watery diarrhea			
1	Norovirus Rotavirus	0.075, 0.1, 0.3, 0.34, 1.6, 2.0	(77-80)
2	*Giardia lamblia*	0, 0.05, 0.1	(18)
3	EPEC	0.01	(66, 81)
4	ETEC	0.01	(66)
5	*Staphylococcus aureus* and other food poisoning bacteria	0 , 0.025, 0.05	(18)
6	*Cryptosporidium* spp.	0.07, 0.6	(18)
			Pert (0, 0.33, 2.0))
Cholera			
	Vibrio cholerae	From year 2009 to 2012, the WHO data shows the CFR = 0 in Rwanda. But to carter for uncertainty due to under diagnosis especially due to close resemblance with ETEC, we selected a CFR between 0% and 0.01%. CFR for ETEC= 0.01 %	(66, 82, 83)
			Pert (0, 0.005, 0.01)

From Table A.7, the average CFR was calculated and taken as the modal: watery diarrhea = **0.33 %,** bloody diarrhea = **5.7%** and cholera = **0.005**% in the pert distribution.

Calculation of mortality rates

We computed as in Equation A.2 the mortality rates (deaths/1000 persons/ year), the population of Rwanda (10,515,973 persons) (29) and the number of deaths due to diarrheal diseases.

Equation A.2

$$Mortality\ rate\ = \frac{deaths}{Total\ population} \div \left(\frac{1}{1000\ persons} \times \frac{1}{year}\right)$$

The minimum, mode and maximum values of mortality rates in the output were selected for further calculations of respective DALY.

To suit the age group settings in the DALY calculator, the deaths proportions (distribution) per age group for watery diarrhea, bloody diarrhea and cholera disease was computed. The deaths estimates (proportions) per age groups from the WHO evidence department (84) were used to distribute the mortality values in Box 2 across different age groups.

Mortality – ORS cases (deaths/1000persons/year)

To calculate mortality for cases of diarrhea which also received ORS treatment, we multiplied the total diarrhea deaths (consideration underreporting) with the % (27.5) use of ORS in Rwanda (67) and thereafter computed the mortality as in the formulae above as follows: The same procedure for distribution mortality rate across age groups was followed as mentioned before.

Box 2. Derivation of mortality due watery diarrhea, bloody diarrhea and cholera using @risk software 7.5, 20,000 iterations

Symbols	Variables	Input	unit	Source
	Case fatality rate			
Cw	Case fatality rates for Watery diarrhea	Risk pert (0, 0.33, 2)	%	Table A.7
Cb	Case fatality rates for Bloody diarrhea	Risk pert (0.1, 5.7, 25)	%	Table A.7
Cc	Case fatality rates for Cholera	Risk pert (0, 0.005, 0.01)	%	Table A.7
	Reported cases with underporting factor			
Rw	Adjusted cases for Watery diarrhea	Riskpert (900605, 1001476, 1201407)	cases	Table A.1
Rb	Adjusted cases for Bloody diarrhea	Riskpert (6654, 9710, 17858)	cases	Table A.1
Rc	Adjusted cases for Cholera	Riskpert (1067, 1920, 3335)	cases	Table A.1
	Derived deaths			
dDw	Derived deaths due to Watery diarrhea	Cw/100*Rw	deaths	Calculated
dDb	Derived deaths due to Bloody diarrhea	Cb/100*Rb	deaths	Calculated
dDc	Derived deaths due to Cholera	Cc/100*Rc	deaths	Calculated
TdD	Total derived deaths	dDw+dDb+dDc	deaths	Calculated
	Reported deaths			
RD	Reported deaths in Rwanda, circa 2013	Risk pert (335, 574, 1147)	deaths	Box 1
CF	Correction factor	TdD/RD (mean ≈10)		Calculated
	Updated deaths			
dW	Updated deaths for Watery diarrhea	dDw/CF	deaths	Calculated
dB	Updated deaths for Blooy diarrhea	dDb/CF	deaths	Calculated
dC	Updated deaths for Cholera	dDc/CF	deaths	Calculated
Pop	Population of Rwanda by 2012	10515973	persons	NISR, 2012
	Mortality rates (deaths/1000 persons/ year			
Mw	Mortality rates due to Watery diarrhea	dW/Pop*1000 persons*1year		Calculated
Mb	Mortality rates due to Bloody diarrhea	dB/Pop*1000 persons*1year		Calculated
Mc	Mortality rates due to Cholera	dC/Pop*1000 persons*1year		Calculated
	Software out put			

	Mortality due to Watery diarrhea
Cell	Sheet1!E28
Minimum	0.000188
Maximum	0.10128
Mean	0.04942
90% CI	± 0.000172
Mode	0.04897
Median	0.04880

	Mortality due to Bloody diarrhea
Cell	Sheet1!E29
Minimum	9.901E-005
Maximum	0.090982
Mean	0.010456
90% CI	± 0.000109
Mode	0.003713
Median	0.007631
Std Dev	0.009401

	Mortality due to Cholera
Cell	Sheet1!E30
Minimum	2.138E-008
Maximum	3.338E-005
Mean	1.382E-006
90% CI	± 1.775E-008
Mode	5.735E-007
Median	9.425E-007
Std Dev	1.526E-006

4.8.2. Typhoid fever

Due to paucity of data for mortality rates for sub-Saharan Africa in previous studies (85) were used. Buckle et al. (2012) reported, modal = **0.004 (** low **= 0.002,** high **= 0.007**) deaths/1000 persons/ year. Deaths per age groups proportions for infectious agents, WHO GHE code 370 (84) were used to distribute the mortality reported by Buckle et al. (85) to different age groups.

4.9. Treatment proportion: (range [0-1])

4.9.1. Watery diarrhea, bloody diarrhea and cholera

Watery diarrhea and Bloody diarrhea

Due to lack of population survey data on medical seeking proportions for watery and bloody diarrhea in Rwanda, we adopted FoodNet –USA population in surveys of year, 2000-2001, 2002-2003 and 2006-2007 for the proportion of survey respondents with non-bloody diarrhea (watery diarrhea) and bloody diarrhea who sought medical care. Proportions (95% credible interval) were, **0.15**, **0.18**, and **0.20 (watery diarrhea) and 0.19, 0.35 and 0.51** (**bloody diarrhea**) for low, modal and high values respectively (23).

Cholera

Always an outbreak spark 100% participation of health care system in Rwanda, therefore treatment proportion assumed to be; **1** (Take A.1)**.**

4.9.2. Typhoid fever

We adopted medical seeking proportions used by Scallan et al.(23) for *Salmonella enterica*, serotype Typhi. Proportions (95% credible interval) were, **0.15, 0.26 and 0.51** for low, modal and high values respectively.

4.10. Duration of disease: (in years)

4.10.1. Watery diarrhea, bloody diarrhea and cholera

We assume that the diarrheal diseases in this study are acute and therefore the duration of chronic diarrhea and their related sequelae are not considered.

Watery diarrhea and bloody diarrhea

We use the durations published in the World Gastroenterology Organisation (WGO) practice guidelines of 2008 (86).

3 (1-14) days **= [0.0082 (0.0027 – 0.038)] years**

Cholera

After infection, the symptoms of cholera can appear from 0.1 to 10 days (87), (88, 89).We therefore adopt the duration of modal = 5 days, low = 0.1 and high= 10 days, **[0.0137 (0.0003 – 0.0274)] years**.

ORS Cases

We used a duration of 3 (0.1-14) days **= [0.0082 (0.0003 – 0.038)] years** for watery diarrhea.

4.10.2. Typhoid fever

We take the duration of typhoid fever to be around 14 (3 - 60) days = 0.0384 (0.0032 – 0.1644) years (18, 65, 90)).

4.11. Disability Weight (DW) of untreated disease: (range [0-1])

4.11.1. Watery diarrhea, bloody diarrhea, cholera and ORS cases

Untreated cases of these diarrheal diseases were assumed to be mild and hence use a DW of 0.061 (0.036–0.093) used in the 2010 global burden of disease study (60).

4.11.2. Typhoid fever

Class 1 DW of **0.096** (91) was adopted for uncomplicated typhoid cases (18).

4.12. Average age at death: age in years

4.12.1. Watery diarrhea, bloody diarrhea and cholera

We use the default age groups in the R-DALY calculator (Tab. A.9) adopted from the 1990 GBD (92). Deaths caused by diarrheal diseases were reported to be more significant for the age groups, 0-4 (93) and +60 years (94, 95) however WHO estimates for sub-Saharan countries (84) indicate significant deaths proportions also in other age groups. Therefore average age at death for diarrheal diseases was adopted as shown in Table A.9

Table A.9. Average age at death for diarrheal diseases.

	Age in years
Age group	modal
0- 4	2
5 - 14	9.5
15 - 44	29.5
45 - 59	52
+ 60	77.5

4.12.2. Typhoid fever

Average age per age groups at death presented in Table A.9 were used.

5. **R-outputs (Estimates for a population of 10,515,973 inhabitants).**

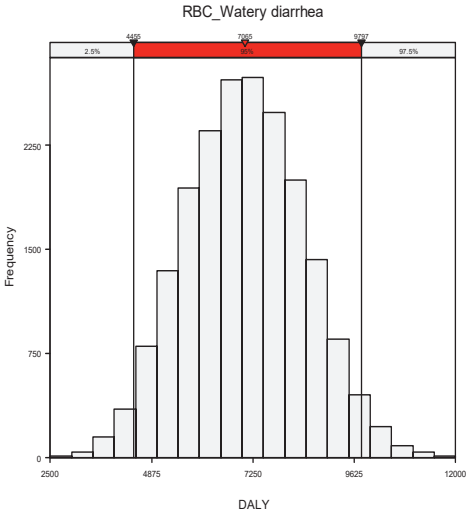
Deaths estimates from different data sources for a given illness are almost equal because of using the same mortality estimates.

DALY Calculator: RBC_Watery diarrhea

	Mean	Median	2.5%	97.5%
DALY	7065	7056	4455	9797
YLD	778	706	226	1717
YLL	6287	6279	3738	8858
cases	1016345	1012790	930438	1120283
deaths	94	94	59	130

YLD/DALY = 11%

YLL/DALY = 89%

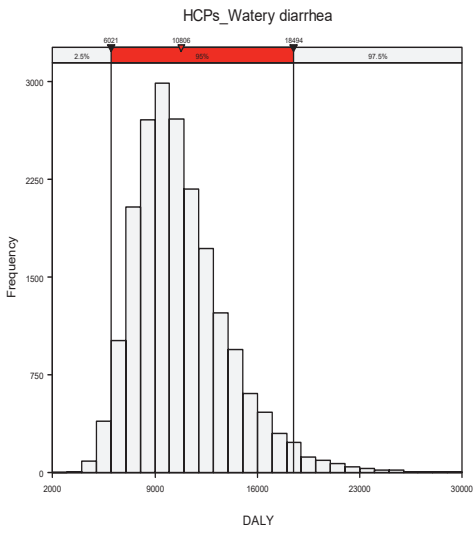


DALY Calculator: HCPs_Watery diarrhea

	Mean	Median	2.5%	97.5%
DALY	10806	10271	6021	18494
YLD	4490	3789	944	11865
YLL	6317	6319	3767	8838
cases	5839672	5668237	2427061	10242476
deaths	95	95	59	130

YLD/DALY = 39%

YLL/DALY = 61%

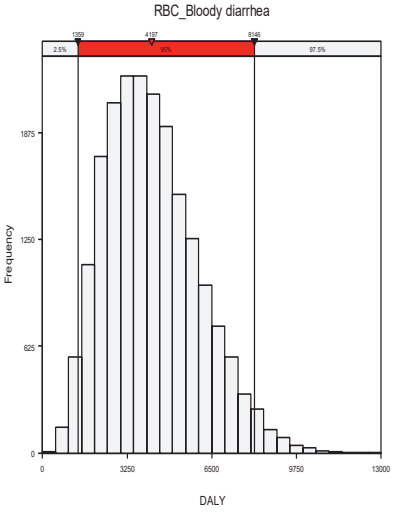


DALY Calculator: RBC_Bloody diarrhea

	Mean	Median	2.5%	97.5%
DALY	2243	2055	525	4997
YLD	8	7	2	19
YLL	2235	2048	516	4989
cases	10539	10380	7457	14502
deaths	34	31	9	70

YLD/DALY = 1%

YLL/DALY = 99%

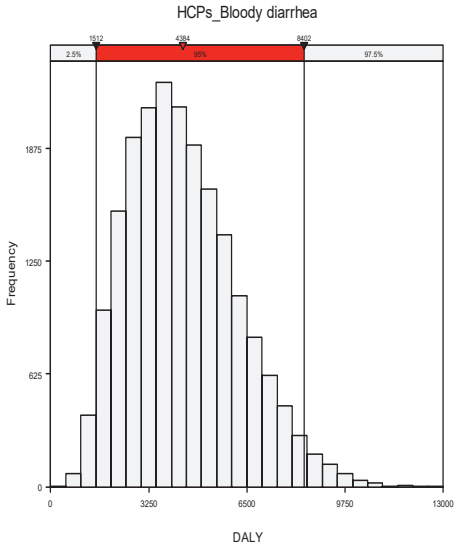


DALY Calculator: HCPs_Bloody diarrhea

	Mean	Median	2.5%	97.5%
DALY	2462	2271	667	5245
YLD	223	136	14	927
YLL	2239	2051	507	4982
cases	290761	209560	31551	953314
deaths	34	31	9	71

YLD/DALY = 10%

YLL/DALY = 90%

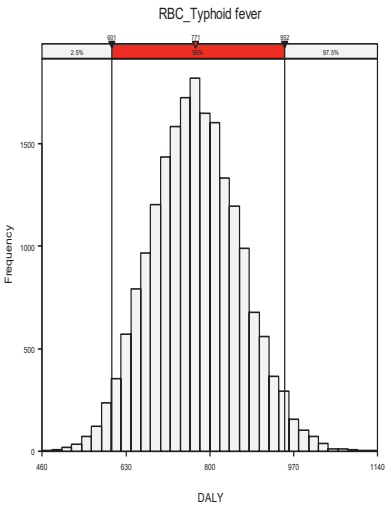


DALY Calculator: RBC_Typhoid fever

	Mean	Median	2.5%	97.5%
DALY	**771**	**770**	**601**	**952**
YLD	**36**	**29**	**6**	**107**
YLL	**735**	**734**	**572**	**905**
cases	**6954**	**6394**	**1887**	**14986**
deaths	**11**	**11**	**8**	**13**

YLD/DALY = 5%

YLL/DALY = 95%

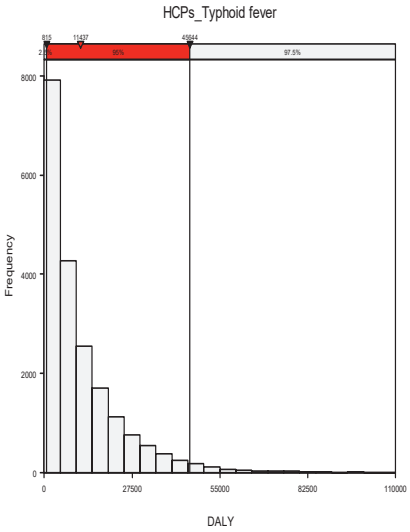


DALY Calculator: HCPs_Typhoid fever

	Mean	Median	2.5%	97.5%
DALY	11437	7062	815	45644
YLD	10703	6333	76	44930
YLL	734	733	571	906
cases	2055633	1433517	18302	7173015
deaths	11	11	8	13

YLD/DALY = 80%

YLL/DALY = 20%

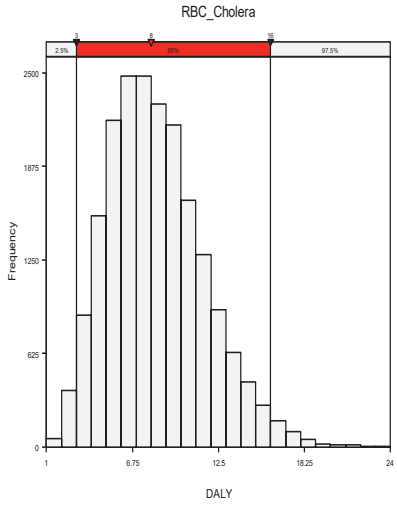


DALY Calculator: RBC_Cholera

	Mean	Median	2.5%	97.5%
DALY	8	8	3	16
YLD	8	8	3	15
YLL	1	0	0	1
cases	2017	2001	1348	2771
deaths	0	0	0	0

YLD/DALY = 93%

YLL/DALY = 7%



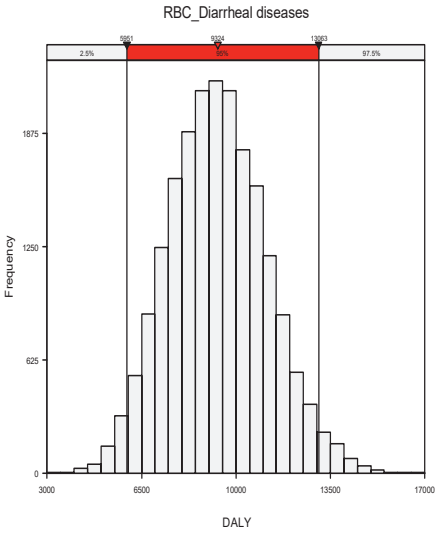
DALY Calculator: RBC_Diarrheal diseases

(cases of watery diarrhea + bloody disease +suspected cases of cholera)

	Mean	Median	2.5%	97.5%
DALY	9324	9266	5951	13063
YLD	796	728	244	1738
YLL	8528	8486	5250	12200
cases	1028839	1025790	942279	1131835
deaths	128	127	82	178

YLD/DALY = 9%

YLL/DALY = 91%

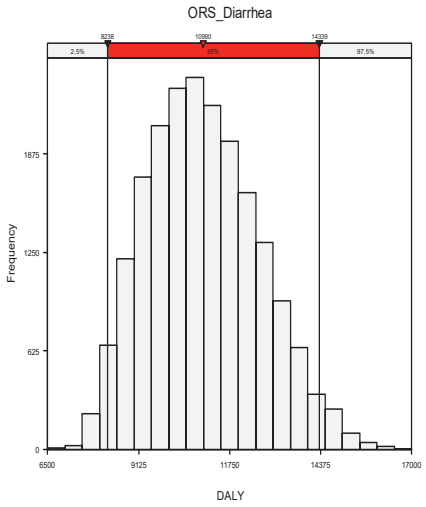


DALY Calculator: ORS_Diarrhea

	Mean	Median	2.5%	97.5%
DALY	10990	10886	8238	14339
YLD	366	337	79	816
YLL	10624	10515	7894	13955
cases	230665	229470	148909	320947
deaths	170	169	133	212

YLD/DALY = 3%

YLL/DALY = 97%



6. Questionnaire administered to health care providers

Opinion survey for Medical practitioners about food-related illnesses in 2013

Symptomatic ranking of food-related illness

How do you rank the prevalence of the following symptoms/clinical feature depending on your experience with patients in the year 2013. (*use the following ranking criteria by ticking* (√) *where applicable*)

Ranking scale

Category	*1*	*2*	*3*	*4*	*5*	*6*	*7*
Percentage (%)	*Not seen (0)*	*0-1*	*1-20*	*20-40*	*40 -60*	*60 -80*	*80 -100*

Clinical feature	*1* *0%*	2 *0-1%*	3 *1-20%*	*4* *20-40%*	5 *40-60%*	*6* *60-80%*	*7* *80-100%*
Bloody diarrhoea	◯	◯	◯	◯	◯	◯	◯
Watery diarrhoea	◯	◯	◯	◯	◯	◯	◯
Typhoid fever	◯	◯	◯	◯	◯	◯	◯

Any comments

.....

Thank you

References

1. **Thomas MK, Murray R, Flockhart L, Pintar K, Pollari F, Fazil A, Nesbitt A, Marshall B.** 2013. Estimates of the burden of foodborne illness in Canada for 30 specified pathogens and unspecified agents, Circa 2006. *Foodborne Pathogens & Disease* 10:639-648.

2. **Havelaar** et al. 2015. World Health Organization global estimates and regional comparisons of the burden of foodborne disease in 2010. *PLoS Medicine* 12:e1001923.

3. **Lasky T.** 2002. Foodborne illness - Old problem, new relevance. *Epidemiology* 13:593-598.

4. **Food and Drug Administration (FDA) of the United States**. 2012. Bad bug book, foodborne pathogenic microorganisms and natural toxins. second edition. Available at: https://www.fda.gov/downloads/food/foodsafety/foodborneillness/foodborneillnessfoodbornepathogensnaturaltoxins/badbugbook/ucm297627.pdf. Accessed 19th May 2017.

5. **Mughini-Gras, L., Schaapveld, M., Kramers, J., Mooij, S., Neefjes-Borst, E. A., van Pelt, W., & Neefjes, J.** (2018). Increased colon cancer risk after severe *Salmonella* infection. *PloS One*, 13: e0189721.

6. **Barton Behravesh C, Jones TF, Vugia DJ, Long C, Marcus R, Smith K, Thomas S, Zansky S, Fullerton KE, Henao OL, Scallan E, Group FW.** 2011. Deaths associated with bacterial pathogens transmitted commonly through food: foodborne diseases active surveillance network (FoodNet), 1996–2005. *Journal of Infectious Diseases* 204:263-267.

7. **Sidibé M, Piot P, Dybul M.** 2012. AIDS is not over. *The Lancet* 380:2058-2060.

8. **Hoffmann S, Maculloch B, Batz M.** 2015. Economic burden of major foodborne illnesses acquired in the United States, p. 1-74, Economic cost of foodborne illnesses in the United States. *Nova Science Publishers*, Inc.

9. **Minor T, Lasher A, Klontz K, Brown B, Nardinelli C, Zorn D.** 2015. The per case and total annual costs of foodborne Illness in the United States. *Risk Analysis* 35(6), 1125-1139.

10. **Montgomery B.** 2015. Economic cost of foodborne illnesses in the U. S. *Nova Science Publishers*, Inc.

11. **Scharff RL.** 2012. Economic burden from health losses due to foodborne illness in the united states. *Journal of Food Protection* 75:123-131.

12. **von Witzke H, Kirschke D, Lotze-Campen H, Noleppa S.** 2005. The Economics of alternative strategies for the reduction of food-borne diseases in developing countries: The case of diarrhea in Rwanda. (No. 72/2005). Working Paper, Wirtschafts-und Sozialwissenschaften an der Landwirtschaftlich-Gärtnerischen Fakultät, Humboldt-Universität zu Berlin. Available at: https://www.econstor.eu/bitstream/10419/96474/1/783959796.pdf. Accessed 05th September 2013.

13. **Wallace RB, Oria M, Council NR.** 2010. Adopting a risk-based decision-making approach to food safety. *National Research Council (US) Committee on the Review of Food and Drug Administration's Role in Ensuring Safe Food*. National Academies Press (US), Washington (DC).

14. **Janati A, Hosseiny M, Gouya MM, Moradi G, Ghaderi E.** 2015. Communicable disease reporting systems in the world: a systematic review article. *Iranian Journal of Public Health* 44: 1453.

15. **Gibbons CL, Mangen M-JJ, Plass D, Havelaar AH, Brooke RJ, Kramarz P, Peterson KL, Stuurman AL, Cassini A, Fèvre EM.** 2014. Measuring underreporting and under-ascertainment in infectious disease datasets: a comparison of methods. *BMC Public Health* 14:147.

16. **MacDougall L, Majowicz S, Dore K, Flint J, Thomas K, Kovacs S, Sockett P.** 2008. Under-reporting of infectious gastrointestinal illness in British Columbia, Canada: who is counted in provincial communicable disease statistics? *Epidemiology & Infection* 136:248-256.

17. **Majowicz SE, Edge VL, Fazil A, McNab WB, Doré KA, Sockett PN, Flint JA, Middleton D, McEwen SA, Wilson JB.** 2005. Estimating the under-reporting rate for infectious gastrointestinal illness in Ontario. *Canadian Journal of Public Health* 1:178-181.

18. **Gkogka E, Reij MW, Havelaar AH, Zwietering MH, Gorris LGM.** 2011. Risk-based estimate of effect of foodborne diseases on public health, Greece. *Emerging Infectious Diseases* 17:1581-1590.

19. **Havelaar AH, Haagsma JA, Mangen M-JJ, Kemmeren JM, Verhoef LPB, Vijgen SMC.** 2012. Disease burden of foodborne pathogens in the Netherlands, 2009. *International Journal of Food Microbiology* 156: 231-238.

20. **Torgerson PR, Devleesschauwer B, Praet N, Speybroeck N, Willingham AL, Kasuga F, Rokni MB, Zhou XN, Fèvre EM, Sripa B, Gargouri N, Fürst T, Budke CM, Carabin H, Kirk MD, Angulo FJ, Havelaar A, de Silva N.** 2015. World Health Organization estimates of the global and regional disease burden of 11 foodborne parasitic diseases, 2010: A data synthesis. *PLoS Medicine* 12.

21. **Murray CJL, Lopez AD.** 1997. Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study. *The Lancet* 349:1436-1442.

22. **Adak G, Long S, O'Brien S.** 2002. Trends in indigenous foodborne disease and deaths, England and Wales: 1992 to 2000. *Gut* 51:832-841.

23. **Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM.** 2011. Foodborne illness acquired in the United States--major pathogens. *Emerging Infectious Diseases* 17:7-15.

24. **The World Health Organisation of the United Nations.** 2012. Manual for integrated foodborne disease surveillance in the WHO Africa Region. Available at: http://apps.who.int/iris/bitstream/10665/170262/1/foodborne-disease-manual.pdf. Accessed 15th November 2013.

25. **Nsubuga P, White ME, Thacker SB, Anderson MA, Blount SB, Broome CV, Chiller TM, Espitia V, Imtiaz R, Sosin D.** 2006. Public health surveillance: a tool for targeting and monitoring interventions. *Disease Control Priorities in Developing Countries* 2:997-1018.

26. **Ukey UU, Chitre DS.** 2013. The various etiological agents in the causation of gastroenteritis. *Annals of Tropical Medicine and Public Health* 6:112-116.

27. **The World Health Organisation of the United Nations.** 2008. Foodborne disease outbreaks: guidelines for investigation and control. Available at: http://www.who.int/foodsafety/publications/foodborne_disease/outbreak_guidelines.pdf. Accessed 05th September 2013.

28. **National Institute of Statistics of Rwanda [Rwanda], Ministry of Health (MOH) [Rwanda], and ICF International.** Rwanda Demographic and Health Survey 2010. Calverton, Maryland, USA: NISR,

MOH, and ICF International. Available at: http://www.statistics.gov.rw/publication/demographic-and-health-survey-2010-hiv-fact-sheet. Accessed 5th January 2014.

29. **National Institute of Statistics of Rwanda, Ministry of finance and economic planning, Rwanda.** Rwanda fourth population and housing Census. Thematic report: Data quality assessment . Available at: http://www.statistics.gov.rw/publication/rphc4-final-report-data-quality-assessment. Accessed 05th May 2014.

30. **Devleesschauwer B, Havelaar AH, Maertens de Noordhout C, Haagsma JA, Praet N, Dorny P, Duchateau L, Torgerson PR, Oyen H, Speybroeck N.** 2014. DALY calculation in practice: a stepwise approach. *International Journal of Public Health* 59:571-574.

31. **Murray** et al**.** 2012. Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. *The Lancet* 380:2197-2223.

32. **Kampstra P.** 2008. Beanplot: A boxplot alternative for visual comparison of distributions. *Journal of Statistical Software* 28:1-9.

33. **Rao MR, Abu-Elyazeed R, Savarino SJ, Naficy AB, Wierzba TF, Abdel-Messih I, Shaheen H, Frenck RW, Svennerholm A-M, Clemens JD.** 2003. High disease burden of diarrhea due to enterotoxigenic *Escherichia coli* among rural Egyptian infants and young children. *Journal of Clinical Microbiology* 41:4862-4864.

34. **The World Health Organisation of the United Nations.** 2008. Weekly epidemiological record (No. 47, 2008, 83, 421–428). Global networks for surveillance of rotavirus gastroenteritis, 2001–2008. Available at: http://www.who.int/wer/2008/wer8347.pdf?ua=1. Accessed 03rd December 2013.

35. **Subekti DS, Lesmana M, Tjaniadi P, Machpud N, Sriwati, Sukarma, Daniel JC, Alexander WK, Campbell JR, Corwin AL, Beecham Iii HJ, Simanjuntak C, Oyofo BA.** 2003. Prevalence of enterotoxigenic *Escherichia coli* (ETEC) in hospitalized acute diarrhea patients in Denpasar, Bali, Indonesia. *Diagnostic Microbiology & Infectious Disease* 47:399-405.

36. **Tabo D-a, Granier SA, Diguimbaye CD, Marault M, Brisabois A, Mama B, Millemann Y.** 2015. Are *Salmonella*-induced gastroenteritis neglected in developing countries? Feedback from microbiological investigations in N'Djamena hospitals, Chad. *PloS one* 10:e0136153.

37. **Pal SC.** 1986. Dysentery: an overview. Still problems to resolve. *Dialogue on diarrhoea* 25, 4

38. **Xiao GG, Fan J, Deng JJ, Chen CH, Zhou W, Li XH, He YW, Li H, Hu B, Qiao Y, Chen GH, Wan CM.** 2012. A school outbreak of *Shigella sonnei* infection in China: Clinical features, antibiotic susceptibility and molecular epidemiology. *Indian Pediatrics* 49:287-290.

39. **Nichols GL.** 2000. Food-borne protozoa. *British Medical Bulletin* 56:209-235.

40. **Osei-Tutu B, Anto F.** 2016. Trends of reported foodborne diseases at the Ridge Hospital, Accra, Ghana: a retrospective review of routine data from 2009-2013. *BMC Infectious Diseases* 16:1-9.

41. **Longini IM, Yunus M, Zaman K, Siddique A, Sack RB, Nizam A.** 2002. Epidemic and endemic cholera trends over a 33-year period in Bangladesh. *Journal of Infectious Diseases* 186:246-251.

42. **Mutonga D, Langat D, Mwangi D, Tonui J, Njeru M, Abade A, Irura Z, Njeru I, Dahlke M.** 2013. National surveillance data on the epidemiology of cholera in Kenya, 1997–2010. *Journal of Infectious Diseases* 208:S55-S61.

43. **Banerjee T, Shukla BN, Filgona J, Anupurba S, Sen MR.** 2014. Trends of typhoid fever seropositivity over ten years in north India. *Indian Journal of Medical Research* 140:310.

44. **Polonsky JA, Martínez-Pino I, Nackers F, Chonzi P, Manangazira P, Van Herp M, Maes P, Porten K, Luquero FJ.** 2014. Descriptive epidemiology of typhoid fever during an epidemic in Harare, Zimbabwe, 2012. *PloS one* 9:e114702.

45. **Oguntoke O, Aboderin OJ, Bankole AM.** 2009. Association of water-borne diseases morbidity pattern and water quality in parts of Ibadan City, Nigeria. *Tanzania Journal of Health Research* 11, 189-195.

46. **Kirby MA, Nagel CL, Rosa G, Umupfasoni MM, Iyakaremye L, Thomas EA, Clasen TF.** 2017. Use, microbiological effectiveness and health impact of a household water filter intervention in rural Rwanda-A matched cohort study. *International Journal of Hygiene & Environmental Health* 220: 1020-1029.

47. **Uwimpuhwe M, Reddy P, Barratt G, Bux F**. 2014. The impact of hygiene and localised treatment on the quality of drinking water in Masaka, Rwanda. *Journal of Environmental Science & Health - Part A Toxic/Hazardous Substances & Environmental Engineering* 49:434-440.

48. **Edge VL, Pollari F, Lim G, Aramini J, Sockett P, Martin SW, Wilson J, Ellis A.** 2004. Syndromic surveillance of gastrointestinal illness using pharmacy over-the-counter sales. A retrospective study of waterborne outbreaks in Saskatchewan and Ontario. *Canadian Journal of Public Health* 95:446-450.

49. **Ao TT, Feasey NA, Gordon MA, Keddy KH, Angulo FJ, Crump JA.** 2015. Global burden of invasive nontyphoidal *Salmonella* disease, 2010. *Emerging Infectious Diseases* 21: 941–949.

50. **IHME, 2016a. Health data for Rwanda.** Institute of Health Metrics, Seattle, USA. Available at: http://www.healthdata.org/rwanda, accessed 08th August 2016.

51. **IHME, 2016b. Health data for Rwanda.** Institute of Health Metrics, Seattle, USA. Available at: http://vizhub.healthdata.org/gbd-compare/, accessed 08th August 2016.

52. **Sinharoy SS, Schmidt WP, Cox K, Clemence Z, Mfura L, Wendt R, Boisson S, Crossett E, Grépin KA, Jack W, Condo J, Habyarimana J, Clasen T.** 2016. Child diarrhoea and nutritional status in rural Rwanda: a cross-sectional study to explore contributing environmental and demographic factors. *Tropical Medicine & International Health* 21:956-964.

53. **The World Health Organisation of the United Nations.** 2016. Department of information, evidence and research. Global Health Estimates 2015: Deaths by Cause, Age, Sex, by Country and by Region, 2000-2015. Available at: http://www.who.int/healthinfo/global_burden_disease/estimates/en/index1.html. Accessed 03rd April 2017.

54. **Nyatani T., Kabeja A., Asiimwe A., Binagwaho A., Koama. J.B., Johnson P., & Kayumba K.** 2014. A National Electronic System for Disease Surveillance in Rwanda (eIDSR): Lessons learned from a successful implementation. *Online Journal of Public Health Informatics* 6: e118.

55. **Scallan E, Jones TF, Cronquist A, Thomas S, Frenzen P, Hoefer D, Medus C, Angulo FJ, Group FW.** 2006. Factors associated with seeking medical care and submitting a stool sample in estimating the burden of foodborne illness. *Foodbourne Pathogens & Disease* 3:432-438.

56. **Keramarou M, Evans MR**. 2012. Completeness of infectious disease notification in the United Kingdom: a systematic review. *Journal of Infection* 64:555-564.

57. **Devleesschauwer B., McDonald S., Haagsma J., Praet N., Havelaar A., Speybroeck N.** 2014. DALY: The DALY Calculator - A GUI for stochastic DALY calculation in R. R package version 1.3.0. Available at: http://cran.rproject.org/package=DALY. Accessed 02nd August 2015.

58. **Murray C, Acharya A.** 1997. Understanding DALYs. *Journal of Health Economics* 16: 703-730.

59. **The World Health Organisation of the United Nations**. 2017. Global task force on Cholera control. Prevention and control of cholera outbreaks: WHO policy and recommendations. Available at: http://www.who.int/cholera/technical/prevention/control/en/index1.html . Accessed 22th January 2017.

60. **Salomon** et al. 2012. Common values in assessing health outcomes from disease and injury: disability weights measurement study for the Global Burden of Disease Study 2010. *The Lancet* 380:2129-2143.

61. **The World Health Organisation of the United Nations.** 2013. Methods and data sources for global burden of disease estimates 2000-2011. Available at: http://www.who.int/healthinfo/statistics/GlobalDALYmethods_2000_2011.pdf?ua=1. Accessed 01st February 2016.

62. **The World Health Organisation of the United Nations and United Nations International Children's fund (UNICEF).** 2006. Oral rehydration salts. Production of the new ORS. Available at: http://apps.who.int/iris/bitstream/10665/69227/1/WHO_FCH_CAH_06.1.pdf?ua=1&ua=1. Accessed 02nd February 2014.

63. **Scallan E, Hoekstra RM, Mahon BE, Jones TF, Griffin PM**. 2015. An assessment of the human health impact of seven leading foodborne pathogens in the United States using disability adjusted life years. *Epidemiology & Infection* 143: 2795-2804.

64. **Kirk M, Ford L, Glass K, Hall G.** 2014. Foodborne illness, Australia, Circa 2000 and Circa 2010. *Emerging Infectious Diseases* 20:1857-1864.

65. **The World Health Organisation of the United Nations.** 2003. Communicable disease surveillance and response vaccines and biologicals. Background document: The diagnosis, treatment and prevention of typhoid fever. Available at: http://www.who.int/rpc/TFGuideWHO.pdf . Accessed 06th May 2016.

66. **Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV.** 1999. Food-related illness and death in the United States. *Emerging Infectious Diseases* 5:607-625.

67. **MoH.** 2015. Ministry of Health, the Republic of Rwanda and National Institute of Statistics of Rwanda. Demographic and Health Survey (DHS) 2014/2015. Key findings. Available at: http://www.moh.gov.rw/fileadmin/templates/Docs/DHS5_KeyFindings_FINAL_FINAL_12_June_2015.pdf. Accessed 09th June 2016. 17th February 2015.

68. **MoH.** 2014. Ministry of Health, the Republic of Rwanda. Monitoring and evaluation plan for the health sector, strategic plan (HSSP III) 2014-2018. Available at: http://www.moh.gov.rw/fileadmin/templates/MOHReports/Final_M_E_plan_for_HSSP_III__A..pdf. Accessed 25th January 2017.

69. **Lamberti LM, Fischer Walker CL, Black RE.** 2012. Systematic review of diarrhea duration and severity in children and adults in low- and middle-income countries. *BMC Public Health* 12:276.

70. **Dewan AM, Corner R, Hashizume M, Ongee ET**. 2013. Typhoid fever and its association with environmental factors in the Dhaka metropolitan area of Bangladesh: a spatial and time-series approach. *PLoS Neglected Tropical Diseases* 7:e1998.

71. **MoH.** 2013. Ministry of Health, the Republic of Rwanda, Annual report, 2012-2013. Available at: http://www.moh.gov.rw/fileadmin/templates/Press_release/MoH_Annual_Report_July_2012-June_2013.pdf . Accessed 05th June 2015.

72. **Bennish ML, Wojtyniak BJ.** 1991. Mortality due to shigellosis: community and hospital data. *Reviews of Infectious Dseases* 13 Suppl 4:S245-251.

73. **Uche IV, MacLennan CA, Saul A.** 2017. A Systematic review of the incidence, risk factors and case fatality rates of invasive nontyphoidal *Salmonella* (INTS) disease in Africa (1966 to 2014). *PLOS Neglected Tropical Diseases* 11:e0005118.

74. **Feasey NA, Dougan G, Kingsley RA, Heyderman RS, Gordon MA.** 2012. Invasive non-typhoidal *Salmonella* disease: an emerging and neglected tropical disease in Africa. *The Lancet* 379:2489-2499.

75. **Ao TT, Feasey NA, Gordon MA, Keddy KH, Angulo FJ, Crump JA.** 2013. Global burden of invasive nontyphoidal *Salmonella* disease, 2010. *On the Cover* 2012:941.

76. **van den Beld M, Reubsaet F.** 2012. Differentiation between *Shigella*, enteroinvasive *Escherichia coli* (EIEC) and noninvasive *Escherichia coli*. *European Journal of Clinical Microbiology & Infectious Diseases* 31:899-904.

77. **Lopman BA, Adak GK, Reacher MH, Brown D.** 2003. Two epidemiologic patterns of norovirus outbreaks: surveillance in England and Wales, 1992-2000. *Emerging Infectious Diseases* 9:71-77.

78. **Lindsay L, Wolter J, De Coster I, Van Damme P, Verstraeten T.** 2015. A decade of norovirus disease risk among older adults in upper-middle and high income countries: a systematic review. *BMC Infectious Diseases* 15:425.

79. **Calderon-Margalit R, Sheffer R, Halperin T, Orr N, Cohen D, Shohat T.** 2005. A large-scale gastroenteritis outbreak associated with norovirus in nursing homes. *Epidemiology & Infection* 133:35-40.

80. **Parashar UD, Hummelman EG, Bresee JS, Miller MA, Glass RI.** 2003. Global illness and deaths caused by rotavirus disease in children. *Emerging Infectious Diseases* 9:565-572.

81. **Vallance BA, Chan C, Robertson ML, Finlay BB.** 2002. Enteropathogenic and enterohemorrhagic *Escherichia coli* infections: emerging themes in pathogenesis and prevention. *Canadian Journal of Gastroenterology & Hepatology* 16:771-778.

82. **Nataro JP, Kaper JB.** 1998. Diarrheagenic *Escherichia coli*. *Clinical Microbiology Reviews* 11:142-201.

83. **The World Health Organisation of the United Nations.** 2013. Weekly epidemiological records. Available at: http://www.who.int/wer/2013/wer8831.pdf. Accessed 21st February 2016.

84. **The World Health Organisation of the United Nations.** 2016**.** Department of information, evidence and research. Global Health Estimates 2015: Deaths by Cause, Age, Sex, by Country and by Region, 2000-2015. Geneva, World Health Organization; 2016.. Available at: http://www.who.int/healthinfo/global_burden_disease/estimates/en/index1.html. Accessed 03rd April 2017.

85. **Buckle GC, Walker C, Black RE.** 2012. Typhoid fever and paratyphoid fever: Systematic review to estimate global morbidity and mortality for 2010. *Journal of Global Health* 2:10401.

86. **World Gastroenterology Organisation (WGO).** 2008. Practice guideline: acute diarrhea. Available at : http://doctor-ru.org/main/1100/1101.pdf . Accessed 20th February 2015.

87. **Azman AS, Rudolph KE, Cummings DA, Lessler J.** 2013. The incubation period of cholera: a systematic review. *Journal of Infection* 66:432-438.

88. **The World Health Organisation of the United Nations**. 2015. Cholera. Available at: http://www.who.int/mediacentre/factsheets/fs107/en/. Accessed 27th July 2016.

89. **Centers for Disease Control and Prevention (CDC).** 2014**.** Cholera. Available at: http://www.cdc.gov/cholera/general/ . Accessed 20th February 2015.

90. **Centers for Disease Control and Prevention (CDC).** 2014.Typhoid and paratyphoid fever. Available at: http://wwwnc.cdc.gov/travel/yellowbook/2016/infectious-diseases-related-to-travel/typhoid-paratyphoid-fever. Accessed on 20th January 2016.

91. **Murray CJ.** 1994. Quantifying the burden of disease: the technical basis for disability-adjusted life years. *Bulletin of the World Health Organization* 72:429-445.

92. **Murray CJ, Lopez AD.** 1996. The global burden of disease and injury series, volume 1: a comprehensive assessment of mortality and disability from diseases, injuries, and risk factors in 1990 and projected to 2020. Cambridge. MA.

93. **Boschi-Pinto C, Velebit L, Shibuya K.** 2008. Estimating child mortality due to diarrhoea in developing countries. *Bulletin of the World Health Organization* 86:710-717.

94. **Ratnaike R.** 1999. Diarrhoea and aging. *Journal of Postgraduate Medicine* 45:60-66.

95. **Holt PR.** 2001. Diarrhea and malabsorption in the elderly. *Gastroenterology clinics of North America* 30:427-444.

3

Indicator microorganisms in fresh vegetables from "farm to fork" in Rwanda

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Abstract

Microbial safety of ready-to-eat vegetables is currently a global concern. We studied indicator microorganisms in fresh vegetables from "farm to fork" in Rwanda, to identify possible trends in microbial counts along the supply chain in a developing country. A total of 453 samples were taken across the vegetable supply chain (farm, market and food service establishment level) and analyzed for indicator microorganisms; *Enterobacteriaceae*, *Listeria* spp., aerobic plate count and coagulase - positive staphylococci. The sampling at farm and market covered 11 types of vegetables commonly eaten raw in salads. Results show that the mean count of *Enterobacteriaceae* and *Listeria* spp. in vegetables were respectively 5.8 and 4.6 log cfu/g at farm, 6.3 and 4.9 log cfu/g at market, 6.0 and 5.1 log cfu/g upon arrival at food service establishments, and finally 3.3 and 2.9 log cfu/g in ready-to-eat salads. Aerobic plate count and coagulase-positive Staphylococci were on average 6.8 and 4.6 respectively at start of salad preparation and 4.9 and 3.0 in the final product. Unit operations like washing with or without sanitizers, trimming and peeling significantly reduced indicator counts by on average 2.1 log cfu/g from start to end of salad preparation. Results also show that 91% (51/56) and 22% (12/56) of ready-to-eat salads prepared by food service establishments met the guidelines for coagulase - positive staphylococci (10^4 cfu/g) and presumptive *Listeria* spp. (10^2 cfu/g). The high counts of these indicator microorganisms along the vegetable supply chain, raises concern about the potential presence of foodborne pathogens. This study calls for improved adherence to GAPs and GHPs in the fresh vegetable supply chain so as to minimize the potential risk from foodborne pathogens.

