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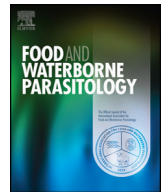
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## Evaluation of a modified method for the detection of *Cryptosporidium* oocysts on spinach leaves

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### ABSTRACT

Despite the infection risk associated with the consumption of contaminated food, techniques for recovering and detecting *Cryptosporidium* oocysts from fruit and vegetables are generally inadequate due to the variable recovery efficiencies and high reagent costs, such as those presented by ISO 18744:2016 "Microbiology of the food chain -Detection and enumeration of *Cryptosporidium* and *Giardia* in fresh leafy green vegetables and berry fruits". Although an improved method for recovering these parasites from Iceberg lettuce, which reported increased recovery efficiency as well as lower costs, has been published, it appears to have limitations for the recovery of *Cryptosporidium* from saponin-rich leaves such as spinach (*Spinacia oleracea*), which have previously been implicated in *Cryptosporidium parvum* outbreaks. In this study, we refined the method to improve its use with these more challenging samples. The use of alkaline elution buffer (1 M glycine) of different pH values was evaluated for their effectiveness in removing *C. parvum* from spinach leaves. The refinement of Utaaker's method showed, from spinach leaves inoculated with 100 oocysts, an increased oocyst recovery rate with an overall mean recovery rate of  $33.79\% \pm 2.82\%$ . The emergence of parasitic foodborne illnesses and outbreaks associated with the consumption of fresh produce demonstrates the need for the development of an optimal recovery process for parasites from suspected foods. Results showed that refinement of existing protocols could improve the retrieval of *Cryptosporidium* oocysts from these more challenging leafy greens.

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## 1. Introduction

Consumption of fruit and vegetables is associated with a healthy lifestyle, as they support the normal functioning of different body systems. The World Health Organization (World Health Organization, 2002) estimated that worldwide, insufficient intake of fruit and vegetables is linked with around 14% of gastrointestinal cancer deaths, 11% of ischaemic heart disease deaths, about 9% of stroke deaths and that 2.6 million deaths could be averted by adequate fruit and vegetable consumption (Lock et al., 2005). Healthier lifestyles have driven changes in eating habits over the last decade, resulting in an increasing demand for leafy green vegetables and ready-to-eat (RTE) salads (Mercanoglu Taban and Halkman, 2011). An increasing number of foodborne illnesses caused by a wide range of pathogens linked to fresh vegetables have been reported (Beuchat, 1996, 2006). Protozoan parasites such as *Giardia duodenalis* (Mintz et al., 1993), *Cryptosporidium parvum* (Åberg et al., 2015; McKerr et al., 2015; Millard, 1994) and *Cyclospora cayetanensis* (Murphy et al., 2017) have caused vegetable or fruit-borne outbreaks of gastro-intestinal illness. At multiple points along the farm-to-fork production/supply chain, fresh produce is susceptible to contamination by human pathogens. Potential sources of contamination include human, livestock and wildlife faeces, soil and amendments (e.g. to improve physical properties), water used in irrigation, chemical applications, washing and processing, and pickers, handlers and food preparation workers (Julien-Javaux et al., 2019; Matthews, 2009).

Despite the health risk associated with the consumption of contaminated food, techniques for recovering *Cryptosporidium* oocysts from fruit and vegetables are generally inadequate, being laborious and time-consuming, presenting variable recovery efficiencies, and high reagent costs (Chalmers et al., 2020). One international standard method currently exists for *Cryptosporidium* in food: ISO 18744:2016 "Microbiology of the food chain – Detection and enumeration of *Cryptosporidium* and *Giardia* in fresh leafy green vegetables and berry fruits" which is largely based on methods for analysing water samples (ISO 15553:2006 and U.S. EPA Method 1623). The method is based on paddle-beating the leaves in buffer to elute the oocysts from the surface, retrieval from the resulting eluate by immunomagnetic separation (IMS), and detection by immunofluorescence microscopy (IFM). In 2015, Utaaker et al. described a cheaper modification, by maximizing the paramagnetic beads used for IMS to retrieve oocysts spiked onto Iceberg lettuce, and incorporating alternative buffer solutions (Utaaker et al., 2015).

