Received: 31 October 2018 | Revised: 1 October 2019 | Accepted: 8 November 2019

DOI: 10.1111/jpn.13263

ORIGINAL ARTICLE

Journal of Animal Physiology and Animal Nutrition | WILEY

Evaluation of microbial contamination and effects of storage in raw meat-based dog foods purchased online

Giada Morelli | Paolo Catellani | Riccardo Miotti Scapin | Sofia Bastianello | Daniele Conficoni | Barbara Contiero | Rebecca Ricci

Department of Animal Medicine, Production and Health, University of Padua, Legnaro, PD, Italy

Correspondence
Giada Morelli, Department of Animal Medicine, Production and Health, University of Padua, Viale dell'Università 16, Legnaro (PD) 35020, Italy.
Email: giada.morelli@phd.unipd.it

Abstract

Feeding raw-meat-based diets to companion animals has become a widespread practice, and many owners are now accustomed to buying frozen ingredients online. The goals of this study were to assess the microbiological quality of raw-meat dog foods obtained from specialized websites and to evaluate the effects of storage at different temperatures for a few days. Twenty-nine raw dog food products were processed for quantitative bacteriology (i.e. total viable count, TVC; *Escherichia coli*; faecal coliforms, FC) and sulphite-reducing clostridia, and analysed for the presence of *Salmonella* spp., *Listeria monocytogenes*, *Yersinia enterocolitica* and *Clostridium difficile*. Every sample was examined right after the delivery (T0), after 24 to 48 hr and after 72 hr, both at 2°C and 7°C. At T0, the mean score for the TVC was 5.9×10^6 cfu/g ($SD = 4.8 \times 10^7$ cfu/g), while those for *E. coli* and FC were 1.1×10^4 cfu/g ($SD = 2.5 \times 10^5$ cfu/g) and 3.3×10^3 cfu/g ($SD = 6.5 \times 10^4$ cfu/g) respectively. The samples stored at 2°C had a significant increase of all parameters (TVC: $p < .01$; *E. coli*: $p = .03$; FC: $p = .04$) through time. Noteworthy differences between the analyses performed at 2°C and 7°C were found for TVC ($p < .01$), being the samples considerably more contaminated at higher temperatures. No sample tested positive for *Salmonella* spp., while *L. monocytogenes* was isolated from 19 products, *Y. enterocolitica* from three products and *Clostridium perfringens* and *C. difficile* from four and six products respectively. The microbiological quality of raw-meat dog foods sold online appears to be poor, carrying considerable amounts of potentially zoonotic bacteria and reaching greater levels of bacterial contaminations if not kept at proper refrigeration temperatures and fed soon after defrosting.

KEYWORDS

dog, microbial contamination, nutrition, raw food, zoonotic

1 | INTRODUCTION

Raw-meat-based diets (RMBDs) for dogs and cats have become noticeably popular over recent years. According to the owners who support such regimes, feeding pets with fresh uncooked animal products and by-products is a safer and healthier alternative to dry or canned pet food (Freeman, Chandler, Hamper, & Weeth, 2013; Morelli, Bastianello, Catellani, & Ricci, 2019; Morgan, Willis, & Shepherd, 2017). However, the claimed benefits of RMBDs have not been clearly supported by research evidence yet, whereas some of the associated animal and

Abbreviations: FC, faecal coliforms; RMBD, raw-meat-based diet; SRC, sulphite-reducing clostridia; TVC, total viable count.

public health risks have been incontrovertibly documented (Freeman et al., 2013; LeJeune & Hancock, 2001). Whether intended for the consumption by humans or pets, raw meat is commonly contaminated with a variety of microbes, some of which could be potentially zoonotic pathogens (Freeman et al., 2013; LeJeune & Hancock, 2001).

Salmonellosis, listeriosis and yersiniosis are some of the most common food-borne zoonoses monitored by the European Food Safety Authority (EFSA, 2018), and the interaction with pets fed raw meat numbers among the infection risk factors (LeJeune & Hancock, 2001). Furthermore, emerging issues of *Clostridium difficile* infections in pets and humans (EFSA, 2018; Weese, Finley, Reid-Smith, Janecko, & Rousseau, 2010; Weese et al., 2001) and the reported isolation of these pathogens from raw meat for human consumption in many European countries (Bouttier et al., 2010; De Boer, Zwartkruis-Nahuis, Heuvelink, Harmanus, & Kuijper, 2011; Jöbstl et al., 2010) raise the question of whether RMBDs might be noteworthy sources of exposure. Indeed, since dogs and cats can become carriers of pathogenic bacteria and spread them through their faeces, RMBDs pose a risk also to the people who handle the contaminated ingredients and who come into contact with subclinically infected pets (Freeman et al., 2013; LeJeune & Hancock, 2001). Major concerns arise especially for individuals with impaired or weakened immune systems (i.e. children, chronically ill people, elderly people and pregnant women) because such category of individuals is more susceptible to developing food-borne infections (Freeman et al., 2013; LeJeune & Hancock, 2001). Previous studies already showed that both home-prepared and commercially available RMBDs can reach high contamination levels and hold potentially pathogenic bacteria (van Bree et al., 2018; Chengappa, Staats, Oberst, Gabbert, & Mcvey, 1993; Finley et al., 2008; Fredriksson-Ahomaa et al., 2017; Freeman & Michel, 2001; Joffe & Schlesinger, 2002; Nemser et al., 2014; Nilsson, 2015; Strohmeyer et al., 2006; Weese, Rousseau, & Arroyo, 2005).

In view of the growing owner demand for raw dog and cat diets, some specialized online shops started selling frozen meat scraps, animal by-products (i.e. internal organs, cartilage and bones) and blended mixtures of these products. The EU regulation about raw pet food states that "effective steps must be taken to ensure that the product is not exposed to contamination throughout the production chain and up to the point of sale" (EC 142/2011, 2011—annex XIII, chapter II, point 1), but concerns may still arise about the wholesomeness of such items at their designated destination.