Introduction

Global production and consumption of fresh vegetables has been increasing for the last three decades (1), concurrently, the reported foodborne outbreaks linked to fresh vegetables have surged (2-5). Pathogens most implicated in these vegetable related outbreaks include Norovirus, *Salmonella* spp., *Escherichia coli* and *Shigella* spp. (2, 6). To minimize the number of these outbreaks internationally, guidelines such as those from the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) (7) have been developed to prevent or control the conditions or factors leading to microbial contamination, survival or growth along the "farm to fork" continuum. To investigate the effectiveness of the control measures in these guidelines, researchers from mainly developed countries have continued to study foodborne pathogens and indicator microorganism (IMOs) at different stages of the vegetable supply chain (8-10). Because pathogens are usually prevalent in low numbers, appear sporadically or absent at times, IMOs like aerobic plate count (APC), faecal coliforms, *Enterobacteriaceae*, *Listeria* spp. can provide more information to the detect the changes in control or preventive measures (11). Indicator microorganisms have been defined as a species of microorganisms or a group of microorganisms that indicate if food has been exposed to conditions that pose an increased risk to be contaminated with a pathogen or has been held under conditions that would allow pathogen proliferation (11). Although researchers (8-10) have used IMOs to investigate the extent of contamination of vegetables, most studies do not cover the whole vegetable supply chain (VSC) *i.e.*. the "farm to fork" continuum. A full overview of microbial levels across the entire supply chain may be of more practical use in preventing foodborne outbreaks at food service level.

In this study, we examined IMOs in the VSC in Rwanda from "farm to fork" to identify possible trends in microbial counts (growth or contamination, inactivation, survival) along the VSC. By investigating the microbial counts of IMOs across the entire VSC, we aim to contribute to practical approaches and information for risk managers in implementing microbial safety guidelines. Three major stages of the VSC were selected for investigation, farm, market and food service establishments (FSEs). To represent the final stage of the VSC, we chose FSEs over households, because in Rwanda, preparation and consumption of raw vegetables salads is more common in FSEs than in households (most people in homes consume cooked vegetables). Four specific objectives were set, (i) determining the difference between counts of IMOs in vegetables at farm and market, (ii) investigating the ability of the different FSEs to eliminate or reduce IMOs counts from start to the end of salad preparation, (iii) benchmarking of the microbial counts in FSE-RTE salads with existing guidelines or regulatory requirements and (iv) comparing the relation between the counts of IMOs at start of salad preparation at FSEs with the counts in the ready-to eat (RTE) salads. *Enterobacteriaceae* and *Listeria* spp. were selected as IMOs along the supply chain based on the expected vast abundance in farm vegetables (12, 13) and hence the ability to provide observable trends (increase or decrease) across the VSC. At FSEs, we included other IMOs viz. aerobic plate count (APC) and coagulase - positive staphylococci (CP. staphylococci) the former, to indicate the exposure of the vegetables to contamination and proliferation of microorganisms in general (14) and the latter to indicate personnel hygiene behaviors (15) during salad preparation.

Foot note: Abbreviations
IMOs (Indicator microorganisms), VSC (Vegetable supply chain), FSEs (Food service establishments), WMV (Whole mixed vegetables), RTE (Ready-to-eat)

Materials and Methods

Study design, sampling points and area

Selected IMOs were analyzed from 453 samples taken along the vegetable supply chain (three major stages: farm, market and FSE) in Rwanda from February to October 2015. The samples at farm and market covered 11 types of vegetables commonly eaten raw, *viz.*; beet root, cabbage, carrot, celery, cucumber, garlic, green pepper, lettuce, onion, parsley and tomato (each vegetable type sampled nine times). At farm, the study concentrated on the vegetable growing regions of Rwanda. Based on availability and the "one farm one sample" approach, we took 30, 26, 21, 16 and 6 samples from the Western, Southern, Northern and Eastern provinces and the peripherals of the City of Kigali respectively. Markets were selected based on the availability of the 11 chosen vegetables sold in built-open markets and supermarkets in the City of Kigali [15], the Southern [3],Western [2], Northern [1] and Eastern [1] provinces of Rwanda. Sampling in FSEs (hotels, restaurants and bars) was done in two cities of Rwanda (Kigali [51] and Musanze [5]). Food service establishments buy whole vegetables from either from markets or from the farms directly and during salad preparation, different vegetables are mixed, washed and cut. One FSE can buy different vegetables from different markets or growing regions depending on the price or availability and no fresh cut vegetables are available before the food service level. The selection of each FSE was based on the maximum transit time of two hours between the FSEs and the laboratory to minimize holding time of prepared salad before analysis. The samples were stored in cooling boxes during transportation. To prepare the FSEs for the study, we organized a consent meeting in which managers of FSEs were briefed about the study and its importance in improving food safety. Out of 280 FSEs invited, 168 FSE managers showed interest to participate in the study and were provided with consent forms to register. To investigate the ability of FSEs to decrease microbial load during salad preparation, a sample was taken at the start and at the end of salad preparation. The samples were provided for free and after the laboratory analysis, we shared the test report and feedback with each individual FSE.

Sample collection

Farms

Each of the 11 types of vegetables was sampled 9 times leading to total of 99 vegetable samples which were purchased randomly from 99 farms. The sampling procedure slightly differed for the three categories of vegetables (fruit, subterranean, leafy). Fruit vegetables (i.e. cucumber, green pepper, tomato) were picked at maturity from the plant. Subterranean vegetables like carrot, beet root, garlic and onion, the vegetable roots, tubers or bulbs were uprooted, hand shaken to remove the attached soil and the aerial part cut off and discarded. Leafy vegetables such as lettuce, cabbage, celery and parsley, the samples consisted of only aerial parts which were cut from the root base. For cabbage, ten heads were collected from each farm. For other farm vegetables, a pooled farm sample (~2 kg) was collected as far apart as possible depending on the farm size. Farm size ranged from around 6 m^2 to over 4000 m^2 and several of these farm units conglomerate to form a vegetable farming area and in each farm one type of vegetable is

grown. Sterile materials such as gloves and knives were used throughout the sampling process and changed between each farm sample.

Markets

Twenty two markets were visited and in each market 11 types of vegetables were purchased leading to a total of 242 samples. To obtain a representative sample for a given market, we randomly purchased small units of vegetables from 6 to 10 vendors to get a pooled sample of about 2 kg for each type of vegetable in retail markets. In supermarkets (single vendors), packaged units were sampled from the shelves of each vegetable type. For cabbage, ten heads were purchased from each market.

Food Service establishments (FSEs)

A total of 56 FSEs (43 hotels and 13 restaurant/bars) were randomly selected and sampled. Each FSE provided 2 samples, one of whole mixed vegetables (FSE-WMV) at start of salad preparation (about 1- 2 kg) and another of ready-to- eat (FSE-RTE) vegetables (about 0.5-1 kg). For the 56 FSEs, a total of 112 (56 × 2) vegetables samples were collected. About 70% (39/56) of the visited FSEs washed vegetables with sanitizers, while others did not use any sanitizer but rinsed vegetables with either boiled water or containerized drinking water. Different sanitizers were used; 2% of FSEs used sodium troclosene (25-75 ppm), 12% used sodium hypochlorite (≈25 ppm), 21% used scouring powder (polyphosphate, sodium hydrogen carbonate, active chlorine; 4 g/l) and 65% used potassium permanganate (0.001-0.003%). Contact time of sanitizers was according to manufacturer instructions but varied between 1 and 10 min, sanitization method was by dipping and all FSEs use tap water to acquire the aqueous sanitizing solution. Food handlers had no specific information on the quantity of vegetables that can sanitized for a given concentration of sanitizer solution.

Sample storage and transportation

After sampling, all samples were placed in sterile zipped polyethylene bags and immediately stored in cooling boxes with ice packs and transported to the laboratory. The transit time was 1-3 h. The samples from farms and markets were analyzed within 24 h while samples from FSEs were analyzed immediately upon reaching the laboratory.

Microbiological analyses

Whole vegetable samples from farm, market and FSEs were first sliced /cut into small pieces (16) on a sterile stainless steel tray using sterilized knives and gloves for each sample, mixed and followed by weighing of the 25ganalytical unit to make the 1:10 dilution with 225 ml of diluent. For the cut RTE vegetables, the samples were hand mixed while still in the field sampling bags and thereafter the 25 g sample was measured into a stomacher bag using sterile tweezers. The culture media and consumables used were from Oxoid (Oxoid Ltd., Basingstoke, UK). The samples were stomached (Model 400 Circulator, Seward, UK) in 225 ml of maximum recovery diluent (MRD) for 1min and this was followed by tenfold serial dilutions of the initial suspension using the same diluent for the enumeration of *Enterobacteriaceae*, APC, and coagulase - positive staphylococci (CP. staphylococci); while for *Listeria* spp. buffered peptone was used as a diluent. The IMOs were enumerated according to ISO methods *i.e.*.;

Enterobacteriaceae [ISO 21528-2: 2004] (17) , APC [ISO 4833-1: 2013] (18) , coagulase - positive Staphylococci (CP. staphylococci) [ISO 6888-2: 1999] (19), *Listeria* spp. [ISO 11290-2: 1998/Amd 1: 2004] (20)at 37°C for 48h (presumptive, typical, blue or blue-green colonies with or without halo were counted as *Listeria* spp.). Selected presumptive *Listeria* strains (n = 99) isolated from farm vegetables were confirmed with API *Listeria* (Biomerieux, France). For quality control of the media and positive controls of the experiments, strains of *E. coli* (LMG 8063) for *Enterobacteriaceae*, *Listeria monocytogenes* (LMG 16783) for *Listeria* spp. and *S. aureus* (LMG 8224) for CP. staphylococci from BCCM (Belgian Coordinated Collection of Microorganisms) were used.

Data analyses

Changes in microbial load from start to end of salad preparation were calculated by subtracting log transformed counts of each IMO in salads (FSE-RTE) from counts in mixed whole vegetables at start of salad preparation process for every FSE. Statistical analyses were performed using IBM SPSS Statistics 22. Pearson's correlation (r) was used to determine the relation between the initial counts of IMOs at start of salad preparation and the counts in ready to eat salads. Paired sample t - tests were used to compare the counts of *Enterobacteriaceae* and *Listeria* spp. in farm and market vegetables, counts of IMOs in vegetables at start of salad preparation and the counts in FSE-RTE. One way ANOVA followed by Tukey's post hoc test, was used to compare the variation in counts between the IMOs during salad preparation. Statistical significance was set at < 0.05.

Results and Discussion

Counts of Indicator microorganisms in farm and market vegetables

Fig. 3.1 and Table 3A.1 (see Appendix) show that from one vegetable to another, mean *Enterobacteriaceae* counts ranged from 4.7 to 7.2 log cfu/g at farm and 5.6 to 6.9 log cfu/g at market while the mean *Listeria* spp. counts ranged from 3.0 to 5.8 and 3.5 to 6.1 log cfu/g at farm and market respectively. For most vegetables (Fig. 1), mean *Enterobacteriaceae* and *Listeria* spp. counts were on average higher by 0.9 log cfu/g ($p = 0.01$) and by 0.5 log cfu/g ($p = 0.18$) respectively at market compared to farm. However, for garlic and onion, the mean *Enterobacteriaceae* and *Listeria* spp. counts were lower in market samples by 0.6 and 0.8 log cfu/g respectively compared to farm samples.

Current guidelines on microbial safety and quality of fresh vegetables do not provide standard limits for counts of *Enterobacteriaceae* and *Listeria* spp. at farm level. In previous studies, Listeria spp. have been reported in vegetable agricultural environment (21) and in retail vegetables. Prevalence can range 0 -100% (13, 22). Our mean counts *Listeria* spp. at farm and market (retail) were higher than most of the counts reported from different countries, Spain (23), Japan (24), UK (25, 26) but comparable to the counts reported in New Zealand (27). For *Enterobacteriaceae*, counts higher than the counts reported in this study have reported in fresh vegetables (12, 28). Nevertheless, in line with our main objective of identifying trends in microbial counts along the VSC, we observed that the counts of these IMOs increased from farm to market, indicating that between farm and market, vegetables are either contaminated or

that there are conditions that allow growth of microorganisms. The results in this study are in agreement with what has been reported elsewhere in developing countries. Studies by Shenge et al. (29) in Nigeria indicated that contamination of tomato fruits with total coliforms and *E. coli*, increased from farm to market. In Jordan, the same trend was observed in fresh vegetables (parsley, lettuce, radish), *E. coli* counts increased by 1 log cfu/g from farm to market (30).

Previous studies have attributed this microbial contamination and or proliferation at farm and market to the pre-and post-harvest factors including soil, irrigation water, green or inadequately composted manure, air (dust), weather conditions, wild and domestic animals, insects, feces, wash water, human handling, among others (30-33). For garlic and onion, the mean counts of *Enterobacteriaceae* and *Listeria* spp. at market were lower compared to the farm level. This trend in microbial counts may be attributed to the reported antimicrobial activity of the organosulphur compounds in these vegetables (34) but also to the practices between farm and market or at market level. In practice, garlic and onion are be kept for longer time at market (more than one week), the outer covers (layers) dry out and keep peeling off, yet the inside fresh parts with nutrients may be undesirable to microorganisms due to the reported antimicrobial compounds. Therefore fresh garlic and onion in open field farms are most likely to have higher microbial counts than the counts from the drying garlic and onion at market level. Further research is needed to investigate the downward trend in microbial counts from farm to market for onion and garlic. If the research outcome points to the reported antimicrobial activity of compounds in onion and garlic, extracts of these compounds may be used in future as antimicrobial ingredients in vegetable salads.

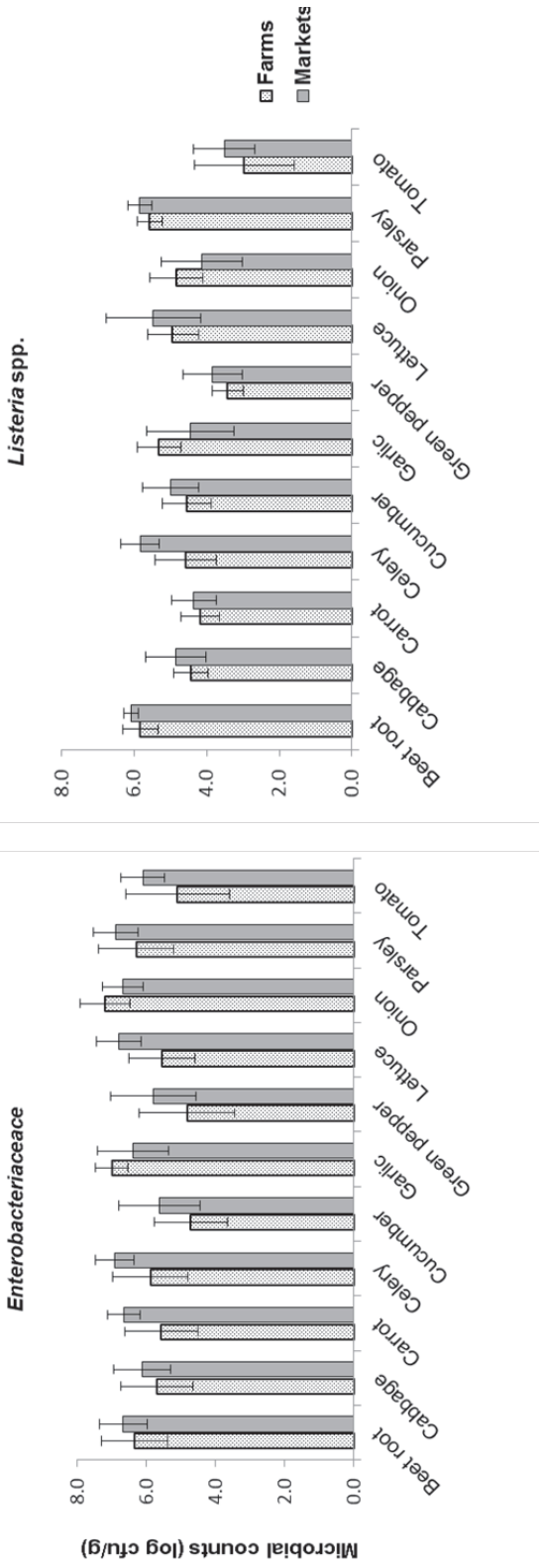

Figure 3.1: Levels of *Enterobacteriaceae* and *Listeria* spp. for different vegetables in farms (n=9 for each vegetable type) and markets (n=22). Error bars represent the standard deviation in microbial counts for a particular vegetable type at different farms and markets.

Counts of indicator microorganisms during salad preparation at FSEs

Fig. 3.2 shows respective pooled mean counts for *Enterobacteriaceae* and *Listeria* spp. in vegetables at farm (5.8 and 4.6 log cfu/g) and at market (6.3 and 4.9 log cfu/g) in addition to mean counts for these IMOs in whole mixed vegetables at FSE level (6.0 and 5.1 log cfu/g respectively). Fig. 3.2 also shows that from start to end of salad preparation, mean counts for *Enterobacteriaceae* and *Listeria* spp. were significantly reduced ($p < 0.001$) on average by 2.5 log cfu/g.

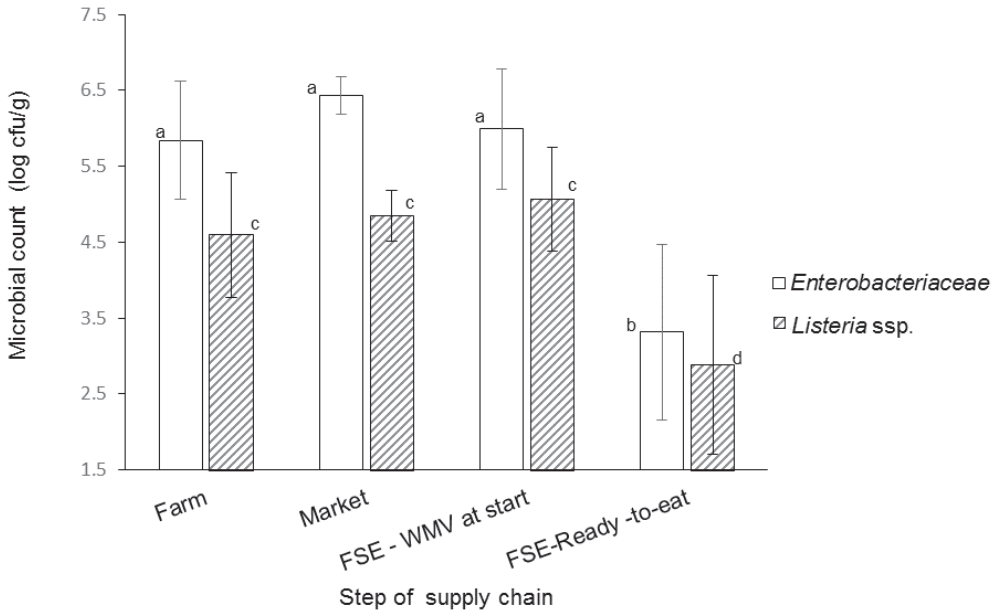

Figure 3.2: Average counts of *Enterobacteriaceae* and *Listeria* spp. at various points of the vegetable supply chain, represented as the mean from different vegetables used for salad preparation. WMV-FSE at start = whole mixed vegetables at start of salad preparation and FSE- Ready-to-eat = garnished ready-to-eat salads at FSEs. Error bars represent the standard deviation in microbial counts at the different stages of the vegetable supply chain. Bars without a common letter show mean microbial counts that differ significantly.

In other results, mean count for aerobic plate count and CP. staphylococci were 6.8, and 4.6 log cfu/g in whole mixed vegetables before salad preparation (FSE-WMV) and 4.9 and 3.0 in ready-to-eat salads (FSE-RTE) respectively. Overall, with all the four IMOs combined, average microbial load reduction was 2.1 log cfu/g from start to end of salad preparation in FSEs. The salad preparation process at FSEs is done manually by food handlers. Salad contact surfaces include knives, shredders, chopping boards, washing and or sanitization sinks. Furthermore Fig. 3.3 shows the changes in counts of different IMOs during salad preparation form one FSE to the other. We observed decreases and few increases in counts of different IMOs separated by the "zero change line" (line for no increase or decrease in IMO counts) at individual FSEs during salad preparation. It can be seen that 88% (49/56) of FSEs show a decrease for all the four IMOs (*Enterobacteriaceae*, *Listeria* spp., APC and CP. staphylococci) during salad preparation. Among the 7 FSEs that were above the "zero change line"; 4/7 of FSEs had

increased counts in CP. staphylococci; 1/7 in APC; 1/7 in *Listeria* spp. and 1/7 in *Enterobacteriaceae*. Using Tukey's method with post hoc tests to compare the ability of FSEs to reduce counts between IMOs, our results indicate that *Enterobacteriaceae* counts were most reduced compared to CP. staphylococci ($p < 0.001$), APC ($p = 0.006$) and *Listeria* spp. ($p = 0.085$).

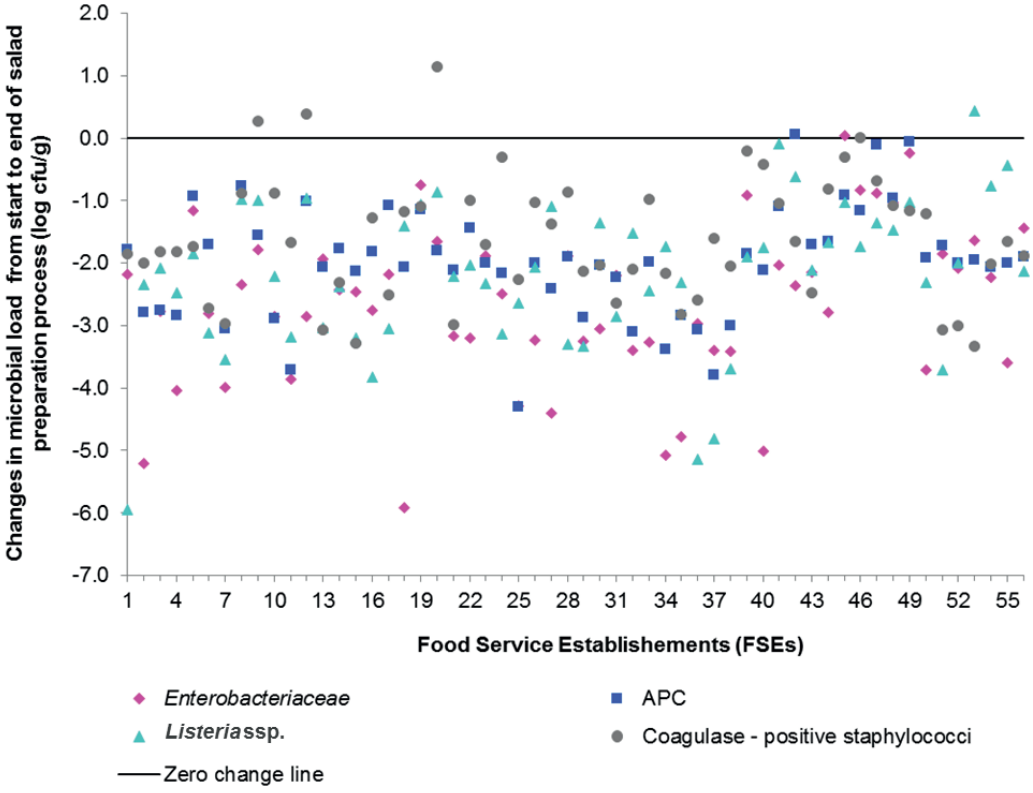

Figure 3.3: Changes of IMOs during salad preparation by 56 Food Service Establishments with average reductions (± standard deviations) of 2.7 (± 1.3) log cfu/g for *Enterobacteriaceae*; 2.0 (± 0.9) APC; 2.2 (± 1.2) of *Listeria* ssp. and 1.6 (± 1.0) of Coagulase-positive staphylococci.

The significant reduction in microbial counts at FSEs (Fig. 3.2) suggests that in the entire VSC in Rwanda, the burden of cleaning vegetables is targeted only at FSEs. This reduction can be attributed to process operations like washing and sanitization, trimming, peeling and cutting that occur at FSEs. However these very process operations such as washing, have also been pointed out as a potential source of contamination if mismanaged (35, 36). In this study, evidence of infrequent contamination is demonstrated by the increase in microbial count during salad preparation shown in Fig. 3.3. These results also show that the ability of FSEs to reduce the microbial load differs among microorganisms indicating that the efficacy of the washing and sanitization process depends on nature of microorganisms present in the vegetables, but also on the effectiveness of the sanitation process. Effectivity of the sanitization process could be improved by selecting and advising reliable sanitation techniques aiming at a lower mean concentration in the final product. In addition the large standard deviation of the counts might

be a target for improved hygienic practices during the salad sanitation and preparation processes. Among the used IMOs, CP. staphylococci increased more dominantly during salad preparation than the other studied IMOs and this contamination may be from food handlers. The observed differences in microbial counts for various vegetable types (Fig. 3.1), the reported high microbial counts in vegetables at start of salad preparation, and the observed possibility of food handlers to contaminate salads during preparation, illustrates the complexity of controlling microbial safety of ready-to eat vegetables.

Benchmarking of microbial counts in salads with regulatory requirements

The ability to reduce the microbial load of incoming vegetables by the majority of FSEs (88%) is encouraging but regulatory requirements may still not be achieved. In Rwanda, guidelines or regulatory requirements for ready-to-eat vegetables are yet to be determined, so we compared our results with the guidelines from the United Kingdom (37). The Health Protection Agency (UK-HPA) classifies total *Listeria* spp. as hygiene indicator with a maximum number of 10^2 cfu/g and CP. staphylococci as pathogen (*S. aureus*) at 10^4 cfu/g. *Enterobacteriaceae* and APC have no set limits since these IMOs are considered to be part of the normal micro flora of vegetables (37). Fig. 3.4 shows that 91% (51/56) and 22% (12/ 56) of FSEs fulfill the guidelines for *S. aureus* and *Listeria* spp. respectively.

Whereas *Listeria* spp. have been commonly associated with agricultural and produce production environments (21, 31), the possibility of pathogenic *Listeria* spp. (*Listeria monocytogenes*) being present cannot be ignored. Indeed confirmation of selected presumptive *Listeria* strains isolated from farm vegetables (data not shown) revealed that 4 out of 99 isolates were *L. monocytogenes*. Chapin et al. (21) reported that half of the samples where *Listeria* spp. was isolated from produce production environment had *L. monocytogenes* and in some cases (6%) all *Listeria* spp. were *L. monocytogenes*. On the other hand, not all presumptive *Listeria* spp. isolates may be truly *Listeria* spp., as Angelidis et al. (38) have reported non *Listeria* spp. bacteria growing with closely similar colonies (bluish green) on the agar we used (Agar *Listeria* according to Ottaviani and Agosti). Nevertheless, the high counts of presumptive *Listeria* spp. reported in this study may indicate exposure to *L. monocytogenes* and risk of listeriosis to consumers of vegetable salads especially the young, old, pregnant and immuno-compromised individuals.

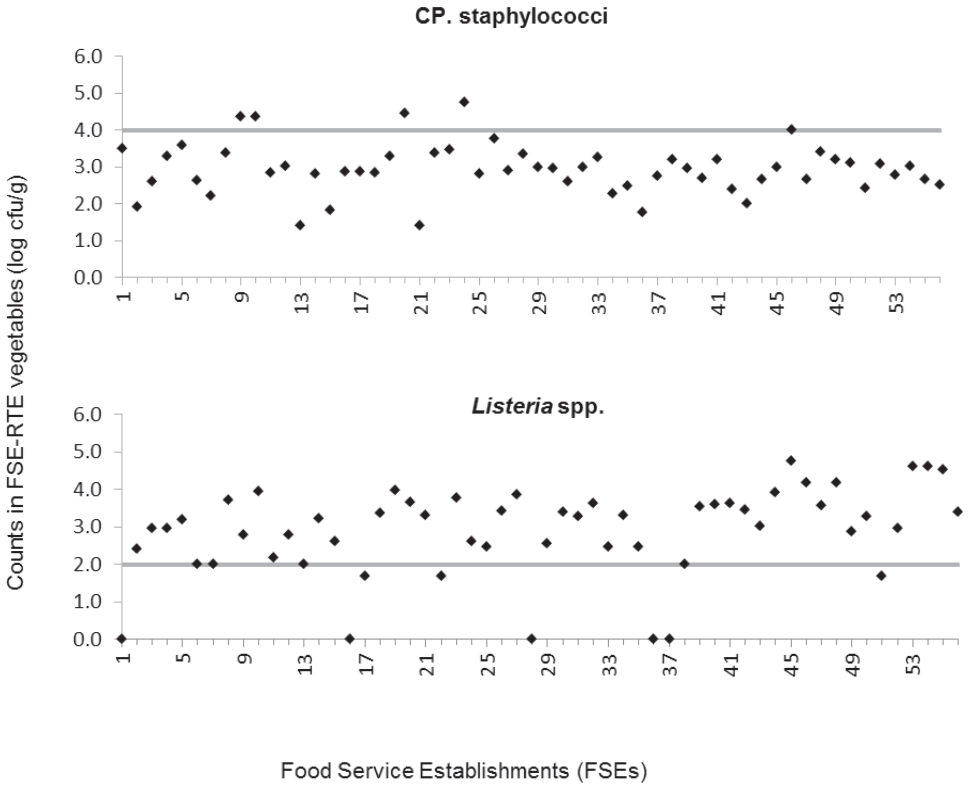

Figure 3.4: Comparison of microbial levels in Ready-to-eat salads prepared in 56 food service establishments in Rwanda with the requirements of the UK-Health Protection Agency for ready-to-eat vegetables: *Listeria* spp. less than 10^2 cfu/g and coagulase-positive staphylococci (CP. staphylococci) less than 10^4 cfu/g. Points on the x- axis were bellow detection limit.

Correlation between microbial counts in vegetables at start and at the end of salad preparation.

In this study we also focused on the microbial load of the vegetables at the start of salad preparation and how it can influence the microbial load in the final product (FSE - RTE). Fig. 3.5 shows the relationship between the initial microbial counts of IMOs in mixed vegetables at the start of salad preparation (FSE-WMV) and the microbial counts in ready- to-eat salads for each FSE. The plots show that almost all points were below the line y=x –axis and no significant correlation (*r*) between the FSE-WMV and FSE – RTE for all the four IMOs, *ie*. CP. staphylococci ($r = 0.04$, $p = 0.974$); APC ($r = 0.086$, $p = 0.528$); *Enterobacteriaceae* ($r = 0.203$, $p = 0.134$) and *Listeria* spp. ($r = 0.245$, $p = 0.073$).

Researchers have highlighted a close relationship between the total mesophilic aerobic counts on lettuce raw material and those on finished shredded lettuce product (39). However, in our study the correlations between counts of in-coming vegetables and the counts of ready-to-eat

were insignificant, suggesting that the quality of washing and sanitization plays an important role in changing the microbial levels of ready-to-eat salads.

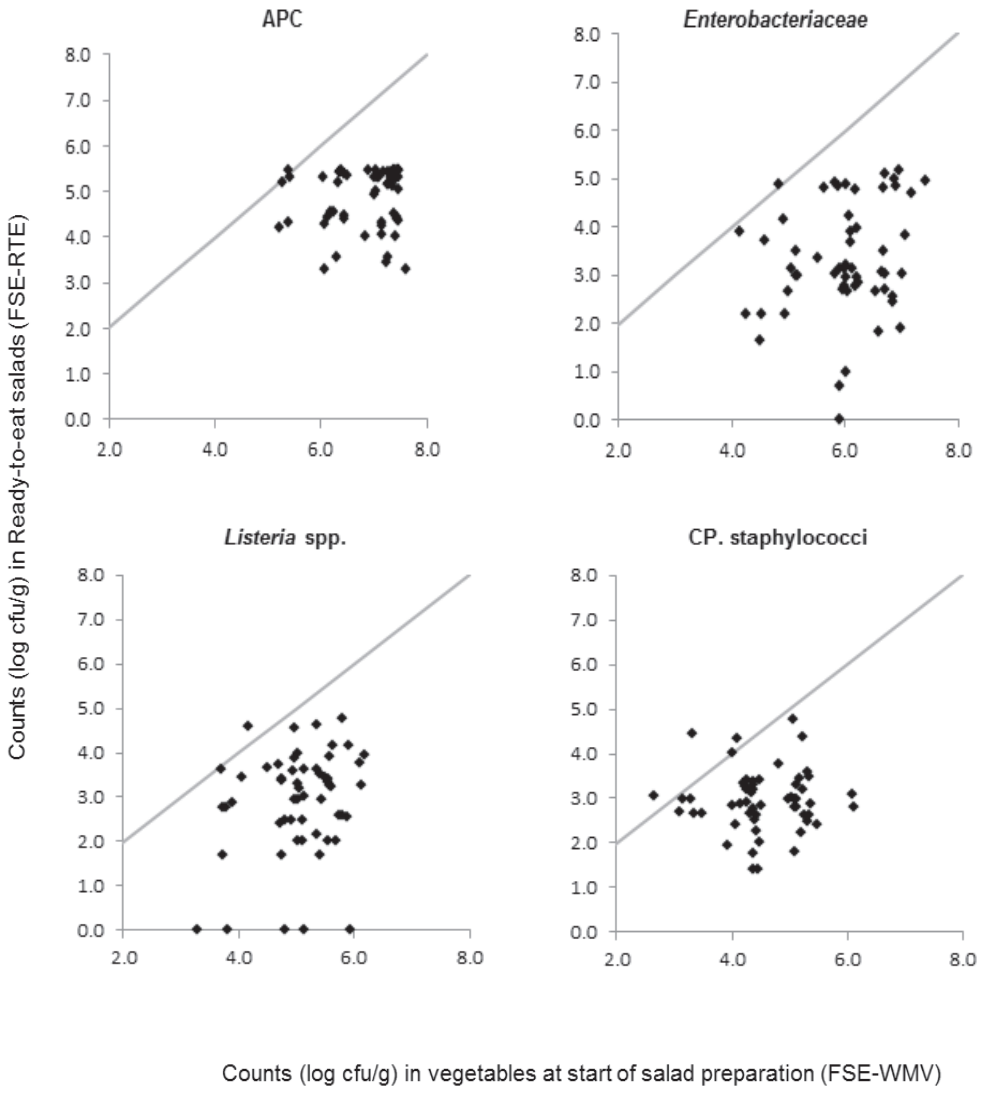

Figure 3.5: Comparison of the initial microbial counts of IMOs in mixed vegetables at the start of salad preparation (FSE-WMV) and the microbial counts in Ready to eat salads (FSE-RTE) for 56 FSEs. The plotted line represents y = x. Points on the x-axis were bellow detection limit.

Conclusion

In this study, we have investigated the trends in counts of IMOs along the VSC (farm, market and FSE) in Rwanda. We have shown that the IMOs counts increased from farm to market vegetables and that IMOs counts did not significantly change from market to FSEs (vegetable before salad preparation). Overall at food service establishments, microbial counts were significantly reduced by unit operations like washing with or without sanitizers, trimming/peeling, with an average reduction of 2.1 log cfu/g from start to end of salad preparation. We have also observed that counts of presumptive *Listeria* spp. and CP. staphylococci in ready-to-eat salads from 78% and 9% of FSEs respectively, exceeded the guideline established by the UK-HPA (37). We acknowledge that exceeding the guideline for counts of these IMOs in ready-to-eat vegetables may not necessarily mean these vegetables are unsafe for consumption. However, the trends in microbial counts presented in this study, should alert the concerned stakeholders, risk managers, and policy makers about the importance of microbial safety in this VSC. Consequently, it would be important in future to study and understand the pre- and post-harvest practices, the mechanisms of contamination and the major pathogens in the context of this VSC.

Appendix

Table 3A.1: Comparison of the levels of *Listeria* spp. and *Enterobacteriaceae* at Farm and Market for different vegetables

		Listeria spp. (log cfu/g)				*Enterobacteriaceae* (log cfu/g)			
		Farms		Markets		Farms		Markets	
S/N	Vegetables	MEAN (n=9)	SD (±)	MEAN (n=22)	SD(±)	MEAN(n=9)	SD(±)	MEAN(n=22)	SD(±)
1	Beet root	5.83	0.48	6.09	0.19	6.35	0.95	6.68	0.69
2	Cabbage	4.42	0.47	4.85	0.84	5.70	1.05	6.13	0.82
3	Carrot	4.17	0.53	4.35	0.61	5.58	1.06	6.67	0.47
4	Celery	4.58	0.84	5.83	0.53	5.90	1.09	6.93	0.55
5	Cucumber	4.55	0.67	4.99	0.76	4.73	1.05	5.63	1.18
6	Garlic*	5.30	0.60	4.45	1.21	7.00	0.47	6.40	1.04
7	Green pepper	3.42	0.43	3.84	0.81	4.84	1.37	5.81	1.24
8	Lettuce	4.93	0.70	5.47	1.31	5.55	0.96	6.81	0.65
9	Onion*	4.83	0.73	4.13	1.12	7.20	0.72	6.69	0.59
10	Parsley	5.57	0.34	5.84	0.33	6.31	1.09	6.90	0.64
11	Tomato	2.96	1.38	3.52	0.85	5.10	1.50	6.11	0.63

*For garlic and onion, the counts of *Enterobacteriaceae* and *Listeria* spp. decreased from farm to market.

References

1. **FAO STAT** (Food and Agriculture Organization of the United Nations, statistics division). 2015. http://faostat3.fao.org/compare/E . Accessed on 03rd May 2016.

2. **Callejon RM, Rodriguez-Naranjo MI, Ubeda C, Hornedo-Ortega R, Garcia-Parrilla MC, Troncoso AM**. 2015. Reported foodborne outbreaks due to fresh produce in the United States and European Union: trends and causes. *Foodborne Pathogens & Disease* 12:32-38.

3. **Herman KM, Hall AJ, Gould LH.** 2015. Outbreaks attributed to fresh leafy vegetables, United States, 1973-2012. *Epidemiology & Infection* 143:3011-3021.

4. **Painter JA, Hoekstra RM, Ayers T, Tauxe RV, Braden CR, Angulo FJ, Griffin PM.** 2013. Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998-2008. *Emerging Infectious Diseases* 19:407-415.

5. **Lynch MF, Tauxe RV, Hedberg CW.** 2009. The growing burden of foodborne outbreaks due to contaminated fresh produce: Risks and opportunities. *Epidemiology & Infection* 137:307-315.

6. **Kozak GK, Macdonald D, Landry L, Farber JM.** 2013. Foodborne outbreaks in Canada linked to produce: 2001 through 2009. *Journal of Food Protection* 76:173-183.

7. **CAC/RCP 53.** 2003. Code of hygienic practice for fresh fruits and vegetables. Codex Alimentarius Commision. http://www.fao.org/ag/agn/CDfruits_en/others/docs/alinorm03a.pdf. Accessed 06th January 2016.

8. **Holvoet K, Sampers I, Seynnaeve M, Uyttendaele M**. 2014. Relationships among hygiene indicators and enteric pathogens in irrigation water, soil and lettuce and the impact of climatic conditions on contamination in the lettuce primary production. *International Journal of Food Microbiology* 171:21-31.

9. **Schwaiger K, Helmke K, Hölzel CS, Bauer J.** 2011. Comparative analysis of the bacterial flora of vegetables collected directly from farms and from supermarkets in Germany. *International Journal of Environmental Health Research* 21:161-172.

10. **Ward M, Dhingra R, Remais JV, Chang HH, Johnston LM, Jaykus L-A, Leon J.** 2015. Associations between Weather and Microbial Load on Fresh Produce Prior to Harvest. *Journal of Food Protection* 78:849-854.

11. **Buchanan RL, Oni R.** 2012. Use of microbiological indicators for assessing hygiene controls for the manufacture of powdered infant formula. *Journal of Food Protection* 75:989-997.

12. **Little C, Roberts D, Youngs E, De Louvois J.** 1999. Microbiological quality of retail imported unprepared whole lettuces: A PHLS food working group study. *Journal of Food Protection* 62:325-328.

13. **Zhu Q, Hussain MA.** 2015. Prevalence of *Listeria* species in Fresh Salad Vegetables and Ready-to-Eat Foods Containing Fresh Produce Marketed in Canterbury, New Zealand. *Advanced in Food Technology and Nutritional Sciences - Open Journal* 1:5-9.

14. **Aycicek H, Oguz U, Karci K.** 2006. Determination of total aerobic and indicator bacteria on some raw eaten vegetables from wholesalers in Ankara, Turkey. *International Journal of Hygiene & Environmental Health* 209:197-201.

15. **Balzaretti CM, Marzano MA.** 2013. Prevention of travel-related foodborne diseases: Microbiological risk assessment of food handlers and ready-to-eat foods in northern Italy airport restaurants. *Food Control* 29:202-207.

16. **Mukherjee A, Speh D, Diez-Gonzalez F**. 2007. Association of farm management practices with risk of *Escherichia coli* contamination in pre-harvest produce grown in Minnesota and Wisconsin. *International Journal of Food Microbiology* 120:296-302.

17. **ISO 21528-2**. 2004. Microbiology of food and animal feeding stuffs -- Horizontal methods for the detection and enumeration of *Enterobacteriaceae* -- Part 2: Colony-count method.

18. **ISO 4833-1.** 2013. Microbiology of the food chain -- Horizontal method for the enumeration of microorganisms -- Part 1: Colony count at 30 degrees C by the pour plate technique.

19. **ISO 6888-2.** 1999. Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) -- Part 2: Technique using rabbit plasma fibrinogen agar medium.

20. **ISO 11290-2:1998/Amd 1.** 2004. Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of *Listeria monocytogenes* — Part 2: Enumeration method AMENDMENT 1: Modification of the enumeration medium.

21. **Chapin TK, Nightingale KK, Worobo RW, Wiedmann M, Strawn LK.** 2014. Geographical and meteorological factors associated with isolation of *Listeria* species in New York state produce production and natural environments. *Journal of Food Protection* 77:1919-1928.

22. **McMahon MAS, Wilson IG.** 2001. The occurrence of enteric pathogens and *Aeromonas* species in organic vegetables. *International Journal of Food Microbiology* 70:155-162.

23. **Selma MV, Allende A, López-Gálvez F, Elizaquível P, Aznar R, Gil MI**. 2007. Potential microbial risk factors related to soil amendments and irrigation water of potato crops. *Journal of Applied Microbiology* 103:2542-2549.

24. **Kaneko KI, Hayashidani H, Ohtomo Y, Kosuge J, Kato M, Takahashi K, Shiraki Y, Ogawa M.** 1999. Bacterial contamination of ready-to-eat foods and fresh products in retail shops and food factories. *Journal of Food Protection* 62:644-649.

25. **Little CL, Mitchell RT.** 2004. Microbiological quality of pre-cut fruit, sprouted seeds, and unpasteurised fruit and vegetable juices from retail and production premises in the UK, and the application of HACCP. *Communicable Disease and Public Health / PHLS* 7:184-190.

26. **Sagoo SK, Little CL, Mitchell RT**. 2001. The microbiological examination of ready-to-eat organic vegetables from retail establishments in the United Kingdom. *Letters in Applied Microbiology* 33:434-439.

27. **Zhu, Qi.** 2015. Assessment of Listeria species in fresh produce grown and sold in Canterbury, New Zealand. https://researcharchive.lincoln.ac.nz/handle/10182/6671?show=full. Accessed 30th August 2015.

28. **Oliveira M, Usall J, Vinas I, Anguera M, Gatius F, Abadias M.** 2010. Microbiological quality of fresh lettuce from organic and conventional production. *Food Microbiology* 27:679-684.

29. **Shenge KC, Whong CMZ, Yakubu LL, Omolehin RA, Erbaugh JM, Miller SA, LeJeune JT.** 2015. Contamination of tomatoes with coliforms and *Escherichia coli* on farms and in markets of Northwest Nigeria. *Journal of Food Protection* 78:57-64.

