Examination of zoonotic bacterial pathogens in canine and feline raw meat-based diets

F.P.J. van Bree, P.A.M. Overgaauw

The feeding of raw meat-based diets (RMBD) to companion animals has become increasingly popular in recent years. However, such diets were demonstrated to be possibly contaminated with various zoonotic bacterial species in several studies and so the feeding of these diets could pose a risk to both animal and human health. Data about the situation in the Netherlands, or Europe in general are not available. Therefore, the purpose of this study was to evaluate the contamination of commercial RMBDs available in the Netherlands with zoonotic bacterial pathogens. Thirty-five commercial RMBDs were evaluated via bacterial culture for *Escherichia coli* O157:H7, extended-spectrum beta-lactamase (ESBL-)producing *E. coli*, *Listeria monocytogenes* and *Salmonella* spp.. *E. coli* O157:H7 was isolated from eight (23%) products, ESBL-producing *E. coli* from twenty-eight (80%) products, *L. monocytogenes* from nineteen (54%) products, other *Listeria* spp. from fifteen (43%) products, and *Salmonella* spp. from seven (20%) products. The results of this study demonstrate the need to pay attention to the potential of RMBDs to be a source for zoonotic bacterial pathogens in the Netherlands. These diets cannot be ruled out as a possible source for bacterial infections in both humans and animals and therefore companion animal owners should be informed about the associated risks.

Keywords: raw meat-based diet; BARF; *E. coli* O157; ESBL; *Listeria monocytogenes*; *Salmonella*; public health

In recent years it has become increasingly popular among dog and cat owners to feed raw meat-based diets (RMBD), instead of the more conventional pet foods, to their pets. It is even estimated that 51% of dog owners in the Netherlands do feed their dog(s) entirely or partially with raw meat-based products. (1) Given that 36% of households in the Netherlands own either a dog, or a cat, it could be estimated that RMBDs are used at some time in 1.3 million Dutch households and so a very large group of people could be exposed to these diets. (2) RMBDs do contain raw animal products and/or by-products and can roughly be divided into three different groups: the raw dried dog and cat treats (e.g. pig ears), the home-prepared RMBDs, and the commercial RMBDs. Ingredients used in home-prepared RMBDs are often purchased from butcheries and more specialized pet shops, and include meat, meaty bones, and organ meats, but also vegetables, eggs, grains, yeast, milk, and yogurt. The BARF (Bones And Raw Food/Biologically Appropriate Raw Food)-diet is probably the most popular example of a home-prepared RMBD. The commercial RMBDs are developed by companies and most of them are intended to provide for a complete diet for dogs and/or cats. Nowadays a variety of products from different brands is available in most pet shops and supermarkets. (3-5)

Proponents of feeding RMBDs have several motivations for feeding RMBDs to their pets. It is claimed that feeding such a diet results in a better overall condition and could have a supportive role in controlling and preventing certain medical conditions in both dogs and cats. (3-7) Other arguments often used to defend the feeding of RMBDs are the claims that nutrients in the conventional pet foods are destroyed or altered by the heat processing, that the consumption of unnecessary additives is avoided in this way, and that it is more natural for carnivore species to eat raw meat products. (3-6,8) However, those benefits attributed to the feeding of RMBDs are mostly anecdotal and are not supported by scientific evidence. On the contrary, scientific research does support a number of risks associated with the feeding of RMBDs. To begin with, the feeding of RMBDs led to the development of clinical conditions (e.g. hyperthyroidism), or injuries (e.g. gastrointestinal tract perforation) in several cases. (3-6,8) In addition, RMBDs are more likely to be nutritionally imbalanced and are often deficient in a number of nutrients. When used for a longer period, such diets can result in serious health problems, especially in animals with specific nutrient requirements (e.g. young animals). (3-6,8,9) But the risk most supported by and discussed in scientific literature at this moment, is the infectious disease risk as a result of the possible contamination of RMBDs with a variety of pathogens, including different zoonotic bacteria.

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The spread of such bacteria in the environment either directly from the contaminated RMBDs, or by infected animals, presents also a risk to the pet owner and other humans in the surroundings. (3-6,8-11)

Up to now, most information is available about the risks associated to the presence of *Salmonella* spp. in RMBDs. The prevalence of *Salmonella* spp. in RMBDs ranges from 5% to even 80%. (11-19) Other bacteria with a possible impact on human health isolated from RMBDs include enterohemorrhagic *Escherichia coli* (including *E. coli* O157:H7 which can cause renal failure in humans) and *Listeria monocytogenes*. (3,17) Those three zoonotic bacterial species account for a total of nearly 63,000 annual reported cases of illness and around 45 deaths a year in the Netherlands. In most of these cases the source of infection was either contaminated food, or contact with an infected animal. (20) Another microbe-related concern receiving more attention in recent years is the occurrence of antibiotic-resistant bacteria, especially Enterobacteriaceae, in companion animals and the associated risk to public health. The presence of antibiotic-resistant bacteria (including extended-spectrum beta-lactamase (ESBL)-producing *E. coli*) in RMBDs has been demonstrated. (13,21-23) Although a number of studies on the bacterial contamination of RMBDs have been conducted, those do all focus on the situation in Canada, the United States and Sweden. So, data about the situation in the Netherlands are not available and data about the European situation in general are scarce. Evaluating the bacterial contamination of RMBDs available in the Netherlands would therefore provide for valuable information, as it is a first step in defining the risks associated to the feeding of RMBDs in this area more accurately.

The purpose of this study was to evaluate the contamination of commercial RMBDs available in the Netherlands with zoonotic bacterial pathogens. In addition the microbiological and hygiene quality of the diets were evaluated.

Materials and Methods

Sample collection and processing

In order to get an indication of the most commonly fed products, an overview was made of all commercial raw-meat based diet brands available in the Netherlands and the number of their retailers. Brands sold by a large number of retailers were assumed to be more commonly bought by pet-owners and thus more commonly fed to dogs and cats. Products from those brands were purchased from a variety of retailers in and around the city of Utrecht, the Netherlands, and stored as per label recommendations until analysis.

Before analysis took place, frozen products were thawed under running tap water at room temperature in a vacuum sealed plastic packaging. All products were processed while still cold (0-4°C) in order to prevent a substantial bacterial growth. Of each product four samples of 25 g each were obtained with a sterile spoon and transferred into a sterile blender bag (BagPage®, Interscience, France). After the addition of 225 mL of a specific solution, depending on the following microbiological method, the samples were homogenized by hand for 90 seconds.

Microbiological methods

All products were analysed for the presence of the following bacterial species: *Escherichia coli* O157:H7 (EHEC), Extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella*. In addition, the products were analysed for their quantity of aerobic bacteria and *Escherichia coli*.

Quantitative bacteriology

For the quantitative bacteriology 225 mL of buffered peptone water (Oxoid Ltd, UK) was added to the samples and a further decimal dilution series was prepared in 0.1% peptone salt solution (Maximum Recovery Diluent, Oxoid Ltd, UK). Concerning the aerobic bacteria, dilutions of 1:1.000, 1:10.000 and 1:1.000.000 were used, whereas for *E. coli* dilutions of 1:10, 1:100 and 1:1.000 were used. Of those dilutions, 1 mL was transferred onto Petrifilm™ Aerobic Count Plates (3M Microbiology, USA) and Petrifilm™ E.coli/Coliform Count Plates (3M Microbiology, USA) for the aerobic bacteria and *E. coli* respectively. The inoculated Petrifilms™ were incubated for 48 ± 3 hours at 35°C ± 1°C according to AOAC Official Method 990.12 (AOAC, 2002) and AOAC Official Method 991.14 (AOAC, 2002) respectively.

Escherichia coli O157:H7

The samples were analysed for the presence of *E. coli* O157:H7 according to ISO 16654:2001 Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Escherichia coli* O157 (ISO, 2001).

ESBL-producing Escherichia coli

For the analysis for the presence of ESBL-producing *E. coli* 225 mL of buffered peptone water (Oxoid Ltd, UK) with cefotaxime (1 mg/L; Cefotaxime sodium salt, Sigma-Aldrich, USA) was added to the samples. For the quantitative analysis a further decimal dilution series was prepared in 0.1% peptone salt solution (Maximum Recovery Diluent, Oxoid Ltd, UK) and 0.1 mL of the dilutions of 1:10, 1:100 and 1:1000 was streaked on MacConkey agar No.3 (Oxoid Ltd., UK) with cefotaxime (1 mg/L; Cefotaxime sodium salt, Sigma-Aldrich, USA). The homogenized suspension itself was incubated at 37ºC overnight, after which it was streaked on MacConkey agar No.3 (Oxoid Ltd., UK) with cefotaxime (1 mg/L; Cefotaxime sodium salt, Sigma-Aldrich, USA) as well. The inoculated plates were incubated at 37ºC overnight. Presumptive *E. coli* colonies were transferred to Tryptone Soya agar plates (Oxoid Ltd, UK) and incubated at 37ºC for 24±3 hours, after which they were confirmed by performing an indole test and an oxidase test.

Listeria monocytogenes

The samples were analysed for the presence of *Listeria monocytogenes* according to ISO 11290-1:1996 Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of *Listeria monocytogenes* – Part 1: Detection method (ISO, 1996), with two modifications. For the primary enrichment UVM Modified Listeria Enrichment Broth (BD Difco™, USA) was used instead of Half Fraser Broth (Oxoid Ltd, UK) and for the selective isolation COMPASS® Listeria Agar (Biokar Diagnostics, France) was used instead of ALOA™ OCLA (ISO) (Oxoid Ltd, UK).

Salmonella species

The samples were analysed for the presence of *Salmonella* spp. according to ISO 6579:2002 Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. (ISO, 2002), with one modification. The presumptive *Salmonella* spp. colonies were only serologically confirmed if their biochemical confirmation was positive and they were only analysed for the presence of O-antigens, with the use of Agglutinating Serum Salmonella Polyvalent-O (Groups A-S) (Remel, USA). Analysis for the presence of Vi-antigens and/or H-antigens was not performed in this study.