The aims of this study were therefore to evaluate the microbiological quality (i.e. total viable count) and the hygiene quality (i.e. *Escherichia coli*, sulphite-reducing clostridia) of commercially available RMBDs for dogs purchased online, as well as to assess the presence of certain pathogenic bacteria (i.e. *Salmonella* spp., *Listeria monocytogenes*, *Yersinia enterocolitica* and *Clostridium difficile*). As suboptimal practices in storing defrosted RMBDs (e.g. inadequate refrigeration temperature, excessive long-term refrigeration) may further contribute to their spoilage, a secondary goal was then to evaluate the microbiological features of the same products kept at different temperatures for multiple days, simulating an improper food handling practice followed by the costumer.

2 | MATERIALS AND METHODS

Twenty-nine commercially available RMBDs produced in Italy and Germany were obtained from three online stores. The items were delivered frozen at −18°C within 48 hr after purchase and were immediately stored in the freezer at −18°C at the Food Microbiology Laboratory of the University of Padua until evaluation. Before storing the samples, the labels were checked to verify that the products were not expired and that all analyses could be concluded prior to the expiration dates.

The collected products, in the form of whole or minced pieces (n = 21/29) (i.e. except for cartilages and bones, all products were made of minced meat and/or offal) and blended mixtures (n = 8/29) included meat and animal by-products derived from a single animal species (n = 27/29) or from two animal species (n = 2/29). More precisely, the animal sources of the analysed products were beef (n = 20/29), turkey (n = 3/29), chicken (n = 2/29), lamb (n = 2/29), duck (n = 1/29), rabbit (n = 1/29), horse (n = 1/29) and salmon (n = 1/29). The beef-based products included meat (n = 1/20), bones (n = 1/20), cartilage (i.e. tracheas, ears, muzzle; n = 5/20), offal (i.e. green tripe, liver, kidney, omasum, heart, spleen, udder; n = 10/20, of which 7 single organs and 3 mixtures) and mixtures of all these (n = 3/20). The poultry-based products included turkey meat (n = 1/5), chicken and turkey offal (n = 1/5), chicken necks (n = 1/5) and duck mixture (n = 1/5); another product was composed of turkey and rabbit mixture (n = 1/5). The lamb-based products included one green tripe (n = 1/2) and a mixture of meat and offal (n = 1/2); the horse-based product included only meat (n = 1/1), while the salmon-based one was a mixture (n = 1/1).

Within 24 hr of delivery (T0), the frozen items were thawed at 2°C for 15 ± 1 hr and subjected to microbiological analyses. The products were processed while still cold and manipulated using sterile tools in an aseptic environment, in order to prevent substantial bacterial growth. One 20-g sample for the quantitative analyses (i.e. total viable count, TVC; faecal coliforms, FC; *E. coli*) and three 25-g samples for the qualitative analyses (i.e. identification of *Salmonella* spp., *Listeria monocytogenes* and *Yersinia enterocolitica*) were obtained from each product. The aliquots were collected from each sample twice more and stored in sterile containers at 2°C or 7°C for the repetition of the analyses in the following 24 or 48 hr (T1 and T2 respectively) and 72 hr (T3). This was performed to mimic a proper refrigerated condition (i.e. 2°C) or an improper one (i.e. 7°C), and to evaluate the changes of the microbial load within few days after thawing.

2.1 | Quantitative microbiology

In a sterile sealed plastic bag, each 20-g sample was mixed to 180 ml of sterile saline solution (0.85% NaCl and 0.15% Pepton) and homogenized in a paddle blender (Stomacher 400, Seward, Worthing, UK) for one minute; the dilutions were then filtered and poured into sterile containers, and serial 10-fold dilutions were performed. Plate

Count Agar acc. ISO 4833:1 (PCA; Oxoid Limited, Basingstoke, UK) was used to culture aerobic bacteria (i.e. TVC), and Chromocult® Coliform agar (CCA; Merck KGaA, Darmstadt, Germany) was used for the simultaneous detection of faecal coliform and *E. coli*.

The inoculated Petri dishes were incubated for 48 ± 3 hr at 31°C ± 1°C and 24 ± 1 hr at 36 ± 1°C for PCA, and McC and CCA respectively. Moreover, Sulphite Polymyxin Sulphadiazine Agar (SPS; Biolife Italiana srl, Milan, Italy) and Cycloserine Cefoxitin Fructose agar (CCFA; Biolife Italiana srl, Milan, Italy) were used to sulphite-reducing clostridia (in 16 samples) and *C. difficile* respectively (in 13 samples), with sample pasteurization treatment, spread plate method and incubation in an anaerobic atmosphere for 48 ± 3 hr at 36°C ± 1°C. Suspicious colonies were identified by biochemical tests (API 20 A, bio-Mérieux).

The microbial counts were determined by applying the formula given in ISO 18593:2004 and expressed as cfu/g.

2.2 | *Salmonella* spp. cultures

The products were analysed for the presence of *Salmonella* species according to ISO 6579:2002. Each 25-g sample was put in a sterile container along with 225 ml of buffered peptoned water (BPW) and incubated at 37°C for 24 hr. Tubes containing 9 ml of Rappaport Vassiliadis broth (RV; Oxoid Limited, Basingstoke, UK) were inoculated with one ml of culture each and subsequently incubated at 42°C for 24 hr. The subculture broth was then inoculated onto Xylose-lysine-tergitol 4 (XLT-4) agar (Merck KGaA, Darmstadt, Germany) and incubated aerobically at 37°C for 24 hr. Suspicious colonies were identified by biochemical tests (API Rapid ID32E, bio-Mérieux) and confirmed by serology for O-antigens detection.

2.3 | Listeria monocytogenes culture

The samples were analysed for the presence of *L. monocytogenes* according to ISO 11290–1:1996. Twenty-five g of each product was put in a sterile container along with 225 ml of Fraser Broth Half Concentration (Merck KGaA, Darmstadt, Germany) and incubated at 30°C for 24 hr. Then, tubes containing 10 ml of Fraser broth were inoculated with 0.1 ml of culture and subsequently incubated at 37°C for 24/48 hr. The subculture broth was inoculated onto Agar Listeria according to Ottaviani and Agosti (ALOA) dishes (Biolife Italiana s.r.l., Milano) and incubated at 37°C for 24/48 hr. The following tests were performed on the suspected strains: Gram staining, haemolytic activity, CAMP test and biochemical identification with API Listeria (bio-Mérieux).