30. **Faour-Klingbeil D, Murtada M, Kuri V, Todd ECD.** 2016. Understanding the routes of contamination of ready-to-eat vegetables in the Middle East. *Food Control* 62:125-133.

31. **Beuchat LR, Ryu JH.** 1997. Produce Handling and Processing Practices. *Emerging Infectious Diseases* 3:459-465.

32. **Gil MI, Selma MV, Suslow T, Jacxsens L, Uyttendaele M, Allende A.** 2015. Pre- and postharvest preventive measures and intervention strategies to control microbial food safety hazards of fresh leafy vegetables. *Critical Reviews in Food Science and Nutrition* 55:453-468.

33. **Marvasi M, Hochmuth GJ, Giurcanu MC, George AS, Noel JT, Bartz J, Teplitski M**. 2013. Factors that affect proliferation of *Salmonella* in tomatoes post-harvest: The roles of seasonal effects, irrigation regime, crop and pathogen genotype. *PLoS ONE* 8.

34. **Wilson EA, Demmig-Adams B.** 2007. Antioxidant, anti-inflammatory, and antimicrobial properties of garlic and onions. *Nutrition & Food Science* 37:178-183.

35. **Gil MI, Selma MV, López-Gálvez F, Allende A**. 2009. Fresh-cut product sanitation and wash water disinfection: Problems and solutions. *International Journal of Food Microbiology* 134:37-45.

36. **Holvoet K, De Keuckelaere A, Sampers I, Van Haute S, Stals A, Uyttendaele M.** 2014. Quantitative study of cross-contamination with *Escherichia coli*, *E. coli* O157, MS2 phage and murine norovirus in a simulated fresh-cut lettuce wash process. *Food Control* 37:218-227.

37. **HPA-UK.** 2009. Health Protection Agency. Guidelines for Assessing the microbiological safety of ready-to-Eat foods. London: UK, Health Protection Agency. https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/363146/Guidelines_for_assessing_the_microbiological_safety_of_ready-to-eat_foods_on_the_market.pdf. Accessed 15th November 2015.

38. **Angelidis AS, Kalamaki MS, Georgiadou SS.** 2015. Identification of non-*Listeria* spp. bacterial isolates yielding a β-d-glucosidase-positive phenotype on Agar *Listeria* according to Ottaviani and Agosti (ALOA). *International Journal of Food Microbiology* 193:114-129.

39. **Barth M, Hankinson TR, Zhuang H, Breidt F.** 2009. Microbiological spoilage of fruits and vegetables, p. 135-183. *In Sperber HW*, Doyle PM (ed.), Compendium of the Microbiological Spoilage of Foods and Beverages. Springer New York, New York, NY.

4

Foodborne pathogens and their risk exposure factors associated with farm vegetables in Rwanda

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Abstract

In this study, we tested farm vegetables and agricultural water for the presence of foodborne pathogens, and evaluated farming practices of vegetable farms in Rwanda. Farm vegetable samples were found to be contaminated with food borne pathogens at considerably high rate (overall 15/99=15%). Specifically, the prevalence of pathogens in farm vegetables varied from 1.0% (1/99) for *Listeria monocytogenes*, 3.0% (3/99) for thermo-tolerant *Campylobacter* spp., 5.1% (5/99) for *Salmonella* spp. to 6.1% (6/99) pathogenic *Escherichia coli.* In agricultural water from rivers, lakes, lagoons, ground and marshlands, prevalence of DNA from pathogens varied from 3.3 % (1/30) for Enteroinvasive *E. coli* (EIEC); 6.7% (2/30) for Enteroaggregative *E. coli* (EAEC); 13.3% (4/30) for Enterotoxigenic *E. coli.* (ETEC) and *Vibrio cholera*; 20.0% (6/30) for *Yersinia pestis*; 26.7% (8/30) for *Francisella tularensis*; 40% (12/30) for *Cyclospora* to 86.7% (26/30) for thermo-tolerant *Campylobacter* spp. DNA of the following pathogens was not detected in water: entero pathogenic *E. coli* (EPEC), shiga toxin producing *E.coli* (STEC), *Salmonella* spp., *L. monocytogenes*, *Burkholderia*, *Rickettsia*, *Toxoplasma gondii*, *Giardia lamblia*, *Cryptosporidium*, *Entamoeba histolytica* and *Hepatitis* E. About farming practices, 60% of the visited vegetable farms practiced irrigation and all the water used was from un-protected sources (from marshlands [70%], rivers [18%], lakes [7%], runoff lagoons [5%]). Over 80% of the farms applied overhead irrigation methods and none of the farms had implemented measures to restrict to access of domestic and wild animals, while 50% of the farms used untreated manure. The high detection rate of foodborne pathogens in agricultural water in combination with the observed several risky farming practices forms a likely explanation for the observed prevalence of pathogens in farm vegetables as reported in this study and is of important public health concern if these vegetables are to be consumed raw.

Introduction

Foodborne illnesses caused by microbial hazards contribute significantly to the global burden of disease (1). In attribution studies, fresh vegetables have been reported among the major food vehicles of foodborne microbial hazards or pathogens (2, 3). Fresh vegetables are known to harbor significant numbers of epiphytic microorganisms, mostly nonpathogenic. However during the "farm to fork" continuum especially in the open field cultivation, fresh vegetables are exposed to environmental conditions or factors that can introduce all kinds of pathogenic microorganisms; bacteria (4), parasites (5) and viruses (6). Once introduced to vegetable plant structures, pathogens can become part of the resident microflora through attachment (7) and internalization (8). With growing popularity of vegetables eaten raw, early detection, control and prevention of factors that contaminate fresh vegetables along the supply chain is paramount.

Beuchat and Rhu (9) classified the sources of contamination for fresh vegetables into pre- and postharvest factors. Pre-harvest factors include: irrigation water, soil, inadequately composted manure, domestic and wild animals, among others, while postharvest factors mostly include: human handling, equipment, containers, wash and rinse water, flying insects to mention a few. In order to reduce the burden of foodborne illnesses due to fresh vegetables, it is important to adopt preventive measures that have shown to be effective. Cognizant of this need, several countries and international organizations like the World Health Organization (WHO) and the Food and Agricultural Organization (FAO) have since developed codes of practice, guidelines and regulations (10-15) with measures that can be used to prevent and control microbial hazards along the fresh vegetable supply chain. While these preventive measures have been documented, the levels of implementation varies from region to region and country to country.

In this study, we investigated the farming practices, prevalence of foodborne pathogens in farms vegetables and agricultural water in Rwanda. First, in vegetable farms, we observed farming practices and also detected foodborne pathogens commonly implicated in outbreaks associated with fresh vegetables (16) and *Listeria monocytogenes*, considered ubiquitous in plant vegetation (17) and having high case fatality rate (18). Second, we analyzed agriculture water for presence of DNA of 19 foodborne pathogens since water has been pointed out as a potential major source of microbial contamination for fresh vegetables through irrigation, washing and flooding (19, 20). The microbial profile of water from surrounding rivers, lakes, ponds/lagoons and marshlands was also used to reflect on the associated environmental activities by humans, livestock and wild life within the vicinity of vegetables fields.

Materials and methods

Study setup

Figure 4.1 shows an overview of the study location. Vegetable samples from Rwandan farms were purchased and investigated for the presence of *Salmonella* spp., *L. monocytogenes*, *Campylobacter jejuni* and pathogenic *E. coli* from February to October 2015. Sampling of farm vegetables was done simultaneously with observation of farming practices with impact on

microbial safety of fresh vegetables. In the second part, we extracted DNA from agricultural water (*i.e.*. irrigation and on-farm postharvest wash water) and investigated the presence of DNA originating from pathogenic microorganisms.

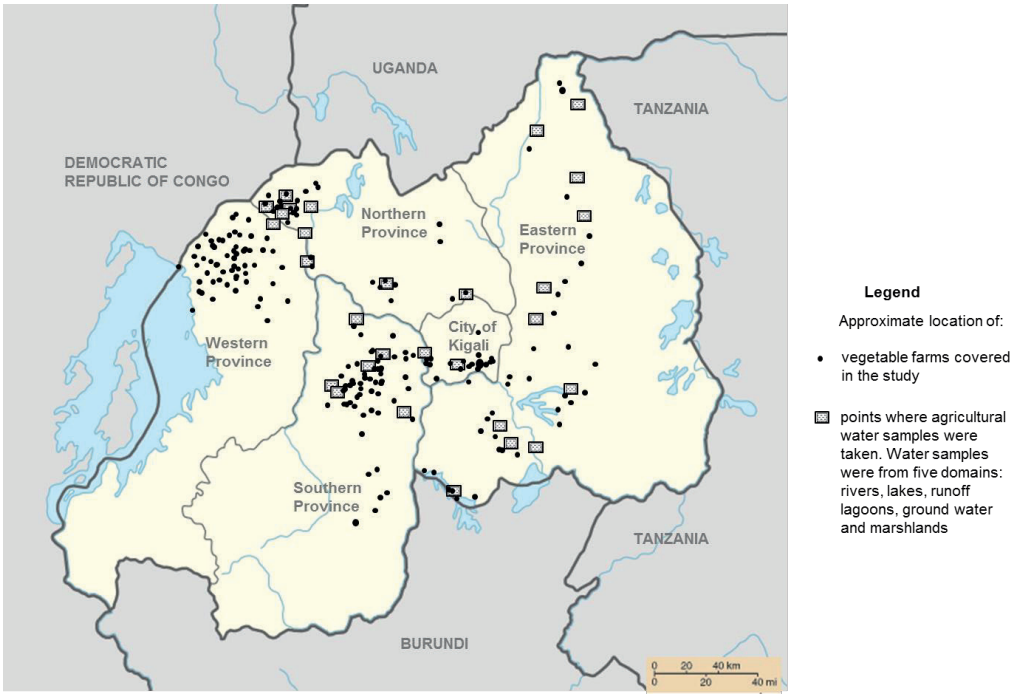

Figure 4.1: Map of Rwanda showing study points in the four provinces and the City of Kigali [Source: Adapted from Wikipedia (21)]

Observation study of farming practices and field conditions

From February to October 2015, a farm review consisting of on-site observations and face to face interviews with vegetable farmers was conducted in Rwanda (Fig. 4.1). The farm review was based on the USA department of Agricultural (USDA) checklist for Good Agricultural Practices (GAPs) and Good Handling Practices (using part1- farm review section, Tab.3) (11). Special focus was accorded to irrigation water, animal and human activity and compost manure as part of the WHO – five keys to growing safer fruits and vegetables (12) and the % number of farms falling in the " yes", "no" or not applicable (NA) answers options was recorded. A total of 198 farms were covered in the study, 29 farms in the Northern province, 59 in the Western province , 34 in the Eastern province , 57 in the Southern province and 19 farms in peripherals of the City of Kigali (Fig. 4.1). Selection of farms for the study was based on the availability of vegetables in farms and one vegetable type was sampled per farm using the same approach as in our previous related study (22). Vegetables understudy were, fruit (*i.e.*. cucumber, green pepper and tomato); subterranean (carrots, beet root, garlic and onion) and leafy vegetables (lettuce, cabbage, celery and parsley).

Sampling of farm vegetables

Each of the 11 types of vegetables were sampled 9 times leading to a total of 99 vegetable samples which were purchased randomly from 99 farms. Sampling of farm vegetables was done in line with the methodology employed in a previous related study (22). Briefly, for subterranean vegetables like carrot, beet root, garlic and onion, the vegetable roots, tubers or bulbs were uprooted, hand shaken to remove the attached soil and the aerial part cut off and discarded. The samples of leafy vegetables such as lettuce, cabbage, celery and parsley consisted of only aerial parts which were cut from the root base. For cabbage, ten heads were collected from each farm. For the other farm vegetables, a pooled farm sample (~ 2kg) was collected as far apart as possible. Sterile materials such as gloves and knives were used throughout the sampling process and changed between each farm sample.

Sampling of agriculture water

A total of 30 samples of agricultural water (Fig. 4.1) *i.e.*., irrigation and on-farm postharvest wash water were taken, *i.e.*. 6 samples from each of the 5 irrigation water domains as listed in the Rwanda irrigation master plan (lakes, rivers, marshlands, ground water, runoff – reservoirs) (23). Water sources or area for each irrigation domain were: Lakes; Muhazi, Mugesera, Cyohoha, Mirayi, Rumira and Birira. Rivers; Mukungwa, Base, Nyabarongo, Akagera, Muvumba and Akanyaru. Marshlands/valleys at; Yanze, Kajevuba, Jenda, Ruhengeri, Kamonyi and Kajeke. Ground water sources (water from natural sand and rocks filtration collected from boreholes and protected wells) at; Mukamira, Nyabihu, Gihinga, Kamonyi, Kigali and Kiramuruzi. Runoff – reservoirs at; Kajevuba, Cyamabuye, Kabgwayi 1, Kabgwayi 2, Kabarore and Rwagitima. Sampling of water was done according to ISO 19458 : 2006 (24) in sterilized Schott glass bottle (DURAN®, Germany) and for each water source, 1L sample was taken.

Handling and transport of samples

All vegetables and water samples were immediately stored in cooling boxes with ice packs and transported for 1-3 h to the laboratory and analyzed within 1h.

Isolation and confirmation of foodborne pathogens from farm vegetables

Preparation of laboratory samples from farm vegetables was done according to the methodology used by Mukherjee et al. (25) with slight modifications. The ~ 2 kg field vegetable samples were first sliced/cut into small pieces on a sterile stainless steel tray using sterilized knives, gloves and thereafter mixed and quartered. Each quarter provided a sample for detecting one of the four pathogens understudy (*Salmonella* spp., *L. monocytogenes*, thermo-tolerant *Campylobacter* spp. and pathogenic *E. coli*). These pathogens were isolated according to ISO methods except for the modification where each vegetable analytical unit was weighed and diluted in a 1:1 weight by volume (w/v) ratio with the enrichment broth/diluent (26) homogenized by hand rubbing for 2 min and incubated at room temperature for 1h. Unless stated otherwise, all culture media and consumables used were from Oxoid (Basingstoke, UK).

Salmonella spp.

Salmonella spp. was isolated from vegetable samples according to ISO 6579:2002 Amd 2007 (27) and confirmation reactions were applied by using the Oxoid Microbact GNB 24E

biochemical identification kit for Gram-negative bacteria (Oxoid Ltd., Basingstoke, UK). Positive controls were performed using *Salmonella* Typhimurium (LMG 14933).

L. monocytogenes

L. monocytogenes was isolated from vegetable samples according to ISO 11290-1: 1998/Amd 1: 2004 (28). Typical blue green colonies, with or without halo were harvested from Listeria Agar according to Ottaviani & Agosti (ALOA). DNA was extracted from the blue green colonies using the 'Wizard genomic DNA purification kit' (Promega Corporation, Madison, U.S.A.) following the manufacturer's instructions for isolation of genomic DNA from Gram-positive bacteria. For confirmation, real-time polymerase chain reaction (qPCR) targeting *L. monocytogenes* characteristic genes was performed. Details of the primers, probes and cycling parameters are listed in Table 4.1.

Thermo-tolerant *Campylobacter* spp.

Detection and confirmation of thermo-tolerant *Campylobacter* spp. from vegetable samples was done according to ISO 10272-1: 2017 (29), except that Bolton broth was replaced with Preston broth (higher sensitivity in presence of contaminants). For confirmation, suspected greyish colonies on modified charcoal cefoperazone deoxycholate (mCCD) agar were picked, streaked on Columbia+ horse blood agar (CAB) and incubated micro-aerobically at 41.5 °C for 48h. Thereafter, five colonies (each sample) were further streaked on Rapid *Campylobacter* agar (Bio-rad, Netherlands) and incubated micro-aerobically at 41.5 °C for 48h. Typical brick red colonies were picked and grown on CAB for confirmation tests that consisted of microscopic observation of morphology and motility test in combination with test for micro-aerobic growth at 25 °C, aerobic growth at 41.5 °C, oxidase and catalase test as described in the ISO10272-1: 2006 (29). Positive controls were performed simultaneously using *C. jejuni* C356 strains (chicken faeces; ASG-WUR, Lelystad, the Netherlands).

Table 4.1: Primer pair sequence and cycling parameters for PCR and qPCR used in the assay of different pathogens target gene(s).

Sample origin / Target organism	Target gene	Primer/ probe name ψ	Primer/ probe sequence, 5'→3' ǂ	Cycling parameters	Reference
Vegetable samples					
Listeria spp. (except *L. grayi*)	Invasion- associated protein (*iap*)	iap-31-deg-F	CA*Y*CCGC*W*AGCAC*W*GTAGTAGT	10 min at 95°C, 30 cycles of 15 s at 95°C and 30 s at 60°C	(30)
		iap-50-deg-R	GCGTC*R*ACAGT*W*GT*S*CC*H*TT		
L. monocytogenes	Listeriolysin O (*hlyA*)	hlyA-177-F	TGCAAGTCCTAAGACGCCA		
		hlyA-177-R	CACTGCATCTCCGTGGTATACTAA		
EPEC	Intimin (*eae*)	eae-F	CATTGATCAGGATTTTTCTGGTGATA		(31, 32)
		eae-R	CTCATGCGGAAATAGCCGTTA		
		eae-P	FAM-ATAGTCTCGCCAGTATTCGCCACCAATACC-TAMRA		
STEC	Shiga toxin 1 (*stx1*)	stx1-F	TTTGT*Y*ACTGT*S*ACAGC*W*GAAGC*Y*TTACG		(31, 33)
		stx1-R	CCCCAGTTCA*R*WGT*R*AG*R*TC*M*AC*R*TC		
		stx1-P	FAM-CTGGATGATCTCAGTGGGCGTTCTTATGTAA-TAMRA		
	Shiga toxin 2 (*stx2*)	stx2-F	TTTGT*Y*ACTGT*S*ACAGC*W*GAAGC*Y*TTACG		
		stx2-R	CCCCAGTTCA*R*WGT*R*AG*R*TC*M*AC*R*TC		
		stx2-P	FAM-TCGTCAGGCACTGTCTGAAACTGCTCC-TAMRA		
EIEC	Invasion plasmid antigen H (*ipaH*)	ipaH-F	CCTTTTCCGCGTTCCTTGA		(34)
		ipaH-R	CGGAATCCGGAGGTATTGC		
		ipaH-P	FAM-CGCCTTTCCGATACCGTCTCTGCA-TAMRA		
ETEC	heat-labile enterotoxin (*lt*)	LT-F	TTCCCACCGGATCACCAA	10 min at 95°C, 40 cycles of 15 s at 95°C and 30 s at 58°C	(35, 36)
		LT-R	CAACCTTGTGGTGCATGATGA		
		LT-P	FAM-CTTGGAGAGAAGAACCCT-TAMRA		
	heat-stable enterotoxin (*st*), human variant	STh-F	GCTAAACCAG*Y*AG*R*GTCTTCAAAA		
		STh-R	CCCGGTAC*AR*GCAGGATTACAACA		
		STh-P	FAM-TGGTCCTGAAAGCATGAA-TAMRA		
	heat-stable enterotoxin (*st*), porcine variant	STp-F	TGAATCACTTGACTCTTCAAAA		
		STp-R	GGCAGGATTACAACAAAGTT		
		STp-P	FAM-TGAACAACACATTTTACTGCT-TAMRA		
EAEC	Transcriptional activator (*aggR*)	aggR-F	GCAATCAGATTAA*R*CAGCGATACA	10 min at 95°C, 40 cycles of 30 s at 95°C and 30 s at 65°C, 10 min at 72°C, 5 min at 15°C	(37)
		aggR-R	CATTCTTGATTGCATAAGGATCTGG		
	Secreted protein (*aaiC*)	aaiC-F	TGGTGACTACTTTGATGGACATTGT		
		aaiC-R	GACACTCTCTTCTGGGGTAAACGA		
Water samples					
EPEC	Intimin (*eae*)	eae-F	CATTGATCAGGATTTTTCTGGTGATA	10 min at 95°C, 40 cycles of 15 s at 95°C and 30 s at 60°C	(31)
		eae-R	CTCATGCGGAAATAGCCGTTA		
		eae-P	FAM-ATAGTCTCGCCAGTATTCGCCACCAATACC-TAMRA		
STEC	Shiga toxin 1 (*stx1*)	stx1-F	TTTGT*Y*ACTGT*S*ACAGC*W*GAAGC*Y*TTACG		(31)
		stx1-R	CCCCAGTTCA*R*WGT*R*AG*R*TC*M*AC*R*TC		
		stx1-P	FAM-CTGGATGATCTCAGTGGGCGTTCTTATGTAA-TAMRA		
	Shiga toxin 2 (*stx2*)	stx2-F	TTTGT*Y*ACTGT*S*ACAGC*W*GAAGC*Y*TTACG		
		stx2-R	CCCCAGTTCA*R*WGT*R*AG*R*TC*M*AC*R*TC		
		stx2-P	FAM-TCGTCAGGCACTGTCTGAAACTGCTCC-TAMRA		

Table 4.1. Continues…..

EIEC	Invasion plasmid antigen H (*ipaH*)	ipaH-F	CCTTTTCCGCGTTCCTTGA		(34)
		ipaH-R	CGGAATCCGGAGGTATTGC		
		ipaH-P	FAM-CGCCTTTCCGATACCGTCTCTGCA-TAMRA		
ETEC	heat-labile enterotoxin (*lt*)	LT-F	TTCCCACCGGATCACCAA	10 min at 95°C, 40 cycles of 15 s at 95°C and 30 s at 58°C	(35, 36)
		LT-R	CAACCTTGTGGTGCATGATGA		
		LT-P	FAM-CTTGGAGAGAAGAACCCT-TAMRA		
	heat-stable enterotoxin (*st*), human variant	STh-F	GCTAAACCAG*Y*AG*R*GTCTTCAAAA		
		STh-R	CCCGGTACA*R*GCAGGATTACAACA		
		STh-P	FAM-TGGTCCTGAAAGCATGAA-TAMRA		
	heat-stable enterotoxin (*st*), porcine variant	STp-F	TGAATCACTTGACTCTTCAAAA		
		STp-R	GGCAGGATTACAACAAAGTT		
		STp-P	FAM-TGAACAACACATTTTACTGCT-TAMRA		
EAEC	Transcriptional activator (*aggR*)	aggR-F	GCAATCAGATTAA*R*CAGCGATACA	10 min at 95°C, 40 cycles of 30 s at 95°C and 30 s at 65°C, 10 min at 72°C, 5 min at 15°C	(37)
		aggR-R	CATTCTTGATTGCATAAGGATCTGG		
	Secreted protein (*aaiC*)	aaiC-F	TGGTGACTACTTTGATGGACATTGT		
		aaiC-R	GACACTCTCTTCTGGGGTAAACGA		
L. monocytogenes	Stress response factor SigmaB (sigB)	(4781) sigB 316F	CATGTGCCGCGCCGAATC	10 min at 95°C, 40 cycles of 15 s at 95°C and 30 s at 60°C	This study
		(4782) sigB 638R	ATCTTCTGTTCTCGCTCATC		
Campylobacter	16s rRNA	UniversalCampy-F	CAACGAGCGCAACCCACG	10 min at 95°C, 40 cycles of 15 s at 95°C and 30 s at 60°C	(38)
		UniversalCampy-R	GCATAAGGGCCATGATGACTTG		
		UniversalCampy	FAM-AGCTCAGACCAAAAGTGACCATC-TAMRA		
Salmonella	ttrRSBCA locus	ttr-6	CTCACCAGGAGATTACAACATGG	10 min at 95°C, 40 cycles of 15 s at 95°C and 30 s at 65°C	(39)
		ttr-4	AGCTCAGACCAAAAGTGACCATC		
		ttr-5	FAM-CACCGACGGCGAGACCGACTTT-BHQ1		
Francisella spp.	Insertion sequence (*ISFtu2*)	ISpri2_f	CAAGCAATTGGTAGATCAGTTGG	5 min at 95°C, 45 cycles of 5 s at 95° and 35 s at 60°C	(40)
		ISpri2_r	GACACCAATATTTCTATTGGATTACCTAAA		
		Tqpro_IS	JOE-ACCACTAAAATCCATGCTATGACTGATG-BHQ1		
Francisella tularensis	Outer membrane protein (*FopA*)	Fapri_f	GCGCTTTGACTAACAAGGACA		
		Fapri_r	CCAGCACCTGATGGAGAGTT		
		Tqpro_FA	6FAM-TGGCCAGTGGTACTTAGGTGTAGATGCTA-BHQ1		
F. tularensis subsp. *tularensis* (type A)	Pathogenicity determinant protein D gene locus (*pdpD*)	pDpri_f	TCAATGGCTCAGAGACATCAATTAAAAGAA		
		pDpri_r	CACAGCTCCAAGAGTACTATTTCC		
		Tqpro_pD	CFR590-ACCAAATCAAAATCCTGCTGAGCAGA-BHQ2		
Vibrio cholerae	Toxin A subunit (*ctxA*)	cta_f	TTTGTTAGGCACGATGATGGAT		(41)
		cta_r	ACCAGACAATATAGTTTGACCCACTAA		
		Tqpro_ctxA	6FAM-TGTTTCCACCTCAATTAGTTTGAGAAGTGCCC-BHQ1		
	Outer membrane protein regulation operon (*toxR*)	txr_f	GTGCCTTCATCAGCCACTGTAG		
		txr_r	AGCAGTCGATTCCCCAAGTTTG		
		Tqpro_toxR	JOE-CACCGCAGCCAGCCAATGTCGT-BHQ1		
Yersinia pestis	Hypothetical protein/ chromosomal signature gene *(ypo0393*)	YPO93pri_f	AGATAGTGTGACTGGTCTTGTTTCA		(40)
		YPO93pri_r	AGATGCAGATTGTATTGTAAACAATGAC		
		Tqpro_YPO093	6FAM-ACTTCCTGATATATTGGAAATCTTCTTCTC-BHQ1		
	F1 antigen (*caf1*)	cafpri_f	CCAGCCCGCATCATC		
		cafpri_r	ATCTGTAAAGTTAACAGATGTGCTAGT		
		Tqpro_caf	JOE-AGCGTACCAACAAGTAATTCTGTATCGATG-BHQ1		

Table 4.1. Continues….

Yersinia pestis	Plasminogen activator/coagulase (*pla*)	plapri2_f	ATGAGAGATCTTACTTTCCGTGAGAA		
		plapri2_r	GACTTTGGCATTAGGTGTGACATA		
		Tqpro2_Pla	CFR590-TCCGGCTCACGTTATTATGGTACCG-BHQ2		
Hepatitis E	Overlapping parts of ORF3 and ORF2	JV_F	GGTGGTTTCTGGGGTGAC	30 min at 50°C, 5 min at 95°C, 50 cycles of 10 s at 95°C and 30 s at 55°C	(42)
		JV_R	AGGGGTTGGTTGGATGAA		
		JV_P	Atto425-TGATTCTCAGCCCTTCGC-BHQ0		
Toxoplasma gondii	*Toxoplasma gondii* Repetititief 529 bp element	Tox-9F	AGGAGAGATATCAGGACTGTAG	10 min at 95°C, 45 cycles of 10 s at 95°C, 20 s at 58°C and 20 s at 72°C	(43)
		Tox-11R	GCGTCGTCTCGTCTAGATCG		
		Tox TP-1	FAM-CCGGCTTGGCTGCTTTTCCT-BHQ1		
Giardia lamblia (+ crypto)	Small ribosomal subunit RNA gene sequence (*ssurRNA*)	Giardia-80F	GACGGCTCAGGACAACGGTT		(44)
		Giardia-127R	TTGCCAGCGGTGTCCG		
		Giardia-105T	FAM-CCCGCGGCGGTCCCTGCTAG-BHQ1		
Cryptosporidium spp.	Cryptosporidium parvum/hominis specifiek 452 fragment	CrF	CGCTTCTCTAGCCTTTTCATGA		(45)
		CrR	CTTCACGTGTGTTTGCCAAT		
		Crypto	Texas red-CCAATCACAGAATCATCAGAATCGACTGGTATC-BHQ2		
Entamoeba histolytica	Small ribosomal subunit RNA gene sequence (*ssurRNA*)	Ehd-239F	ATTGTCGTGGCATCCTAACTCA		(44)
		Ehd-88R	GCGGACGGCTCATTATAACA		
		Histolytica-96T	Texas red-TCATTGAATGAATTGGCCATTT-BHQ2		
Cyclospora cayetanensis	Small ribosomal subunit RNA gene sequence (*ssurRNA*)	Cyclo250F	TAGTAACCGAACGGATCGCATT		(46)
		Cyclo350R	AATGCCACGTAGGCCAATA		
		Cyclo	FAM-CCGGCGATAGATCATTCAAGTTTCTGACC-BHQ1		
Burkholderia mallei/ pseudomallei	ISBma2, transposase	Bumcpri_f	GCGGAAGCGGAAAAAGGG	5 min at 95°C, 45 cycles of 5 s at 95°C and 35 s at 60°C	(47)
		Bumcpri_r	GCGGGTAGTCGAAGCTG		
		Tqpro_Bumc	6FAM-TCRCCAGACGCAGCAGCAT-BHQ1		
Burkholderia pseudomallei	Hypothetical protein	psupri_f	GCGCGATCCGTCGAG		
		psupri_r	AGCCGCTACGACGATTATG		
		Tqpro_psu	JOE-CCGCGACAATACGACCATCC-BHQ1		
Burkholderia mallei	Hypothetical protein	maupri3_f	GGCGAAAGAACGCGAAC		
		maupri3_r	GCGTTCCACGATCAACTCT		
		Tqpro2_mau	CFR590-CATCCCGCACCGTCCG-BHQ2		

ψ F: forward, R: reverse, P: probe

ǂ In italics are degenerated nucleotides: R = A or G, Y = C or T, W = A or T, S = C or G, M = A or C

Pathogenic *E. coli*

Escherichia coli were isolated from farm vegetables as blue green colonies on tryptone-bile-glucuronide agar (TBX) based on ISO 16649-2: 2001 (48). The isolated colonies were further tested for pathogenic *E. coli*; entero pathogenic *E. coli* (EPEC), shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC) using PCR based methods. STEC was specifically examined according to ISO/TS 13136:2012 (31). Details of the PCR methodology are presented in Table 4.1.

DNA extraction and detection of pathogens from agricultural water

DNA was extracted from agricultural water using the Power Water DNA isolation kit (MO BIO Laboratories/QIAGEN Company, Carlsbad, CA, USA) following the manufacturer's instructions. Controls of sterile deionized water (1 L) and sterile deionized water (1 L) inoculated with 1 mL of *E. coli* (LMG 8063) were included to evaluate the effectiveness of the DNA isolation procedure. Using PCR based methods, the extracted DNA samples were investigated for target genes of pathogenic *E. coli* (EPEC, STEC, EIEC, EAEC and ETEC), *Salmonella* spp., thermo-tolerant *Campylobacter* spp., *L. monocytogenes, Burkholderia*, *Francisella* spp. and *Francisella tularensis*, *Vibrio cholera*, *Yersinia pestis*, *Hepatitis* E, *Rickettsia*, *Toxoplasma gondii*, *Giardia lamblia*, *Cryptosporidium*, *Entamoeba histolytica* and *Cyclospora*. Details of the PCR methodology are presented in Table 1.

Results and discussion

Foodborne pathogens in farm vegetables

Table 4.2 shows detailed results for the tested pathogens in different farm vegetables. Out of 99 farm vegetables samples, *L.* monocytogenes was detected in 1.0% (1/99) of the samples, thermo-tolerant *Campylobacter* spp., in 3.0% (3/99), *Salmonella* spp. in 5.1% (5/99) *E. coli* pathotypes detected in 6.1% (6/99) of the samples. Generally one pathogen was isolated per farm except for two farms where two pathogens were detected in each *ie. Salmonella* spp. and thermo-tolerant *Campylobacter* spp. together in a lettuce farm from the southern province (Fig. 4.1) and another farm of parsley with Shiga toxin-producing *E. coli* (STEC) thermo-tolerant *Campylobacter* spp. in the Northern province. Furthermore, generic E. coli as an indicator of pathogenic microorganisms was isolated in vegetable samples from 76 out of 99 farms.

In farm vegetables, various researchers have reported differing prevalence for these pathogens. Microbial risk differs from one locality (region or country), season to another and also greatly influenced by level of implementation of GAPs in vegetable farms. For example, pathogenic *E. coli,* serotype O157:H7 was reported at 0% [0/605] in USA (49) and [0/36] in Brazil (50) and 5% [3/60] in Greece (51). *Salmonella* spp. was at 2.7% [1/36] in Brazil (50), 15% [35/238] in Nigeria (52), 50% [10/20] in Burkina Faso (53). Thermo-tolerant *Campylobacter* spp. were reported at 3.0 [1/33] and 19 % [3/16] (54), 35% [90/255] in Malaysia (55). *L. monocytogenes* in farm vegetables was reported at 0 % [0/33] in Canada (56) and 14 % [16/118] in Korea (57, 58).

Table 4.2: Prevalence of foodborne pathogens in farm vegetables

Pathogens	Number of samples with positive detection out of n = 99.	Number of positive samples for each vegetable type
Salmonella spp.	5	celery (1), beet root (1), lettuce (1), onion (1), Garlic (1)
Thermo-tolerant *Campylobacter* spp.	3	celery (1), parsley (1), lettuce (1)
Pathogenic *E. coli*	6	carrot (1), parsley (1), onion (3), tomato (1)
- Enteropathogenic *E. coli* (EPEC)	0	--
- Shiga toxin-producing *E. coli* (STEC)	2	parsley (1), tomato (1)
- Enteroinvasive *E. coli* (EIEC)	0	--
- Enterotoxigenic *E. coli* (ETEC)	4	carrot (1), onion (3)
- Enteroaggregative *E. coli* (EAEC)	0	--
Listeria monocytogenes	1	lettuce (1)

Pre-harvest factors and risk exposure assessment

Several pre-harvest factors have been reported to affect the microbial safety of fresh vegetables in farms (9, 15). In this study, it also observed that the vegetable farming practices, settings and conditions were closely similar (over 50 % score as shown in Table 4.3) throughout the studied area and hence it was difficult to conclude from this study the exact contributing factor(s) of the pathogens isolated in some farms. Further studies will be required to establish causal relationship between the prevalence and trends of specific pathogens isolated in fresh vegetables along the supply chain and their predisposing factors. Nevertheless, the high detection of generic *E. coli* in vegetable samples from 77% of farms can already be an indication of the significant contribution of zoonotic sources where vegetables in farms can be contaminated via water usage, human activities, livestock and wild life, and compost manure application as further discussed in our qualitative assessment in the following sections.

Water usage

Vegetables are commonly grown in marshlands and valleys to make use of the entrapped water for irrigation and the available arable land since Rwanda is a high altitude country with the lowest point at 950 m above sea level (59). About 60% of the visited farmers irrigated their vegetables during the dry season, while the rest entirely relied on rainfall. Irrigation water was sourced directly from marshlands (70%), rivers (18%), lakes (7%), runoff lagoons (5%) without any further treatment. No farmer irrigated with ground water. Farm vegetables are mainly irrigated by a combination of furrow and manual watering cans (62 % [78/125] of the famers who irrigate) while in 11 % and 27% of farms, only furrow and manual watering cans was singly used. The climate in Rwanda is temperate tropical with a temperature range of 12°C to 27°C, two rainy seasons from February to May and September to December and a dry season from June to September (23). Figure 4.1a shows the prevalence of pathogen DNA in irrigation water samples for the different water sources. Prevalence for EIEC was 3.3 % (1/30); 6.7% (2/30) for EAEC; 13.3% (4/30) for ETEC and *Vibrio cholera*; 20.0% (6/30) for *Yersinia pestis*; 26.7% (8/30) for *Francisella tularensis*; 40% (12/30) for *Cyclospora* and 86.7% (26/30) for thermo-tolerant *Campylobacter* spp. Figure 4.2b shows that overall prevalence of pathogens DNA (number of positive samples) was highest for water from rivers (28%) followed by marshland and run off/ lagoons (22%) and lowest in ground water (10%). The following pathogens were not detected in any of the 30 water samples: EPEC, STEC, *Salmonella* spp., *L. monocytogenes*, *Burkholderia*, *Rickettsia*, *T. gondii*, *G. lamblia*, *Cryptosporidium*, *E. histolytica* and *Hepatiti*s E. Comparing the results of this study to other studies in the African region (although different test methods were used), *Salmonella* spp. was 19 % (37/200) in well, piped and sachet water in Nigeria (60). In South Africa, Ijabadeyini (61) reported 53% for *Listeria monocytogenes*, and 42 % for *Salmonella* spp. from 36 samples taken 3 rivers/canal. This study did not focus on the comparison of pathogens in farm vegetables and the nearby agricultural water because only a few (four) pathogens were investigated in farm vegetables compared to the 19 pathogens studied agricultural water. However in the Northern province, ETEC was isolated in onion and carrot samples which corresponded with the ETEC DNA detected in the nearby water sources.

Table 4.3: Farms review checklist for good agricultural practices (GAPs) and good handling practices for fresh vegetables. Number farms surveyed (n) = 198.

Question No.	Farm aspect/ /GAP/Activity question	% number of farms under each answer category		
		Yes	No	NA
	Water usage			
1.	A water quality assessment has been performed to determine the quality of water used for irrigation purpose on the crop(s) being applied.	0 %	63 %	37 %
2.	A water quality assessment has been performed to determine the quality of water use for chemical application or fertigation method.	0 %	63 %	37 %
3.	If necessary, steps are taken to protect irrigation water from potential direct and nonpoint source contamination.	0 %	63 %	37 %
	Sewage Treatment			
4.	The farm sewage treatment system/septic system is functioning properly and there is no evidence of leaking or runoff.	0 %	0 %	100 %
5.	There is no municipal/commercial sewage treatment facility or waste material landfill adjacent to the farm.	0 %	0 %	100 %
	Animals/Wildlife/Livestock			
6.	Crop production areas are not located near or adjacent to dairy, livestock, or fowl production facilities unless adequate barriers exist.	70 %	30 %	0 %
9.	Manure lagoons located near or adjacent to crop production areas are maintained to prevent leaking/overflowing, or measures have been taken to stop runoff from contaminating the crop production areas.	0 %	0 %	100 %
7.	Manure stored near or adjacent to crop production areas is contained to prevent contamination of crops.	0 %	7 %	93 %
8.	Measures are taken to restrict access of livestock to the source or delivery system of crop irrigation water.	0 %	100 %	0 %
9.	Crop production areas are monitored for the presence or signs of wild or domestic animals the entering the land.	0 %	100 %	0 %
10.	Measures are taken to reduce the opportunity for wild and/or domestic animals from entering crop production areas.	0 %	100 %	0 %
	Manure and Municipal Biosolids **Option A: Raw Manure**			
11.	When raw manure is applied, it is incorporated at least 2 weeks prior to planting or a minimum of 120 days prior to harvest.	62 %	0 %	38 %
12.	Raw manure is not used on commodities that are harvested within 120 days of planting.	62 %	0 %	38 %
13.	If both raw and treated manure are used, the treated manure is properly treated, composted or exposed to reduce the expected levels of pathogens.	10 %	53 %	38 %
14.	Manure is properly stored prior to use.	7 %	56 %	38 %

Table 4.3 Continues…

	Manure and Municipal Biosolids **Option B: Composted Manure**			
15.	Only composted manure are used as a soil amendment.	28 %	34 %	38 %
16.	Composted manure are properly treated, composted, or exposed to environmental conditions that would lower the expected level of pathogens.	4 %	24 %	38 %
17.	Composted manure are properly stored and are protected to minimize recontamination.	0 %	28 %	38 %
18.	Analysis reports are available for composted manure/treated biosolids.	0 %	62 %	38 %
	Manure and Municipal Biosolids **Option C: No Manure/Biosolids Used**			
19.	No animal manure are used.	38 %	62 %	0 %
	Soils			
20.	A previous land use risk assessment has been performed.	0 %	100 %	0 %
21.	When previous land use history indicates a Soils Questions possibility of contamination, preventative measures have been taken to mitigate the known risks and soils have been tested for contaminants and the land use is commensurate with test results.	0 %	100 %	0 %
22.	Crop production areas that have been subjected to flooding are tested for potential microbial hazards.	0 %	100 %	0 %
	Traceability			
24.	Each production area is identified or coded to enable traceability in the event of a recall.	0 %	100 %	0 %

NA, response denotes those GAPs or GHPs that are not applicable or practical to the farm situation in Rwanda

DNA detection in agricultural water (irrigation and on-farm washing water) has revealed strikingly high prevalence of thermo-tolerant *Campylobacter* spp. (86.7%). In other studies, the prevalence of thermo-tolerant *Campylobacter* spp. in environmental water sources was reported at 19.3 % [50/260] in USA (62), 30.9% [37/120] in Belgium (63) and 55.3 % [162/293] in New Zealand (64). Detecting DNA from *Campylobacter* and other pathogens may not necessarily represent the presence of their living cells, but these results indicate a high risk of microbial contamination and using this water to irrigate and or wash vegetables after harvesting may present a major risk of microbial hazards.

This study revealed that rivers and marshlands had the highest prevalence of pathogen DNA, implying that if this water is used for overhead irrigation of leafy vegetables (10), contamination of the edible part of the vegetables may occur. Ground water had the lowest prevalence of pathogens DNA, but this water is hardly used for irrigation in most developing countries. Instead it is mainly targeted for household use (65).

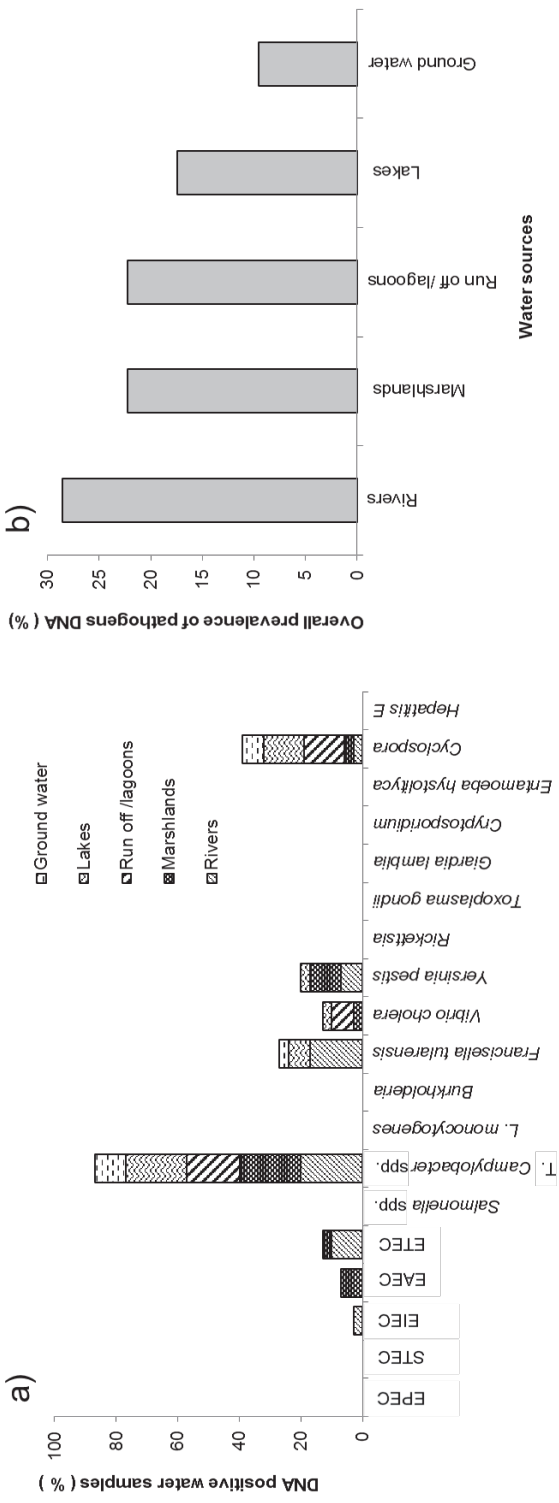

Figure 4.2: a) Occurrence of pathogen DNA in different water sources. **b)** Overall prevalence of pathogens DNA detected in different water sources. Six samples (n=6) were taken from different sources for every water domain (lakes, rivers, marshlands, lagoons and ground water). EPEC = Entero pathogenic *E. coli*, STEC= shiga toxin producing *E. coli*, EIEC= Enteroinvasive *E. coli*, EAEC= Enteroaggregative *E. coli*, ETEC= Enterotoxigenic *E. coli*, T. *Campylobacter* spp. =Thermo-tolerant *Campylobacter* spp.

