Deliverable D-JRP-TOXOSOURCES-WP3.1

Report on available analytical procedures for detection of *Toxoplasma gondii* in fresh produce and list of promising analytical procedures

Workpackage 3 of JRP22-FBZ4.1-TOXOSOURCES

Responsible Partners:
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D-JRP-TOXOSOURCES-WP3.1

REPORT ON AVAILABLE ANALYTICAL PROCEDURES FOR DETECTION OF *TOXOPLASMA GONDII* IN FRESH PRODUCE AND LIST OF PROMISING ANALYTICAL PROCEDURES

BACKGROUND

This is a public deliverable of One Health EJP Joint Research Project:
JRP22-FBZ4.1-TOXOSOURCES – *Toxoplasma gondii* sources quantified
(https://onehealthejp.eu/jrp-toxosources/);

Work Package:
JRP-TOXOSOURCES-WP3 Multicentre survey to fill the key existing gap: role of fresh produce (i.e. Ready-to-Eat salads);

Task:
JRP-TOXOSOURCES-WP3-T1 Selection, evaluation and implementation of detection procedure for *T. gondii* oocysts in fresh produce.

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TOXOSOURCES addresses the research question – **What are the relative contributions of the different sources of *T. gondii* infection?** – by using several multidisciplinary approaches and novel and improved methods to yield the most robust estimates possible that can inform risk management and policy makers.

TOXOSOURCES WP3 aims to fill the knowledge gap concerning the relevance of fresh produce contamination by *T. gondii* oocysts as an infection source for humans. Objectives of TOXOSOURCES WP3:

- ✓ To identify and assess the most appropriate procedure to detect *T. gondii* oocysts in fresh produce.
- ✓ To provide an overview of *T. gondii* oocysts in fresh produce and the environment.
- ✓ To conduct a risk-based pilot study based on available prevalence data (literature review), data on food production chains, EU trade patterns of selected fresh produce and available consumption data (WP2).
- ✓ To evaluate *T. gondii* oocyst contamination in selected fresh produce commodities by a multicentre pilot survey in representative EU regions.

This deliverable reports on the first objective of TOXOSOURCES WP3. Based on this work and an inter-laboratory comparison, WP3 willwill select the most reliable methods for the molecular detection of *T. gondii* oocysts in fresh produce. Harmonised detection method(s) will be implemented among the partners of WP3 by providing a standard operating procedure (SOP) and organizing a technical workshop, and then be applied in a multicentre pilot survey on *T. gondii* in fresh produce.

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INTRODUCTION

Toxoplasmosis is a parasitic disease of global public health and veterinary relevance. The food- and waterborne transmission routes are complex: humans can become infected by ingestion of oocysts in soil, water or in contaminated fruits, vegetables and molluscs, or by ingestion of raw/undercooked meat of infected animals containing infective stages of the parasite. Despite the uncertainty of the relative contribution of human infection sources, *Toxoplasma gondii* is recognized as a foodborne parasite. (Bouwknegt et al., 2018; FAO/WHO, 2014; Torgerson et al., 2015). The typically low numbers of *T. gondii* tissue cysts in meat or oocysts in fresh produce makes the parasite difficult and expensive to detect by direct methods and, at present, no specific regulations nor ISO standards are available for detection of *T. gondii* in food. Evaluation of contamination of fruits and vegetables, especially pre-washed, ready-to-eat (RTE), bagged salad leaves, with *T. gondii* oocysts is of particular concern for both public health and the food industry. It is of particular relevance at present considering increasing preferenceconsumer preference for pre-washed RTE salads. Although scientific literature supports an association between *T. gondii* infection and the consumption of unwashed raw fruits and vegetables, the relative importance of fresh produce as a foodborne infection source for humans has not yet been clearly assessed. This lack of information is mainly attributable to the low numbers of expert laboratories working in this research field. Detection of *T. gondii* in vegetables, as for other foodborne parasites (e.g. *Cryptosporidium* spp. and *Giardia duodenalis*), is challenging due to thethe low sensitivity of existing methods for detection of low numbers of oocysts in vegetable samples. IIt is further hampered by the lack of validated detection methods. The ISO method (ISO 18744:2016) for the detection of (oo)cysts of *Cryptosporidium* and *Giardia* on leafy greens and berry fruits requires costly and time-consuming processing as well as visual detection by immunofluorescence microscopy. This is expensive, laborious, and not amenable to high throughput testing. In order to address food safety risk assessment challenges with respect to foodborne parasites, it is essential that food testing is standardised and improved through the validation of molecular assays, such as e.g. US FDA - BAM 19b for “Molecular Detection of *Cyclospora cayetanensis* in Fresh Produce Using Real-Time PCR”.

