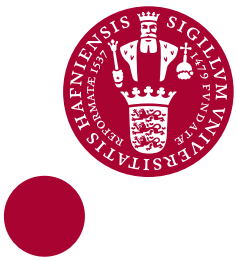

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Current status and future needs for validation

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Parasite detection in food: Current status and future needs for validation

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ABSTRACT

Background: Many parasites (protozoa and helminths) can be transmitted through food and lead to infections with high morbidity, as well as disease outbreaks. Although the importance of foodborne parasites (FBP) is recognised by many sectors of the food industry, standardized analytical methods and validation procedures for testing food for FBP are lacking.

Scope and approach:Current methods for detection of FBP, and their validation, are critically reviewed, focusing on priority FBP in Europe: the helminths *Echinococcus multilocularis, Echinococcus granulosus, Taenia saginata, Trichinella* spp., and Anisakidae, and the protozoa *Toxoplasma gondii, Cryptosporidium* spp., and *Giardia duodenalis*.

Key findings and conclusions:Standard methods exist for detection of *T. saginata* in beef, and *Trichinella* spp. in meat (and are mandatory at meat inspection in Europe), Anisakidae in fish, and *Cryptosporidium* spp. and *G. duodenalis* in leafy green vegetables and berry fruits. For other FBP or foods, methods used in sample surveys have been described, but validation data are generally absent; limits of detection are not provided, ring trials have rarely been performed, and for most FBP quality control materials, proficiency schemes, and reference standards are lacking. The use of surrogate particles or organisms for method development or validation purposes needs to be carefully considered. Documented procedures for validation, such as ISO17468 and ISO16140-2:2016 that were established for bacteria, are mostly inappropriate for FBP. The development and application of standardized and validated detection methods would enhance understanding of the foodborne route of transmission, improve risk assessments, and help identify and verify critical control points.

Abbreviations: FBP, foodborne parasites; CCP, critical control point; lpg, larvae per gram; LOD, limit of detection; HACCP, Hazard Analysis Critical Control Point; IFM, immunofluorescence microscopy; Ab-ELISA, antibody detection enzyme linked immunosorbent assay; Ag-ELISA, antigen detection enzyme linked immunosorbent assay; MS, mass spectrometry

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1. Introduction

Parasitic protozoa and helminths are hugely diverse groups of eukaryotic organisms that can cause disease in humans and animals. Transmission to people can occur through a variety of routes and vehicles, including animal to person or person to person contact, water, soil, and food (Ortega & Sterling, 2018). The global burden of human parasitic disease has been estimated at 407 million cases annually, of which 91.1 million cases (22%) and about 52 thousand deaths are thought to be foodborne (Torgerson et al., 2015). Despite their impact on public health, awareness of foodborne parasites (FBP) is highly variable (EFSA BIOHAZ Panel et al., 2018). For example, the analysis of FBP in foods is only mandated in Europe as part of general visual meat inspection for *Taenia saginata* in beef according to Regulation (EC) No 854/2004 (European-Union, 2002), and for *Trichinella* spp. in meat of susceptible species according to Regulation (EC) No 1375/2015 (European-Union, 2015). At present, there are no EU microbiological criteria published specifically for protozoa in food. It is only recently that the fresh produce industry has started to pay attention to *Cryptosporidium* spp. following widespread outbreaks of disease, involving hundreds of cases, linked to the consumption of ready-to-eat salad in the UK (McKerr et al., 2015) and Finland (Aberg et al., 2015). The lack of suitable detection methods, coupled with few criteria for these organisms, limits the analysis of most FBP carried out in foods.

Risk assessments and identification of critical control points (CCP) benefit from evidence provided by sample surveys, as does risk-based sampling. Where robust laboratory methods are available, statistical designs can be made for consistent detection (or not) of a defined level of infection/contamination at a determined level of confidence; such an approach can guide a proportionate approach to testing. However, to ensure robust detection of FBP for these purposes, validated methods are essential. Current guidelines for standardization and validation, such as ISO16140-2, 2016, were devised for monitoring bacteria, and are not directly applicable to FBP. Unlike for bacteria, *in vitro* culture cannot be used for detection of FBP. Other considerations are the very diverse nature of FBP (ranging from microscopic protozoa to macroscopic parasitic worms), and differences in their biology (e.g. complex life cycles, hosts, transmission routes, and survival characteristics). There are also variations in food vehicles in which they may be present and transmitted to consumers, as well as differences in sampling methods, preparation procedures, and detection targets used. The food matrices analysed for FBPs can variously include undercooked meat, fish, and shellfish, untreated milk and fruit juices, and contaminated fruit or vegetables.

In light of the increasing internationalization of food supply chains, and the considerable number and size of outbreaks caused by FBP, focused efforts were made within COST Action Euro-FBP (FA1408) to rank and prioritize FBP for Europe (Bouwknegt, Devleesschauwer, Graham, Robertson, van der Giessen, & The Euro-Fbp Workshop P, 2018). Here we have reviewed the methods for detection of those FBP ranked in the top eight in Europe overall and/or those in the top four in the individual European regions i.e. the helminth cestodes (tapeworms) *Echinococcus multilocularis, Echinococcus granulosus,* and *T. saginata,* the nematodes (roundworms) *Trichinella* spp. and Anisakidae, and the protozoa *Toxoplasma gondii, Cryptosporidium* spp., and *Giardia duodenalis* (Table 1). Trematodes were not ranked highly in that exercise and therefore are not included in this paper. Of additional note, although it is not within the scope of this review to assess methods for prevention of contamination/infection, removal or inactivation of FBP in the food chain and refer the reader to other work (Franssen et al., 2019; Gerard et al., 2019; Paulsen, Franssen, Gerard, La Carbona, & Robertson, 2019), these are an essential part of food safety.

Reporting of human cases of illness caused by most of these FBP (echinococcosis, trichinellosis, congenital toxoplasmosis, cryptosporidiosis and giardiosis) is under mandatory EU-wide surveillance using data collected in The European Surveillance System (TESSy) and reported annually (Surveillance Atlas of Infectious Diseases) (Table 1). Nevertheless, there is substantial variation in ascertainment and reporting of cases caused by each parasite, both within and between countries, and estimates of the proportions of disease that are foodborne are not included. Therefore, estimates of the disease burden, and that which is foodborne, provide useful comparators (Torgerson et al., 2015) (Table 1). Results of mandatory inspection for *T. saginata* in beef and *Trichinella* spp. in meat from susceptible species including pigs, horses, wild boar and bear, are collected electronically to the EFSA zoonoses database, through EFSA's Data Collection Framework (DCF), and reported annually (EFSA/ECDC, 2017) (Table 1).

This work aims to provide a critical review of the detection methods applied to food for the selected FBP based on literature review (see details in Supplementary Table 1). The foods investigated were those identified globally as most likely vehicles of human infection with these parasites (FAO/WHO, 2014) (Table 1). A brief description of the selected parasites is provided below; for a comprehensive overview of parasite biology, geographical distribution, disease in humans, relevance for trade and impact on economically vulnerable populations, the reader is referred to Annex 7 of the FAO/WHO "Multi-criteria based ranking for risk management of foodborne parasites" (FAO/WHO, 2014). It is important to note that these parasites have a wide range of transmission routes, and therefore those life cycle stages that are found in food, and provide detection targets, vary considerably (Table 1). Additionally, there is no environmental reproduction, outside of hosts (animals, humans), and infectious doses are low, so detection of small numbers of parasites is important. Most detection methods do not indicate whether the parasite is either viable or infectious to humans.

2. Brief description of the selected parasites

An overview for each of the selected FBP, their most likely food vehicles, human health impact and the context for food testing in Europe is provided in Table 1. For comparative purposes, this includes Disability Adjusted Life Years (DALYs), defined by the World Health Organization as the sum of years of potential life lost due to premature mortality and the years of productive life lost due to disability.

