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Viability of *Cryptosporidium parvum* oocysts in experimentally contaminated parsley and strawberries when subjected to different freezing regimes

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Master in Food Science

1. Preface

This master thesis is part of a global research collaboration on foodborne parasites. The master project thesis objectives were presented by the Department of Food Safety and Infection Biology of Norwegian University of Life Science (NMBU) in Norway and Nestlé Research Institute in Switzerland. Laboratory work was performed at the Faculty of Veterinary Medicine, Department of Food Safety and Infection Biology, Section for Microbiology, Immunology and Parasitology of NMBU, at the Norwegian School of Veterinary Science at Adamstuen in Oslo. Main supervisor was Post doctor Kristoffer Relling Tysnes. Professor Lucy Robertson was additional supervisor. They have been the best supervisors possible, and I am enormously grateful for all advice and support. Kjersti Selstad Utaaker has also been of great support, guiding me in the universe of *Cryptopsopridium* spp. I would also like to thank all other employees at the laboratory, contributing to a positive and inclusiveness work environment.

Thanks to Lone Berg for help with the visual presentation of the overview of the experiments.

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Ane Elen Holbø

2. Summary

Cryptosporidium spp. are parasites causing gastroenteritis. They are associated with waterborne outbreaks and childhood diarrhea in developing countries. Cryptosporidiosis is normally a self-limiting infection, but it can be life threatening to immunocompromised individuals. The infection is highly underdiagnosed and underreported. An increasing number of outbreaks have been associated with foodborne transmission in the last decades. FAO/WHO ranked *Cryptosporidium* spp. number five in a multicriteria-based ranking for parasites from a food safety perspective. As the parasite is being an increasing threat to food safety worldwide, it is necessary to get an overview over factors that can be applied for inactivation. Little research has been conducted regarding freezing of the parasite using food matrices. The aim of this study was to investigate the amount of time needed for *C. parvum* oocysts to be inactivated, when subjected to - 20 ^{o}C on experimentally contaminated fresh produce.

C. parvum oocysts from young calves, were used for contamination of parsley and strawberry samples. Stock suspensions of oocysts were diluted in water prior to contamination, with between 7 500 and 20 000 oocysts spiked on each sample in droplets of 10 µl. Parsley samples were kept in the freezer at - 20 ^{o}C and in the refrigerator at + 4 ^{o}C for 1, 4, 8, 12, 24 and 72 hours. Oocysts suspended in water were held in the refrigerator the same time intervals. Contaminated strawberry samples were kept in the freezer at - 20 ^{o}C for 4, 24 and 72 hours.

Oocysts on fresh produce were eluted in glycine buffer, and concentrated by centrifugation and immunomagnetic separation using immunomagnetic anti-*Cryptosporidium* beads, before staining with the fluorescent nuclear dyes, 4'.6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI). Viability was assessed based on inclusion/exclusion of DAPI and PI as determined, using a fluorescent microscope.

The results were analyzed using linear regression. For parsley samples, viability of oocysts inoculated on parsley and stored in the freezer were compared with identical samples stored in the refrigerator. The model showed a clear difference in viability between refrigerated parsley samples and frozen parsley samples after 8 hours ($p<0.001$). Difference between parsley samples were statistically significant ($p<0.001$) for 12, 24 and 72 hours. There was 98.8 % inactivation of oocysts after 12 hours of freezing. For strawberry samples, viability of oocysts inoculated on strawberries stored in the freezer, were compared with reference samples of oocysts suspended in water. The

model showed a significant reduction in viability already after 4 hours ($p<0.001$). There was inactivation of 94.3 % of oocysts after 24 hours. A small proportion of oocysts remained viable after 72 hours of freezing in both parsley (2 %) and strawberry (4 %) samples. There is a possibility that the results over-estimated infectivity as the method used for viability assay in this study has been linked to over-estimation in previous studies.

These findings indicate that *C. parvum* oocysts excreted from the same host are not necessarily homogenous, and some may be more resistant than others. A small proportion of the oocysts were viable after 72 hours, indicating that they are much more resistant to freezing than the 'average' oocyst. Further research is needed to determine what time interval is needed for a total inactivation of *Cryptosporidium* spp. oocysts by freezing at -20 ^{o}C, or related industrial freezing temperatures.

3. Sammendrag (Norwegian summary)

Cryptosporidium spp. er parasitter som forårsaker gastroenteritt. De er ofte assosiert med vannbårne utbrudd i industrielle land og er en vanlig årsak til diaré hos barn i utviklingsland. Cryptosporidiose er vanligvis en selvbegrensende infeksjon, men den kan være livstruende hos immunsvekkede individer. Infeksjonen er under-diagnostisert og under-rapportert. Økende antall matbårne utbrudd er rapportert de siste tiårene. FAO/WHO har rangert *Cryptosporidium* spp. nummer fem i en rangering basert på kriterier assosiert med mattrygghet og parasitter. Parasitten er en økende trussel til den globale matsikkerheten, og det er både ønskelig og nødvendig å kartlegge ulike faktorer som kan inaktivere den. Det er lite dokumentasjon på frysebehandling av parasitten ved bruk at mat-matriser. Målet med denne studien var å undersøke hvor lenge *C. parvum* oocyster må fryses ved - 20 ^{o}C på eksperimentelt kontaminerte vegetabiler, for å bli inaktivert.

C. parvum fra unge kalver ble brukt til kontaminering av persille- og jordbærprøver. Fortynnet stokkløsning av parasitter ble påført råvarene i dråper på 10 µl, med mellom 7 500 og 20 000 parasitter per prøve. Persilleprøver ble oppbevart i fryser ved - 20 ^{o}C og i kjøleskap ved + 4 ^{o}C i 1, 4, 8, 12, 24 og 72 timer. Oocyster suspendert i vann ble oppbevart i kjøleskap ved samme tidsintervaller. Kontaminerte jordbær ble oppbevart i fryser ved -20 ^{o}C i 4, 24, og 72 timer.

Oocystene ble eluert i glycinbuffer, før konsentrasjon ved sentrifugering og immunomagnetisk separasjon ved bruk av anti-*Cryptosporidium* beads. Vurdering av oocystenes viabilitet ble gjort

med fluoriserensmikroskop etter farging med fargestoffene, 4'.6-diamidino-2-phenylindole (DAPI) og propidium iodide (PI). Viabiliteten ble fastsatt ved bruk av fluoriserende mikroskop og inklusjon/eksklusjon av DAPI og PI i oocystene.

Resultatene ble analysert ved bruk av lineær regresjon. For persille prøver ble viabiliteten til oocyster inokulert på fryselagret persille sammenlignet med identiske prøver for persille oppbevart i kjøleskap. Modellen viste en tydelig forskjell i viabilitet mellom oocyster på kjølte og fryste prøver lagret i 8 timer ($p<0.001$). Det var også signifikante forskjeller ($p<0.001$) mellom tilsvarende persilleprøver kjølt og fryst i 12, 24 og 72 timer. Etter 12 timers frysebehandling var 98.8 % av oocystene inaktivert. Fryste jordbærprøver ble sammenlignet med referanseprøver av oocyster suspendert i vann. Det var signifikant reduksjon i viabiliteten allerede etter 4 timer ($p<0.001$). Etter 24 timers fryselagring var 94.3 % av oocystene på jorbærene inaktivert. En liten andel oocyster var levedyktige etter 72 timers fryselagring på både persille (2 %) og jordbær (4 %).

Resultatene indikerer at *C. parvum* oocyster utskilt fra samme vert ikke nødvendigvis er homogen, noen oocyster kan være mer resistente mot frysing enn andre. En liten andel av oocystene var levedyktige og mulig infektive etter 72 timer. Videre forskning er nødvendig for å fastsette hvilken tidslengde som er nødvendig for en total inaktivering av *Cryptosporidium* spp. oocyster lagret ved - 20 ^{o}C, eller nærliggende temperaturer brukt ved industriell frysebehandling av næringsmidler.

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4. Introduction

4.1.1 Introduction

Cryptosporidium spp. are protozoan zoonotic endoparasites that are found in humans and domestic and wild animals worldwide. It can cause cryptosporidiosis, an enteric infection affecting the epithelial cells of the distal small intestines, with watery diarrhea as the main symptom.

The genus *Cryptosporidium* was discovered in 1907 by Ernest Edward Tyzzer, but human infection was first identified in 1976 (White 2010). In the 1980s *Cryptosporidium* was recognized as an important cause of persistent diarrhea in patients with AIDS (Current et al. 1983). The parasite was gradually linked to malnutrition and death caused by diarrhea in children in developing countries (Kotloff et al. 2013; Sallon et al. 1988). *Cryptosporidium* spp. is currently a major cause of waterborne outbreaks worldwide, reported in 239 waterborne outbreaks between 2011 and 2016 (Efstratiou et al. 2017). Some species of *Cryptosporidium* spp. are also linked to zoonotic and foodborne transmission.

In a report from 2014 from Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO), *Cryptosporidium* spp. was ranked number five in a multicriteria-based ranking of foodborne parasites from a food safety perspective (FAO/WHO 2014). Out of 95 parasites, 24 was ranked by criteria such as prevalence, severity of illness, impact on community and global distribution. *Cryptosporidium* spp. had a high ranking because it has been linked to numerous foodborne outbreaks in different types of food worldwide, it has a high level of severity in immunocompromised individuals, and the oocysts can be viable after chlorination of water, a method commonly used to inactivate pathogenic microorganisms in water.

There has also been a European risk ranking of foodborne parasites that is to be presented on the 18.05.2017 in Rome, at the European Union Reference Laboratory for Parasites for the annual meeting of European National Reference Laboratories for parasitology. In this risk-ranking *Cryptosporidium* spp. was ranked as the second most important foodborne parasite in Northern and Western Europe, based on expert elicitation at a Cost Action EURO-FBP (FA 1408) workshop in 2016, using multi-criteria decision analyses.

4.1.2 Morphology and life cycle

Sporulated oocysts are the infective stage of the parasite. They are 4-6 µm with a thick wall in a spherical to ovoid shape, containing four sporozoites (Fayer & Xiao 2008). Taxonomic classification of the genus *Cryptosporidium* is; family of Cryptosporidiidae that has a homoxenous life cycle and endogenous sporogony; class of Coccidea with sexual and asexual reproduction stages and development of infective sporozoites; phylum of Apicomplexa (Fayer & Xiao 2008)

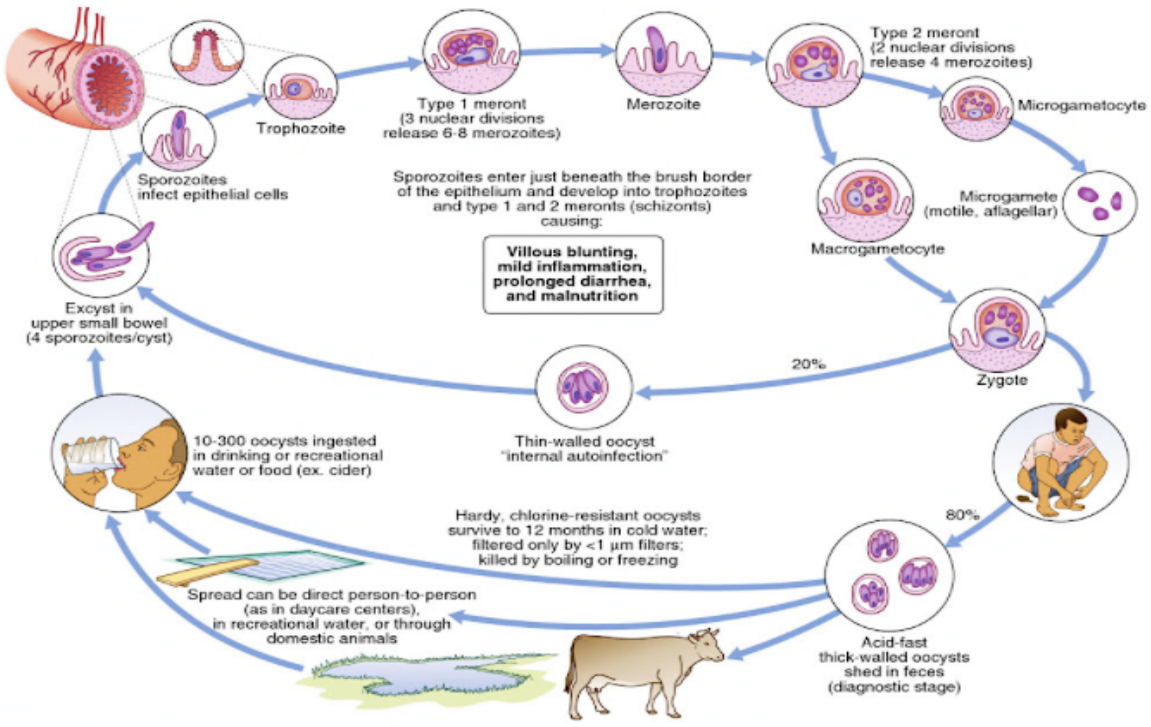

Figure 1. Life cycle of *Cryptosporidium* (Lima et al. 2011)

Cryptosporidium spp. completes its life cycle in a single host, after asexual and sexual stages, producing oocysts (Fayer & Xiao 2008; Rossle & Latif 2013). The life cycle starts with the ingestion of infectious oocysts by the host. When the oocysts reach the small intestine, sporozoites are released by excystation, and these invade epithelial cells. There they have a paracellular location, being intracellular but extra-cytoplasmic. The asexual stage of the life cycle consists of differentiation into trophozoites that develop into type l meronts. Type l meronts produce 6-8 merozoites, a structure similar to sporozoites, that infect epithelial cells and develop into type l or type ll meronts. Type ll meronts produce four merozoites, that develop into micro- or macrogamonts. The sexual stages of development occur when microgamonts develop into mirogametes and penetrate macrogamonts, creating a zygote. The zygotes develop into oocysts

with four sporozoites. Two types of oocysts develop, one type with a thick wall that are shed with the feces, and another type with a thin wall that may re-infect the original host without being excreted. If the thin-walled oocysts are excreted they are likely to be less robust. See figure 1.