2.4 | Yersinia enterocolitica cultures

The initial sample suspensions were put in sterile containers with Yersinia Irgasan Ticarcillin Chlorate Broth (ITC; Biolife Italiana s.r.l., Milano) to obtain 1:100 dilutions and incubated at 25°C for 48 hr. Then, the subculture broths were inoculated onto Yersinia Selective Agar Base dishes (CIN agar; Biolife Italiana s.r.l., Milano) and incubated at 30°C for 24 hr. Colonies with a typical bulls-eye appearance were identified by the use of biochemical tests (API Rapid ID32E, bio-Mérieux).

2.5 | Statistical analyses

The data collected from the analyses were transferred into a spreadsheet (Excel, Microsoft) and underwent descriptive analysis. Statistical analyses were performed with SAS 9.4 (SAS Institute Inc., Cary, NC, USA). When the microbial counts were below the detection limit, a reference value was assumed (i.e. 50 if < 100). The data converted to Log10 were processed by non-parametric analysis (Mann–Whitney *U* test for independent samples) to test the effects of time and temperature using an ANOVA linear model. Post hoc pairwise comparisons were performed using Bonferroni correction, and significance levels were defined as $p < .05$.

3 | RESULTS

Immediately after thawing (T0) at 2°C, the mean score for TVC in the 29 analysed RMBDs resulted 5.9×10^6 cfu/g ($SD = 4.8 \times 10^7$ cfu/g). The most contaminated products were lamb meat mixture (ground meat and green tripe; 1.1×10^8 cfu/g) and bovine green tripe (6.0×10^7 cfu/g); the least contaminated products were bovine muzzle (5.0×10^4 cfu/g) and horse meat (9.0×10^4 cfu/g).

The quantitative scores for *E. coli* ranged from 8.0×10^2 to 8.2×10^6 cfu/g (mean value = 1.1×10^4, $SD = 2.5 \times 10^5$) and those for FC ranged from 1.0×10^2 to 1.8×10^6 cfu/g (mean value = 3.3×10^3, $SD = 6.5 \times 10^4$). Values below the detectable level were found in three products, namely bovine trachea, muzzle and bone.

The presence of SRC was investigated in 16 samples and bovine green tripe registered the highest amount (>10^4 cfu/g); *C. sporogenes* was recognized by the subsequent biochemical identification. *C. difficile* was searched in 13 samples, and the positivity was found in six samples, of which four included poultry (turkey meat, turkey and chicken offal, chicken mixture and duck mixture) and two beef (meat and mixture); *C. difficile* ranged from 2.0×10^2 to 6.0×10^2 cfu/g (mean value = $4{,}0 \times 10^2$; $SD = 2{,}8 \times 10^2$) at T0, and from 6.0×10^2 to 2.0×10^3 cfu/g (mean value = $9{,}8 \times 10^2$, $SD = 6{,}5 \times 10^2$) at T3. The biochemical test confirmed the presence of *C. difficile* in all samples, but the isolates were not submitted to PCR and neither assayed for the production of toxin A/B. Other biochemical tests performed on the strains isolated from SPS and CCFA media identified *C. perfringens* ($n = 4$), *C. bifermentans* ($n = 1$), *C. sordellii* ($n = 1$) and *C. tertium* ($n = 1$), and four strains remained unidentified.

Table 1 shows the results of the storage tests performed in time and temperature abuse conditions. The analysed microflora grew

TABLE 1 Microbiological changes (log_{10} cfu/g; LS mean ± *SE*, 95% CI)[‡] of raw-meat dog foods stored at 2°C and 7°C

	T0	T1	T2	T3	*p*
TVC, 2°C	6.77 ± 0.17[b]	7.44 ± 0.22[b]	7.53 ± 0.28[ab]	8.32 ± 0.18 [a]	<.0001
TVC, 7°C	\	7.93 ± 0.17[b]	8.32 ± 0.21[ab]	8.83 ± 0.15 [a]	.0008
E. coli, 2°C	4.05 ± 0.27[b]	4.30 ± 0.37[ab]	4.71 ± 0.46[ab]	5.23 ± 0.29 [a]	.027
E. coli, 7°C	\	5.03 ± 0.35	5.97 ± 0.44	5.11 ± 0.32	>.05
FC, 2°C	3.52 ± 0.23[b]	3.81 ± 0.32[ab]	3.99 ± 0.40[ab]	4.48 ± 0.25 [a]	.040
FC, 7°C	\	4.59 ± 0.33	4.52 ± 0.41	4.84 ± 0.30	>.05
SRC[§], 2°C	1.83 ± 0.17	\	2.24 ± 0.20	2.31 ± 0.21	>.05
SRC[§], 7°C	2.09 ± 0.32	1.94 ± 0.32	\	1.85 ± 0.32	>.05
C. difficile[§], 2°C	\	\	2.24 ± 0.23	1.70 ± 0.42	>.05
C. difficile[§], 7°C	\	1.84 ± 0.29	\	1.74 ± 0.29	>.05

Abbreviations: LS, least squares; SE, standard error; CI, confidence interval; TVC, total viable count; FC, faecal coliforms; SRC, sulphite-reducing clostridia.

[‡]Different significance levels were denoted with different letters (a, b).

[§]SRC were analysed in 16/29 samples; *C. difficile* was analysed in 13/29 samples.

TABLE 2 Microbiological loads (log_{10} cfu/g; LS $mean_{T1\text{-}T3}$ ± $SE_{T1\text{-}T3}$, 95% CI) of raw-meat dog foods stored at 2°C or 7°C

	2°	7°	*p*
TVC	7.76 ± 0.12	8.36 ± 0.12	.0007
E. coli	4.75 ± 0.22	5.37 ± 0.23	.052
FC	4.10 ± 0.19	4.65 ± 0.20	.047
SRC[a]	1.96 ± 0.18	1.86 ± 0.25	>.05
C. difficile[a]	1.12 ± 0.12	1.93 ± 0.21	>.05

Abbreviations: CI, confidence interval; FC, faecal coliforms; LS, least squares; SE, standard error; SRC, sulphite-reducing clostridia; TVC, total viable count.

[a]SRC were analysed in 16/29 samples; *C. difficile* was analysed in 13/29 samples.