2.1. *Echinococcus granulosus and Echinococcus multilocularis*

Echinococcosis is a global disease caused when humans are infected either as aberrant dead-end hosts by ingesting *Echinococcus* eggs in contaminated food, water or soil, or during direct contact with infected definitive hosts, mainly canids. Two *Echinococcus* species are most important in humans, *E. granulosus,* which causes cystic echinococcosis (CE, hydatid disease or hydatidosis) and *E. multilocularis,* which causes alveolar echinococcosis (AE). Eggs in definitive host faeces may contaminate food or feed, and might be ingested by people or by intermediate hosts. Following ingestion, the parasite is transported into various organs, often the liver and the lungs, where they develop into metacestodes. Protoscolices, the embryonic tapeworms, develop inside the metacestodes in huge numbers, which, when ingested by definitive hosts, develop into adult worms in the small intestine. Both AE and CE are serious diseases that can be life-threatening; the parasites persist in humans and surgery or lifelong treatment with benzimidazole drugs are currently the only treatment options, depending on the parasite species and disease stage. The large number of DALYs associated with AE (Table 1) are due to the high mortality associated with this disease in untreated cases. Although foodborne transmission of both *E. multilocularis* and *E. granulosus* is both plausible and probable, the long prepatent period (years or even decades) between infection and onset of symptoms means that foodborne transmission has not yet been proven (EFSA BIOHAZ Panel et al., 2018). Perhaps for this reason, despite the potential severity associated with infection with these parasites, there is a lack of concerted efforts regarding development of methods for identifying contamination of "high risk" food stuffs with eggs (berries,

Table 1
Selected foodborne parasites, human health impact, their most likely food vehicles, and the context for food testing in Europe.

Parasite	Disease (Human symptoms)	Cases reported in Europe 2017[a]	Median rate per 100,000 of foodborne DALY's in Europe; Proportion of disease that is foodborne[c]	Priority ranking overall in Europe; and by region[d]	Most likely food vehicle(s)[e] FAO/WHO (2014)	Detection target in food	Requirement for testing food	Detections in food reported in EU/EEA to EFSA in 2017 (EFSA/ECDC, 2018) Number positive samples or animals/number tested (%)
Cestodes								
Echinococcus multilocularis	Alveolar echinococcosis (Prolonged asymptomatic incubation period; varying symptoms possible depending on cyst location; fatal if untreated)	832 for both *Echinococcus* species (no species differentiation available)	1 (0.4–8); 0.48 (0.01–0.76)	1 In top 4 in 4 European regions (N, E, SW, and SE).	Fresh produce	Tapeworm eggs	To assess human exposure to parasites in fresh produce	No European-wide surveillance
Echinococcus granulosus	Cystic echinococcosis (Prolonged asymptomatic incubation period, varying symptoms possible depending on cyst location)	See comment for *E. multilocularis*	0.8 (0.3–2); 0.21 (0.15–0.29)	4 In top 4 in 4 European regions (W, E, SW, and SE). 4	Fresh produce	Tapeworm eggs	To assess human exposure to parasites in fresh produce	No European-wide surveillance
Taenia saginata	Taeniosis (Mild digestive symptoms)	No European-wide surveillance	Not available; 1	15 In top 4 in 1 European region (SE).	Beef	Cysticerci	Mandatory as part of general meat inspection according to Regulation (EC) No 2004/854	For cattle inspected in Belgium: 1,375/922,797 (0.14%) Slovenia: 8/18,235 (0.007%): Sweden: 0/406,030 (0.0%): Spain: 1/107,419 (0.0009%): Luxembourg: 19/26,173(0.07%)
Nematodes								
Trichinella spp.	Trichinellosis (acute clinical cases with varying symptoms, e.g. fever, diarrhoea and muscle pain; in fatal cases also endo- or myocarditis)	168	0.04 (0.02–0.07); 1	*T. spiralis*: 3 In top 4 in 3 European regions (W, E, SE). Other *Trichinella* spp.: 6 In top 4 in 3 European regions (N, E, SW).	Pork, horse, game meat	Larvae	Mandatory as part of general meat inspection according to regulation (EC) No 2004/854 and specifically according to regulation (EU) 2015/1375	In EU: 132/17,799 (0.74%) farmed wild boar, 1,228/1,404,565 (0.08%) hunted or not specified wild boar, 224/125,982,161 (< 0.01%) fattening pigs from not controlled housing conditions or not specified housing conditions, 19/752 (5.2%) brown bear
Anisakidae	Anisakiasis, (Gastric, intestinal, ectopic or gastro-allergic symptoms)	No European-wide surveillance	Not available, 1	7 In top 4 in 1 European region (SW).	Marine fish, Crustaceans, Cephalopods	Third-stage larvae (L3)	Mandatory as part of general fisheries products inspection for parasites according to regulation (EC) No 2004/854 and regulation (EC) No 2074/2005	Only Spain reported data: 101/366 (27.6%) raw fish at retail

(*continued on next page*)

Table 1 (*continued*)

Parasite	Disease (Human symptoms)	Cases reported in Europe 2017[a]	Median rate per 100,000 of foodborne DALY's in Europe; Proportion of disease that is foodborne[c]	Priority ranking overall in Europe; and by region[d]	Most likely food vehicle(s)[e] FAO/WHO (2014)	Detection target in food	Requirement for testing food	Detections in food reported in EU/EEA to EFSA in 2017 (EFSA/ECDC, 2018) Number positive samples or animals/number tested (%)
Protozoans								
Toxoplasma gondii	Congenital toxoplasmosis (Abortion, newborn hydrocephalus and seizures, juvenile ocular toxoplasmosis) Acquired toxoplasmosis (mostly non-specific clinical symptoms, ocular toxoplasmosis, infection in immunocompromised individuals can be fatal),	Congenital toxoplasmosis 40[b] No European-wide surveillance for acquired toxoplasmosis	2 (1–3); 0.49 (0.40–0.58) 6 (4–10); 0.49 (0.39–0.59)	Congenital toxoplasmosis: 2 Acquired toxoplasmosis: 6 Toxoplasmosis in top 4 in 2 European regions (N, W).	Meat from small ruminants, pork, beef, horse, game (red meat and organs)	Tissue cysts/ bradyzoites	To assess human exposure to parasites in meat	No European-wide surveillance
					Dairy products	Tachyzoites,	To assess human exposure to parasites in milk or dairy products	No European-wide surveillance
					Fresh produce, seafood	Oocysts	To assess human exposure to parasites in fresh produce and seafood.	No European-wide surveillance
Cryptosporidium spp.	Cryptosporidiosis (Diarrhoeal disease)	11,418	0.2 (0.03–0.6); 0.14 (0.06–0.28)	5 In top 4 in 2 European regions (N, W)	Fresh produce, fruit juice, milk and dairy products, shellfish	Oocysts	To assess human exposure to parasites in food	No European-wide surveillance
Giardia duodenalis	Giardiosis (Diarrhoeal disease)	19,437	0.03 (0.009–0.1); 0.15 (0.07–0.27)	8 Not in top 4 in any European regions	Fresh produce, shellfish	Cysts	To assess human exposure to parasites in fresh produce	No European-wide surveillance

[a] Human cases reported in EU/EEA to ECDC in 2017 (TESSy data) (web reference: Surveillance Atlas of Infectious Diseases).

[b] France did not report in 2017, but in 2015, reported 273 confirmed cases of congenital toxoplasmosis as a result of the active screening of pregnant women, accounting for 90% of all confirmed cases in the EU/EEA (https://ecdc.europa.eu/sites/portal/files/documents/AER_for_2015-toxoplasmosis.pdf).

[c] Median rate per 100,000 of foodborne Disability Adjusted Life Years (DALYs) by region, with 95% uncertainty intervals (Torgerson et al., 2015).

[d] Priority ranking overall in Europe and by region (Bouwknegt et al., 2018). N, Northern Europe; W, Western Europe; E, Eastern Europe; SE, South-Eastern Europe; SW, South-Western Europe.

[e] Most likely food vehicle(s) (FAO/WHO, 2014).

salad vegetables, and other fresh produce eaten raw that may be contaminated with *Echinococcus* eggs).

2.2. Taenia saginata

Infection with the tapeworm *T. saginata* causes taeniosis; humans are the definitive host and gravid segments (proglottids) of the intestinal worms exit the gut or release eggs in the faeces. *Taenia saginata* is also called the beef tapeworm, as cattle are the most common intermediate host. They become infected when they ingest *T. saginata* eggs, from which oncosphere larvae hatch that migrate to muscle tissue, including, the heart, the tongue, oesophagus, diaphragm and striated muscles. Here the larva forms a cysticercus – a fluid-filled cyst enclosed in a fibrous capsule. People become infected by ingestion of viable cysticerci in undercooked beef; the infectious dose is not known but is theoretically one viable cysticercus. Most human infections with *T. saginata* are asymptomatic, but mild abdominal symptoms may occur, including pain, loss of appetite, weight loss, nausea, and proglottids may be passed through the anus, causing pruritis. Although European directives regulating meat inspection for bovine cysticercosis have been in place for decades, *T. saginata* is still present in Europe, persisting partly due to the very low sensitivity of meat inspection and the environmental spread of eggs from sewage. In addition, there is a lack of prioritisation as the clinical symptoms are mild and the public health burden is low. Taeniosis is still reported in two thirds of the European countries, especially in Eastern Europe (Trevisan et al., 2018).