Infected individuals shed high numbers of oocysts that can remain viable for months in humid and temperate environments, such as soil and surface waters (FAO/WHO 2014). A study on infected calves in Japan showed a mean shedding of 6×10^7 oocysts per g feces, giving a total excretion of up to 6×10^{11} oocysts from one calf (Uga et al. 2000). In a study where oocysts were placed in chambers containing soil that were placed in natural environments at a dairy farm, 5-30 % of *C. parvum* oocysts remained viable after 120 days at temperatures between +1 and 0 ^{o}C, with a few short intervals at - 2 ^{o}C, and one short interval at - 8 ^{o}C (Kato et al. 2004). The high number of oocysts shed in feces, their ability to survive and stay viable for a prolonged time in water and soil, combined with the low number needed to induce an infection, makes transmission of the parasite effective.

4.1.3 Global distribution

The genus *Cryptosporidium* can cause infection in all classes of vertebrates (Fayer & Xiao 2008). The different species of *Cryptosporidium* are pathogenic to various species of vertebrates. Some have only one host species, while others may infect different hosts. Host and associated major species of *Cryptosporidium* in animals are presented in table 1. For species and genotypes causing human infection, see section 4.1.4 Cryptosporidiosis.

Table 1. Major and minor species infecting selected domesticated animals and wildlife*

Host	Major species	Minor species
Camel	*C. andersoni, C. parvum*?	
Cat	*C. felis*	
Cattle	*C. parvum, C. bovis, C. andersoni*	*C. suis*
Chicken	*C. baileyi*	*C. melagridis, C. galli*
Deer (Red)	*C. parvum, C. ubiquitum*	
Dog	*C. canis*	
Fish	*C. scophthalmi, C. molnari*	
Goat	*C. parvum, C. xiaoi*	
Mouse	*C. muris*, mourse genotype	
Pig	*C. suis, C. scrofarum*	Pig genotype ll
Rabbits	*C. cuniculus*	
Sheep	*C. ubiquitum, C. xiaoi*	
Snake	*C. serpentis*	*C. varanii*, snake genotype
Squirrel	*C. muris*, squirrel genotype	
Turkey	*C. meleagridis, C. baileyi*	

* Modified from Fayer & Xiao 2008, and Robertson 2014

Cryptosporidium spp. are distributed worldwide, with cryptosporidiosis reported in 106 countries located in Africa, the Pacific, Europe, Middle-East, Asia, Caribbean, North-, Central- and South America (Fayer & Xiao 2008). The number of infections is believed to be higher in developing countries, compared to developed countries, although current prevalence data is somewhat incomplete (Cacciò & Widmer 2014). Africa, Asia, Latin America and Australia are areas documented with a high prevalence (Erickson & Ortega 2006). Death of children up to 5 years of age is caused by diarrhea in approx. 10 % of cases worldwide (Liu et al. 2012). In a study including 9439 ill children younger than 5 years from sub-Sahara and south-Asia, areas with the highest number of child deaths caused by diarrhea, *Cryptosporidium* was the second most common cause of moderate to severe diarrhea in infants (0-11 months) (Kotloff et al. 2013). *Cryptosporidium* was significantly associated with moderate to severe diarrhea in children at all locations in this study. In the United States of America (USA) *Cryptosporidium* is responsible for approx. 1 % of cases of diarrhea (Erickson & Ortega 2006). Immunological testing for antibodies has, however, been positive in 30 % of the healthy adult population in USA (Ungar et al. 1989). Infection by *Cryptosporidium* spp. is highly under-diagnosed and under-reported in countries both with and without surveillance systems for detection (Caccio & Chalmers 2016; Cacciò & Widmer 2014). Outbreaks and contamination detected in fresh produce worldwide, is reflecting the global distribution of the parasite (table 2 and 3). Products such as milk and meat can also be contaminated with *Cryptosporidium* spp. oocysts from feces, if hygiene is poor (Budu-Amoako et al. 2011).

Table 2. Surveillance studies reporting the presence of *Cryptosporidium* on fresh produce worldwide*

Country	Produce testing positive	Prevalence	Reference
Cambodia	Water spinach	8 %	(Vuong et al. 2007)
Canada	Packaged leafy greens	1.7 %	(Dixon et al. 2013)
Costa Rica	Cilantro leaves, cilantro roots, lettuce, carrots, cucumbers, radishes, tomatoes.	1.2-8.7 %	(Monge & Chinchilla 1996)
Costa Rica	Lettuce, parsley, cilantro, blackberries	4-24 %	(Abou el Naga 1999)
Egypt	Rocket, lettuce, parsley, leek, green onions	29.3 %	(Said 2012)
Iran	Mint, leek, cress, green onions, coriander, basil	1.1-14.8 %	(Ranjbar-Bahadori et al. 2013)
Nepal	Radishes, cabbage, mustard leaves	3.3-16.7 %	(Ghimire et al. 2005)
Norway	Lettuce, mung bean sprouts	4 %	(Robertson & Gjerde 2001)
Norway	Mung bean sprouts	8 %	(Robertson et al. 2002)
Peru	Cabbage, celery, cilantro, green onions, green chili, leek, lettuce, parsley, yerba buena, basil	14.5 % and 19.4 %	(Ortega et al. 1997)

* Modified from Dixon 2015

4.1.4 Cryptosporidiosis

Cryptosporidiosis is a gastrointestinal infection caused by *Cryptosporidium* spp. Parasites cause destruction and increased permeability of the epithelial layer of the intestine, resulting in self-limiting diarrhea in immunocompetent individuals, and life-threatening persistent diarrhea in immunocompromised (Rossle & Latif 2013). The infection can cause symptoms like abdominal pain, nausea, low-grade fever, fatigue and vomiting (Caccio & Chalmers 2016). Infants, young children and elderly may also experience dehydration and malnutrition (Fayer & Xiao 2008). Cryptosporidiosis can also be asymptomatic.

The immune system is the most important factor when it comes to susceptibility of hosts to cryptosporidiosis (Bouzid et al. 2013). A weakened immune system gives a higher probability of infection, and increased burden of disease.

Human infection is associated with at least 20 different species and genotypes of *Cryptosporidium*, but is primarily caused by *C. parvum* and *C. hominis* (Cacciò & Widmer 2014; Ryan et al. 2014). Collected data shows that these two species are responsible for over 90 % of cases of cryptosporidiosis (Xiao 2010). *C. parvum* is the most common zoonotic species, reported in 152 mammalian hosts (Fayer et al. 2000). Other species and genotypes infectious to humans are *C. meleagridis, C. felis, C. canis, C. ubiquitum, C. cuniculus, C. viatorum, C. muris, C. suis, C. fayeri, C. andersoni, C. bovis, C. scrofarum, C. tyzzeri, C. erinacei* and horse, skunk and chipmunk l genotypes of *Cryptosporidium* (Ryan et al. 2014).

C. parvum has a low infectious dose, with ID_{50} of 132 oocysts for one strain in a study including 29 healthy volunteers (DuPont et al. 1995). *C. hominis* also has a low infectious dose, estimated to ID_{50} 10-83 oocysts in a study with 21 healthy volunteers (Chappell et al. 2006). With the high number of oocysts shed by animal and human infected hosts, one gram feces possibly containing as much as 10 billion *C. parvum* oocysts, the risk of infection by *Cryptosporidium* spp. is present when in contact with infected people (Caccio & Chalmers 2016; Carey et al. 2004).

Diagnosis is usually performed by detection of oocysts in feces of the infected individual. Oocysts are too small to be seen in feces without the use of a microscope (Caccio & Chalmers 2016). No consensus has been reached regarding which diagnostic method is superior, and several methods are available. Therefore, different diagnostic practices are used at different laboratories. See section 4.1.7 Methods for diagnosis and detection of *Cryptosporidium.*

Cryptosporidiosis can be treated with nitazoxanide in immuncompetent individuals, but it is not effective in individuals with a weakened immune response (Gargala 2008).

4.1.5 Outbreaks

There have been numerous waterborne outbreaks of cryptosporidiosis, the largest being in Milwaukee in USA in 1993 where 403 000 people were infected (Mac Kenzie et al. 1994). More recently, a large outbreak of waterborne cryptosporidiosis resulted in approx. 27 000 infections in Östersund, Sweden in 2010 (Widerström et al. 2014).

Foodborne outbreaks of cryptosporidiosis are most frequently reported in Europe and USA, which could reflect a higher degree of resources applied to detection and reporting compared to developing countries (Robertson & Chalmers 2013). Foodborne outbreaks of cryptosporidiosis are presented in table 3.

Table 3. Foodborne outbreaks of cryptosporidiosis*

Country	Source	No. of cases	Reference
Mexico	Cow's milk	22	(Sterling et al. 1986)
USA	Chicken salad	15	(CDC 1996)
USA	Cow's milk	50	(Gelletlie et al. 1997)
UK	Apple cider	154	(Blackburn et al. 2006)
USA	Green onions	54	(CDC 1998)
USA	Apple cider	31	(CDC 1998)
Spain	Clams/mussels/oysters	-	(Freire-Santos et al. 2000)
Spain	Mussels/cockles	-	(Gomez-Bautista et al. 2000)
USA	Fruit/vegetables	148	(Quiroz et al. 2000)
Australia	Cow's milk	8	(Cowell et al. 2002)
USA	Mussels	-	(Miller et al. 2005)
USA	Apple cider	23	(CDC 1996)
Japan	Raw meat and raw liver dish	4	(Yoshida et al. 2007)
Denmark	Salad buffet	99	(Ethelberg et al. 2009)
Finland	Salad	72	(Pönka et al. 2009)
Chile	Snails	-	(Neira et al. 2010)
Sweden	Fresh herbs	-	(Gherasim et al. 2012)
UK	Pre-cut mixed salad leaves	>300	(McKerr et al. 2015)
Finland	Salad	>250	(Åberg et al. 2015)
UK	Pre-cut salad leaves	>300	(Anonymous 2013)

-: unknown
* Modified from Rossle et al. 2013, and Robertson et al. 2014

4.1.6 Routes of transmission

Cryptosporidium oocysts are usually transmitted indirectly through water, or directly from an infected to a healthy individual (Dixon 2016; FAO/WHO 2014). Transmission can also be

foodborne. In USA, 8 % of annual disease caused by transmission from food is caused by *Cryptosporidium* spp. (Scallan et al. 2011).

There are several ways that oocysts can enter the food chain. Food or equipment can be contaminated directly from infected individuals or by food handlers who are in contact with infected people (Dixon 2015). This can happen throughout the food chain. Washing of fresh produce and equipment with contaminated water is another transmission route, as is irrigation with contaminated water. Water used on fresh produce can contain oocysts if contaminated with human sewage or agricultural runoff. Oocysts can contaminate fresh produce if livestock has access to fields and production areas, or if manure is used as fertilizer. Climatic changes may affect the rate of transmission of *Cryptosporidium* spp. With increased precipitation comes the possibility of increased fecal runoff into water and flooding, so that viable oocysts can be spread to a greater extent (EEA 2007 ; Semenza et al. 2012).

Although food is not the major transmission route of *Cryptosporidium* spp. infection, it is important to take into consideration from a food safety perspective. With the growing rate of globalization, travelling and international food distribution, comes an increased possibility of transmission from food, also in developed countries (Dixon 2015; Hoorfar 2011). Trends of eating natural and minimally processed food is also a contributing factor to the increased risk of foodborne transmission of *Cryptosporidium* spp.

Good agricultural practice, good manufacturing practice and food safety measures to avoid contamination by *Cryptosporidium* should be implemented in production and handling of fresh produce (Dawson 2005). Measures at the farm level should involve that water free of *Cryptosporidium* oocysts is used for irrigation and distribution of pesticides (FAO/WHO 2014). UV irradiation, ozone-treatment or membrane ultrafiltration are methods effective in reducing oocyst viability in wastewater (de Velásquez et al. 2006; Kalisvaart 2004; Lonigro et al. 2006). Livestock and wild animals should be prevented from entering farmland and being in contact with water used for production. Runoff from animal excrement should be prevented from entering surface waters and animal excrements should not be used as fertilizer. In a study of irrigation waters in USA and several countries in Central America, 36 % of irrigation waters tested positive of *Cryptosporidium* spp. oocysts (Thurston-Enriquez et al. 2002). These irrigation waters were used in the production of fresh produce traditionally eaten raw. *Cryptosporidium* have also been found in water used for irrigation of fresh produce in South-Africa (Duhain et al. 2012). In another

study from Mexico evaluating surface water used for irrigation plus equipment used for washing, 48 % of water samples and 16 % of equipment samples tested positive for *Crypsosporidium* oocysts (Chaidez et al. 2005). A study was conducted in Spain investigating the occurrence of *Cryptosporidium* in irrigation water and on the fresh produce irrigated with this water (Amoros et al. 2010). The water came from a natural source used for irrigation, which also contained wastewater. All samples of the irrigation water were positive for *Cryptosporidium*, and 63.1 % of the salad products tested, were positive. This indicates that irrigation water can be a vehicle for transmission of *Cryptosporidium* to fresh produce.