Considering that the oocyst stage of *T. gondii* is resistant to environmental factors and does not multiply in the environment, isolation of the parasite from fresh produce is the first and key step to allow an efficient detection. Molecular detection relies on efficient DNA extraction from the robust oocysts, with a simultaneous reduction of possible contaminants that could inhibit the DNA amplification step. Finally, amplification must be specific and sensitive enough to detect low numbers of oocysts (ideally down to 1 oocysts/sample).

Detection of *T. gondii* oocyst contamination in different matrices has been attempted and achieved with multiple molecular techniques, including conventional and nested PCR, qPCR and LAMP (loop mediated isothermal amplification). Distinct procedures have been described, leading to a huge variability of methods

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with different oocyst recovery/detection limits, while a widely accepted optimized method remains to be defined.

The main objective of TOXOSOURCES WP3 is to perform a multicentre survey to fill the knowledge gap on the role of fresh produce as a relevant source of human *T. gondii* infections in Europe. To achieve this goal, WP3-Task1 will define a standard operating procedure (SOP) to be applied by the laboratories of the consortium involved in the multicentre survey. To achieve this, an extensive literature review and multi-attribute assessment of the different methodological steps (oocyst recovery, DNA isolation, and DNA amplification) were performed in order to select the most suitable molecular method for *T. gondii* detection in fresh produce. In addition, a questionnaire on *T. gondii* molecular (DNA) detection (with a focus on oocysts) was developed based on outlines of the project IMPACT (Standardising molecular detection methods to IMprove risk assessment capacity for foodborne protozoan Parasites, using Cryptosporidium in ready-to-eat salad as a model organism"; Partnering Grant Project Grant Agreement Number GP/EFSA/ENCO/2018/03 – GA03). The questionnaire was administered online (Microsoft Forms application) within the TOXOSOURCES consortium and to experts involved in past or ongoing research projects with similar or complementary objectives. The main goal was to collect information on current practices for molecular detection and characterization of *T. gondii* in food and non-food matrices. This combined approach – extensive literature review and collection of expert opinion – was selected to ensure the method selected will be optimal for the purpose.

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METHODS

1. Extensive literature review – Molecular detection methods for *Toxoplasma gondii* oocysts

We conducted an extensive literature review on molecular detection methods for *T. gondii* oocysts, irrespective of the matrix (e.g. faeces, water, fresh produce). We searched online international databases, PubMed (all fields) and Scopus (title, abstract, and key words), for all published records on the topic following PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines. The search strategy was presented at the TOXOSOURCES Kick-off meeting held in Copenhagen, Denmark, on February 3th and 4th, 2020 (M-JRP-TOXOSOURCES-01). The search terms aimed to capture all potentially relevant studies, and they were grouped into 14 combinations (Figure 1).

The databases were searched for papers in English published up to 12th February 2020. Papers were initially screened for eligibility based on title and abstract by three independent reviewers – Marco Lalle (ML); Iva Slana (IS) and Nadja Bier (NB). The exclusion criteria were: i) letters, editorials, notes, comments, reviews; ii) studies describing methods not applicable for *T. gondii*; iii) methods using unavailable material for isolation/concentration of oocysts (i.e. antibodies). Papers were excluded, or included, if the judgement of two reviewers was in accordance. Papers were further screened for eligibility based on full text by six independent reviewers – ML; IS; NB; Barbora Bartosova (BB); Alessia Possenti (AP) and Gianluca Marucci (GM). Exclusion criteria for this second screening were: i) full text was unavailable; ii) study did not describe methods applicable to any stage (oocyst recovery, DNA extraction from oocysts, parasite identification including target and primer selection, non nucleic acid-based method) useful for a *T. gondii* oocyst molecular detection procedure. From each eligible document, data (Figure 1) were extracted in predefined tables. Six independent reviewers (ML, IS, NB, BB, AP and GM) performed the data extraction.

2. Questionnaire

The questionnaire and a preliminary list of selected experts with experience in *T. gondii* detection in food and non-food matrices were presented during the TOXOSOURCES Kick-off meeting held in Copenhagen, Denmark, on February 3th and 4th, 2020 (M-JRP-TOXOSOURCES-01). After the meeting, the questionnaire was further amended to its final version (ANNEX 1). At the Kick-off meeting, consortium partners were invited to provide further contacts of *T. gondii* experts worldwide. International experts were identified among TOXOSOURCES partners, participants of the EURO-FBP COST Action (https://www.euro-fbp.org/), partners of the projects IMPACT and SafeConsume (http://safeconsume.eu/), INETS (International Network for Environmental Toxoplasma Studies) as well as among scientists outside EU.

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The request to contribute to the online questionnaire was delivered by email on February 6th 2020 (ANNEX 2), explaining the reason of the questionnaire and providing the link (https://forms.office.com/Pages/ResponsePage.aspx?id=_ccwzxZmYkutg7V0sn1ZEhhXCGP7SSRChysEdN0YUABUMVQ3QzQ2S09HQ0hBRjNLVVZDT0xOVzBLSC4u). The questionnaire was open from February 6th to February 21st 2020. If the request was sent to several contacts of the same laboratory, only one representative per laboratory was invited to complete the questionnaire. The questionnaire (54 questions) included: demographic information (questions 1-3), information about current practices, details and facilities for molecular testing of *T. gondii* in different matrices (questions 4-47); opinions on molecular methods for *T. gondii* oocyst molecular detection in food (other than meat; questions 48-54).