2.3. Trichinella spp.

Trichinellosis (syn. Trichinosis) is a global disease caused by the consumption of inadequately cooked meat containing viable larvae of the nematode *Trichinella* spp. Pork is the most important source of human infection, but meat from horses, wild boars, bears, walruses and badgers has also been the source of outbreaks (Gottstein, Pozio, & Nockler, 2009). Upon ingestion of *Trichinella*-infested striated muscle tissue, the infective larvae are released, enter the enterocytes of the small intestine, and undergo four moults to the adult stage. The adult worms mate and produce larvae, which migrate via the circulatory system to the striated muscles of the new host (Gottstein et al., 2009). Infection can cause serious disease in humans, which is characterized by diarrhoea, fever, periorbital oedema and myalgia, and possible complications such as myocarditis, thromboembolic disease, and encephalitis (Gottstein et al., 2009). *Trichinella spiralis* is the cause of most human infections worldwide, followed by *Trichinella britovi*. In addition, ten other taxa are less often associated with human disease and are found in different regions of the world, usually in wild animals (Pozio & Murrell, 2006). In wildlife, a substantial infection pressure persists, and is increasing in some areas, raising concern of potential spillover to outdoor, free-ranging pigs (Murrell, 2016). The human trichinellosis incidence in the EU has decreased steadily in the past decades; but in 2017, 168 confirmed human trichinellosis cases were reported, an increase of 65% compared with 2016 (101 confirmed human cases). This was mainly due to an increased number of cases in Romania (+38) and Bulgaria (+20) largely due to outbreak events. In 2017, Bulgaria had the highest notification rate in the EU (0.77 cases per 100,000), followed by Croatia, Lithuania, and Romania with 0.51, 0.32 and 0.24 cases per 100,000 population, respectively (EFSA/ECDC, 2018).

2.4. Anisakidae

Nematodes belonging to the family Anisakidae employ zooplankton as intermediate hosts, fish and cephalopods as paratenic hosts, and marine mammals as definitive hosts. Humans are accidental dead-end hosts, becoming ill following consumption of thermally unprocessed fish and seafood harbouring infective third-stage larvae (L3). Anisakiasis can manifest as gastric, intestinal, ectopic or gastro-allergic forms, although its incidence in Europe is still speculative and lacks reliable epidemiological data (D'Amico et al., 2014). Regulation (EC) 2074/2005 (European-Union, 2005) requires visual inspection of fish products for parasites. The main human pathogens in the Anisakidae family are *Anisakis simplex*, *Anisakis pegreffii* and *Pseudoterranova decipiens*. Morphological identification is not as accurate as genetic analysis. The prevalence of Anisakidae in wild fish and retail markets varies depending on genus, species, and geographic area (EFSA/ECDC, 2016) (Levsen et al., 2018). In the EU, in the period 2009–2013, the Rapid Alert System for Food and Feed (RASFF, web reference) reported a total of 333 notifications for the presence of parasites: of these, 262 (78.5%) were due to *Anisakis* spp. These notifications were mainly forwarded by Italy (147) and Spain (49) and refer to 108 border rejections, 86 information reports and 68 alerts (D'Amico et al., 2014).

2.5. Toxoplasma gondii

T. gondii is a protozoan parasite for which felids, especially domestic cats, are the definitive hosts. All warm-blooded animals, including livestock and humans, can act as intermediate hosts. Infected cats shed oocysts in their faeces, which, if ingested after sporulation, can infect intermediate hosts, developing into rapidly multiplying tachyzoites that spread throughout the body (EFSA BIOHAZ Panel et al., 2018). In pregnant women, tachyzoites can pass through the placenta and infect the foetus. After localising in muscle tissues and the central nervous system, tachyzoites convert to tissue cysts (bradyzoites). A foodborne *T. gondii* infection can thus be acquired either through the ingestion of tissue cysts in raw or undercooked meat or through ingestion of oocysts via consumption of contaminated vegetables, water, or shellfish (EFSA BIOHAZ Panel et al., 2018). Another source of infection for humans is unpasteurised milk or milk products that may contain tachyzoites. The relative importance of tissue cysts, tachyzoites, or oocyst-mediated infection is largely unknown; however, meat consumption is regarded as an important risk factor (Belluco, Simonato, Mancin, Pietrobelli, & Ricci, 2017; Cook et al., 2000), indicating the importance of bradyzoites. Anyone can become infected with *T. gondii* and the outcome depends on both the person and infecting strain. Most infections in otherwise healthy people are asymptomatic or result in flu-like symptoms. Occasionally, more severe illness may develop with fever, headaches, nausea, and muscle and joint aches. Ocular toxoplasmosis may develop, especially following infection with a more virulent strain, and is more common in South America than Europe. Of note, increased animal and meat trading might also increase the risk of spread of more virulent, genotypically diverse *T. gondii* strains (Pomares et al., 2011). During pregnancy, primary infection with *T. gondii* can cause abortion or birth of a congenitally infected child with serious health problems (Table 1). An acute or chronic *T. gondii* infection is especially serious for people who have weakened immune systems, and life-threatening complications may develop (e.g., encephalitis).

2.6. Cryptosporidium spp.

Infection with the protozoan parasite *Cryptosporidium* spp. causes acute gastroenteritis (cryptosporidiosis) in a wide range of animals, as well as humans. Of the 40 or so currently recognised *Cryptosporidium* spp., *Cryptosporidium hominis* and *Cryptosporidium parvum* are the major causative agents of human cryptosporidiosis, the former being predominant in humans and the latter zoonotic and also highly prevalent in livestock. Transmission is via the oocyst stage shed in faeces, transmitted directly to animals or people, or that can then contaminate water or food. Water is likely a vehicle in crop contamination and in food processing. Ingestion of even a single oocyst carries a probability of infection. Long-term sequelae following acute disease is increasingly reported, including gastrointestinal upset, irritable bowel syndrome (IBS), or IBS-like symptoms (Carter et al., 2019). *Cryptosporidium* is one of the most frequent causes of moderate-to-severe diarrhoea in young

children in sub-Saharan Africa and Southeast Asia, with a significant risk of death in toddlers (Kotloff et al., 2013). Despite this, many cases are undiagnosed, and identification and reporting of outbreaks is highly variable. Globally, 25 foodborne cryptosporidiosis outbreaks were reported between 1984 and 2017, the main vehicles being fresh produce (11 outbreaks), unpasteurised milk and dairy products (7 outbreaks) and fruit juice (3 outbreaks) (EFSA BIOHAZ Panel et al., 2018).

2.7. Giardia duodenalis

Infection with the protozoan *G. duodenalis* (syn. *G. lamblia, G. intestinalis*) can cause the gastrointestinal illness giardiosis, the most common parasite-caused diarrhoeal disease in humans (Cacciò & Lalle, 2015). Symptoms range from sub-clinical to acute or chronic disease, can lead to post-infectious long-term sequelae in naïve patients, and are associated with malabsorption and growth retardation in children experiencing recurrent infection (Cacciò & Lalle, 2015). *Giardia* poses serious concerns to both veterinary and human health, although zoonotic transmission is not the main route for human infections. In Europe, *Giardia* is often associated with foreign travel, whereas locally acquired infections are less often considered and therefore may remain undiagnosed. Infection occurs by ingestion of cysts, either present in contaminated water or food, or by direct contact with cyst-containing faeces (Cacciò & Lalle, 2015). Waterborne transmission can be an important route of infection, with several outbreaks documented worldwide, and irrigation using contaminated wastewater has been recognised as a source of crop contamination. The impact of *G. duodenalis* as foodborne pathogen is under debate, however, a recent review highlights its potential importance (Ryan, Hijjawi, Feng, & Xiao, 2018). In the rarely reported foodborne outbreaks, contaminated fresh produce (raw vegetables, salads, fruits and fruit juice) was the most commonly implicated food category (Adam, Yoder, Gould, Hlavsa, & Gargano, 2016). According to a recent systematic review on worldwide prevalence of human pathogens on fresh produce at the farm or packing facility, *Giardia* had the highest median prevalence among parasites investigated (Van Pelt et al., 2018). Contamination may occur throughout the food-production chain, and direct contamination by infected food handlers has been highlighted as a risk factor and associated directly with foodborne giardiosis (Figgatt et al., 2017) and a high proportion of the outbreaks (Ryan et al., 2018).