Measures at the food handler level should ensure that water free of *Cryptosporidium* oocysts is used for production and washing of fresh produce and equipment (FAO/WHO 2014). Information and control measures for personal hygiene and cross-contamination should be implemented in food safety protocols for food handlers both pre- and post-harvest. Good hygienic practice should be implemented. Food needs to be handled with a high level of hygiene by infected food-handlers, infected consumers, or people in contact with infected people, to avoid contamination. Although washing of fresh produce might not remove all infectious oocysts, washing of fresh produce is advisable.

4.1.7 Methods for diagnosis and detection of *Cryptosporidium*

A number of laboratory methods are available for diagnosis of *Cryptosporidium* oocysts: staining with fluorescent or acid fast stains, enzyme immunoassays, immunochromatographic methods and molecular methods (Caccio & Chalmers 2016). A study by Chalmers et al. shows that immunofluorescence microscopy is more sensitive than enzyme immunoassay and immunochromatographic lateral flow, as well as being more sensitive than UK standard methods of staining with auramine phenol and modified Ziehl-Neelsen (Chalmers et al. 2011). Immunofluorescence microscopy is the overall preferred method for detection of *Cryptosporidium* spp. in Europe and in USA (Checkley et al. 2015). Molecular methods have been developed extensively in research laboratories in the last decade, yielding polymerase chain reaction (PCR) methods of high sensitivity and specificity for detection of *Cryptosporidium* spp. (Hadfield et al. 2011). Molecular methods may become the preferred diagnostic tool in the future, as they become automated and cheaper and need not rely on the microscopic skills of laboratory technicians. PCR and subsequent sequencing are common methods used for

diagnosis. Amplification of the small subunit (SSU) 18s rRNA can be used for determination of species, while GP60 is used for subtyping of *Cryptosporidium* (Checkley et al. 2015).

With regards to detection in environmental matrices, the U.S. Environmental Protection Agency (EPA) have standard methods for detection of *Cryptosporidium* spp. in water: Methods 1622 and 1623 (US EPA 1622:2005 ; US EPA 1623:2005). The International Organization for Standardization (ISO) has also published a method 15553 for detection of *Cryptosporidium* and *Giardia* in water (ISO 15553:2006). In addition there is an ISO Standard Method (18744) for detection of *Cryptosporidium* and *Giardia* in fresh leafy green vegetables and berry fruits (ISO 18744:2016). From this ISO standard, a reduced cost laboratory protocol (Veg-i-trade Method) has been developed, using less expensive buffers and fewer IMS-beads (Utaaker et al. 2015). The standardized methods and the reduced-cost method are used for detection and enumeration of oocysts. They do not identify species, and they cannot be used for viability assessment of the oocysts.

ISO Standard Method 18744 and the Veg-i-trade method is based on elution of potential oocysts from the fresh produce, concentration and extraction of *Cryptosporidium* oocysts by immunomagnetic separation (IMS), then detection by staining by immunofluorescent antibody testing (IFAT), analysis by fluorescent microscopy and differential interference contrast (DIC) microscopy.

Elution is when oocysts are removed from the fresh produce. By adding a deteargent-based buffer and mechanical treatment, oocysts are removed from the food matrix and collected in the eluate. The eluate is concentrated by centrifugation before IMS.

IMS is a method used to specifically separate and concentrate oocysts from residual material in the eluate, by means of *Cryptosporidium* spp. oocysts wall protein monoclonal antibodies (mAb) conjugated to paramagnetic beads (Fayer & Xiao 2008). This step is important as various organic and inorganic elements in the sample matrix might disturb or inhibit detection of the oocysts. When placed in a magnet, the magnetic beads will be attracted to the magnet, allowing removal of the residual solution while retaining the *Cryptosporidium* spp. oocysts at the magnet. Kits used for IMS with buffers, magnetic beads, and instructions from the manufacturer are commercially available.

Immunofluorescent antibody testing (IFAT) is based on labelling the *Cryptosporidium* oocysts with a wall-specific mAb conjugated with a fluorochrome. Fluorescein isothiocyanate

(FITC) is often used (Fayer & Xiao 2008). FITC is visible in fluorescent light at 480 nm excitation, 520 nm emission (ISO 18744:2016). After FITC staining, samples are analysed by immunofluorescent microscopy to detect the *Cryptosporidium* oocysts, as the FITC-C-mAb binding to the wall makes the outer contour of the oocyst visible in fluorescent light. A number of kits for staining of *Cryptopsporidium* spp. with FITC are commercially available.

4.1.8 Methods for assessment of *Cryptosporidium* viability and infectivity

Several methods are available to determine viability in oocysts. These methods are based on different principles, and can cause variation in viability results as some methods are more sensitive than others (King & Monis 2007). Animal bioassays, like neonatal mouse assays, are considered the gold standard, as these methods give a secure observation of animal infectivity induced by the oocysts. They have been widely used assessing *C. parvum* oocyst infectivity (Bukhari et al. 2000; Rochelle et al. 2002). These methods are however expensive, and comprehensive in regards to workload. In addition, ethical issues are associated with these methods, plus neonatal mouse assays cannot be used to assess viability of *C. hominis* oocysts.. Cell culture methods is combining *in vitro* excystation with viability assay based on invasion of monolayers of cells. These methods are associated with a higher degree of variability than animal bioassays (Robertson 2014). For viability assays of the monolayer, IFAT, *in situ* hybridisation and molecular methods like reverse transcriptase PCR and real-time PCR have been used. Fluorescent *in situ* hybridisation (FISH) is another method of oocyst viability that has been widely used. It is suited for *C. parvum* assays, but has been reported to give a rather unprecise determination of oocyst infectivity of *C. hominis* (Jenkins et al. 2002; Rochelle et al. 2002). A method based on detecting mRNA transcripts from viable oocysts, single *in vitro* excystation method and vital dye staining are considered less sensitive methods for assessment of viability in *Cryptosporidium* spp. oocysts compared with animal bioassays (Robertson 2014). The last two indicating a falsely high level of infectivity.

One of these viability assays of *Cryptosporidium* spp. oocysts using vital dyes is based on inclusion/exclusion of fluorescent dyes 4'.6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) (Campbell et al. 1992). DAPI is used to visualize the presence of cellular nuclei, as DAPI colour binds to A- and T-rich regions of deoxyribonucleic acid (DNA), making them visible by fluorescent microscopy. Coloured nuclei have 20 times brighter fluorescence compared to unstained material (Kubista et al. 1987). DAPI-stained material can be seen at 375 nm excitation,

> 420 nm emission (ISO 18744:2016). Propidium iodide (PI) is a fluorescent stain that can only penetrate disrupted cell membranes. It is visible by fluorescent microscopy at 555 nm excitation, 630 emission (ISO 18744:2016). Inclusion or exclusion of PI and DAPI in *Cryptosporidium* spp. oocysts are used in viability assays to determine if the oocysts are dead, alive, viable at assay or viable after further triggering. Criteria for viability assessment are presented in table 4. It is based on the viability criteria described in a study on how inclusion or exclusion of fluorogenic vital dyes correlate to *in vitro* excystation (Campbell et al. 1992).

Table 4. Criteria for viability assessment of *Cryptosporidium* oocysts*

	Dead	**Viable** at assay	**Viable** after further trigger	**Ghost**
DAPI	Positive	Positive	Negative	Negative
PI	Positive	Negative	Negative	Negative
DIC	Visible content	Visible content	Visible content	Not visible content

- DAPI-positive: dotted sky blue colour inside the oocyst at DAPI filter
- DAPI-negative: oocyst has a continuous monochrome light blue colour without dots at DAPI filter
- PI-positive: red colour inside the oocyst at PI filter
- PI-negative: red colour visible on the outer membrane of the oocyst, not inside the oocyst

* Modified from Campbell et al. 1992

4.1.8 Inactivation of oocysts

Though *Cryptosporidium* spp. oocysts are highly robust, a number of physical factors and chemicals are reported to reduce the infectivity of oocysts (Fayer & Xiao 2008). Heat treatment, freezing, gamma irradiation, UV irradiation, high pressure, drying, addition of chemical disinfectants like chlorine and hydrogen peroxide are all examples of vehicles that have been shown to reduce oocyst viability. In the food industry, where chemicals toxic to humans cannot remain on the edible products, physical treatments are preferred. In this regard, heat treatment, freezing, and drying are methods already used to a large extent by the food industry today. They are applied to inactivate or to inhibit the proliferation of general pathogenic microorganisms in the food. There has been little focus on parasites in the food safety context compared with bacteria and virus. Critical values (temperature and duration) for inactivation of *Cryptosporidium* spp. by heat treatment, drying and freezing has not yet been fully established.

When it comes to freezing, most laboratory experiments on *Cryptosporidium* spp. have involved oocysts suspended in water. There have been few experiments on fresh produce.

Industrial freezing of food is the process where the temperature of food is lowered to - 18 ^{o}C or lower, for preservation (Owen et al. 1973). Freezing is considered the most natural way of

preserving food. Lowering the temperature reduces the activity of enzymes, microorganisms and metabolic processes. At the same time, the products maintain their texture, taste and nutrients.

Which mechanisms in the freezing process that cause inactivation of microorganisms is not fully known, since freezing can cause damage to microorganisms by several mechanisms. When ice crystals form, residual solutes and microorganisms are concentrated, possibly inducing diffusion of water from the microbial cells (Archer 2004). Freezing can affect the viability by a high concentration of solutes inside or outside of the microorganism, by mechanical damage by ice crystals, or the low temperature itself (El-Kest & Marth 1992). Several factors could have a contributing role in the inactivation of microorganisms: rate of freezing, storage temperature, storage medium, presence of cryoprotectants, and nutritional status of the microorganism.

There are different types of equipment used in industrial freezing of food; for example, air blast freezers, tunnel freezers, belt freezers, fluidized bed freezers, contact freezers, plate freezers etc. (Barbosa-Cánovas et al. 2005). The method of freezing used depends on, and is customized to the product that is to be frozen, leading to a huge variety in freezing techniques.

With *Cryptosporidium* spp. being an increased threat to food safety worldwide, information about the duration of freezing needed to inactivate oocysts is of high importance.

5. Aim of the study

The aim of this study was to investigate the duration of time needed for *Cryptosporidium parvum* oocysts to be inactivated, when subjected to temperatures of - 20 ^{o}C on experimentally contaminated parsley and strawberry samples.

6. Materials and methods

6.1. Fresh produce and water

6.1.1 Origin

Potted parsley (*Petroselinum crispum*) from Rosnes and Ravnsborg gård in Norway was bought in the supermarket. It was kept in the refrigerator at approx. + 4 ^{o}C during the experiments, and watered as required.

Strawberries (*Fragaria* x *ananassa)* from Frutas Borja in Spain were bought in the supermarket. They were kept in the refrigerator at approx. + 4 ^{o}C during the experiments.

Water used during the experiments was filtered by Direct-Q 3 with pump from Millipore, and was therefore free of *Cryptosporidium* spp.

6.1.2 Analysis of fresh produce for contamination of *Cryptosporidium*

To investigate if the surface of the fresh produce was contaminated with *Cryptosporidium* spp. oocysts before the spiking experiments, samples were analysed using the ISO Method 18744, with modifications according to Utaaker et all 2015 (ISO 18744:2016 ; Utaaker et al. 2015). The main principle of this analysis is the IFAT method that is based on labelling the *Cryptosporidium* oocysts with mAb conjugated to fluorescein isothiocyanate (FITC). After FITC staining, samples are analysed by immunofluorescent microscopy to detect *Cryptosporidium* oocysts.

Two batches of parsley were analysed on two separate occasions, as the parsley experiments were done over a long period of time. The first parsley sample had a total weight of 30 g and consisted of leaves and stems from a whole potted parsley, plus two leaves from five other potted parsleys. The second parsley sample had a total weight of 9.7 g, derived from a single potted parsley. Strawberries from four different packages were analysed in one day. Two berries, one from each package, were analysed together (63.6 g and 58.0 g).

Prior to IFAT, fresh produce had to be eluted in glycine buffer, and possible oocysts concentrated by IMS. The same protocol as for section 6.3.4 ‘Elution and concentration of contaminated samples’ (main experiment) was followed, except in the end the sample plus 5 µl of NaOH was placed on a one welled slide (Novakemi, Handen, Sweden), instead of in a 1.5 ml centrifuge tube.

Post-IMS samples (55 µl) were dried in an incubator at + 37 ^{o}C, fixated with one drop of methanol, and stained with 50 µl of 1X FITC conjugated mAb (Aqua-Glo, Waterborne Inc., New Orleans, USA). The slides were placed in a humid chamber at + 37 ^{o}C for 30 minutes. Surplus mAb was tapped off by placing the slide in a vertical position, before counterstaining with 50 µl diluted DAPI (0.5 µl/ml) (Sigma-Aldrich, Saint Louis, USA). Slides were incubated at room temperature for 3-4 minutes. After incubation, slides were rinsed with a drop of filtered water, by tilting the slides in a vertical position and placing one drop of water above the well. With the slide in a horizontal position, one drop of mounting medium was added, before the well was covered with a cover slip (VWR, Leuven, Belgium).

Samples were analysed with immunofluorescence on a Leica DMCB microscope with FITC at 480 excitation, 520 emission, DAPI at 350 excitation, 450 nm emission, plus DIC microscopy at x20 and x40 magnification. Vegetables were considered positive of *Cryptosporidium* spp. if fluorescent oocysts at the right size (4-6 µm) were detected under the FITC filter, and they had the correct internal structures when viewed by DIC. The whole well was analysed for oocysts. For further details on assessment on fluorescent microscope, se section 6.3.6 'Viability assessment by fluorescent microscopy'.