Human activity, livestock and wildlife

Population density is characteristically high in the farming areas, *i.e.*. in the main vegetable growing areas of Musanze and Rubavu, it was reported in the national census in 2012 that there were 694 and 1,039 inhabitants per sq.km (66). Mixed farming is common; domestic animals (goats, sheep and cattle) were observed in the vegetable growing areas. We observed that measures to restrict domestic and wild animals (15) from accessing crop production areas and irrigation water were not implemented. In other countries, unrestricted animal access to vegetable farms was observed in 60% [6/10] of the vegetable farms in Nigeria (67) and 23 % [57/246] in USA (68).

The presence of zoonotic pathogens in environmental water indicates the possibility of human, livestock and wildlife activities to introduce zoonotic pathogens either directly or indirectly through runoff and erosion of land surfaces after precipitation (69). The high prevalence of *Campylobacter* spp. in the studied agricultural water may be linked to the reported high population (66) and livestock density per km^2 (70) but also due to the un controlled roaming of livestock and wild animals in the environment (71), especially since this bacterium has been detected frequently in fecal droppings (72). In addition, the pathogens detected in farm vegetables (section 3.1) can also be attributed to humans and animals in vegetable growing areas.

Compost manure application

With the increasing amount of livestock (70) , the use of animal manure is popular in Rwanda. Compost and or raw manure application was used in 60% of the visited vegetable farms while recommended storage (12) and treatment (15) of this manure was not yet implemented by over 50% of the farmers. Similar findings were observed in Nigeria (67) whereas only 2% of the farmers in Minnesota applied raw manure, USA (68). Improperly treated animal manure has been reported to harbor human pathogens (73), and in order to minimize these microbial hazards in compost manure, measures such as active and passive treatment of manure have been recommended (15).

Conclusion

Like in studies from countries with settings similar to Rwanda, *i.e.*. Nigeria (74), the Middle East (75), farming practices that can expose fresh vegetables to a high microbial contamination (15) have been observed in this study. With the growing population and the increasing amount of livestock and production of fresh vegetables, risk managers will have to adopt sustainable approaches (76) (77) to prevent or control the spread of pathogens in the human-plant-animal interface. Efforts to address the challenges of microbial safety in farm vegetables will require strict adherence to GAPs and GHPs. Farming practices are critical to control and prevention of microbial hazards and in this study, serious gaps have been observed. Farmers will have to be trained and or provided with an economic incentive/ legislation to impact behavior change and improve on hygienic farming. In Nigeria, it was reported that farmers were generally unware of the link between food safety practices and microbial contamination of fresh vegetables (74) and their findings seem to be in line with the current study. Recommendations from organization like the FAO (13, 15) and the WHO (12) of the United Nations should be adopted

to guide practices on water use, compost manure, handling domestic and wild animals, human activities and general environmental hygiene in vegetable farms.

References

1. **Havelaar** et al. 2015. World Health Organization global estimates and regional comparisons of the burden of foodborne disease in 2010. *PLoS Medicine* 12:e1001923.

2. **Batz MB.** 2013. The foods most often associated with major foodborne pathogens: attributing illnesses to food sources and ranking pathogen/food combinations. In Morris G. J., Potter M. (4th Edn). *Foodborne Infections & Intoxications* (pp 19–35). London: Academic Press.

3. **Painter JA, Hoekstra RM, Ayers T, Tauxe RV, Braden CR, Angulo FJ, Griffin PM**. 2013. Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998-2008. *Emerging Infectious Diseases* 19:407-415.

4. **Holvoet K, Sampers I, Callens B, Dewulf J, Uyttendaele M.** 2013. Moderate prevalence of antimicrobial resistance in *Escherichia coli* isolates from lettuce, irrigation water, and soil. *Applied & Environmental Microbiology* 79:6677-6683.

5. **Adamu NB, Adamu JY, Mohammed D.** 2012. Prevalence of helminth parasites found on vegetables sold in Maiduguri, Northeastern Nigeria. *Food Control* 25:23-26.

6. **Aw TG, Wengert S, Rose JB.** 2016. Metagenomic analysis of viruses associated with field-grown and retail lettuce identifies human and animal viruses. *International Journal of Food Microbiology* 223:50-56.

7. **Gorski L, Palumbo JD, Nguyen KD.** 2004. Strain-specific differences in the attachment of *Listeria monocytogenes* to alfalfa sprouts. *Journal of Food Protection* 67:2488-2495.

8. **Macarisin D, Patel J, Sharma VK.** 2014. Role of curli and plant cultivation conditions on *Escherichia coli* O157:H7 internalization into spinach grown on hydroponics and in soil. *International Journal of Food Microbiology* 173:48-53.

9. **Beuchat LR, Ryu JH.** 1997. Produce handling and processing practices. *Emerging Infectious Diseases* 3:459-465.

10. **Gil MI, Selma MV, Suslow T, Jacxsens L, Uyttendaele M, Allende A.** 2015. Pre- and postharvest preventive measures and intervention strategies to control microbial food safety hazards of fresh leafy vegetables. *Critical Reviews in Food Science & Nutrition* 55:453-468.

11. **USDA.** 2014. United States department of agriculture. Good Agricultural Practices, Good Handling Practice audit verification checklist. Available at: https://www.ams.usda.gov/sites/default/files/media/GAPGHP_Checklist_no_spell_Checklist_Enabled%5B1%5D.pdf. Accessed 15th November 2014.

12. **WHO.** 2012. World Health Organization of the United Nations. Five keys to growing safer fruits and vegetables. Promoting health by decreasing microbial contamination. Available at: http://apps.who.int//iris/bitstream/10665/75196/1/9789241504003_eng.pdf?ua=1. Accessed 15th November 2014.

13. **FAO.** 2012. Food and Agriculture Organization of the United Nations. On-farm practices for the safe use of wastewater in urban and peri-urban horticulture. A training handbook for farmer field schools. Available at: http://www.fao.org/docrep/016/i3041e/i3041e.pdf. Accessed 17th July 2013.

14. **FAO.** 2008a. Food and Agriculture Organisation (FAO) of the United Nations. Contributing to "One World, One Health". A strategic framework for reducing risks of infectious diseases at the animal–

human–ecosystems interface. Available at ftp://ftp.fao.org/docrep/fao/011/aj137e/aj137e00.pdf. Accessed 01st October 2016.

15. **FAO.** 2008b. Food and Agriculture Organization and the World Health Organization (WHO) of the United Nations. Microbiological hazards in fresh leafy vegetables and herbs. Meeting report. Available at : http://www.fao.org/3/a-i0452e.pdf. Accessed 15th November 2015.

16. **Callejon RM, Rodriguez-Naranjo MI, Ubeda C, Hornedo-Ortega R, Garcia-Parrilla MC, Troncoso AM.** 2015. Reported foodborne outbreaks due to fresh produce in the United States and European Union: trends and causes. *Foodborne Pathogens & Disease* 12:32-38.

17. **Beuchat LR.** 1996. *Listeria monocytogenes*: incidence on vegetables. *Food Control* 7:223-228.

18. **Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV.** 1999. Food-related illness and death in the United States. *Emerging Infectious Diseases* 5:607-625.

19. **Beuchat LR.** 2006. Vectors and conditions for preharvest contamination of fruits and vegetables with pathogens capable of causing enteric diseases. *British Food Journal* 108:38-53.

20. **Uyttendaele M, Jaykus L-A, Amoah P, Chiodini A, Cunliffe D, Jacxsens L, Holvoet K, Korsten L, Lau M, McClure P, Medema G, Sampers I, Rao Jasti P**. 2015. Microbial hazards in irrigation water: standards, norms, and testing to manage use of water in fresh produce primary production. *Comprehensive Reviews in Food Science & Food Safety* 14:336-356.

21. **Wikipedia**. 2016. Template: Location map of Rwanda. Available at: https://en.wikipedia.org/wiki/Template:Location_map_Rwanda. Accessed 12th December 2017.

22. **Ssemanda JN, Reij M, Bagabe MC, Muvunyi CM, Joosten H, Zwietering MH.** 2017. Indicator microorganisms in fresh vegetables from "farm to fork" in Rwanda. *Food Control* 75:126-133.

23. **Malesu MM, Oduor A, Chrogony K, Nyolei D, Gachene C, Biamah E, O'Neil M, Ilyama M, Mogoi J.** 2010. Rwanda irrigation master plan. Nairobi, Kenya: The Government of Rwanda, Ministry of agriculture and animal resources, *Ebony Company Limited & World Agro-forestry Centre* (ICRAF).

24. **ISO 19458:** 2006. Water quality -- Sampling for microbiological analysis. *International Organization for Standardization* (ISO), Geneva, Switzerland.

25. **Mukherjee A, Speh D, Diez-Gonzalez F.** 2007. Association of farm management practices with risk of *Escherichia coli* contamination in pre-harvest produce grown in Minnesota and Wisconsin. *International Journal of Food Microbiology* 120:296-302.

26. **Pagadala S, Marine SC, Micallef SA, Wang F, Pahl DM, Melendez MV, Kline WL, Oni RA, Walsh CS, Everts KL, Buchanan RL.** 2015. Assessment of region, farming system, irrigation source and sampling time as food safety risk factors for tomatoes. *International Journal of Food Microbiology* 196:98-108.

27. **ISO 6579:** 2002/Amd 1: 2007. Microbiology of food and animal feeding stuffs -- Horizontal method for the detection of *Salmonella* spp. Annex D: Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage. *International Organization for Standardization*, Geneva, Switzerland.

28. **ISO 11290-1:**1996/Amd 1:2004. Microbiology of food and animal feeding stuffs -- Horizontal method for the detection and enumeration of *Listeria monocytogenes* -- Part 1: Detection method. Amendments: Modification of the isolation media and the haemolysis test, and inclusion of precision data. *International Organization for Standardization*, Geneva, Switzerland.

29. **ISO 10272-1**: 2017. Microbiology of food and animal feeding stuffs -- Horizontal method for detection and enumeration *of Campylobacter* spp. -- Part 1: Detection method. *International Organization for Standardization*, Geneva, Switzerland.

30. **Barbau-Piednoir E, Botteldoorn N, Yde M, Mahillon J, Roosens NH.** 2013. Development and validation of qualitative SYBR® Green real-time PCR for detection and discrimination of *Listeria* spp. and *Listeria monocytogenes*. *Applied Microbiology & Biotechnology* 97:4021-4037.

31. **ISO/TS 13136:** 2012. Microbiology of food and animal feed -- Real-time polymerase chain reaction (PCR)-based method for the detection of food-borne pathogens -- Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) and the determination of O157, O111, O26, O103 and O145 serogroups. *International Organization for Standardization*, Geneva, Switzerland.

32. **Nielsen EM, Andersen MT.** 2003. Detection and characterization of verocytotoxin-producing *Escherichia coli* by automated 5′ nuclease PCR assay. *Journal of Clinical Microbiology* 41:2884-2893.

33. **Perelle S, Dilasser F, Grout J, Fach P.** 2004. Detection by 5′-nuclease PCR of Shiga-toxin producing *Escherichia coli* O26, O55, O91, O103, O111, O113, O145 and O157: H7, associated with the world's most frequent clinical cases. *Molecular and Cellular Probes* 18:185-192.

34. **Thiem VD, Sethabutr O, von Seidlein L, Van Tung T, Chien BT, Lee H, Houng H-S, Hale TL, Clemens JD, Mason C.** 2004. Detection of *Shigella* by a PCR assay targeting the ipaH gene suggests increased prevalence of shigellosis in Nha Trang, Vietnam. *Journal of Clinical Microbiology* 42:2031-2035.

35. **EU.** 2013. European Union reference laboratory for *E. coli*. Detection of Enterotoxigenic *Escherichia coli* in food by Real Time PCR amplification of the lt, sth, and stp genes, encoding the heat-labile and heat-stable enterotoxins. Department of veterinary public health and food safety, unit of foodborne zoonoses, (Istituto Superiore di Sanità). Available at: http://www.iss.it/binary/vtec/cont/EU_RL_VTEC_Method_08_Rev_0.pdf. Accessed 04th June 2015.

36. **Liu J, Gratz J, Amour C, Kibiki G, Becker S, Janaki L, Verweij JJ, Taniuchi M, Sobuz SU, Haque R.** 2013. A laboratory-developed TaqMan Array Card for simultaneous detection of 19 enteropathogens. *Journal of Clinical Microbiology* 51:472-480.

37. **Boisen N, Scheutz F, Rasko DA, Redman JC, Persson S, Simon J, Kotloff KL, Levine MM, Sow S, Tamboura B.** 2012. Genomic characterization of enteroaggregative *Escherichia coli* from children in Mali. *Journal of Infectious Diseases* 205:431-444.

38. **Keramas G, Bang DD, Lund M, Madsen M, Rasmussen SE, Bunkenborg H, Telleman P, Christensen CBV.** 2003. Development of a sensitive DNA microarray suitable for rapid detection of Campylobacter spp. *Molecular & Cellular Probes* 17:187-196.

39. **Malorny B, Paccassoni E, Fach P, Bunge C, Martin A, Helmuth R.** 2004. Diagnostic real-time PCR for detection of *Salmonella* in food. *Applied & Environmental Microbiology* 70:7046-7052.

40. **Janse I, Hamidjaja RA, Bok JM, van Rotterdam BJ.** 2010. Reliable detection of *Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis* by using multiplex qPCR including internal controls for nucleic acid extraction and amplification. *BMC Microbiology* 10:314.

41. **Schets F, Van den Berg H, Marchese A, Garbom S, de Roda Husman A.** 2011. Potentially human pathogenic vibrios in marine and fresh bathing waters related to environmental conditions and disease outcome. *International Journal of Hygiene & Environmental Health* 214:399-406.

42. **Vasickova P, Kralik P, Slana I, Pavlik I.** 2012. Optimisation of a triplex real time RT-PCR for detection of hepatitis E virus RNA and validation on biological samples. *Journal of Virological Methods* 180:38-42.

43. **Reischl U, Bretagne S, Krüger D, Ernault P, Costa J-M.** 2003. Comparison of two DNA targets for the diagnosis of Toxoplasmosis by real-time PCR using fluorescence resonance energy transfer hybridization probes. *BMC Infectious Diseases* 3:7.

44. **Verweij JJ, Blangé RA, Templeton K, Schinkel J, Brienen EA, van Rooyen MA, van Lieshout L, Polderman AM.** 2004. Simultaneous detection of *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum* in fecal samples by using multiplex real-time PCR. *Journal of Clinical Microbiology* 42:1220-1223.

45. **Fontaine M, Guillot E.** 2002. Development of a TaqMan quantitative PCR assay specific for *Cryptosporidium parvum*. *FEMS Microbiology Letters* 214:13-17.

46. **Verweij JJ, Laeijendecker D, Brienen EA, van Lieshout L, Polderman AM.** 2003. Detection of *Cyclospora cayetanensis* in travellers returning from the tropics and subtropics using microscopy and real-time PCR. *International Journal of Medical Microbiology* 293:199-202.

47. **Janse I, Hamidjaja RA, Hendriks AC, van Rotterdam BJ**. 2013. Multiplex qPCR for reliable detection and differentiation of *Burkholderia mallei* and *Burkholderia pseudomallei*. *BMC Infectious Diseases* 13:86.

48. **ISO 16649-2:**2001. Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli* -- Part 2: Colony-count technique at 44 degrees C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide. *International Organization for Standardization*, Geneva, Switzerland.

49. **Mukherjee A, Speh D, Dyck E, Diez-Gonzalez F.** 2004. Preharvest evaluation of coliforms, *Escherichia coli*, *Salmonella*, and *Escherichia coli* O157: H7 in organic and conventional produce grown by Minnesota farmers. *Journal of Food Protection* 67:894-900.

50. **de Quadros Rodrigues R, Loiko MR, Minéia Daniel de Paula C, Hessel CT, Jacxsens L, Uyttendaele M, Bender RJ, Tondo EC.** 2014. Microbiological contamination linked to implementation of good agricultural practices in the production of organic lettuce in Southern Brazil. *Food Control* 42:152-164.

51. **Pinaka O, Pournaras S, Mouchtouri V, Plakokefalos E, Katsiaflaka A, Kolokythopoulou F, Barboutsi E, Bitsolas N, Hadjichristodoulou C**. 2013. Shiga toxin-producing *Escherichia coli* in Central Greece: Prevalence and virulence genes of O157:H7 and non-O157 in animal feces, vegetables, and humans. *European Journal of Clinical Microbiology & Infectious Diseases* 32:1401-1408.

52. **Abakpa GO, Umoh VJ, Ameh JB, Yakubu SE, Kwaga JKP, Kamaruzaman S.** 2015. Diversity and antimicrobial resistance of *Salmonella enterica* isolated from fresh produce and environmental samples. *Environmental Nanotechnology, Monitoring & Management* 3:38-46

53. **Traoré O, Nyholm O, Siitonen A, Bonkoungou IJO, Traoré AS, Barro N, Haukka K.** 2015. Prevalence and diversity of *Salmonella enterica* in water, fish and lettuce in Ouagadougou, Burkina Faso. *BMC Microbiology* 15:1-7.

54. **Chai LC, Ghazali FM, Bakar FA, Lee HY, Suhaimi LRA, Talib SA, Nakaguchi Y, Nishibuchi M, Radu S.** 2009. Occurrence of thermophilic *Campylobacter* spp. contamination on vegetable farms in Malaysia. *Journal of Microbiology and Biotechnology* 19:1415-1420.

55. **Khalid MI, Tang JYH, Baharuddin NH, Rahman NS, Rahimi NF, Radu S.** 2015. Prevalence, Antibiogram, and cdt Genes of Toxigenic *Campylobacter jejuni* in Salad Style Vegetables (Ulam) at farms and retail outlets in Terengganu. *Journal of Food Protection* 78:65-71.

56. **Dallaire R, LeBlanc DI, Tranchant CC, Vasseur L, Delaquis P, Beaulieu C.** 2006. Monitoring the microbial populations and temperatures of fresh broccoli from harvest to retail display. *Journal of Food Protection* 69:1118-1125.

57. **Tango CN, Choi NJ, Chung MS, Oh DH.** 2014. Bacteriological quality of vegetables from organic and conventional production in different areas of Korea. *Journal of Food Protection* 77:1411-1417.

58. **Thapa SP, Kim SS, Hong SS, Park DS, Lim CK, Hur JH.** 2008. Monitoring of bacterial pathogens in agricultural products and environments at farms in Korea. *Journal of Applied Biological Chemistry* 51:128-135.

59. **MINAFFET.** 2017. Ministry of foreign affairs and cooperation of the Republic of Rwanda. Geography. Available at: http://www.minaffet.gov.rw/rwanda/geography/. Accessed 02nd February 2017.

60. **Akinyemi KO, Iwalokun BA, Foli F, Oshodi K, Coker AO.** 2011. Prevalence of multiple drug resistance and screening of enterotoxin (stn) gene in *Salmonella* enterica serovars from water sources in Lagos, Nigeria. *Public Health* 125: 65-71

61. **Ijabadeniyi OA, Debusho LK, Vanderlinde M, Buys EM.** 2011. Irrigation water as a potential preharvest source of bacterial contamination of vegetables. *Journal of Food Safety* 31:452-461.

62. **Gu G, Luo Z, Cevallos-Cevallos JM, Adams P, Vellidis G, Wright A, van Bruggen AHC.** 2013. Occurrence and population density of *Campylobacter jejuni* in irrigation ponds on produce farms in the Suwannee River watershed. *Canadian Journal of Microbiology* 59:339-346.

63. **Holvoet K, Sampers I, Seynnaeve M, Uyttendaele M.** 2014. Relationships among hygiene indicators and enteric pathogens in irrigation water, soil and lettuce and the impact of climatic conditions on contamination in the lettuce primary production. *International Journal of Food Microbiology* 171:21-31.

64. **Devane M, Nicol C, Ball A, Klena J, Scholes P, Hudson J, Baker M, Gilpin B, Garrett N, Savill M.** 2005. The occurrence of *Campylobacter* subtypes in environmental reservoirs and potential transmission routes. *Journal of Applied Microbiology* 98:980-990.

65. **Jimmy DH, Sundufu AJ, Malanoski AP, Jacobsen KH, Ansumana R, Leski TA, Bangura U, Bockarie AS, Tejan E, Lin B, Stenger DA.** 2013. Water quality associated public health risk in Bo, Sierra Leone. *Environmental Monitoring & Assessment* 185:241-251.

66. **NISR.** 2012. National Institute of Statistics of Rwanda, Ministry of Finance and Economic Planning, Rwanda. Rwanda fourth population and housing census. Thematic Report: Data quality assessment . Available at: http://www.statistics.gov.rw/publication/rphc4-final-report-data-quality-assessment. Accessed 05th February 2015.

67. **Oyinlola LA, Obadina AO, Omemu AM, Oyewole OB.** 2017. Prevention of microbial hazard on fresh-cut lettuce through adoption of food safety and hygienic practices by lettuce farmers. *Food Science & Nutrition* 5:67-75.

68. **Hultberg A, Schermann M, Tong C.** 2012. Results from a mail survey to assess Minnesota vegetable growers' adherence to good agricultural practices. *HortTechnology* 22:83-88.

69. **Cooley M, Quiñones B, Oryang D, Mandrell R, Gorski L.** 2014. Prevalence of shiga toxin producing *Escherichia coli*, *Salmonella enterica*, and *Listeria monocytogenes* at public access watershed sites in a California Central Coast agricultural region. *Frontiers in Cellular & Infection Microbiology* 4:30-43

70. **MINAGRI.** 2012. Ministry of Agriculture and Animal Resources. Republic of Rwanda. Strategic and investment plan to strengthen meat industry in Rwanda. Final report, July, 2012. Available at: http://www.minagri.gov.rw/fileadmin/user_upload/documents/STRAT.PLC/Final_report_Rwanda_Meat_industry_final.pdf. Accessed 04th January 2017.

71. **Chatikobo P, Manzi M, Kagarama J, Rwemarika J, Umunezero O.** 2009. Benchmark study on husbandry factors affecting reproductive performance of smallholder dairy cows in the Eastern Province of Rwanda. *Livestock Research for Rural Development* 21:83.

72. **Ogden ID, Dallas JF, MacRae M, Rotariu O, Reay KW, Leitch M, Thomson AP, Sheppard SK, Maiden M, Forbes KJ, Strachan NJC.** 2009. *Campylobacter* excreted into the environment by animal sources: Prevalence, concentration shed, and host association. *Foodborne Pathogens & Disease* 6:1161-1170.

73. **Strawn LK, Gröhn YT, Warchocki S, Worobo RW, Bihn EA, Wiedmann M.** 2013. Risk factors associated with *Salmonella* and *Listeria monocytogenes* contamination of produce fields. *Applied & Environmental Microbiology* 79:7618-7627.

74. **Shenge KC, Whong CMZ, Yakubu LL, Omolehin RA, Erbaugh JM, Miller SA, LeJeune JT.** 2015. Contamination of tomatoes with coliforms and *Escherichia coli* on farms and in markets of Northwest Nigeria. *Journal of Food Protection* 78:57-64.

75. **Faour-Klingbeil D, Murtada M, Kuri V, Todd ECD.** 2016. Understanding the routes of contamination of ready-to-eat vegetables in the Middle East. *Food Control* 62:125-133.

76. **Nyatanyi T, Wilkes M, McDermott H, Nzietchueng S, Gafarasi I, Mudakikwa A, Kinani JF, Rukelibuga J, Omolo J, Mupfasoni D.** 2017. Implementing One Health as an integrated approach to health in Rwanda. *BMJ Global Health* 2:e000121.

77. **Fresco, L.O., Bouwstra, R.J., de Jong, M.C.M., van der Poel, W.H.M., Scholten, M.C.T., Takken, W. and the global one health task force of Wageningen University and Research Centre.** 2015. Global one health – a new integrated approach Available at: http://www.wur.nl/upload_mm/e/a/c/d734f322-b66d-4ec0-aa5b3ceb31001fe0_GOH_Final_20150602.pdf. Accessed 20th September 2016.

5

Reduction of microbial counts during kitchen scale washing and sanitization of salad vegetables

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Abstract

Washing with or without sanitizers is one of the important steps designated to reduce or eliminate microbial hazards in fresh vegetables but the settings, conditions and effectiveness of this step remain contentious. In this study, we investigated kitchen scale salad preparation practices in a field study in Rwandan food service establishments (FSEs) and conducted laboratory trials to identify treatments that can improve reduction of microbial counts during washing and sanitization. In the field study, vegetable samples (n=112) were taken from 56 FSEs before and after washing with or without sanitizer(s) to determine reduction of counts of *Enterobacteriaceae, Listeria* spp., and coagulase positive (CP)-staphylococci coupled with observation of the salad preparation practices from start to end. Based on the results obtained during the field study, 8 sanitizers were evaluated in the laboratory to optimize the efficacy of washing of leafy vegetables (corn salad, *Valerianella locusta*). Findings in the field study revealed that about 61% of the visited FSEs used sanitizers during washing of fresh vegetables, in particular, potassium permanganate ($KMnO_4$) in 39 % of FSEs, sanitizing powder (a mixture of polyphosphate, sodium hydrogen carbonate and active chlorine), 13%; sodium hypochlorite (NaClO), 7 %; and sodium dichloroisocyanurate (NaDCC) in 2%. Average inactivation ranged from 1.0 log ($KMnO_4$) to 3.1 log (NaDCC). In the laboratory study, average inactivation observed with *Listeria* spp., *Escherichia coli* and Aerobic plate count (APC) ranged from 0.7 log (water alone) to 3.0 log (NaDCC). Out of the 8 sanitizers that were evaluated, 5 sanitizers (NaDCC [90 ppm], NaClO [200 ppm], lemon juice [98%], acetic acid [2 %] and sanitizing powder [4 g/L]) resulted in significantly higher inactivation compared to water alone. A contact time of 5 min and salad-sanitizer ratio of 1: 20 were considered optimal for kitchen based washing of the studied leafy vegetables with NaDCC and NaClO sanitizers.

Introduction

A diet rich in vegetables has been associated with health benefits like reduced risk to cancers and cardiovascular diseases (1). Consumption of fresh vegetables is increasing year by year (2) and reports indicate that a large portion of these vegetables are consumed raw (3). At the same time, the number of reported foodborne illnesses linked to fresh vegetables has been increasing. In the United States between 1998 and 2008, produce (fresh vegetables and nuts) accounted for 46% of foodborne illness (4) while a high number of outbreaks has also been reported in the European Union (5). Etiological agents range from pathogenic bacteria (5) (6) to parasites (7) and viruses (5, 8).

Washing with water is a crucial postharvest step designated to reduce or eliminate field dirt and their associated microorganisms from fresh vegetables but this step also increases the chances for microbial hazards to spread in the entire batch (9, 10). Chemical sanitizers can be added to increase the efficacy *i.e.*. by preventing cross contamination (11), but maximum reduction rates are typically around 3 logs (10). To date, efforts to further improve washing and sanitization of fresh vegetables are ongoing especially in countries with a developed commercial fresh cut industry (12-15). Such efforts include pilot and laboratory studies to evaluate the efficacy of different vegetable sanitizers and washing techniques. In countries with limited fresh cut industry, the washing and sanitization of fresh vegetables is mainly done in kitchens of food service establishments (FSEs) and households during salad preparation. However, it has been acknowledged that commercial washing and sanitization conditions are not suitable for food service or home use, because the users lack technical skills, knowledge, and equipment to apply treatments safely and effectively (9). So far few studies (16-18) have targeted kitchen based washing and sanitization of fresh vegetables, but also do not compare microbial inactivation in the field (FSEs or households) and in the laboratory.

In this study, we seek to identify sanitizers, conditions, treatments and techniques that enable targeted microbial reduction during washing and sanitization of fresh vegetables to propose guidelines for FSEs and households. Our study consisted of a field study in which practices for preparing vegetable salads were investigated with a focus on microbial inactivation and a laboratory simulation of the washing and sanitization of vegetables in FSEs based on the results from the field study to identify alternatives for improvement. The field study was conducted in Rwanda, a country where vegetable washing and sanitization is mainly done in FSEs and household level (19).

Foot note 1, Abbreviations
L (litres), FSEs (Food Service Establishments), NaDCC (Sodium dichloroisocyanurate), OWA (Organic washing aid), SDBS (Sodium dodecylbenzenesulfonate), AA= Acetic acid (2%), $KMnO_4$ = Potassium permanganate, SP = Sanitizing powder, NaClO = Sodium hypochlorite.

Materials and methods

Field study

Study description and sampling

The field study was conducted in food service establishments (FSEs) in Rwanda from February to October 2015. We interacted face to face with managers and food handlers in FSEs during salad vegetable preparations, observed the unit operations (especially washing and sanitization steps) and took samples of vegetables for microbiological analysis. Efficacy of washing and sanitization of fresh vegetables in FSEs was evaluated based on the changes in counts of indicator microorganisms; APC (aerobic plate count), *Enterobacteriaceae, Listeria* spp. and coagulase positive staphylococci (CP. staphylococci).

FSEs were prepared for the study in a way reported in our previous related study (Ssemanda et al., 2017). In summary, out of the 280 FSEs managers invited, 168 showed interest and we were able to cover 56 of these FSEs in this study. Each FSE provided 2 samples, one sample (1 - 2 kg) of whole mixed vegetables was taken before washing and another sample (0.5 - 1 kg) after washing treatment, before cutting. Vegetables commonly used for salad making were beet root, cabbage, carrot, celery, cucumber, garlic, green pepper, lettuce, onion, parsley and tomato. Using sterile hand gloves, the 2 samples were placed and closed in sterile plastic zip bags and thereafter, all samples were stored in cooling boxes with ice packs and transported for 1-3 h to the laboratory and analyzed immediately.

Microbiological analysis

The 1-2 kg whole vegetable samples from FSEs as described in section 2.1.1 of different types were sliced /cut into small pieces (20) on a sterile stainless steel tray using sterilized knives and gloves for each sample and thereafter mixed. Then 25 g of analytical unit of these samples were thereafter stomached (Model 400 Circulator, Seward, UK) in 225 mL of maximum recovery diluent (MRD) for 1 min. Thereafter, tenfold serial dilutions were prepared using MRD for the enumeration of *Enterobacteriaceae,* APC and CP. staphylococci and buffered peptone for *Listeria* spp. The culture media and consumables used were from Oxoid (Oxoid Ltd., Basingstoke, UK). The enumeration was conducted according to ISO methods *i.e.*.; *Enterobacteriaceae*, ISO 21528-2:2004 (21); APC, ISO 4833-1:2013 (22) ; CP. staphylococci, ISO 6888-2:1999 (23); and *Listeria* spp., ISO 11290-2:1998/Amd 1:2004 (24). For quality control of the media and positive controls of the experiments in the field study, the following strains from the Belgian Coordinated Collection of Microorganisms were used: *i.e.. E. coli* (LMG 8063) for *Enterobacteriaceae*, *Listeria monocytogenes* (LMG 16783) for *Listeria* spp. and *Staphylococcus aureus* (LMG 8224) for CP. staphylococci.

Laboratory study

Preparing vegetables for the laboratory study.

Corn salad (*Valerianella locusta*) was selected in this study because leafy vegetables are known for their high microbial attachment (25) and because they are eaten raw, easy to

handle (require no cutting during washing) and available year round. For every experimental set up, prewashed, ready to eat corn salads in unit plastic packages of 75g were purchased from local supermarkets in Wageningen. At the start of every experiment, samples of corn salad were taken for microbial analysis before artificial contamination (inoculation) to examine the counts of *Listeria* spp., *Escherichia coli*, and APC originally present.

Preparing strains and inoculum

Unless stated otherwise, the strains of nonpathogenic *E. coli* and *Listeria* spp. used in this study were from the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures. *E. coli* strains were DSM 498, DSM 1756 and O2K (from Laboratory of Food Microbiology, Wageningen University). *Listeria* spp. strains were *L. seeligeri* (DSM 20751), *L. welshimeri* (DSM 20650), *L. innocua* (DSM 20649). Methodology for preparing of the inoculum was based on previous studies (26, 27) with slight modifications. The stock culture of each bacterial strain from cryovials (-80ºC) were streaked separately on brain heart infusion (BHI) agar (Oxoid Ltd., Basingstoke, UK) and incubated at 30ºC for 24 h. Thereafter, a colony from each strain was inoculated in 100 mL BHI broth and incubated at 30ºC for 24 h with agitation at 160 rpm. The cell cultures (10 mL each) were transferred into sterile tubes and concentrated by centrifugation at 11000 x *g* for 2 min at 20ºC and thereafter the supernatant was removed and washed in 10 mL of peptone physiological salt solution (PPS). The washing step was repeated twice and followed by resuspension with 10 ml of PPS. Microbial population of each cell suspension was determined by spiral plating (Eddy jet spiral plater, Spain) 50 µL portions (-5, -7 dilutions) on BHI agar and incubated at 30°C for 24 h (yielded levels of 6.5 to 9.5 cfu/mL). The suspensions were stored at 4°C for further use and freshly prepared every week. Equal numbers of each strain were combined to give a cocktail of strains containing approximately 9 log cfu/mL of either *E. coli* or *Listeria* spp. To prepare the final inoculum, 5 mL of each cocktail were mixed with 1 L of sterile deionized water.

Inoculating corn salads

For each experimental unit, 365 g of corn salads were dipped for 10 min in 5 L of the final inoculum prepared as described in section 2.2.2. Thereafter, the corn salads were drained and kept overnight in sterile plastic bags for 24 h at 4°C to allow microbial attachment (26, 28). This step was followed by taking three analytical corn salad samples of 10 g to determine the counts of *E. coli*, *Listeria* spp. and APC attached to the leaves prior to the washing and sanitization treatments (laboratory trials).

Screening sanitizers

Eight different sanitizers (Table 5.1) were procured, including those observed in the field study in FSEs (Rwanda). Sanitizer solutions were prepared by mixing with tap water at room temperature (20°C) in 15 L plastic vessels (disinfected with 70% ethanol). The tap water used in the laboratory was from the Dutch supply system, reported to distribute potable water (29) with a purification system that does not use chlorine disinfectants (29, 30). Unless stated otherwise the sanitization treatments were done by dipping 100 g of

inoculated corn salad into 2 L of sanitizer solution (1: 20 (w/v)) for 10 min while stirring manually with sterilized gloves. The corn salads were removed, drained and taken for microbial analysis without rinsing.

Effect of rinsing after sanitization

The possible effect of rinsing after sanitization on microbial counts was determined for the 100 ppm of NaClO and 10 min contact time condition. After the treatment, 20 g of samples were dipped in 800 mL of tap water at 25°C in sterile zip lock plastic bags and constantly shaken for 30 s mimicking the practical situation in FSEs.

Effect of sanitizer concentration and contact time

Sodium hypochlorite (NaClO) was selected as sanitizer for this part of the study. Inoculated corn salad (100 g) were dipped into 2 L of sanitizer solution. Occasional agitation by hand using sterile gloves during sanitization was done for the contact time of 2, 5, 10 and 15 min and concentration of 50, 75, 100, 150 and 200 ppm. Tap water was used as control.

Effect of salad to sanitizer solution ratio

Ratios evaluated were 1:10 (50 g salad in 500 mL solution), 1:20 (50 g salad in 1000 mL solution) and 1:50 (50 g in 2500 mL).

Table 5.1. List and description of sanitizers screened in the laboratory study

Sanitizer	Origin	Classification	Concentration used	Reference
Acetic acid	Merck, Germany	Organic acid	2%	(31)
Lemon juice	Polenghi, Italy	Organic acid	98%	(32)
Organic washing aid (OWA) consisting of citric pulp extract, citric acid, glycerin and demineralized water	Polypan , Greece	Organic acid	0.5%	(33)
Potassium permanganate ($KMnO_4$)	Fluka, Germany	Oxidizer	25 ppm	(34)
Sanitizing powder (polyphosphate, sodium hydrogen carbonate and active chlorine)	Ecolab East Africa, Kenya	Chroline + Surfactant	4 g/L	ψ
Sodium dodecylbenzenesulfonate (SDBS)	Sigma-Aldrich, Germany	Surfactant	111 ppm	(35)
Sodium hypochlorite (NaClO)	Sigma-Aldrich, Germany	Chlorine	200 ppm	(10)
Sodium troclosene or sodium dichloroisocyanurate + adipic acid (NaDCC)	Diversey, UK	Chlorine	90 ppm	ǂ

ψ Concentration obtained from product manufacturer's instructions, not available online

ǂ A maximum concentration of 100ppm NaDCC was recommended (36) but due to adverse effect on the appearance of the treated vegetables, 90 ppm was used in this study.

Potential for cross contamination through used sanitizer solutions

Five batches of inoculated corn salad (each 50 g) were prepared and dipped one batch at a time in the same sanitizer solution of 1 L NaDCC (90 ppm) for 5 min. Samples of the 1st, 3rd and 5th batch of corn salad were taken for microbiological analysis.

Microbiological analysis

In the laboratory study, 10 g of corn salad were weighed into the stomacher bag and stomached in 90 mL of MRD for 2 min. Thereafter, tenfold serial dilutions were prepared as in section 2.1.2. and so was the enumeration of *Listeria* spp. and APC. For *E. coli*, enumeration was conducted according to ISO 16649-2:2001(37).

Data analyses

Changes in microbial counts of each indicator microorganism were computed by subtracting log transformed counts before and after washing treatment. Statistical analyses were performed in IBM SPSS Statistics 22. In the field study, one way ANOVA with Scheffe's post hoc tests was used to compare the efficacy of sanitizers used by different FSEs in washing salad vegetables. In the laboratory study, experiments were repeated three times on different days and the error bars on the generated figures represent standard deviation. One-way ANOVA followed by Tukey's post hoc tests was used to analyze the difference in efficacy of different washing treatments. Independent and paired t-tests were used to analyze the effect of contact time and rinsing respectively. Statistical significance was set at 0.05.

Results and discussion

Field study (salad washing and sanitization at FSEs in Rwanda)

At reception, the FSE chefs grade and sort the different vegetables (beet root, cabbage, carrot, celery, cucumber, garlic, green pepper, lettuce, onion, parsley and tomato) to remove those vegetables which are not fit for salads (the bruised, rotten and broken). All vegetables are received as whole vegetables, no fresh cut vegetables are sold at the markets in Rwanda currently. Average initial microbial counts in vegetables received at FSEs before salad preparation were 6.8 ± 0.7, 6.0 ± 0.8, 5.1 ± 0.7, 4.6 ± 0.7 log cfu/g for APC, *Enterobacteriaceae*, *Listeria* spp. and CP. staphylococci respectively. We identified three major unit operations during salad preparation in which water was involved: prewashing, washing with or without sanitizers (sanitization step) and rinsing.

Prewashing step

The prewashing step was practiced in 56 % of the visited FSEs, while the rest of FSEs skipped this step and straightaway proceeded to the sanitization or main wash step. Food handlers used running municipal tap water to wash whole vegetables.

Sanitization or main wash step

Practices varied from one FSE to another (Fig. 5.1); about 61% of the visited FSEs washed vegetables with sanitizers, while others did not use any sanitizer but washed

vegetables with either boiled water or containerized drinking water. The salad treatment methods and the nature and concentration of sanitizers applied varied, some were used much more often (in 22 FSES) than others (even sometimes only once).

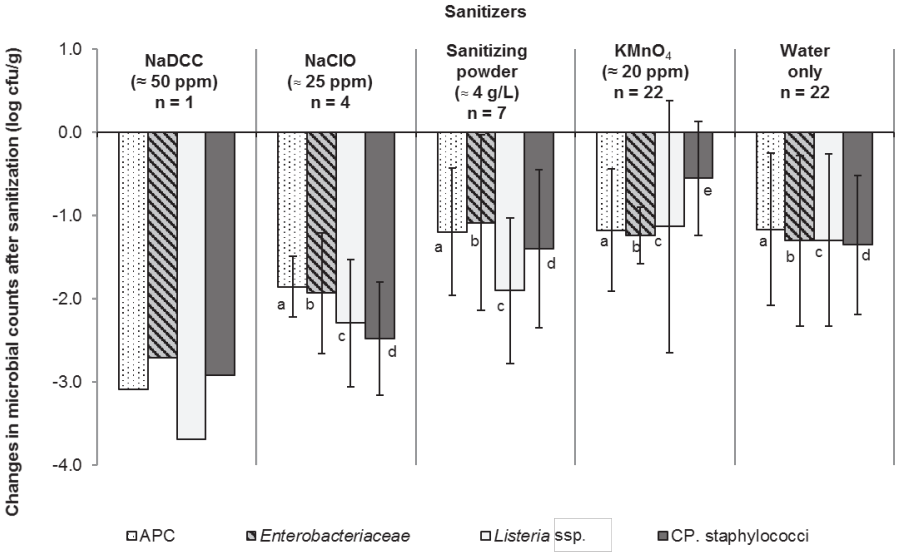


Figure 5.1: Microbial reduction due to washing with or without sanitizers during vegetable salad preparation in food service establishments (FSEs) (n= 56). Error bars represent the standard deviation in changes of microbial counts from one FSE to another. NaDCC = sodium dichloroisocyanurate, mixture of 20-30 % weight of adipic acid; NaClO = sodium hypochlorite; Sanitizing powder is a mixture of polyphosphate, sodium hydrogen carbonate and active chlorine; $KMnO_4$ =potassium permanganate. Bars of the same microbial indicator with a common letter under the different sanitizers do not differ significantly. Significant differences in microbial counts for NaDCC sanitizer are not available (n = 1).

Sanitizers used were NaDCC (50 ppm), NaClO (20 – 30 ppm), sanitizing powder (polyphosphate, sodium hydrogen carbonate, active chlorine; 4 g/L) and $KMnO_4$ (10 – 30 ppm), applied in 2 %, 7%, 13% and 39% of the visited FSEs respectively. Contact time of sanitizers was according to manufacturer instructions but varied between 1 to 10 min. The sanitization method was by dipping and all FSEs used tap water (same as in prewashing step) to acquire the aqueous sanitizing solution. Food handlers had no information on the quantity of vegetables that can be sanitized for a given amount of sanitizer solution. Our investigation on the microbial efficacy of the applied sanitizers (Fig. 5.1) revealed that the highest overall microbial reduction in the field study was achieved with NaDCC (average, 3.1 log cfu/g) followed by NaClO (average, 2.1 log cfu/g), sanitizing powder (1.4 log cfu/g), water only (1.3 log cfu/g) and the lowest for $KMnO_4$ (on average, 1.0 log cfu/g). Figure 5.1 also shows that reduction in counts of CP. staphylococci was significantly lower for FSEs that used $KMnO_4$ compared to those FSEs that washed with NaClO.

Rinsing step

Rinsing was the final step in which water was involved during salad preparation. From one FSE to another, there were variations in the type and quality of water used. Out of the visited FSEs, 54 % rinsed vegetables with boiled water, 6% with containerized drinking water and 40% used municipal kitchen tap water (for microbiological quality see section 3.2.3).

Laboratory trials (Laboratory study)

Microbial counts in corn salads

Sanitizing and washing of vegetables salads in the field study was simulated in laboratory settings using artificially contaminated corn salad. Counts of APC in corn salad before inoculation ranged from 6.5 to 8.0 log cfu/g. In 7 out of 15 samples of corn salad, *Listeria* spp. (not *L. monocytogenes*) were detected in the range of 2.3 to 3.5 log cfu/g while *E. coli* was not detected in any of the samples. Average initial microbial counts in vegetables before washing treatments were 6.3 ± 0.3, 6.6 ± 0.1, and 7.1 ± 0.4 log cfu/g for *Listeria* spp., *E. coli* and APC respectively.

