Selected viral, protozoan and bacterial agents on minimally processed vegetables and sprouts at point of sale

MONIKA MORAVKOVA – PETRA VASICKOVA – MICHAL SLANY – IVANA KOLACKOVA – JAKUB HRDY – RENATA KARPISKOVA – PETR KRALIK

Summary

Epidemiological data linked to cases of food-borne infection from fresh produce are scarce and usually underestimated. The present study reports the occurrence of food-borne pathogens such as viruses (norovirus, hepatitis A virus) and protozoa (*Giardia intestinalis*, *Cryptosporidium parvum*/*hominis*) in 175 samples of fresh-cut vegetables, frozen vegetables and sprouts that originated in the Czech Republic and in other European countries, as detected using real-time PCR and reverse transcription real-time PCR. In addition, samples were analysed for *Escherichia coli* as an indicator of fecal contamination and for *Staphylococcus aureus* as an indicator of possible contamination by food handlers, using conventional culture methods. Norovirus was detected in two samples (1.1 %) of rocket and frozen mixed vegetables. *G. intestinalis* was detected in two samples (1.1 %) of mixed fresh vegetable salads and mixed frozen vegetables. The occurrence of norovirus did not correlate with *E. coli* presence. *Staph. aureus* was detected in 3 samples (1.7 %) but without genes for classical enterotoxins production. Our results highlight the need for microbiological analysis of food of non-animal origin for specific protozoan and viral agents, which are being less frequently monitored in these food category types, but their presence may pose risk for consumers.

Keywords

food-borne pathogens; vegetables; *Giardia*; norovirus; *Staphylococcus aureus*

Ready-to-eat vegetables and sprouts pose a higher risk of food-borne infections resulting from consumption of fresh produce as they are more commonly contaminated with infectious pathogenic agents than processed foodstuffs [1, 2]. Although in the past, attention was mainly focused on the study of bacterial pathogens contaminating foodstuffs, in the last few years, more attention has been paid to viruses and, most recently, also to parasites [2–5].

Norovirus (HuNoV) is the most common cause of non-bacterial gastroenteritis worldwide [6]. The virus is highly infectious with an incubation period of one to three days and only several units or tens of viral particles are necessary to elicit an infection [7]. This is in contrast with the long incubation period of two to four weeks for hepatitis A virus (HAV) infection, which further complicates tracking of infection sources for contaminated food. The World Health Organization identified both HuNoV and HAV in fresh produce including vegetables as a priority virus/commodity combination for which control measures should be considered [8].

Protozoan diseases are widely distributed in developing countries [9, 10] but food-borne parasites are increasingly being recognized as a cause of health problems in humans in high-income countries as well [11]. This is due to cosmopolitan markets with food, changes in climate and agriculture, and the increasing number of immunocompromised humans worldwide [4]. *Cryptosporidium* spp. and *Giardia intestinalis* (syn. *G. lamblia* or *G. duodenalis*) are ranked among the most

Monika Moravkova, Petra Vasickova, Michal Slany, Ivana Kolackova, Renata Karpiskova, Petr Kralik, Department of Microbiology and Antimicrobial Resistance, Veterinary Research Institute, Hudcova 70, 62100 Brno, Czech Republic.
Jakub Hrdy, Department of Microbiology and Antimicrobial Resistance, Veterinary Research Institute, Hudcova 70, 62100 Brno, Czech Republic; Department of Experimental Biology, Faculty of Science, Masaryk University, Kamenice 753/5, 62500 Brno, Czech Republic.

Correspondence author:
Monika Moravkova, e-mail: moravkova@vri.cz

commonly reported enteric protozoa in developed countries. The most often affected patients are young children and immunocompromised patients who experience clinical disease more frequently and with higher severity. Cryptosporidiosis is the second most common diarrheal infection in infants under two years in developing countries [12]. In industrialized countries, reported cases of cryptosporidiosis are not so frequent. However the number of diagnosed cases is increasing, likely due to improved laboratory diagnostics and physician education. In the European Union, 10915 cases of cryptosporidiosis were confirmed in 2015, which was by 41 % more than reported in 2014 [13]. In the same year, giardiasis was confirmed in 18031 cases with an increase of 4.4 % from 2014 [14]. Out of *Cryptosporidium* spp., *C. parvum* and *C. hominis* are responsible for vast majority of human cases [2]. Both infections are transmitted via the fecal-oral route when people may be infected via ingestion of cysts/oocysts through the consumption of contaminated food or water, or during direct contact with infected humans and animals.