Storing bacterial isolates

At the end of the study all confirmed isolates of *E. coli* O157:H7, ESBL-producing *E. coli*, *Listeria* spp., and *Salmonella* spp. were suspended into Tryptone Soya broth (Oxoid Ltd., UK) and incubated at 37ºC for 24 hours. Subsequently, 0.6 mL of the bacterial suspensions was mixed with 0.6 mL of a 21.25% glycerol solution (Glycerol 85%, Merck, Germany) and stored at -80ºC.

A more detailed description of the microbiological methods used in this study is given in the supplementary file: Microbiological methodology.

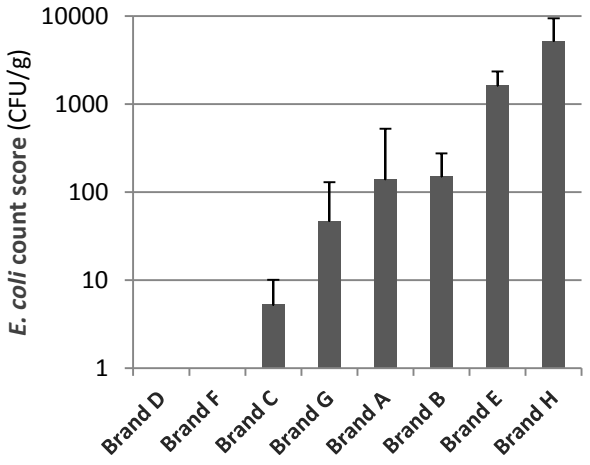

FIG 1. Bar chart showing the mean *Escherichia coli* count scores for the different brands of commercial raw meat-based diets tested in this study.

Results

Sample collection

Thirty-five commercial raw-meat based diets from eight different brands (1-9 products/brand) were analysed. Fifteen (43%) products contained only meat and animal by-products, of which five contained meat and by-products of a single animal species (i.e. chicken and rabbit). The remaining twenty (57%) products contained other ingredients (e.g. rice, vegetables) in addition to meat and animal by-products. The meat and animal by-product sources included in the analysed products were chicken (n=20), beef (n=18), lamb (n=6), duck (n=2), rabbit (n=2), horse (n=1), and turkey (n=1). A complete overview of the analysed products is given in the supplementary file: Table S1.

Bacterial analysis

The quantitative scores for aerobic bacteria ranged from 7.9×10^2 to 5.0×10^6 CFU/g (mean value (MV)=2.3×10^5, standard deviation (SD)=8.6×10^5). In three products the score was lower than 1.0×10^3 CFU/g and in two products it exceeded 1.0×10^6 CFU/g. *Escherichia coli* was isolated from thirty (86%) products and its quantitative scores ranged from less than 2.6 to 1.1×10^4 CFU/g (MV=8.9×10^2, SD=2.2×10^3). In seven products the score exceeded 5.0×10^2 CFU/g. The mean scores for *E. coli* varied among the different brands and from the products from two brands this bacterial species was not isolated at all (**Figure 1**).

Eight (23%; 95% confidence interval (CI): 9-37) products from three different brands (n=5, n=2, n=1) were confirmed to be positive for *Escherichia coli* O157:H7 (**Table 1**). Meat and animal by-product sources included in these products were chicken (n=5), beef (n=4), lamb (n=1), and turkey (n=1). Extended- spectrum beta-lactamase (ESBL)-producing *E. coli* was isolated from twenty-eight (80%; 95% CI: 67-93) products from seven different brands (**Table 1**). The meat and animal by-product sources of these products included chicken (n=17), beef (n=14), lamb (n=6), rabbit (n=1), and turkey (n=1). In one of the products the quantity of ESBL-producing *E. coli* could be determined, which was 12.0 CFU/g.

Listeria monocytogenes was isolated from nineteen (54%; 95% CI: 37-71) products from five different brands, in addition fifteen (43%; 95% CI: 27-59) other products from all different brands were confirmed to be positive for

TABLE 1 Results of the examination of 35 commercial raw meat-based diets for the presence of *Escherichia coli* O157:H7, ESBL-producing *E. coli*, *Listeria monocytogenes*, other *Listeria* species, and *Salmonella* species.

Brand	Products positive for (%):										Total tested
	E. coli O157:H7		ESBL-producing *E. coli*		*L. monocytogenes*		Other *Listeria* spp.		*Salmonella* spp.		
A	0	(0)	6	(67)	7	(78)	2	(22)	1	(11)	9
B	5	(63)	7	(88)	5	(63)	3	(37)	4	(50)	8
C	1	(20)	4	(80)	2	(40)	3	(60)	1	(20)	5
D	0	(0)	0	(0)	0	(0)	1	(100)	0	(0)	1
E	0	(0)	2	(100)	0	(0)	2	(100)	0	(0)	2
F	0	(0)	1	(50)	0	(0)	1	(50)	0	(0)	2
G	2	(50)	4	(100)	3	(75)	1	(25)	0	(0)	4
H	0	(0)	4	(100)	2	(50)	2	(50)	1	(25)	4
Total	8	(23)	28	(80)	19	(54)	15	(43)	7	(20)	35

other *Listeria* spp. (**Table 1**). All meat and animal by-product sources present in this study were included in both these groups of products. *Salmonella* spp. was isolated from seven (20%; 95% CI: 7-33) products from four different brands (n=4, n=1, n=1, n=1) (**Table 1**). Meat and animal by-product sources included in these products were chicken (n=4), beef (n=3), duck (n=2), horse (n=1), and lamb (n=1).

One product (3%) was confirmed to be positive for all four bacterial species (i.e. *E. coli* O157:H7, ESBL-producing *E. coli*, *L. monocytogenes*, and *Salmonella* spp.), eight (23%) products were confirmed to be positive for three of them, thirteen (37%) products for two of them and eight (23%) products for one of them. From the five (14%) remaining products none of the four bacterial species could be isolated.

Discussion

The results of this study showed that commercial raw meat-based diets may be contaminated with a variety of zoonotic bacterial pathogens, including *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* spp.. Therefore, these diets can be a source of some bacterial species of public health significance, especially with regard to immunocompromised individuals like young children, the elderly, and pregnant women. In addition, extended-spectrum beta-lactamase (ESBL)-producing *E. coli* were isolated from the commercial RMBDs in this study. So RMBDs could be a source for an infection with ESBL-producing *E. coli* in companion animals and thus play a role in the subsequent propagation of such antibiotic-resistant bacteria in the environment.

The overall microbiological quality of the commercial RMBDs tested in this study was not bad since none of the diets contained over 5 x 10^6 CFU/g and only two diets contained over 5 x 10^5 CFU/g, the two limit values applicable to both minced and mechanically separated meat intended for human consumption. (24) However, the hygiene quality of the tested RMBDs was worse. Seven diets contained over 500 CFU/g *E. coli* and would therefore have been qualified of unsatisfactory hygiene quality when intended for human consumption. Seven other diets contained between 50 and 500 CFU/g *E. coli*. When two of five samples from one batch contain this amount of *E. coli* the batch would have been qualified of unsatisfactory hygiene quality for human consumption. (24) However, these findings were not unexpected as previous studies reported comparable or even higher frequencies and quantities of *E. coli* in RMBDs. (18,19,22) Besides, RMBDs are meant to be pet foods and often contain animal by-products, which are not restricted to such strict hygiene regulations as products intended for human consumption.

Escherichia coli O157:H7 is a gram-negative, rod-shaped, facultative anaerobic bacteria belonging to the family Enterobacteriaceae. Like the other enterohemorrhagic *E. coli* serotypes, this bacteria produces verocytotoxins (VT) which are released during bacteriolysis. (25) Although it has been reported that both dogs and cats can become infected with *E. coli* O157:H7, illness in these animals as a result of such an infection rarely occurs and thus most of them are asymptomatic carriers. (10,25,26) However, *E. coli* O157:H7 infections in humans have been associated with serious diseases like hemorrhagic colitis and haemolytic uremic syndrome (HUS). Moreover, the infective dose of this pathogen in humans is very low, even the ingestion of a single CFU has been reported to be able to result in illness. (25,27) Most of human cases are associated with the consumption of contaminated food and especially raw or undercooked beef, since cattle is reported to be the main reservoir of this bacterial species. Although dogs and cats are also suggested to be possible reservoirs for human infection and contact with dogs has even been suggested to result in the transmission of *E. coli* O157:H7 to humans, these companion animals are rather considered to be short-term vectors of these bacteria. (25,26,28) Therefore, the presence of *E. coli* O157:H7 in RMBDs is a substantial risk, especially to public health, as it could serve as a source for infection in both dogs and cats. Based on previous studies, the isolation frequency in this study was not expected to be as high as 23%. Until now only one homemade RMBD was reported to be positive for *E. coli* O157:H7 and one other study reported only 4% of the raw foods tested to be positive for non-O157 VT-producing *E. coli* (**Table 2**). (3,17) Furthermore, *E. coli* O157 was exclusively isolated from one meat category in a Dutch microbiological examination of food products in 2008, namely from 0.4% of minced or prepared beef samples. (29) European data also show a much lower isolation frequency than found in this study, as only 0.1% of meat samples tested positive for *E. coli* O157 in 2014. (30) Besides the high isolation frequency in this study, it is also remarkable that five of the eight products positive for *E. coli* O157:H7 in this study were from the same brand and only one of these five products contained beef. This could suggest that these products were contaminated during the production process at the commercial RMBD facility.