Sanitizer screening

Figure 5.2, shows the reduction in counts of *Listeria* spp., *E. coli* and APC after sanitization of corn salad with different sanitizers and tap water. The highest mean microbial reduction was induced by NaDCC (3.4, 2.8 and 2.9 log cfu/g for *Listeria* spp., *E. coli* and APC respectively) followed by lemon juice (3.0 and 2.4 log cfu/g for *Listeria* spp. and APC respectively) and sanitizing powder (1.9 log cfu/g for *E. coli*) while the lowest reduction was registered for tap water (0.9, 0.9 and 0.4 log cfu/g for *Listeria* spp., *E. coli* and APC respectively). The mean microbial reduction due to washing corn salad with tap water alone, was significantly lower ($p < 0.05$) than that of the sanitizers; NaDCC (90 ppm), lemon juice (98%), NaClO (200 ppm), acetic acid (2%) and sanitizing powder (4 g/L) for *Listeria* spp. and APC. For *E. coli*, only NaDCC (90 ppm) and sanitizing powder (4 g/L) contributed to a significant higher reduction when compared to washing with only tap water.

Results from our field and laboratory study on washing with tap water (without sanitizers) were close to the 0.8 log reduction in aerobic mesophilic bacteria reported by Nascimento et al. (31) in their study with lettuce leaves. Vijayakumar and Wolf-Hall (32) also reported a 0.6 log reduction for *E. coli* when iceberg lettuce was washed with distilled water. In experimental studies, microbial quality and safety of water can be controlled but the situation may be different in practice. For example, in our field study different water sources were used during the prewashing and rinsing of salad vegetables in FSEs, yet studies (38) have reported that some water types were not potable in Rwanda.

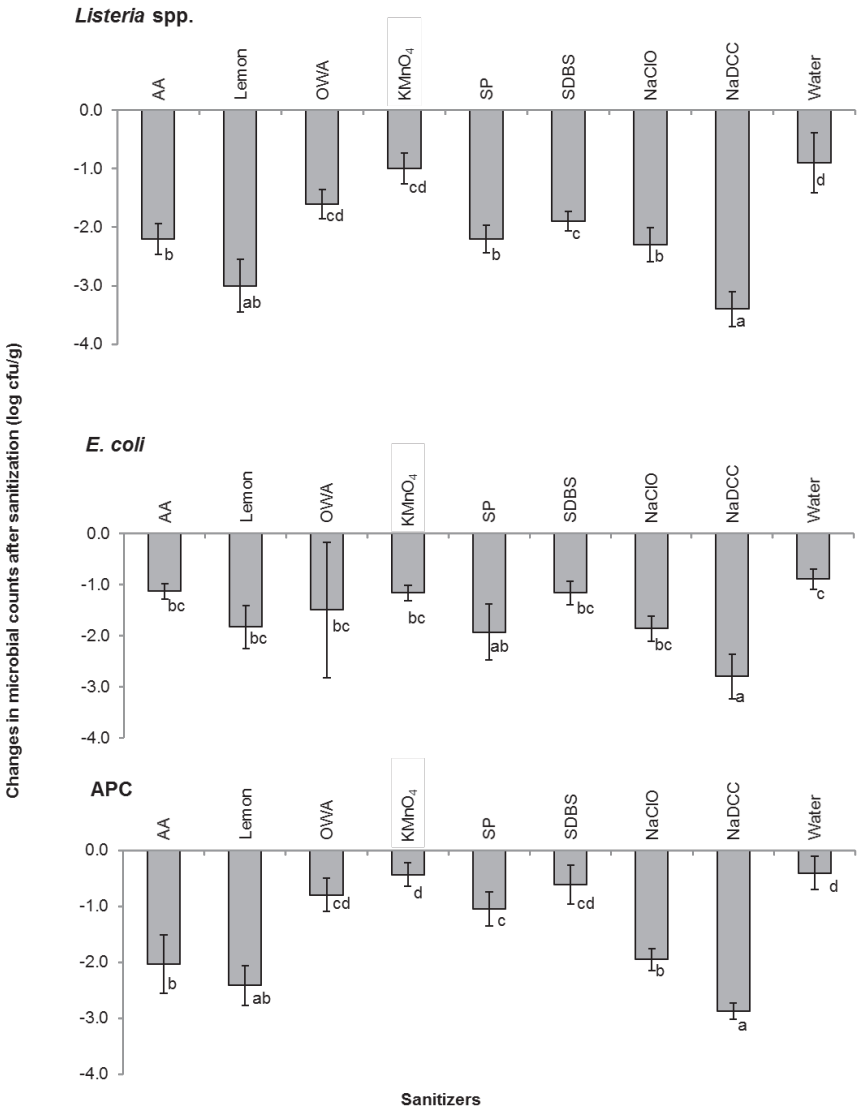


Figure 5.2: Laboratory trials for the efficacy of different sanitizers on *Listeria* spp., *E. coli* and APC (aerobic plate count) during the sanitization of corn salad for 10 min contact time and vegetable to sanitizer ratio of 1: 20 (w/v), without rinsing. Sanitizers AA= Acetic acid (2%), Lemon = lemon juice (98%), OWA = Organic washing aid (0.5%) consists of citric pulp extract, citric acid, glycerin and demineralized water, $KMnO_4$ = Potassium permanganate (25 ppm), SP = Sanitizing powder (4g/L) is a compound of polyphosphate, sodium hydrogen carbonate and active chlorine, SDBS = Sodium dodecyl benzene sulphonate (111 ppm), NaClO = Sodium hypochlorite (200 ppm), NaDCC = Sodium dichloroisocyanurate (90 ppm), contains 20-30% weight of adipic acid. Bars with a common letter are not statistically significant.

In our field study, $KMnO_4$ (mean 20 ppm) was the most applied sanitizer during salad preparation in FSEs yet reduction in all counts were comparable to that of washing with only tap water. Similar results were obtained with $KMnO_4$ (25 ppm) in the laboratory

study. Sukul and Sheth (39) reported the effect of $KMnO_4$ (50-100 ppm) after washing coriander leaves and microbial reduction varied from 0.4 to 1.4 log cfu/g. With a higher concentration (200 ppm $KMnO_4$), Amoah et al. (16) reported a 2.5 log reduction after washing lettuce. For all the sanitizers applied, $KMnO_4$ had the lowest inactivation and was not significantly different from washing vegetables with potable tap water.

In this study, acetic acid (2%) and lemon juice (98%) were the organic sanitizers that caused significant reduction in microbial counts. For acetic acid (2%), our results were in line with the study by Park et al. (40) where after washing lettuce, *L. monocytogenes* and *E. coli* O157: H7 counts were reduced by 1.7 and 1.4 log cfu/g respectively. In other studies, a 5 and over 2 log reduction was achieved for *E. coli* and APC counts respectively with 35% white vinegar (1.9% acetic acid) (32). Our results obtained with lemon juice as a sanitizers were in range with the study by Santos et al. (41) where a 2 log reduction of *E. coli* O157:H7 was achieved by washing lettuce for 15 min. In contrast, Sengun and Karapinar (42) reported that 100% lemon juice reduced counts of *Y. enterocolitica* on carrot from 7.2 log cfu/g to an undetectable level after 15 min treatment. Antimicrobial effect of organic acids depend on the pH, type of acid and strain of microorganism (43) and these acids have GRAS status (10). However using higher concentrations of organic acids and long contact time targeting maximum microbial reduction, can also result to sour and wilted appearance of especially leafy vegetables (32), creating acceptability problems.

NaDCC gave the highest microbial inactivation rate (average 3 log cfu/g) for all the studied indicator microorganisms, compared to other sanitizers in both the field and laboratory study. These results are in line with a previous study (31) that found NaDCC (200 ppm) was able to cause 3.2 log cfu/g reduction in aerobic microorganisms from lettuce. In Western Africa, the use of NaDCC (100 ppm) to wash lettuce, led to a 2.7 log reduction of faecal coliforms (16). NaDCC was more effective than other chlorine based sanitizers (NaClO, sanitizing powder) in both the field and laboratory study. This advantage has been attributed to the ability of NaDCC to slowly decompose and liberate HOCl and the capacity to maintain an appropriate level of active chlorine without affecting the pH of the water (44, 45).

Rinsing after sanitization

In Figure 5.3, rinsing corn salads with potable water after applying a treatment of 100 ppm NaClO did not result to further significant reduction ($p > 0.05$) in the counts of *Listeria* spp., *E. coli* and APC. Nevertheless, even though rising may not contribute to further reduction in microbial counts, it is considered to be essential for other purposes such as avoiding product quality deterioration (46) and removing undesirable sanitizer by-products (47). However, rinsing and prewashing of salad vegetables should be done with potable water to avoid recontamination. In many developing countries, water used for drinking and preparing food is not microbiologically safe (48). A recent national study in Rwanda has reported thermophilic total coliforms in the range of 4.3 (95% CI: 1.9 – 8.5) TTC/100mL for piped water and also raised concerns for possible microbial safety problems in drinking water sources (38). On site measures to disinfect or kill

pathogenic microorganisms in the water before salad preparation should be uniformly practiced by all food handlers in these settings. In our field study some FSEs boil the water before salad preparation and this approach seems to be practical for small scale operations.

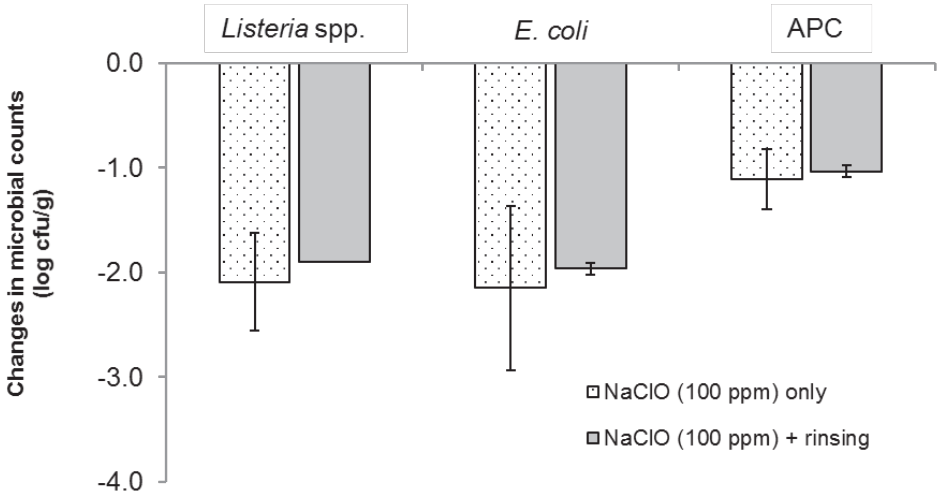


Figure 5.3: Effect of washing corn salads in sodium hypochlorite (NaClO) with and without further rinsing on the counts of *Listeria* spp., *E. coli* and APC (aerobic plate count) for 10 min and vegetable to sanitizer solution ratio (1: 20 w/v).

Sanitizer concentration

Different batches of inoculated corn salads were washed in tap water and in 50, 75, 100, 150 and 200 ppm of NaClO for 2, 5, 10 and 15 min at 25°C and pH 6.5 (Fig. 5.4). At constant contact time, increasing concentration of NaClO from 50 to 200 ppm did not result to significant higher inactivation. At the lowest contact time (2 min), there was no significant effect of washing corn salad with either tap water or NaClO (all concentrations) for APC, while for *Listeria* spp. and *E. coli*, the 100 – 200 ppm and the 50- 200 ppm NaClO respectively had a significant effect compared to tap water. APC inactivation was the lowest compared to other indicators and this resistance to sanitizers has been linked to the sufficient time of attachment and biofilm formation of natural microflora in leaf matrix (32, 49, 50).

From our field study, a much lower concentration of 25 ppm was applied for NaClO compared to the 50- 200 ppm in the laboratory study and inactivation levels were in the same range for both studies. Since increasing sanitizer concentration and washing time has limited effect on microbial inactivation, sanitizer concentration ranging from 25 ppm for chlorine based sanitizers (NaClO and NaDCC) can best be adopted.

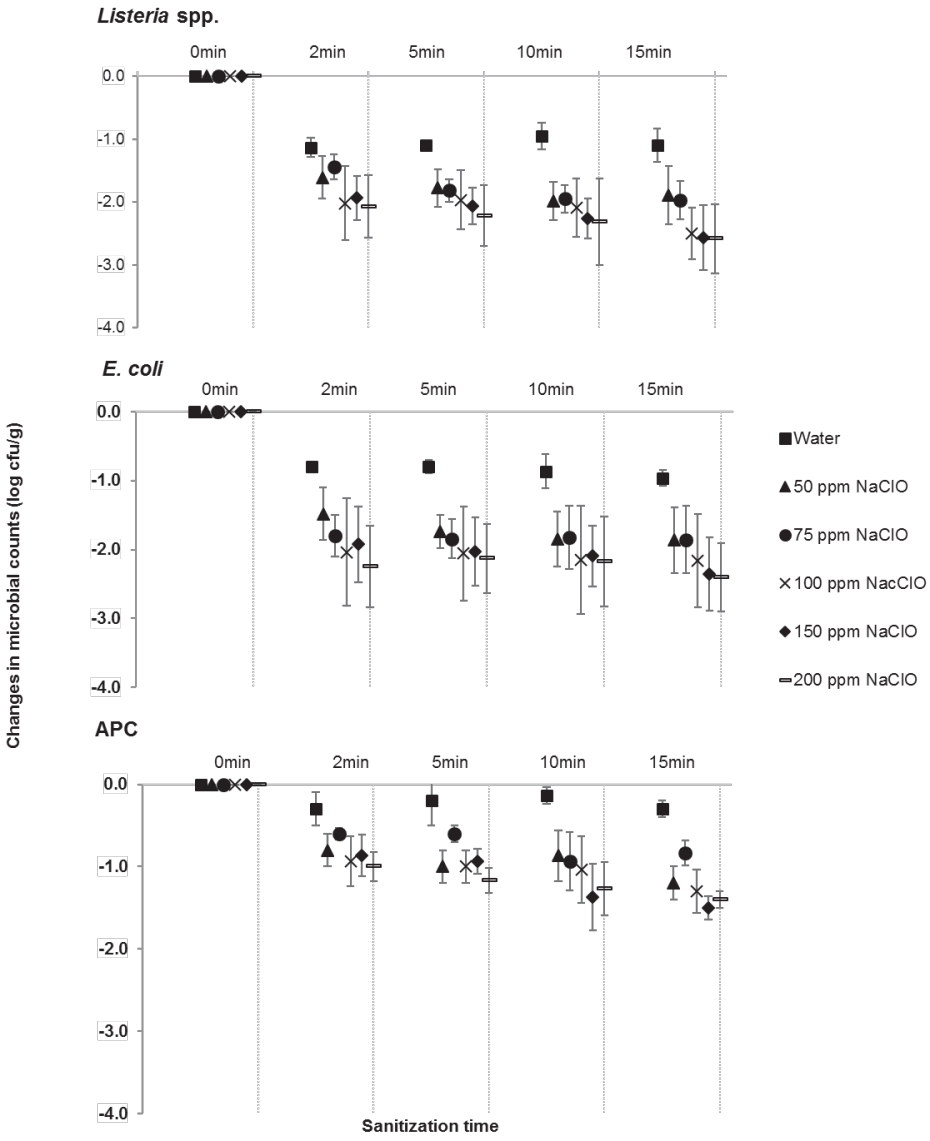


Figure 5.4: Effect of sodium hypochlorite (NaClO) on counts of *Listeria* spp., *E. coli* and APC (aerobic plate count) for different concentration and contact time with the same amount of vegetable to sanitizer solution (1:20 w/v).

Sanitizer contact time

Increasing contact time from 2, 5, 10 to 15 min did not significantly increase reduction in counts of *Listeria* spp. and *E. coli* (Fig. 5.4) by NaClO. In other studies, a marginal influence of longer exposure time was observed for chlorine sanitizer against *L. monocytogenes* counts on lettuce (51). Since varying sanitizer concentration from 50 to 200 ppm NaClO was still important at 2 min to have a significant difference between water and sanitizer, a contact time 5 min would be favorable for sanitization.

Salad: sanitizer solution ratio

Application of different ratios (1:10, 1:20, and 1:50) of corn salad to sanitizer solution (NaDCC or acetic acid) did not result in different inactivation of *Listeria* spp., *E. coli* and APC (Fig. 5.5). However, at the 1: 10 ratio the leaves could not be submerged completely, which is why this dilution rate cannot be recommended. Consequently, targeting efficacy with minimal amount of sanitizer solution and avoiding excess waste water, a 1: 20 ratio, can be adopted with respect to leafy vegetables. Further studies are needed to determine the vegetable to sanitizer solution ratio for the different types of vegetables like tomatoes.

Multiple batch sanitization

Figure 5.6 shows that no significant difference in the inactivation for the three microbial indicators (*Listeria* spp. [$p = 0.903$], *E. coli* [$p = 0.817$] and APC [$p= 0.082$]) was observed when 1 up to 5 consecutive batches of corn salad (1:20 salads to sanitizer solution (90 ppm NaDCC) were washed for 5 min in the same sanitizer solution. Studies have shown that washing vegetables in water alone may result in transfer of pathogens from vegetables to wash water (52-54). Sanitizers have been reported to inactivate microorganisms in wash solutions once detached from vegetables surfaces (54) and also can prevent cross contamination.

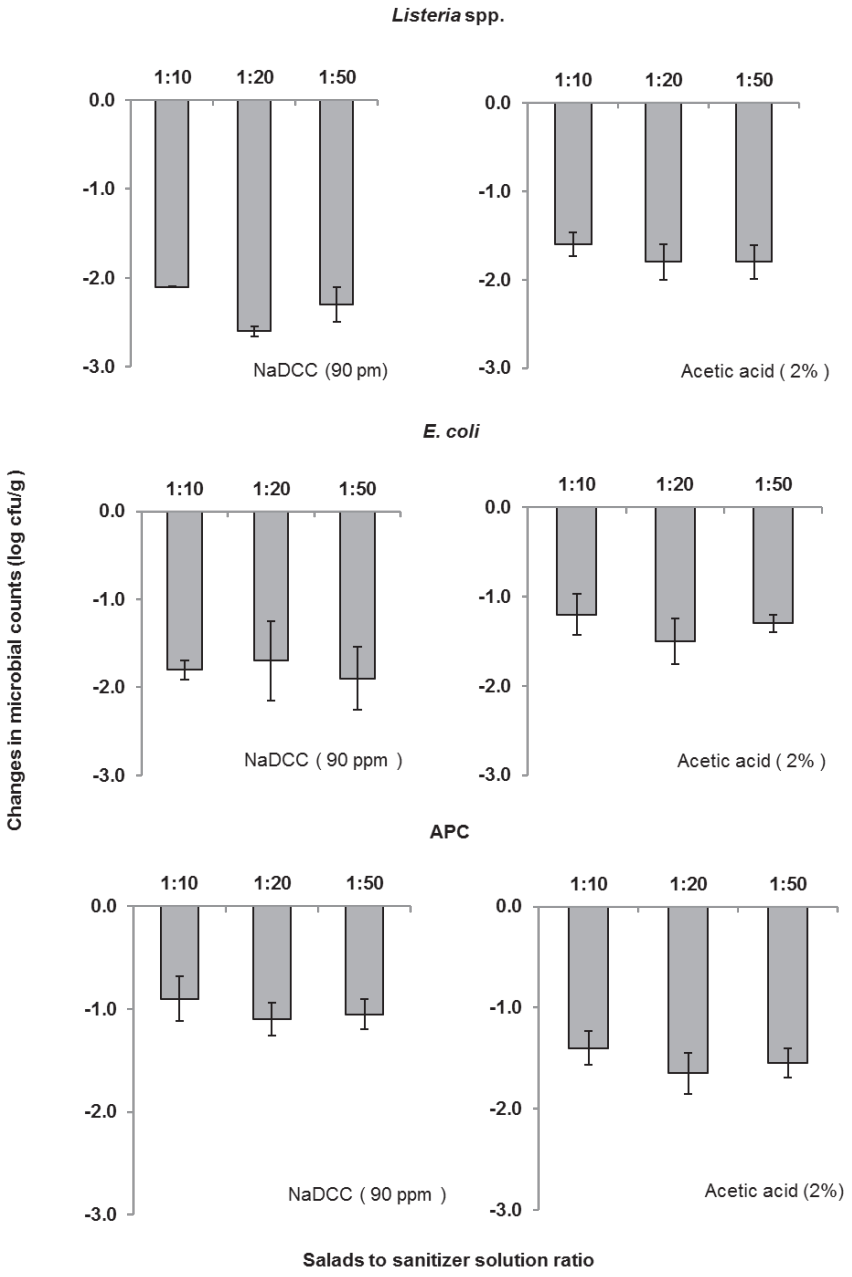


Figure 5.5: Effect of washing a specified quantity of vegetables in varying volumes of sanitizer solution on counts of *Listeria* spp., *E. coli* and APC (aerobic plate count). Two sanitizers were used, sodium dichloroisocyanurate, (NaDCC, 90 ppm) and acetic acid (2%).

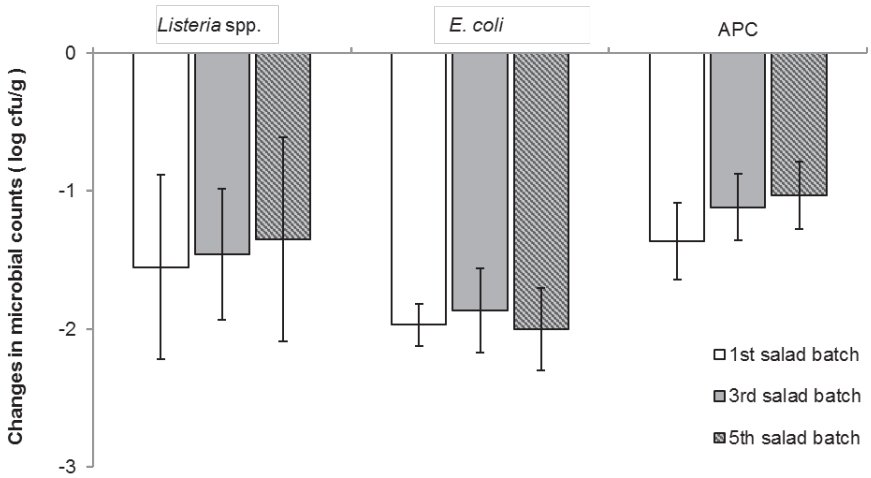


Figure 5.6: Reduction in counts of *Listeria* spp., *E. coli* and APC (aerobic plate count) after washing different batches of salads in the same quantity of sanitizer solution (sodium dichloroisocyanurate, 90 ppm) for 5 min.

Recommendations for salad preparation in FSEs

Whereas this study has mainly focused on washing, sanitization and rinsing of fresh vegetables, it is important to note that other factors can also affect microbial safety during salad preparation in FSEs. Such factors include food handlers' food safety knowledge (55), health status (56) and personal behaviors like handwashing (57), cross contamination from other food items like meat (58), hygiene of food contact surfaces (59) and microbial safety of salad dressing ingredients.

As washing and sanitization is also affected by type of vegetables prepared (9), it is important to note that the findings in this study may be more relevant for leafy vegetables than for fruit vegetables. The practice at FSEs to mix different vegetables during salad preparation will also require that these vegetables are treated separately to carter for their structural differences that may affect the efficacy of washing and sanitization. This study also mainly focused on bacterial indicators but different results may be obtained with parasites and viruses that are reported to be more resistant to especially chlorine based sanitizers (60). We have observed that salad preparation practices vary from one FSE to another in the field study. To support harmonization of these FSE practices and guideline development, the steps for salad preparations have been reorganized as in Figure 5.7.

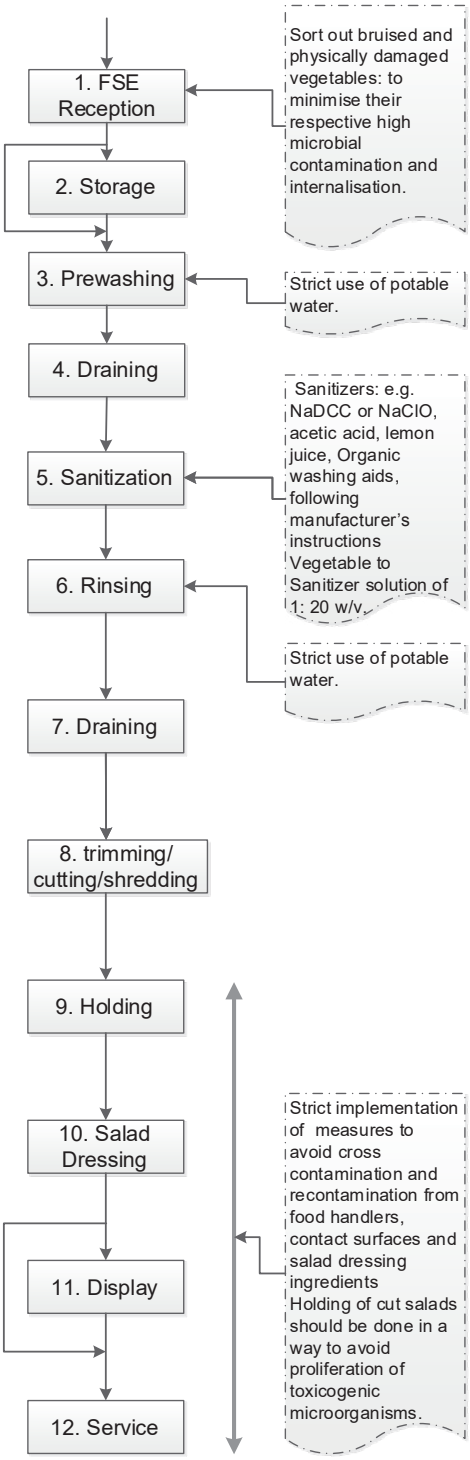


Figure 5.7: Harmonized flow diagram that can be adopted during kitchen scale salad preparation in food service establishments.

At the reception step of FSEs, vegetables with damaged tissues should be removed as they can provide nutrients for microbial proliferation and or allow entry of pathogens to the interior of vegetable tissues (61) where this internalization can render washing and or sanitization less effective. Storage of salad vegetables at FSEs should be done in way to minimize cross contamination and growth of microbial hazards. Prewashing with potable water should be done on whole vegetables to remove soils and organic load as they can render the sanitization less effective (62).

As a major objective for FSEs during preparation, vegetable salads are supposed to be fresh, organoleptically attractive and microbiologically and chemically safe. To achieve these requirements during salad preparation, FSEs need to balance sanitization settings such as sanitizer concentration and contact time. From this study it can be concluded that for corn salads, a model for leafy vegetables, use of NaDCC (≈ 50 ppm) and NaClO (25 – 50 ppm), sanitizing powder (4 g/L), lemon juice (98%) and acetic acid (2%) can be applied for 5 min and a vegetable to sanitizer ratio of 1: 20. If FSEs are using tap water, we strongly recommend onsite disinfection or boiling the water before sanitization and rinsing of salad vegetables in developing countries.

References

1. **He FJ, Nowson CA, Lucas M, MacGregor GA.** 2007. Increased consumption of fruit and vegetables is related to a reduced risk of coronary heart disease: meta-analysis of cohort studies. *Journal of Human Hypertension* 21:717-728.

2. **FAO STAT.** 2015. Food and Agriculture Organization of the United Nations, Statistics Division. Available at: http://faostat3.fao.org/compare/E. Accessed on 03rd May 2016.

3. **Cook R.** 2011. Tracking demographics and US fruit and vegetable consumption patterns. Department of Agricultural and Resource Economics, University of California, Davis.

4. **Painter JA, Hoekstra RM, Ayers T, Tauxe RV, Braden CR, Angulo FJ, Griffin PM.** 2013. Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998-2008. *Emerging Infectious Diseases* 19:407-415.

5. **Callejon RM, Rodriguez-Naranjo MI, Ubeda C, Hornedo-Ortega R, Garcia-Parrilla MC, Troncoso AM.** 2015. Reported foodborne outbreaks due to fresh produce in the United States and European Union: trends and causes. *Foodborne Pathogens & Disease* 12:32-38.

6. **Soon JM, Seaman P, Baines RN.** 2013. *Escherichia coli* O104:H4 outbreak from sprouted seeds. *International Journal of Hygiene & Environmental Health* 216:346-354.

7. **Bohaychuk VM, Bradbury RW, Dimock R, Fehr M, Gensler GE, King RK, Rieve R, Barrios PR.** 2009. A microbiological survey of selected alberta-grown fresh produce from farmers' markets in Alberta, Canada. *Journal of Food Protection* 72:415-420.

8. **Laura S, Irene R, Roberta B, Maria G, Serena S, Gabriella DM, Carlo E.** 2012. Potential Risk of Norovirus Infection Due to the Consumption of "Ready to Eat" Food. *Food & Environmental Virology* 4:89-92.

9. **Sapers GM.** 2009. Chapter 16 - Disinfection of contaminated produce with conventional washing and sanitizing technology, p. 393-424. In Sapers GM, Solomon EB, Matthews KR (ed.), *The Produce Contamination Problem*. Academic Press, San Diego.

10. **Warriner K, Namvar A.** 2014. 6 - Postharvest washing as a critical control point in fresh produce processing: alternative sanitizers and wash technologies A2 - Hoorfar, J, p. 71-102, *Global Safety of Fresh Produce.* Woodhead Publishing.

11. **Banach JL, van Bokhorst-van de Veen H, van Overbeek LS, van der Zouwen PS, van der Fels-Klerx HJ, Groot MNN.** 2017. The efficacy of chemical sanitizers on the reduction of *Salmonella* Typhimurium and *Escherichia coli* affected by bacterial cell history and water quality. *Food Control* 81:137-146.

12. **Abadias M, Usall J, Oliveira M, Alegre I, Vinas I.** 2008. Efficacy of neutral electrolyzed water (NEW) for reducing microbial contamination on minimally-processed vegetables. *International Journal of Food Microbiology* 123:151-158.

13. **Davidson GR, Buchholz AL, Ryser ET.** 2013. Efficacy of commercial produce sanitizers against nontoxigenic *Escherichia coli* O157:H7 during processing of iceberg lettuce in a pilot-scale leafy green processing line. *Journal of Food Protection* 76:1838-1845.

14. **Holvoet K, Jacxsens L, Sampers I, Uyttendaele M.** 2012. Insight into the prevalence and distribution of microbial contamination to evaluate water management in the fresh produce processing industry. *Journal of Food Protection* 75:671-681.

15. **Kinsinger NM, Mayton HM, Luth MR, Walker SL.** 2017. Efficacy of post-harvest rinsing and bleach disinfection of *E. coli* O157:H7 on spinach leaf surfaces. *Food Microbiology* 62:212-220.

16. **Amoah P, Drechsel P, Abaidoo RC, Klutse A**. 2007. Effectiveness of common and improved sanitary washing methods in selected cities of West Africa for the reduction of coliform bacteria and helminth eggs on vegetables. *Tropical Medicine & International Health* 12:40-50.

17. **Beuchat LR, Harris LJ, Ward TE, Kajs TM.** 2001. Development of a proposed standard method for assessing the efficacy of fresh produce sanitizers. *Journal of Food Protection* 64:1103-1109.

18. **Kilonzo-Nthenge A, Chen F-C, Godwin SL.** 2006. Efficacy of home washing methods in controlling surface microbial contamination on fresh produce. *Journal of Food Protection* 69:330-334.

19. **Ssemanda JN, Reij M, Bagabe MC, Muvunyi CM, Joosten H, Zwietering MH.** 2017. Indicator microorganisms in fresh vegetables from "farm to fork" in Rwanda. *Food Control* 75:126-133.

20. **Mukherjee A, Speh D, Diez-Gonzalez F**. 2007. Association of farm management practices with risk of *Escherichia coli* contamination in pre-harvest produce grown in Minnesota and Wisconsin. *International Journal of Food Microbiology* 120:296-302.

21. **ISO 21528-2:** 2004. Microbiology of food and animal feeding stuffs -- Horizontal methods for the detection and enumeration of *Enterobacteriaceae* -- Part 2: Colony-count method. *International Organization for Standardization*, Geneva, Switzerland.

22. **ISO 4833-1:** 2013. Microbiology of the food chain -- Horizontal method for the enumeration of microorganisms -- Part 1: Colony count at 30 degrees C by the pour plate technique. *International Organization for Standardization*, Geneva, Switzerland.

23. **ISO 6888-2:** 1999. Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) -- Part 2: Technique using rabbit plasma fibrinogen agar medium. *International Organization for Standardization*, Geneva, Switzerland.

24. **ISO 11290-2:1998/Amd 1:** 2004. Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of *Listeria monocytogenes* — Part 2: Enumeration method AMENDMENT 1: Modification of the enumeration medium. *International Organization for Standardization*, Geneva, Switzerland.

25. **Berger CN, Shaw RK, Brown DJ, Mather H, Clare S, Dougan G, Pallen MJ, Frankel G.** 2009. Interaction of *Salmonella enterica* with basil and other salad leaves. *ISME Journal* 3:261-265.

26. **López-Gálvez F, Gil MI, Truchado P, Selma MV, Allende A.** 2010. Cross-contamination of fresh-cut lettuce after a short-term exposure during pre-washing cannot be controlled after subsequent washing with chlorine dioxide or sodium hypochlorite. *Food Microbiology* 27:199-204.

27. **Venkitanarayanan KS, Ezeike GO, Hung Y-C, Doyle MP. 1999**. Efficacy of electrolyzed oxidizing water for inactivating *Escherichia coli* O157: H7, *Salmonella enteritidis*, and *Listeria monocytogenes*. *Applied & Environmental Microbiology* 65:4276-4279.

28. **Beuchat LR, Adler BB, Lang MM.** 2004. Efficacy of chlorine and a peroxyacetic acid sanitizer in killing *Listeria monocytogenes* on iceberg and romaine lettuce using simulated commercial processing conditions. *Journal of Food Protection* 67:1238-1242.

29. **Vitens.** 2016. Drinking water supply company for the Gelderland province, the Netherlands. Available at: https://www.vitens.com/. Accessed 28th August 2016.

30. **Smeets P, Medema G, Van Dijk J.** 2009. The Dutch secret: how to provide safe drinking water without chlorine in the Netherlands. *Drinking Water Engineering & Science* 2:1-14.

31. **Nascimento M, Silva N, Catanozi M, Silva K.** 2003. Effects of different disinfection treatments on the natural microbiota of lettuce. *Journal of Food Protection* 66:1697-1700.

32. **Vijayakumar C, Wolf-Hall CE.** 2002. Evaluation of household sanitizers for reducing levels of *Escherichia coli* on iceberg lettuce. *Journal of Food Protection* 65:1646-1650.

33. **Citrox.** 2016. Citrox 14W Plus - produce. Technical data sheet. Available at: http://www.citrox.co.nz/files/tech.sheet-14wp-produce-april-2013.pdf. Accessed 18th July 2016.

34. **Soriano JM, Rico H, Moltó JC, Mañes J.** 2000. Assessment of the microbiological quality and wash treatments of lettuce served in University restaurants. International *Journal of Food Microbiology* 58:123-128.

35. **FDA.** 2016a. The Food and drug administration of the United States, department of health and human services. Code of federal regulations. Part 173 - Secondary direct food additives permitted in food for human consumption. Subpart D-Specific usage additives. 21CFR173.405. Silver Spring, Maryland, United States. Available at: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=173.405. Accessed 02nd September 2016.

36. **FDA.** 2016b. The Food and drug administration of the United States, department of health and human services. Code of federal regulations. Part 178-indirect food additives: adjuvants, production aids, and sanitizers. Subpart B-substances utilized to control the growth of microorganisms. 21CFR178.1010. Silver Spring, Maryland, United States. Available at: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=178.1010. Accessed 02nd September 2016.

37. **ISO 16649-2:** 2001. Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli*-- Part 2: Colony-count technique at 44 degrees C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide. *International Organization for Standardization*, Geneva, Switzerland.

38. **Kirby MA, Nagel CL, Rosa G, Iyakaremye L, Zambrano LD, Clasen TF**. 2016. Faecal contamination of household drinking water in Rwanda: A national cross-sectional study. *Science of the Total Environment* 571:426-434.

39. **Sukul S, Sheth M.** 2012. Can sanitizers reduce microbial load of coriander leaves? *Nutrition and Food Science* 42:12-20.

40. **Park SH, Choi MR, Park JW, Park KH, Chung MS, Ryu S, Kang DH**. 2011. Use of organic acids to inactivate *Escherichia coli* O157: H7, *Salmonella Typhimurium*, and *Listeria monocytogenes* on organic fresh apples and lettuce. *Journal of Food Science* 76:M293-M298.

41. **Santos YO, Almeida RCC, Guimarães AG, Almeida PF.** 2010. Hygienic-sanitary quality of vegetables and evaluation of treatments for the elimination of indigenous *E. coli* and *E. coli* O157: H7 from the surface of leaves of lettuce (*Lactuca sativa L.*). *Food Science & Technology (Campinas*) 30(4), 1083-1089.

42. **Sengun IY, Karapinar M.** 2004. Effectiveness of lemon juice, vinegar and their mixture in the elimination of *Salmonella* Typhimurium on carrots (Daucus carota L.). *International Journal of Food Microbiology* 96:301-305.

43. **Parish M, Beuchat L, Suslow T, Harris L, Garrett E, Farber J, Busta F.** 2003. Methods to reduce/eliminate pathogens from fresh and fresh-cut produce. *Comprehensive Reviews in Food Science & Food Safety* 2:161-173.

44. **Clasen T, Edmondson P.** 2006. Sodium dichloroisocyanurate (NaDCC) tablets as an alternative to sodium hypochlorite for the routine treatment of drinking water at the household level. International *Journal of Hygiene & Environmental Health* 209:173-181.

45. **Hammond BG, Barbee SJ, Inoue T, Ishida N, Levinskas GJ, Stevens MW, Wheeler AG, Cascieri T.** 1986. A review of toxicology studies on cyanurate and its chlorinated derivatives. *Environmental Health Perspectives* 69:287.

46. **Szabo E, Simons L, Coventry M, Cole M.** 2003. Assessment of control measures to achieve a food safety objective of less than 100 CFU of *Listeria monocytogenes* per gram at the point of consumption for fresh precut iceberg lettuce. *Journal of Food Protection* 66:256-264.

47. **Van Haute S, Sampers I, Holvoet K, Uyttendaele M.** 2013. Physicochemical quality and chemical safety of chlorine as a reconditioning agent and wash water disinfectant for fresh-cut lettuce washing. *Applied & Environmental Microbiology* 79:2850-2861.

48. **Bain R, Cronk R, Wright J, Yang H, Slaymaker T, Bartram J.** 2014. Fecal contamination of drinking-water in low-and middle-income countries: a systematic review and meta-analysis. *PLoS medicine* 11:e1001644.

49. **Neo SY, Lim PY, Phua LK, Khoo GH, Kim S-J, Lee S-C, Yuk H-G.** 2013. Efficacy of chlorine and peroxyacetic acid on reduction of natural microflora, *Escherichia coli* O157:H7, *Listeria monocyotgenes* and *Salmonella* spp. on mung bean sprouts. *Food Microbiology* 36:475-480.

50. **Ölmez H, Temur SD.** 2010. Effects of different sanitizing treatments on biofilms and attachment of *Escherichia coli* and *Listeria monocytogenes* on green leaf lettuce. *LWT - Food Science and Technology* 43:964-970.

51. **Zhang S, Farber JM.** 1996. The effects of various disinfectants against *Listeria monocytogenes* on fresh-cut vegetables. *Food Microbiology* 13:311-321.

52. **Jensen DA, Friedrich LM, Harris LJ, Danyluk MD, Schaffner DW.** 2015. Cross contamination of *Escherichia coli* O157:H7 between lettuce and wash water during home-scale washing. *Food Microbiology* 46:428-433.

53. **Holvoet K, De Keuckelaere A, Sampers I, Van Haute S, Stals A, Uyttendaele M.** 2014. Quantitative study of cross-contamination with *Escherichia coli*, *E.coli* O157, MS2 phage and murine norovirus in a simulated fresh-cut lettuce wash process. *Food Control* 37:218-227.

54. **Zhang G, Ma L, Phelan VH, Doyle MP**. 2009. Efficacy of antimicrobial agents in lettuce leaf processing water for control of *Escherichia coli* O157: H7. *Journal of Food Protection* 72:1392-1397.

55. **Abdullah Sani N, Siow ON.** 2014. Knowledge, attitudes and practices of food handlers on food safety in food service operations at the Universiti Kebangsaan Malaysia. *Food Control* 37:210-217.

56. **Kamau P, Aloo-Obudho P, Kabiru E, Ombacho K, Langat B, Mucheru O, Ireri L.** 2012. Prevalence of intestinal parasitic infections in certified food-handlers working in food establishments in the City of Nairobi, Kenya. *Journal of Biomedical Research* 26:84-89.

57. **Robinson AL, Lee HJ, Kwon J, Todd E, Rodriguez FP, Ryu D.** 2016. Adequate hand washing and glove use are necessary to reduce cross-contamination from hands with high bacterial loads. *Journal of Food Protection* 79:304-308.

58. **Redmond EC, Griffith CJ, Slader J, Humphrey TJ.** 2004. Microbiological and observational analysis of cross contamination risks during domestic food preparation. *British Food Journal* 106:581-597.

59. **Gorman R, Bloomfield S, Adley CC.** 2002. A study of cross-contamination of food-borne pathogens in the domestic kitchen in the Republic of Ireland. *International Journal of Food Microbiology* 76:143-150.

60. **CDC.** 2012. Center for Disease Control and prevention, Atlanta, Georgia, USA. Effect of Chlorination on Inactivating Selected Pathogen. Available at: https://www.cdc.gov/safewater/effectiveness-on-pathogens.html. Accessed on 25th June 2017.

61. **Beuchat LR.** 2006. Vectors and conditions for preharvest contamination of fruits and vegetables with pathogens capable of causing enteric diseases. *British Food Journal* 108:38-53.

62. **Shen C, Luo Y, Nou X, Wang Q, Millner P.** 2013. Dynamic effects of free chlorine concentration, organic load, and exposure time on the inactivation of *Salmonella*, *Escherichia coli* O157: H7, and non-O157 Shiga toxin–producing *E. coli*. *Journal of Food Protection* 76:386-393.

6

General discussion, conclusion and future perspectives

Globalization has led to increased international travel, trade and tourism, implying that people from different countries now interact more often. This interaction among people has resulted to drastic changes in cultures, life styles, eating behavior and food preferences (1). Changes in life style include an increase in the consumption of convenient, ready to-eat foods and eating away from home in canteens, restaurants and hotels. Trends in eating behavior and food preferences show that a vegetable based diet is gaining popularity (2-4) year by year and a significant proportion of these vegetables are consumed raw.

While the exchange of life style, eating behavior and food preferences is global, the level of implementing food safety standards and guidelines (*i.e.*. GAPs, GHPs, HACCP) continue to vary from one region or country to another. All countries have limited resources and at times experience a lapse in implementing food safety preventive and control measures leading to food contamination which may later emerge as foodborne illnesses and or outbreaks. Food service establishments (FSEs) have been implicated in foodborne outbreaks (5-10), yet it is not always clear whether contamination takes place in these eating places or elsewhere along the food supply chain.

This study was commissioned to analyze the microbial risk from "farm to fork" along the fresh vegetable supply chain in Rwanda to explore microbial safety options that can contribute to an integrated system to detect, control and prevent foodborne infections. In approaching this study, specific objectives were developed. The first objective was to estimate the burden of foodborne infectious illnesses using the available data to obtain insight into the general plight of food safety issues and to develop a framework for future investigations (Chapter 2). Second, an investigation into the microbial safety status, handling practices and risk exposure factors was conducted along the fresh vegetable supply chain (Chapters 3 to 5). This chapter discusses the link between the previous chapters (1 to 5) and activities critical to microbial safety and opportunities for improvement. Furthermore, the methodology and the outcome of analysing the microbial risk from "farm to fork" are presented next to the suggestions for approaching microbial safety of fresh vegetables and the way forward for an integrated food chain system.