Unfortunately, epidemiological data linked to cases of alimentary infections originating from fresh produce are scarce and likely underestimated. Therefore, the current study aimed to assess the preliminary data on presence of viral and protozoan food-borne pathogens, particularly HuNoV, HAV, *G. intestinalis* and *C. parvum/hominis* in leafy greens, frozen vegetables and sprouts collected from supermarkets in the Czech Republic. To correlate the results with conventional methods, *Escherichia coli* as an indicator of fecal contamination and *Staphylococcus aureus* as an indicator of contamination by food handlers were assessed by conventional culture methods.

MATERIALS AND METHODS

Sample collection

A total of 175 samples from 32 producers were collected from February to November 2014 from eight supermarket chains in the Czech Republic. Samples were collected according to availability in various seasons usually in two runs per month. Samples included minimally processed vegetables such as packed fresh vegetable mixed salads and leafy greens (97 samples; e.g. rocket, spinach, lamb's lettuce), frozen vegetables (43 samples; e.g. peas, carrots, maize and mixed) and sprouts (35 samples; alfalfa sprouts, mung bean sprouts, radish sprouts, lentil sprouts; Tab. 1). Seventy-six samples originated from the Czech Republic, 75 samples originated from other European Union countries, namely, Austria, Belgium, France, Italy, Germany, Netherlands, Poland and Slovakia, two samples were from United Kingdom, six samples from Israel and no country of origin was specified for 16 samples. Information about processing from the producer was available only for few samples of fresh vegetable mixed salads and leafy greens. Sixteen samples were labelled as washed or processed but no further information was added. Thirteen samples were labelled as non-washed or washing before consumption was recommended. All samples were transported to the laboratory in a cool boxes, stored in a refrigerator at 6 ± 2 °C or, in case of frozen vegetables, in a freezer at –18 °C. Samples were processed within 24 h from purchase, all during the expiration period.

Viral and protozoan detection

To reduce the cost and time of preparation, a unified procedure for processing of samples for both viral agents and protozoa was applied [15]. Initially, 100 g of each sample was homogenized in Trizma base [2-amino-2-(hydroxymethyl)-1,3-propanediol] glycine beef extract (TGBE washing buffer; 12.1 g Trizma base, 3.8 g glycine, 10 g beef extract dissolved in 1 l of distilled water, pH 9.5, prepared in house) and centrifuged. The supernatant was used for further analyses focusing on the presence of food-borne viruses and the sediment was used for detection of protozoan agents. The presence of viral agents in samples was determined according to ISO/TS 15216-2:2013 [16] with slight modifications regarding the amount of supernatant at the beginning of the analysis

Tab. 1. Collected vegetable samples.

Produce type	Number	Sample	Number
Fresh pre-cut products	97	Vegetable mixed salads	57
		Spinach	19
		Rocket	12
		Lamb's lettuce	9
Frozen vegetables	43	Frozen vegetable mix	22
		Green peas	9
		Carrot	4
		Frozen corn	3
		Spinach	3
		Green beans	2
Sprouts	35	Mung bean sprouts	19
		Mix of mung beans, alfalfa and lentil sprouts	11
		Mix of mung beans, pea and lentil sprouts	4
		Radish sprouts	1

(200 ml of supernatant was used instead of the recommended 40 ml to ensure rinsing of the entire sample). RNA isolation was carried out with the NucliSENS Magnetic Extraction Reagent (BioMérieux, Marcy-l'Étoile, France) according to the manufacturer's instructions with a final repeated elution resulting in 100 μl of nucleic acid solution. The isolated nucleic acids were assayed immediately.