Listeria monocytogenes is a pathogenic *Listeria* species, which are gram-positive, rod-shaped, facultative anaerobic bacteria. A special feature of these bacteria species is that they are able to grow over a wide range of temperatures, even in the refrigerator, and thus do easily replicate in the environment. (25,31) *L. monocytogenes* has been isolated from the faeces of both dogs and cats, however infection with this pathogen in these animal species is rare and usually asymptomatic. (25,32) Nevertheless, clinical listeriosis in dogs and cats has been reported in some cases and one case report even identified *L. monocytogenes* to be the cause of an abortion in a bitch. Moreover, in the abortion case raw meat products were considered to be the source of infection. (25,33) Unlike in companion animals, *L. monocytogenes* can cause serious illness in humans. Although healthy adults usually only show clinical signs comparable to influenza symptoms, an infection can be life-threatening in immunocompromised individuals. Especially neonates and pregnant women, in whom it can lead to abortion, are at risk. (25,31) In both animals and humans contaminated food products, including raw meat, have been identified as common sources for an infection with *L. monocytogenes*. (25) Up till now, only one other study evaluated RMBDs for the presence of *Listeria* species. In that study 16% of the raw food samples tested positive for *L. monocytogenes* and 17% tested positive for other *Listeria* spp. (**Table 2**). (17) The isolation frequencies in this study were thus twice as high as previously reported, namely 54%

TABLE 2 Proportions of raw meat-based diets contaminated with the bacterial species which were of interest in this study, as reported by the other studies on the bacterial contamination of raw meat-based diets available at this moment.

Bacterial species	Country	Proportion of contaminated RMBDs	Reference
Escherichia coli O157	United States	20%	(3)
VT-producing *Escherichia coli* (non-O157)	United States	4%	(17)
Listeria monocytogenes	United States	16%	(17)
Other *Listeria* species	United States	17%	(17)
Salmonella species	Canada	5%	(11)
		20%	(19)
		21%	(13)
		37%	(12)
		80%	(14)
	United States	7%	(16)
		7%	(18)
		8%	(17)
		45%	(35)
ESC-producing *Escherichia coli*	Sweden	23%	(22)

RMBD – raw meat-based diet; VT – verocytotoxin; ESC – extended-spectrum cephalosporinase

and 43% for *L. monocytogenes* and other *Listeria* spp. respectively. This study also showed a higher isolation frequency of *L. monocytogenes* compared to both Dutch and European data about meat products in general. (29,30) Given the fact that *L. monocytogenes* does easily replicate in the environment and can cause serious illness in certain human beings, the presence of this bacterial species in RMBDs could pose a substantial risk to public health.

Concerning the bacterial contamination of RMBDs, *Salmonella* species have received most attention in scientific research so far. *Salmonella* spp. are gram-negative, rod-shaped, and usually motile bacteria belonging to the family Enterobacteriaceae. Of this bacterial species more than 2,500 different serotypes have been identified. (25,31) Although subclinical infections do frequently occur, salmonellae are able to cause gastroenteritis and even septicemic disease, as a result of which other organs can also become affected, in both humans and companion animals. Many serotypes are known to be able to infect a wide range of animal species including humans and it has even been reported that the serotypes isolated from dogs are similar to those isolated from humans. (25,34) Common sources of infection are contaminated foods and water, but *Salmonella* spp. can also be widespread in the environment since they are able to survive for a respectively long period outside their hosts. In addition, direct transmission from companion animals to humans and the other way around have been mentioned. (25,34) *Salmonella* spp. have been previously isolated from RMBDs in several studies, so it was not surprising to find this bacterial species in this study. The isolation frequency of 20% was also comparable to the prevalence found in other studies. The prevalence reported in Canadian studies ranged from 7% to 80%, whereas studies conducted in the United States reported a prevalence ranging from 5% to 45% (**Table 2**). (11-14,16-19,35) Furthermore, it has been reported that salmonellae were isolated from raw meat products, especially poultry, intended for human consumption, although with a lower isolation frequency than reported in this study. In the Netherlands around 8% of broiler meat samples were reported to be contaminated with *Salmonella* spp. in 2008 and 2009, whereas an overall prevalence of 2.8% was reported in 2014 for Europe as a whole. (29,30) Although the presence of *Salmonella* spp. in RMBDs may not be surprising, it is still of concern, since such diets could thus be a source of infection with these bacteria in both dogs and cats.

In addition to merely evaluating the presence of *Salmonella* spp. in RMBDs, further research has been conducted in an attempt to define its associated risk. In the beginning of the 1990s, contaminated RMBDs were already identified as a source for gastroenteritis in greyhounds, based on the fact that the *Salmonella* strains isolated from the dogs with clinical signs were related to those isolated from their diets. (35,36) A more recent study evaluating *Salmonella* infections at a greyhound breeding facility also identified raw meat to be the source of some of the isolated *Salmonella* strains. (37) Moreover, several studies have reported that RMBD fed dogs are more likely to shed *Salmonella* spp. in their faeces. In 2002 a study pointed out that 30% of the faecal samples from homemade RMBD fed dogs were contaminated with different *Salmonella* serotypes, whereas this bacterial species could not be isolated from the faeces of non RMBD fed dogs. One of the dogs in this study was even found to shed the same *Salmonella* serotype as isolated from its diet. (14) In one experiment dogs were fed a RMBD previously shown to be contaminated with *Salmonella* spp. in order to evaluate the shedding of salmonellae by dogs after the consumption of such a diet. Forty-four percent of these dogs were actually shedding salmonellae in their faeces and it was reported that this shedding, after the feeding of a contaminated RMBD only once, lasted up to one week. (12) However, in another study it was reported that dogs, which did consume RMBDs over a longer period of time, were shedding salmonellae for up to eight months. This same study also demonstrated that

61% of RMBD fed dogs were shedding *Salmonella* spp., in comparison with only 8% of the other dogs. (15) In parallel, one other study reported salmonellae shedding in 14.3% of the RMBD fed dogs and in none of the non RMBD fed dogs. Moreover, this study reported vacuum cleaner waste from households with RMBD fed dogs to be more frequently contaminated with *Salmonella* spp. than the vacuum cleaner waste from other households. (11) From these four studies it could be concluded that RMBD fed dogs are actually more likely to shed *Salmonella* spp. and consequently are able to contaminate their household environment.

Although RMBDs are likely to be a source for *Salmonella* infections, the consumption of these diets has not yet been proven to be a direct cause of salmonellosis in dogs. However, it has been reported that the feeding of *Salmonella*-contaminated RMBDs resulted in septicemic salmonellosis in two cats as a result of which both animals had to be euthanized. (38) And in addition to the aforementioned gastroenteritis in greyhounds, one case of diarrhoea in a litter of pups has been linked to the feeding of a contaminated RMBD. (39) Since RMBD fed dogs are likely to shed salmonellae in their faeces and thus contaminate their environment, humans in their surroundings are also at risk. Transmission of *Salmonella* from dogs to humans has been reported and also contact with cats has been associated with human *Salmonella* infections. (40,41) Apart from being an indirect source by infecting companion animals, RMBDs could also be a more direct source for *Salmonella* infections in humans. It has been reported that *Salmonella* spp. could persist in food bowls, which were inoculated with a contaminated RMBD, at room temperature. This fortifies the assertion that *Salmonella* can contaminate and survive in the environment, which subsequently becomes an infective source itself. Furthermore, cleaning and disinfection of the contaminated food bowls were shown to be hardly effective. (42) However, commercial and homemade RMBDs have not been identified as a direct source for human salmonellosis so far. On the other hand, human outbreaks of *Salmonella* infections have been associated to both contaminated dried pig ears and contaminated chicken jerky pet treats and direct contact with these pet treats has been proposed as a possible route of transmission. (43,44) Besides animal health, public health is thus likely to be also at risk when feeding RMBDs to companion animals.

Another major problem related to the occurrence of bacteria in general, is the emergence and increase in antibiotic resistance. Several studies on the bacterial contamination of RMBDs have also paid attention to this subject. Different *Salmonella* strains isolated from RMBDs were reported to be resistant to a number of different antibiotics. In some studies even multidrug (>3) resistance was observed, including both a potential extended-spectrum cephalosporinase (ESC)-producing strain and a potential ESBL-producing strain. (11-13,15,18) It has been proposed that the resistance of these salmonellae is a result of the transfer of genes from other bacteria, like *E. coli*. In one study it was even mentioned that the resistance phenotype of a *Salmonella* strain isolated from one dog was comparable to that of an *E. coli* strain isolated from the diet fed to this dog. (12,15) In another study the resistance phenotypes of the *E. coli* isolates were reported to be generally similar to those of the *Salmonella* isolates and resistance to a number of different antibiotics was reported. (18) A recent Swedish study demonstrated 23% of the RMBD samples to be positive for ESC-producing *E. coli* (**Table 2**). (22) The results of this study also indicated the presence of antibiotic resistant *E. coli* in a large number (80%) of the tested RMBDs. In addition, ESBL-producing Enterobacteriaceae are reported to be isolated from companion animals more often in recent years and the isolated types seem to resemble those found in humans. Owning companion animals has even been proposed to be a risk factor for infection with ESBL-producing bacteria in humans. (21,23,45) A study into antibiotic resistant *E. coli* isolated from healthy dogs conducted in the UK, reported the consumption of raw meat to be a significant risk factor for such resistance. (46) On top of that, eating raw meat has been indicated as a risk factor for the shedding of ESBL-producing Enterobacteriaceae by dogs in a recent Dutch study. (45) The presence of antibiotic bacteria in RMBDs could thus be a serious risk to both animal health and public health. Not only because infections with these bacteria are hard to treat, but also because it could contribute to a more widespread occurrence of such bacteria.