Table 2
Summary of standard and most commonly used methods for detection of FBP and key issues identified.

Parasite	Standard or most commonly used method	Key issues for food detection methods	Requirements for method development and validation
Echinococcus multilocularis *Echinococcus granulosus*	No current standard method. Washing and elution of eggs from fresh produce, flotation, sedimentation, and/or sieving followed by microscopy.	Sample preparation methods are cumbersome and need streamlining. Microscopy is non-specific as all Taeniid eggs have similar morphology; molecular methods are required for differentiation, even of genera.	These are highly pathogenic organisms and the use of an appropriate surrogate is needed for validation, quality control and proficiency testing. The use of other Taeniid eggs should be explored for this purpose
Taenia saginata	Regulation (EC) No 2004/854 requires visual inspection of whole or incised organs for cystircerci.	Visual inspection for cysticerci is subjective and lacks both sensitivity and specificity. Serology (Ag Elisa) has been developed but is not used as sensitivity is lacking.	There is no proficiency scheme, and ring trials have not been performed. The necessity for testing at all has been questioned and a risk-based sampling and testing approach may be more cost-effective.
Trichinella spp.	ISO 18743:2015 Artificial digestion/magnetic stirrer preparation of muscle samples, filtration, sedimentation, and direct detection of *Trichinella* larvae by microscopy or agglutination.	Food surveillance data of pork meat should be comparable across countries as monitoring and surveillance have been successfully harmonised between the EU member states. EURL have undertaken ring trials.	Test methods for other meats and meat products need validation. PCR needs further work and is not in Standard Method. There are guidelines for critical points.
Anisakidae	Regulation (EC) No 2074/2005 requires visual inspection of fish and fish products. Artificial digestion or compression and UV-press are used by most reference labs and seem to be comparable. ISO standards are currently being developed (ISO/CD 23036-1; ISO/CD 23036-2). Artificial digestion of some fish species for viability (FAO CODEX STAN 190–1995, FAO CODEX STAN 244–2004, FAO CODEX STAN 311–2013).	Methods are destructive. Visual inspection and candling lack accuracy, artificial digestion precision and UV-press speed optimisation. Molecular and mass spectrometry methods might be suitable for large-scale testing but at reference labs or on-shore facilities.	The ISO methods being developed await a ring trial.
Toxoplasma gondii	No current standard method for tissue cysts/bradyzoites. A method for pork meat based on enzymatic digestion, specific DNA magnetic capture and detection by PCR requires further validation for different meat samples and needs tested with other *T. gondii* genotypes from different regions of the world. In scientific studies mouse bioassays, and PCR, are used. No current standard method for tachyzoites. In scientific studies mouse bioassays, and PCR, are used. No current standard method for oocysts. In scientific studies mouse bioassays, and PCR or LAMP, are used.	No single method due to different food items and different parasite stages that require specific considerations and raise variable issues (e.g., *in vitro* culture, parasite purification, DNA concentration. There are ethical concerns around the use of bioassays for viability, and the requirement for cats for oocyst production (for which facilities are also lacking).	Molecular methods for detection and viability assays need further evaluation and validation. Serological assays may provide cost-effective tools to monitor and control the exposure of livestock to *T. gondii*.
Cryptosporidium spp. and *Giardia duodenalis*	ISO 18744 for detection of oocysts on leafy green vegetables and red berry fruits, based on surface elution, concentration and isolation by immuno-magnetic separation (IMS), detection and quantification by immuno-fluorescence microscopy.	It is not suitable for routine analyses, but could help food operators in their monitoring plan and/or in the validation of their HACCP system. For *Giardia* only, storage temperature is important (keep it cool).	The ISO method requires further validation as it does not cover the range of leaf types which may influence performance. Applicability to fruit juice needs to be explored. No proficiency scheme; no viability or infectivity assessment; no molecular characterisation.

3. Findings of the literature review

To gain a better understanding of which detection methods have been developed and validated for each parasite, and to provide some context of the requirement for testing, literature reviews were conducted up to 2017 or 2018. Due to the different legal requirements and varying amounts of literature available, the approaches ranged from systematic reviews to non-systematic selection of key relevant references (see details in Supplementary Table 1). The standard and most commonly used methods for detection, and key issues relating to these, are summarised for each parasite in Table 2.

3.1. *Echinococcus granulosus and Echinococcus multilocularis*

Microscopy has been widely used, but detects eggs of the family Taeniidae (which includes *Echinococcus* spp. and *Taenia* spp.) without differentiation of genus or species. One review (Alvarez Rojas, Mathis, & Deplazes, 2018) described 12 articles in which detection of Taeniidae eggs on fresh produce was described: four studies were from Iran, one from Jordan, one from Libya, four from Nigeria and two from Turkey. Diverse methods were used, but tended to be based on elution from the fresh produce, usually in sodium chloride solution, sometimes including a surfactant such as 0.1% Tween-80 or 1% sodium dodecyl sulphate, followed by concentration, either by sedimentation (some with centrifugation) or sucrose flotation or formol-ether concentration, followed by detection by microscopy. In all these articles, important data such as limit of detection (LOD) or reproducibility were lacking. No efforts had been made to consider validation or to include internal or external QC samples or information.

A further three articles concentrated specifically on detection of *Echinococcus* spp. eggs using PCR-based methods. One article described the large-scale processing of 14 kg fruit, root vegetables, and lettuce, individually washed in 240 L tap water, concentrated by serial filtration through decreasing mesh apertures (final stage 21 µm aperture) and centrifugation, and detection by both microscopy and a multiplex PCR for the discrimination of *E. granulosus* and *E. multilocularis* from other cestodes (e.g. *Taenia* spp., *Mesocestoides* spp.). This was achieved following DNA extraction using alkaline lysis, neutralization and a QIAamp DNA mini kit (Qiagen, Hilden, Germany), along with rotation for 30 min with Chelex beads, and PCRs targeting two mitochondrial genes; the NADH dehydrogenase subunit 1 (Cest1/Cest2 primer pairs that amplify a 395 bp target of *E. multilocularis*) and the small subunit ribosomal RNA gene (Cest3/Cest5 amplify a 117 bp target of *Echinococcus* spp. causing CE, and Cest4/Cest5 amplify 267 bp target of other cestodes). However, validation data and LOD information were lacking (Federer et al., 2016).

Two articles from Poland used a single method to prepare eluates from a more manageable sample size of 0.3–0.5 kg berries, mushrooms, root vegetables, lettuce (one head), or herbs (two bunches): samples were washed in 2 L of 0.05% Tween-80, sedimented by gravity to 100 ml, sieved (50 µm), frozen at −70 °C for 3 days to kill the eggs, thawed and concentrated by $ZnCl_2$ flotation on to a slide, washed into a 2 ml tube using distilled water, centrifuged, and stored at −20 °C. After freeze-thawing (3 x −70 °C to +30 °C), the DNA was extracted and samples analysed by a nested PCR targeting the 314 bp of the 12S ribosomal RNA (rRNA) gene for specific detection of *E. multilocularis* (Lass, Szostakowska, Myjak, & Korzeniewski, 2015, 2016). Validation data were not provided, but LOD was reported to be 100 eggs based on spiking raspberries, radishes, and mushrooms with 10, 100, and 1000 eggs (Lass et al., 2015).

The infectious dose of *Echinococcus* spp. for humans is not known, but is theoretically one egg; thus whether a LOD of 100 eggs is sufficient is questionable. Critical stages in detection are sampling (the large sample aliquots used in Federer et al. (2016) required very laborious processing that would be unsuitable for normal routine analytical laboratories), and concentration (the method used by Lass et al. (2015; 2016) involved considerable sample manipulation that is likely to risk considerable analyte loss). In addition, as Lass et al. (2015, 2016) used only PCR analyses, it is unclear whether eggs or free DNA were detected, although the flotation procedure is presumably designed with eggs in mind.

In conclusion, current methods are cumbersome and need streamlining. Microscopy could be supplemented with PCR to identify the genus, at least. Validation and proficiency data are lacking, but could be generated using other Taeniid eggs as a surrogate for the highly pathogenic *Echinococcus* spp. in spiking trials, for performance monitoring and for quality control.