Burden of foodborne illnesses

While foodborne illnesses have been described as an old and known problem (11, 12), these illnesses continue to cause substantial morbidity and mortality worldwide, burdening public health systems and impeding social-economic development (13). Reasons highlighted for the continuing burden of foodborne illnesses include: the insufficient implementation of effective control measures, the emergence and re-emergence of foodborne pathogens, the increasing potential of spread due to globalization, the changing patterns of microbial resistance to antibiotics and the surging number of susceptible population (11, 12). Consequently, estimating and reporting of foodborne illnesses is required to assess the burden nationally and globally to direct and prioritize food safety policies and interventions (14). In line with estimating the burden of foodborne illnesses, the World Health Organisation initiated a task group (FERG) in 2007 and also advised national governments to continually conduct studies on the burden of

foodborne illnesses. To date, national studies on the burden of foodborne illnesses come from few countries; England (15), United States (16), Netherlands (17), Greece (18), Canada (19). The limited numbers of these studies has been associated with the challenges of operating an elaborate disease surveillance systems and lack of required expertise.

In order to support efforts of estimating burden of foodborne illnesses in Rwanda and other developing countries, we estimated the burden of food-related illnesses based on syndromic surveillance data (Chapter 2) which is commonly available and affordable in resource scarce settings. Study findings indicate that for the year 2013, watery diarrhea occurred all year round as by the national notifiable surveillance system data, resulting to an estimated 672 (95% credible interval [CrI] 424 – 932) DALY per million inhabitants, bloody diarrhea was seasonal coinciding with the rainy months and caused an estimated 213 (95% CrI 50 – 475) DALY per million, typhoid fever and cholera were sporadic with an estimated 73 (95% CrI 57 – 91) and 1 (95% CrI 0 – 2) DALY per million respectively. Data gaps always characterise these studies on estimates of burden of foodborne illnesses (20) and in Chapter 2 of this thesis, these data gaps have also been presented. One of the major data gaps in the present study was that we could not attribute the estimates of food related illnesses to any food vehicle based on the available data. However, foodborne pathogens and or their indicators have been detected and isolated from various food items in Rwanda, for example milk and milk products (21), raw meat (22) and drinking water (23, 24). In the present study (Chapter 4), foodborne pathogens (*Salmonella* ssp*., Listeria monocytogenes*, *Campylobacter* spp. and pathogenic *Escherichia coli*) were detected in farm vegetables. Investigating the microbial safety of different food sources along the supply chains and coupling with epidemiology data will improve burden of foodborne illness estimates.

Microbial safety concern of fresh vegetables

Attribution studies have shown fresh vegetables among the major upcoming food vehicles for foodborne pathogens leading to foodborne illnesses, but most studies and data come from developed countries (25, 26). While production and consumption of fresh vegetables is increasing in developing countries (4), there is limited information on their safety and attribution to the burden of foodborne diseases. Farm vegetables in open fields can be contaminated with human zoonotic pathogens through agronomic factors such as irrigation water, flooding, soil, manure, human biosolids, dust, domestic and wild animals, human activities (27-30). Studies indicate that, once introduced, human pathogens can attach (31, 32) and or internalize in- (33, 34) and persist on the vegetable structures. In Chapter 4 we have reported the prevalence of pathogens, *Salmonella* spp., *Campylobacter jejuni*, pathogenic *E. coli* and *L. monocytogenes* in farm vegetables in Rwanda in comparison with studies from studies from other countries. Although there can be postharvest contamination, it has been argued that pathogens once introduced to vegetables during primary production, are difficult to remove later by washing and sanitization (35). In chapter 3, we used indicator microorganisms to investigate what happens to the microbial counts in fresh vegetables from “farm to fork”. Our results show that microbial counts slightly increased from farm to market and to reception at FSEs, but while at FSEs, salad preparation led to an average reduction of 2.1 log cfu/g. For

pathogens like *Salmonella* spp. (where ingestion of one cell can cause *Salmonellosis*) (36) and selected *E. coli* strains, their presence in farm vegetables even in low numbers means that they can present a health risk to the consumers. Moreover in Chapter 5, field and laboratory trials to identify the most effective sanitizer and washing technique showed that the maximum possible microbial inactivation was around 3 logs with sodium dichloroisocyanurate as a sanitizer. Pathogens, however, may be present in high numbers, so it is important not to rely on only washing and sanitization but on a range of measures across the whole supply chain to prevent and control pathogens from contaminating fresh vegetables. In order to estimate the risk to consumers, microbial safety data along the vegetable supply chain was gathered and an analysis of the microbial risk was conducted from "farm to fork" for the vegetable supply chain in Rwanda as presented in the next section.

"Farm to fork" risk analysis along the fresh vegetable supply chain

Risk analysis as fostered internationally by the WHO and FAO, is a systematic, disciplined approach for making evidence based food safety decisions by competent authorities. Risk analysis comprises of three overlapping components (Fig. 6.1); risk assessment, risk management and risk communication (37). In risk assessment, science based information is generated for risk managers to develop food safety policies from the available scientific options (risk management). While exchange of information between risk assessors, risk managers, consumers and other stakeholders is referred to as risk communication (37).

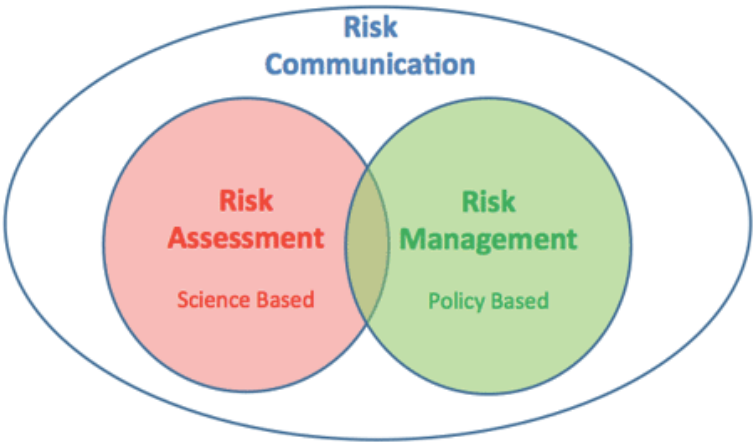


Figure 6.1: Components of Risk analysis [Source: FAO (38)].

Risk assessment

A quantitative microbial risk assessment was conducted in this study by using the data gathered in Chapters 3 to 5. The processes of hazard identification, exposure assessment, hazard characterization and risk characterization that are involved in risk assessment were followed and presented in the following sub-sections of this chapter.

Hazard identification

In the "farm to fork" continuum, fresh vegetables can be exposed to three categories of pathogenic microorganisms; bacteria (39), parasites (40) and viruses (41). In Chapter 4, we tested farm vegetables and agricultural water for the presence of foodborne pathogens. Prevalence of pathogens in farm vegetables varied from 1.0 % (1/99) for *L. monocytogenes*, 3.0 % (3/99) for thermo-tolerant *Campylobacter* spp., 5.1 % (5/99) for *Salmonella* spp. to 6.1 % (6/99) pathogenic *E. coli.* In agricultural water from rivers, lakes, lagoons, ground and marshlands, prevalence of DNA from pathogens varied from 3 % (1/30) for Enteroinvasive *E. coli* (EIEC); 7 % (2/30) for Enteroaggregative *E. coli* (EAEC); 13 % (4/30) for Enterotoxigenic *E. coli.* (ETEC) and *Vibrio cholera*; 20 % (6/30) for *Yersinia pestis*; 27 % (8/30) for *Francisella tularensis*; 40 % (12/30) for *Cyclospora* to 87 % (26/30) for thermo-tolerant *Campylobacter* spp. Table 6A.1 shows a summary of the pathogens detected in farm vegetables and agriculture water in Rwanda and their associated health outcomes.

The findings on the prevalence of foodborne pathogens associated with farm vegetables in Rwanda as presented in Chapter 4 can point to several pathogens as candidates for risk assessment. The risk assessment in this study focused on pathogenic *E. coli* due to their high prevalence, public health impact and association with the vegetable supply chain. Further details on hazard identification of pathogenic *E. coli* are presented in the Appendix section of this chapter.

Exposure assessment

Figure 6.2 shows the various routes in the vegetable supply chain in Rwanda. In chapters 4 and 5 of this thesis, findings are presented for the microbial risk exposure factors of fresh vegetables from "farm to fork". Based on these findings and other information from literature, such as that from the Pang et al. study (42), a quantitative microbial risk assessment (QMRA) model was developed for pathogenic *E. coli* from "farm to fork". This QMRA was based on leafy vegetables (lettuce) model developed in previous studies (42-45). The exposure assessment part of the QMRA model was built in 4 modules: at farm, during transport, at market and at food service establishments as discussed in details in the next sub-sections of this chapter. The summary of variables, distributions, formula and data sources are presented in Table 6.1. All simulations were conducted in @ risk 7.5 software (Palisade Corp., Ithaca, NY)) using 100,000 iterations making use of the Monte Carlo simulation technique (44).

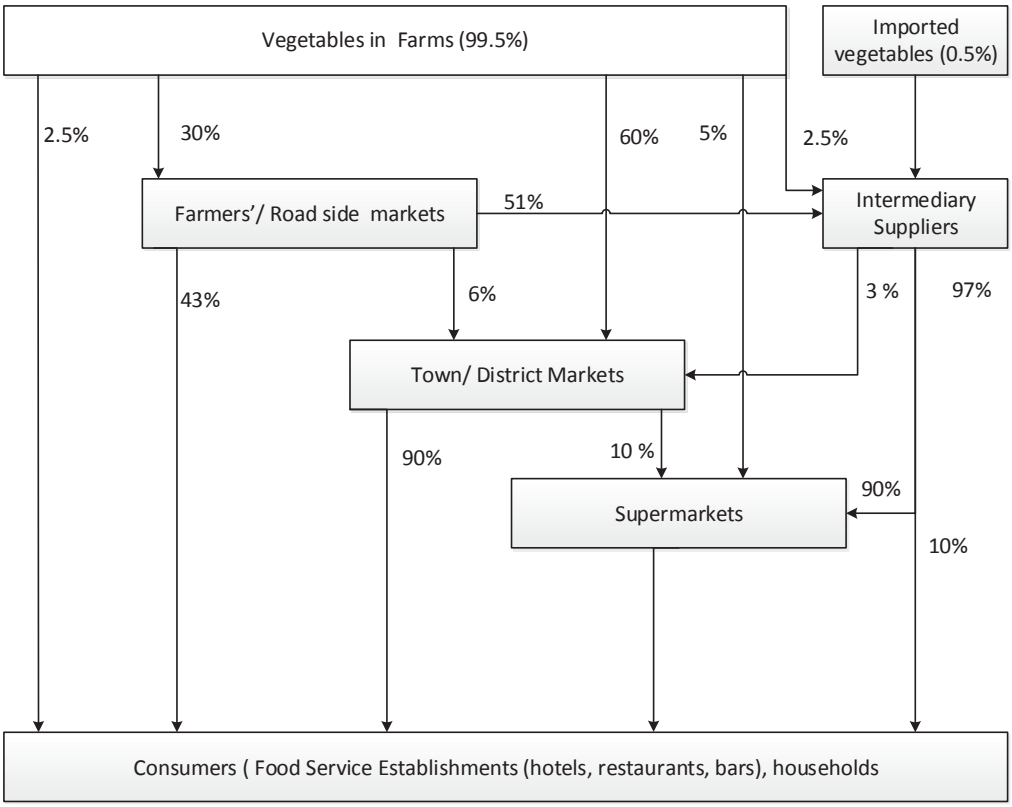


Figure 6.2: Structure of the vegetable supply chain in Rwanda. The consumers (hotels, restaurants, households) can receive vegetables from any step of the chain. The percentages indicate the proportions of vegetables channeled through specific routes.

At farm

Details of our evaluation of microbial hazards and risk exposure factors at farm level were largely taken from Chapter 4 of this thesis. To estimate the prevalence and concentration of pathogenic *E. coli* in vegetables at farm level, the approach was as follows:

In the QMRA model, there was no individual quantification of the risk from pre-harvest factors like irrigation water, manure, and environmental pollution (Tab. 6.1), like it was in the study of Ding et al (45). We assumed that these pre-harvest factors contributed to 6.1 % (6/99) prevalence of pathogenic *E. coli* in farm vegetables reported in Chapter 4. Initial concentrations of *E. coli* in farm leafy vegetables were adopted from a previous study in Lebanon (46, 47) a country with conditions of the vegetable supply chain similar to Rwanda. The *E. coli* (generic) levels (in positive vegetables) were reorganized into a Risk Pert distribution with the mean (1.3 log cfu/g) as the most likely value while the lowest and highest value were 0.7 and 7 log cfu/g respectively. Furthermore, microbial counts in farm vegetables are reported to reduce with time till harvest (43). In their study, Maffei et al.(43) modelled log reduction in *Salmonella* spp. as

a function of 60 days between the initial contamination and the time of harvest. The approach of Maffei et al. (43) was also applied in this study. Risk Uniform and Risk Normal distributions were respectively used to model the days in the field after contamination and the log reduction in the field.

At harvest, the prevalence and concentration of *E. coli* can also be affected by cross contamination from contact surfaces of harvest containers and harvesters hands, on-farm washing and refreshing of vegetables. These on-farm practices at harvest were also modelled in this QMRA study (summarised in Table 6.1). During on-farm washing using the surrounding surface waters, it is also possible there can be reduction in *E. coli* counts. Water alone led to a 0.9 ± 0.2 log reduction during the washing of leafy vegetables (Chapter 5, this thesis) and this log reduction was described by a Normal distribution. On the other side, on farm washing and refreshing can also result to contamination of farm vegetables. In the study of Pang et al. (42) as shown in Table 6.1, this contamination was computed as a function of levels of *E. coli* in refreshing water, water holding capacity on a lettuce model (Risk Normal(0.108, 0.019, Risk Truncate (0,)) ml/g) and the ratio of pathogenic *E. coli* to generic *E.coli* in washing water. Levels of *E. coli* in surface water surrounding farm vegetables in Rwanda were reorganized into a risk Pert distribution with the mean (2.0 log cfu/100ml) as the most likely value while the lowest and highest value were 0.0 and 3.6 log cfu/100ml respectively (unpublished results, this study). The ratio of pathogenic *E. coli* to generic *E. coli* in agricultural water in Rwanda used in the study was 7/27, from Chapter 4 where the prevalence of pathogenic *E. coli* was 7/30 and from unpublished results where 3/30 samples had 0.0 cfu/100ml levels of generic *E.coli*. The algorithm also caters for cross contamination by estimating and quantifying the number of cells of *E. coli* transferred from and to farm vegetables (Tab. 6.1). Transfer rates of *E. coli* from contaminated leafy vegetables to hands were adopted from Verhaelen et al. (48) by taking a Risk Pert distribution of the transfer rate estimates of human norovirus from lettuce to hands. A risk triangle distribution was applied to the transfer rates of *E. coli* form leafy vegetables to harvest surfaces by assuming that the these surfaces are similar to those of the conveyor belt surfaces in the Pang et al. study (42). Chen et al. (49) quantified transfer rates of bacterial cross contamination from hand to lettuce in a RiskWeibull distribution, these transfer rates were also adopted for this study. In their study, Jensen et al. (50) quantified transfer rates of *E. coli* O157: H7 between fresh cut produce and common kitchen surfaces. This study adopted the transfer rates from contaminated plastic surfaces in the Jensen et al. (50) study to represent harvest containers to lettuce and described in a Risk Pert distribution. Furthermore, due to cross contamination, for each washing or refreshing step the prevalence of pathogenic *E. coli* in farm vegetables was multiplied by a factor of spread of cross contamination with a Risk Pert (1,1.2, 2) distribution from the Pang et al. study (42). A summary of associated functions and calculations is presented in Table 6.1.

Transportation

Vegetables are transported by farmers, suppliers or traders using several means, non-specialized vehicles, bicycles, carts and head carrying, and no refrigeration is involved. Vegetables are transported at temperatures between 14 to 25˚C [common temperature range in Rwanda] (51) and in this study a Risk Pert distribution of 14 (low), 20 (most likely) and 25 (high) ˚C was

applied. Figure 6.2 shows the transportation routes. Major transportation routes include the transportation of vegetables from farms to markets to FSEs (97.5%), while in some cases vegetables are directly transported from farms to FSEs (2.5%).

In this QMRA study, the transportation time was described by a Risk Uniform distribution as from previous studies (45). It was assumed that during transport there is growth and like from previous studies (42, 44), primary and secondary models were used:

The primary model used to describe the growth of pathogenic *E. coli* was as shown in Equation 1.

$$\log N_t = \log N_0 + \mu \times t \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots \text{Eq. 1}$$

where N_t is the concentration at time t (CFU/g), N_0 is the concentration at time 0 (CFU/g) and μ is the growth rate at a specific temperature (log CFU/g/h). To determine μ, a secondary model was used as in equation (Eq. 2) proposed by Ratkowsky et al. (52)

$$\sqrt{\mu} = b\,(T - T_0) \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{Eq. 2}$$

where T is the temperature (in degrees Celsius), and b and T_0 are regression constants. These growth equations (Eq. 1 and Eq. 2) were used where applicable in other modules of this QMRA study.

It was also assumed that the microbial growth rate due to transportation of vegetables between farms and markets was equal to that between markets and FSEs. Transportation time from farms to markets was about 0.5 to 3 h and the same time was assumed to be spent between markets and FSEs. Therefore, the microbial growth due to transportation between farms and markets was assumed to be equal to that growth due to transportation between market and FSEs. Microbial contamination of fresh vegetable during transportation was considered minimal and not included in the QMRA study.

At market

At market level, about 90 % of vegetables are sold in town and farmer markets, which are open air structures with raised platforms and more than 20 vendors while the rest go through supermarkets where vegetables are sold under refrigeration (This study). The overall concentration of *E. coli* at market was generated from three modules; growth during display, contamination during refreshing of fresh vegetables and cross contamination. The contribution of these three modules to the concentration and prevalence of *E. coli* at market stage was thereafter computed.

Depending on the vegetable type and market infrastructure, vegetables can be retained at market from 1- 5 days and display temperatures are between 14 and 25 ˚C (51) for open air markets (This study). Growth in *E. coli* levels during display at market was also modelled in a similar way as during transportation. In these markets, vendors also refresh leafy vegetables with water from nearby water sources (streams, rivers and tap water). Like during harvest at farm level, this contamination was computed as a function of levels of *E. coli* in refreshing water, water

holding capacity on a lettuce model (Risk Normal(0.108, 0.019, RiskTruncate (0,)) ml/g) and the ratio of pathogenic *E. coli* to generic *E. coli* in washing water (Tab. 6.1). Cross contamination can occur at market between fresh vegetables and market handlers and display surfaces. The same distributions and variables for cross contamination at harvest in the farm module were used for the market module (Tab. 6.1). In addition due to cross contamination, the prevalence of pathogenic *E. coli* in vegetables at market stage was obtained by multiplying the prevalence after the farm stage by a factor of spread of cross contamination as used in the Pang et al. study (42).

At food service establishments (FSEs)

Figure 6A.1 summarises the steps involved at FSEs (This study). Details of the unit operations involved at FSEs are presented as findings from the field study in Chapter 5. In the QMRA study, growth rate of *E. coli* during storage at FSEs was modelled using the same approach as in the previous steps (Eq. 1 and Eq. 2). The storage temperatures and time were around 2 – 8˚C and 2 to 48 h and were described by a Risk Pert distribution (Tab. 6.1). In previous studies(42, 53), a die off model (Equation 3) was applied for *E. coli* O157:H7 at temperature below 5 ˚C and the same approach was used where applicable in this study.

$$\log(N_t / N_0) = -k \times t \cdots\cdots\cdots\cdots\cdots\cdots \text{Eq. 3}$$

where N_t, N_0 and t are as described in Eq. 1, while k is the death rate (log cfu/g/h).

Washing and sanitization is a crucial step in reducing microbial counts as was observed in Chapter 3 and 5. A Risk Normal distribution was used to model the reduction of counts of *Enterobacteriaceae* reported in Chapter 5 due to washing and sanitization of vegetables in the field study with FSEs and it was assumed that the *E. coli* would be reduced similarly as *Enterobacteriaceae*. A cross contamination module was also added in the same way as at harvesting (Farm level) to represent possible exchange of *E. coli* during salad preparation in kitchen surfaces and hands of handlers. Contamination due to washing water was not included in the QMRA study, it was also assumed that the water used for washing and sanitization was fit for purpose. The concentration of *E. coli* after the FSE stage was computed as presented in Tab. 6.1. All counts of *E. coli* above log 7 cfu/g at FSES were truncated in further steps of the QMRA study (Tab.6.1) as from previous studies (42, 43) to minimize unrealistic estimates of the risk. It was assumed that all *E. coli* behaved like *E. coli* O157: H7 on fresh vegetables which was reported to seldom grow above 7 cfu/g at room temperature [25 °C] (54). The contribution of cross contamination was computed using the same approach as at market and farm modules.

Table 6.1: Summary of variables and parameters used in the QMRA model for pathogenic *E. coli*

Variable symbol	Variable description	Distribution/ formula	Unit	Data source/ reference
	FARM			
Ce	Initial levels of generic *E. coli* in farm leafy vegetables (leafy vegs. ψ.)	Risk Pert (0.7, 1.3, 7.0)	log cfu/g	(46, 47)
tf	Days in the field after contamination	Risk Uniform (1,60)	days	(43)
Rf	Log reduction in the field	Risk Normal(0.0175,0.00862)	log cfu/g/day	(43, 55)
Ch"	Contamination levels at time of harvest	Ce- (tf *Rf)	log cfu/g	Calculated
Ch	Contamination levels at time of harvest	10^Ch"	cfu/g	Calculated
Fc	Prevalence of pathogenic *E.coli* in farm vegetables	(6/99*100)	%	Chapter 4
	Harvester's hands and contact surfaces (harvest boxes, baskets)			(42)
TR LH	Transfer rates from contaminated leafy vegs. to hands	Risk Pert (3.9, 6.0, 9.0)	%	(48)
TR LS	Transfer rates from contaminated leafy vegs. to harvest surfaces	Risk Triangle (0.00, 0.10,0.24)	%	(42, 56)
TR HL	Transfer rates from contaminated hands to leafy vegs.	Risk Weibull (0.75, 11.75)	%	(49)
TR SL	Transfer rates from contaminated harvest surfaces to leafy vegs.	Risk Pert (2.27, 18.64, 73.77)	%	(50)
N tfarm	No. of cells transferred from leafy vegs. to hands & harvest surfaces	Ch * (TR LH +TR LS)/100	cfu/g	Calculated
N hs	No. of cells transferred from hands & harvest surfaces to leafy vegs.	N tfarm * (TR HL +TR SL)/100	cfu/g	Calculated
Nfc	Levels of *E. coli* after cross contamination at farm	Ch + Nhs - N tfarm	cfu/g	Calculated
	Farm washing and refreshing			
Dw"	Log reduction by washing with water	Risk Normal (0.9, 0.2)	log cfu/g	Chapter 5
Clw	Levels after washing	10^(logNfc-DW")	cfu/g	Calculated
	Contamination due to on farm washing and refreshing			(42)
Cin"	Log *E. coli* levels in refreshing water	Risk Pert (0.0, 2.0, 3.6)	log cfu/100 ml	This study
Cin	*E. coli* levels in refreshing water	10^ Cin"	cfu/100 ml	
Rref	Ratio of pathogenic *E. coli* to generic *E. coli* in refreshing water	7/27		This study
W	Water holding for leafy vegs.	Risk Normal (0.108,0.019,RiskTruncate(0,))	ml/g	(42)
Cref	Levels of *E. coli* in leafy vegs. from refreshing water	Cin * Rref* W*100	cfu/g	Calculated
Cfw	Levels *E. coli* in leafy vegs. after farm washing	Clw + Cref	cfu/g	Calculated
S	Spread of contamination due to cross contamination	Risk Pert(1, 1.2, 2)		(42)
Pfc	Prevalence after cross-contamination	S*Fc	%	

Table 6.1 Continues…

	TRANSPORTATION FROM FARMS TO MARKETS			
tr1	Transportation time	Risk Uniform (0.5, 3.0)	h	This study
Tr1	Transportation temperature	Risk Pert (14, 20, 25)	°C	(51)
	Growth during transportation			
b	Growth model parameter (b)	0.0243	√log cfu/g/h/°C	(42, 43, 53)
T_0	Growth model parameter (T_0)	2.628	°C	(44)
μG1	Growth rate during transport	(b*(Tr1-T0))^2	log cfu/h	Calculated
Nr1	Growth during entire of transportation time from farm to market	μG1 * tr1	log cfu/g	Calculated
Ltrapt1	Growth during transportation from farm to market	logCfw+Nr1	log cfu/g	(42, 57)
	VEGETABLES AT MARKET			
	Growth during display			
td2	Display time	Risk Pert (0.5, 12, 48)	h	This study
Td2	Display temperature	Risk Pert (14, 20, 25)	°C	(51)
b	Growth model parameter (b)	0.0243	√log cfu/g/h/°C	(42, 43, 53)
T_0	Growth model parameter (T_0)	2.628	°C	(44)
μGd2	Growth rate during display	(b*(Tr1-T_0))^2	log cfu/g/h	Calculated
NdT	Growth during entire of display time	μGd2* td2	log cfu/g	Calculated
	Contamination during refreshing at traditional market			
Cin"	Log *E. coli* levels in refreshing water	Risk Pert (0.0, 2.0, 3.6)	log cfu/100 ml	This study
Cin	*E. coli* levels in refreshing water	10^ Cin"	cfu/100 ml	
Rref	Ratio of pathogenic *E. coli* to generic *E. coli* in refreshing water	7/27		This study
W	Water holding for leafy vegs.	Risk Normal(0.108,0.019,RiskTruncate(0,))	ml/g	(42)
Crem	Levels from *E. coli* from refreshing water	Cin * Rref* W*100	cfu/g	Calculated
	Cross contamination during sale			(42)
TR1	Transfer rates from contaminated leafy vegs. to hands	Risk Pert (3.9, 6.0, 9.0)	%	(48)
TR2	Transfer rates from contaminated leafy vegs. to display surfaces	Risk Triangle (0.00, 0.10,0.24)	%	(42, 56)
TR3	Transfer rates from contaminated hands to leafy vegs.	Risk Weibull (0.75, 11.75)	%	(49)
TR4	Transfer rates from contaminated display surfaces to leafy vegs.	Risk Pert (2.27, 18.64, 73.77)	%	(50)
N tmarkt	No. of cells transferred from leafy vegs. to market surfaces	10^Ltrapt1* (TR1+TR2)/100	cfu/g	Calculated
Ntrans	No. of cells transferred from market surfaces to leafy vegs.	N tmarkt * (TR3+TR4)/100	cfu/g	Calculated
Ncc1	Levels of pathogenic *E. coli* after cross contamination at traditional Market	10^Ltrapt1+ Ntrans – N tmarkt	cfu/g	Calculated
	Overall Levels of pathogenic *E. coli* after market			
Lm	Levels of pathogenic *E. coli* after market	NdT+ log(Crem + Ncc1)	log cfu/g	Calculated
S	Spread of contamination due to cross contamination	Risk Pert (1, 1.2, 2)		(42)

Table 6.1: Continues…

Pm	Prevalence after cross-contamination	S*Pfc	%	Calculated
	TRANSPORTATION FROM MARKETS TO FSEs			
Nr2	Growth during entire transportation time from market to FSEs	Nr2 =Nr1=(G1 * tr1)	log cfu/g	Assumption
Ltrapt2	Growth during transportation from market to FSEs	Lm+Nr2	log cfu/g	Calculated
	VEGETABLES AT FOOD SERVICE ESTABLISHMENTS (FSES)			
	Growth/die off during holding at FSEs			
ht	Holding time	Risk Pert (2.0, 24.0 , 48.0)	h	This study
hTp	Holding temperature	Risk Pert (2.0, 4.0, 8.0)	°C	This study
b	Growth model parameter (b)	0.0243	√log cfu/g/h/°C	(42, 43, 53)
T_0	Growth model parameter (T_0)	2.628	°C	(44)
k	Die-off rate	RiskLognorm(0.013,0.001,RiskShift(0.001))	log cfu/g/h	(42)
μGdh	Growth rate during holding	(b*(hTp-T0))^2	log cfu/g/h	Calculated
Ndh	Growth during holding time	μGdh* ht	log cfu/g	Calculated
Ddh	Die-off during holding time	-k*ht	log cfu/g	Calculated
Qh	Growth or die off during holding	IF(hTp >5,1,0)		(42)
Gh	Change in levels of E. coli during holding	IF (Qh=1, Ndh,Ddh)	log cfu/g	Calculated
Nfse1	Levels of *E. coli* after holding at FSEs	Lrapt2 + Gh	log cfu/g	Calculated
	Washing and sanitization			
FSE wash	Reduction by washing with or without sanitizers	Normal (1.7, 0.6)	log cfu/g	Chapter 5
Nfse2	Levels after washing	Nfse1 - FSE wash	log cfu/g	Calculated
	Cross contamination at Food service establishments (FSEs)			(42)
TR1	Transfer rates from contaminated leafy vegs. to hands	Risk Pert (3.9, 6.0, 9.0)	%	(48)
TR2	Transfer rates from contaminated leafy vegs. to contact surfaces	Risk Triangle (0.00, 0.10,0.24)	%	(42, 56)
TR3	Transfer rates from contaminated hands to leafy vegs.	Risk Weibull (0.75, 11.75)	%	(49)
TR4	Transfer rates from contaminated display surfaces to leafy vegs.	Risk Pert (2.27, 18.64, 73.77)	%	(50)
L fse	No. of cells transferred from leafy vegs. to FSE contact surfaces in a unit batch	10^Nfse 2 * (TR1+TR2)/100	cfu/g	Calculated
Ntrans 3	No. of cells transferred from FSE contact surfaces to leafy vegs. in a unit batch	L fse * (TR3+TR4)/100	cfu/g	Calculated
Nfinal	Levels of pathogenic *E. coli* after cross contamination at FSE	10^N fse 2 + N trans 3 – L fse	cfu/g	Calculated
Lfinal	Limit of level if Nfse final > 10^7 (7 log CFU/g)	IF(Nfse final<10^7, Nfse final,10^7)		Calculated
S	Spread of contamination due to cross contamination	Risk Pert (1, 1.2, 2)		(42)
Pfse	Prevalence after cross-contamination at FSE	S*Pm	%	Calculated

Table 6.1: Continues…

	Serving			
Ser	Serving size	Risk Normal (75, 25,RiskTruncate(1,100))	g	This study
D	Dose per contaminated serving (CFU/serving)	L_{final} * Ser	CFU/serving	Calculated
	Hazard characterization			
	Dose response			
α	Dose-response parameter	0.267		
β	Dose-response parameter	229.2928		(42)
P	Probability of illness per serving	(1- (1 + D/β)^ -α) * Pfse/100		Calculated
	Risk Characterization			
Rp	Risk population (Urban residents of Rwanda from 4 years and above)	1,505, 870	persons	(58)
A	Annual per person leafy vegs. consumption in Rwanda	5,888	g/yr	(42)
Nser	Number of servings per person in 1 year	A/Ser		Calculated
RW ser	No. of servings consumed per year in target population in Rwanda	Nser * Rp		Calculated
Ncases	Number of illness cases per year	Rwser * P		Calculated

Ψ Leafy vegs. = Leafy vegetables, a lettuce modal was used to represent the leafy vegetables in this study

Dose-response relationship and risk characterization

A serving size of 50 to 100g per person was used in the QMRA based on observation from the field study with FSEs (This study). The serving size was described by a Risk Normal distribution with average set at 75g and truncated at 1 and 100 to minimize extremely low and high estimate outcomes. To obtain the number of pathogenic *E. coli* cells ingested per contaminated serving of leafy vegetables, the levels of pathogenic *E. coli* after FSEs salad preparation was multiplied by the serving size (42). The dose response model used in this study (Equation 4) was according to previous related studies (42, 44) which were based on *E. coli* O157:H7 strain behavior in leafy vegetables. This study assumed that all pathogenic *E. coli* strains behaved like *E. coli* O157:H7 on leafy vegetables.

$$P = 1 - (1 + D / \beta)^{\alpha} \quad \text{.....Eq. 4}$$

where P is the probability of illness per contaminated serving, D is the number of organisms ingested per contaminated serving, α and β are model parameters.

Annual per capita estimates for lettuce consumption in the United States of 5,888g used by Pang et al. (42) were adopted for this study. In Rwanda, the majority of the work force especially in urban centers have meals in FSEs (canteens, restaurants and hotels) during the day. It was assumed that salad serving is mainly concentrated in urban centers in Rwanda, so the urban population of Rwanda (58) for age of 4 and above were used in the calculations of the annual number of servings consumed per year. Children below 4 years of age were most likely assumed not be served with salads at FSEs. All further calculations for hazard and risk characterization are presented in Table 6.1.

Intervention strategies (What if scenarios)

In the previous sections of this QMRA study, modules were developed based on the description of conditions in which over 90% of the fresh vegetables go through along the supply chain and herein referred to as the baseline model or route 1. Based on the pre-harvest and postharvest factors as described in previous studies (28, 29), intervention strategies (what if scenarios) summarized in Table 6.2 were assumed so as to observe the fold changes in the predicated risk (estimated probability of illness and number of illnesses) compared to the baseline model.

Table 6.2: Summary of measures and intervention (what if scenarios) as alternatives to the base line model

No.	What if scenarios	Assumption
1	Modifying supply chain (Route 2)	All vegetables are channelled straight from farms to food service establishments (FSEs) without going through markets
2	Modifying supply chain (Route 3)	All vegetables are channelled from farms via supermarkets (built closed markets with specialized refrigeration systems) to food service establishments (FSEs)
3	Modifying supply chain (Introduction of cold chain)	All vegetables are kept under refrigeration temperatures (2 and 8°C) from "farm to fork". Introduction of a die off model (Eq. 3)
4	Improving washing and sanitization at FSEs	All vegetables are effectively washed and sanitized. A 3 ± 0.5 log reduction was used in the simulation compared to 1.7 ± 0.6 log reduction applied in the baseline model (Chapter 5 of this thesis).
5	Avoiding cross contamination along the supply chain	Assuming no contamination and cross contamination between vegetables and other surfaces at farm harvest, market, and at FSEs.
6	Farm interventions	Assuming that preventive measures and interventions (29) are implemented at farm level to reduce prevalence and levels of pathogenic *E. coli* in the base line model by 90%.
7	Farm to fork measures and interventions	Assuming that the scenarios 4, 5, 6 are combined.

Risk estimates

From the baseline model (represents over 90% of the current vegetable supply chain), the number of cases per year and the probability of illness per serving of leafy vegetables contaminated pathogenic *E. coli* were estimated with a mode of 12.1 million and 0.1 respectively. Compared to risk estimates from previous studies (Tab. 6.3), the estimates of the probability of illness per serving in this study were high. In Colombia, the probability of illness was set at a propounded benchmark 10^{-4} per year for *Salmonella* ssp. with lettuce, cabbage and broccoli (60). However it should be acknowledged that these estimates include mild illness from less harmful pathotypes of pathogenic *E. coli viz.* enterotoxigenic *E. coli* (ETEC) and enteropathogenic *E. coli* (59). The estimates in this study were in close range with estimates from countries with settings similar to Rwanda for other pathogens *ie.* Ghana (for Norovirus) and Colombia (for *Salmonella* spp.) (Tab 6.3).

Table 6.3: Comparison of the estimates for the probability of illness per serving of fresh vegetables due to various foodborne pathogens in different countries

Foodborne pathogen	Average probability of illness per serving	Country	Reference
Pathogenic *E. coli*	1.0×10^{-1}	Rwanda	This study
Norovirus	8.6×10^{-1}	Ghana	(61)
E. coli O157: H7	9.87×10^{-8}	USA	(42)
Salmonella spp.	7.4×10^{-1}	Colombia	(60)
Norovirus	3×10^{-4}	EU	(62)
Hepatitis A	3×10^{-8}	EU	(62)
Salmonella spp.	$1.9 \times 10^{-4*}$	Brazil	(63)
L. monocytogenes	$2.7 \times 10^{-7*}$	Brazil	(63)
L. monocytogenes	1.42×10^{-7}	Korea	(45)
E. coli O157: H7	6.04×10^{-6}	Netherlands	(64)
Salmonella spp.	6.83×10^{-6}	Netherlands	(64)
L. monocytogenes	1.23×10^{-8}	Netherlands	(64)

* Estimates of probability of illnesses adjusted to per serving

Figure 6.3 and Table 6.4 summarise the results of the QMRA conducted in this study, while further graphs are provided as supplementary material (risk assessments outputs).

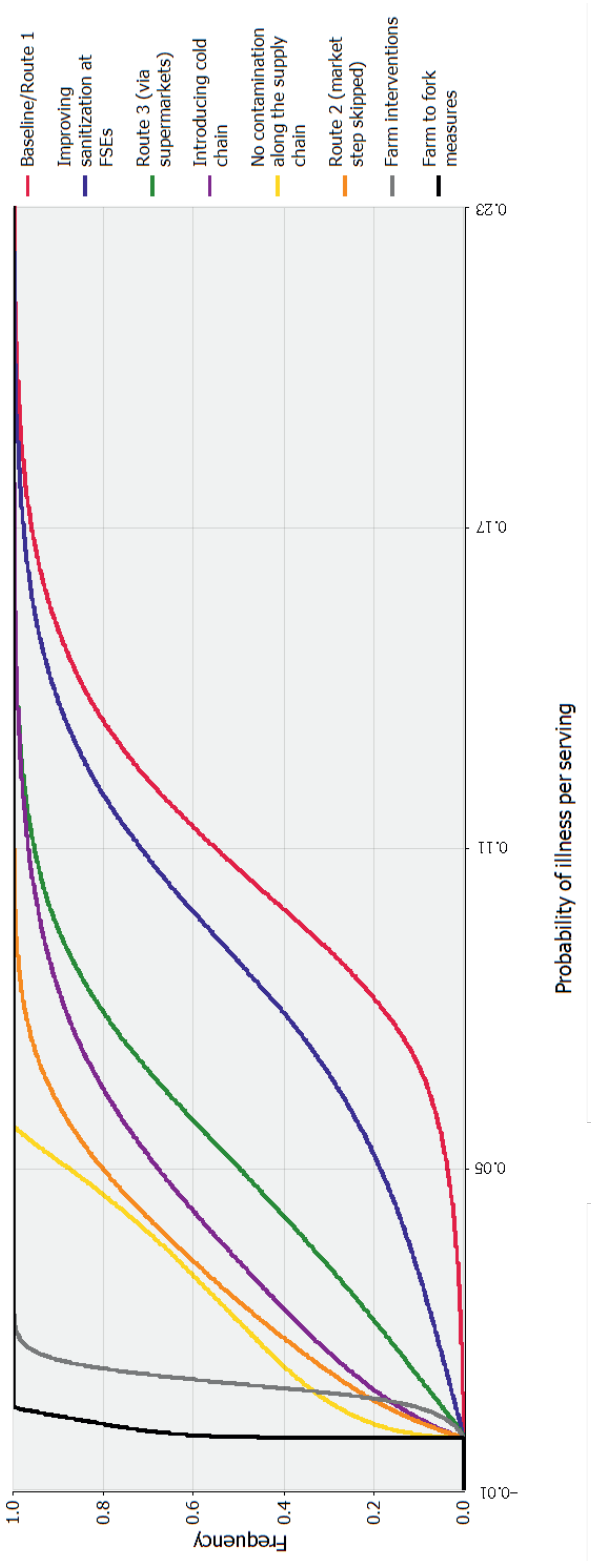


Figure 6.3: Probability of illnesses from different what if scenarios (1-7) compared to the baseline model

Table 6.4: Number of illnesses per year and probability of illness per serving after 100, 000 iterations of the baseline model and the what if scenarios

What if scenarios [b]	No. of illnesses per year (in millions)		Probability of illness per serving		Fold change [#]
	Mode	5^{th}, 95^{th} Percentile	mode	5^{th}, 95^{th} Percentile	
Baseline/ Route 1 [a]	12.1	6.96, 32.6	0.100	0.0572, 0.169	—
Improving washing and sanitization at FSEs	10.63	2.13, 27.8	0.1039	0.0151, 0.156	1.14
Route 3	6.26	0.828, 17.3	0.0535	0.0395, 0.0057	1.93
Farm interventions	1.13	0.517, 3.101	0.01029	0.00395, 0.0165	10.71
Introduction of cold chain	0.288	0.218, 15.1	0.00042	0.0015, 0.1016	42.01
Route 2 (market step skipped)	0.139	0.195, 10.87	0.000455	0.0013, 0.0728	87.1
No contamination and cross contamination along the supply chain	0.00272	0.00339, 9.4	0.0000183	0.0002, 0.0564	4,449
Farm to fork measures and interventions	0.00001108	0.0000144, 0.694	7.33×10^{-8}	0.000, 0.00494	1.1×10^{6}

[a] Baseline model or Route 1 represents a simulation of the supply chain through which about 90% of the vegetables are channelled from farms via traditional markets to food service establishments (FSEs)

—, not applicable

#, Fold change were calculated by diving the mode for the numbers of illness per year in the baseline model with the mode for the numbers of illness per year in the what if scenarios.

[b] What if scenarios arranged in descending of order of the number of illnesses per year and probability of illness per serving.

Compared to the baseline model, simulation of the 7 what if scenarios resulted to varying fold changes in the predicted microbial risk (Tab. 6.4). The what if scenario of improving washing and sanitization at FSEs resulted to a less than 2 fold change in the predicted microbial risk. While a twofold change was observed for the what if scenario of channelling all vegetables through supermarkets instead of traditional markets. Farms interventions to reduce the prevalence and levels of pathogenic *E. coli* in the base line model by 90%, introducing a cold chain and skipping the market step (Route 2) resulted to a tenfold reduction in predicted microbial risk. The what if scenario of reducing of avoiding contamination and cross contamination along the supply chain led to 1000 fold reduction in the predicted microbial risk. Lastly, combing what if scenarios 4, 5, 6 in Tab. 6.2 (farm to fork measures) resulted to the highest fold reduction of 1 million.

Food safety management options

Risk estimates should be analyzed and weighted in order to prioritize risk management measures and interventions, not only for their effectiveness in improving public health, but also for their feasibility and affordability. From the predicted risk results of this QMRA study, approaches to change the type of supply chain in Rwanda may not be given priority by risk managers except for the scenario of skipping the market step so that FSEs get vegetables straight from farms. It can be observed from this study that changing the supply chain by introducing a cold chain or refrigeration at market (supermarkets) may not address the microbial risk associated with fresh vegetables. Instead, risk managers can focus on addressing the factor leading to contamination and cross contamination from "farm to fork". In Chapter 3, it was reported that the washing and sanitization of salad vegetables at FSEs was the only step along the supply chain where significant inactivation of indicator organisms was observed. From this QMRA study and Chapter 5, it has been demonstrated this sanitization step alone cannot be enough to address microbial safety concerns. Consequently, risk assessors and risk manager should identify all the possible sources of microbial hazards along the entire supply chain and device measures and interventions to address them and spearhead risk communication strategies among stakeholders.

Aspects for risk communication along the vegetable supply chain

Farm level

Several factors such as irrigation water (65), organic manure (66), domestic and wild animal (35) and human activity have been documented among the factors that can expose fresh vegetables to microbial risk at farm level in various studies (28, 29), the WHO (30) and the FAO (27, 67, 68). In Chapter 4 of this thesis, risky farming practices were observed in addition to detection foodborne pathogens in farm vegetables and their DNA markers in agricultural water. Moreover the findings in Chapter 3 with indicator microorganisms suggest that the microbial levels at farm level greatly influence the microbial levels in ready-to-vegetables at FSEs. In the QMRA study, applying farm measures and interventions targeting reduction of the factors of spread and contamination by 90%, resulted to a tenfold decrease in the estimates of the number of illnesses per year due to pathogenic *E. coli*.