- **Reference**
- **Item (e.g. food, water, faeces, …)**
- **Food type (be specific, e.g. type of lettuce including additional information such as organic production or ready-to eat/unwashed**
- **Part and amount tested (e.g. grams, …)**
- **If field samples Positive/Total**
- **Sample preparation method (e.g. elution, concentration, IMS, …)**
- **DNA extraction method**
  - classification (i.e. chemical, beat beating, etc.)
  - Description (if kit report name and brand)
- **DNA Amplification**
  - cPCR
  - qPCR (TaqMan or SYBR green)
  - Other PCR (specify)
  - Other molecular method (specify)
  - IAC (internal amplification control) Y/N (specify)
- **Target gene**
  - Gene amplified
  - primer sequence
  - primer binding region
  - Size of the amplicon (bp)
- **For spiking studies**
  - How many oocysts were seeded?
  - What was the recovery?
  - From how many samples?
- **Method parameters**
  - Analytical sensitivity
  - LoD - Limit of detection,
  - LoQ - Limit of quantification
  - LoA - Limit of absence, the lowest concentration of analyte that the method can differentiate from zero
  - Analytical specificity (also any cross reactions/non-specific amplifications identified)
  - Amplification efficiency
  - Linearity
  - Repeatability
  - Reproducibility
  - Duration of assay (the whole assay/PCR)
  - Specialist equipment needed
  - Applicability
  - Practicability
  - ❑ Costs
  - ❑ Need for training of the stuff
  - Ring trial
  - ❑ Ring trial (Y/N)
  - ❑ Results
  - Validation status (are there validation data for the methods used? Y/N)
- **Problems (e.g. PCR inhibition for specific matrices) or specific recommendations**
- **Critical steps in the performance (e.g. sampling, concentration, separation, …) highlighted by the authors**
- **General comments**

Figure 1. List of the data extracted from full text documents.

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RESULTS

1. Extensive literature review – Molecular detection methods for *Toxoplasma gondii* oocysts.

Of an initial list of 494 papers, 77 papers were included in the review after the screening process using the inclusion/exclusion criteria set for the purpose of this study. The included papers tested different matrices (either individually or in combination). The matrices tested were: faeces (12 studies), soil samples (15 studies), water (27 studies), edible and non-edible bivalves (16 studies), fresh produce (14 studies) and other matrices (3 studies).

Data extracted from the selected papers were collated in an Excel spreadsheet. Data concerning the three main steps (oocyst recovery, DNA isolation and DNA amplification) required for the molecular detection of *T. gondii* oocyst in fresh produce were analyzed independently. Taking into account relevant differences among the various matrices where *T. gondii* oocysts can be detected, for the oocyst recovery step, we focused on papers on fresh produce, the matrix relevant for the SOP development.

Available analytical procedures for detection of *Toxoplasma gondii* in fresh produce. Of the 13 papers reporting analytical procedures for the molecular detection of *T. gondii* in fresh produce, eight reported on method evaluation using fresh produce artificially contaminated with sporulated *T. gondii* oocysts (purified from feline faeces) and one using *Eimeria papillata* oocysts (Chandra et al. 2014; Lass et al. 2012; Hohweyer et al. 2016; Lalle et al. 2018; Shapiro et al. 2019; de Souza et al. 2016; Marchioro et al. 2016; Temesgen et al. 2019; Lalonde and Gajadhar 2016a). Two papers additionally reported testing of field samples (Lass et al. 2012; Marchioro et al. 2016). The remaining four papers described only prospective surveys (Lalonde and Gajadhar 2016b; Caradonna et al. 2019; Lass et al. 2019; Slany et al. 2019) The fresh produce types, the amount of tested matrix, sample preparation methods, as well as spiking protocol and number of spiked oocysts varied considerably by study, limiting the possibility to compare the reported procedures.

Oocyst recovery step. Spiking studies were considered to provide information of great relevance, under controlled experimental conditions, to evaluate the performance of methods. For the spiking studies identified, different types of berries (strawberries, raspberries, blackberries, blueberries and cranberries), leafy greens (basil, lettuce, spinach, cilantro, dill, mint and parsley) and/or other vegetables (radish, thyme, and green onions) were used as sample types. The sample amount used varied between 10 g to 60 g. In six studies, spiking was done by randomly pipetting the oocysts suspension onto the vegetable surface (dripping method) to mimic vegetable contamination by irrigation, while two studies used immersion method (i.e. vegetables were immersed in a large volume of water containing a known amount of oocysts). A post-spiking

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incubation time to allow oocyst adherence to the matrix of a minimum of 30 min up to overnight (either at room temperature or a +4°C) was reported for dripping experiments.