Detection and identification of HuNoV (HuNoV GI and HuNoV GII) and HAV genomes were carried out by one-step duplex reverse transcription real-time PCR (RT-qPCR) as described by ISO/TS 15216-2:2013 [16] with utilization of an internal amplification control (IAC) [17]. Protozoan DNA was isolated from the sediments using PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, California, USA) following the manufacturer's instructions. Detection of *G. intestinalis* and *C. parvum/hominis* was performed using two separate duplex qPCR assays, both including IAC [15] to facilitate identification of false negative results. The primers and probes used for the detection of *G. intestinalis* (targeting β-giardin gene) and *C. parvum/hominis* (targeting *hsp70* gene) were adopted from a previous study [18]. The primers for *C. parvum/hominis* have broader specificity, they are able to detect primarily *C. parvum* and *C. hominis,* which are responsible for vast majority of human cases, but also other species related to human infection, e.g. *C. meleagridis*, *C. ubiquitum* and *C. viatorum*. The PCR mixture contained 1× LightCycler 480 Probes Master (Roche, Mannheim, Germany), 250 nmol·l^{-1} of each primer, 100 nmol·l^{-1} of each probe, 1 U of uracil DNA glycosylase (EC 3.2.2.28; Sigma, St. Louis, Missouri, USA), 5×10^2 copies of IAC and 5 μl of DNA solution in a total reaction volume of 20 μl. Detection of viral and protozoan agents was performed in LightCycler 480 (Roche), the subsequent analysis of results was carried out using the "Fitpoint analysis" option of the LightCycler 480 Software release 1.5.0 (version 1.5.0.39, Roche). Quantification of process control virus (PCV) as well as calculation of the virus extraction efficiency (estimate of PCV recovery) for viruses was done in the same way as in the previous study [19]. Samples with a processing efficiency lower than 1% were not considered valid results and were not included in further analyses. The limit of detection (*LOD*) and the efficiency of protozoan DNA isolation was determined by testing hexaplicates of 10-fold serially diluted suspension of *G. intestinalis* cysts, which ranged from 10^5 to 10^1 cysts per 100 g of mixed leafy greens. *LOD* was specified as the lowest concentration of cysts that could be detected with 95% probability. The efficiency of protozoan DNA isolation from vegetable samples ranged between 41.1 % and 60.8 %, *LOD* was determined as 65 cysts per gram. Positive, negative and isolation controls of qPCR were included in each analysis run.

Detection and enumeration of *Staph. aureus*

For enumeration of coagulase-positive staphylococci, a portion of 25 g of sample was homogenized in 225 ml of buffered peptone water (BPW; Oxoid, Basingstoke, United Kingdom) using a stomacher homogenizer. According to ISO 6888-1(1999) [20], 200 μl of the suspension was directly streaked onto Baird-Parker medium (BioRad, Hercules, California, USA) in triplicate and incubated at 37 °C for 18–24 h. To increase detection sensitivity, the homogenized suspension was further incubated at 37 °C overnight and then inoculated on Baird-Parker medium. After incubation at 37 °C for 18–24 h, the occurrence of colonies with morphology typical for coagulase-positive staphylococci was assessed. Three typical colonies were plated on blood agar (LabMediaServis, Jaroměř, Czech Republic) and incubated overnight at the same conditions. Species identity was confirmed using MALDI-TOF mass spectrometry equipment AutoFlex speed (Bruker Daltonics, Bremen, Germany) with the use of Biotyper software (version 3.1, Bruker Daltonics) with a score above 2.0. Detection of genes encoding production of classical enterotoxins (*sea-see*) in *Staph. aureus* was performed using PCR as described previously [21].

Detection and enumeration of *E. coli*

Detection of *E. coli* was performed after homogenization of 10 g sample in 90 ml of BPW and enrichment at 37 °C overnight, followed by culture on tryptone bile glucuronic (TBX) agar (BioRad) simultaneously at 44 °C and 37 °C for 24 h. For enumeration, homogenized samples were directly decimally diluted and streaked in triplicate on TBX agar plates and incubated at 44 °C and 37 °C for 24 h. Characteristic colonies were subcultured on MacConkey medium (BioRad). Identity of *E. coli* was confirmed by detection of *uid*A gene by qPCR as described previously [22].

Statistical analysis

Statistical analysis was performed to confirm association between produce type and occurrence of *E. coli* using Fisher-Freeman-Halton exact test. Differences were considered statistically significant if $P < 0.05$.

RESULTS AND DISCUSSION

Ensuring the safety of food is a long-standing and crucial task for public health authorities. In order to address and manage food safety, it is crucial to have knowledge of current situation and trends with regard to occurrence and spread of food-borne pathogens in the production chain. The highest number of food-borne outbreaks from fresh produce in EU and USA result from contamination with HuNoV, *Salmonella* spp. and Shiga toxin-producing *E. coli* [1]. In addition to the above-mentioned pathogens, vegetables may also be contaminated with other less studied agents that may result in food-borne infection. Therefore, our study focused on screening of HuNoV as the most common pathogen and also on other viral (HAV) and protozoan (*C. parvum*/*hominis* and *G. intestinalis*) pathogens in fresh and frozen produce. Out of 175 samples examined, four separate samples (2.2 %) were positive for viral or protozoan pathogens (Tab. 2). HuNoV GI was detected in one sample of raw vegetables (rocket) and one sample of frozen mixed vegetables (containing cabbage, mung sprouts and bamboo shoots). This relatively low rate of HuNoV contamination of fresh and frozen vegetables is in general agreement with data reported in previous European studies in which HuNoV was detected in less than 0.1 % (1/1372) samples of fresh leafy vegetable products and in none of 1160 samples of ready-to-eat vegetables [23]. Despite the generally low rate of HuNoV contamination of food, viral particles can be preserved by freezing or refrigeration and can be able to persist from days to months during such storage [24]. An increase in food-borne outbreaks caused by HuNoV has been noted and many outbreaks were traced to food that was handled by infected workers during food preparation. The impact of vegetables contaminated by HuNoV was outlined, for example, in annual European Food Safety Authority (EFSA) reports [3].