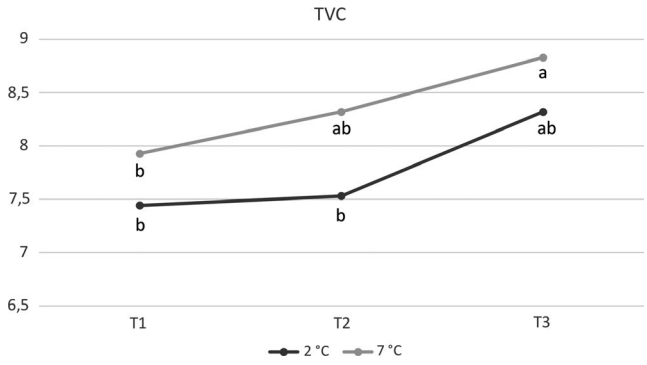

FIGURE 1 Effects of time and temperature on the TVC (log_{10} cfu/g) of the analysed raw-meat pet foods. Different significance levels were denoted with different letters (a, b)

abundantly even at low temperatures, as its mean size exceeded 10^8 cfu/g after three days of storage at 2°C. Starting from 24 hr after thawing, the contamination of RMBDs significantly increased with time, both at 2 and 7°C. The signs of spoilage were less delayed and more severe at 7°C; however, *E. coli* and FC remained constant with time. Overall, the storage at higher temperatures significantly affected the microbial growth in the analysed samples (Table 2). The statistical differences in time-temperature abuse conditions are reported in Figure 1.

No *Salmonella* spp. were isolated from the collected samples, while *L. monocytogenes* was found in 19 products (12 bovine, 4 poultry, 1 lamb, 1 fish, 1 horse) and *Y. enterocolitica* in 3 products (bovine offal mixture, turkey and rabbit mixture, lamb meat and green tripe mixture). Sixteen RMBDs also tested positive for other *Listeria* species (12 bovine, 2 lamb, 1 poultry, 1 poultry and rabbit; *L. innocua*, *L. seeligeri*, but none was *L. ivanovii*), and none of the three above-mentioned bacteria could be isolated from two products only (bovine liver and kidney).

4 | DISCUSSION

The purchase of pet food from the Internet has become very common in the last few years, and many online stores sell now raw-meat-based products intended for the domestic consumption by dogs and cats. From a survey in Italy (Morelli et al., 2019), it emerged that 28% of the interviewed RMBD-feeding dog owners was used to obtain raw ingredients through some websites. It is well known that particular care should be taken when processing, transporting and storing raw-meat-based products in order to prevent inappropriate contamination levels and to minimize microbial growth (Lambert, Smith, & Dodds, 1991). The maintenance of the cold chain and the respect of strict hygiene criteria should be guaranteed by raw pet food manufacturers who must in fact abide by specific EU legislation (EC 142/2011, 2011—annex XIII, chapter II). However, the vagueness of the current law and the insufficient monitoring may raise questions about the microbiological quality

of RMBDs, especially when such products are sold online and undergo a further shipment.

For what concerns the quantitative microbiology of the analysed RMBDs, the high values detected immediately after thawing suggested a deteriorating quality from the beginning. Since the European Union has not imposed a maximum value for the aerobic colony count allowed to be detected on raw pet foods, EC 2073/2005—Annex I, Chapter 2.118 was taken into account and 18 products evaluated in this study surpassed the threshold for minced meat intended for human consumption (i.e. 5×10^6 cfu/g). Similarly, considering the hygiene criteria for *E. coli* and faecal coliforms whose upper limit is 5×10^2 cfu/g (EC 2073/2005, 2005—Annex I, Chapter 2.1), 26 and 17 products respectively would be judged unacceptable. Among the RMBDs collected for this research, the most contaminated ones included green tripe, while the least contaminated were bones and cartilaginous parts.

In a recent Dutch study by van Bree et al. (2018), 35 commercial RMBDs were tested for total bacterial count and a mean value of 2.3×10^5 cfu/g was found. The microbiological quality of those diets was considered acceptable since none surpassed the abovementioned maximum value for the aerobic colony count (EC 2073/2005, 2005—Annex I, Chapter 2.1); however, 40% of the products did not meet the thresholds for *E. coli* (van Bree et al., 2018). A higher percentage of commercial RMBDs not complying with the European legal limit for *E. coli* was observed by Nilsson (2015) in Sweden, as 59% of the analysed products (total $n = 39$) showed values greater than 5×10^2 cfu/g. It is noteworthy to mention that the latter study included some raw-meat-based products purchased from the Internet. Overall, the prevalence and abundance of *E. coli* found in the present study are similar or higher than those obtained in other studies performed worldwide (van Bree et al., 2018; Nilsson, 2015; Strohmeyer et al., 2006; Weese et al., 2005), while the average and the maximum TVC values are instead the highest recorded in commercially available RMBDs so far, seen the scores of 2.5×10^5 cfu/g reached by Freeman and Michel (2001) and 5.0×10^6 cfu/g by Van Bree et al. (2018). Unfortunately, comparisons based on meat type and animal source were not possible because the RMBDs composition in other studies were not described in detail.

Meat is commonly recognized as one of the most perishable foods by virtue of its chemical composition which favours microbial growth and speeds up the spoilage process (Doulgeraki, Ercolini, Villani, & Nychas, 2012). The microbial load of the analysed products grew indeed significantly over the short period considered, suggesting that defrosted RMBDs should be consumed within one day, as usually reported on their labels. Furthermore, higher temperatures predictably developed a more suitable environment for bacterial proliferation in the samples examined. Storage temperature is the paramount concern relative to proper meat conservation, and food safety authorities worldwide recommend that refrigerated foods be stored between 4 and 5°C in order to prevent or inhibit the growth of spoilage and pathogenic microorganisms (Roccato et al., 2015). In fact, the literature clearly demonstrated that storing meat at 4°C or below immediately after slaughtering and during transportation is critical for its hygiene, safety and shelf life (Dave & Ghaly, 2011). The growth of the major mesophilic bacteria of public health significance (e.g. *Salmonella* spp., *L. monocytogenes*, *Y. enterocolitica*) is limited at normal refrigerated storage conditions (0–4°C), but they may pose a potential public health threat if meat is subjected to temperature abuse, particularly during storage and transportation (Lambert et al., 1991; Palumbo, 1986). Time-temperature abuse due to improper consumer food handling practices has been reported as one of the most common contributory factors in outbreaks of food-borne bacterial infections (Roccato et al., 2015). Given the high initial microbial load of the products analysed in this study, particular care must be taken by owners handling and storing commercial RMBDs purchased from the Internet in order to prevent further and faster contaminations.