With respect to leafy greens, all procedures for oocyst recovery involved washing and pelleting by centrifugation of the post-wash eluate. Additional steps reported prior to centrifugation included: i) immunomagnetic separation (IMS) using an in-house developed anti-*T. gondii* oocyst antibody (not commercially available); ii) overnight flocculation using $CaCO_3$ solution when a large volume (≥ 2 L) of wash buffer was used; iii) filtration through cellulose ester membrane; iv) flotation with Sheather's solution. Filtration, flocculation and flotation might have the advantage of avoiding the use of a centrifuge for processing large volumes of wash buffer or a large number of samples. Moreover, flocculation and flotation might reduce the number of soil particles and other contaminants that could potentially inhibit DNA amplification. However, limited information on the impact of these additional steps on recovery rate could be extracted from the analysed articles. Addition of a *T. gondii* IMS step did not improve recovery rate (quantified by qPCR) (Hohweyer et al., 2016). One study underlined that if flotation is used, residual Sheather's solution could inhibit downstream PCR reactions (Lalonde and Gajadar, 2016a). Similarly, flocculation with $Fe_2(SO_4)_3$ resulted in inhibition of a PCR targeting the 18S-rDNA (Kourenti and Karanis, 2004). The high risk of oocyst loss following $NaNO_3$ flotation was highlighted in one study, for detecting *T. gondii* in soil by PCR (Lass et. al., 2009), suggesting that $NaNO_3$ flotation is suitable when oocyst contamination is expected to be $\geq 10^3$/40 g sample (soil). A paper using wastewater as matrix discussed that while flocculation is simple and inexpensive, filtration is more robust for processing turbid water (that could be compared to wash solution from fresh produce) and that filtrates are less likely to contain PCR inhibitors, which appeared to be eliminated by using 1 µm-pore sized polyethersulfonate membrane filters (Villena et al., 2004).

Washing of vegetables either by hand or automatic horizontal orbital shaking (from 15-30 sec to 60 min) or by stomaching was reported. The washing buffers commonly used were water solution of 1 M glycine pH 5.5 (4 studies) and 0.1-1% Tween 80 (4 studies) with a mean ratio of a 4-6 ml of wash buffer/g of sample. Two systematic validation studies compared washing in plastic filter bags by stomaching vs washing by hand or horizontal orbital shaking using either spinach or other various leafy herbs (cilantro, dill, mint and parsley). For a similar spiking level (≥100 oocysts/g) by dripping, stomaching with 1 M glycine pH 5.5 buffer provided a higher recovery rate (in %) than horizontal orbital shaking to recover oocysts from leafy herbs, whereas manual shaking with 0.1% Tween 80 was more effective for spinach as matrix (Lalonde and Gajadhar, 2016a; Shapiro et al. (2019) One study highlighted that use of washing buffers containing a surfactant or detergent is not recommended for fresh produce types to be processed by stomaching, since the excessive amount of bubbles produced during the homogenization seems to interfere with oocyst recovery (Lalonde and Gajadar, 2016a). Beyond differences in the washing buffers used, it is worth to note that spinach spiking was done with heat-inactivated (80 °C for 20 min) *T. gondii* oocysts whereas leafy herbs were contaminated with *E. papillata* oocysts, thus accounting for eventual differences in oocyst adherence to the matrices.

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Evaluation of the efficacy of different wash buffers was reported in two studies. Although different washing protocols were used (hand vs automatic shaking or stomaching), both reported that 1 M glycine pH 5.5 performed better with leafy greens compared to PBS (Chandra et al., 2014; Lalonde and Gajadhar, 2016a).

In the six prospective survey studies, a larger variety of leafy greens were tested in comparison to spiking studies, including mixed salads (RTE or not). Two of the surveys (from the same laboratory; Lass et al., 2012 and 2019) used a large amount of the tested sample as well as a large volume of wash buffer (≥2 L) followed by flocculation. In the other four studies, the amount of tested samples was between 35 g and 100 g, with an average amount of wash buffer of 2 ml/g of sample. Use of an automatic shaker (15-20 min) for sample washing followed by centrifugation, was the most often used method (three studies), irrespective of the type of leafy green tested. Although the four studies reported the use of different washing buffers, all buffers contained either Tween-80 (0.1-1%) or glycine. In the largest survey (with almost 1200 samples), 35 g of sample was tested, washing was done with 200 ml of 1 M glycine pH 5.5 using an orbital shaker or stomacher (depending on the type of leafy green) and oocysts were recovered by centrifugation and flotation with Sheather's sucrose solution (Lalonde and Gajadhar, 2016b).