3.2. *Taenia saginata*

Currently, the only control method applied for *T. saginata* is meat inspection according to Regulation (EC) No 854/2004 (European-Union, 2004a), which requires that every carcass from all bovines above 6 weeks of age is examined. This consists of a visual inspection of the carcass surface and predilection sites (tongue, oesophagus, and diaphragm) for cysticerci. In addition, the external and internal masseters, as well as the pericardium and heart, are visually inspected in a systematic manner following standard incisions. If suspected lesions are identified, various confirmation methods are available, although usually not used, including gross stereomicroscopic, histological, immunohistochemical, or molecular methods (Ogunremi, MacDonald, Geerts, & Brandt, 2004). Meat inspection is time consuming and costly, and when infected carcasses are detected, substantial economic losses due to downgrading and condemnation are incurred, making bovine cysticercosis primarily an economic constraint (Blagojevic et al., 2017; Dorny & Praet, 2007tion requires condemnation of the whole carcass if there is generalised infection. In cases of localised infection (cysts in one predilection site), the affected part is removed and the carcass, or deboned/jointed meat, held at temperatures below −10 °C or −7 °C for a minimum of two or three weeks, respectively (Hill et al., 2014).

Although visual inspection is mandated by the EU Regulation, the diagnostic sensitivity is below 30%, and can be especially low (down to 1%) for very light infections, and, consequently, official figures are probably hugely underestimated (Jansen et al., 2017; Jansen, Dorny, Gabriel, Eichenberger, & Berkvens, 2018) and continued transmission of the parasite has to be expected. In addition, specificity is also questionable due to possible misdiagnosis (Ogunremi et al., 2004). In recent years, it has been controversially discussed whether modifications of the visual meat inspection could increase sensitivity, also in a possible risk-based approach (Allepuz et al., 2012; Eichenberger, Stephan, & Deplazes, 2011; Jansen, Dorny, Trevisan, et al., 2018). However, as the method is subjective, dependent on the experience and skills of the inspector, and very labour-intensive (WHO/FAO/OIE, 2005), there is a clear need for applying more sensitive techniques to detect infected cattle (Jansen, Dorny, Gabriel, et al., 2018). The EU legislation allows alternatives, if: (i) specific serological tests indicate that the cattle have not been exposed to *T. saginata* eggs during rearing and/or (ii) the cattle originate from a farm officially certified to be free of cysticercosis (Dorny et al., 2010). However, to date serological tests are not used as an alternative to meat inspection. EFSA suggested a move to a risk-based approach to meat inspection (by inspection of "high risk" animals only), which would move away from a visual-only inspection (EFSA BIOHAZ Panel, 2013). However, as *T. saginata* is considered to be of low priority due to the assumed low human incidence and low health impact (Dorny et al., 2010), alternative improved post-mortem diagnostic techniques, such as antibody detection by enzyme linked immunosorbent assay (Ab-ELISA) in meat juice and serum (Abuseir, Kuhne, Schnieder, Klein, & Epe, 2007), antigen detection by enzyme linked immunosorbent assay (Ag-ELISA) on serum (Jansen, Dorny, Gabriel, et al., 2018), antigen detection by immunohistochemical methods in lesions (Ogunremi et al., 2004), and biomolecular assays (Chiesa et al., 2010) have not yet been

implemented.

In conclusion, current methods rely on subjective visual examination, for which there is no proficiency scheme, ring trials have not been performed, and there is a demonstrable lack of sensitivity. The necessity for testing at all has been questioned. If testing was more infection-site specific and included the use of an Ag ELISA and targeted high-risk populations, the total number of inspections would be reduced and thereby lower the costs to the beef industry, increase the number of infected carcasses found, lower microbial contamination of beef products, and improve public health outcomes.

3.3. *Trichinella spp.*

EU legislation has established special rules for the control of *Trichinella* in pig, horse, wild game and other meat that could contain this parasite (Regulation (EU) No 2015/1375, European-Union, 2015). Trichinellosis prevention is based on mandatory inspection and testing of all slaughtered pigs and horses, except in pigs from holdings officially recognised as applying controlled-housing conditions. Methods specified in the legislation are based on the enzymatic digestion of muscle samples and subsequent filtration and sedimentation steps, followed by the direct detection of *Trichinella* larvae by microscopy or agglutination. The internationally accepted reference method is the artificial digestion/magnetic stirrer method, described in ISO 18743:2015 (ISO 18743, 2015). Alternative methods are also provided in the EU legislation (Regulation (EU) No 2015/1375, European-Union, 2015), but positive results obtained in other methods have to be confirmed by digestion. None of the digestion methods enable identification of the *Trichinella* species or genotype; this can be carried out subsequently by molecular methods.

The majority (70%) of the 81 studies identified in the systematic review used an artificial digestion/magnetic stirrer method, and 25% used molecular methods. The food matrices tested were mostly pork, followed by wild boar, game, horse and other meats. Depending on trade obligations and national legislation, a multitude of small variations in the general protocol of the method were identified, with differing validation statuses. The artificial digestion/magnetic stirrer method has been the most extensively validated and showed the most reliable results (Gamble, 1996; Gayda, Reckinger, Thaben, Nockler, & Mayer-Scholl, 2016; Riehn et al., 2013). Validation parameters reported were mostly sensitivity, specificity, and efficiency of detection; reproducibility and repeatability were less commonly reported. Based on these validation studies the following test characteristics have been determined. Sensitivity is dependent on the sample size and sample type used; a 1 g sample of pork reliably allows for detection of ≥3 larvae per g (lpg) in muscle tissue, whereas ≥ 1.5 lpg and ≥1 lpg can be reliably detected in 3 and 5 g sample sizes, respectively (Forbes & Gajadhar, 1999; Gamble, 1996). This test sensitivity allows detection of the lowest number of larvae that can cause clinical symptoms in humans (Dupouy-Camet & Bruschi, 2007). To compensate for the lower digestibility of game meat, larger sample sizes should be used to improve sensitivity (Regulation (EU) No 2015/1375, European-Union, 2015). The specificity of the test is dependent on the skill of the operator in recognizing *Trichinella* larvae and should reach 100%. Molecular determination of the *Trichinella* species detected is undertaken at national reference laboratories (NRLs) or the EU Reference Laboratory for Parasites (EURLP).

All laboratories carrying out regulatory *Trichinella* testing must regularly participate in proficiency tests. The percentage of European NRLs that passed the proficiency test increased from 83% to 100% over an eight-year period (Marucci et al., 2016). The performance on national level was more heterogeneous, but a general improvement over time was also demonstrated, with, on average, more than 80% of all positive samples correctly identified (Glawischnig, Schleicher, Griesbacher, Stadlmuller, & Dablander, 2014; Johne, Bahn, Thaben, Nockler, & Mayer-Scholl, 2018; Petroff, Hasenclever, Makrutzki, Riehn, & Lucker, 2014).

Furthermore, to improve the performance of the testing laboratories, critical stages of the artificial digestion/magnetic stirrer method have been identified and recommendations made for sample collection and preparation, equipment and consumables, assay performance, results verification, and documentation. These minimum standards are available in the International Commission on Trichinellosis guidelines (ICT guidelines). It should be stressed that *Trichinella*-testing laboratories should adhere to the guidelines and EU legislation, as deviations from these protocols have not been sufficiently validated.

In conclusion, *Trichinella* monitoring and surveillance have been successfully harmonised between the EU member states. This includes mandatory inspection and testing as well as laboratory methods for detection of *Trichinella* spp. in pork, but to a lesser extent in game or other meat products. The majority of the data obtained for pork are thus comparable between the member states, and the results based on these data interpretable at EU-level. Test methods for other meats and meat products need validation.

3.4. *Anisakidae*

Visual inspection for parasites is mandatory before fish enter the food chain, in accordance with the Commission Regulation (EC) No 853/2004 and No 2074/2005 (European-Union, 2004b, 2005). The whole, unprocessed abdominal cavity (liver, gonads, and egg mass) must be inspected. This should be done continuously during manual evisceration and washing, or on a representative number of samples (not less than 10 fish per batch) if the process is mechanised. When implicated fish species are to be processed, or where a high infestation of parasites is suspected, FAO CODEX STAN 190 (1995) recommends that fish flesh should also be inspected for L3 larvae by candling (shining a bright light through a fillet in a dark room, such that the larvae show up as dark shadows). Visual inspection and candling are of low efficiency for Anisakidae detection (Llarena-Reino et al., 2013); only 7–10% of L3 larvae are detected in fillets by candling, dependent on fillet thickness, size, texture, colour, and fish species, and largely depends on the training and skills of inspectors (Levsen, Lunestad, & Berland, 2005). The use of a UV-press (i.e., compression of the edible portion followed by freezing and UV visualisation of the pigment lipofuscin in L3 larval cuticle at λ 366 nm), could be readily implemented by industrial operators with no experience of detecting of nematode larvae. Although the method is destructive, it could be used on batch samples. Although samples could be prepared on a fishing vessel, the UV visualisation is better suited to land-based fish-processing plants (Gomez-Morales et al., 2018).