In this study, no sample was positive for hepatitis A virus.

Although parasites such as *Cryptosporidium* spp. and *G. intestinalis* may be present in produce and can be the cause of food-borne diseases [2], standardized molecular methods for their detection in food are not available [25]. Until now, several methods based on microscopy or molecular approaches with variable recovery efficiencies and variable detection limits were applied for protozoan detection [25]. Most studies monitoring the foodborne protozoa come from developing countries where poor hygiene is prevalent [9, 10, 26]. The number of studies addressing the significance

Tab. 2. Prevalence of studied viral, protozoan and bacterial agents in minimally processed vegetable and frozen produce, and sprouts.

Produce type	Total number of samples	Viruses				Protozoa				Bacteria					
		HAV		HuNoV		*Cryptosporidium parvum/hominis*		*Giardia intestinalis*		*Staphylococcus aureus*		*Escherichia coli*		*Escherichia coli* $>10^2$ CFU·g^{-1}		*Escherichia coli* $>10^3$ CFU·g^{-1}	
		Number	[%]	Number	[%]	Number	[%]	Number	[%]	Number	[%]	Number	[%]	Number	[%]	Number	[%]
Fresh pre-cut products*	97	0	0.0	1	1.1	0	0.0	1	1.0	1	1.0	33	34.0	6	6.2	3	3.1
Frozen vegetables**	43	0	0.0	1	2.3	0	0.0	1	2.3	2	4.7	17	39.5	0	0.0	0	0.0
Sprouts	35	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	26	74.3	19	54.3	18	51.4
Total	175	0	0.0	2	1.1	0	0.0	2	1.1	3	1.7	76	43.4	25	14.3	21	12.0

* – ready to eat product such as fresh leafy green salads, spinach, rucola or lamb's lettuce (one sample of rucola was positive for norovirus, one sample of fresh leafy green mixed salads was positive for *Giardia intestinalis* and one sample of fresh leafy green mixed salads *Staphylococcus aureus*)

** – mixed frozen vegetables, frozen peas, frozen maize or frozen spinach (one sample of mixed frozen vegetables was positive for norovirus and one sample of mixed frozen vegetables was positive for *Giardia intestinalis*, two samples from different producers were positive for *Staphylococcus aureus*).

HAV - hepatitis A virus; HuNoV – norovirus (NoV GI).

of foodborne parasites in developed countries have arisen in recent years [5, 11].

In the present study, monitoring of *C. parvum*/*hominis* and *G. intestinalis* in produce was conducted using two duplex qPCR assays. According to the results, occurrence of parasites in fresh-cut vegetables, frozen vegetables and sprouts was low. *G. intestinalis* was detected in two samples (mixed fresh vegetable salads and mixed frozen vegetables) and no sample was positive for *C. parvum*/*hominis*. This low positivity might have been caused by the limited number of samples examined but also by the relatively high level of *LOD* of our detection method [27]. Only limited data on survival of *Giardia* cysts in frozen vegetables are available, however, most of the studies generally confirmed adverse effect of freezing on viability of protozoa oocyt/cyst [2]. A similar rate of contamination by *G. intestinalis* (1.8 %) was revealed in packed pre-cut salads and leafy greens sold in Canada [28]. On the other hand, occurrence of *Cryptosporidium* spp. in Canada was higher, reaching 5.9 %. For comparison, *Giardia* spp. was found in 8.8 % of fresh vegetables in Egypt [26]. Duedu et al. [9] detected *G. intestinalis* in 6.0 % and *Cryptosporidium* spp. in 17.0 % of fresh vegetables sold in Ghana. In our previous study, *G. intestinalis* was observed in 2.8 % of vegetable samples derived from farms in comparison to 1.1 % of the same type of vegetable samples from markets. Surprisingly, C. *parvum*/*hominis* was detected in 16 out of 433 (3.7%) of vegetable sampled on farms, but no sample collected in market was confirmed positive [15]. The water-borne route and manure application to vegetable fields is probably the most important means of transmission of these parasites [11]. In some cases, transmission of these pathogens to vegetables were attributed to infected food handlers [29].