DNA extraction. In the spiking studies, following oocyst recovery from fresh produce and prior to DNA extraction, all protocols included a step to break the robust wall of the oocysts to facilitate the release of parasite DNA and maximize downstream molecular detection techniques. Bead-beating (BB) using a commercial mix of beads (with different sizes and compositions) and a high-speed mechanical homogenizer were used in four of the studies (Chandra et al. 2014; Lass et al. 2012; Lalle et al., 2018; Temesgen et al. 2019). Homogenization was achieved with single or double cycles at speeds in the 4-6.5 m/s range for 30 s up to 2 min. In the other four studies(Hohweyer et al. 2016; Shapiro et al. 2019; de Souza et al. 2016; Marchioro et al. 2016)., the freeze and thaw (FT) method was used with variable number of cycles (from 1 to 10), temperature range (-80/-196 °C to 65/100°C) and incubation times (1-5 min). A direct comparison of the efficacy of FT or ultrasound (US) vs no pre-treatment in improving DNA detection provides evidence that FT and US are equally effective (de Souza et al., 2016). US or incubation with Proteinase K at 56°C have been used as additional step after FT cycles (two studies).

Performance of different DNA extraction procedures including FT and/or BB were evaluated and compared in three spiking studies using non-vegetable matrices (i.e. bivalves and faeces). Use of commercial kits including sample homogenization by BB performed better compared to a procedure using sedimentation/flotation in combination with FT followed by in-house phenol-chloroform extraction (Herrmann et al., 2011) or to a DNA extraction kit without BB even when an additional step using glass beads was performed . In one study, the combination of FT, BB and proteinase K treatment together with a commercial DNA extraction kit showed increased sensitivity compared to vortexing and BB alone followed by DNA extraction using another commercial kit (Staggs et al., 2015). Concerning FT, one study suggested that increasing numbers of FT cycles did not enhance oocyst DNA detection and may have resulted in decreased sensitivity due to degradation of DNA with repeated FT cycles (Manore et al., 2019).

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Fifteen studies reported on the use of BB for DNA extraction from *T. gondii* oocysts (Durand et al., 2020; Géba et al., 2020; Escotte-Binet et al., 2019; Galvani et al., 2019; Slanyet al. 2019; Temesgen at al., 2019; Lalle et al., 2018; Staggs et al., 2015; Ribeiro et al., 2015; Lass et al., 2012; Herrmann et al., 2011; Yang et al., 2009; Lass et. al., 2009; Chandra et al., 2014; Salant et al., 2007), and two commercial kits were mainly cited: FastDNA-SPIN Kit for Soil from MP Biomedicals (7 studies); and DNeasy PowerSoil from Qiagen (4 studies). Whether performance of these commercial kits was comparable was not specifically assessed in any of the selected reports but might be presumed by comparability of NucleoSpin Soil (Marcherey-Nagel, Germany) vs ZymoResearch fecal DNA Kit (Zymo, USA) (Herrmann et al., 2011), as all these commercial kits use similar protocols. FT associated with silica spin-column kits from different brands is reported in the majority of the reports (58 studies). This procedure has the advantage of not requiring expensive equipment (i.e. beat-beater). However, the requirement of several cycles of FT is time consuming and might be difficult to standardize, especially when a large panel of samples must be tested. Kits using pre-packed silica spin column require the subsequent transfer of only a fraction of the supernatant obtained from the initial sample lysis. This leads to considerable loss, as only a portion of the original material is used for the final DNA isolation step, and might require multiple loading of columns with increasing risk of cross-contamination and time loss. Commercial kits based on a mechanical disruption step of the sample have been proven to be the most suitable to detect contamination with *Hammondia* and *T. gondii* oocysts by PCR (Hermann et al., 2011), and have the advantage of ingusing larger sample volumes, with silica matrix being loaded onto empty columns.

DNA amplification. In the 13 papers reporting analytical procedures for the molecular detection of *T. gondii* in fresh produce, DNA detection was done by qPCR (10 studies), either using Taqman-assays (7 studies) or High Resolution Melting (HRM) analysis (3 studies). The assays targeted individually the multicopy genes *18S-rDNA* (2 studies), *B1* (4 studies) or the *529 bp* (529 RE) multiple repeated element (4 studies), whereas one used a multiplex qPCR targeting both *B1* gene and the *529 RE*. One *T. gondii* genome harbours about 30 copies of the *B1* gene and 200-300 copies of the *529 RE*, being both suitable targets to increase assay sensitivity. None of the studies reported on a full validation process and no ring-trial has been performed to assess the reproducibility of the molecular assay.

From all included papers, conventional PCR was used in 17 studies (22%), nested or semi-nested PCR in 20 studies (25%) and two papers reported on loop-mediated isothermal amplification (LAMP). As nested-PCR and LAMP, despite the higher sensitivity and specificity compared to conventional PCR, suffer from a high risk of background and cross-contamination (even if for different reasons) and as nested PCR requires two consecutive rounds of amplification, we did not further consider these assays as suitable for the purpose of a mid-large scale survey in fresh produce.