Although the number of samples in this study is comparable to those in most other studies into the bacterial contamination of RMBDs, the low sample size (n=35) in this study may have influenced the reported isolation frequencies. Nevertheless, it can still be concluded that the commercial RMBDs could be contaminated with zoonotic bacterial pathogens and the computed 95% confidence intervals showed all isolation frequencies to be above 5%, which is still substantial. Another limitation of this study is the fact that only three suspicious colony forming units of each bacterial species per product were subcultured for further confirmation. This could have resulted in missing positive colonies and therefore false negative results. Furthermore, the presence of the pathogenic bacterial species was not quantified in this study and thus it could not be concluded if the products did contain a sufficient quantity of these bacteria to be able to result in illness directly. Besides, confirmation of the different bacterial isolates was carried out according to the aforementioned standardized protocols and did not include further serotyping and/or genetic confirmation. It could thus be possible that the positive isolates are from non- or less-pathogenic serotypes, or from other bacterial subspecies due to the sensitivity of the confirmation tests. Also antibiotic susceptibility testing was not performed in this study and thus the presence of actual ESBL-producing *E. coli* in the RMBDs could not be properly qualified, although the results indicated a substantial number of RMBDs to be possibly contaminated with such *E. coli*.

RMBDs are a possible source for a variety of zoonotic bacterial pathogens, as demonstrated in this study. Therefore, the feeding of such diets to companion animals could result in infections and in some cases it could even cause disease. Although these diets are not intended for human consumption, contaminated RMBDs are very likely to present a risk to public health as well. Not only the shedding of pathogenic bacteria by infected companion animals, but also the handling of contaminated RMBDs is a

possible route of transmission to humans. Furthermore, the household environment is likely to become contaminated by infected dogs and/or cats and the handling of the RMBDs in the kitchen could result in the cross-contamination of food intended for human consumption. The latter could constitute a direct source for human infection with bacteria originating from the RMBDs. Given the fact that RMBDs are likely to be fed in a large number of households and could possibly result in human diseases with serious health consequences, it is important to raise awareness of the possible risks associated with the feeding of these diets to companion animals. Undoubtedly, the best solution to prevent the transmission of zoonotic bacteria from RMBDs is to quit feeding these diets to companion animals or to heat the raw meat before consumption. However, once feeding RMBDs, pet owners are likely to be very committed to this practice and will not just quit doing so. (6) Moreover, the heating of RMBDs is unfavourable since the meat could not be considered as raw and/or natural after doing this. For this same reason, other processing techniques (e.g. high pressure pasteurization, dehydration, and radiation) are not commonly used and not likely to be deployed by the manufacturers of RMBDs. Therefore it would be unattainable to prevent bacterial contamination during the manufacturing process, since the most likely route of RMBD contamination is the supply and use of already contaminated raw meat and animal-by products from slaughter houses. It could be possible to screen and where necessary to reject already contaminated ingredients, but economically this would likely be unfavourable. It is therefore important to provide companion animal owners, who wish to feed RMBDs to their animals, with clear and sufficient information concerning the possible risks to both animal and public health. Veterinarians should not recommend the feeding of RMBDs, but if pet owners already do or are very eager to feed such diets a veterinarian should be the one to properly educate these people. They have to be made aware of the possible health risks related to the feeding of RMBDs and it should be emphasized that it does not only pose a risk to themselves and their animals, but also to other people and animals in their surroundings. Immunocompromised individuals, including young children, elderly, pregnant women, and those who are ill, should especially be brought into focus. The feeding of RMBDs in households with immunocompromised members and the contact between RMBD fed animals and such individuals should be strongly discouraged. Furthermore, pet owners should be educated about personal hygiene and proper handling of RMBDs. The RMBDs should be stored and prepared apart from food products intended for human consumption, the best option would be to handle and feed these diets in a separate room. Also, children should be supervised during the preparation and feeding of RMBDs, so they could not come into contact with these diets. Besides, the importance of proper handwashing after handling and feeding RMBDs, but also after each contact with a companion animals, should be emphasized. Not only veterinarians should have the responsibility to educate pet owners, the RMBD manufacturers should contribute to this as well. Warnings and handling instructions should be present on product labels and/or packages more often, since in this study the product packages of only one brand were provided with a warning about the potential bacterial contamination of the product.

Following a number of American and Canadian studies, this study is the first one into the contamination of commercial RMBDs in the Netherlands and the first European one to evaluate a broader range of bacterial species. The reported results demonstrate the need to pay attention to the potential of RMBDs to be a source for a variety of zoonotic bacterial pathogens in this country as well and reinforces the warning recently published in the Dutch veterinary journal. (47) However, the actual public health risk associated with the feeding of RMBDs to companion animals cannot be properly defined, because of inadequate available information at this moment. For this reason, further research is of interest. Building on this study, a bigger number of samples should be tested and besides qualitative isolations it should also be useful to perform quantitative isolations of the zoonotic bacterial pathogens. Moreover, further serotyping and/or genetic confirmation of the bacterial isolates could provide more information about their pathogenicity and actual risk to both animal and human health. It is also desirable to perform antibiotic susceptibility testing with the bacterial isolates which are presumed to be antibiotic resistant, in order to determine their resistance phenotype. Subsequently, research into the transfer of the bacterial species from the RMBDs to companion animals and the shedding of these bacteria following animal infection is necessary. It would also be desirable to evaluate the contamination of a household environment with a RMBD fed animal member. Finally, RMBDs should be kept in mind and investigated as a possible source in clinical cases of foodborne infections in both animals and humans. So further research is definitely necessary to define the actual health risk associated with the feeding of RMBDs. For now it can be stated that these diets cannot be ruled out as a possible source for bacterial infections in both humans and animals, and thus people should be made aware of the possible risks.

Supplementary files

Microbiological methodology Microbiological methods for bacterial enumeration, isolation and identification used in this study.

Table S1 Complete overview of raw-meat-based diets purchased to analyse for the presence of bacterial pathogens.

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Supplementary file: Microbiological methodology

Microbiological methods for bacterial enumeration, isolation, and identification used in "Examination of zoonotic bacterial pathogens in canine and feline raw meat-based diets"

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Contents

Introduction

This file gives an overview of the microbiological methods used in the study "Examination of zoonotic bacterial pathogens in canine and feline raw meat-based diets". The aim of this study was to evaluate the prevalence of a variety of zoonotic bacterial pathogens in raw meat-based diets for companion animals, in which the bacterial species of interest were: *Escherichia coli* O157:H7, extended spectrum beta-lactamase (ESBL)-producing *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* species. In addition, the diets were analysed for their quantity of aerobic bacteria and *Escherichia coli* in general.

All methods used in the study were based on previously used and/or standardized procedures, although a few modifications were made. The methods used for the different bacterial species will be described in the following sections. The descriptions include a complete overview of the materials and when necessary instructions how to prepare them, and a description and explanation of the procedure.

1 Enumeration of aerobic bacteria; i.e. total aerobic count (1,2)

The numbers of aerobic bacteria present in the samples were determined to get an indication of the overall bacteriological quality of the tested products.

1.1 Materials

Buffered peptone water

Buffered peptone water (BPW) was used to prepare a suspension of the solid sample. In this study Buffered Peptone Water - BK018HA (Biokar Diagnostics, France) was used. The dehydrated medium was suspended in distilled water (25.5 g/L) and after complete dissolution, the solution was dispensed into bottles (225 mL). The filled bottles were sterilized at 121°C for 15 minutes and after cooling down they were stored in the fridge (± 3°C) until use.

Peptone salt solution

A 0.1% peptone salt solution was used to prepare a decimal dilution series of the sample suspension. In this study prefilled 9 mL tubes of Maximum Recovery Diluent - CM0733 (Oxoid Ltd, UK) were used.

Aerobic count plates

Aerobic count plates were used to isolate aerobic bacteria from the sample suspension. In this study 3M™ Petrifilm™ Aerobic Count Plates (3M Microbiology, USA) were used.

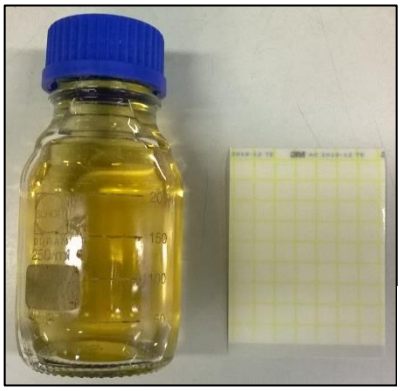

Figure 1 Materials used for the enumeration of aerobic bacteria. (From left to right: buffered peptone water (BPW) and an aerobic count plate.)

1.2 Procedure

In this study the samples were analysed for their quantity of aerobic bacteria using the 3M™ Petrifilm™ Aerobic Count Plates according to the product instructions and the AOAC Official Method 990.12 (AOAC, 2002).

- Obtain a 25 g sample from the product with a sterile spoon and transfer it into a sterile blender bag.
- Add 225 mL of BPW and homogenize the sample by hand for 90 seconds.
- Add 1 mL of the sample suspension to a test tube filled with 9 mL of peptone salt solution and mix it by vortexing, in order to prepare a second decimal dilution. In the same way, prepare a third decimal dilution from the second one and repeat these actions for the preparation of a fourth, a fifth and a sixth decimal dilution.

- Pipet 1 mL of the third decimal dilution, lift the top film and dispense the suspension onto the centre of the bottom film of the aerobic count plate.
- Distribute the sample over the surface of the count plate by placing a spreader (recessed side down) on the centre of this plate and by pressing gently on the centre of this spreader.
- Leave the count plate undisturbed for at least one minute after the removal of the spreader.
- Repeat the three previous actions for the fourth and the sixth decimal dilutions as well.
- Incubate the aerobic count plates for 48±3 hours at 35°C.
- Count the number of bacterial colonies (i.e. all red colonies) present inside the circle on the count plate. When more than 300 colonies are present, the number of bacterial colonies is estimated by counting the number of colonies in a number of squares, determining the average number per square and multiplying this number by twenty.