The true incidence of staphylococcal food poisoning is unknown for a number of reasons, including the short period of illness usually not requiring seeing a physician. *Staph. aureus* is part of the natural microbiota of humans, and therefore, contamination with *Staph. aureus* represents the most common way for produce contamination through direct contact with food handlers [29].

In the present study, the counts of coagulase-positive staphylococci were under the detection limit of our method ($< 5.10^1$ CFU·g^{-1}), with *Staph aureus* being confirmed in 1.7 % of samples after enrichment (one mixed salad and two frozen peas packets). Genes for production of classical enterotoxins (*sea–see*) were not detected in any of the tested strains. Our results show a lower prevalence of *Staph. aureus* than revealed in Thailand [30] or in China [31]. Ananchaipattana et al. [30] reported *Staph. aureus* in 67 % of leafy vegetable samples from open markets and 25 % from supermarkets in Thailand. Xing et al. [31] detected *Staph. aureus* in 27.6 % salad vegetable samples. On the other hand, no samples positive for *Staph. aureus* were detected in fresh produce from Italian supermarkets and greengrocer's shops [32]. This may be explained by more strict regulations and good hygiene and manufacturing practices implemented routinely in the European Union (e.g. EC 2073/2005 [33]).

In the present study, 43.4 % of samples were found to be positive for *E. coli*, 14.3 % of samples exceeded a level of 10^2 CFU·g^{-1} and 12.0 % of samples exceeded a level of 10^3 CFU·g^{-1} (Tab. 2). As expected, sprouts were the samples with the highest level of contamination ($p < 0.01$; adjusted Pearson's residuals). Twenty percent of samples exceeded a level of 10^5 CFU·g^{-1}. Sprout manufacturing provides excellent conditions for bacterial growth and any pathogen contamination at the beginning may produce high counts in the final product and pose a health risk to humans. Therefore, the high number of *E. coli*-positive sprout samples with high counts of these bacteria suggest primary contamination of seeds and insufficient decontamination procedures of seeds prior to sprouting. In cut leafy greens and salads originating in the Czech Republic, Italy and Austria, *E. coli* counts exceeded a level of 10^2 CFU·g^{-1} and 10^3 CFU·g^{-1} in 6.2 % and 3.1 % of samples, respectively (Tab. 2). These results show a relatively high level of contamination in comparison to other studies from Canada or Italy where *E. coli* counts were mostly below the detection limit [34, 35].

The routine monitoring of fecal indicator bacteria such as *E. coli* was traditionally used to predict enteric virus and protozoan pathogens in water and food samples. Recently, it has been recognized [36] and confirmed also by our study that the occurrence of enteric viral and protozoan pathogens often does not correlate with indicator *E. coli* presence. Both HuNoV positive samples were negative for the presence of *E. coli* and both *G. intestinalis* positive samples harboured *E. coli,* but only at a low level of less than 10^2 CFU·g^{-1}. Therefore, *E. coli* cannot be considered as the appropriate fecal indicator for determination of viruses and protozoa in food.

CONCLUSIONS

Our results revealed that viral and protozoan pathogens, namely, HuNoV and *G. lamblia* were

present on minimally processed vegetables available on the market and their detection was not correlated with indicator *E. coli*. Noticeable is also detection of high levels of the indicator *E. coli* in sprouts and higher number of fresh vegetable samples (leafy greens and salads) with the counts above 10^2 CFU·g^{-1}. Findings of this study indicate that there is a need for better prevention strategies and increased levels of knowledge related to the occurrence of food-borne viral and protozoan pathogens in this group of food products. Indicators of fecal contamination of produce appropriate to viral and protozoal pathogens should be introduced.

Acknowledgements

This work was supported by the grant No. 17–31921A provided by the Ministry of Health of the Czech Republic and by grants from the Ministry of Agriculture of the Czech Republic (QK1810212 and MZE–RO0518). This study was also carried out with financial support from the Ministry for Education, Youth and Sport (MEYS) of the Czech Republic CZ.1.05./2.1.00/19.0385. Dr. Vladimir Babak is thanked for statistical analysis.