6.2. Parasites

6.2.1 Origin

Parasites were extracted from bovine fecal samples. The fecal samples were collected from Norwegian Red cattle calves at Bygdøy Kongsgård. The calves were all less than five weeks old. Fecal samples were stored in a refrigerator at approx. + 4 ^{o}C during detection and extraction.

Oocysts were extracted from samples derived from two different calves. At the first collection one out of six samples tested positive for *Cryptosporidium* spp. oocysts (calf number 2600). This fecal sample was approx. 20 g and contained a high number of oocysts. This sample is presented in figure 2. At the second collection one out of three samples tested positive (calf number 2614). This fecal sample was approx. 90 g, but contained fewer oocysts than the fecal sample from calf 2600.

Figure 2. Fecal sample of calf 2600 used for isolation of *Cryptosporidium* oocysts for parsley samples.

6.2.2 Detection of oocysts in fecal samples

Detection of *Cryptosporidium* spp. oocysts in fecal samples was done by IFAT on direct smears.

Approximately 15 µl of fecal matter from each calf was placed on a microscopic slide (Elka, Assistent, Sondheim, Germany) using a soft loop. The samples were left to dry in an incubator (+ 37 ^{o}C), fixed with a drop of methanol and stained with 20 µl FITC mAb (Aqua-Glo, Waterborne Inc., New Orleans, USA) before incubation for 45 minutes at + 37^{o}C in a humid chamber. After incubation slides were placed in a vertical position while a drop of water was added over the smear, to remove surplus mAb. Next 5 µl of DAPI (Sigma-Aldrich, Saint Louis, USA) was added and the slides were incubated in room temperature for one minute. A drop of water was added to the smear with the slides in a horizontal position, before samples were covered with a cover glass (VWR, Leuven, Belgium).

Similar to the fresh produce samples, fecal samples were analysed with an immunofluorescent microscope at x20 and x40 magnification at two different filter settings; FITC at 480 excitation, 520 emission, DAPI at 350 excitation, 450 nm emission, plus DIC. Samples were considered positive of *Cryptosporidium* if fluorescent oocysts of the right size (4-6 µm) were detected under the FITC filter. For further details on assessment using fluorescent microscopy, see section 6.3.6 ‘Viability assessment by Fluorescent microscopy’.

6.2.3 Isolation of oocysts from fecal samples

Cryptosporidium oocyst were isolated from the fecal samples within 1-2 days after collection. Isolation of *Cryptosporidium* from fecal samples was based on salt flotation of the oocysts, after separating and removing the fatty parts of the feces using ethyl acetate (Fayer & Xiao 2008).

Initially, 12 g of feces was the basis for isolation for fecal sample 2600, split between four 50 ml plastic tubes. There were two tubes with 3 g of feces isolated on day one (2600-1) and two tubes on isolated on day two (2600-2). Fecal samples for isolation on the second day were filled with water, vortexed and stored in the refrigerator approx. + 4 ^{o}C from day one to day two.

Sample 2614 consisted of approx. 90 g feces. Because of the low number of oocysts in this sample, all fecal matter was divided between four 50 ml plastic tubes for isolation.

All fecal sample tubes used for isolation were filled half way up with tap water, vortexed, then filled with tap water to the 45 ml mark, before centrifugation at 3000 rpm for 10 minutes. The supernatant was discarded. Tubes were filled with approximately 10 ml wash buffer (as explained in the draft of EPA Method 1622 from 1997) vortexed, filled with 35 ml tap water and centrifuged at 3000 rpm for 10 minutes. These washing steps were repeated four times on fecal samples 2600-1, two times on fecal samples 2600-2 and three times on fecal samples from calf 2614. The washing step was repeated one last time with tap water on all samples. Fecal samples 2600-1 were filled with 20 ml ethyl acetate and vortexed, before they were filled with 30 ml water and vortexed. All the other samples were filled with water first, then ethyl acetate. Otherwise same amounts and procedure for vortexing. Vortexed samples were centrifuged at 3000 rpm for 10 minutes. After centrifugation, samples were separated into four different phases; pellet, aqueous phase, fatty phase, ethyl acetate. Ethyl acetate was removed using an automatic pipette, while the fatty phase was aspired using a plastic pipette, and the watery supernatant was discarded. Tubes were then filled with 25 ml saturated NaCl solution (36 g NaCl for every 100 ml filtered water), and vortexed to re-suspend the pellet before carefully placing a layer of filtered water on top. This was done by tilting the plastic tubes while adding filtered water by a plastic dropper, keeping the two phases separate. The samples were centrifuged at 3000 rpm for 10 minutes, and the upper layer (down to 25 ml) were transferred to a new tube by an automatic pipette. This suspension contained the oocysts and was washed twice; the tubes were filled with tap water and vortexed; samples centrifuged at 3000 rpm for 10 minutes; and supernatant discarded. All supernatant was discarded

the first time, second time discarded down to desired volume at approx. 5 ml. Samples were vortexed and placed in the refrigerator at approx. + 4 ^{o}C overnight for further purification.

6.2.4 Purification of oocysts from fecal samples

To ensure a clean isolate of *Cryptosporidium* oocysts, the fecal isolates were further processed by IMS. This enables extraction of oocysts by using paramagnetic beads coated with *Cryptosporidium* antibodies to separate the oocysts from residual fecal matter. See also section 6.3.4 'Elution and concentration of oocysts from contaminated samples' (main experiments).

Isolated oocysts from sample 2600-1 and 2600-2 were transferred to two separate L10 tubes. One of four tubes with fecal isolations from calf 2614 contained more fat and turbidity than the three others, and were transferred to a single L10 tube. The three other samples were free of fat and collected in another L10 tube. Samples are referred to as 2614-1 and 2614-2.

Samples 2600-1 and 2600-2 were mixed with 1 ml buffer A, 1 ml buffer B and 50 µl *Cryptosporidium* beads all from a kit called Dynabeads® GC-Combo (Life Technologies, Oslo, Norway) then sealed and placed on a rotator for one hour. After rotation tubes were placed in a magnet holder and manually rocked for one minute. The supernatant was discarded. Beads from each sample were resuspended in 400 µl diluted buffer A (1:10), and transferred to a 1.5 ml tube by using a glass Pasteur pipette. This was repeated two times. The 1.5 ml sample tubes were once again placed in MPC-M magnet, manually rocked for one minute and supernatant was discarded. Beads were mixed with 100 µl of 0.1M HCl. To separate oocysts from magnetic beads, samples were vortexed vigorously for 30 seconds, then left on the bench for 10 minutes, before another round of vigorously vortexing for 30 seconds. Samples were placed in a magnet holder and manually rocked for 30 seconds, before the supernatant containing the oocysts was transferred to a 15 ml plastic tube. Washing with HCl was repeated two times. Supernatant from both samples (200 µl) were collected in the same 15 ml tube for the first two HCl washing steps (400 µl), and mixed with 40 µl 1M NaOH to neutralize the acid. It is referred to as Stock 1. Supernatant from the last HCl washing step (200 µl) was collected in a separate 15 ml plastic tube, added 20 µl 1M NaOH and vortexed. This is referred to as Stock 2, and was a backup. Both Stock 1 and 2 were washed once with PBS, and the final volume adjusted to 5 ml PBS.

Sample 2614-1 and 2614-2 followed the same procedure for purification, but had different amounts of some solutions. Sample 2614-1 was mixed with 20 µl *Cryptosporidium* beads and washed with 300 µl diluted buffer A. Only 50 µl 0.1M HCl and 15 µl 1M NaOH was used for

oocyst-bead dissociation and neutralization, while sample 2614-2 was mixed with 80 µl *Cryptosporidium* beads, washed with 500 µl diluted buffer A, and 150 µl 0.1M HCl and 15 µl 1M NaOH was used for oocyst-bead dissociation and neutralization. Both purified samples were washed with PBS and transferred to 50 ml plastic tubes referred to as Stock 3 and Stock 4.

Antibiotics was added to the stock suspensions to avoid bacterial overgrowth: 100 U/ml penicillin (Life Technologies, Bleiswijk, Netherlands), 100 µl/ml streptomycin (Life Technologies, Bleiswijk, Netherlands) and 0.1 µg/ml amphotericin B (Life Technologies, Bleiswijk, Netherlands). Stock suspensions were stored in the refrigerator at approx. + 4.5 ^{o}C for storage.

Oocyst concentration was estimated in Stock 1, by counting oocysts in sub-samples diluted 1:100 and 1:1000. Using a slide with 10 wells (Novakemi, Handen, Sweden), 5 µl from each suspension was placed in 5 wells. IFAT was performed, using the same protocol as for vegetable samples explained in section 6.1.2, but using only 5 µl mAb and 2 µl DAPI (Sigma-Aldrich, Saint Louis, USA). Oocysts were counted in a fluorescent microscope, giving an approx. average content of 2.16 million oocysts/ml in Stock 1.

Similarly, oocyst concentration in Stock 3 and 4 was estimated by IFAT, but this was performed on 20 µl sub-samples from each stock (undiluted) on a one welled slide. The same protocol as for vegetable samples (section 6.1.2) was used, with 15 µl mAb and 5 µl DAPI. Oocysts were counted in a fluorescent microscope. Stock 3 had 428 oocysts per 20 µl. Oocysts in this stock were concentrated by centrifugation (3000 rpm for 10 min) and removal of supernatant (4.3 ml out of approx. 5 ml). This gave Stock 3 a rough estimate of 153 000 oocysts/ml.

Stock 4 had 509 oocysts per 20 µl counted in the well, giving approx. 25 000 oocysts/ml. This stock was used for contamination at the beginning of strawberry experiments without being further concentrated. But as it was difficult to count samples because of insufficient numbers of oocysts, Stock 4 was later concentrated. See section 6.3.2 ‘Experimental contamination of vegetable and water samples’ for further details.

Stock 1 was finalized on 13.01.2017. This is categorized as day 0. Likewise Stock 3 and 4 was finalized on 03.03.2017. It was noted how many days after oocyst isolation the different experiments were performed, as time from excretion could affect the viability of the oocysts and thereby the results.

6.2.5 DNA isolation, PCR and sequencing of oocysts

DNA from oocysts derived from calf 2600 and 2614 was isolated using a commercial spin column kit for DNA isolation (QIAamp® DNA Mini Kit Qiagen, Hilden, Germany) on a semi-automated system (Qiacube® Qiagen, Hilden, Germany) according to the manufacturer's instructions with a few modifications. Before proteinase K lysing, 100 µl from Stock 1 and Stock 4 was mixed with 150 µl TE-buffer (100 mM Tris and 100 mM EDTA) and the samples subjected to five rounds of freeze-thawing in liquid nitrogen and boiling water after which they were incubated on a heating block at + 90 ^{o}C for one hour. The samples were cooled for 10 minutes and centrifuged for 2-3 seconds. 100 µl ATL buffer (QIAamp® DNA Mini Kit) was added, and the tubes vortexed before addition of 20 µl Proteinase K. The tip of the pipette was used to mix the contents incubated at + 56 ^{o}C overnight. The tubes were centrifuged for one minute, and spin column isolation performed automatically on QIAcube instrument using a standard protocol for the QIAamp DNA Mini Kit Tissue with a final elution volume of 200 µl.

Samples were stored in the refrigerator at approx. + 4 ^{o}C before further processing by PCR.

For PCR the SSU and GP60 genes was targeted using Xiao Internal primers. PCR MIX for one PCR sample was 8.3 µl PCR water, 1 µl forward primer, 1 µl reverse primer, 0.2 µl BSA 20 mg/ml and 12.5 µl HotStarTaq. DNA isolate of 2 µl was added in the primer solution, before the sample was placed in the PCR machine iCycler Biorad.

For PCR for GP60 protocol of Glaberman et al. 2002 was used (Glaberman et al. 2002).

After PCR, samples were run on a 1 % agarose gel containing syber safe (Life Technologies, Oslo, Norway). Samples at a volume of 5 µl, were mixed with 1 µl loading dye (Life Technologies, Oslo, Norway) before application to the wells.

Samples were visualized with GeneGenious imaging system. Samples that showed strong bonds were further purified for sequencing using ExoSAP-IT (Life Technologies, Oslo, Norway). It was sent for Sanger sequencing at GATC Biotech AB.

Sequences were manually checked and aligned using Geneious software, and further compared with sequences in GenBank using BLAST, and the sub-genotype determined according to Fayer and Xiao (Fayer & Xiao 2008).

6.2.6 Viability assessment of oocysts in stock suspensions

The viability of the stock suspensions of oocysts (referred to as reference samples) were assessed. This was performed to obtain a reference for the number oocysts that were alive, dead, viable or ghost in the stock suspensions before the experiments. The protocol for viability assessment is described further in section 6.3.5/6 (main experiments).

The reference sample for parsley was analysed by counting oocysts from a 200 µl sub-sample from Stock 1, which was concentrated to 50 µl by centrifugation (14 100 rpm for 3 minutes). This was performed before the parsley experiments started, 21 days after oocyst isolation from the fecal matter. A set of six reference samples were also analysed at the end of most parsley experiments, 49 days after isolating the oocysts. By counting oocysts from 30 µl Stock 1 diluted in 70 µl filtered water, it was approx. 64 800 oocysts in each sample.

Reference samples for strawberry consisted of 30 µl Stock 4 diluted in 70 µl filtered water. A total of six reference samples were analysed, three days after the oocyst were isolated from the fecal matter.

'Elution and concentration of oocysts from contaminated samples' section 6.3.4 was not performed on the reference samples. These samples were only exposed to viability staining as described in section 6.3.5 'Viability staining of oocyst from contaminated samples'.