Most other methods are applied as confirmatory tests or to quantify parasitic infections by specialised laboratories; they are not suitable in routine examination. These include artificial digestion, magnetic resonance imaging (MRI), and molecular methods (EFSA BIOHAZ Panel, 2010; Llarena-Reino et al., 2013). Parasite counting and determination of viability in un-processed or lightly-processed fish fillets (e.g., smoked fish) can be undertaken by artificial digestion followed by visual inspection for viability (FAO CODEX STAN 244–2004 and amendments). Improvements to artificial digestion include the use of liquid pepsin, reported to increase sensitivity, efficiency and accuracy (Llarena-Reino et al., 2013). A comparative study performed on anchovies, showed that artificial digestion performed better than visual inspection, with recovery of 91.7%, sensitivity and specificity of 93–100%, and accuracy of 97% (Guardone et al., 2016). In multicentre studies (CORDIS EU research results, 2016; Gomez-Morales et al., 2018), UV-press was less variable and more reproducible than artificial digestion, although there were no differences in accuracy and specificity. Based on this evidence, an ISO standard “Microbiology of the food chain – Methods for the detection of Anisakidae L3 larvae in fish and fishery products – Part 1: UV-press method and Part 2: Artificial digestion method” is under development (ISO/CD 23036-1 and ISO/CD 23036-2). Since 2013, an

accredited proficiency testing scheme for confirmatory analysis of L3 in fish fillets has been provided by the EU Reference Laboratory for Parasites. Results largely support the comparable and higher performance of artificial digestion and UV-press versus candling (see web reference: Proficiency testing, EURLP).

Among the newest and sensitive non-destructive methods, MRI has been shown to detect *A. simplex* accumulations and movements in the herring viscera. This method is genus-specific and enables larvae detection in live fish. However, it requires expensive hardware and maintenance, specialised training, data acquisition is slow (13 min/fish), and is not applicable in frozen products (Bao et al., 2017).

None of the methods listed so far allow determination of parasite species or genotypes and final identification can be done by morphological examination of the larvae and/or by molecular methods. As the genus *Anisakis* comprises a complex of sibling species, morphological discrimination is difficult. PCR-based methods usually target the ITS or mitochondrial genes (Abe, Ohya, & Yanagiguchi, 2005; D'Amelio et al., 2000; Mattiucci, Paoletti, Damiano, & Nascetti, 2007; Umehara, Kawakami, Araki, & Uchida, 2008; Zhu et al., 2002). A multiplex real-time PCR reported LODs of 0.32 pg/µl (~2.4 genomic copies/µl) for *Anisakis* spp. and 1.6 pg/µl (~12 genomic copies/µl) for *Pseudoterranova* spp. (Cavallero et al., 2017); a TaqMan assay was able to detect 0.1 pg of *A. simplex* DNA (Lopez & Pardo, 2010).

Anisakis spp. can cause gastro-allergic reactions due to hypersensitivity to allergens, even in processed fish. In this respect, detection of allergens is preferable as they are more stable than DNA following heat treatment (e.g., pasteurisation of baby food) or freezing. Allergens are usually detected by ELISA. The efficiency of IgG and IgE-based ELISA is largely dependent on the tested sample with the recovery being 59–74% in whitefish pudding and 106–110% in pepper mackerel (Faeste, Plassen, Lovberg, Moen, & Egaas, 2015). Sensitivity was high, with reported LOD of the antigen Ani s 4 of < 1 ppm (recovery > 65% and no cross-reactivity) (Rodriguez-Mahillo, Gonzalez-Munoz, de las Heras, Tejada, & Moneo, 2010). ELISA has also been used to quantify *A. simplex* proteins in fish products and seafood. Intra- and inter-assay precisions for a sandwich-format ELISA were < 11 and < 25% respectively, with reported LOD of 0.3 µg A. *simplex* protein/g food and LOD of 1.1 µg A. *simplex* protein/g food but varied by fish product with reported recoveries ranging from 72 to 101% (Werner, Faeste, Levsen, & Egaas, 2011).

Another approach for the detection of *A. simplex* allergens is mass spectrometry (MS) but so far only for research purposes. High-resolution MS using Orbitrap showed a LOD comparable to ELISA (Faeste et al., 2016). Further adaptations of MS approaches such as collision-induced dissociation (CID) liquid chromatography-tandem mass spectrometry (LC-MS/MS) have also been described (Faeste et al., 2015).

In conclusion, for fish industries visual inspection and candling remain the only non-destructive options for detection of Anisakidae, although they need well-trained operators. For confirmation, artificial digestion seems promising and more data will indubitably become available following application of the forthcoming ISO standard; further evaluation of artificial digestion precision will be necessary. Automation of the UV-press is required to reduce test time and enable transfer to fisheries. Molecular methods are suitable for large-scale application and random sampling of food at point of sale and for quality standardisation (Cavallero et al., 2017). Antigen detection by either ELISA or MS needs standardization for confirmative and quantitative tests by industry. Risk-based sampling is not performed, but could be considered, such as where a considerably lower Anisakidae prevalence is identified (e.g., in farm-reared sea bass) (Cammilleri et al., 2018).

3.5. *Toxoplasma gondii*

There is no single method for the detection of *T. gondii* in food as different food matrices (meat, fruit, vegetables, shellfish, milk, or milk products) often contain different parasite life cycle stages. *T. gondii* has a low infectious dose; single viable oocysts can lead from acute to chronic infections or to the death of infected mice, depending on the *T. gondii* strain (Dubey, Ferreira, Martins, & McLeod, 2012), meaning that detection methods need to include concentration steps to ensure high analytical sensitivity. Different processing techniques are required to concentrate either *T. gondii* parasitic stages or DNA, due to the variant nature of the food items and the varying robustness of the different lifecycle stages of the parasite within them. If assays aim at the detection of infective, rather than dead, parasites, additional considerations during storage and processing of food samples are necessary to guarantee that the relevant *T. gondii* lifecycle stages are not inactivated (e.g., by freezing samples prior to analysis). Methods show varying degrees of sensitivity and specificity. However, to the best of our knowledge, no country has adopted mandatory or routine testing of any food items for *T. gondii*; no generally accepted, validated, standard methods exist, and, consequently, there are no proficiency schemes for testing food for the presence of *T. gondii* parasites.

Based on a recent EFSA scientific opinion (EFSA BIOHAZ Panel et al., 2018) and on an extensive literature review for detection methods of *T. gondii* by an EFSA-funded consortium, it was concluded that bioassays (experimental inoculation of cats and mice with sample material) are the most sensitive methods of detecting viable, infective *T. gondii* in meat samples (EFSA BIOHAZ Panel et al., 2018; (Opsteegh, Maas, Schares, & van der Giessen, 2016). However, bio-assays are not a practical method to screen food products because they may take several weeks to complete, require specialist facilities and expertise, and raise serious ethical concerns. Nevertheless, bio-assays are considered the reference standard for the evaluation of detection and viability assays for *T. gondii* (Gisbert Algaba et al., 2017). Defining an alternative benchmark method would be useful. Currently, other viability assays (avoiding animal experimentation) such as *in vitro* culture (Koethe, Schade, Fehlhaber, & Ludewig, 2017; Rousseau et al., 2018; Villegas et al., 2010; Ware et al., 2010) or the molecular SporoSAG reverse transcriptase RT-PCR assay (Villegas et al., 2010) lack sensitivity or suitability, but could be further developed. At present, *in vitro* culture is challenging for meat or even impossible for other types of samples such as fresh produce contaminated by *T. gondii* oocysts.