It is no surprise that raw meat, whether sold for human or animal consumption, can be also contaminated with a variety of pathogenic bacteria despite the care used during its processing (LeJeune & Hancock, 2001). The risk of food-borne illnesses in pet animals consuming RMBDs is therefore a concern, but the public health risk of infections should be also plentifully considered since zoonotic pathogens may be transmitted directly by handling the raw ingredients or indirectly by the faecal-oral route. Microorganisms of the genus *Salmonella* are the ones which received the most attention in pet food investigations because of their well-known zoonotic potential. Even if *Salmonella*-related gastroenteritis in dogs (Chengappa et al., 1993; Morley et al., 2006; Stone et al., 1993) and fatal septicaemia in cats (Stiver, Frazier, Mauel, & Styer, 2003) have been reported, pets can more commonly become subclinical carriers and the main concern is thus related to the possible infection of humans as a result of strict contacts or environmental contaminations. The literature demonstrated that *Salmonella* is frequently found in faeces from dogs consuming contaminated raw meat (Finley et al., 2007; Joffe & Schlesinger, 2002; Lefebvre, Reid-Smith, Boerlin, & Weese, 2008; Leonard et al., 2011; Morley et al., 2006; Stone et al., 1993), and pet-to-person transmission of this pathogen may happen if carrier animals are handled without observing proper hygienic practices (LeJeune & Hancock, 2001). No *Salmonella* spp. were found in the RMBDs collected for this study, in line with the results obtained in a previous investigation by Freeman and Michel (Freeman & Michel, 2001). However, many works reported the presence of *Salmonella* in both homemade and commercial RMBDs instead, with prevalence rates ranging from 2% to 100% of the tested samples (van Bree et al., 2018; Chengappa et al., 1993; Finley et al., 2008; Fredriksson-Ahomaa et al., 2017; Joffe & Schlesinger, 2002; Lenz, Joffe, Kauffman, Zhang, & Lejeune, 2009; Morley et al., 2006; Nemser et al., 2014; Stone et al., 1993; Strohmeyer et al., 2006; Weese et al., 2005). Interestingly, a recent publication attributed the onset of salmonellosis in two cats to the feeding of frozen poultry-based RMBDs bought on the Internet (Giacometti, Magarotto, Serraino, & Piva, 2017).

Listeria monocytogenes was previously found in 16% and 54% of the RMBDs evaluated by Nemser and colleagues (Nemser et al., 2014) and by Van Bree and colleagues (van Bree et al., 2018) respectively;

in the same studies, 17% and 43% of the samples confirmed to be positive also for other *Listeria* species. In the present investigation, the prevalence of these bacteria was higher as *L. monocytogenes* and other *Listeria* species were found in 65% and 55% of the analysed products. So far as known, a low prevalence of *Listeria* has been detected in dogs' faeces (Weber, Potel, Schäfer-Schmidt, Prell, & Datzmann, 1995) and few clinically manifest infections caused by *L. monocytogenes* have been documented (Palerme et al., 2016; Pritchard et al., 2016; Schroeder & van Rensburg, 1993), one of which was a case of abortion in a bitch fed raw meat products (Weber & Plagemann, 1991). Despite the mainly asymptomatic role in pets and the relatively limited spreading potential through faeces, *L. monocytogenes* can cause a severe disease in humans even several weeks after exposure and symptoms range from flu-like to more serious infections (e.g. meningitis) and other potentially life-threatening complications, especially in people with a weakened immune system and pregnant women where it may cause abortion (van Bree et al., 2018). Seen the high prevalence of *L. monocytogenes* found in this study, the handling of RMBDs purchased online may become a concrete source of environmental contamination and a noteworthy infection risk factor for pet owners if appropriate hygiene precautions are not taken.

Yersinia enterocolitica is another microorganism frequently isolated from raw meat, and dogs can be subclinically infected with serotypes that are pathogenic to their species but also to humans (LeJeune & Hancock, 2001). In fact, the development of canine *Yersinia*-related enteritis as well as the transmission of this pathogen from dogs to people has been reported in the literature (David Wilson, McCormick, & Feeley, 1976; Fenwick, Madie, & Wilks, 1994; Fredriksson-Ahomaa, Korte, & Korkeala, 2001; Gutman, Ottesen, Quan, Noce, & Katz, 1973). Although *Y. enterocolitica* is more frequently detected in pork meat (Fredriksson-Ahomaa et al., 2017, 2001; LeJeune & Hancock, 2001), it was found in three products obtained from different animal sources (i.e. bovine, turkey mixed with rabbit, lamb) in the present research. Fredriksson-Ahomaa et al. (2017) recently identified *Y. enterocolitica* in 10 out of 88 RMBDs, namely those containing pork, as well as in the faeces of one dog (out of 29) and one cat (out of 2) fed with those diets.

Clostridium difficile and *C. perfringens* were isolated in 46% and 25% of the RMBDs samples collected for this study, respectively, and SRC were isolated in 75% of the samples. The identification of potentially pathogenic spore-forming bacteria is noteworthy since both *C. difficile* and *C. perfringens* are well recognized causes of enteric disease in dogs and of food poisoning and diarrhoea in humans (Marks & Kather, 2003; Weese et al., 2005).

The research of *C. difficile* was performed for the possibility of human exposure through direct or indirect contact (e.g. environmental contamination through faeces) with pets that are eliminators or carriers of this pathogen (Hensgens et al., 2012). In fact, *C. difficile* was recently isolated from the faeces of dogs and cats (Andrés-Lasheras et al., 2018; Hussain et al., 2015; Rabold et al., 2018), and molecular epidemiology studies have shown a substantial identity between these strains and those isolated in humans (Rabold et al., 2018).