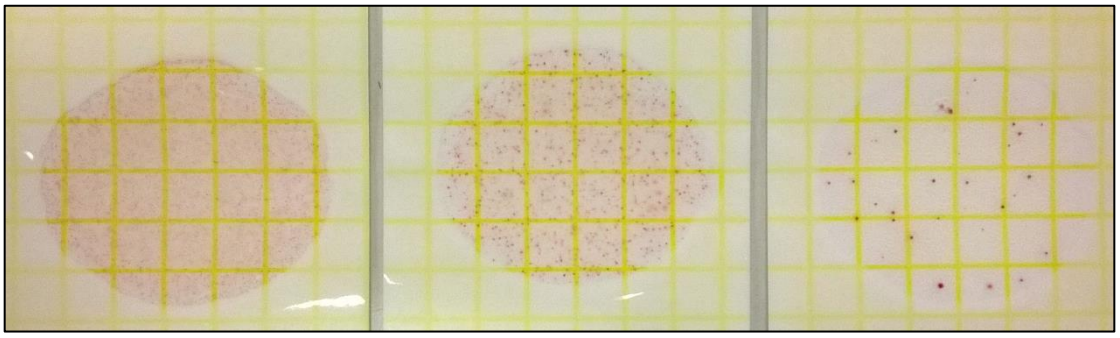

Figure 2 **Aerobic count plates inoculated with a third (left), a fourth (middle), and a sixth (right) decimal dilution of the sample suspension, after incubation for 48 hours at 35°C. Aerobic bacterial colonies are visible as red spots.**

2 Enumeration of *Escherichia coli* (1,3)

The numbers of *Escherichia coli* present in the samples were determined to get an indication of the hygiene status of the samples and their production processes. Since *E. coli* is normally found in the intestines, presence of this bacterial species in samples can be used as an indication for faecal contamination.

2.1 Materials

Buffered peptone water

Buffered peptone water (BPW) was used to prepare a suspension of the solid sample. In this study Buffered Peptone Water - BK018HA (Biokar Diagnostics, France) was used. The dehydrated medium was suspended in distilled water (25.5 g/L) and after complete dissolution, the solution was dispensed into bottles (225 mL). The filled bottles were sterilized at 121°C for 15 minutes and after cooling down they were stored in the fridge (± 3°C) until use.

Peptone salt solution

A 0.1% peptone salt solution was used to prepare a decimal dilution series of the sample suspension. In this study prefilled 9 mL tubes of Maximum Recovery Diluent - CM0733 (Oxoid Ltd, UK) were used.

E. coli count plates

E. coli count plates were used to isolate *E. coli* from the sample suspension. In this study 3M™ Petrifilm™ E.coli/Coliform Count Plates (3M Microbiology, USA) were used.

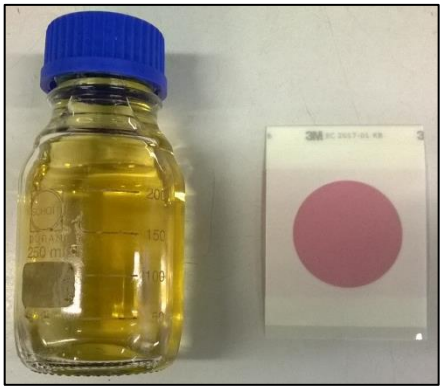

Figure 3 **Materials used for the enumeration of *Escherichia coli*. (From left to right: buffered peptone water (BPW) and an *E. coli* count plate.)**

2.2 Procedure

In this study the samples were analysed for their quantity of *E. coli* using the 3M™ Petrifilm™ E.coli/Coliform Count Plates according to the product instructions and the AOAC Official Method 991.14 (AOAC, 2002).

- Obtain a 25 g sample from the product with a sterile spoon and transfer it into a sterile blender bag.
- Add 225 mL of BPW and homogenize the sample by hand for 90 seconds.
- Add 1 mL of the sample suspension to a test tube filled with 9 mL of peptone salt solution and mix it by vortexing, in order to prepare a second decimal dilution. In the same way, prepare a third decimal dilution from the second one.
- Pipet 1 mL of the sample suspension (i.e. the first decimal dilution), lift the top film and dispense the suspension onto the centre of the bottom film of the *E. coli* count plate.
- Distribute the sample over the surface of the count plate by placing a spreader (flat side down) on the centre of this plate and by pressing gently on the centre of this spreader.
- Leave the count plate undisturbed for at least one minute after the removal of the spreader.
- Repeat the three previous actions for the other decimal dilutions as well.
- Incubate the *E. coli* count plates for 48±3 hours at 35°C.
- Count the number of *E. coli* colonies (i.e. (red-)blue colonies associated with entrapped gas) present inside the circle on the count plate. When more than 150 colonies are present, the number of *E. coli* colonies is estimated by counting the number of colonies in a number of squares, determining the average number per square and multiplying this number by twenty.

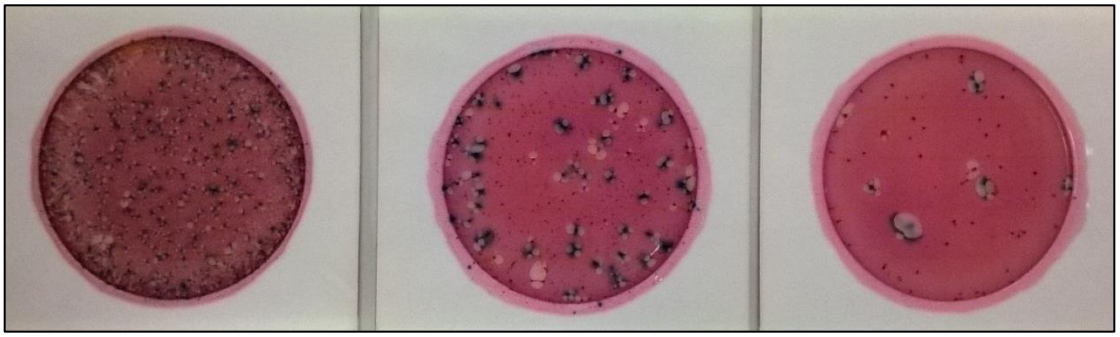

Figure 4 ***E. coli*** **count plates inoculated with a first (left), a second (middle), and a third (right) decimal dilution of the sample, after incubation for 48 hours at 35°C.** ***E. coli*** **colonies are visible as (red-)blue spots associated with entrapped gas.**

3 Detection of *Escherichia coli* O157:H7 (4-12)

In this study the product samples were analysed for the presence of *Escherichia coli* O157:H7, a Gram-negative, rod-shaped bacteria belonging to the family Enterobacteriaceae. This bacterial species produces verocytotoxins which are released after bacteriolysis. *E. coli* O157:H7 infections in humans have been associated with serious diseases like hemorrhagic colitis and haemolytic uremic syndrome (HUS). Moreover, the infective dose of this pathogen in humans is very low. On the contrary, illness as a result of a *E. coli* O157:H7 in companion animals rarely occurs. Most cases are associated with the consumption of contaminated food products, however companion animals are also suggested to be possible reservoirs for human infection.

3.1 Materials

Modified tryptic soy broth with novobiocin

Modified tryptic soy broth with novobiocin (TSBm+n) was used for both preparing a suspension of the solid sample, and the subsequent selective enrichment. The selectivity of this broth is a result of the addition of novobiocin, an antibiotic which inhibits the growth of gram positive bacteria. In this study prefilled 225 mL bottles of TSB modified + Novobiocine - T431.78.0225 (Tritium Microbiologie, The Netherlands) were used.

Immunomagnetic beads

Immunomagnetic beads are magnetic, polystyrene beads, which are coated with antibodies against *E. coli* O157:H7. These beads were used to remove a large proportion of the bacterial species which were not of interest, and thus to concentrate the enriched sample suspension. In this study Dynabeads® anti-*E.coli* O157 (Life Technologies, Norway) was used.

Peptone salt solution with Tween® 20

The washing buffer used in this study to purify the remaining bacteria bound to the immunomagnetic beads, was a peptone salt solution with Tween® 20. To prepare this solution, Tween® 20 (Sigma-Aldrich, USA) was added to a peptone salt solution (1 ml/L), after which it was sterilized at 121°C for 15 minutes.

Sorbitol MacConkey agar with cefixime and tellurite

Sorbitol MacConkey agar with cefixime and tellurite (CT-SMAC) was used for the isolation of *E. coli* O157:H7 from the enriched and purified suspension. Sorbitol can be fermented by most *E. coli* serotypes, which results in the formation of pink colonies on SMAC plates. However, *E. coli* O157:H7 is not able to ferment this sugar alcohol and therefore its colonies will be colourless on SMAC plates. The addition of both cefixime, and potassium tellurite contribute to the selectivity of CT-SMAC by inhibiting the growth of other non-sorbitol fermenting bacteria. Cefixime, an antibiotic, inhibits the growth of *Proteus* spp., whereas potassium tellurite inhibits the growth of non-O157 *E. coli* serotypes, *Aeromonas* spp., and *Providencia* spp.. In this study Sorbitol MacConkey Agar - CM0813 (Oxoid Ltd, UK) and Cefixime-Tellurite Supplement - SR0172 (Oxoid Ltd, UK) were used. 25.75 g of the dehydrated SMAC-medium was suspended in 500 mL of distilled water, which was brought to the boil for complete dissolution. Subsequently, it was sterilized at 121°C for 15 minutes. After cooling down to 50°C, the solution was supplemented with one vial of CT (the dehydrated content of the vial had to be suspended in 2 mL of sterile distilled water) and after mixing it was poured into sterile Petri dishes. The plates were left to solidify, afterwards they were turned upside-down and left overnight at room temperature to check for contamination (i.e. bacterial growth). Subsequently, they were stored in the fridge (± 3°C) until use.