For the conventional PCR (cPCR) assay, most studies targeted the *B1* gene and the *529 RE*. Six studies compared the sensitivity of cPCR targeting *B1* vs *529 RE*, expressed as the limit of oocysts providing a positive amplification. Contradictory results have been expected as a result from differences in primer sequences, binding sites and amplicon sizes. In fresh produce, *B1* proved to be 10 times more sensitive than

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529 RE (de Souza et al., 2016) with an limit of detection (LoD) of 10 and 100 oocysts/head of lettuce, respectively. In soil and faeces , the results were the opposite (Du et al., 2012; Schares et al., 2008). Of course, the sensitivity of the *529 RE*-cPCR is also affected by the efficiency of the DNA extraction method (BB increased sensitivity in faeces sample, Herrmann et al., 2011) and results improved as the amplicon size reduces (Schares et. al. 2008). Sensitivity can also be hampered by inhibition of DNA amplification due to the high amount of matrix DNA and inhibitors co-extracted from the sample with parasitic DNA. Therefore, sensitivity is generally higher in water or relatively simple matrices (e.g. mussel haemolymph) compared to complex matrices (e.g. mussel tissue; Staggs et al., 2015). Addition of bovine serum albumin (BSA; in the range of 0.4-10 mg/ml) to the amplification reaction was reported in several publications (12 studies) to alleviate the effect of potential PCR inhibitors present in different sample types (e.g. mussels or drinking/surface water).The addition of $MgCl_2$ (up to 5 mM) has also been reported to improve the performance of a B1-qPCR assay (Galvani et al., 2019) to detect *T. gondii* oocysts in water. Comparison of the sensitivity among the different assays (e.g. qPCR vs cPCR, B1 vs 529 RE) was hampered due to inconsistency in the spiking protocols and reporting of the LoD.

Assays relying on qPCR account for almost 50% of studies (38 studies), and were mainly qualitative, with Taqman-assays targeting the *529 RE* being the most often used. Among others, the use of a specific Taqman-probe in a qPCR assay has the advantage of combining detection with confirmation of the amplification products without the need of further amplicon sequencing. Two assays targeting the *529 RE* (Lèlu et al., 2011; Staggs et al., 2015) and two assays detecting the *B1* gene (Villena et al., 2004; Lass et al., 2012) were the most often used .

Only one of the B1-qPCR assays was used for the analysis of fresh produce providing a LoD of 100 oocysts/radish (Lass et al., 2012). An increased sensitivity of less than 1 oocyst/g of fresh produce has been reported for the two different 529 RE qPCR assays (Hohweyer et al., 2016; Lalle et al., 2018; Temesgen et al., 2019). The analytical and diagnostic performance of the endpoint 529 RE cPCR, using the primer Tox5 and Tox8 (Schares et. al. 2008), and two 529 RE qPCR assays, one of which used the Tox-9F and Tox-11R primers and the probe Tox-TP1 (originally described in Reischl et al., 2003 and Opsteegh et al., 2010), were evaluated in a recent publication (Bier et al., 2019) for *T. gondii* DNA detection in pork meat samples. Both qPCRs provide similar sensitivity and specificity higher than the cPCR (Bier et al., 2019). For any qPCR assay, it is important to report the performance characteristics according to the MIQE guidelines (Bustin et al. 2009 and 2010; Taylor et al., 2019). This includes: i) a standard curve (ten-fold serial dilution of at least five template concentrations) prepared with background matrix (e.g., washings from uncontaminated food matrix); ii) efficiency and linearity with a R^2 value ≥0.98. The dynamic range should be reported over at least 3 orders of magnitude. Supported by the spiking studies, the LoD should be determined and reported; iii) an internal amplification control (IAC) should be included to check for PCR inhibition; iv) the LoD should be determined and reported to help with interpretation of sensitivity and the results from naturally contaminated samples.

Noteworthy, performance characteristics of qPCR assays were reported only in four of the reviewed studies. In Temesgen et al. 2019, the performance of the 529 RE Taqman assay (Opsteegh et al., 2010)

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applied to fresh produce (berries) was fully evaluated, including: specificity, efficiency, linearity, LoD, repeatability, intermediate precision, and robustness. The original assay was further improved with the use of MGBEQ-labelled probe instead of BHQ1.

Few studies reported the use of an IAC, including a competitive IAC (CIAC, Wells et al., 2015) or synthetic targets (Lass et al., 2019; Slany et al., 2019). Use of a CIAC has been reported to reduce the assay sensitivity, so it is not recommended. Synthetic IACs, ideally as a sequence with no homology with neither the target parasitic DNA nor with the matrix have the advantage to generally not compete with target amplification when used in low concentrations.