REFERENCES

1. Callejon, R. M. – Rodriguez–Naranjo, M. I. – Ubeda, C. – Hornedo–Ortega, R. – Garcia–Parrilla, M. C. – Troncoso, A. M.: Reported foodborne outbreaks due to fresh produce in the United States and European Union: trends and causes. Foodborne Pathogens and Disease, *12*, 2015, pp. 32–38. DOI: 10.1089/fpd.2014.1821.
2. Multicriteria based ranking for risk management of foodborne parasites. Microbiological risk assessment series No. 23. Rome : Food and Agriculture Organization/World Health Organization, 2014. ISSN: 1726-5274. ISBN: 978-92-5-108199-0. <http://www.fao.org/3/a-i3649e.pdf>
3. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food–borne outbreaks in 2015. EFSA Journal, *14*, 2016, e4634. DOI: 10.2903/j.efsa.2016.4634.
4. Robertson, L. J. – Sprong, H. – Ortega, Y. R. – van der Giessen, J. W. – Fayer, R.: Impacts of globalisation on foodborne parasites. Trends in Parasitology, *30*, 2014, pp. 37–52. DOI: 10.1016/j.pt.2013.09.005.
5. Trevisan, C. – Torgerson, P. R. – Robertson, L. J.: Foodborne parasites in Europe: Present status and future trends. Trends in Parasitology, *35*, 2019, pp. 695–703. DOI: 10.1016/j.pt.2019.07.002.
6. WHO estimates of the global burden of foodborne diseases: foodborne disease burden epidemiology reference group 2007–2015. Geneva : World Health Organization, 2015. ISBN: 9789241565165. <https://www.who.int/iris/bitstream/10665/199350/1/9789241565165_eng.pdf>
7. Atmar, R. L. – Opekun, A. R. – Gilger, M. A. – Estes, M. K. – Crawford, S.E. – Neill, F. H. – Graham, D. Y.: Norwalk virus shedding after experimental human infection. Emerging Infectious Diseases, *14*, 2008, pp. 1553–1557. DOI: 10.3201/eid1410.080117.
8. Microbiological hazards in fresh leafy vegetables and herbs: Meeting report. Microbiological risk assessment series No. 14. Rome : Food and Agriculture Organization/World Health Organization, 2008, ISSN: 1726-5274. ISBN: 978-92-4-156378-9. <http://www.fao.org/3/i0452e/i0452e.pdf>
9. Duedu, K. O. – Yarnie, E. A. – Tetteh–Quarcoo, P. B. – Attah, S. K. – Donkor, E. S. – Ayeh–Kumi, P. F.: A comparative survey of the prevalence of human parasites found in fresh vegetables sold in supermarkets and open–aired markets in Accra, Ghana. BMC Research Notes, *7*, 2014, article 836. DOI: 10.1186/1756-0500-7-836.
10. Karshima, S. N.: Parasites of importance for human health on edible fruits and vegetables in Nigeria: a systematic review and meta–analysis of published data. Pathogens and Global Health, *112*, 2018, pp. 47–55. DOI: 10.1080/20477724.2018.1425604.
11. EFSA Panel on Biological Hazards (BIOHAZ), Koutsoumanis, K. – Allende, A. – Alvarez–Ordonez, A. – Bolton, D. – Bover–Cid, S. – Chemaly, M. – Davies, R. – De Cesare, A. – Herman, L. – Hilbert, F. – Lindqvist, R. – Nauta, M. – Peixe, L. – Ru, G. – Simmons, M. – Skandamis, P. – Suffredini, E. – Caccio, S. – Chalmers, R. – Deplazes, P. – Devleesschauwer, B. – Innes, E. – Romig, T. – van der Giessen, J. – Hempen, M. – Van der Stede, Y. – Robertson, L.: Public health risks associated with food–borne parasites. EFSA Journal, *16*, 2018, e05495. DOI: 10.2903/j.efsa.2018.5495.
12. Kotloff, K. L. – Nataro, J. P. – Blackwelder, W. C. – Nasrin, D. – Farag, T. H. – Panchalingam, S. – Wu, Y. – Sow, S. O. – Sur, D. – Breiman, R. F. – Faruque, A. S. – Zaidi, A. K. – Saha, D. – Alonso, P. L. – Tamboura, B. – Sanogo, D. – Onwuchekwa, U. – Manna, B. – Ramamurthy, T. – Kanungo, S. – Ochieng, J. B. – Omore, R. – Oundo, J. O. – Hossain, A. – Das, S. K. – Ahmed, S. – Qureshi, S. – Quadri, F. – Adegbola, R. A. – Antonio, M. – Hossain, M. J. – Akinsola, A. – Mandomando, I. – Nhampossa, T. – Acacio, S. – Biswas, K. – O'Reilly, C. E. – Mintz, E. D. – Berkeley, L. Y. – Muhsen, K. – Sommerfelt, H. – Robins–Browne, R. M. – Levine, M. M.: Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case–control study. Lancet, *382*, 2013, pp. 209–222. DOI: 10.1016/S0140-6736(13)60844-2.
13. Cryptosporidiosis – Annual epidemiological report for 2015. In: European Centre for Disease Prevention and Control [online]. Solna : European Centre for Disease Prevention and Control, 2018. <https://www.ecdc.europa.eu/en/publications-data/cryptosporidiosis-annual-epidemiological-report-2015>
14. Giardiasis – Annual epidemiological report for