Weese Rousseau and Arroyo (2005) reported the presence of toxigenic *C. difficile* in a turkey-based commercially available RMBD. More recently, *C. difficile* was not detected in any of the 20 feline raw foods purchased in France (Bouttier et al., 2010). The isolation of *C. difficile* from raw meat for human consumption has been well documented (Bouttier et al., 2010; De Boer et al., 2011; Jöbstl et al., 2010), and recent studies showed that the contamination of carcass and offal surfaces can already occur during from the slaughter of livestock and poultry (Bakri, 2018; Guran & Ilhak, 2015; Knight, Putsathit, Elliott, & Riley, 2016). Therefore, the consumption by pets of raw meat, raw offal and other animal by-products may be a possible source of *C. difficile* intestinal colonization. However, the role of pet animals in the spread of human *C. difficile* infections is still unclear (Clooten, Kruth, Arroyo, & Weese, 2008; Hensgens et al., 2012; Stone et al., 2016).

There are some limitations that should be noted when considering the results of this study. The design is limited in that only traditional microbiological methods have been used, whose sensitivity is inferior when compared to modern molecular analyses. Moreover, not all major food-borne pathogens have been tested for presence in the collected products. However, these limitations do not hinder the importance of this study which showed that the microbiological quality of RMBDs purchasable online may be low and that such products may carry zoonotic pathogenic bacteria whose prevalence is more of a concern from a public health standpoint. Other risks posed by the consumption of RMBDs (e.g. nutritional imbalances, physical damages) have been widely discussed in a recent paper by Morelli et al. (2019).

5 | CONCLUSIONS

Feeding RMBDs is a current trend among pet owners and purchasing frozen ingredients from the Internet has become a common practice. This study showed that RMBDs sold online could reach very high levels of bacterial contamination that may further increase if such products are not stored properly at home. Moreover, zoonotic meat-borne bacteria like *Listeria monocytogenes* and *Yersinia enterocolitica* and food-borne pathogens (*C. perfringens* and *C. difficile*) were found in the analysed products, making the feeding and handling of RMBDs a potential health hazard both for animals and in-contact humans. Online RMBDs sellers should take further measures to minimize adulteration and prevent the growth of pathogens. Also, RMBDs feeders should be educated about the sanitary risks to animal and human health posed by the handling and the administration of such products, and they should be encouraged to improve kitchen hygiene rules and domestic cleaning standards.

CONFLICT OF INTEREST

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors. No pet food

producer played any role in the study design, samples collection or analysis and interpretation of data. None of the authors has any financial or personal relationship that could inappropriately influence or bias the content of the paper.

ANIMAL WELFARE STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. The authors confirm that they have followed EU standards for feed legislation.

ORCID

Giada Morelli https://orcid.org/0000-0001-5424-4164
Rebecca Ricci https://orcid.org/0000-0001-5678-9555

DATA AVAILABILITY STATEMENT

The data sets analysed in the current study are available from the corresponding author on reasonable request.

REFERENCES

Andrés-Lasheras, S., Martín-Burriel, I., Mainar-Jaime, R. C., Morales, M., Kuijper, E., Blanco, J. L., … Bolea, R. (2018). Preliminary studies on isolates of *Clostridium difficile* from dogs and exotic pets. *BMC Veterinary Research*, *14*(1), 77. https://doi.org/10.1186/s12917-018-1402-7

Bakri, M. (2018). Prevalence of *Clostridium difficile* in raw cow, sheep, and goat meat in Jazan, Saudi Arabia. *Saudi Journal of Biological Sciences*, *25*, 783–785. https://doi.org/10.1016/j.sjbs.2016.07.002

Bouttier, S., Barc, M. C., Felix, B., Lambert, S., Collignon, A., & Barbut, F. (2010). Clostridium difficile in ground meat, France. *Emerging Infectious Diseases*, *16*(4), 733.

Chengappa, M. M., Staats, J., Oberst, R. D., Gabbert, N. H., & Mcvey, S. (1993). Prevalence of *Salmonella* in raw meat used in diets of racing greyhounds. *Journal of Veterinary Diagnostic Investigation*, *5*, 372–377. https://doi.org/10.1177/104063879300500312

Clooten, J., Kruth, S., Arroyo, L., & Weese, J. S. (2008). Prevalence and risk factors for *Clostridium difficile* colonization in dogs and cats hospitalized in an intensive care unit. *Veterinary Microbiology*, *129*(1–2), 209–214. https://doi.org/10.1016/j.vetmic.2007.11.013

Dave, D., & Ghaly, A. E. (2011). Meat spoilage mechanisms and preservation techniques: A critical review. *American Journal of Agricultural and Biological Science*, *6*(4), 486–510. https://doi.org/10.3844/ajabssp.2011.486.510

David Wilson, H., McCormick, J. B., & Feeley, J. C. (1976). *Yersinia enterocolitica* infection in a 4-month-old infant associated with infection in household dogs. *The Journal of Pediatrics*, *89*(5), 767–769. https://doi.org/10.1016/S0022-3476(76)80799-8

De Boer, E., Zwartkruis-Nahuis, A., Heuvelink, A. E., Harmanus, C., & Kuijper, E. J. (2011). Prevalence of *Clostridium difficile* in retailed meat in the Netherlands. *International Journal of Food Microbiology*, *144*(3), 561–564.

Doulgeraki, A. I., Ercolini, D., Villani, F., & Nychas, G. J. E. (2012). Spoilage microbiota associated to the storage of raw meat in different conditions. *International Journal of Food Microbiology*, *157*(2), 130–141. https://doi.org/10.1016/j.ijfoodmicro.2012.05.020

EC 142/2011 (2011). Commission Regulation (EU) No 142/2011 of 25 February 2011 implementing Regulation (EC) No 1069/2009 of the European Parliament and of the Council laying down health rules as regards animal by-products and derived products not intended for human consumption. *Official Journal of the European Union*, *50*, 1–245. Retrieved from https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32011R0142&from=EN

EC 2073/2005 (2005). Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. *Official Journal of the European Union*, *L322*(2073), 1–19. https://doi.org/10.1016/B978-0-12-385007-2.00012-7

European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA and ECDC) (2018). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017. *Efsa Journal*, *16*(12), e05500.