To contribute to the design of a low cost, robust method for *Cryptosporidium* detection in food, COST Action FA1408 funded a Short-Term Scientific Mission (STSM) at the *Cryptosporidium* Reference Unit (Swansea, UK). Utaaker's method was modified and applied to a wider variety of leafy greens (see the STSM report at [https://www.euro-fbp.org/uploads/6/5/9/0/65903825/stsm\\_report\\_romy\\_2018.pdf](https://www.euro-fbp.org/uploads/6/5/9/0/65903825/stsm_report_romy_2018.pdf)). However, this preliminary work showed that recovery from spinach leaves was 9%, lower than the mean of 53% (range 4% to 88%) which had been previously reported for Iceberg lettuce (Utaaker et al., 2015). While oocysts might be trapped in the stomata of spinach leaves (Macarasin et al., 2010), other factors also appeared to have influenced the poor recovery, such as foaming that occurred during processing. Chandra et al. (2014) mentioned that the foam produced during the recovery process might contribute to a loss of oocysts and we observed that fleshy leaves such as spinach produced more foam (see the STSM report at [https://www.euro-fbp.org/uploads/6/5/9/0/65903825/stsm\\_report\\_romy\\_2018.pdf](https://www.euro-fbp.org/uploads/6/5/9/0/65903825/stsm_report_romy_2018.pdf)). Spinach leaves contain relatively large amounts of the surface-active glycoside saponin which has detergent properties and can cause distinctive foaming during processing. It has been reported that 1 kg of dried spinach leaves contain 100 g saponin (Cho et al., 2011) and that just 150 g fresh spinach had the equivalent haemolytic activity of 0.1 g pure saponin (Kofler, 1931). Saponins are characterized by their structure containing triterpene or steroid aglycone and one or more sugars, and may act as an anionic detergent like sodium dodecyl sulfate (SDS) which, at concentrations above 0.2% w/v has been reported to inhibit the reaction between antigen and antibody by more than 90% (Dimitriadis, 1979). These results may explain the low recovery (9%) obtained during the preliminary work, as both the oocyst recovery by IMS and detection by IFM rely on such reactions. A spiking experiment of spinach eluate (obtained from the paddle-beating step of the process), to exclude the possibility of trapping oocysts in stomata, also showed poor recovery at 13% compared to 86% from reagent grade water. These data prompted the further refinements of the Utaaker method described here to reduce the foaming and improve oocyst recovery from saponin-rich leaves (Kregiel et al., 2017). Stabilization of foams strongly depends on the intrinsic properties and concentration of the surface-active substances and the environmental conditions such as pH and temperature (Narsimhan and Xiang, 2018; Qiao et al., 2020). In this present study, influence of pH on the recovery of *Cryptosporidium* oocysts from spinach leaves were assessed by replacing the acidic 1 M glycine buffer (pH 5.5) with 1 M glycine buffer at the alkaline pH range.

## 2. Materials and methods

During a preliminary study, different variety of leafy greens (including spinach leaves) were spiked with approximately 100 oocysts and left to dry overnight at ambient temperature. Elution of *Cryptosporidium* oocysts was performed using 1 M glycine buffer pH 5.5. Utaaker's method was modified by reducing the elution buffer volume from 240 to 200 ml to facilitate centrifugation (see the STSM report at [https://www.euro-fbp.org/uploads/6/5/9/0/65903825/stsm\\_report\\_romy\\_2018.pdf](https://www.euro-fbp.org/uploads/6/5/9/0/65903825/stsm_report_romy_2018.pdf)). Specific results from spinach leaves, during this preliminary study, were used as a control to evaluate the further refinements of Utaaker's method described in this present study.

## 2.1. Parasites

*C. parvum* oocysts were passaged in neonatal calves (INRA, Nouzilly, France) and faeces collected and stored without preservatives at 4 °C for less than 3 months until oocyst purification by IMS (Isolate®, TCS Biosciences) as per the manufacturer's instructions.

Working suspensions of oocysts were prepared by dilution in PBS to contain approximately 100 oocysts per 5 µl. The mean concentration of the working suspension was checked by spotting 5 µl suspension onto each of 10 single-welled glass microscope slides, fixing with methanol, and staining with Crypto-Cel FITC stain (TCS Biosciences) according to the manufacturer's instructions. The nuclear fluorochrome 4', 6-diamidino-2-phenylindole (DAPI) was also used to aid the identification of *C. parvum* oocysts. Slides were examined using an epifluorescence microscope fitted with 450–490 nm excitation and 520 nm emission filters; for DAPI observation, the excitation wavelength was changed to 330–380 nm. *Cryptosporidium* oocysts were counted under a  $\times 20$  objective.

## 2.2. Matrix spiking

Spinach leaves (*Spinacia oleracea*) were purchased as sealed bags labelled “washed” or “ready-to-eat”. For sample spiking and for optimal recovery, according to Robertson and Gjerde (2001), 30 g of spinach leaves were weighed into a homogenizer bag (Bagpage R) with a filter porosity < 250 µm (Interscience) and the 5 µl spike containing 100 oocysts was deposited by spotting across the leaves. Inoculated leaves were incubated at room temperature ( $21\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ ) for 1 h and then at 4 °C overnight with the bag sealed by securing the folded openings with binder clips. Non-spiked leaves were incubated in the same way and tested as a control for natural contamination. As an oocyst recovery control, 20 ml of reverse osmosis (RO) water was spiked with 5 µl containing 100 oocysts in filter bags and processed the same way as the bags containing spinach leaves.