2015. In: European Centre for Disease Prevention and Control [online]. Solna : European Centre for Disease Prevention and Control, 2018. <https://www.ecdc.europa.eu/en/publications-data/giardiasis-annual-epidemiological-report-2015>
15. Huvarova, V. – Kralik, P. – Vasickova, P. – Kubankova, M. – Verbikova, V. – Slany, M. – Babak, V. – Moravkova, M.: Tracing of selected viral, bacterial, and parasitic agents on vegetables and herbs originating from farms and markets. Journal of Food Science, *83*, 2018, pp. 3044–3053. DOI: 10.1111/1750-3841.14385.
16. ISO/TS 15216–2:2013. Microbiology of food and animal feed – Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR – Part 2: Method for qualitative detection. Geneva : International Organization for Standardization, 2013.
17. Vasickova, P. – Kralik, P. – Slana, I. – Pavlik, I.: Optimisation of a triplex real time RT-PCR for detection of hepatitis E virus RNA and validation on biological samples. Journal of Virological Methods, *180*, 2012, pp. 38–42. DOI: 10.1016/j.jviromet.2011.12.007.
18. Helmi, K. – Skraber, S. – Burnet, J. B. – Leblanc, L. – Hoffmann, L. – Cauchie, H. M.: Two–year monitoring of *Cryptosporidium parvum* and *Giardia lamblia* occurrence in a recreational and drinking water reservoir using standard microscopic and molecular biology techniques. Environmental Monitoring and Assessment, *179*, 2011, pp. 163–175. DOI: 10.1007/s10661-010-1726-7.
19. Mikel, P. – Vasickova, P. – Tesarik, R. – Malenovska, H. – Kulich, P. – Vesely, T. – Kralik, P.: Preparation of MS2 phage–like particles and their use as potential process control viruses for detection and quantification of enteric RNA viruses in different matrices. Frontiers in Microbiology, *7*, 2016, article 1911. DOI: 10.3389/fmicb.2016.01911.
20. ISO 6888–1:1999. Microbiology of food and animal feed – Horizontal method for the enumeration of coagulase–positive staphylococci (*Staphylococcus aureus* and other species) — Part 1: Technique using Baird–Parker agar medium. Geneva : International Organization for Standardization, 1999.
21. Monday, S. R. – Bohach, G. A.: Use of multiplex PCR to detect classical and newly described pyrogenic toxin genes in staphylococcal isolates. Journal of Clinical Microbiology, *37*, 1999, pp. 3411–3414. DOI: 10.1128/JCM.37.10.3411-3414.1999.
22. Chern, E. C. – Siefring, S. – Paar, J. – Doolittle, M. – Haugland, R. A.: Comparison of quantitative PCR assays for *Escherichia coli* targeting ribosomal RNA and single copy genes. Letters in Applied Microbiology, *52*, 2011, pp. 298–306. DOI: 10.1111/j.1472-765X.2010.03001.x.
23. Losio, M. N. – Pavoni, E. – Bilei, S. – Bertasi, B. – Bove, D. – Capuano, F. – Farneti, S. – Blasi, G. – Comin, D. – Cardamone, C. – Decastelli, L. – Delibato, E. – De Santis, P. – Di Pasquale, S. – Gattuso, A. – Goffredo, E. – Fadda, A. – Pisanu, M. – De Medici, D.: Microbiological survey of raw and ready–to–eat leafy green vegetables marketed in Italy. International Journal of Food Microbiology, *210*, 2015, pp. 88–91. DOI: 10.1016/j.ijfoodmicro.2015.05.026.
24. Butot, S. – Putallaz, T. – Sánchez, G.: Effects of sanitation, freezing and frozen storage on enteric viruses in berries and herbs. International Journal of Food Microbiology, *126*, 2008, pp. 30–35. DOI: 10.1016/j.ijfoodmicro.2008.04.033.