Fenwick, S. G., Madie, P., & Wilks, C. R. (1994). Duration of carriage and transmission of *Yersinia enterocolitica* biotype 4, serotype 0:3 in dogs. *Epidemiology and Infection*, *113*(3), 471–477. https://doi.org/10.1017/S0950268800068485

Finley, R., Reid-Smith, R., Ribble, C., Popa, M., Vandermeer, M., & Aramini, J. (2008). The occurrence and antimicrobial susceptibility of salmonellae isolated from commercially available canine raw food diets in three Canadian cities. *Zoonoses and Public Health*, *55*(8–10), 462–469. https://doi.org/10.1111/j.1863-2378.2008.01147.x

Finley, R., Ribble, C., Aramini, J., Vandermeer, M., Popa, M., Litman, M., & Reid-Smith, R. (2007). The risk of salmonellae shedding by dogs fed *Salmonella*-contaminated commerical raw food diets. *Canadian Veterinary Journal*, *48*(1), 69–75.

Fredriksson-Ahomaa, M., Heikkilä, T., Pernu, N., Kovanen, S., Hielm-Björkman, A., & Kivistö, R. (2017). Raw meat-based diets in dogs and cats. *Veterinary Sciences*, *4*(3), 33. https://doi.org/10.3390/vetsci4030033

Fredriksson-Ahomaa, M., Korte, T., & Korkeala, H. (2001). Transmission of *Yersinia enterocolitica* 4/O:3 to pets via contaminated pork. *Letters in Applied Microbiology*, *32*(6), 375–378. https://doi.org/10.1046/j.1472-765X.2001.00922.x

Freeman, L. M., Chandler, M. L., Hamper, B. A., & Weeth, L. P. (2013). Current knowledge about the risks and benefits of raw meat–based diets for dogs and cats. *Journal of the American Veterinary Medical Association*, *243*(11), 1549–1558. https://doi.org/10.2460/javma.243.11.1549

Freeman, L. M., & Michel, K. E. (2001). Evaluation of raw food diets for dogs. *Journal of the American Veterinary Medical Association*, *218*(5), 705–709. https://doi.org/10.2460/javma.2001.218.705

Giacometti, F., Magarotto, J., Serraino, A., & Piva, S. (2017). Highly suspected cases of salmonellosis in two cats fed with a commercial raw meat-based diet: Health risks to animals and zoonotic implications. *BMC Veterinary Research*, *13*(1), 1–5. https://doi.org/10.1186/s12917-017-1143-z

Guran, H. S., & Ilhak, O. I. (2015). *Clostridium difficile* in retail chicken meat parts and liver in the Eastern Region of Turkey. *Journal Für Verbraucherschutz Und Lebensmittelsicherheit*, *10*(4), 359–364.

Gutman, L. T., Ottesen, E. A., Quan, T. J., Noce, P. S., & Katz, S. L. (1973). An inter-familial outbreak of *Yersinia enterocolitica* enteritis. *New England Journal of Medicine*, *288*(26), 1372–1377.

Hensgens, M. P., Keessen, E. C., Squire, M. M., Riley, T. V., Koene, M. G., de Boer, E., … Kuijper, E. (2012). *Clostridium difficile* infection in the community: A zoonotic disease? *Clinical Microbiology and Infection*, *18*(7), 635–645.

Hussain, I., Sharma, R. K., Borah, P., Rajkhowa, S., Hussain, I., Barkalita, L. M., … Saikia, G. K. (2015). Isolation and characterization of *Clostridium difficile* from pet dogs in Assam, India. *Anaerobe*, *36*, 9–13. https://doi.org/10.1016/j.anaerobe.2015.09.006

Jöbstl, M., Heuberger, S., Indra, A., Nepf, R., Köfer, J., & Wagner, M. (2010). Clostridium difficile in raw products of animal origin. *International Journal of Food Microbiology*, *138*(1–2), 172–175.

Joffe, D. J., & Schlesinger, D. P. (2002). Preliminary assessment of the risk of *Salmonella* infection in dogs fed raw chicken diets. *Canadian Veterinary Journal*, *43*(6), 441–442.

Knight, D. R., Putsathit, P., Elliott, B., & Riley, T. V. (2016). Contamination of Australian newborn calf carcasses at slaughter with Clostridium difficile. *Clinical Microbiology and Infection*, *22*(3), 266 e1–7. https://doi.org/10.1016/j.cmi.2015.11.017

Lambert, A. D., Smith, J. P., & Dodds, K. L. (1991). Shelf life extension and microbiological safety of fresh meat—a review. *Food Microbiology*, *8*(4), 267–297.

Lefebvre, S. L., Reid-Smith, R., Boerlin, P., & Weese, J. S. (2008). Evaluation of the risks of shedding salmonellae and other potential pathogens by therapy dogs fed raw diets in Ontario and Alberta. *Zoonoses and Public Health*, *55*(8–10), 470–480. https://doi.org/10.1111/j.1863-2378.2008.01145.x

LeJeune, J. T., & Hancock, D. D. (2001). Public health concerns associated with feeding raw meat diets. *Journal of the American Veterinary Medical Association*, *219*(9), 1222–1225. https://doi.org/10.2460/javma.2001.219.1222

Lenz, J., Joffe, D., Kauffman, M., Zhang, Y., & Lejeune, J. (2009). Perceptions, practices, and consequences associated with foodborne pathogens and the feeding of raw meat to dogs. *Canadian Veterinary Journal*, *50*(6), 637–643.

Leonard, E. K., Pearl, D. L., Finley, R. L., Janecko, N., Peregrine, A. S., Reid-Smith, R. J., & Weese, J. S. (2011). Evaluation of pet-related management factors and the risk of *Salmonella* spp. carriage in pet dogs from volunteer households in Ontario (2005–2006). *Zoonoses and Public Health*, *58*(2), 140–149. https://doi.org/10.1111/j.1863-2378.2009.01320.x

Marks, S. L., & Kather, E. J. (2003). Bacterial-associated diarrhea in the dog: A critical appraisal. *The Veterinary Clinics of North America. Small Animal Practice*, *33*(5), 1029–1060.

Morelli, G., Bastianello, S., Catellani, P., & Ricci, R. (2019). Raw meat-based diets for dogs: Survey of owners' motivations, attitudes and practices. *BMC Veterinary Research*, *15*(1), 74.