## 2.3. Recovery and isolation of *Cryptosporidium* oocysts

The method published by Utaaker et al. (2015) was used with the following modifications designed to reduce foaming and improve oocyst recovery. The 1 M glycine elution buffer (pH 5.5) was replaced with 1 M alkaline glycine-NaOH buffer at a range of pH values (pH = 7.2; 8; 8.5; 9 and 10), each tested in sextuplicate. The volume of buffer added to homogenizer bags was reduced from 200 ml to 160 ml. Bags were vigorously shaken by hand in an upside-down manner until leaves were completely wetted, before paddle-beating for 1 min. The eluate was distributed evenly between four 50 ml tubes, the bags rinsed out twice with an additional 20 ml of buffer and squeezed by hand to remove as much of the eluate as possible, which was distributed between the 50 ml tubes. Samples were centrifuged at 1550 rcf for 10 min at 4 °C and the supernatant discarded by pipetting and leaving a volume of 10 ml. Pellets were resuspended, combined in a single 50 ml tube and again subjected to centrifugation at 1550 rcf for 10 min, after which the supernatant was aspirated to a volume of 10 ml. A weak detergent solution (2 ml) containing 1% SDS (71736, Sigma-Aldrich), 0.1% Tween 80 (P1754, Sigma-Aldrich) and 0.001% antifoam Y (A5758, Sigma-Aldrich) was added, and the pellet resuspended. Antifoam Y is an aqueous emulsion of antifoam A concentrate, also known as a defoamer and more easily dispersed in aqueous systems than Antifoam A.

The total volume of suspension was adjusted to 50 ml with RO water, mixed, centrifuged as above and the supernatant discarded. The final sample, with a packed pellet volume of less than 2 ml, was resuspended in 7 ml of RO water and oocysts were recovered by IMS (Isolate®, TCS Biosciences) with the following modification to the manufacturer's instructions based on the method described by Utaaker et al. (2015). Reagent A (1000 µl) was replaced with 200 µl reagent A mixed with 800 µl of 0.05% PBS-Tween 20, 1000 µl of reagent B buffer was replaced with 200 µl of reagent B mixed with 800 µl of StabilZyme AP buffer (SM-SZ, SurModics) and only 20 µl anti-*Cryptosporidium* coated magnetic beads were used per sample.

The bead-oocyst complexes were resuspended in 50 µl 0.1 N HCl, vortexed thoroughly for 10 s to separate the oocysts from beads, incubated for 5 min at room temperature, and re-vortexed for 10 s. Beads were captured by a magnet, and oocysts in suspension transferred to single-welled glass microscope slides containing 5 µl 1.0 N NaOH. This dissociation procedure was repeated as per the manufacturer's instructions. The staining procedure, microscopic detection and oocyst enumeration were performed as described above.

## 2.4. Statistical analysis

The recovery rates (range, median, mean, standard deviation) obtained with the different pH buffers were compared by the Kruskal-Wallis test with Bonferroni correction for multiple testing and unpaired Student's *t*-test was used for comparison between groups using R (version 3.6.3). A *P*-value of 0.05 or less was considered significant.

## 3. Results and discussion

The emergence of parasitic foodborne illnesses and outbreaks associated with the consumption of fresh produce, highlights the need for validated detection methods (Chalmers et al., 2020). We used a spiking study to investigate a range of elution buffers and measures aimed to reduce foaming and improve oocyst recovery from spinach leaves. For spiking studies, guidance is being developed for standardisation of incubation conditions (Chalmers et al., in preparation). While Robertson and Gjerde



**Table 1**Comparison of oocyst recovery efficiencies from spinach leaves seeded with 100 *Cryptosporidium* oocysts using 1 M glycine buffers of different pH values.

Buffer	Mean % ( $\pm$ SD)	Range	P-value
pH 5.5 (n = 2)	6.5 (3.53)	4–9	–
pH 7.2 (n = 6)	33.33 (6.05)	27–44	<0.001
pH 8 (n = 6)	36.33 (5.53)	30–43	<0.001
pH 8.5 (n = 6)	30.00 (6.87)	21–38	<0.001
pH 10 (n = 6)	35.50 (5.20)	29–40	<0.001

(2000, 2001) mention 1 h, Utaaker et al. (2015) opted for at least 5 h or overnight at ambient temperature, and the US Food and Drug Administration suggested longer time under refrigerated conditions (U.S. Food and Drug Administration, Office of Foods, 2019). In the current study, instead of letting the samples dry overnight at ambient temperature, inoculated spinach leaves were kept at room temperature ( $21\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ ) for an hour to dry and then stored at  $4\text{ }^{\circ}\text{C}$  overnight, replicating the normal storage conditions for these food products, before testing the recovery efficiency.