25. Chalmers, R. M. – Robertson, L. J. – Dorny, P. – Jordan, S. – Karssin, A. – Katzer, F. – La Carbona, S. – Lalle, M. – Lassen, B. – Mladineo, I. – Rozycki, M. – Bilska–Zajac, E. – Schares, G. – Mayer–Scholl, A. – Trevisan, C. – Tysnes, K. – Vasilev, S. – Klotz, C.: Parasite detection in food: Current status and future needs for validation. Trends in Food Science and Technology, *99*, 2020, pp. 337–350. DOI: 10.1016/j.tifs.2020.03.011.
26. Eraky, M. A. – Rashed, S. M. – Nasr Mel, S. – El-Hamshary, A. M. – Salah El-Ghannam, A.: Parasitic contamination of commonly consumed fresh leafy vegetables in Benha, Egypt. Journal of Parasitology Research, *2014*, 2014, article 613960. DOI: 10.1155/2014/613960.
27. Berrouch, S. – Escotte–Binet, S. – Harrak, R. – Huguenin, A. – Flori, P. – Favennec, L. – Villena, I. – Hafid, J.: Detection methods and prevalence of transmission stages of *Toxoplasma gondii*, *Giardia duodenalis* and *Cryptosporidium* spp. in fresh vegetables: a review. Parasitology, *147*, 2020, pp. 516–532. DOI: 10.1017/S0031182020000086.
28. Dixon, B. – Parrington, L. – Cook, A. – Pollari, F. – Farber, J.: Detection of *Cyclospora*, *Cryptosporidium*, and *Giardia* in ready–to–eat packaged leafy greens in Ontario, Canada. Journal of Food Protection, *76*, 2013, pp. 307–313. DOI: 10.4315/0362-028X.JFP-12-282.
29. Saeed, H. A. – Hamid, H. H.: Bacteriological and parasitological assessment of food handlers in the Omdurman area of Sudan. Journal of Microbiology, Immunology, and Infection, *43*, 2010, pp. 70–73. DOI: 10.1016/S1684-1182(10)60010-2.
30. Ananchaipattana, C. – Hosotani, Y. – Kawasaki, S. – Pongsawat, S. – Latiful, B. M. – Isobe, S. – Inatsu, Y.: Prevalence of foodborne pathogens in retailed foods in Thailand. Foodborne Pathogens and Disease, *9*, 2012, pp. 835–840. DOI: 10.1089/fpd.2012.1169.
31. Xing, X. – Li, G. – Zhang, W. – Wang, X. – Xia, X. – Yang, B. – Meng, J.: Prevalence, antimicrobial susceptibility, and enterotoxin gene detection of *Staphylococcus aureus* isolates in ready-to-eat foods in Shaanxi, People's Republic of China. Journal of Food Protection, *77*, 2014, pp. 331–334. DOI: 10.4315/0362-028X.JFP-13-301.
32. Cardamone, C. – Aleo, A. – Mammina, C. – Oliveri, G. – Di Noto, A. M.: Assessment of the microbiological quality of fresh produce on sale in Sicily, Italy: preliminary results. Journal of Biological Research-Thessaloniki, *22*, 2015, article 3. DOI: 10.1186/s40709-015-0026-3.
33. Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Official Journal of the European Union,

48, 2005, L338, pp. 1-26. ISSN: 1725-2555. <http://data.europa.eu/eli/reg/2005/2073/oj>

34. Arienzo, A. – Murgia, L. – Fraudentali, I. – Gallo, V. – Angelini, R. – Antonini, G.: Microbiological quality of ready-to-eat leafy green salads during shelf and home-refrigeration. Foods, *9*, 2020, article 1421. DOI: 10.3390/foods9101421.
35. Zhang, H. – Yamamoto, E. – Murphy, J. – Locas, A.: Microbiological safety of ready-to-eat fresh-cut fruits and vegetables sold on the Canadian retail market. International Journal of Food Microbiology, *335*, 2020, article 108855. DOI: 10.1016/j.ijfoodmicro.2020.108855.
36. Flannery, J. – Keaveney, S. – Dore, W.: Use of FRNA bacteriophages to indicate the risk of norovirus contamination in Irish oysters. Journal of Food Protection, *72*, 2009, pp. 2358–2362. DOI: 10.4315/0362-028X-72.11.2358.

Received 11 March 2021; 1st revised 20 April 2021; accepted 8 May 2021; published online 15 June 2021.