6.3. Experiments

6.3.1 Overview over experimental set-up

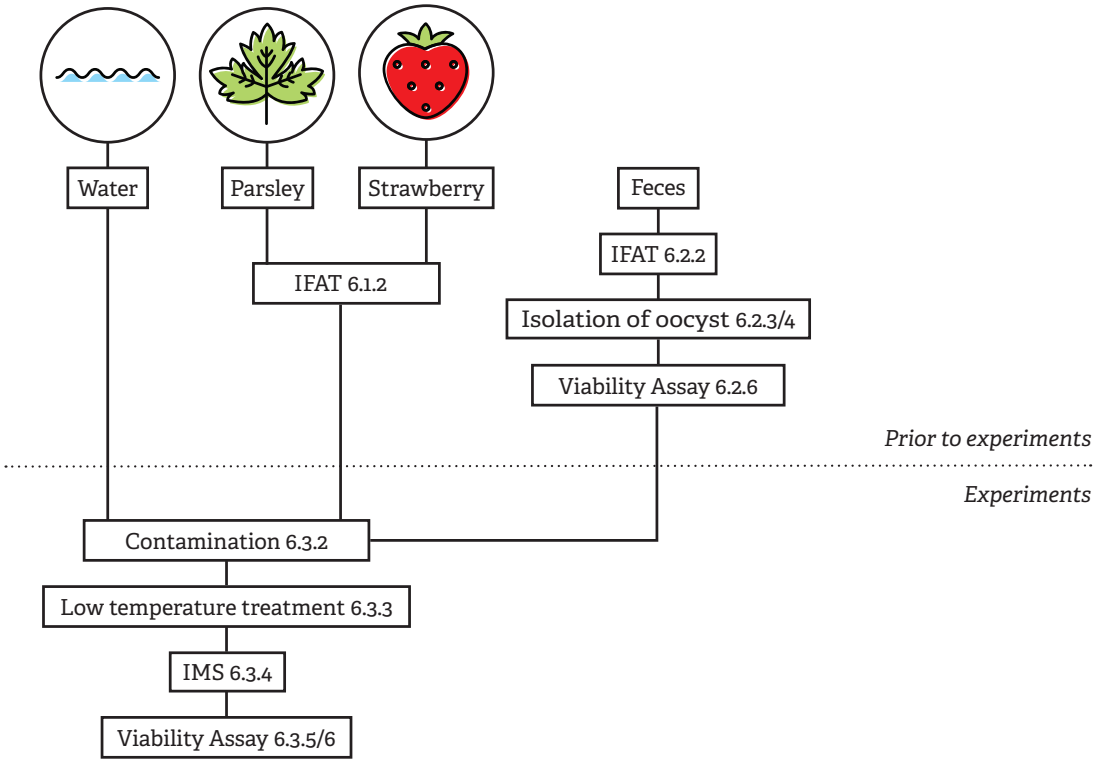

Figure 3. Overview over experiments

6.3.2 Experimental contamination of vegetable and water samples

Samples of 3 g (2.99-3.01) parsley or two strawberries (approx. 55 g) were placed in plastic containers prior to contamination. See figure 4 and 5. Vegetable samples were unwashed and dry. The contamination was applied in droplets, imitating droplets of naturally contaminated irrigation water. In addition, water samples with oocysts suspended in filtered water were kept in 1.5 ml centrifuge tubes.

Figure 4. Parsley sample.

Figure 5. Strawberry samples.

Stock 1 was used to contaminate parsley, reference samples for parsley and water samples. Stock 3 and 4 were used for strawberry samples and reference sample for strawberry.

Parsley samples were contaminated with approx. 20 000 oocysts, by diluting 10 µl Stock 1 in 90 µl filtered water and placing 10 droplets of 10 µl on different areas of the leaves.

Stock 3 and 4 was used for contamination of strawberries. Due to the low oocyst concentration in these stocks, they were used undiluted. Strawberries frozen for 4 hours were contaminated with 100 µl of Stock 4, in droplets of 10 µl, without addition of water. There were insufficient oocysts to count all samples, and Stock 4 was therefore concentrated. At this point Stock 4 was 3429 ml, and approx. half of the supernatant (1700 ml) was removed. This gave a new Stock 4 solution with approx. 50 000 oocysts/ml. The next samples (24 hours incubation and three of the 4 hours incubation samples that had too few oocysts to be counted earlier) were contaminated with 15 droplets of 10 µl Stock 4. This gave a contamination of approx. 7 500 oocysts to every sample. Stock 3 was used for the last strawberry samples that was frozen for 72 hours. Samples were

contaminated with 10 droplets of 10 µl Stock 3, giving a contamination of approx. 15 000 oocysts to every sample.

Water samples were made up of 10 µl Stock 1 and 90 µl filtered water. All six water samples were kept in one 1.5 ml centrifuge tube in the refrigerator, and divided before vortexing.

6.3.3 Low temperature treatment

All samples were placed in the freezer or refrigerator set at - 20 °C and + 4.5 °C directly after contamination. Freezing was performed in a Bosch GSN32A23 freezer, calibrated with a VWR EU620-0918 digital thermometer. Refrigeration was performed in an Electrolux ER8892C refrigerator, calibrated with a PA-54 digital thermometer.

Contaminated vegetables were kept in plastic boxes without lids throughout the cooling/freezing period. An overview over experiments and contaminated samples including fresh produce, temperature and time intervals is shown in table 5. There were six samples for all separate treatments.

Table 5. Overview over freezing and cooling regimes of contaminated vegetable and water samples.

Fresh produce	*Temperature*	*Time interval*
Parsley	Freezing - 20 °C	1 hour
		4 hours
		8 hours
		12 hours
		24 hours
		72 hours
	Cooling + 4.5 °C	1 hour
		4 hours
		8 hours
		12 hours
		24 hours
		24 hours + 1 hour freezing
		72 hours
Strawberry	Freezing - 20 °C	4 hours
		24 hours
		72 hours
Water	Cooling + 4.5 °C	1 hour
		4 hours
		8 hours
		12 hours
		24 hours
		72 hours

After freezing and before analysis, parsley samples were thawed for 15 minutes at room temperature, while strawberry samples were thawed for 25 minutes.

6.3.4 Elution and concentration of oocysts from contaminated samples

Elution, concentration and isolation of *Cryptosporidium* was based on the Veg-i-trade approach for IMS described by Utaaker et al. (Utaaker et al. 2015). The protocol was used with the intent of collecting *Cryptosporidium* spp. oocysts from the contaminated samples. This was performed by elution in glycine buffer, then centrifugation and IMS of oocysts, to concentrate them into a small volume for viability assessment.

Glycine buffer was added to the contaminated samples, 100 ml for parsley and water samples, 150 ml for strawberries and 200 ml for reference samples. The parsley samples were placed in filtered stomacher bags (Stomacher® 400 classic bags, Seward, West Sussex, UK) and stomached in a stomacher machine for one minute, while strawberry samples were put on an orbital shaker at speed 600 rpm for 10 minutes. Positive control parsley water samples were exposed to the same treatment as parsley. After the samples had been subjected to elution procedures, the glycine buffer was poured into 50 ml plastic tubes (two tubes for parsley and water samples, three for strawberries) and centrifuged at 3000 rpm for 10 minutes. The supernatant was aspired to 10 ml, before resuspension by vortexing. Each sample was combined in a single 50 ml plastic tube before another centrifugation step at 3000 rpm for 10 minutes. After centrifugation samples were aspired to approx. 7.5 ml before vortexing, and transmission to L10 tubes.

For IMS, 1 ml 10X Q4 buffer and 800 µl StabilZyme AP buffer (SurModics, Eden Prairie, USA) was added to the L10 tubes. The 10X Q4 buffer was made from 100 ml 10X Phosphate Buffered Saline (PBS) (Amresco, VWR, Solon, USA) and 50 µl Tween-20 (Sigma-Aldrich, Saint Louis, USA). Next 200 µl SL buffer B and 20 µl anti-*Cryptosporidium* beads from the commercially available Dynabeads® GC-Combo kit (Life Technologies, Oslo, Norway) was added to the L10 tubes. Tubes were rotated on a mixer (Dynabeads MX Mixer) at 20 rpm for one hour. Samples were placed in a MPC-1 magnet holder and manually rocked for two minutes. While holding the tube in the magnet, fluid was discarded by inverting it over a waste container. The glass tubes were removed from the magnet and 1.5 ml 1X Q4 buffer was added. The beads were gently resuspended (manually) before transmission to a plastic 1.5 ml eppendorf tube using a glass Pasteur pipette. The tubes were placed in a MPC-M magnet holder and manually rocked for one

minute before removing the supernatant using a glass Pasteur pipette. The tubes were removed from the magnet before addition of 50 µl 0,1M HCl and vigorously vortexing for 30 seconds. Tubes were left on the bench for 10 minutes, then vortexed for 30 seconds another time. Tubes were once again placed in the MPC-M magnet holder, and manually rocked for 10 seconds. Suspensions containing oocysts were transferred to a clean 1.5 ml centrifuge tube with 5 µl 1M NaOH.

6.3.5 Viability staining of oocysts from contaminated samples

The protocol for viability assessment of *Cryptosporidium* spp oocysts described here is based on inclusion/exclusion of immunofluorescent dyes, DAPI and PI. In addition FITC-labelled mAb was used for identification of the oocysts.

Samples from IMS of vegetable and water samples were added 900 µl HBSS (Biochrom GmbH, Berlin, Germany) and 10 µl 0.1M HCl, then vortexed and incubated at + 37 ^{o}C for 30 minutes. After incubation, samples were centrifuged at 14 100 rpm for 3 minutes. The supernatant was discarded, and the pellet resuspended and vortexed in 100 µl HBSS. The washing step, with centrifugation, removal of supernatant, resuspension and vortexing, was repeated. To assess the viability, 10 µl of DAPI (Thermo Scientific, Rockford, Germany) solution (2mg/ml) and 15 µl of PI solution (1mg/ml) was added. The samples were vortexed before incubation for 1,5 hours at + 37 ^{o}C. After incubation, 10 µl of 20X mAb (Aqua-Glo, Waterborne Inc., New Orleans, USA) was added, before another incubation period of 30 minutes at + 37 ^{o}C. Tubes were filled with 1400 µl of 1X PBS, vortexed and centrifuged at 14100 rpm for 3 minutes. The supernatant was removed until approx. 50 µl was left in the tubes. At the end samples were vortexed.

6.3.6 Viability assessment by fluorescent microscopy

Viability of the oocysts can be determined by inclusion or exclusion of DAPI and PI, and the presence of visible intact and non-deformed cell content (Campbell et al. 1992). Identification of *Cryptosporidium* oocysts based on mAb-binding, DAPI-staining and DIC microscopy optics is described in ISO 18744 (ISO 18744:2016).

To assess the *Cryptosporidium* spp. oocysts, 9 µl of each sample was placed on a microscopic slide without well (Elka, Assistent, Sondheim, Germany), and a cover glass applied (VWR, Leuven, Belgium). The cover slide was sealed around the edges with nail polish, to avoid

evaporation. All samples were kept in a closed sample collector in a dark room at ambient temperature until viewing by fluorescence microscopy.

The samples were assessed using a Leica DMCB fluorescent microscope, with DAPI (350 nm excitation, 450 nm emission), PI (500 nm excitation, 630 nm emission), FITC (480 nm excitation, 520 nm emission) filters and DIC optics.

One hundred oocysts were counted on each slide for each assay. Identification of oocysts was based on mAb staining, size and morphology. Exclusion criteria were different factors that inhibited a sufficient assessment including oocyst in a cluster or oocyst in areas of high turbidity. A few oocysts (approx. 10) were difficult to determine positive or negative using the vital dyes, and were therefore excluded from the assessment.

Criteria for viability assessment is based on the viability criteria described in a study on how inclusion or exclusion of fluorogenic vital dyes DAPI and PI correlate with *in vitro* excystation (Campbell et al. 1992). (See introduction section 4.1.7, table 4). Examples of oocysts on FITC filter and some of the different viability assessment criteria are presented in figures 6 to 9. Pictures are taken of oocysts from Stock 1 on the Leica DMCB fluorescent microscope, using program LAS X from Leica Microscopy. In addition DIC microscopy was used to assess cell content and morphology.

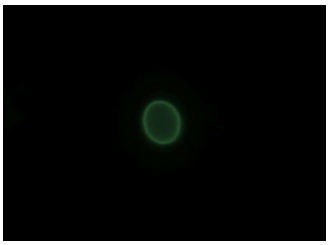

Figure 6. A *Cryptosporidium* oocyst on FITC filter.

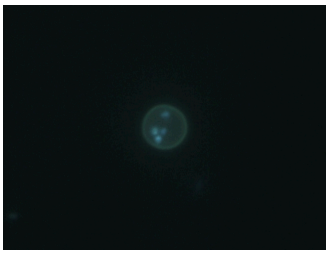

Figure 7. A DAPI-positive *Cryptosporidium* oocyst.

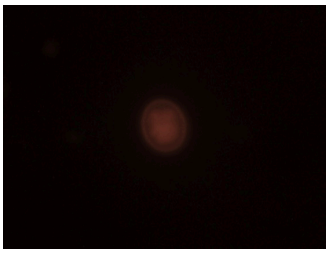

Figure 8. A PI-positive *Cryptosporidium* oocyst. Colours faded fast during photography due to high exposure. PI-positive oocysts appeared much brighter under the microscope.

Figure 9. A PI-negative *Cryptosporidium* oocyst. If the inside of the oocyst had a red colour density stronger than this, the oocyst was considered PI positive. If colour density was lower than this, the oocyst was considered negative.

6.4. Statistical analysis

Strawberry data were analyzed using linear regression with viability as the dependent variable and time in hours as the explanatory variable.