2. Questionnaire results

A total of 24 experts completed the questionnaire (ANNEX 3). Ten (42.0%) experts were part of the TOXOSOURCES partnership (either full or associated partners), while 14 (48.0%) were additional invited experts. The participants were from Health Agencies (n=6), Universities/Research Institutes (n=15) and other Organizations (n=3). The majority of participants work in organizations with main focus on Animal Health and/or the Environment (n=9), One Health (n=12) and Human Health (n=3). Seven of the participants do not use molecular methods for *T. gondii* detection, but test meat (n=4), faeces (n=3) or other samples by microscopy (3) or by bioassay (1) or did not provide any further comment. The 17 experts that do use molecular methods for *T. gondii* detection also report on the use of microscopy (n=9), serology (n=2) and bioassay (n=1).

Here, only answers to questions that could most thoroughly affect the final decision on the selection of the most suitable molecular method for *T. gondii* detection in food are presented. Only 7 participants reported testing different kinds of fresh produce (e.g. fruits, salads). For these, sample processing is performed by stomaching; washing and pelleting and centrifugation (n=6). In addition, two participants report the use of density gradient purification with or without filtration. The reported washing buffers (n=5) were glycine 1 M pH 5.5, PBS with 0.01% Tween 80, PBS Trypsin 0.25% (n=2); Tris-glycine 1% beef extract (TGBE) (n=1), Alconox 0.1% (n=1) or 1% Tween 80 (n=1).

Treatment of the sample before DNA extraction is performed by 18 participants mainly by BB (n=9) or FT (n=6). Bead-beating was used by 5 out of 7 participant testing fresh produce. Manual DNA extraction in contrast to automatic extraction was the common procedure except for 1 participant. The most common DNA extraction kits for those testing fresh produce were: Power-Soil DNA Isolation Kit (Qiagen); FAST DNA spin kit for soil (MP Biochemicals); DNeasy Blood & Tissue Extraction Kit (Qiagen, Germany).

qPCR was the most used molecular assay (n=18), with a TaqMan-qPCR being used by 15 participants. Irrespective of the molecular assay, the target was mainly the 529 RE (n=17), which was detected by qPCR by 9 participants. The applied methods were those already identified in the literature review. Remarkably, IAC

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was reported to be used only by 8 out of 15 participants that used TaqMan-qPCRs. Out of these, 2 participants used a commercial IAC. The qPCR assay was used for oocyst quantification by 7 participants using a standard curve.

Eighteen of 24 (81.8 %) participants were fully satisfied with the molecular methods they are currently using for the detection of *T. gondii*. Those that were not (n=5) mentioned that methods are time-consuming, have sometimes poor specificity (cross-reactivity with unintended parasites or matrices), give no information on oocyst viability, or have problems with PCR inhibition. According to the participants, commercial products to specifically enrich or capture oocysts or DNA are needed to optimize molecular detection of *T. gondii* oocysts. Moreover, molecular detection should be standardised with regards to the recovery of *T. gondii* oocysts from food (i.e. the wash procedure) and identification of relevant target sequences associated with high sensitivity in food. An efficient, robust and accessible DNA extraction should be selected. For quantification of *T. gondii* in fresh produce, the need to define a consensus toward the way to quantify the target (DNA amount vs number of oocysts/copies) as well as a standardized and harmonized procedure to convert this information into an equivalent number of oocysts emerged as one of the issue to be defined. Indeed this information is of great relevance for food stakeholders. Ring trials among different laboratories would be also required for optimizing and standardizing the chosen method. The most important features of molecular methods for *T. gondii* detection in food identified by the multi-attribute scores were sensitivity, transferability of method between laboratories, biological robustness and high level of characterization.

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CONCLUSIONS AND HIGHLIGHTS

Each food matrix displays specific characteristics that may interfere with protozoa extraction/elution (trapping, adhesion force) and molecular detection (inhibitors), and hence may require the implementation of different methods to properly detect the target parasite. PCR inhibitors are important confounders that must be addressed in any PCR-based detection effort.

There are a number of available analytical procedures for detection of *T. gondii* in fresh produce, and analytical procedures that are suitable for the SOP were identified. The combined approach of literature review and expert opinions proved useful.

Based on the reviewed literature and the opinions of the experts collected via the questionnaire, the following decisions were taken regarding analytical procedures for the development of the SOP:

- **Amount of sample to be tested:** 50 g. This was the average amount tested.
- **Type of sample:** Leafy greens, mixed salad (excluding carrots). As single types of leafy greens cannot be independently tested, the use of mixed salad will ensure to include all the variables associated with specific properties of each vegetable.
- **Washing buffer to be used:** Glycine 1M pH 5.5. The use of this buffer for leafy vegetables (herbs and salads) has been reported as the most suitable in terms of efficacy and reproducibility. Furthermore, as highlighted from IMPACT project participants, the addition of antifoam to the buffer will be considered as some leafy greens might release high amount of saponins following homogenization.
- **Washing protocol:** Stomaching in filter-bags. Although orbital shaking has been also reported as suitable for leafy greens, high variability in terms of shaking times was the reason to exclude this approach. More consistent settings were reported for stomaching.
- **Oocyst recovery:** Washing and pelleting by centrifugation. Despite centrifugation being time consuming, filtration through a membrane requires more specialized equipment and is therefore not applicable for a large set of samples.
- **DNA extraction**: Beat-beating based commercial kit for soil. This method provided thefastest and best results in many different matrices, both in terms of DNA yield and removal of PCR inhibitors. However, since freeze and thaw procedure was largely adopted in combination with silica spin columns kits, different commercial kits for each procedure will be tested in parallel. A freezing step of the vegetable pellet at -20°C for 48-72h, minimum, prior to DNA extraction

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will be included sincesince in the multicentresurvey the vegetable pellet will be frozen before being delivered from satellite laboratories to main laboratories.