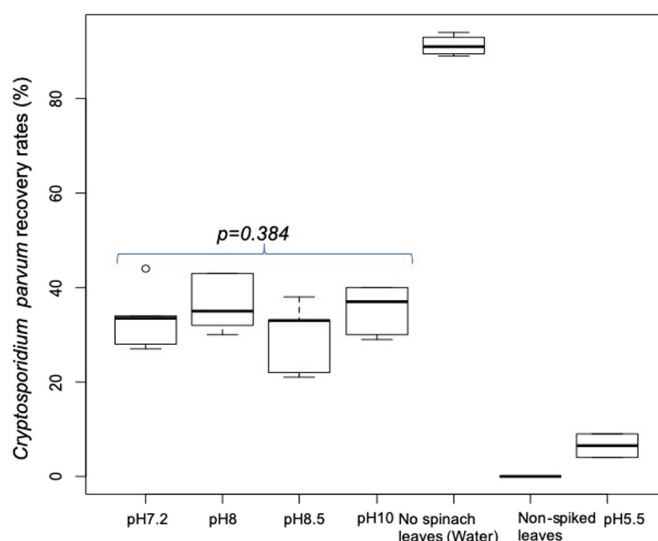
The recovery rates using different pH buffers are shown in Table 1. No natural occurrence of *Cryptosporidium* oocyst contamination was detected in any spinach samples, as all non-spiked leaves were negative. The highest mean recovery rate was obtained using buffer at pH 8 but this was not significantly different from the baseline (pH 7.2).

Recovery of *Cryptosporidium* from spiked water resulted in a mean recovery of  $91.25 \pm 2.21\%$ . These data highlight the effect of the matrix and its impact on the recovery rate. Indeed, the difference between the spinach leaves and the water matrix recovery rate was highly significant ( $P = 0.013$ ). Reduced foam formation during the elution process was observed in bags containing alkaline buffer (Fig. 1 right), compared to those using acidic buffer (Fig. 1 left).

Although data from this study indicate no significant differences between the recovery rate among alkaline 1 M glycine buffer solutions ( $P = 0.384$ ), compared to the preliminary research using pH 5.5, the recovery efficiencies were significantly improved ( $P < 0.001$ ) (Fig. 2).

In addition to the composition of wash solutions, differences in food matrices and the surface morphology could influence the oocysts adherence, which can affect the efficacies of the removal process (Chandra et al., 2014). Macarisin et al. (2010) reported that *Cryptosporidium* oocysts were capable of firmly adhering to spinach leaves after being sprayed with a spiking suspension (1000 *C. parvum* oocysts/ml) and were also internalized within the stomata. They also stated that prolonged and intensive washing of spinach roots and leaves in an elution buffer developed and recommended for testing fresh produce for *Cryptosporidium* spp. and *Giardia duodenalis* contamination was unable to dislodge all oocysts. Stomata crucially permit plants to regulate transpirational water loss from leaves during the uptake of  $\text{CO}_2$  for photosynthesis. Oocyst recovery may be influenced by stomata behaviour (opening and closing the stomata), and this might be different between types of leaves. Temperature is one of the most variable factors in the environment, and it affects many plant physiological processes, yet little is known about its effect on stomatal behaviour. However, studies provided conflicting results. While some evidence suggested that stomata opening increased with increasing temperature (Lu et al., 2000; Mott and Peak, 2010), other studies found that temperature had no effect on stomata (Cerasoli et al., 2014; von Caemmerer and Evans, 2015), or that increased temperature triggered stomatal closure. Further research should focus on verifying these results in a controlled environmental condition as well as discovering the principles of temperature dependency of stomatal regulation. In Winter Spinach, stomata are large with an elliptical shape, and the average number is more than  $100/\text{mm}^2$  of the leaf surface. In contrast, other lettuce, such as the butterhead

**Fig. 1.** Effect of glycine buffer pH on foam formation with spinach leaves during the elution process.



**Fig. 2.** Boxplot distribution of *C. parvum* recovery with buffers of different pH. In each boxplot, the solid horizontal line denotes the median value, while the upper and lower edges of the boxes represent the.

lettuce (belonging to the same complex “*Lactuca sativa*” as the Iceberg lettuce), has smaller stomata and the density is lower (<50/mm<sup>2</sup>) (Lawson, 2014).

The results obtained in this study from spiking experiments showed significantly improved recovery from spinach leaves, using the alkaline 1 M glycine buffer. Further refinement of the elution buffer may yet show additional improvements in the sensitivity of detection of *Cryptosporidium* from leaves with different characteristics. Each leafy green with a potential to be associated with foodborne outbreaks has their own characteristics and chemical compositions (lipids, saponins). Therefore, more studies are needed to determine the recovery efficiencies from other leafy green matrices.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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