Parsley data were analyzed using a linear regression model comparing refrigerated parsley samples to frozen parsley samples with viability as the dependent variable and time in hours as the explanatory variable.

Water data were analyzed using linear regression with time as the variable.

p-values <0.05 was regarded as statistically significant.

7. Results

7.1. Fresh produce

7.1.1 Analysis of fresh produce

Parsley and strawberries were clean by appearance, with no visual soil or dirt. IFAT was performed to see if they were contaminated with *Cryptosporidium* oocysts before the experimental contamination. Both parsley samples tested negative for *Cryptosporidium* spp. oocysts. The two strawberry samples were found to be positive, with a total of 11 and 199 oocysts. These oocysts all had blurry and patchy walls. Although strawberries tested positive of *Cryptosporidium* spp. oocysts, visual appearance of the oocysts detected were substantially different from the oocysts isolated to be used in the experiments, and it was decided to proceed with these strawberries in the experiments.

7.2. Parasites

7.2.1 PCR and sequencing

Samples had strong bonds for both SSU and GP60, but only GP60 was successfully sequenced.

GP60 gave this sequence:
GTTCCTGTTGAGGGcTCATCATCGTCATCATCATCATCATCATCATCATCATCATCATCATCATCATCATCAT
CATCATCATCATCATCAACATCaacCGTCGCACCAgCAAataanGCAAgAactGgAgAAGAcgcanAanGCA
GTCAagATtctggtGgtactGAagCTtctGGTagccagGGttctGAAGAgGAagGtagtGAagGcgATGGcCAAActAntG
CTGCTTCCCaaCCCACTACTCCagCTcaaAgtGaAgGcgCAACTACCGAAACcatAgAAgCTACTCCAaAAG
AAgAatGCGG

The tandem repeats contained 22 TCA and one TCG. There was one ACATCA after the tandem repeat. This gave the subtype *Cryptosporidium parvum* IIaA22G1R1.

7.2.2 Viability assessment of oocysts in stock suspensions

Viability assessment of reference samples was performed to have an overview of oocyst viability. Results are presented in table 6.

Table 6. Reference samples. Viability of *Cryptosporidium* oocysts in stock suspensions 1 and 4

	Dead	*Ghost*	***Inactivated***	*Viable* at assay	*Viable* by stimuli	***Viable***	*DAI*
**Stock 1*	22.6	6.8	**29.4**	36.8	33.8	**70.6**	21
Stock 1	61.9	3.3	**65.2**	32.0	2.8	**34.8**	49
Stock 4	17.2	0.1	**17.3**	66.5	16.2	**82.7**	3

- Average values of 6 samples (n = 100)
- * Values based on 1 sample of 133 oocysts. Values presented as a percentage
- DAI: days after isolation

7.3. Experiments

7.3.1 Viability assessment of contaminated samples

Parsley samples were contaminated with droplets that contained *C. parvum* oocysts, before storage in the refrigerator or freezer for different lengths of time. Water samples spiked with oocysts from Stock 1 were kept in the refrigerator for the same lengths of time. Viability results of these samples are presented in table 7.

Table 7. Effect of freezing and refrigeration of *Cryptosporidium parvum* oocysts on contaminated parsley samples

	Hours	*Dead*	*Ghost*	***Inactivated***	*Viable* *at assay*	*Viable* *by stimuli*	***Viable***	*DAI*
Frozen	1 [b]	62.0	7.0	**69.0**	20.0	11.0	**31.0**	27
- 20 °C	4 [a]	54.7	14.3	**69.0**	19.5	11.5	**31.0**	25
	8 [a]	80.1	13.2	**93.3**	5.7	1.0	**6.7**	34
	12 [a]	87.0	11.8	**98.8**	0.7	0.5	**1.2**	31/34
	24 [a]	79.8	18.7	**98.5**	1.0	0.5	**1.5**	41
	72 [a]	79.5	18.5	**98.0**	1.8	0.2	**2.0**	66
Refrigerated	1 [b]	37.0	4.8	**41.8**	21.8	36.4	**58,2**	27
+ 4.5 °C	4 [a]	33.6	4.2	**37.8**	30.3	31.9	**62,2**	25
	8 [a]	55.3	7.0	**62.3**	17.9	19.8	**37,7**	34
	12 [a]	55.0	18.1	**73.1**	14.7	12.2	**26,9**	31
	24 [a]	62.0	17.3	**79.3**	6.7	14.0	**20,7**	41
	72 [a]	63.0	33.2	**96.2**	2.0	1.8	**3,8**	66
Water	1 [a]	57.0	5.0	**62.0**	25.0	13.0	**38.0**	45
+ 4.5 °C	4 [a]	59.3	4.4	**63.7**	25.8	10.5	**36.3**	45
	8 [a]	64.3	5.7	**70.0**	19.2	10.8	**30.0**	47
	12 [a]	65.0	5.2	**70.2**	23.2	6.6	**29.8**	47
	24 [a]	66.5	6.5	**73.0**	13.7	13.3	**27.0**	47
	72 [a]	69.8	6.2	**76.0**	20.3	3.7	**24.0**	66
R + F	24 + 1[a]	63.3	20.7	**84.0**	7.3	8.7	**16.0**	41

a: Average values based on 6 samples (N=6, n=100)
b: Average values based on 5 samples (N=5, n=100)
- DAI: days after isolation, R: refrigerated, F: frozen
- A few oocysts (approx. 5) was DAPI negative, but had a strong PI, and were counted as dead
- Viable at assay refers to oocysts that are DAPI-positive and PI-negative
- Viable by stimuli refers to oocysts that are DAPI-negative, PI-negative, with intact cell content seen on DIC microscopy

Statistical analysis was performed, comparing viability of oocysts on refrigerated parsley with oocysts on frozen parsley. A linear regression model with viability as the dependent variable and time in hours as the explanatory variable was used. The model explained 88 % of the variability, and showed a clear difference in viability between refrigerated parsley samples and frozen parsley samples after 8 hours ($p<0.001$). Difference between parsley samples were statistically significant ($p<0.001$) for all increasing time intervals (12, 24 and 72 hours). Figure 10 shows the predicted values of viability in oocysts in frozen and refrigerated parsley samples.

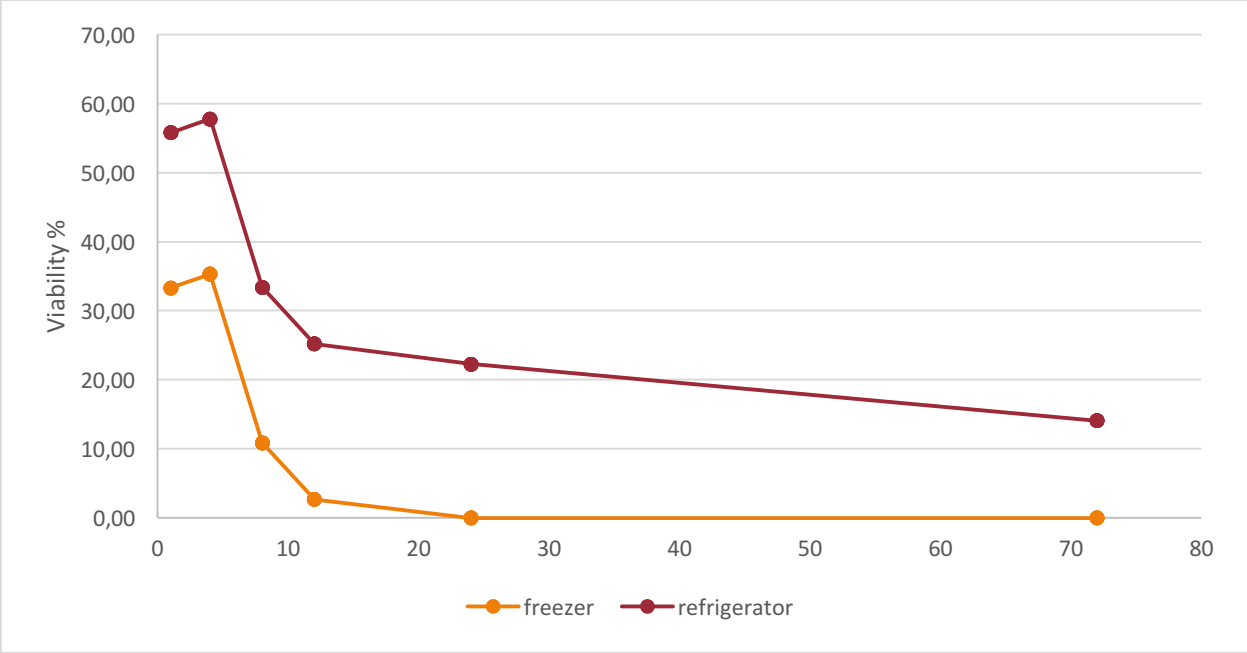

Figure 10. Predicted viability of *Cryptosporidium parvum* oocysts in parsley samples frozen at - 20 °C and refrigerated at + 4.5 °C for 1, 4, 8, 12, 24 and 72 hours

Strawberry samples were contaminated with droplets that contained *C. parvum* oocysts, before storage in the freezer at - 20 °C for 4, 24 and 72 hours. Viability results after treatment is presented in table X. Viability of frozen strawberry samples were compared to viability in reference samples of Stock 4. Strawberry data were analysed using linear regression with viability as the dependent variable and time in hours as the explanatory variable. As viability dropped to very low levels already after 24 hours, time was defined as a categorical variable in the regression. The model explained 92 % of the variability, and showed a clear drop in viability already after 4 hours ($p<0.001$). No difference was observed in viability between oocysts on strawberry samples frozen

for 24 and 72 hours (p=0.77). Figure 11 shows the raw data and the predicted line. p-values are presented in table 8.

Viability in water samples were analysed using time as a variable. The model only explained 29 % of the variability, giving a statistical decrease in viability after 24 hours (p=0.008).

Table 8. Effect of freezing of *Cryptosporidium parvum* oocysts on contaminated strawberry

	Hours	*Dead*	*Ghost*	***Inactivated***	*Viable* *at assay*	*Viable* *by stimuli*	***Viable***	*DAI*	*p*
Frozen - 20 °C	4 [b]	41.5	2.6	**44.1**	32.0	23.9	**55.9**	4/7	<0.001
	24 [a]	89.7	4.6	**94.3**	5.2	0.5	**5.7**	7	<0.001
	72 [a]	90.8	5.2	**96.0**	3.7	0.3	**4.0**	10	<0.001

a: Average values based on 6 samples (N=6, n=100)
b: Average values based on 7 samples, five samples with 100 oocysts, one with 98 oocysts, one with 94 oocysts, with values presented as a percentage
- DAI: days after isolation
- p: p-value of difference in viability between frozen strawberry samples and reference samples of Stock 4

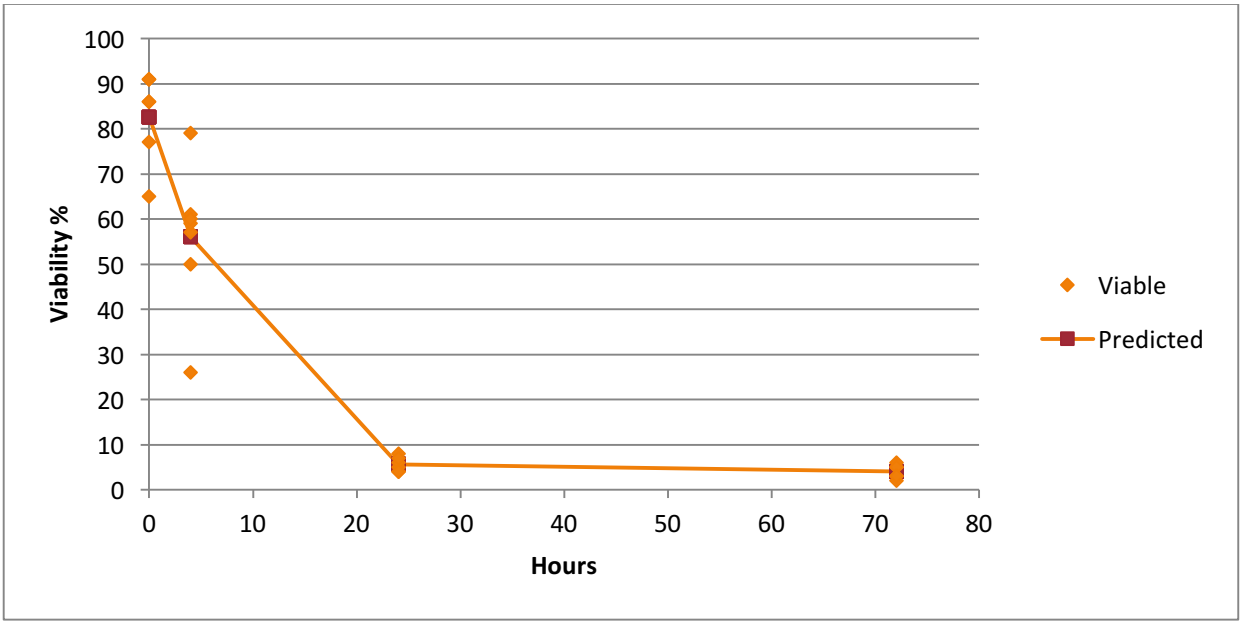

Figure 11. Viability of *Cryptosporidium parvum* oocysts on strawberry reference samples and strawberry samples frozen at - 20 °C for 4, 24 and 72 hours