It is therefore important for stakeholders to implement guidelines that have been reported to be effective and efficient in preventing microbial hazards at farm level. Various countries such as Ireland (69) and United States (70) have developed guidelines while internationally, the WHO (30) and the FAO (27, 67, 68) have continued to provide mitigation measures in addressing the microbial hazards associated with farm vegetables. For each microbial risk exposure factor, mitigation measures have been provided. For example, compost manure can only be used after passive and or active treatment (67), agricultural water has to be protected from contamination (68), domestic and wild animals have to be restricted from accessing the vegetable farms (27, 30). Ways of controlling the possible microbial contamination from vegetable harvest equipment, containers and storage facilities have also been provided (30). Because majority of the vegetable farmers in Rwanda are small scale, competent authorities should take a lead in:

- Developing microbial safety standards and guidelines for agricultural water, soils and manure,
- Enforcing farm hygiene policies and provide training in sanitation to all farmers,
- Registering vegetable farmers for targeted extension services and identifying farming areas to enable traceability in case of aggravated field microbial contamination,
- Assessing the vulnerability of agricultural water to microbial contamination to ascertain the suitability of water for the intended use,
- Conducting continuous field research to understand the trends in pathogens by seasons or the factors leading to their variation,
- Dissemination of information about microbial safety of vegetables at farm level.

Vegetable markets

In Chapter 3, it was reported that counts of indicator microorganisms increased slightly (<1.0 log cfu/g) at market compared to the counts at farm level. In the QMRA study skipping the market resulted to 100 fold decrease in the microbial risk, indicating that this step also presents a major avenue for the contamination of fresh vegetables. To address the possible microbial safety concerns, it is important to implement GHPs aimed at hygiene of vendors and market infrastructure. Avoiding cross contamination from other food items especially those of animal origin. Measures to keep dust and flies away from open markets, wash fresh vegetables with potable water, are all essential to improve the microbial safety of fresh vegetables at market level

Food service establishments (FSEs) level

In this study, it has been reported in Chapter 3 and Chapter 5 that FSE is the only current step where washing and sanitization of salad vegetables is done in addition to preparation steps (Fig. 6A.1 in the Appendix). Whereas it was reported in Chapter 3 that there was a significant reduction in microbial counts of indicators microorganisms at FSEs, the salad preparation processes varied greatly from one FSEs to another (Chapter 5). Major issues of concern to the microbial safety of salad vegetables were, the type and conditions of sanitization application (Chapter 5); microbial quality of vegetable wash water, cross- contamination and contamination of salad vegetables from kitchen surfaces. To prevent and control microbial safety problems

that can occur during salad preparation at FSEs, existing international (71, 72) and countries guidelines (73) can be adopted.
In the QMRA study, improving washing and sanitization at FSEs by assuming that vegetables were effectively washed and sanitized (3 ± 0.5 log reduction) resulted 1 fold decrease in the number of illnesses per year. However, the 3 log reduction during washing and sanitization was only achieved by less than 2% of the visited FSEs. In chapter 5, potassium permanganate ($KMnO_4$) was reported to be the most applied sanitizer yet field and laboratory studies showed that it resulted in the lowest microbial inactivation during salad vegetable washing. Chapter 5 also presents further recommendations which can be adopted during washing and sanitization of fresh vegetables. For example, a contact time of 5 min and salad-sanitizer ratio of 1: 20 were considered optimal for kitchen based washing of the studied leafy vegetables using Sodium dichloroisocyanurate (NaDCC) and Sodium hypochlorite (NaClO) sanitizers. Therefore, competent authorities should develop a policy on the use of sanitizers and a guideline for kitchen based preparation of vegetables salads.

Competent authorities should also develop regulation for the design of kitchens in FSEs so that they can comply to the minimum standards during construction and installation. These standards and regulations will help in implementing measures to prevent cross contamination. For example there should be a sufficient kitchen size to allow space for a separate salad preparation area for the area were other food items like meat and fish are prepared.

Conclusion and future perspective

In Chapter 2 of this thesis, the estimates of the burden of illnesses from food-related pathogens are presented and these estimates indicate the potential threat associated with foodborne illnesses in Rwanda, as was reported in other parts of the Globe (13, 18, 74-76). Different food sources can be responsible for these burden estimates (21, 23, 24, 77) but this study focused on the potential contribution of microbial hazards associated with fresh vegetables. It has been reported in Chapter 3 that there was an upward trend in pathogens' indicator microorganisms for fresh vegetables from farm to FSEs. Moreover zoonotic foodborne pathogens were detected in farm vegetables at a prevalence of 15/99 (Chapter 4) in addition to the detection of pathogen DNA markers in agricultural water some of which are known to be highly virulent. Investigations of pre-harvest (Chapter 4) and postharvest (Chapter 5) sources of microbial hazards along the vegetable supply chain reveal a lapse in implementing GAPs and GHPs. Also in Chapter 3, we indicated that of the three main stages of the vegetable supply chain, farm, market and FSEs, measures and interventions to prevent microbial threats are concentrated at FSEs during washing and sanitization. In Chapter 5, however, it has was demonstrated through field and laboratory trials that the settings, conditions and efficacy of washing and sanitization at FSEs remain contentious. Following our findings in this study and the continued global reports (27) and studies (25, 26, 78) about the potential threat posed by microbial hazards in fresh vegetables, it is inevitable that every country should put in place measures and intervention towards the assurance of microbial safety of fresh vegetables.
To attain microbial safety of vegetables eaten raw, the sources and factors that introduce human pathogens into fresh vegetables in the "farm to fork" continuum have to be identified and

targeted for preventive measures and interventions. In Chapter 1, the pre-harvest and postharvest sources of human pathogens in fresh vegetables were discussed. What is characteristic with these sources of human pathogens along the vegetable supply chain is that they can all be traced back to the complex interconnectedness between plants, animals, humans, and their ecosystem. Consequently, microbial safety of fresh vegetables cannot be isolated from public and animal health. Zoonotic human pathogens crossover from domestic and wild animals to fresh vegetables via direct contact, fecal droppings, manure and biosolids, sewage, irrigation and other pathways and establish a new niche with epiphytic microorganisms. There is therefore a need for strategic approaches to manage these pathogens in terms of drivers and sources, factors of spread and persistence, and biology in different systems. One of the suggested approaches so far include the "Global One Health" (GOH) (79), proposed by a group of researchers at Wageningen university (Fig. 6.18) and the Manhattan Principles on "One World One Health" (OWOH) (80).

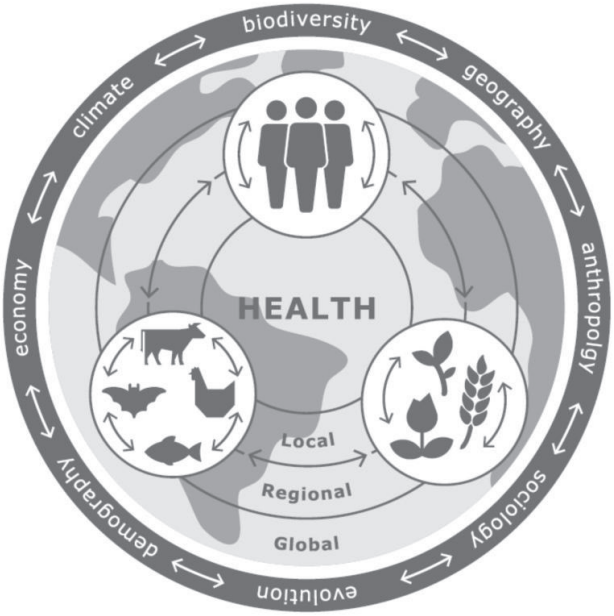


Figure 6.4: Determinants of Global One Health and their interactions [source: Fresco et al. 2015 (79)].

The GOH and the OWOH approaches both propose the need for combined multidisciplinary efforts to improve the health of humans, animals and plants within sustainable ecosystems at global level by using an integrated system to come to transnational and global policy, research and practices (79, 80). As shown in Figure 6.4, the determinants of GOH interconnect; the animal, plant and human interfaces are intra and extra linked and offsets at any level/factor, can trigger a cascade of effects that can lead to highly undesirable health situations at local, regional, continental and global scale (79). For example, due to excessive use of antibiotics in livestock, antibiotic resistant human zoonotic pathogens have been introduced in the environment some of which can now be traced in fresh vegetables. The encroachment on forest reserves have

increased the wandering of wildlife into farming areas and as a consequence zoonotic pathogens contaminate crops and irrigation water. It is global because the world is now a small village, trade in fresh vegetables is international, travels have increased and tourism is a major economic activity, vegetables salads are now very popular part of the menu in hotels and in households especially in family celebrations. One meal with vegetable salads if contaminated has high probability of causing illnesses to many people at once.

To achieve Global One Health (GOH) and move towards microbial safety, there is need for political awareness and international agreements to bring together governments, the private sector, researchers and educationists at both at national and international level to develop an integrated system. Key areas for these actors include trade, health, agriculture, environment, water management and rural development. This integrated system should aim at early warning, monitoring, risk management and prevention of diseases at all stages of the vegetable supply chain and the entire ecosystem. Key activities include:

- understanding the role of biodiversity in maintaining healthy environments and the functioning of ecosystems;
- determining the impact of land and water use in aiding the shifts in patterns of pathogens;
- conducting wild health surveillance and management;
- linking public health management to environmental conservation;
- developing new tools for diagnosis, estimating the burden- and prevention of foodborne pathogens;
- solving institutional complexities and variability to identify synergies and areas of overlap and stimulate institutional collaboration.

Currently in Rwanda, there is a need to establish or mandate an existing institution to co-ordinate all public and private stake holders in matters relating to this integrated food chain system. Education and awareness raising of stakeholders towards influencing social-economic/cultural practices *i.e.* there are many challenges that will require a major shift in what is considered normal to the culture in Rwanda. The culture of animal moving freely in farms and sharing water with irrigation sources and wash water, hygiene in farms, the culture of washing vegetables with surface and tap water yet people know that this water is not safe for drinking, adjusting land tenure systems and settlements.

In conclusion, the study involved detecting foodborne pathogens and an investigation of the handling practises and risk exposure factors. Indicator microorganisms were detected from "farm to fork" and their counts can be used as signal for possible contamination at a given level of the supply chain for urgent interventions. The food safety information generated from any food chain should be relevant to food safety policy makers in developing evidence based policies. In this study, a methodology to estimate the burden of foodborne illness was proposed in Chapter 2 besides the QMRA study in this Chapter. This study focused on bacterial foodborne pathogens commonly implicated in outbreaks attributed to fresh vegetables. Future studies should focus on parasites and viruses and how handling practices affect their prevalence and levels from farm to fork. Further work remains to be done regarding bacterial pathogens such as the current global concerns of the raising trends in antibiotic resistance. Over all the

approach used in this study can be adopted as a research framework for the integrated food chain system to detect control and prevent foodborne illnesses along the fresh vegetables supply chain and other food supply chains.

Appendix

Further details on Hazard identification: Pathogenic *E. coli*

In public health, pathogenic *E. coli* are among the major agents contributing to the global burden of disease. In 2010, the WHO estimates show that pathogenic *E. coli* (ETEC, EPEC and STEC) resulted to about 110 million illnesses, 60,000 deaths and 5 million DALYs (13). Epidemiologically, ETEC has been highlighted among and the main causes of traveller's diarrhea and diarrhea in children under 5 years of age in developing countries (81, 82) and also listed among foodborne pathogens associated with raw vegetables (83) and other unsafely prepared foods. In a review of leafy vegetables associated outbreaks between 1973 and 2012, the Centres for Disease Control and Prevention (CDC) reported that in the United States, EHEC was the leading cause of leafy vegetable outbreaks with a confirmed aetiology (84). In 2011, there was a large outbreak (total of 3816 cases, including 54 deaths) in Germany characterized by haemolytic-uremic syndrome (HUS) and bloody diarrhea caused by EHEC O104: H4 with the food vehicle being sprouts (85). A number of recent multistate outbreaks in the USA associated with fresh vegetables includes the outbreak linked to organic spinach and spring mix blend in which 33 person were infected and two persons suffered from HUS (86) and another outbreak of *E. coli* O157:H7 infections linked to alfalfa sprouts reported by the CDC in March 2016 during which eleven people were infected and two hospitalized (87). In 2007, there was a shiga toxin-producing *E. coli* (STEC) outbreak in The Netherlands and Iceland linked to contaminated lettuce, shredded and pre-packed in a Dutch food processing plant (88), and a lettuce outbreak in Sweden in 2005 (89). Furthermore, in July 2016, the WHO was notified about the *E. coli* O157: H7 outbreaks related to mixed leafy salads in United Kingdom where 105 patients were confirmed, four patients were hospitalized and two deaths (90).

Escherichia coli are Gram negative, facultative anaerobic bacteria of the family *Enterobacteriaceae*, commonly associated with the intestinal micro flora of humans and other warm blooded animals. Most *E. coli* are harmless, however in immunosuppressed individuals or when gastrointestinal barriers are disrupted, even non-pathogenic strains can cause infection (59, 91). The infection due to pathogenic *E. coli* are generally classified into three syndromes; urinary tract infection, sepsis/meningitis, and enteric/diarrheal disease (59). Pathogenic *E. coli* strains can be identified in several ways. Most commonly, *E. coli* are serotyped on the basis of their O (somatic), H (flagellar), and K (capsular) surface antigen profiles, for example *E. coli* O157: H7 (59) or according to their virulence properties, mechanisms of pathogenicity, clinical syndrome and or antigenic characteristics for which the six pathotypes of *E. coli* have been categorized. In these six pathotypes, *E. coli* are further classified into enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (59). The strategy of infection for these *E. coli* pathotypes is similar to that of other mucosal pathogens *i.e.*. colonization of a mucosal site, evasion of host defences, multiplication, and host damage (59). Specifically, diarrhea as one of the major illnesses due to pathogenic *E. coli*, is caused through enterotoxin production (ETEC and EAEC), invasion (EIEC), and intimate adherence with membrane signalling (EPEC and EHEC), although the pathogen-host interaction is quintessential of each pathotype.

Table 6A.1: Possible public health outcome(s) of the pathogens detected in farm vegetables and agricultural water this study

Pathogen	Study sample	Associated illnesses	References
Salmonella spp.	Farm vegetables	Two types of illness, depending on the serotype: Non typhoidal *Salmonellosis*. Acute: nausea, vomiting, abdominal cramps, diarrhea, fever, headache. Long-term sequelae: reactive arthritis, septicaemia, bacteremia. Typhoidal illness. High fever, diarrhea or constipation, aches, headache, and lethargy.	(13, 36)
Shiga toxin-producing *E. coli* (STEC)	Farm vegetables	Acute: diarrheal disease. Long-term sequelae: hemolytic uremic syndrome, end-stage renal disease	(13, 92)
Enteroinvasive *E. coli* (EIEC)	Agricultural water	Acute: mild dysentery; abdominal cramps, diarrhea, vomiting, fever, chills	(36)
Enteroaggregative *E. coli* (EAEC)	Agricultural water	Acute and persistent diarrhea, inducing chronic intestine inflammation in the absence of dysentery and leading to stunting and malnutrition in children.	(93)
Enterotoxigenic *E. coli* (ETEC)	Agricultural water, farm vegetables	Sudden onset of watery diarrhea	(36)
Listeria monocytogenes	Farm vegetables	Listeriosis Major risks to the young, old, pregnant women and individuals with weakened immune systems. Complications include non-invasive gastrointestinal illness, neonatal death, Infant septicemia, meningitis, CNS infection, neurological sequelae, in pregnant women ; flu-like illness, spontaneous abortions and stillbirths.	(13, 94-96) (36)

Table 6A.1. Continue….

Pathogen	Study sample	Associated illnesses	References
Thermo-tolerant *Campylobacter* spp.	Agricultural water , farm vegetables,	Campylobacteriosis. Acute: fever, abdominal cramping, and diarrhea, with or without blood in stools. Long term sequelae: Guillain Barré Syndrome and or Miller Fischer Syndrome.	(13, 97, 98)
Vibrio cholera	Agricultural water	Cholera	(99)
Francisella tularensis	Agricultural water	Tularemia: suppuration and skin eruptions, less commonly pneumonia and meningitis	(100)
Yersinia pestis	Agricultural water	Bubonic, septicemic plague and pneumonic plague	(101)
Cyclospora.	Agricultural water	Acute: profuse watery diarrhea, anorexia, nausea, flatulence, fatigue, abdominal cramping, low-grade fever and weight loss. Long term sequelae: Guillain-Barre´ syndrome (GBS) and Reiter syndrome.	(36, 102)

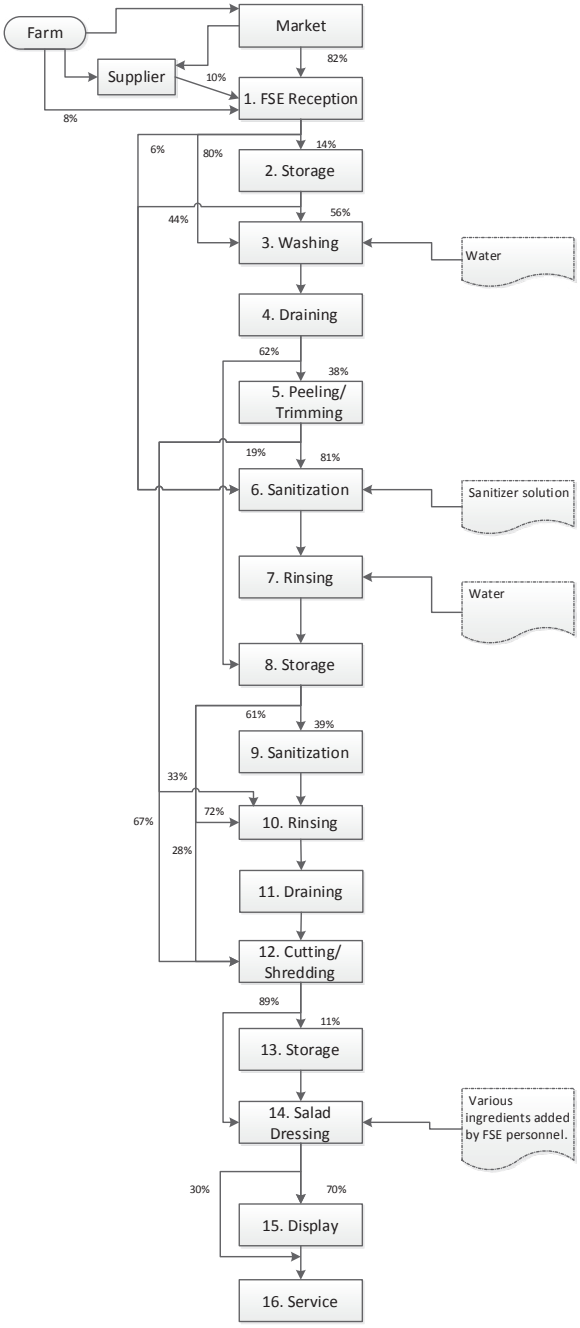


Figure 6A.1: Flow diagram for salad preparation in Food Service Establishments (FSEs). Percentages indicate the proportion of FSEs which practise a specific process- step or route.

Stakeholders' roles to address microbial safety issues along the fresh vegetable the supply chain

In this section, some important activities for a typical integrated food chain system are suggested with focus on the vegetable supply chain.

At farm level

- Registration of vegetable farmers for targeted extension services, identifying farming areas to enable traceability in case of aggravated field microbial contamination.
- Assessment of proposed vegetables growing area for possible microbial hazards by looking at the topography, climate and geology.
- Assessing the vulnerability of agricultural water to microbial contamination to ascertain the suitability of water for the intended use.
- Developing microbial standards and guidelines for agricultural water, soils and manure
- Conducting corrective action activities to address soil and water microbial contamination
- Ensuring that on-farm washing and rinsing of vegetables is done hygienically.
- Ensuring farmers health and growing field sanitation
- Reinforcing GAPs at farm level
- Training of farmers on land preparation, manure use, good irrigation practices, and handling of domestic and wild animals on farms
- Enforcing and initiating measures to restrict access of livestock to the source of crop irrigation water.
- Monitoring vegetable farms for the presence or signs of wild or domestic animals entering the growing area.
- Assessing the microbial quality of water used for irrigation.
- Proper treatment and storage of manure prior to use in the farms to lower the expected level of pathogens
- Conducting microbial analysis of composted manure/treated biosolids.
- Conducting continuous field research to understand the trends in pathogens by seasons or the factors leading to their variation.
- Dissemination of information about microbial safety of vegetables at farm level

At market level

- Infrastructural development including market structures, sanitation facilities, microbiologically safe water and measure to restrict cross contamination.
- Registering of vegetable vendors to enable food safety training programmes and traceability along the supply chain.
- Training of market vendors about food safety.
- Developing and enforcing hygiene standards and guidelines.
- Research and disseminating information about food safety at market.

At food service level

- Infrastructural development including hygienic design of FSEs, sanitation facilities, microbiologically safe water and measure to restrict cross contamination in kitchens.
- Registering of FSEs to enable food safety training programmes and traceability along the supply chain.
- Training of food handlers about food safety.
- Periodic medical screening of food handlers
- Developing and enforcing hygiene standards and guidelines.
- Research and disseminating information about microbial food safety at FSEs.

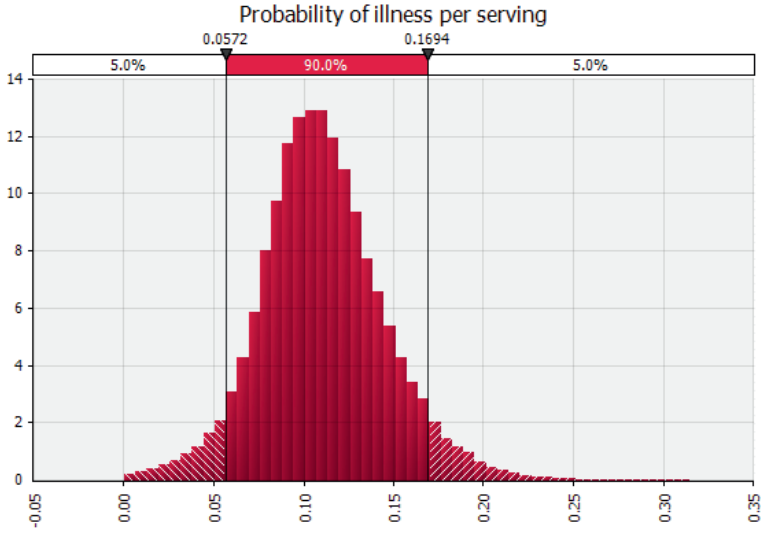
At consumer level

- Conducting consumer studies to understand food consumption trends and behavior.

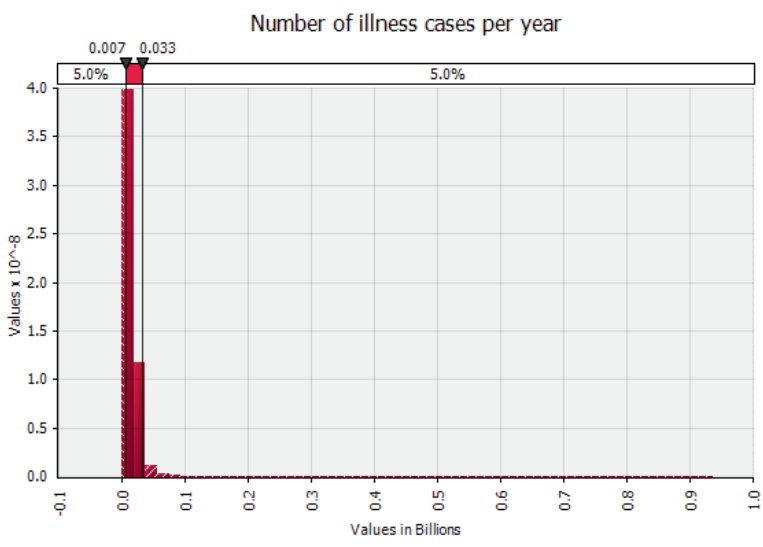
- Training the general public about food safety and safe handling of food.
- Educating general population to provide an essential function in the recognizing and reporting of foodborne illness to public health authorities.
- Surveillance of foodborne illnesses in the general population.
- Estimating the burden of foodborne illness and their contribution to overall burden of disease.

Other risk assessments outputs and graphs

1. Route 1

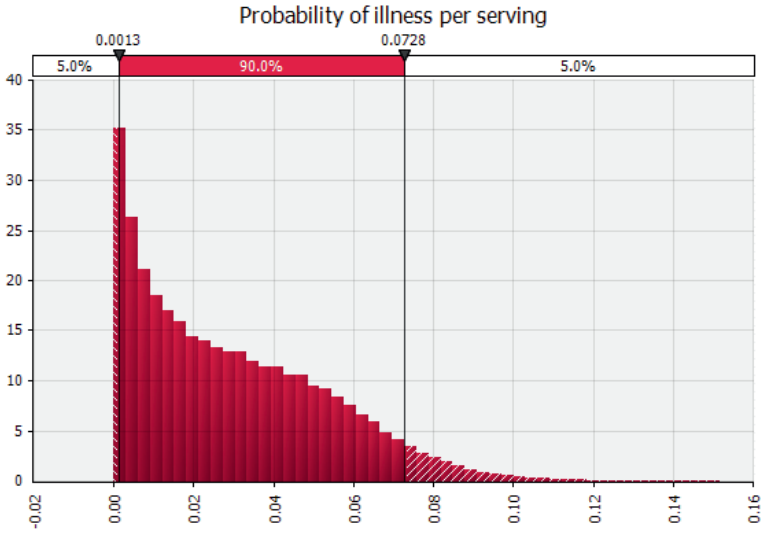


	Probability of illness per serving
Cell	Route 1 most commo..
Minimum	0.000233
Maximum	0.31497
Mean	0.11063
90% CI	± 0.000178
Mode	0.10000
Median	0.10884
Std Dev	0.03426
Skewness	0.2561
Kurtosis	3.7415
Values	100000
Errors	0
Filtered	0
Left X	0.0572
Left P	5.0%
Right X	0.1694
Right P	95.0%
Dif. X	0.11220
Dif. P	90.0%

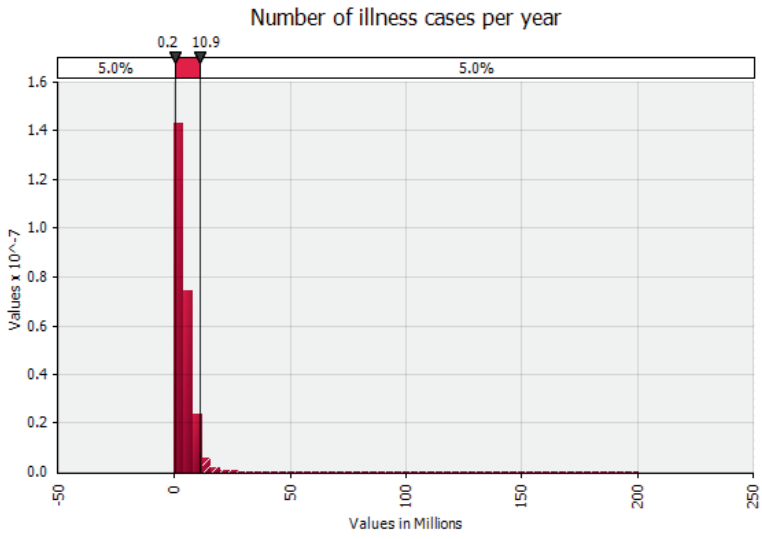


	Number of illness cases per year
Cell	Route 1 most commo..
Minimum	93,694.20
Maximum	937,360,737.19
Mean	16,706,662.08
90% CI	± 79,979.68
Mode	12,050,095.95
Median	14,136,935.56
Std Dev	15,376,176.52
Skewness	18.8517
Kurtosis	703.4876
Values	100000
Errors	0
Filtered	0
Left X	6,959,424.80
Left P	5.0%
Right X	32,639,813.90
Right P	95.0%
Dif. X	25,680,389.10
Dif. P	90.0%

2. Route 2

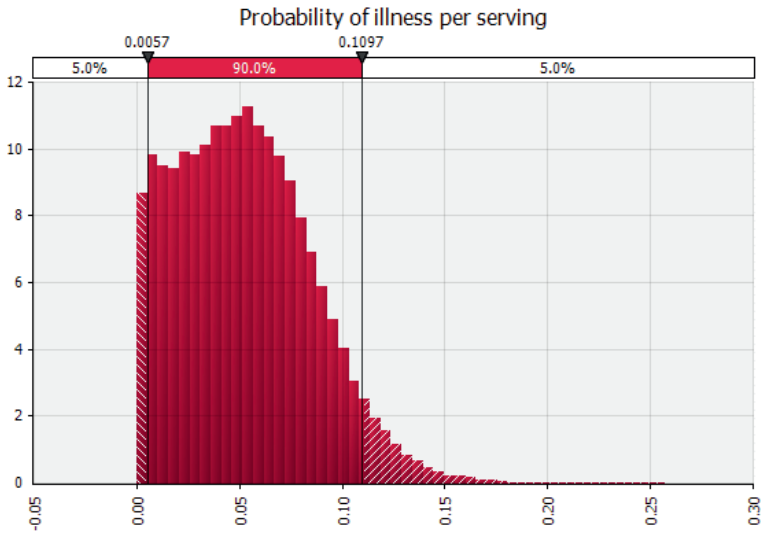


	Probability of illness per serving
Cell	Route 2 Farm direct t..
Minimum	7.710E-006
Maximum	0.15166
Mean	0.02942
90% CI	± 0.000122
Mode	0.000455
Median	0.02481
Std Dev	0.02338
Skewness	0.7521
Kurtosis	2.9183
Values	100000
Errors	0
Filtered	0
Left X	0.0013
Left P	5.0%
Right X	0.0728
Right P	95.0%
Dif. X	0.07145
Dif. P	90.0%

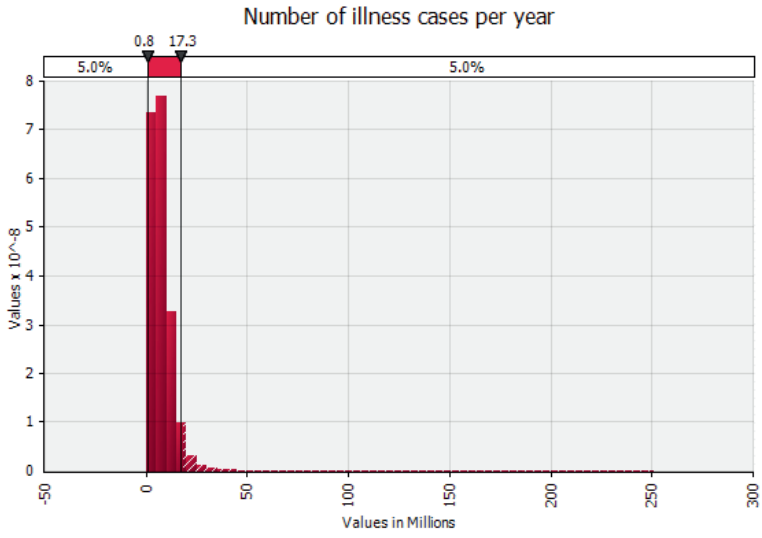


	Number of illness cases per year
Cell	Route 2 Farm direct t..
Minimum	1,184.45
Maximum	200,526,301.42
Mean	4,165,629.14
90% CI	± 21,114.49
Mode	139,044.90
Median	3,299,284.89
Std Dev	4,059,283.39
Skewness	5.1354
Kurtosis	121.5701
Values	100000
Errors	0
Filtered	0
Left X	194,752.99
Left P	5.0%
Right X	10,873,042.78
Right P	95.0%
Dif. X	10,678,289.79
Dif. P	90.0%

3. Route 3

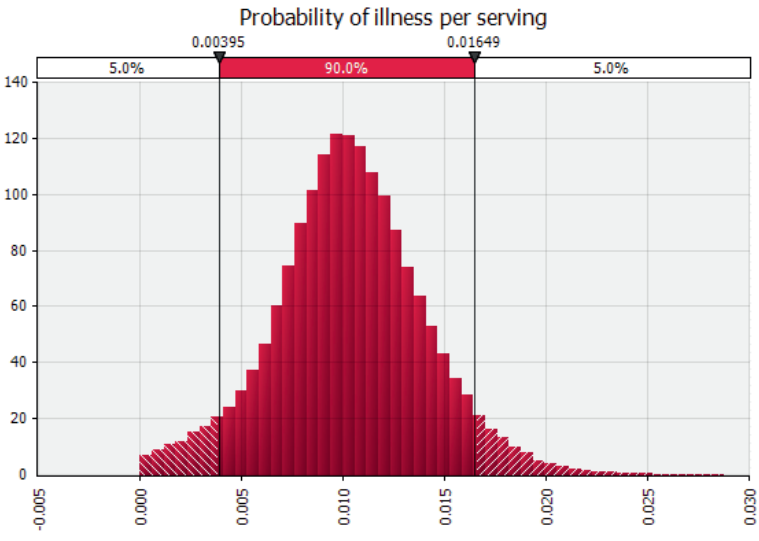


	Probability of illness per serving
Cell	Route 3!E109
Minimum	3.363E-005
Maximum	0.25822
Mean	0.05259
90% CI	± 0.000169
Mode	0.05349
Median	0.05042
Std Dev	0.03251
Skewness	0.5265
Kurtosis	3.0064
Values	100000
Errors	0
Filtered	0
Left X	0.0057
Left P	5.0%
Right X	0.1097
Right P	95.0%
Dif. X	0.10394
Dif. P	90.0%

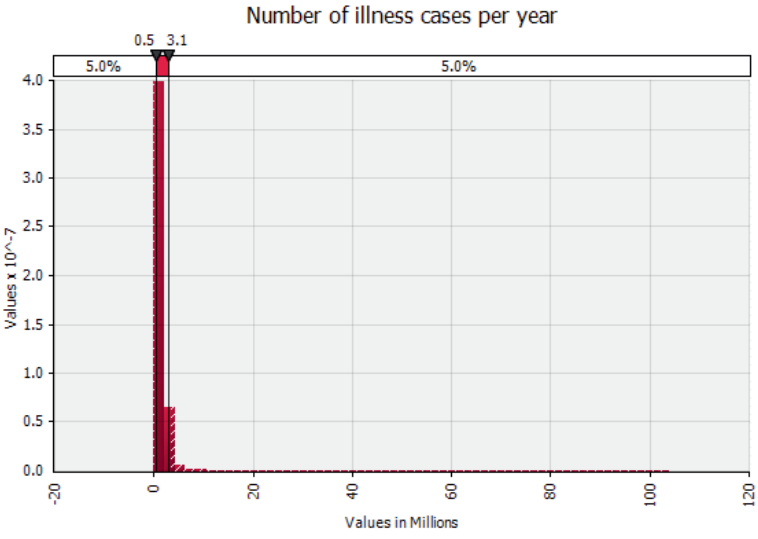


	Number of illness cases per year
Cell	Route 3!E115
Minimum	6,708.26
Maximum	251,306,797.36
Mean	7,529,762.94
90% CI	± 32,921.82
Mode	6,263,563.63
Median	6,556,583.60
Std Dev	6,329,253.54
Skewness	6.1536
Kurtosis	131.4340
Values	100000
Errors	0
Filtered	0
Left X	828,430.44
Left P	5.0%
Right X	17,258,582.81
Right P	95.0%
Dif. X	16,430,152.37
Dif. P	90.0%

4. Farm interventions (reduction of prevalence of pathogenic *E. coli* by 90%)

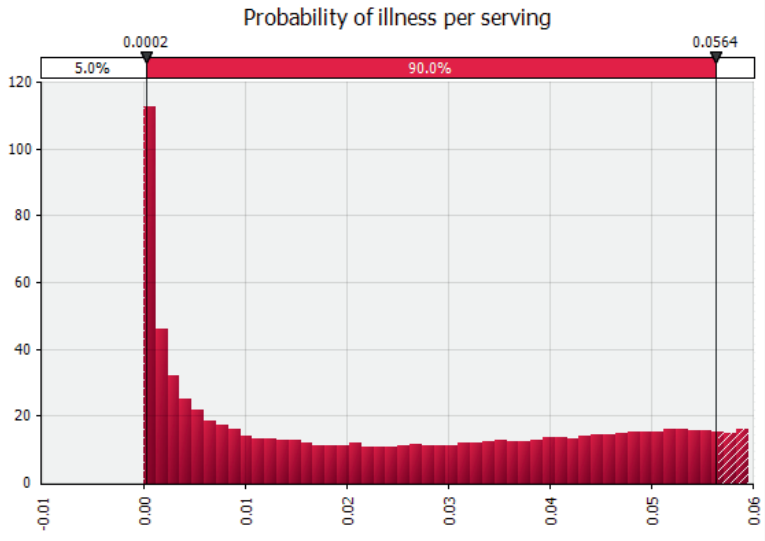


	Probability of illness per serving
Cell	Farm interventions!E..
Minimum	1.417E-005
Maximum	0.028765
Mean	0.010338
90% CI	± 2.160E-005
Mode	0.010331
Median	0.010293
Std Dev	0.003718
Skewness	0.0899
Kurtosis	3.4877
Values	80157
Errors	19843
Filtered	0
Left X	0.00395
Left P	5.0%
Right X	0.01649
Right P	95.0%
Dif. X	0.012541
Dif. P	90.0%

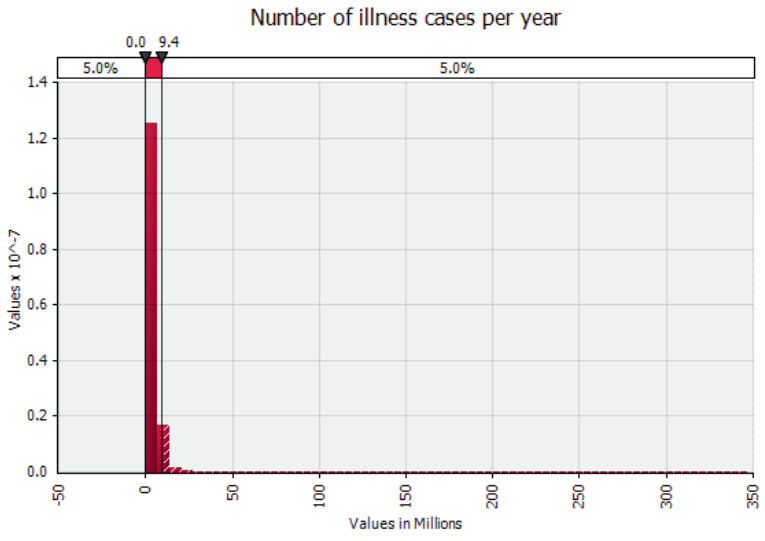


	Number of illness cases per year
Cell	Farm interventions!E..
Minimum	2,006.46
Maximum	103,855,232.86
Mean	1,554,796.78
90% CI	± 8,395.37
Mode	1,132,175.61
Median	1,329,212.26
Std Dev	1,445,034.84
Skewness	18.6326
Kurtosis	747.7264
Values	80157
Errors	19843
Filtered	0
Left X	517,244.25
Left P	5.0%
Right X	3,100,988.33
Right P	95.0%
Dif. X	2,583,744.08
Dif. P	90.0%

5. Assuming no contamination and cross contamination along the supply chain

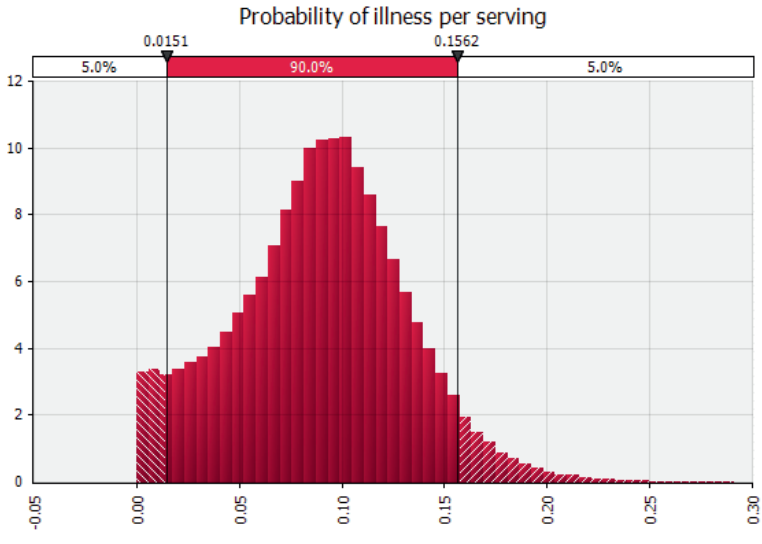


	Probability of illness per serving
Cell	No contamin at all le..
Minimum	1.117E-007
Maximum	0.059583
Mean	0.024612
90% CI	± 0.000103
Mode	1.827E-005
Median	0.022349
Std Dev	0.019832
Skewness	0.2369
Kurtosis	1.5923
Values	100000
Errors	0
Filtered	0
Left X	0.0002
Left P	5.0%
Right X	0.0564
Right P	95.0%
Dif. X	0.056114
Dif. P	90.0%

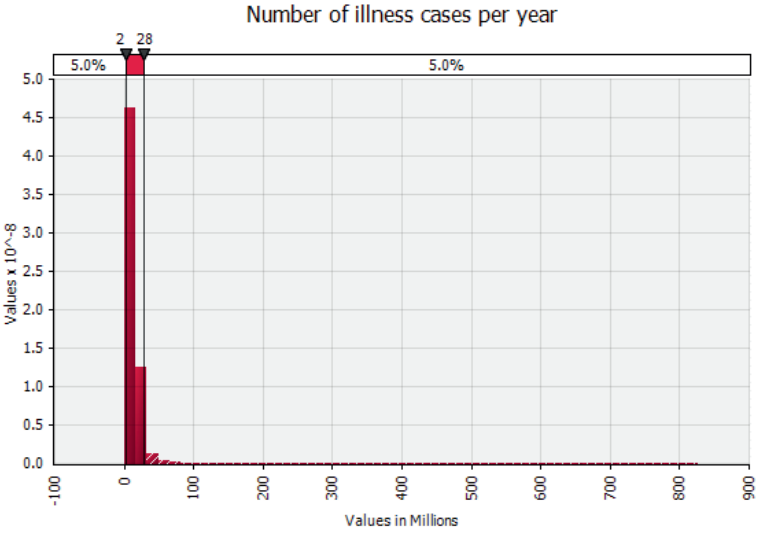


	Number of illness cases per year
Cell	No contamin at all le..
Minimum	49.03
Maximum	346,749,144.34
Mean	3,616,466.19
90% CI	± 23,688.70
Mode	2,719.19
Median	2,928,958.39
Std Dev	4,554,177.48
Skewness	16.8606
Kurtosis	830.1690
Values	100000
Errors	0
Filtered	0
Left X	33,937.61
Left P	5.0%
Right X	9,398,825.16
Right P	95.0%
Dif. X	9,364,887.55
Dif. P	90.0%

6. Maximizing Sanitization at FSEs

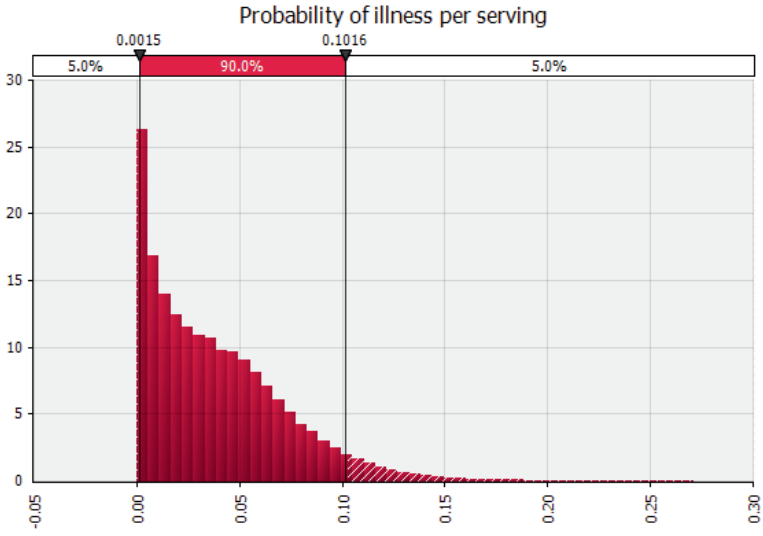


	Probability of illness per serving
Cell	Improving sanitizatio..
Minimum	4.273E-005
Maximum	0.29159
Mean	0.08907
90% CI	± 0.000217
Mode	0.10394
Median	0.09081
Std Dev	0.04180
Skewness	0.0454
Kurtosis	2.8793
Values	100000
Errors	0
Filtered	0
Left X	0.0151
Left P	5.0%
Right X	0.1562
Right P	95.0%
Dif. X	0.14112
Dif. P	90.0%