Sorbitol MacConkey agar with cefixime and rhamnose

Sorbitol MacConkey agar with cefixime and rhamnose (CR-SMAC) was used for the isolation of *E. coli* O157:H7 from the enriched and purified suspension. Sorbitol can be fermented by most *E. coli* serotypes, which results in the formation of pink colonies on SMAC plates. In addition, rhamnose can be fermented by around 60% of the non-sorbitol fermenting *E. coli* serotypes, which also results in the formation of pink colonies on SMAC plates. However, *E. coli* O157:H7 is not able to ferment both these substances and therefore its colonies will be straw-coloured on CR-SMAC plates. The addition of cefixime also contributes to the selectivity of CR-SMAC. This antibiotic inhibits the growth of non-sorbitol fermenting *Proteus* spp.. In this study Sorbitol MacConkey Agar - CM0813 (Oxoid Ltd, UK), L-Rhamnose monohydrate - R3875 (Sigma-Aldrich, USA), and Cefixime Supplement - SR0191 (Oxoid Ltd, UK) were used. 25.75 g of the dehydrated SMAC-medium, together with 2.5 g of rhamnose was suspended in 500 mL of distilled water, which was brought to the boil for complete dissolution. Subsequently, it was sterilized at 121°C for 15 minutes. After cooling down to 50°C, the solution was supplemented with one vial of cefixime (the dehydrated content of the vial had to be suspended in 2 mL of sterile distilled water) and after mixing it was poured into sterile Petri dishes. The plates were left to solidify, afterwards they were turned upside-down and left overnight at room temperature to check for contamination (i.e. bacterial growth). Subsequently, they were stored in the fridge (± 3°C) until use.

Nutrient agar

Nutrient agar (NA) was used to subculture and store bacterial colonies which were suspected to be *E. coli* O157:H7. In this study Nutrient Agar - CM0003 (Oxoid Ltd, UK) was used. The dehydrated medium was suspended in distilled water (28 g/L), which was brought to the boil for complete dissolution. Next, it was sterilized at 121°C for 15 minutes and after cooling down to 50°C, the solution was poured into sterile Petri dishes. The plates were left to solidify, afterwards they were turned upside-down and left overnight at room temperature to check for contamination (i.e. bacterial growth). Subsequently, they were stored in the fridge (± 3°C) until use.

Tryptone water

Tryptone water (TW) was used for the indole test, one of the tests used for the confirmation of bacterial isolates which were suspected to be *E. coli* O157:H7. The tryptophan present in this medium can be converted into indole by certain bacterial species. In this study Tryptone Water - CM0087 (Oxoid Ltd, UK) was used. The dehydrated medium was suspended in distilled water (15 g/L) and after complete dissolution, the solution was dispensed into test tubes (5 mL). The filled test tubes were sterilized at 121°C for 15 minutes and after cooling down they were stored in the fridge (± 3°C) until use.

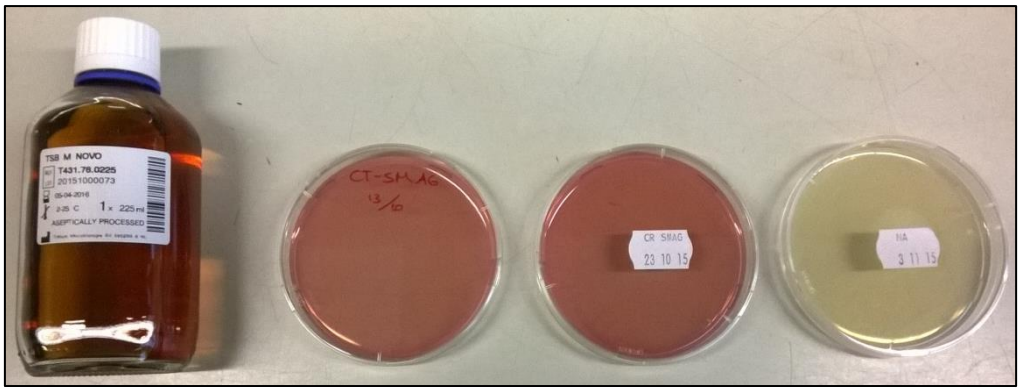

Figure 5 Materials used for the detection of *Escherichia coli* O157:H7. (From left to right: modified tryptic soy broth with novobiocin (TSBm+n), MacConkey sorbitol agar with cefixime and tellurite (CT-SMAC), MacConkey sorbitol agar with cefixime and rhamnose (CR-SMAC), and nutrient agar (NA).)

Kovac's reagent

Kovac's reagent was used for the detection of indole during the indole test, one of the tests used for the confirmation of bacterial isolates which were suspected to be *E. coli* O157:H7. This reagent contains p-aminobenzaldehyde, which can react with tryptophan resulting in the formation of a pink-red complex. In this study Kovac's Reagent for indoles - 60983 (Sigma-Aldrich, USA) was used.

E. coli O157-antiserum

E. coli O157-antiserum was used for the serological confirmation of bacterial isolates which were suspected to be *E. coli* O157:H7. In this study a Prolex™ *E. coli* O157 Latex Test Reagent Kit - PL.071B (Pro-Lab Diagnostics, Canada) was used. This test kit includes a dropper bottle containing latex particles which are coated with purified rabbit IgG with affinity for *E. coli* O157 serotypes, a dropper bottle containing latex particles which are coated with purified rabbit IgG without affinity for *E. coli* O157 serotypes (i.e. negative control), and a dropper bottle with a suspension of *E. coli* O157:H7 antigens (i.e. positive control).

Peptone salt solution

A 0.1% peptone salt solution was used to make a suspension of a bacterial isolate which was suspected to be *E. coli* O157:H7, in order to perform the confirmatory serologic test. In this study Maximum Recovery Diluent - CM0733 (Oxoid Ltd, UK) was used.

3.2 Procedure

In this study the samples were analysed for the presence of *E. coli* O157:H7 according to ISO 16654:2001 Microbiology of food and animal - Horizontal method for the detection of *Escherichia coli* O157 (ISO, 2001). The procedure can roughly be divided into two phases, starting with sample enrichment and selective isolation of the bacteria from these samples. Subsequently, presumptive *E. coli* O157:H7 colonies were confirmed using two confirmatory tests, which were the indole test and serological confirmation. Since *E. coli* O157:H7 is able to cause severe human disease, it is classified into risk group 3 in the directive on the protection of workers from risks related to exposure to biological agents at work of the European Union. Therefore, the procedure described here should be performed in a ML-II laboratory.

3.2.1 Enrichment and isolation

Sample enrichment

- Obtain a 25 g sample from the product with a sterile spoon and transfer it into a sterile blender bag.
- Add 225 mL of TSBm+n and homogenize the sample by hand for 90 seconds.
- Incubate the sample suspension for 18-24 hours at 41.5°C, during which the suspension stays in motion by the use of a shaker (100 rpm).

Immunomagnetic separation

- Pipet 1 mL of the enriched sample suspension and dispense into an Eppendorf tube.
- Pipet 20 µL of the immunomagnetic bead suspension (after vortexing it) and dispense into the Eppendorf tube as well.
- Mix the sample suspension with the immunomagnetic beads for 30 minutes by using a rotating device, in order to achieve good adhesion of the bacteria to the immunomagnetic beads.
- Concentrate the immunomagnetic beads on the side of the Eppendorf tube by placing the tube into a magnetic particle concentrator and inverting it several times.
- Aspirate and discard the supernatant of the sample suspension with a pipet (also check the tube cap for remaining liquid).
- Pipet 1 mL of the washing buffer and dispense into the Eppendorf tube.
- Repeat the concentration of the immunomagnetic beads, the aspiration of the supernatant, and the addition of washing buffer three more times. However, add only 0.1 mL of the washing buffer at the end.
- Suspend the immunomagnetic beads in the added washing buffer by briefly vortexing it.

Selective isolation

- Dip a sterile cotton swab into the purified sample suspension and strike it off onto one third of both a CT-SMAC, and a CR-SMAC plate.
- Streak with a sterile inoculation loop in order to dilute the applied sample, resulting in the obtaining of isolated bacterial colonies.
- Incubate the CT-SMAC and CR-SMAC plates for 18-24 hours at 37°C.
- Read the agar plates for the presence of morphological characteristic bacterial colonies.

  Since both rhamnose, and sorbitol cannot be fermented by *E. coli* O157:H7, the colonies of this bacterial species will appear colourless on the CT-SMAC plates and straw-coloured on the CR-SMAC plates. Otherwise, the colonies of bacterial species which are able to ferment these two substances will appear pink.

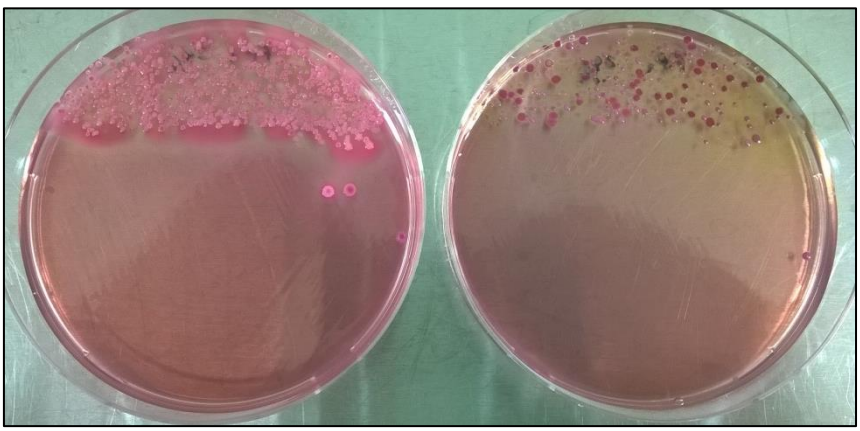

Figure 6 A CT-SMAC (left) and a CR-SMAC (right) plate inoculated with a purified enriched sample suspension, after incubation for 18-24 hours at 37°C. On both plates *E. coli* O157:H7 colonies are not clearly visible.