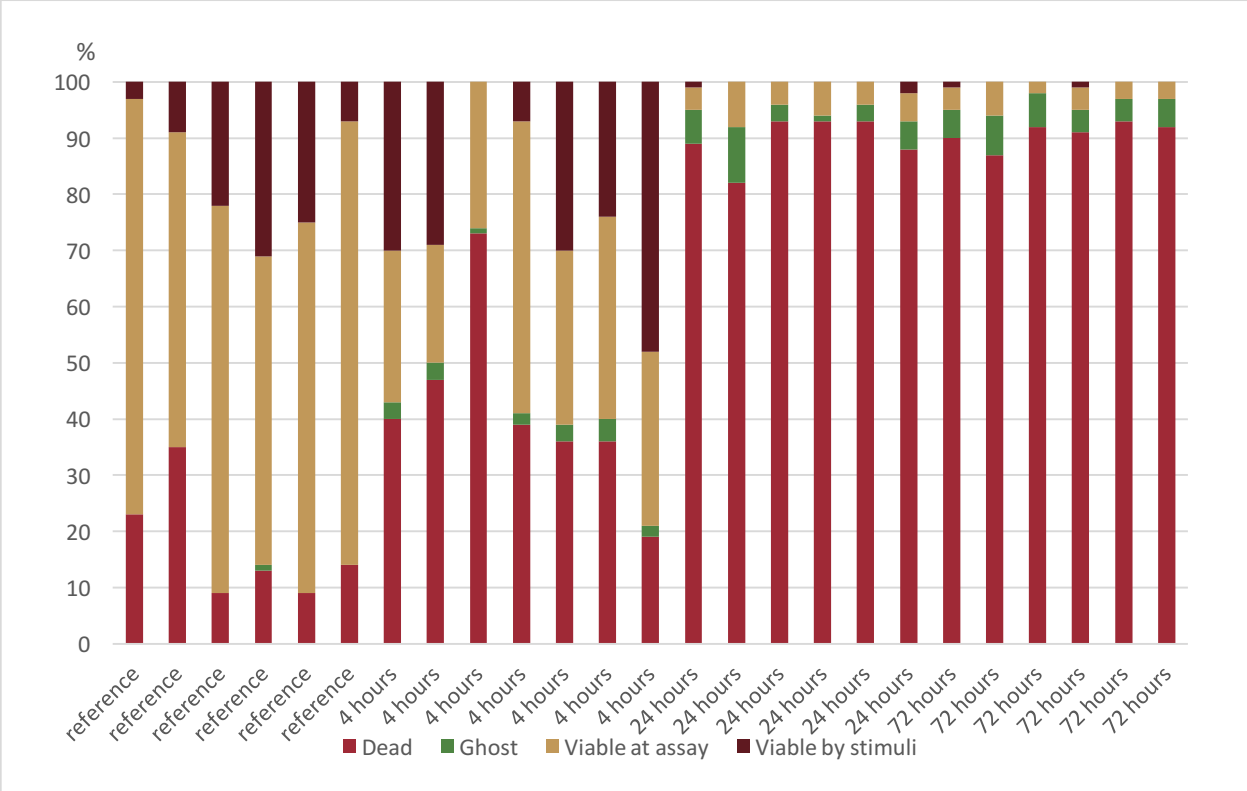

Figure 12. Viability of *Cryptosporidium parvum* oocysts in reference and frozen strawberry samples

8. Discussion

8.1. Experiments

Viability of *C. parvum* oocysts had a significant decline in both parsley and strawberry samples when exposed to freezing at - 20 ^{o}C. There was a significant difference between oocyst viability in strawberry reference samples and strawberry samples frozen for 4 hours or more. There was a significant difference in viability between oocysts of refrigerated parsley samples compared to frozen parsley samples after 8 hours or more, making freezing significantly more effective in inactivating oocysts than refrigerating. There was also a greater inactivation of oocysts in frozen and refrigerated parsley samples compared to refrigerated oocysts in suspension in water.

Although a complete endpoint for oocyst inactivation could not be determined based on these experiments, it is interesting to observe the decline in oocyst viability between food matrices when exposed to freezing. There were only 1.2 % viable oocysts in parsley samples after 12 hours in the freezer, but 5.7 % viable oocysts in strawberry samples after 24 hours in the freezer. The difference observed in viability between these food matrices is most likely due to the difference in oocyst viability of the stock suspensions used in the experiments.

In addition to the decline in oocyst viability observed, the results indicate that a small proportion of oocysts may remain viable for a prolonged time after the majority of oocysts were inactivated in both parsley and strawberry samples. The decline in viability observed in this study is similar to the logarithmical inactivation of bacteria by heat treatment, where a small fraction of the total bacteria will stay alive for a prolonged time (Fellows 2009). With a small fraction of *C. parvum* oocysts remaining stable and viable from 12 to 72 hours, this could indicate that oocysts excreted from the same host are not necessarily homogenous, and there could be a small sub-population of oocysts more resistant to freezing than others. A study by Su et al. has detected persistent subpopulations of *C. parvum* oocysts after disinfection treatments (heat and silver nanoparticles) using quantitative dielectrophoretic tracking (Su et al. 2014). Existence of *C. parvum* subpopulations that are more resistant to freezing than the 'average' oocyst cannot be ruled out. Further research that includes prolonged storage in the freezer and characterization of subpopulations are necessary to confirm this.

A study by Robertson et al. also using exclusion and inclusion of fluorogenic vital dyes for assessment of viability, found that *C. parvum* suspended in water exposed to - 22 ^{o}C for 21 hours,

resulted in a 67 % inactivation of oocysts (Robertson et al. 1992). After 775 hours at - 22 ^{o}C, there was a 98.2 % inactivation of oocysts. This is similar to the results in this study, where a small proportion of oocysts remain viable for a prolonged time, after the majority of oocysts are inactivated. There is however an extended time interval for the majority of oocysts suspended in water in the study by Robertson et al. to be inactivated, compared to this study. There were 97.1 % inactivated oocysts after 436 hours in - 22 ^{o}C in the study by Robertson et al., while 98.8 % of oocysts inoculated on parsley and frozen at - 20 ^{o}C in this study was inactivated after 12 hours. These results are most likely affected by the viability level of stock suspensions prior to freezing. In the study by Robertson et al. 79 % of oocysts in stock suspensions were viable before freezing. In this study 71 % (based on counting only 133 oocysts) were viable before experiments. This declined to only 35 % viable oocysts before performing the last parsley experiment where samples were frozen for 72 hours. The large difference in time interval needed for a close to total inactivation of oocysts suspended in water in the Robertson et al. study, compared to oocysts inoculated on parsley in this study, could however indicate that the storage matrix may have a significant effect on the inactivation rate of *C. parvum* oocysts during freezing. *Cryptosporidium* spp. oocysts are most likely found on the outer surface of the fresh produce, or sheltered in the outer layer in the stoma of plants (FAO/WHO 2014). Liquid food matrices where oocysts are in suspension throughout the product may need a prolonged freezing time to inactivate *Cryptosporidium* spp. oocysts compared to fresh produce where oocysts are situated on the outer layer of the product. Juice and milk have both contained viable *C. parvum* oocysts after three weeks of freezing at - 20 ^{o}C (Erickson & Ortega 2006). In liquid food matrices, where oocysts can be situated in the center of the product, freezing rates could be of high importance. The rate of freezing throughout fresh produce could be of less importance, as the low temperature are effective on the outer areas where oocysts are situated at a rapid pace.

A study by Macarisin et al. investigating oocyst attachment to apples by low-temperature scanning electron microscopy, found that *C. parvum* oocysts could be found both in surface crevices and on the smooth surface of apples (Macarisin et al. 2010). On the smooth surface it appeared as the oocysts were attached by an extracellular matrix. A glycocalyx-like layer around *C. parvum* has also been documented in a study by Nanduri et al. (Nanduri et al. 1999). This indicates that oocysts have the ability to adhere to vegetable surfaces, and that they might be difficult to wash off. If oocysts adhering to vegetables can be more resistant to freezing and have

a higher degree of viability is uncertain, as this study only examined oocysts that was eluted by glycine buffer.

Fayer and Nerad has conducted a comprehensive study investigating infectivity of *C. parvum* oocysts suspended in water and stored at + 5, - 10, - 15, - 20 and - 70 ^{o}C for different lengths of time (Fayer & Nerad 1996). After storage, oocysts were given to 5-7 BALB/c mice for each type of treatment, to see if the oocysts were viable and infective. Oocysts stored at + 5 ^{o}C (168 hours), -10 ^{o}C (8-168 hours), -15 ^{o}C (8-24 hours) and -20 ^{o}C (1-5 hours) caused developmental stages of *C. parvum* in all mice. Oocysts stored at -15 ^{o}C (168 hours), -20 ^{o}C (24-168 hours) and - 70 ^{o}C (1-24 hours) were not infective to any mice. Oocysts stored at - 20 ^{o}C for 8 hours caused a few developmental stages of *C. parvum* in one out of six mice. This confirms the viability and infectivity of oocysts stored at - 20 ^{o}C for up to 8 hours, by the gold standard neonatal mouse assay.

The study by Robertson et al. using exclusion and inclusion of fluorogenic vital dyes for assessment of viability, found that *C. parvum* suspended in water exposed to rapid freezing by liquid nitrogen resulted in a total inactivation of the oocysts (Robertson et al. 1992). The study by Fayer and Nerad also included freezing at - 70 ^{o}C for one hour, inducing inactivation of oocysts in all samples. Oocysts were still viable after freezing at 20 ^{o}C for 775 hours in the Robertson et al. study, and after 72 hours in this study. Oocysts were still infective after 8 hours freezing at - 20 ^{o}C in the Fayer and Nerad study. In a study by Dunhain et al., based on viability assessment using PI and a flow cytometer, blast freezing for four minutes, imitating the industrial freezing at - 20 ^{o}C only inactivated a small percentage of *C. parvum* oocysts inoculated on green peppers (Duhain et al. 2012). These findings indicate that lower temperatures induce higher degree of inactivation of oocysts compared to higher temperatures.

In this study, a great reduction in viability was observed between refrigerated parsley samples stored for 24 and 72 hours, with 79.2 and 96.3 % inactivated oocysts respectively. This reduction in viability could have been influenced by desiccation (Warnes & Keevil 2003). Parsley samples stored in the refrigerator for 12 hours still had droplets of contamination, while parsley samples stored for more than 24 hours had no visible droplets of contamination. Samples stored in the refrigerator for 72 hours showed signs of drying parsley leaves. The withering of parsley leaves gave an indication of desiccation.

A number of factors are involved in the presence of oocysts and their viability on contaminated fresh produce. Primary goals in the food industry should be preventing the original contamination. This can be done by adding preventive measures that hinders contamination via already documented transmission routes. Source of transmission could be essential to oocyst viability, as oocysts from the environment possibly could have low viability levels, while an infected food-handler that has poor hygiene can contaminate fresh produce with oocysts that has a high level of viability just before sale. Industrial production of fresh produce is most often time-consuming with harvesting, processing, packaging, transportation and storage, which could induce a natural decline in viability of oocysts dependent on environmental factors. With irrigation water as the source of contamination, oocysts could naturally lose viability or be inactivated before consumption if droplets of irrigation water dried out, and oocysts were exposed to desiccation. In a study by Robertson et al. investigating various environmental pressures to *C. parvum* oocysts, desiccation of 50 µl on glass slides that was air dried were found to induce 100 % inactivation of oocysts after 4 hours (Robertson et al. 1992). Fresh produce in the industrial food production is however preferably stored in cool and humid environments to reduce transpiration and respiration, and this could possibly help maintain the viability of oocysts (Fellows 2009). This is confirmed in a study on apples contaminated with *C. parvum* oocysts, stored at + 6 ^{o}C under conditions characteristic for fruit storage, which found oocysts to be infective to mice after 4 weeks of storage (Macarisin et al. 2010).

Viability of oocysts can be reduced by different industrial production steps, such as temperature treatment. Blanching is a common pre-treatment used for most vegetables that are to be frozen. It is used to inactivate enzymes and microorganisms. This is done by using steam or hot water in a time-temperature combination specific for the vegetables, usually between 70-100 ^{o}C for 1-15 minutes (Fellows 2009). In a study by Dunhain et al. investigating the effect of pre-treatment methods such as washing with chlorinated water (100 and 200 ppm) and blanching (+ 96 ^{o}C water bath for 3 min), plus blast freezing (blast freezer - 20 ^{o}C for 4 min) on viability of *C. parvum* on green peppers, washing with chlorine did not affect the viability, 93 % of the oocysts were inactivated after blanching, while 20 % were inactivated after blast-freezing (Duhain et al. 2012). In a study by Fayer, *C. parvum* oocysts became non-infectious to mice after heat treatment at + 72.4 ^{o}C for one minute (Fayer 1994). As *Cryptosporidium* spp. oocyst walls contains proteins, heat treatment causes detrimental effects to the oocyst due to denaturation. Heat treatment before

freezing is however usually not performed on berries and herbs, and freezing would be the main inactivation step for parasites. Freezing regimes sufficient to inactivate all *Cryptosporidium* spp. oocysts are currently not established. Whether some freezing regimes are better than others, is uncertain. Determining a single threshold with one freezing temperature for a set time that are to be used on all frozen vegetables could be challenging, as equipment and type of freezing is adapted to separate products. It could also be difficult to imitate the industrial freezing regimes in a laboratory setting, as equipment requires a lot of space. The industry would most likely not be willing to provide production facilities and equipment that involves experiments with heavily contaminated products.