An overall workflow for the detection of *T. gondii* in various foods needs to include a concentration step dependent on the type of test sample (Supplementary Table 2), followed by an efficient DNA extraction and a sensitive *T. gondii* DNA detection method. Various protocols to release and concentrate the parasitic stages have been applied, including homogenisation (e.g. mechanically or enzymatically) or washing followed by sieving or flocculation and subsequent centrifugation (Supplementary Table 2). For samples containing oocysts, in-house immunomagnetic separation (IMS) of oocysts from leafy vegetables has been successfully applied (Supplementary Table 2). For other parasitic stages, such as bradyzoites and tachyzoites, IMS methods are not available.

Optimally, parasite concentration is followed by DNA extraction and a *T. gondii*-specific PCR, such as those based on the *T. gondii* 529 bp repeat, which, having up to 200–300 copies per genome of *T. gondii,* is more abundant than other PCR targets (Homan, Vercammen, De Braekeleer, & Verschueren, 2000). Reports on proficiency tests or comparisons of various DNA detection methods applied to human diagnostic samples, e.g., (Belaz, Gangneux, Dupretz, Guiguen, & Robert-Gangneux, 2015; Morelle et al., 2012; Sterkers et al., 2010; Varlet-Marie et al., 2014), may provide valuable information for the selection of optimal DNA detection methods applied to analyse DNA extracted from food matrices.

An alternative approach for meat, seafood, milk and potentially milk-products would be (i) digesting total samples (e.g., with Proteinase K), and then (ii) using a specific DNA magnetic capture (MC) method to concentrate target DNA that can then be tested by PCR. This approach has been refined and validated using the ISO/IEC 17025, 2017 standard for use in meat (Gisbert Algaba et al., 2017); pork from experimentally

infected pigs was used to validate a MC-qPCR method to enrich target DNA from tissue lysates in comparison to bio-assay. The assay was reported to have a LOD of 65.4 tachyzoites per 100 g of pork at a sensitivity level of 99%. A non-competitive PCR control was included to test for PCR inhibition. The assay was assessed for intra- and inter-lab repeatability, two operators and two labs respectively (Gisbert Algaba et al., 2017). This is the most validated method for the detection of *T. gondii* in food so far but it requires further validation for 1) different meat samples, 2) potentially for other food samples, such as shellfish, milk or milk products and 3) strains/clonal types circulating in other parts of the world (for example South America) than Europe and North America.

In conclusion, there are currently no standard methods available to detect *T. gondii* in a variety of different food matrices and contaminated with different parasitic stages. The MC-qPCR protocol for meat (i.e., pork containing tissue cysts) (Gisbert Algaba et al., 2017) is not widely used as yet. Applicability for routine application also needs to be shown. In this respect, due to the high prevalence in some meat sources, it should be carefully evaluated under which circumstances routine testing of meat products is desirable at all. For other matrices, IMS procedures represent promising alternatives but are under development. Although ethically problematic, bioassays continue to represent a reference method; in contrast to other methods, bioassays are able to show not only the presence, but also the viability, of the parasite. Serological assays are able to demonstrate the exposure of some livestock species to *T. gondii*; however, the presence of specific antibodies is not a reliable proxy to predict the presence of viable *T. gondii* in livestock deemed for meat production, in particular in cattle or equids (Opsteegh et al., 2016). Nevertheless, serological tests are interesting and cost-effective tools to monitor and control the exposure of some livestock species to *T. gondii*.

3.6. *Cryptosporidium spp.*

There is one International standard method for testing food for *Cryptosporidium*, ISO 18744: 2016 (ISO 18744, 2016), based on retrieval of oocysts from leafy greens and berry fruits by surface elution, concentration by centrifugation, and isolation by immuno-magnetic separation (IMS), and enumeration by immunofluorescent microscopy (IFM). A ring trial had variable results, despite being limited to iceberg lettuce and raspberries (Cook et al., 2006a; 2006b). Considering the differences in biochemical composition and structure of other leafy greens and berries, the applicability of this standard requires further validation. Furthermore, there is currently no proficiency scheme. The method is rarely used, most likely because: i) of the limited number of laboratories with appropriate expertise; ii) it is time-consuming, iii) it is expensive to perform, iv) the long incubation period between ingestion and illness, combined with the perishability of fresh produce, limits its usefulness for source attribution in outbreaks. Two steps are critical: 1) Elution: oocyst recovery depends on the physical structure of the food matrix and whether oocysts are retained, and the chemistry of the food matrix and buffers (Amoros, Alonso, & Cuesta, 2010; Chandra, Torres, & Ortega, 2014; Cook et al., 2006b, 2006a; Robertson and Gjerde, 2001, 2000; Rzezutka et al., 2010; Shields, Lee, & Murphy, 2012), and 2) microscopy detection: reliant on the expertise of microscopists and this uncertainty of measurement questions the quantitative nature of ISO18744. Modification, especially of the IMS step by reducing the numbers of paramagnetic beads and using in-house buffers, reduced the cost; and a mean recovery rate of 53% ± 28 was achieved in a ring trial (Utaaker, Huang, & Robertson, 2015). Other modifications such as elimination of the IMS step (Caradonna et al., 2017; Sim et al., 2017; Utaaker et al., 2015; Utaaker, Kumar, Joshi, Chaudhary, & Robertson, 2017), a new isolation step based on aptamer binding (Iqbal et al., 2015), and detection by molecular assays (Hohweyer et al., 2016; Hong et al., 2014) may reduce cost and time-to-result, both of which are crucial for industrial self-monitoring plans and foodborne outbreak investigations. However, few studies determined method performance: mean recovery rates of 11% and 14% in basil and raspberries respectively, and limits of detection of ≤1 oocyst/g in raspberries and 12 oocysts/g in pineapple or mango have been reported (Hohweyer et al., 2016; Iqbal et al., 2015). Sample surveys showed lower occurrence where IMS was not used (EFSA BIOHAZ Panel et al., 2018).

Published methods for fruit juice included an isolation step (IMS, flotation, sucrose gradient, or microfiltration) followed by molecular detection (based on conventional or real-time PCR) or IFM (Deng & Cliver, 2000; Deng, Lam, & Cliver, 2000; Frazar & Orlandi, 2007; Garcia, Henderson, Fabri, & Oke, 2006; Minarovicova, Kaclikova, & Kuchta, 2010; Robertson et al., 2019). No ring trials were performed and limit of detection was the only assessed performance criterion (from 10 to 500 oocysts/100 ml). The main critical steps are: 1) sample preparation (volume, isolation method which may be affected by acidity); and 2) DNA extraction efficacy. Microfiltration and a real-time nested PCR led to the lowest LOD, 4 oocysts/100 ml (Minarovicova et al., 2010).

Most of the studies of milk and dairy products have been performed on liquid milk. Only one study also assessed ice cream and yogurt (Deng and Cliver, 1999). Depending on the fat content, sample preparation may include defatting (using ether, trypsin, or detergents) followed by oocyst isolation (by IMS, flotation or microfiltration). Detection was performed by microscopy of stained concentrates, or, more commonly, by conventional or real-time PCR. Limits of detection (Deng et al., 2000; Di Pinto & Tantillo, 2002; Frazar & Orlandi, 2007; Laberge, Ibrahim, Barta, & Griffiths, 1996; Minarovicova, Lopasovska, Valik, & Kuchta, 2011) and recovery rates from 1 to 98% (Deng and Cliver, 1999) were described, but there have been no ring trials. Reducing fat content is critical for the recovery rate, affecting the sample preparation and DNA extraction. Considering LOD, the most promising methods in milk appears to be an IMS-PCR, regardless of the fat content (10–20 oocysts per 100 ml) (Deng et al., 2000; Di Pinto & Tantillo, 2002), or microfiltration followed by real-time PCR detection in low fat milk (10 oocysts per 100 ml) (Minarovicova et al., 2011).

One advantage of PCR is the possibility of genotyping and subtyping, which may indicate the source of contamination and may also indicate the potential risk to consumers. However, a challenge for PCR detection of *Cryptosporidium* spp. in food, and, especially real-time PCR seeking all *Cryptosporidium* spp., is the design of primers that don't amplify sequences from the food matrix. This has been observed with some primers in the 18S gene, requiring confirmation of positive PCR reactions, for example by sequencing (Staggs et al., 2013).

In conclusion, ISO 18744 supports the growing need to collect *Cryptosporidium* occurrence data in food and is a good starting point for sample surveys, but requires further validation to refine the range of its applicability. More importantly, it is not suitable for routine analyses to help food operators in their monitoring plan and/or in the validation of their HACCP system. Alternative methods need to be developed and validated, keeping in mind the various food matrices, cost, time-to-results, and practicability. This could be achieved in part by transition from IFM to molecular detection.