- Transfer a maximum of six presumptive *E. coli* O157:H7 colonies (i.e. max. three presumptive colonies from CT-SMAC and max. three from CR-SMAC) with a sterile inoculation loop onto a NA plate and strike them on one quarter of this plate (i.e. max. four presumptive colonies per NA plate).
- Incubate the NA plate for 18-24 hours at 37°C.
- Store the NA plate in the fridge (± 3°C) until the confirmation of the bacterial isolates.

3.2.2 Confirmation

Indole test

- Transfer some material of the presumptive *E. coli* O157:H7 isolate into a test tube filled with 5 mL of tryptone water and emulsify it by vortexing.
- Incubate the test tube for 24-48 hours at 35°C.
- Pipet 0.2-0.5 mL of Kovac's reagent and dispense into the incubated test tube.

The development of a pink-red coloration at the top of the tryptone water within one minute is regarded as a positive reaction. In case of a negative reaction no colour change is visible and the Kovac's reagent at the top of the tryptone water remains yellow. Since *E. coli* possesses tryptophanase activity, this bacterial species is able to split indole from the tryptophane present in the tryptone water. Therefore, the presence of *E. coli* O157:H7 in the culture results in a positive reaction.

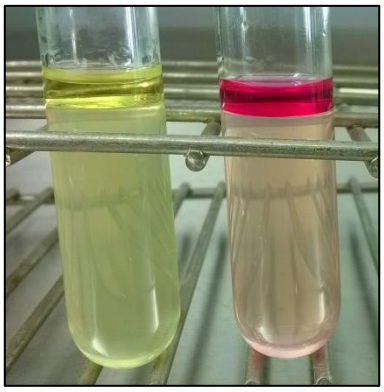

Figure 7 **A negative (left) and a positive (as for *E. coli* O157:H7; right) indole test. The formation of a pink-red coloration at the top of the tryptone water within one minute is regarded as a positive reaction.**

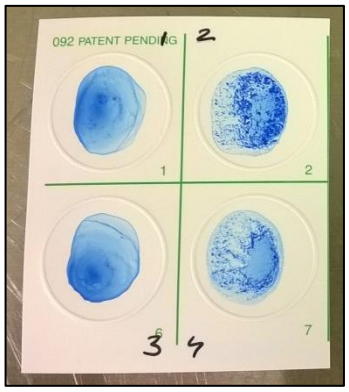

Figure 8 **Negative (left) and positive (right) serological confirmatory tests. In the two circles at the right agglutination is visible as the agglomeration of blue particles.**

Serological confirmation

- Put a drop of peptone salt solution and one drop of *E. coli* O157-antiserum in the test circle on a provided test card.
- Touch the presumptive *E. coli* O157:H7 isolate on the NA plate with a sterile inoculation loop and subsequently, emulsify the sample in the drop of peptone salt solution.
- Mix the two drops with the inoculation loop.
- Rock the test card for up to two minutes and examine the drop mixture.
- When a positive result is visible, the procedure is repeated with a drop of negative control-serum instead of a drop of *E. coli* O157:H7-antiserum.

The antiserum reacts with the antigens of *E. coli* O157:H7 and therefore, the presence of these bacterial species in the culture results in agglutination. In this case the agglutination is visible as the agglomeration of tiny blue particles. The negative control-serum will not react with *E. coli* O157:H7 and so no agglutination is visible.

4 Detection and enumeration of ESBL-producing *Escherichia coli* (1,5,9,10,12-17)

The emergence and increase in antibiotic resistance is a major problem related to the occurrence of bacteria. Extended spectrum beta-lactamase (ESBL-)producing *E. coli* strains are an example of bacteria which acquired multidrug resistance. In recent years, ESBL-producing Enterobacteriaceae are reported to be isolated from companion animals more often and the consumption of raw food by these animals has been reported as a risk factor for the carrying of such antibiotic resistant bacteria. In addition, the isolated types seem to resemble those found in humans. In this study the products were analysed for the presence of ESBL-producing *E. coli*.

4.1 Materials

Cefotaxime solution

Cefotaxime solution was used as a supplement to both buffered peptone water, and MacConkey agar which were used for the examination for ESBL-producing *E. coli*. Cefotaxime is a broad spectrum third generation cephalosporin antibiotic. In this study Cefotaxime sodium salt - C7912 (Sigma-Aldrich, USA) was used to prepare a stock solution. The medium was suspended in distilled water at a ratio of one to one (i.e. 1 mg/1 mL). The solution was filter sterilized and dispensed into Eppendorf tubes (1 mL). The filled tubes were frozen at -20°C and stored until use.

Buffered peptone water with cefotaxime

Buffered peptone water (BPW) with cefotaxime was used for both preparing a suspension of the solid sample, and the subsequent pre-enrichment. Cefotaxime is added to the medium in order to inhibit the growth of both gram-positive, and gram-negative bacterial strains which are not resistant to this antibiotic. In this study Buffered Peptone Water - BK018HA (Biokar Diagnostics, France) was used. The dehydrated medium was suspended in distilled water (25.5 g/L) and after complete dissolution, the solution was dispensed into bottles (225 mL). The filled bottles were sterilized at 121°C for 15 minutes and after cooling down they were stored in the fridge (± 3°C) until use. Just before use, 250 µL of the self-prepared cefotaxime stock solution was added to 225 mL of BPW.

Peptone salt solution

A 0.1% peptone salt solution was used to prepare a decimal dilution series of the sample suspension. In this study prefilled 9 mL tubes of Maximum Recovery Diluent - CM0733 (Oxoid Ltd, UK) were used.

MacConkey agar with cefotaxime

MacConkey agar (MCA) with cefotaxime was used for the isolation of ESBL-producing *E. coli* from the (enriched) sample suspension. The lactose present in this medium can be fermented by some bacterial species, resulting in an acidic alteration of the pH. To make this acidification visible, neutral red is incorporated in the medium, which serves as a pH indicator with a colour range from red to yellow (acid-alkaline). Both bile salts, and crystal violet are added in order to inhibit the growth of gram-positive cocci. Cefotaxime is added to the medium in order to inhibit the growth of both gram-positive, and gram-negative bacterial strains which are not resistant to this antibiotic. In this study MacConkey Agar No.3 - CM0115 (Oxoid Ltd, UK) was used. The dehydrated medium was suspended in distilled water (51.5 g/L), which was brought to the boil in order to achieve complete dissolution. The solution was sterilized at 121°C for 15 minutes and after cooling down to 50°C, the cefotaxime stock solution was added (1 mL/L). After mixing, the solution was poured into sterile Petri dishes and the plates were left to solidify, after which they were turned upside down and left overnight at room temperature to check for contamination (i.e. bacterial growth). Subsequently, they were stored upside-down in the fridge (± 3°C) until use.

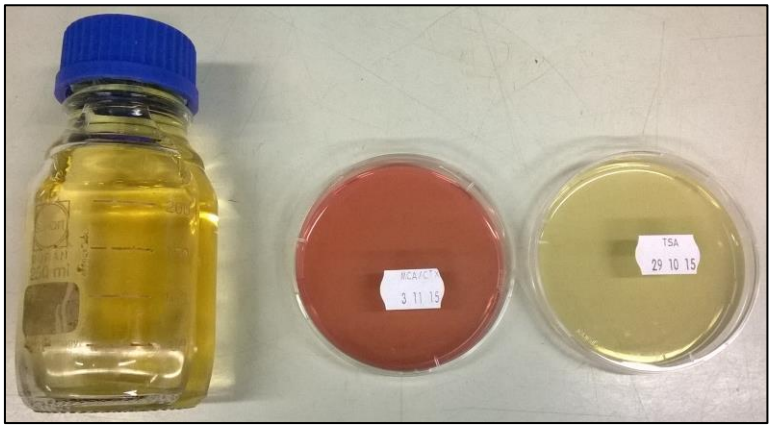

Figure 9 **Materials used for the detection and enumeration of extended spectrum beta-lactamase (ESBL)-producing *Escherichia coli*. (From left to right: buffered peptone water (BPW), MacConkey agar with cefotaxime (MCA+CTX), and tryptone soya agar (TSA).)**

Tryptone soya agar

Tryptone soya agar (TSA) was used to subculture and store bacterial colonies which were suspected to be ESBL-producing *E. coli*. In this study Tryptone Soya Agar - CM0131 (Oxoid Ltd, UK) was used. The dehydrated medium was suspended in distilled water (40 g/L), which was brought to the boil in order to achieve complete dissolution. The solution was sterilized at 121°C for 15 minutes and after cooling down to 50°C, it was poured into sterile Petri dishes. The plates were left to solidify, afterwards they were turned upside-down and left overnight at room temperature to check for contamination (i.e. bacterial growth). Subsequently, they were stored in the fridge (± 3°C) until use.

Tryptone water

Tryptone water (TW) was used for the indole test, one of the tests used for the confirmation of bacterial isolates which were suspected to be ESBL-producing *E. coli*. The tryptophan present in this medium can be converted into indole by certain bacterial species. In this study Tryptone Water - CM0087 (Oxoid Ltd, UK) was used. The dehydrated medium was suspended in distilled water (15 g/L) and after complete dissolution, the solution was dispensed into test tubes (5 mL). The filled test tubes were sterilized at 121°C for 15 minutes and after cooling down they were stored in the fridge (± 3°C) until use.

Kovac's reagent

Kovac's reagent was used for the detection of indole during the indole test, one of the tests used for the confirmation of bacterial isolates which were suspected to be ESBL-producing *E. coli*. This reagent contains p-aminobenzaldehyde, which can react with tryptophan resulting in the formation of a pink-red complex. In this study Kovac's Reagent for indoles - 60983 (Sigma-Aldrich, USA) was used.