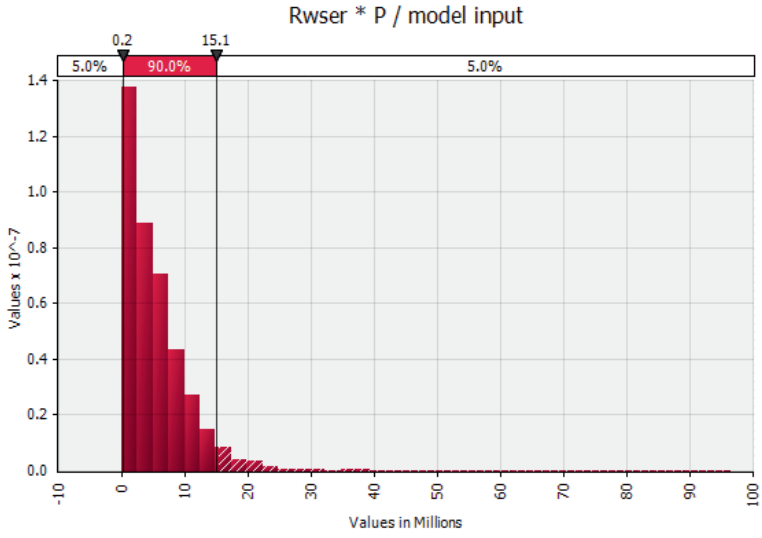


	Number of illness cases per year
Cell	Improving sanitizatio..
Minimum	6,688.61
Maximum	826,600,678.80
Mean	13,260,914.83
90% CI	± 66,786.21
Mode	10,630,852.39
Median	11,579,754.25
Std Dev	12,839,719.10
Skewness	16.0634
Kurtosis	577.5804
Values	100000
Errors	0
Filtered	0
Left X	2,127,656.80
Left P	5.0%
Right X	27,790,117.59
Right P	95.0%
Dif. X	25,662,460.79
Dif. P	90.0%

7. Introducing cold chain

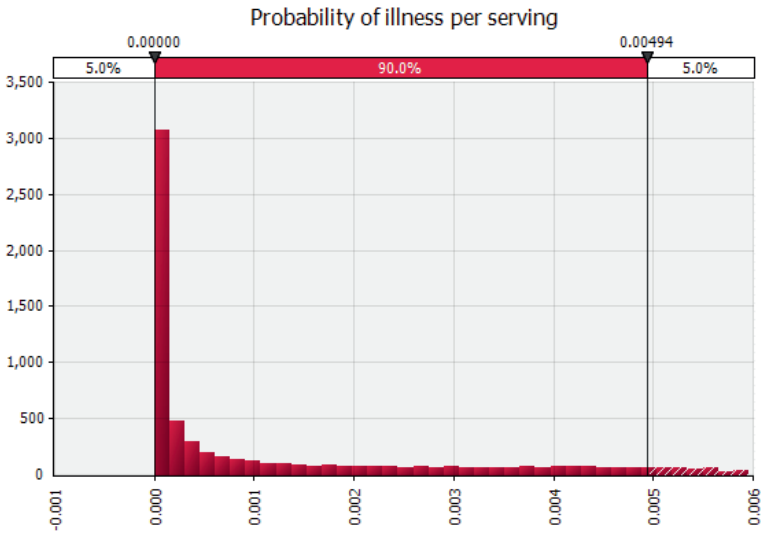


	Probability of illness per serving
Cell	Introducing cold cha..
Minimum	7.354E-008
Maximum	0.27127
Mean	0.03904
90% CI	± 0.000179
Mode	0.000420
Median	0.03235
Std Dev	0.03272
Skewness	1.0832
Kurtosis	4.2730
Values	90576
Errors	9424
Filtered	0
Left X	0.0015
Left P	5.0%
Right X	0.1016
Right P	95.0%
Dif. X	0.10008
Dif. P	90.0%

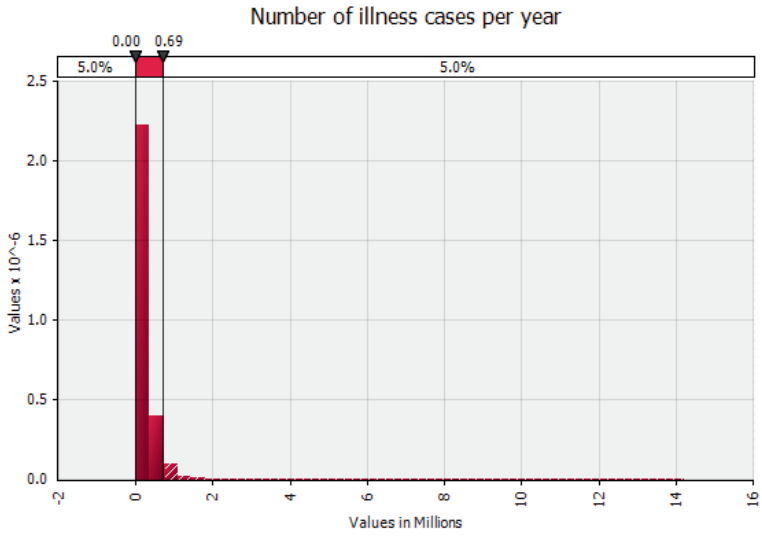


	Rwser * P / model input
Cell	Introducing cold cha..
Minimum	5.13
Maximum	96,694,756.21
Mean	5,555,122.36
90% CI	± 106,475.44
Mode	288,249.80
Median	4,189,461.72
Std Dev	5,850,020.30
Skewness	4.0240
Kurtosis	39.5025
Values	8169
Errors	831
Filtered	0
Left X	217,858.65
Left P	5.0%
Right X	15,097,368.88
Right P	95.0%
Dif. X	14,879,510.23
Dif. P	90.0%

8. Combing farm to fork measures



	Probability of illness per serving
Cell	Farm to Fork measur..
Minimum	3.742E-009
Maximum	0.0059573
Mean	0.0011641
90% CI	± 2.888E-005
Mode	7.331E-008
Median	0.0002046
Std Dev	0.0016655
Skewness	1.3708
Kurtosis	3.5304
Values	9000
Errors	0
Filtered	0
Left X	0.00000
Left P	5.0%
Right X	0.00494
Right P	95.0%
Dif. X	0.0049423
Dif. P	90.0%



	Number of illness cases per year
Cell	Farm to Fork measur..
Minimum	0.409
Maximum	14,213,813.96
Mean	170,683.29
90% CI	± 5,628.80
Mode	11.08
Median	28,301.57
Std Dev	324,612.52
Skewness	12.2390
Kurtosis	425.0486
Values	9000
Errors	0
Filtered	0
Left X	143.54
Left P	5.0%
Right X	694,913.93
Right P	95.0%
Dif. X	694,770.40
Dif. P	90.0%

References

1. **Broglia A, Kapel C.** 2011. Changing dietary habits in a changing world: Emerging drivers for the transmission of foodborne parasitic zoonoses. *Veterinary Parasitology* 182:2-13.

2. **Satia JA, Kristal AR, Patterson RE, Neuhouser ML, Trudeau E**. 2002. Psychosocial factors and dietary habits associated with vegetable consumption. *Nutrition* 18:247-254.

3. **Jacxsens L, Ibañez IC, Gómez-López VM, Fernandes JA, Allende A, Uyttendaele M, Huybrechts I**. 2015. Belgian and Spanish consumption data and consumer handling practices for fresh fruits and vegetables useful for further microbiological and chemical exposure assessment. *Journal of Food Protection* 78:784-795.

4. **Miller V, Mente A, Dehghan M, Rangarajan S, Zhang X, Swaminathan S, Dagenais G, Gupta R, Mohan V, Lear S**. 2017. Fruit, vegetable, and legume intake, and cardiovascular disease and deaths in 18 countries (PURE): a prospective cohort study. *The Lancet 390*: 2037-2049.

5. **Lowry PW, Levine R, Stroup DF, Gunn RA, Wilder MH, Konigsberg Jr C**. 1989. Hepatitis A outbreak on a floating restaurant in Florida, 1986. *American Journal of Epidemiology* 129:155-164.

6. **Jackson LA, Keene WE, McAnulty JM, Alexander ER, Diermayer M, Davis MA, Hedberg K, Boase J, Barrett TJ, Samadpour M, Fleming DW**. 2000. Where's the beef? The role of cross-contamination in 4 chain restaurant-associated outbreaks of *Escherichia coli* O157:H7 in the pacific northwest. *Archives of Internal Medicine* 160:2380-2385.

7. **Kimura AC, Mead P, Walsh B, Alfano E, Gray SK, Durso L, Humphrey C, Monroe SS, Visvesvera G, Puhr N, Shieh WJ, Eberhard M, Hoekstra RM, Mintz ED.** 2006. A large outbreak of Brainerd diarrhea associated with a restaurant in the Red River Valley, Texas. *Clinical Infectious Diseases* 43:55-61.

8. **Bradley KK, Williams JM, Burnsed LJ, Lytle MB, McDermott MD, Mody RK, Bhattarai A, Mallonee S, Piercefield EW, McDonald-Hamm CK, Smithee LK.** 2012. Epidemiology of a large restaurant-associated outbreak of Shiga toxin-producing *Escherichia coli* O111:NM. *Epidemiology & Infection* 140:1644-1654.

9. **Raj P, Tay J, Ang LW, Tien WS, Thu M, Lee P, Pang QY, Tang YL, Lee KY, Maurer-Stroh S, Gunalan V, Cutter J, Goh KT.** 2016. A large common-source outbreak of norovirus gastroenteritis in a hotel in Singapore, 2012. *Epidemiology & Infection* 145(3), 535-544.

10. **Angelo KM, Nisler AL, Hall AJ, Brown LG, Gould LH.** 2016. Epidemiology of restaurant-associated foodborne disease outbreaks, United States, 1998–2013. *Epidemiology & Infection* 145(3), 523-534.

11. **Lasky T.** 2002. Foodborne illness - Old problem, new relevance. *Epidemiology* 13:593-598.

12. **Kasowski EJ, Gackstetter GD, Sharp TW.** 2002. Foodborne illness: new developments concerning an old problem. *Current Gastroenterology Reports* 4:308-318.

13. **Havelaar AH, Kirk MD, Torgerson PR, Gibb HJ, Hald T, Lake RJ et al.** 2015. World Health Organization global estimates and regional comparisons of the burden of foodborne disease in 2010. *PLoS Medicine* 12:e1001923.

14. **Wallace RB, Oria M, Council NR.** 2010. Adopting a risk-based decision-making approach to food safety. *National Research Council (US) Committee on the Review of Food and Drug Administration's Role in Ensuring Safe Food*. National Academies Press (US). Washington (DC)

15. **Adak G, Long S, O'Brien S.** 2002. Trends in indigenous foodborne disease and deaths, England and Wales: 1992 to 2000. *Gut* 51:832-841.

16. **Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM**. 2011. Foodborne illness acquired in the United States--major pathogens. *Emerging Infectious Diseases* 17:7-15.

17. **Havelaar AH, Haagsma JA, Mangen MJJ, Kemmeren JM, Verhoef LPB, Vijgen SMC, Wilson M, Friesema IHM, Kortbeek LM, van Duynhoven YTHP, van Pelt W.** 2012. Disease burden of foodborne pathogens in the Netherlands, 2009. *International Journal of Food Microbiology* 156:231-238.

18. **Gkogka E, Reij MW, Havelaar AH, Zwietering MH, Gorris LGM.** 2011. Risk-based estimate of effect of foodborne diseases on public health, Greece. *Emerging Infectious Diseases* 17:1581-1590.

19. **Thomas MK, Murray R, Flockhart L, Pintar K, Pollari F, Fazil A, Nesbitt A, Marshall B.** 2013. Estimates of the burden of foodborne illness in Canada for 30 specified pathogens and unspecified agents, Circa 2006. *Foodborne Pathogens & Disease* 10:639-648.

20. **Lake RJ, Cressey PJ, Campbell DM, Oakley E.** 2010. Risk ranking for foodborne microbial hazards in New Zealand: Burden of disease estimates. *Risk Analysis* 30:743-752.

21. **Kamana O, Ceuppens S, Jacxsens L, Kimonyo A, Uyttendaele M.** 2014. Microbiological quality and safety assessment of the Rwandan milk and dairy chain. *Journal of Food Protection* 77:299-307.

22. **Niyonzima E, Ongol MP, Brostaux Y, Korsak Koulagenko N, Daube G, Kimonyo A, Sindic M.** 2017. Consumption patterns, bacteriological quality and risk factors for *Salmonella* contamination in meat-based meals consumed outside the home in Kigali, Rwanda. *Food Control* 73, 546-554.

23. **Kirby MA, Nagel CL, Rosa G, Iyakaremye L, Zambrano LD, Clasen TF**. 2016. Faecal contamination of household drinking water in Rwanda: A national cross-sectional study. *Science of the Total Environment* 571:426-434.

24. **Uwimpuhwe M, Reddy P, Barratt G, Bux F**. 2014. The impact of hygiene and localised treatment on the quality of drinking water in Masaka, Rwanda. *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances & Environmental Engineering* 49:434-440.

25. **Painter JA, Hoekstra RM, Ayers T, Tauxe RV, Braden CR, Angulo FJ, Griffin PM.** 2013. Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998-2008. *Emerging Infectious Diseases* 19:407-415.

26. **Callejon RM, Rodriguez-Naranjo MI, Ubeda C, Hornedo-Ortega R, Garcia-Parrilla MC, Troncoso AM**. 2015. Reported foodborne outbreaks due to fresh produce in the United States and European Union: trends and causes. *Foodborne Pathogens & Disease* 12:32-38.

27. **The World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the united nations.** 2008. Microbiological hazards in fresh leafy vegetables and herbs. Meeting report. Available at : http://www.fao.org/3/a-i0452e.pdf , Accessed 15th November 2015.

28. **Beuchat LR, Ryu JH.** 1997. Produce handling and processing practices. *Emerging Infectious Diseases* 3:459-465.

29. **Gil MI, Selma MV, Suslow T, Jacxsens L, Uyttendaele M, Allende A.** 2015. Pre- and postharvest preventive measures and intervention strategies to control microbial food safety hazards of fresh leafy vegetables. *Critical Reviews in Food Science & Nutrition* 55:453-468.

30. **The World Health Organisation (WHO) of the United Nations.** 2012. Five keys to growing safer fruits and vegetables. Promoting health by decreasing microbial contamination. Available at: http://apps.who.int//iris/bitstream/10665/75196/1/9789241504003_eng.pdf?ua=1. Accessed 15th November 2014.

31. **Gorski L, Palumbo JD, Nguyen KD.** 2004. Strain-specific differences in the attachment of *Listeria monocytogenes* to alfalfa sprouts. *Journal of Food Protection* 67:2488-2495.

32. **Solomon EB, Sharma M.** 2009. Chapter 2 - Microbial attachment and limitations of decontamination methodologies, p. 21-45. In Sapers GM, Solomon EB, Matthews KR (ed.), *The Produce Contamination Problem*. Academic Press, San Diego.

33. **Warriner K, Ibrahim F, Dickinson M, Wright C, Waites WM.** 2003. Internalization of human pathogens within growing salad vegetables. *Biotechnology & Genetic Engineering Reviews* 20:117-136.

34. **Deering AJ, Mauer LJ, Pruitt RE.** 2012. Internalization of *E. coli* O157:H7 and *Salmonella* spp. in plants: A review. *Food Research International* 45:567-575.

35. **Beuchat LR.** 2006. Vectors and conditions for pre-harvest contamination of fruits and vegetables with pathogens capable of causing enteric diseases. *British Food Journal* 108:38-53.

36. **Food and Drug Administration of the United States** (US FDA). 2012. Bad Bug Book, Foodborne Pathogenic microorganisms and natural toxins. *Second edition*. New Hampshire, United States.

37. **The World Health Organisation (WHO)/ Food and Agriculture (FAO) of the United Nations**. 2006. Food safety risk analysis: A guide for national food safety authorities. Available at: http://www.fao.org/docrep/012/a0822e/a0822e.pdf. Accessed 10th September 2013.

38. **Food and Agriculture (FAO) of the United Nations**. 2017. Capacity development: Risk analysis. Available at: http://www.fao.org/food/food-safety-quality/capacity-development/risk-analysis/en/. Accessed 02nd July 2017.

39. **Holvoet K, Sampers I, Callens B, Dewulf J, Uyttendaele M**. 2013. Moderate prevalence of antimicrobial resistance in *Escherichia coli* isolates from lettuce, irrigation water, and soil. *Applied & Environmental Microbiology* 79:6677-6683.

40. **Adamu NB, Adamu JY, Mohammed D.** 2012. Prevalence of helminth parasites found on vegetables sold in Maiduguri, Northeastern Nigeria. *Food Control* 25:23-26.

41. **Aw TG, Wengert S, Rose JB.** 2016. Metagenomic analysis of viruses associated with field-grown and retail lettuce identifies human and animal viruses. *International Journal of Food Microbiology* 223:50-56.

42. **Pang H, Lambertini E, Buchanan RL, Schaffner DW, Pradhan AK**. 2017. Quantitative microbial risk assessment for *Escherichia coli* O157:H7 in fresh-cut lettuce. *Journal of Food Protection* 80:302-311.

43. **Maffei DF, Sant'Ana AS, Franco BDGM, Schaffner DW.** 2017. Quantitative assessment of the impact of cross-contamination during the washing step of ready-to-eat leafy greens on the risk of illness caused by Salmonella. *Food Research International* 92:106-112.

44. **Danyluk MD, Schaffner DW.** 2011. Quantitative assessment of the microbial risk of leafy greens from farm to consumption: Preliminary framework, data, and risk estimates. *Journal of Food Protection* 74:700-708.

45. **Ding T, Iwahori Ji, Kasuga F, Wang J, Forghani F, Park M-S, Oh D-H.** 2013. Risk assessment for *Listeria monocytogenes* on lettuce from farm to table in Korea. *Food Control* 30:190-199.

46. **Faour-Klingbeil D, Murtada M, Kuri V, Todd ECD**. 2016. Understanding the routes of contamination of ready-to-eat vegetables in the Middle East. *Food Control* 62:125-133.

47. **Wood JL, Chen JC, Friesen E, Delaquis P, Allen KJ.** 2015. Microbiological survey of locally grown lettuce sold at farmers' markets in Vancouver, British Columbia. *Journal of Food Protection* 78:203-208.

48. **Verhaelen K, Bouwknegt M, Carratalà A, Lodder-Verschoor F, Diez-Valcarce M, Rodríguez-Lázaro D, de Roda Husman AM, Rutjes SA**. 2013. Virus transfer proportions between gloved fingertips, soft berries, and lettuce, and associated health risks. *International Journal of Food Microbiology* 166:419-425.

49. **Chen Y, Jackson KM, Chea FP, Schaffner DW.** 2001. Quantification and variability analysis of bacterial cross-contamination rates in common food service tasks. *Journal of Food Protection* 64:72-80.

50. **Jensen DA, Friedrich LM, Harris LJ, Danyluk MD, Schaffner DW**. 2013. Quantifying transfer rates of *Salmonella* and *Escherichia coli* O157: H7 between fresh-cut produce and common kitchen surfaces. *Journal of Food Protection* 76:1530-1538.

51. **Rwanda Meteorology Agency.** 2017. Climatology of Rwanda. Available at: http://www.meteorwanda.gov.rw/index.php?id=30. Accessed 05th June 2017.

52. **Ratkowsky D, Olley J, McMeekin T, Ball A.** 1982. Relationship between temperature and growth rate of bacterial cultures. *Journal of Bacteriology* 149:1-5.

53. **McKellar RC, Delaquis P.** 2011. Development of a dynamic growth–death model for *Escherichia coli* O157: H7 in minimally processed leafy green vegetables. *International Journal of Food Microbiology* 151:7-14.

54. **Koseki S, Isobe S.** 2005. Prediction of pathogen growth on iceberg lettuce under real temperature history during distribution from farm to table. *International Journal of Food Microbiology* 104:239-248.

55. **Islam M, Doyle MP, Phatak SC, Millner P, Jiang X.** 2004. Persistence of enterohemorrhagic *Escherichia coli* O157: H7 in soil and on leaf lettuce and parsley grown in fields treated with contaminated manure composts or irrigation water. *Journal of Food Protection* 67:1365-1370.

56. **Pérez Rodríguez F, Campos D, Ryser ET, Buchholz AL, Posada-Izquierdo GD, Marks BP, Zurera G, Todd E.** 2011. A mathematical risk model for *Escherichia coli* O157:H7 cross-contamination of lettuce during processing. *Food Microbiology* 28:694-701.

57. **Buchanan R, Whiting R, Damert W.** 1997. When is simple good enough: a comparison of the Gompertz, Baranyi, and three-phase linear models for fitting bacterial growth curves. *Food Microbiology* 14:313-326.

58. **National Institute of Statistics of Rwanda (NISR), Ministry of Finance and Economic Planning, Rwanda.** 2012. Rwanda Fourth Population and Housing Census. Thematic Report: Data quality assessment . Available at: http://www.statistics.gov.rw/publication/rphc4-final-report-data-quality-assessment. Accessed 05th February 2015.

59. **Nataro JP, Kaper JB.** 1998. Diarrheagenic *Escherichia coli*. *Clinical microbiology reviews* 11:142-201.

60. **Henao-Herreño LX, López-Tamayo AM, Ramos-Bonilla JP, Haas CN, Husserl J.** 2017. Risk of illness with *Salmonella* due to consumption of raw unwashed vegetables irrigated with water from the Bogotá river. *Risk Analysis* 37:733-743.

61. **Owusu-Ansah Ed-GJ, Sampson A, Amponsah SK, Abaidoo RC, Dalsgaard A, Hald T.** 2017. Probabilistic quantitative microbial risk assessment model of norovirus from wastewater irrigated vegetables in Ghana using genome copies and fecal indicator ratio conversion for estimating exposure dose. *Science of The Total Environment* 601-602:1712-1719.

62. **Bouwknegt M, Verhaelen K, Rzeżutka A, Kozyra I, Maunula L, von Bonsdorff C-H, Vantarakis A, Kokkinos P, Petrovic T, Lazic S, Pavlik I, Vasickova P, Willems KA, Havelaar AH, Rutjes SA, de Roda Husman AM.** 2015. Quantitative farm-to-fork risk assessment model for norovirus and hepatitis A virus in European leafy green vegetable and berry fruit supply chains. *International Journal of Food Microbiology* 198:50-58.

63. **Sant'Ana AS, Franco BDGM, Schaffner DW.** 2014. Risk of infection with *Salmonella* and *Listeria monocytogenes* due to consumption of ready-to-eat leafy vegetables in Brazil. *Food Control* 42:1-8.

64. **Franz E, Tromp SO, Rijgersberg H, Van Der Fels-Klerx HJ. 2010.** Quantitative microbial risk assessment for *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in leafy green vegetables consumed at salad bars. *Journal of Food Protection* 73:274-285.

65. **Amponsah-Doku F, Obiri-Danso K, Abaidoo RC, Andoh LA, Drechsel P, Kondrasen F. 2010.** Bacterial contamination of lettuce and associated risk factors at production sites, markets and street food restaurants in urban and peri-urban Kumasi, Ghana. *Scientific Research & Essays* 5:217-223.

66. **Strawn LK, Gröhn YT, Warchocki S, Worobo RW, Bihn EA, Wiedmann M.** 2013. Risk factors associated with *Salmonella* and *Listeria monocytogenes* contamination of produce fields. *Applied & Environmental Microbiology* 79:7618-7627.

67. **Joint Institute for Food Safety and Applied Nutrition, University of Maryland and Food and Drug Administration (US FDA).** 2002. Improving the safety and quality of fresh fruits and vegetables: a training manual for trainers. Available at: http://www.fao.org/ag/agn/CDfruits_en/others/docs/maryland_manual.pdf. Accessed 4th September 2014.

68. **The Food and Agriculture Organization (FAO) of the united nations.** 2012. On-farm practices for the safe use of wastewater in urban and peri-urban horticulture. A training handbook for farmer field schools. Available at: http://www.fao.org/docrep/016/i3041e/i3041e.pdf. Accessed 17th July 2013.

69. **Food Safety Authority of Ireland.** 2016. Guidance Note No. 31. Fresh Produce Safety in Primary Production in Ireland. Available at: https://www.fsai.ie/publications_GN31_fresh_produce/. Accessed 20th October 2016.

70. **U.S. Department of Health and Human Services Food and Drug Administration, Center for Food Safety and Applied Nutrition (CFSAN).** 1998. Guidance for industry. Guide to minimize microbial food safety hazards for fresh fruits and vegetables. Available at: http://wcmorris.com/gap/files/prodguid.pdf. Accessed 17th July 2013.

71. **The World Health Organisation (WHO)/ Food and Agriculture (FAO) of the United NAtions, Codex Alimentarius Commission, international standards.** 1969. General principles of food hygiene. Available at: http://www.fao.org/fao-who-codexalimentarius/shproxy/ar/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCAC%2BRCP%2B1-1969%252FCXP_001e.pdf. Accessed 30th November 2015.

72. **The World Health Organisation (WHO)/ Food and Agriculture (FAO) of the United Nations, Codex Alimentarius Commission, international standards.** 2003. Code of hygienic practice for fresh fruits and vegetables (CAC/RCP 53- 2003). Available at: www.fao.org/input/download/standards/10200/CXP_053e_2013.pdf. Accessed 30th November 2015.

73. **Royal United Hospital Bath (RUH), NHS Trust**. 2010. Hygiene and food safety policy for food production and food service. Available at: http://www.ibrarian.net/navon/paper/HYGIENE_AND_FOOD_SAFETY_POLICY_FOR_FOOD_PRODUCTIO.pdf?paperid=20779658. Accessed 06th February 2014.

74. **Havelaar AH, Haagsma JA, Mangen M-JJ, Kemmeren JM, Verhoef LPB, Vijgen SMC.** 2012. Disease burden of foodborne pathogens in the Netherlands, 2009. *International Journal of Food Microbiology* 156: 231-238.

75. **Adak GK, Meakins SM, Yip H, Lopman BA, O'Brien SJ.** 2005. Disease risks from foods, England and Wales, 1996-2000. *Emerging Infectious Diseases* 11:365-372.

76. **Scallan E, Kirk M, Griffin PM.** 2013. Estimates of disease burden associated with contaminated food in the United States and globally, p. 3-18. In: J. G. Morris, Jr. and M. E. Potter (Eds.) Foodborne Infections and Intoxications (Fourth Edition), Elsevier Inc.

77. **Niyonzima E, Ongol MP, Brostaux Y, Koulagenko NK, Daube G, Kimonyo A, Sindic M.** 2016. Daily intake and bacteriological quality of meat consumed in the households of Kigali, Rwanda. *Food Control* 69:108-114.

78. **Herman KM, Hall AJ, Gould LH.** 2015. Outbreaks attributed to fresh leafy vegetables, United States, 1973-2012. *Epidemiology & Infection* 143:3011-3021.

79. **Fresco, L.O., Bouwstra, R.J., de Jong, M.C.M., van der Poel, W.H.M., Scholten, M.C.T., Takken, W. and the global one health task force of Wageningen University and Research Centre.** 2015. Global one health – a new integrated approach Available at: http://www.wur.nl/upload_mm/e/a/c/d734f322-b66d-4ec0-aa5b-3ceb31001fe0_GOH_Final_20150602.pdf. Accessed 20th September 2016.

80. **The Food and Agriculture Organisation of the United Nations (FAO)**. 2008. Contributing to "One World, One Health". A strategic framework for reducing risks of infectious diseases at the animal–human–ecosystems Interface. Available at: ftp://ftp.fao.org/docrep/fao/011/aj137e/aj137e00.pdf. Accessed 01st October 2013.

81. **Daniels NA**. 2006. Enterotoxigenic *Escherichia coli*: traveler's diarrhea comes home. The University of Chicago Press.

82. **Qadri F, Svennerholm A-M, Faruque A, Sack RB.** 2005. Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. *Clinical Microbiology Reviews* 18:465-483.

83. **Beuchat LR.** 1996. Pathogenic microorganisms associated with fresh produce. *Journal of Food Protection* 59:204-216.

84. **Herman KM, Hall AJ, Gould LH.** 2015. Outbreaks attributed to fresh leafy vegetables, United States, 1973–2012. *Epidemiology & Infection* 143:3011-3021.

85. **Frank** et al**.** 2011. Epidemic profile of Shiga-toxin–producing *Escherichia coli* O104: H4 outbreak in Germany. *New England Journal of Medicine* 365:1771-1780.

86. **Center for Disease Control and Prevention of the United States**. 2012. Multistate outbreak of shiga toxin-producing *Escherichia coli* O157:H7 infections linked to organic spinach and spring mix blend. Available at: http://www.cdc.gov/ecoli/2012/O157H7-11-12/index.html. Accessed 22nd October 2016.

87. **Center for Disease Control and Prevention of the United States**. 2016. Foodborne outbreak tracking and reporting. Available at: https://www.cdc.gov/foodsafety/fdoss/. Accessed on 23rd October 2016.

88. **Friesema I, Sigmundsdottir G, Van Der Zwaluw K, Heuvelink A, Schimmer B, De Jager C, Rump B, Briem H, Hardardottir H, Atladottir A.** 2008. An international outbreak of Shiga toxin-producing *Escherichia coli* O157 infection due to lettuce, September-October 2007. Euro surveillance: *European Communicable Disease Bulletin* 13:3029-3035.

89. **Söderström** et al**.** 2008. A large *Escherichia coli* O157 outbreak in Sweden associated with locally produced lettuce. *Foodborne Pathogens & Disease* 5:339-349.

90. **The World Health Organization of the United Nation (WHO)**. 2016. Enterohaemorrhagic *Escherichia coli* – United Kingdom. Available at: http://www.who.int/csr/don/20-july-2016-ehec-uk/en/. Accessed 10th October,2016.

91. **Adams, Moss.** 2008. Food Microbiology, Third ed. The Royal Society of Chemistry, Thomas Graham House, Science Park, Milton Road, Cambridge CB4 0WF, UK.

92. **Tozzi AE, Caprioli A, Minelli F, Gianviti A, De Petris L, Edefonti A, Montini G, Ferretti A, De Palo T, Gaido M.** 2003. Shiga toxin‐producing *Escherichia coli* infections associated with hemolytic uremic syndrome. *Emerging Infectious Diseases* 9:107.

93. **Okhuysen PC, DuPont HL**. 2010. Enteroaggregative *Escherichia coli* (EAEC): A cause of acute and persistent diarrhea of worldwide importance. *Journal of Infectious Diseases* 202:503-505.

94. **Farber JM, Peterkin PI.** 1991. *Listeria monocytogenes*, a food-borne pathogen. *Microbiological Reviews* 55:476-511.

95. **Jemmi T, Stephan R.** 2006. *Listeria monocytogenes*: Food-borne pathogen and hygiene indicator. *Scientific & Technical Review of the Office International des Epizooties* 25:571-580.

96. **Wang S, Orsi RH.** 2013. *Listeria*, Chapter 11, p. 199-216. Elsevier Inc.

97. **Epps SVR, Harvey RB, Hume ME, Phillips TD, Anderson RC, Nisbet DJ.** 2013. Foodborne *Campylobacter*: infections, metabolism, pathogenesis and reservoirs. *International Journal of Environmental Research & Public Health* 10:6292-6304.

98. **Silva J, Leite D, Fernandes M, Mena C, Gibbs PA, Teixeira P.** 2011. *Campylobacter* spp. as a Foodborne Pathogen: A Review. *Frontiers in Microbiology* 2:200.

99. **Murugaiah C.** 2011. The burden of cholera. *Critical Reviews in Microbiology* 37:337-348.

100. **Petersen JM, Mead PS, Schriefer ME.** 2009. *Francisella tularensis*: an arthropod-borne pathogen. *Veterinary Research* 40:07.

101. **Perry RD, Fetherston JD**. 1997. *Yersinia pestis*--etiologic agent of plague. *Clinical Microbiology Reviews* 10:35-66.

102. **Ortega YR, Sanchez R.** 2010. Update on *Cyclospora cayetanensis*, a food-borne and waterborne parasite. *Clinical Microbiology Reviews* 23:218-234.

Summary

Global consumption of vegetables and the associated reported foodborne illnesses have been increasing in tandem. This study was commissioned to analyze the microbial risk from "farm to fork" along the fresh vegetable supply chain in Rwanda to explore microbial safety options that can contribute to an integrated system to detect, control and prevent foodborne infections. Specific study objectives were developed, the first being to estimate the burden of foodborne infectious illnesses using the available data to obtain insight into the general plight of food safety issues and to also develop a framework for future investigations. Second, to investigate the microbial safety status, handling practices and risk exposure factors along the vegetable supply chain.

To understand the overall impact of foodborne illnesses on human health in Rwanda the burden of food related illnesses in year 2013 was estimated using the DALY (disability adjusted life year) metric, as encouraged by the World Health Organization. DALY is a measure that combines years of life lost due to premature death and healthy years lost due to disability during sickness. Study findings indicate that for the year 2013, watery diarrhea occurred all year round as by the surveillance system data, resulting to an estimated 672 (95% credible interval [CrI] 424 – 932) DALY per million inhabitants, bloody diarrhea was seasonal coinciding with the rainy months and caused an estimated 213 (95% CrI 50 – 475) DALY per million, typhoid and cholera manifested as outbreaks with an estimated 73 (95% CrI 57 – 91) and 1 (95% CrI 0 – 2) DALY per million respectively. These data show that the health burden is high and we suspect that a large proportion is caused by consumption of contaminated food.

Also investigated in this study, was the prevalence of foodborne pathogens in farm vegetables and agricultural water (used for irrigation and on-farm washing of vegetables) in Rwanda. In agricultural water from rivers, lakes, lagoons, ground and marshlands, traces in the form of DNA from a wide variety of virulent pathogenic organisms were detected, including enteroinvasive, enteroaggregative, and enterotoxigenic *E. coli, Vibrio cholera*, *Yersinia pestis* and the parasite *Cyclospora*. DNA from thermo-tolerant *Campylobacter* spp. was found in 87% of the samples. Although this does not mean that all these pathogens were still alive by the time of detection; presence of DNA is an indication that the pressure of pathogens in agricultural water and the environment is high. Indeed from 99 samples of farm vegetables, different viable foodborne pathogens were isolated *viz. Listeria monocytogenes* (1%), *Campylobacter* spp. (3%), *Salmonella* spp. (5%) and pathogenic *E. coli* (6%).

In addition to tracing the pathogens at farm level, selected indicator microorganisms were investigated, to get an impression of their survival, growth and/or inactivation along the vegetable supply chain. The latter was complemented with a detailed observation of handling practices along the supply chain. The mean count of *Enterobacteriaceae* in 11 types of vegetables increased slightly from farm to markets to reach an average of 6.0 log cfu/g upon arrival at FSEs. During food preparation microbial counts were significantly reduced by washing with or without sanitizers, trimming/peeling, with an average of 2.1 log cfu/g from start to end of salad preparation. Ready-to-eat salads prepared by FSEs met the guidelines by

91% and 22% for coagulase-positive staphylococci (10^4 cfu/g) and presumptive *Listeria* spp. (10^2 cfu/g) respectively. Because washing and sanitization procedures differ from one FSE to another, a laboratory study was designed to mimic the practices at FSEs with the aim to select optimal washing and sanitization procedures. Findings in the field study with FSEs revealed that about 61% of the visited FSEs used sanitizers during washing of fresh vegetables, in particular, potassium permanganate ($KMnO_4$) in 39 % of FSEs, sanitizing powder (a mixture of polyphosphate, sodium hydrogen carbonate and active chlorine), 13%; sodium hypochlorite (NaClO), 7 %; and sodium dichloroisocyanurate (NaDCC) in 2%. Average reduction ranged from 1.0 log (for $KMnO_4$) to 3.1 log (for NaDCC). In the laboratory study, average inactivation observed with indicator microorganisms ranged from 0.7 log (for water alone) to 3.0 log (for NaDCC). Out of the 8 sanitizers that were evaluated, 5 sanitizers (NaDCC [90 ppm], NaClO [200 ppm], lemon juice [98%], acetic acid [2 %] and sanitizing powder [4 g/L]) resulted in significantly higher inactivation compared to water alone. A contact time of 5 minutes and a salad-sanitizer ratio of 1: 20 were considered optimal for kitchen based washing of the studied leafy vegetables with NaDCC and NaClO sanitizers. This study also reveals that the most widely used sanitizer (25ppm $KMnO_4$) was not more effective than washing with only water and an indication that a policy, guideline or regulation on kitchen based washing and sanitization of salad vegetables should be enacted.

The high prevalence (15%) of foodborne pathogens associated vegetables at farm level and increasing trends in levels of indicator microorganisms from farm to FSEs, raises concern about the potential presence of foodborne pathogens in ready-to-eat salads. By using @risk software (Palisade Corporation, Ithaca, NY, USA) and Monte Carlo simulation (100,000 iterations), the number of cases due to serving leafy vegetables contaminated pathogenic *E. coli* have been estimated with a mode of 12 million cases of illness per year and 0.1 probability of illness per serving. These estimates can be considered high compared to previous risk assessments in other countries. However it should be acknowledged that these estimates also include mild illness from less harmful pathotypes of pathogenic *E. coli. viz.* enterotoxigenic *E. coli* (ETEC) and enteropathogenic *E. coli.* To further advise risk managers, seven "what if scenarios" were simulated to compare with the baseline model. The scenario of improving washing and sanitization (3.0 log reduction) at FSEs resulted to a less than 2 fold change in the predicted microbial risk. While a 2 fold change was observed for the scenario of channelling all vegetables through supermarkets instead of traditional markets. Farm interventions reducing the prevalence and levels of pathogenic *E. coli* in the base line model by 90%, the introduction of a cold chain and skipping the market step, all resulted to a 10 fold reduction in predicted microbial risk. The scenario of reducing or avoiding contamination and cross contamination along the supply chain led to 1000 fold reduction in the predicted microbial risk. Lastly, farm to fork measures combining three different scenarios (avoiding contamination from farm to fork, farm interventions (90% reduction) and improving sanitization) were predicted to reduce microbial risk by a factor of 1 million.

Risk estimates should be analyzed and weighted in order to prioritize risk management measures and interventions, not only for their effectiveness in improving public health, but also for their feasibility, acceptability and affordability. From the predicted microbial risk in this

study, approaches to change the routes or temperatures of supply chain in Rwanda may not be given priority by risk managers except for the scenario of skipping the market step so that FSEs get vegetables straight from farms. It can be observed from this study that changing the supply chain by introducing a cold chain or refrigeration at market (supermarkets) may not address the microbial risk associated with fresh vegetables. Instead, risk managers can focus on addressing the factors leading to contamination and cross contamination from "farm to fork". It has been demonstrated in this study that washing and sanitization at FSEs alone (current practise) is not enough to address microbial safety concerns. Consequently, risk assessors and risk manager should identify all the possible sources of microbial hazards along the entire supply chain and devise measures and interventions to address them and spearhead risk communication strategies among stakeholders. This study recommends embracing the concepts of "Global One Health" in order to move towards sustainable microbial safety of fresh vegetables in Rwanda and the Globe.

We hope that the results from this study will be helpful for policy makers and risk managers, not only in approaching the microbial safety concerns of vegetables along the supply chain but also in developing national integrated food chain systems.

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James Noah

About the author

Born 19th March 1981 in Mpigi, Uganda, James Noah Ssemanda graduated at Makerere University Kampala, Uganda in 2005 with a BSc. of Food Science and Technology. In the same year, he was recruited at Rwanda Standards Boards (RSB) in what would later turn out to be an extended career in food quality and safety. James Noah has since contributed to the enhancement of the quality infrastructure in Rwanda and East Africa. While at RSB, James Noah has served in different roles; as a Food quality and safety inspector for local industries and imported foods, Laboratory analyst, Laboratory quality manager and as a HACCP/FSMS certification officer. James Noah is a trained auditor of conformity assessment standards and quality management systems (ISO 17025:2005 ISO 17021:2006 and ISO 22000:2005). In 2008, James Noah was a recipient of the VLIR-UOS scholarship to study at KU Leuven in cooperation with Ghent University, Belgium and graduated *cum laude* with a Masters of Food Technology in 2010. Thereafter, James Noah returned to RSB where he became team leader in documenting the laboratory quality system in preparation for accreditation. In 2012, James Noah was a coordinator of a RSB project to develop local capacity in HACCP and GMPs in collaboration with the British Standard Institute and Trade Mark East Africa (NGO). In 2013, James Noah accepted a PhD study entitled, 'Towards microbial safety of fresh vegetables in Rwanda', at the Laboratory of Food Microbiology of Wageningen University and Research, Kingdom of the Netherlands. The results of his PhD work are described in this thesis. Currently, James Noah has returned to RSB, to further contribute to the development and implementation of microbial safety guidelines and standards along the food chain in Rwanda and East Africa.

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List of publications

James Noah Ssemanda, Martine Reij, Mark Cyubahiro Bagabe, Claude Mambo Muvunyi, José Nyamusore, Han Joosten, Marcel H. Zwietering. (2018). Estimates of the burden of illnesses related to foodborne pathogens as from the syndromic surveillance of 2013 in Rwanda. *Microbial Analysis* (https://doi.org/10.1016/j.mran.2018.02.002).

James Noah Ssemanda, Martine Reij, Gerrieke van Middendorp, El Bouw, Rozemarijn van der Plaats, Eelco Franz, Claude Mambo Muvunyi, Mark Cyubahiro Bagabe, Marcel H. Zwietering, Han Joosten. (2018). Foodborne pathogens and risk exposure factors associated with farm vegetables in Rwanda. *Food Control* 89: 86-89.

James Noah Ssemanda, Han Joosten, Mark Cyubahiro Bagabe, Marcel H. Zwietering, Martine Reij. (2018). Reduction of microbial counts during kitchen scale washing and sanitization of salad vegetables. *Food Control*.85: 495- 503.

James Noah Ssemanda, Martine Reij, Mark Cyubahiro Bagabe, Claude Mambo Muvunyi, Han Joosten, Marcel H. Zwietering. (2017). Indicator microorganisms in fresh vegetables from "farm to fork" in Rwanda. *Food Control* 75: 126 – 133.

Muyanja C, Birungi S, Ahimbisibwe M, Ssemanda J and BS Namugumya. (2010). Traditional processing, microbial and physicochemical changes during fermentation of Malwa. *African Journal of Food Agriculture and Nutrition* 10:4124-4138.

Overview of completed training activities

Discipline specific courses			
Governance and Food safety in international food chains	CDI	Wageningen	2012
Genetics and physiology of food-associated microorganisms	VLAG	Wageningen	2013
Management of Microbial Hazards Foods	VLAG	Wageningen	2014
Multivariate analysis for food data	VLAG	Wageningen	2014
Applied Statistics	VLAG	Wageningen	2016
Meeting and conferences			
Epidemiology of foodborne illnesses	RIVM/WHO	Amsterdam	2015
Global Burden of Disease: Foodborne Pathogens, The FERG Approach	KNVM	Wageningen	2016
General courses			
Information Literacy including endnote introduction	WGS	Wageningen	2013
Philosophy and Ethics of Food science	WGS	Wageningen	2014
Scientific Writing	WGS	Wageningen	2016
Writing Grant Proposals	WGS	Wageningen	2016
Project and Time Management	WGS	Wageningen	2016
Presenting with impact	WGS	Wageningen	2016
Research integrity	WGS	Wageningen	2016
Optional courses			
Preparation of research proposal	WUR	Wageningen	2013
PhD study tour to Ireland	FHM	Cork, Dublin	2014
PhD study tour to Italy	FHM	Bolgna, Parma, Milan, Alba, Turin	2017
Food Microbiology department seminars	FHM	Wageningen	2013-2017

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