- **DNA amplification assay:** qPCR targeting the 529 RE and the B1 as reported in Slany et al., 2019, and developed at VRI. This assay includes the two most frequently used qPCR assays that have also been applied to test fresh produce and have the advantage of testing for both loci giving a more robust result. The assay will be evaluated both in single and duplex format. The improvement reported for the 529 RE qPCR in Temesgen et al., 2019 will be implemented for the test. The test will also include the IAC reported in Slany et al., 2019.

To develop the SOP, spiking experiments will be conducted by dripping (done in parallel by ISS and VRI), according to the spiking guidelines developed in the framework of the IMPACT project (see the workflow in Figure 2). This experimental approach will allow us to evaluate qPCR sensitivity (LoD either in terms of DNA and oocyst amount) in combination with DNA extraction performance. Characteristics such as sensitivity, reproducibility, repeatability will be evaluated in parallel in the two laboratories.

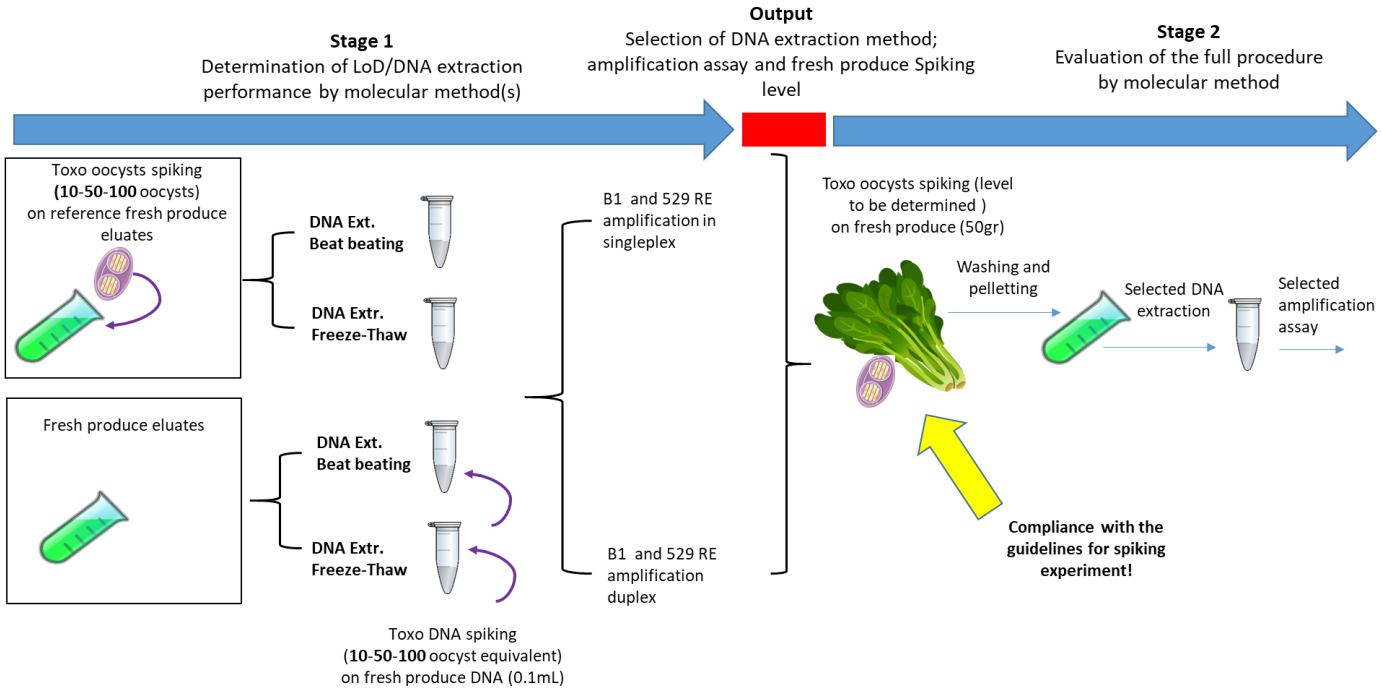

Figure 2. Workflow for the SOP development.

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ANNEX 1. Questionnaire.

ANNEX 2. Invitation letter.

ANNEX 3. Questionnaire results.