3.7. *Giardia duodenalis*

ISO 18744: 2016, discussed above for *Cryptosporidium*, includes detection of *Giardia* cysts in fresh produce, but is performed rarely, having the same limitations. Few studies have focused on further development and/or improvement of detection methods. Various vegetables have been tested using similar methods (mainly mixed salads, tomato, parsley, lettuce and cabbage) and to a lesser extent berries (Hohweyer et al., 2016; Robertson & Gjerde, 2000; Robertson and Gjerde, 2001) or fresh fruit juices (Mossallam, 2010). The overall *Giardia* prevalence ranged from 0.2% to 60%, depending on the setting and the type of fresh produce. Validation data was scarcely provided; in one study, reproducibility was assessed but mean recovery was only 2%

for basil (LOD of 3 cysts/g) and 21% for raspberries (LOD < 1 cyst/g) (Hohweyer et al., 2016). A single ring trial was conducted using the modified ISO 18744 discussed above (Utaaker et al., 2015), but considerable inter-laboratory variability in the recovery efficiency of 50 cysts (mean 18 ± 20 cysts) was reported. Exclusion of labs that had reported sample problems increased the mean recovery efficiency to 33 ± 22 cysts.

Some studies (Supplementary Table 3) used cheaper isolation strategies than IMS, such as filtration and discontinuous sucrose gradient centrifugation or sugar flotation (Armon, Oron, Gold, Sheinman, & Zuckerman, 2002). High variability in cyst recovery rate has been reported (2–70%) largely depending on the foodstuff (leafy greens vs berries and smooth skinned vegetables) and to a lesser extent on the isolation method (Amoros et al., 2010; Hohweyer et al., 2016; Keserue et al., 2012; Ramirez-Martinez et al., 2015; Robertson & Gjerde, 2000, Robertson and Gjerde, 2001). Parasites were mainly detected by light microscopy or IFM and/or PCR. Higher sensitivity of PCR vs. IFM has been reported (Dixon, Parrington, Cook, Pollari, & Farber, 2013; Ramirez-Martinez et al., 2015; Tiyo et al., 2016). In one study, PCR targeting the β giardin (bg) gene, which also allows genotyping, was evaluated and revealed high amplification efficiency (90%), and compared to microscopy, high sensitivity (100%) and specificity (81%) (Ramirez-Martinez et al., 2015).

Since there is no proficiency scheme, the selected literature highlighted some critical steps for further method development. Long-term storage of samples without refrigeration, the type of fresh produce, and different washing buffers and procedures can all affect cyst recovery (Utaaker et al., 2017). Although rigorous comparative analysis of detection methods has not been performed, IFM should be considered more sensitive than light microscopy. To assess applicability and reliability of molecular methods, in particular qPCR (with or without IMS), different target loci and primer combinations should be evaluated. Efficiency of DNA extraction methods was only preliminarily assessed (with alkaline lysis more efficient than freeze-thawing or thermal shock) (Ramirez-Martinez et al., 2015), and other protocols (e.g. bead beating) and the effect of multiple fresh produce types should be evaluated in order to develop a robust detection method.

In conclusion, despite the availability of an ISO standard, further developments are needed for a robust method applicable to various fresh produce types, including fresh juice. To foster transition from IFM to molecular detection, robust evidence of the advantage (genotyping; faster results) and disadvantage (contamination with DNA) especially for laboratories performing routine analysis are still needed.

4. General points and concluding remarks

The development and application of standardized and validated detection methods for these selected FBP would enhance our understanding of their foodborne routes of transmission, improve risk assessment, and help identify and verify critical control points. By reviewing the methods that have been used for detecting some FBPs in their most important food items, and their validation, we have identified that the requirements for testing for FBP differ widely, varying by detection targets, transmission pathways, potential impact, and legislation. Development of a universally applicable detection procedure for FBP is therefore unrealistic.

For one FBP, *Trichinella* spp., both the legislative requirements and analytical tools are well defined, and are supported by a proficiency scheme that has reported improved performance; data are now largely comparable across the EU. For others, even where testing is mandatory (*T. saginata* as part of general meat inspection; Anisakidae by visual inspection), potential for improvement has been identified. A risk-based approach has been proposed for *T. saginata*, possibly in combination with Ag-ELISA, to prevent heavily-infected carcasses entering the food chain. With the EFSA recommendations to move away from masseter incisions (EFSA BIOHAZ Panel, 2013), such an approach may be even more relevant. For Anisakidae, the challenge is how to incorporate improved alternatives to visual inspection (UV-press and artificial digestion) into the fish-processing line.

To date, no regulations or microbiological standards exist for most FBP in foods. Nevertheless, these pathogens also fit into the regulatory framework of EC No. 178/2002 (Food law, Article 14). The publication of a standard method in 2016 (ISO 18744) for *Cryptosporidium* and *Giardia* in leafy green vegetables and red berry fruits, initiated work in this direction. However, this method is not widely used, particularly routinely, probably largely because there is no mandatory requirement. Furthermore, convincing validation data are lacking. Awareness of the public health relevance of foodborne *T. gondii* is increasing, and although it is envisioned that risk-based testing of meat will be more frequently practised in the near future, a practical alternative to the bioassay for the detection of viable tissue cysts remains challenging. Although there is increased awareness that contamination of vegetables, salads or fruits by helminth eggs (such as *Echinococcus* spp.) or *T. gondii* oocysts are of potential relevance for public health, there are no ISO methods and no common strategy for method development.

For most FBP, the key aspects of detection assays include sample preparation, separation of the parasite from background material, and either visualisation of the parasite itself - for which microscopy is often needed - or detection of antigens, DNA, or other specific markers. Since FBP do not multiply in the environment, assays need to be able to detect low numbers. Unlike bacterial assays, they do not rely on growth or culture, so neither viability nor infectivity are indicated. Another shortcoming of some detection methods for FBP is that genus and/or species are not identified, unless molecular methods are used. Whether for detection or genotyping, DNA extraction efficacy becomes a critical element and sample representativeness is crucial (e.g., homogenisation and concentration). Furthermore, efforts should be made to overcome challenges related to PCR inhibitors in the sample matrix. The potential of further characterization of contaminating FBP that DNA-based analyses provide, can improve source attribution and comparison with infections in humans, which is essential in outbreak investigations, as illustrated by *Cryptosporidium*. In addition, species identification by genotyping of parasitic contaminants along the food chain might be used as an indicator of human or animal faecal contamination.

If standard methods are to be developed, proper validation is necessary to ensure robust detection assays that can be readily performed by analytical laboratories. One problem is that documented procedures for validation, such as ISO 17468:2016 and 16140-2:2016, were originally established for bacteria. Although protozoa are now acknowledged in ISO 16140-2:2016, few recommendations are indicated and further developments are required to fit with parasite specificities. The gaps identified in validation meant most methods fail to meet technical requirements for the establishment of a reference method, the absence of which, in itself, may cause difficulties. For example, limited sample types are tested (e.g., restricted range of salad leaves), limits of detection are not provided, ring trials are rarely performed, and, for most FBP, quality-control materials, proficiency schemes, and reference standards are lacking. Efforts are needed to harmonize performance requirements (based on ISO16140-2 recommendations) and the cut-off values. The use of surrogate particles may be necessary for method development or validation purposes, but their use should be critically evaluated.

Future strategies should focus on developing methods that are amenable to standardisation, provide for validation, and ensure they are accessible to analytical laboratories, with reduced cost and time-to-result. However, such methods can probably not be harmonised with those for detection of some other pathogens (such as bacteria and viruses) in food, due to the diversity of FBP, their low abundance, and lack of amenability to *in vitro* culture. Furthermore, implementation of risk-based testing strategies should be carefully considered. In the future, techniques should be assessed in the ISO 17468 framework to propose a standard suitable for the food products considered here.

Finally, selected methods should be assessed on a wide range of appropriate food matrices at risk, keeping in mind that any single method may be not suitable for the detection of a specific parasite in different foods. Several European initiatives are underway to improve and harmonize detection methods and validation for FBPs. For example, the EFSA-supported project IMPACT for "standardising molecular detection methods to improve risk assessment capacity for foodborne protozoan parasites, using *Cryptosporidium* in ready to eat salad as a model" and several projects within the One Health European Joint Programme include FBPs highlighted in this paper, such as *Echinococcus*, *T. gondii*, *Giardia,* and *Cryptosporidium* (see web reference OHEJP).

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Appendix A. Supplementary data

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