Several factors affecting transmission and viability of oocysts in the food industry leads to great variety in concentration and viability of oocysts on fresh produce. Studies detecting *Cryptosporidium* spp. oocysts on fresh produce have reported between 1 and 156 oocysts per 100 g, on lettuce and celery respectively (Robertson & Gjerde 2001; Rzeżutka et al. 2010). *Cryptosporidium* spp. on fresh produce have most frequently been reported on vegetables that are usually not exposed to freezing, like different types of salads. Contamination on fresh herbs like parsley, cilantro and basil have also been reported. Of all the foodstuffs reported with *Cryptosporidium* spp. that are commonly exposed to freezing, the majority would most likely be exposed to heat treatment, for example boiling or frying, after freezing. These types of heat treatment will most likely be sufficient for inactivation of the oocysts. *Cryptosporidium* spp. has however also been detected in blackberries that have a high probability of being eaten without heating, neither before or after freezing (Abou el Naga 1999). Concentration of oocyst on the blackberries were not reported. If irrigation water was the source of contamination, level of oocysts could be the same in lettuce and berries. Based on the highest number of oocysts detected on lettuce from three different studies (0.6, 6 and 30 oocysts per 100 g), the average number of oocysts per 100 g was 12 oocysts (Amoros et al. 2010; Keserue et al. 2012; Robertson & Gjerde 2001). If the same number of oocysts were contaminated on 100 g berries, and these berries were exposed to freezing at - 20 ^{o}C for 24 hours with the same decline in viability as oocysts inoculated on strawberries in this study (from 82.7 to 5.7 % viable oocysts), number of viable oocyst would be 0.7 per 100 g of berries. With the reported ID_{50} of 132 oocysts for *C. parvum* and 10 oocysts for *C. hominis*, freezing would be sufficient in making oocysts on the contaminated berries non-infective, as long as the consumer ate 100 g of berries. This calculation is only an indication, as it

is based on non-specific documentation. Further research on freezing time and temperature needed for inactivation of *Cryptosporidium* spp. oocysts, detection of oocyst numbers on individual types of berries, and calculations of these are needed. It is worth mentioning that no cryptosporidiosis outbreaks have been associated with frozen products (Dixon 2015).

The report from FAO/WHO placing *Cryptosporidium* spp. parasite number 5 in a food safety perspective states: 'Although oocysts are somewhat resistant to freezing, they can be inactivated by storing produce at - 20 ^{o}C for >24 hours, or at - 15 ^{o}C for at least a week' (FAO/WHO 2014). The endpoint for oocyst inactivation could not be determined in this study, and it is therefore not possible to give any specific recommendations on freezing-time for fresh produce that can be implemented in a HACCP-plan to secure a product that is non-infectious by *Cryptosporidium* spp. However, this study showed that the FAO guidelines may need to be modified. Not all oocysts were inactivated after storage at - 20 ^{o}C for 72 hours. Vegetables that are to be frozen without blanching as a pre-treatment, should be exposed to low freezing temperature, industrial packaging with no further possibility for contamination, and prolonged storage at minimum - 20 ^{o}C. Further research is needed to find what time interval and what temperature of freezing is needed for a total inactivation of *Cryptosporidium* spp. oocysts on fresh produce.

8.2. Fresh produce

IFAT of strawberries gave a positive result of 11 and 199 oocysts seen as blurry circles with patchy walls on the FITC filter of the fluorescent microscope. The size of the circles in addition to them being visual on the FITC filter indicated that it was *Cryptosporidium* spp. oocysts. But they were visually not alike the oocysts observed in the main experiments, because of the blurry and patchy walls. There were only one oocyst similar to them seen in the experiments, and it was excluded (not counted) because of the substantial difference in morphology.

With the high number of 199 oocysts from one sample of two berries, indication of laboratory contamination was strong. L10 tubes were expected as a possible source of contamination to IFAT strawberry samples. L10 tubes were all kept on the rotator for one hour containing up to 20 000 oocysts in each experimental sample, and millions of oocysts in IMS samples of isolated feces. L10 tubes have lids with cardboard inside. This material could have been a possible source of

contamination, as it is assumed oocysts could get attached to the cardboard and transferred to the next sample although L10 tubes were treated with chlorinated water and washed in a dishwasher at + 65 ^{o}C between use. The blurry and patchy walls could indicate insufficient chlorination of *Cryptosporidium* oocyst. A possible explanation of why both strawberry samples tested positive, while no parsley samples were positive could be that the first IFAT sample of parsley was performed at the beginning of parsley experiments, while the IFAT strawberry samples were performed after the majority of the parsley experiments had been done. A total of 18 L10 tubes were in circulation. There was a higher probability of contaminated L10 tubes used for IFAT samples of strawberries compared the first IFAT sample of parsley, as a higher number of L10 tubes had been in use. Laboratory routines changed after the positive IFAT strawberry samples. L10 lids were then treated with undiluted hypochlorite (sodium hypochlorite solution with 4 % active chlorine) overnight. The last IFAT sample of parsley was done after the new routine of chlorination was implemented.

8.3. Parasites

Subtype *Cryptosporidium parvum* IIaA22G1R1 belongs to subtype family IIa that has been detected in humans in Ireland, Portugal, Slovenia, Netherlands, Australia, Japan, Kuwait, Canada, USA (Xiao 2010). This subtype family of *C. parvum* is not common in humans in developing countries (Fayer & Xiao 2008). The subtype has been detected in infected cattle in Germany, Argentina, Czech republic and Sweden (Broglia et al. 2008; Del Coco et al. 2014; Kváč et al. 2011; Silverlås et al. 2013). Even though subtype IIaA22G1R1 has not yet been reported in any foodborne outbreaks, it was considered a suitable subtype of *C. parvum* for these experiments.

8.4. Limitations of the study

Viability assays based on exclusion/inclusion of vital dyes on oocysts are associated with an over-estimation of infectivity. This is better than being associated with an under-estimation of infectivity, seen from a food safety perspective. Viability methods under-estimating infectivity of oocysts could result in treatments for inactivation of *Cryptosporidium* spp. oocysts that were

insufficient. This will most likely not be the case when results are associated with an over-estimation of infectivity.

Viability assays based on exclusion/inclusion of vital dyes are much faster and cheaper compared to animal bioassays. This allows a more comprehensive study that includes a larger variety and number of experiments, compared to what could have been done in an animal bioassays in the same time-interval. Viability assays are also better from an ethical point of view.

In this study, parsley experiments were more comprehensive compared to strawberry experiments. The time span between oocyst isolation and the different parsley experiments was longer compared to the strawberry experiments. Viability assessments of oocysts are time-consuming, and the viability of stock suspensions could not be measured frequently. The single reference sample of viability of Stock 1 was taken before the experiments started, 21 days after isolation. Parsley experiments started 25 days after isolation. The six reference samples for parsley was performed 49 days after isolation. They should clearly have been taken before the experiments started, as the oocyst viability decreased over time. Commercial oocysts (WaterborneTM Inc., New Orleans, USA) are recommended to be used within 30 days for them to remain viable (Hohweyer et al. 2016). In comparison, viability assessment of six reference samples of oocysts in Stock 4 used for strawberry experiments was performed three days after isolation. This gave a higher level of viable oocysts in the stock suspension used for strawberry samples, as all strawberry samples in the experiments were contaminated within 10 days after isolation of the oocysts. This is why strawberry samples were the only fresh produce samples that were statistically compared to reference samples. Frozen parsley samples were only compared to their related refrigerated parsley samples.

Reference samples were not exposed to elution, concentration and IMS like all experimental samples were. The statistical results for strawberry samples that were statistically compared to reference samples could therefore be inaccurate, as the elution, concentration and IMS steps probably could have induced a reduction in oocyst viability in experimental strawberry samples only, and not in the reference samples.

Water samples were initially included in this study with the intent of being a basis for which frozen and refrigerated parsley samples could be compared. As the experiments were time-consuming, with water samples performed 20 days after parsley samples, the natural decline in

oocyst viability made water samples not comparable to refrigerated and frozen samples, and they were excluded in the statistical analysis.

With insufficient data on viability, it was difficult to compare the inactivation of oocysts in strawberry, parsley and water samples, and it was not possible to determine if there was a significant difference in inactivation rate or time between food matrices.

Viability of oocyst in parsley samples frozen for 12, 24 and 72 hours was increasing from 1.2 to 2.0 %. This is probably due to natural sample variation, as oocysts have not been reported to regain viability after inactivation.

As already mentioned, experiments in this study were time-consuming. There were increasing working-day-lengths as the time-interval of freezing went up, and experiments were carried out according to what was practically most convenient, and not in a chronological order after length of freezing. Freezing and refrigeration of parsley samples for 4 hours was the first experiment performed. This was performed 25 days after the isolation of the oocysts. A mixed solution residue of PI remaining after other research studies was used. On all subsequent experiments, a batch of freshly mixed PI was used. There was a higher range of viability in oocysts frozen and refrigerated for 4 hours, than 1 hour. Difference in intensity of the PI colours used could have been a contributing factor to these results, as the viability of oocysts is assessed by the intensity of the PI-colour on the microscope. A weaker colour intensity of PI of the oocysts may increase the number of false viable oocysts, as could have happened for the 4 hour parsley samples. One hour parsley samples were performed two days after the 4 hour samples. Even though viability of oocysts naturally decrease over time, two days are most likely not sufficient to cause the level of reduced viability observed in 1 hour parsley samples compared to 4 hour samples.

Assessment of viability of *Cryptosporidium* oocysts using exclusion/inclusion of vital dyes is based on visual appearance and colour intensity and is therefore subjective and operator dependent. When looking in the fluorescent microscope as the light was exposed to the samples, a rapid decline in intensity of fluorescent colour/light from the oocysts was observed. This is especially critical on the PI filter, as PI is used to evaluate if the oocysts are dead or could be viable. The person assessing the oocysts has his or her own perception of when an oocyst is considered dead or not dead, and the oocysts have a gradual transition in colour from a viable to a dead stage. In addition to the PI colour in the fluorescent microscope gradually loosing intensity, the intensity of the colour staining of the oocyst slides themselves can also vary. In view of all this,

the subjective assessment could sometimes be challenging. DAPI can also be difficult to determine positive or negative, as it was experienced that the intensity of the DAPI-positive areas could sometimes be very weak. However, DAPI assessment was not as critical for the viability results as PI was, since both DAPI-positive and DAPI-negative oocysts can be viable. DIC microscopy was used to reveal if a DAPI-negative oocyst was viable at further assay or if it was a ghost oocyst. Therefore, PI assessment was considered a critical point in viability assessment in this study, and a possible source of over-estimation of infectivity.

Animal bioassays measures infectivity, whereas vital dye assays, such as the one used in this study measures viability. Thus, direct comparison from these two types of studies is problematic, as they are based on different principles. When performing animal bioassays a large number of oocysts is needed to establish infection in the experimental animals. In a study by Peeters et al., a minimum of 1000 oocysts were needed to cause 100 % infection in mice (Peeters et al. 1989). Assays using vital dyes enable assessment of viability of individual oocysts. These methods can therefore be used to examine environmental samples, such as food samples, that are likely to contain low numbers of oocysts. However, the main limitation of vital dye assays is that it can only indicate the potential for infective potential of oocysts, i.e. oocysts that are infective will be viable, but viable oocysts are not necessarily infective. Infectivity of oocysts is host dependent, as the immunological status of a person/animal is essential for whether an infection occurs. The number of oocyst needed to cause an infection can also vary. In the Fayer and Nerad study, oocysts stored at - 20 ^{o}C for 8 hours caused a few developmental stages of *C. parvum* in one out of six mice. In the Robertson et al. study and in my study, a small proportion of *C. parvum* oocysts were viable after 775 hours and 72 hours storage at - 20 ^{o}C. However, this does not necessarily mean that these oocysts are infective and will cause an infection in a susceptible host. Methods assessing viability based on inclusion/exclusion of vital dyes show a high correlation with *in vitro* excystation (Campbell et al. 1992). These methods have both been associated with an over-estimation of *C. parvum* oocyst viability in studies including treatment with disinfectants (Black et al. 1996; Bukhari et al. 2000; Jenkins et al. 1997; Joachim et al. 2003; Korich et al. 1990). One reason for this over-estimation of viability in studies of disinfectants could be that this type of treatment may result in alterations of the oocyst wall that inhibits the infective potential of the oocyst by injuring attachment or invasion mechanisms, without causing structural damage that increases dye penetration (Bukhari et al. 2000). However, the study by Korich et al. show

acceptable correlation between excystation methods and mouse assays (Korich et al. 1990). As there is a high correlation between viability assessment based on inclusion/exclusion of vital dyes and based on *in vitro* excystation, vital dye assays would be expected to correlate well with mouse infectivity assays. An overestimation of viability in the experiments performed here cannot be dismissed. Although the results in this study may have over-estimated the oocyst infectivity, the indication of a few viable oocysts and a subpopulation of *C. parvum* oocysts which are more robust to freezing than others, is however, still strong. And even if this study over-estimated viability/infectivity, it would give a good indication of trends, as a possible over-estimation would occur in all samples.

9. Concluding remarks and future perspectives

Cryptosporidium spp. have been found in irrigation water and on the surface of vegetables worldwide. Measures preventing contamination of fresh produce by this parasite should be implemented globally. Measures to inactivate the parasite should also be implemented in food safety programs in the food industry, to ensure safe products.

C. parvum oocysts inoculated on parsley and strawberry had a significant decline in infectivity with only 1.2 % viable oocysts in parsley samples after 12 hours, and 5.7 % viable oocysts in strawberry samples after 24 hours, when stored at - 20 ^{o}C. The level of viable oocyst at this point seemed to retain their viability also in samples stored for 72 hours in the freezer at the same temperature. This indicates that a small subpopulation is more resistant to freezing than the 'average' oocyst.

Total inactivation of oocysts was not observed at any time interval in this study. More research is needed to determine the time-interval needed for total inactivation of *Cryptosporidium* spp. oocysts.

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