Morgan, S. K., Willis, S., & Shepherd, M. L. (2017). Survey of owner motivations and veterinary input of owners feeding diets containing raw animal products. *PeerJ*, *5*, e3031. https://doi.org/10.7717/peerj.3031

Morley, P. S., Strohmeyer, R. A., Tankson, J. D., Hyatt, D. R., Dargatz, D. A., & Fedorka-Cray, P. J. (2006). Evaluation of the association between feeding raw meat and *Salmonella enterica* infections at a greyhound breeding facility. *Journal of the American Veterinary Medical Association*, *228*(10), 1524–1532. https://doi.org/10.2460/javma.228.10.1524

Nemser, S. M., Doran, T., Grabenstein, M., McConnell, T., McGrath, T., Pamboukian, R., … Reimschuessel, R. (2014). Investigation of *Listeria*, *Salmonella*, and toxigenic *Escherichia coli* in various pet foods. *Foodborne Pathogens and Disease*, *11*(9), 706–709. https://doi.org/10.1089/fpd.2014.1748

Nilsson, O. (2015). Hygiene quality and presence of ESBL-producing *Escherichia coli* in raw food diets for dogs. *Infection Ecology & Epidemiology*, *5*(January), 28758. https://doi.org/10.3402/iee.v5.28758

Palerme, J. S., Pan, P. C., Parsons, C. T., Kathariou, S., Ward, T. J., & Jacob, M. E. (2016). Isolation and characterization of atypical *Listeria monocytogenes* associated with a canine urinary tract infection. *Journal of Veterinary Diagnostic Investigation*, *28*(5), 604–607. https://doi.org/10.1177/1040638716661381

Palumbo, S. A. (1986). Is refrigeration enough to restrain foodborne pathogens? *Journal of Food Protection*, *49*, 1003–1009.

Pritchard, J. C., Jacob, M. E., Ward, T. J., Parsons, C. T., Kathariou, S., & Wood, M. W. (2016). *Listeria monocytogenes* septicemia in an immunocompromised dog. *Veterinary Clinical Pathology*, *45*(2), 254–259. https://doi.org/10.1111/vcp.12363

Rabold, D., Espelage, W., Abu Sin, M., Eckmanns, T., Schneeberg, A., Neubauer, H., … Lübke-Becker, A. (2018). The zoonotic potential of *Clostridium difficile* from small companion animals and their owners. *PLoS ONE*, *13*(2), e019341. https://doi.org/10.1371/journal.pone.0193411

Roccato, A., Uyttendaele, M., Cibin, V., Barrucci, F., Cappa, V., Zavagnin, P., … Ricci, A. (2015). Effects of domestic storage and thawing practices on salmonella in poultry-based meat preparations. *Journal of Food Protection*, *78*(12), 2117–2125.

Schroeder, H., & van Rensburg, I. B. (1993). Generalised *Listeria monocytogenes* infection in a dog. *Journal of the South African Veterinary Association*, *64*(3), 133–136. Retrieved from http://europepmc.org/abstract/MED/8176688

Stiver, S. L., Frazier, K. S., Mauel, M. J., & Styer, E. L. (2003). Septicemic salmonellosis in two cats fed a raw-meat diet. *Journal of the American Animal Hospital Association*, *39*(6), 538–542. https://doi.org/10.5326/0390538

Stone, G. G., Chengappa, M. M., Oberst, R. D., Gabbert, N. H., Mcvey, S., Hennessy, K. J., … Staats, J. (1993). Application of polymerase chain reaction for the correlation of *Salmonella* serovars recovered from greyhound feces with their diet. *Journal of Veterinary Diagnostic Investigation*, *5*, 378–385. https://doi.org/10.1177/104063879300500313

Stone, N. E., Sidak-Loftis, L. C., Sahl, J. W., Vazquez, A. J., Wiggins, K. B., Gillece, J. D., … Wagner, D. M. (2016). More than 50% of Clostridium difficile isolates from pet dogs in Flagstaff, USA, carry toxigenic genotypes. *PLoS ONE*, *10*, 11(10), e0164504. https://doi.org/10.1371/journal.pone.0164504

Strohmeyer, R. A., Morley, P. S., Hyatt, D. R., Dargatz, D. A., Scorza, A. V., & Lappin, M. R. (2006). Evaluation of bacterial and protozoal contamination of commercially available raw meat diets for dogs. *Journal of the American Veterinary Medical Association*, *228*(4), 537–542. https://doi.org/10.2460/javma.228.4.537

van Bree, F. P. J., Bokken, G. C. A. M., Mineur, R., Franssen, F., Opsteegh, M., van der Giessen, J. W. B., … Overgaauw, P. A. M. (2018). Zoonotic bacteria and parasites found in raw meat-based diets for cats and dogs. *Veterinary Record*, *182*(2), 50–50. https://doi.org/10.1136/vr.104535

Weber, A., & Plagemann, O. (1991). *Listeria monocytogenes* as cause of abortion in dogs. *Kleintierpraxis (Germany, F.R.)*.

Weber, A., Potel, J., Schäfer-Schmidt, R., Prell, A., & Datzmann, C. (1995). Studies on the occurrence of *Listeria monocytogenes* in fecal samples of domestic and companion animals. *Zentralblatt Für Hygiene Und Umweltmedizin = International Journal of Hygiene and Environmental Medicine*, *198*(2), 117–123 Retrieved from http://europepmc.org/abstract/MED/9376045

Weese, J. S., Finley, R., Reid-Smith, R. R., Janecko, N., & Rousseau, J. (2010). Evaluation of Clostridium difficile in dogs and the household environment. *Epidemiology & Infection*, *138*(8), 1100–1104.

Weese, J. S., Rousseau, J., & Arroyo, L. (2005). Bacteriological evaluation of commercial canine and feline raw diets. *Canadian Veterinary Journal*, *46*, 513–516.

Weese, J. S., Staempfli, H. R., Prescott, J. F., Kruth, S. A., Greenwood, S. J., & Weese, H. E. (2001). The roles of *Clostridium difficile* and enterotoxigenic *Clostridium perfringens* in diarrhea in dogs. *Journal of Veterinary Internal Medicine*, *15*(4), 374–378.

How to cite this article: Morelli G, Catellani P, Miotti Scapin R, et al. Evaluation of microbial contamination and effects of storage in raw meat-based dog foods purchased online. *J Anim Physiol Anim Nutr*. 2019;00:1–8. https://doi.org/10.1111/jpn.13263