Oxidase reagent

Oxidase reagent was used for the oxidase test, one of the tests used for the confirmation of bacterial isolates which were suspected to be ESBL-producing *E. coli*. The N,N,N',N'-tetramethyl-1,4-phenylenediammonium dichloride incorporated in this reagent can be oxidized by the enzyme oxidase, which results in a purple-blueish discoloration. In this study N,N,N',N'-Tetramethyl-1,4-phenylenediammonium dichloride - 8.21102 (Merck, Germany) was used. The dehydrated medium was suspended in distilled water (0.1 g/L). After mixing, the solution had to be used directly.

4.2 Procedure

Since no international standardized method is available for the analysis of food samples for the presence of ESBL-producing *E. coli*, the method commonly used at the microbiological laboratory of the Institute for Risk Assessment Sciences at Utrecht University was used in this study as well. This method is somewhat similar to the one described by Dierikx et al. (2013). In this study the samples were both quantitatively, and qualitatively analysed for the presence of ESBL-producing *E. coli* and therefore the procedure can roughly be divided into three phases. First, the quantitative bacteriology was performed, followed by the sample enrichment and the selective isolation of the bacteria. Subsequently, presumptive ESBL-producing *E. coli* colonies were confirmed using two confirmatory tests, which were the indole test and the oxidase test.

4.2.1 Enumeration

- Obtain a 25 g sample from the product with a sterile spoon and transfer it into a sterile blender bag.
- Add 225 mL of BPW with cefotaxime and homogenize the sample by hand for 90 seconds.
- Add 1 mL of the sample suspension to a test tube filled with 9 mL of peptone salt solution and mix it by vortexing, in order to prepare a second decimal dilution. In the same way, prepare a third decimal dilution from the second one.
- Pipet 0.1 mL of the sample suspension (i.e. the first decimal dilution), dispense onto a dried MCA with cefotaxime plate and distribute the sample over the entire surface of the agar plate with a sterile spreader. Repeat these actions for the other decimal dilutions as well.
- Incubate the MCA with cefotaxime plates overnight at 37°C.
- Read the agar plates for the presence of morphological characteristic bacterial colonies and count the number of these CFU's.

  (See selective isolation.)

4.2.2 Enrichment and isolation

Enrichment

- Incubate the sample suspension overnight at 37°C.

Selective isolation

- Inoculate the enriched sample suspension onto a MCA with cefotaxime plate. Do this by streaking with a sterile inoculation loop in order to dilute the applied sample, resulting in the obtaining of isolated bacterial colonies.
- Incubate the agar plate overnight at 37°C.
- Read the agar plate for the presence of morphological characteristic bacterial colonies.

  Due to the fermentation of the lactose present in the medium, ESBL-producing *E. coli* colonies will appear pink on the MCA with cefotaxime plates. In addition, these colonies will appear well circumscribed and will have a whitish gloss.

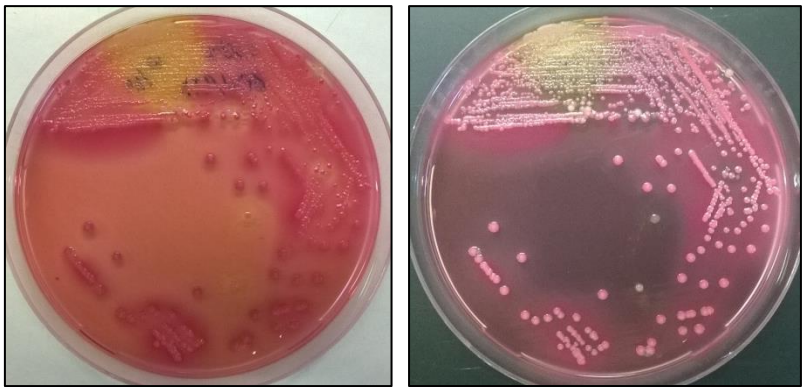

Figure 10 A MCA with cefotaxime plate inoculated with an enriched sample suspension, after incubation overnight at 37°C. Presumptive ESBL-producing *E. coli* colonies are visible as well circumscribed, pink colonies. The whitish gloss of these colonies is clearly visible against a dark background (right).

- Transfer a maximum of three presumptive ESBL-producing *E. coli* colonies with a sterile inoculation loop from the MCA with cefotaxime plate onto a TSA plate and strike them on one quarter of this plate (i.e. max. four presumptive colonies per TSA plate).
- Incubate the TSA plate for 18-24 hours at 37°C.
- Store the TSA plate in the fridge (± 3°C) until the confirmation of the bacterial isolates.

4.2.3 Confirmation

Indole test

- Transfer some material of the presumptive ESBL-producing *E. coli* isolate into a test tube filled with 5 mL of tryptone water and emulsify it by vortexing.
- Incubate the test tube for 24-48 hours at 35°C.
- Pipet 0.2-0.5 mL of Kovac's reagent and dispense into the incubated test tube.

The development of a pink-red coloration at the top of the tryptone water within one minute is regarded as a positive reaction. In case of a negative reaction no colour change is visible and the Kovac's reagent at the top of the tryptone water remains yellow. Since *E. coli* possesses tryptophanase activity, this bacterial species is able to split indole from the tryptophane present in the tryptone water. Therefore, the presence of ESBL-producing *E. coli* in the culture results in a positive reaction.

Oxidase test

- Put one drop of oxidase reagent on a sterile microscopic slide.
- Touch the presumptive ESBL-producing *E. coli* isolate on the TSA plate with a sterile cotton swab and subsequently, dip this swab into the drop of oxidase reagent.

The development of a deep blue-purple coloration at the inoculated area of the swab within ten seconds is regarded as a positive reaction. In case of a negative reaction the swab is purple as well, however a darkening of this colour does not occur. Since *E. coli* does not possess oxidase activity, this bacterial species is not able to oxidize the N,N,N',N'-tetramethyl-1,4-phenylenediammonium dichloride incorporated in the reagent. Therefore, the presence of ESBL-producing *E. coli* in the culture results in a negative reaction.

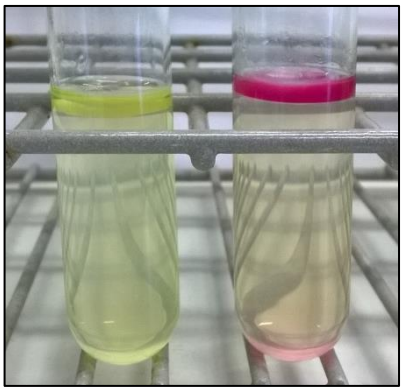

Figure 11 A negative (left) and a positive (as for ESBL-producing *E. coli*; right) indole test. The formation of a pink-red coloration at the top of the tryptone water within one minute is regarded as a positive reaction.

Figure 12 A negative (as for ESBL-producing *E. coli*; left) and a positive (right) oxidase test. When a darkening of the purple colour does not occur within ten seconds, the reaction is regarded as negative.

5 Detection of *Listeria monocytogenes* and other *Listeria* species (5,12,18-25)

The product samples were analysed for the presence of *Listeria* spp., in particular *Listeria monocytogenes*, in this study. *Listeria* spp. are Gram-positive, short rod-shaped, motile bacteria, which are able to grow over a wide range of temperatures including those in a refrigerator. *L. monocytogenes* is a pathogenic species and can cause serious illness in humans, especially neonates and pregnant women. In contrast to the mild influenza-like symptoms occurring in healthy adults, an infection with *L. monocytogenes* could result to an abortion in a pregnant woman and could be life-threatening to a neonatal individual. Clinical listeriosis in companion animals is rare. Contaminated food products have been identified as common sources for an infection with this bacterial species in both humans, and animals.

5.1 Materials

UVM Listeria enrichment broth

UVM *Listeria* enrichment broth was used for both preparing a suspension of the solid sample, and the subsequent primary enrichment. The selectivity of this medium is caused by the incorporation of both acriflavine hydrochloride, which inhibits the growth of a large number of gram-positive bacterial species, and nalidixic acid, which inhibits the growth of gram-negative species. In this study UVM Modified Listeria Enrichment Broth - 222330 (BD Difco™, USA) was used. The dehydrated medium was suspended in distilled water (52 g/L), which was heated up to boiling temperature in order to achieve complete dissolution. Subsequently, the solution was dispensed into bottles (225 mL), which were sterilized at 121°C for 15 minutes. After cooling down, the filled bottles were stored in the fridge (± 3°C) until use.

Fraser broth

Fraser broth was used for the secondary enrichment of the enriched sample suspension. Like UVM *Listeria* enrichment broth, this medium contains acriflavine hydrochloride and nalidixic acid, however in higher concentrations. In addition, this medium contains lithium chloride, which inhibits the growth of *Enterococcus* spp.. The aesculin incorporated in this medium can be hydrolysed by *Listeria* spp., resulting in the formation of aesculetin. The aesculetin reacts with the ferric ammonium citrate present in the medium, which results in a black discoloration of this medium. In this study prefilled 10 mL tubes of Fraser Broth - K644B010AA (bioTRADING, The Netherlands) were used.

COMPASS® Listeria agar

COMPASS® *Listeria* agar was used for the isolation of *Listeria* spp. and *Listeria monocytogenes* in particular, from the enriched sample suspensions. The growth of a large number of other microorganisms in this medium is inhibited by the incorporation of lithium chloride, the antibiotics ceftazidime, nalidixic acid and polymyxin B, and the fungicide cycloheximide. The 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside present in the medium can be hydrolysed by *Listeria* spp. to a substance which reacts with oxygen, resulting in the formation of a blue coloured precipitate. In addition, *L. monocytogenes* is able to degrade the phosphatidyl-inositol incorporated in this medium, which results in the formation of an untransparent precipitate. Therefore, colonies of *L. monocytogenes* appear blue-greenish and are surrounded with an untransparent zone on these plates. In this study pre-poured Petri dishes with COMPASS® *Listeria* Agar - BM12308 (Biokar Diagnostics, France) were used.

PALCAM agar

PALCAM agar was used for the isolation of *Listeria* spp. and *Listeria monocytogenes* in particular, from the enriched sample suspensions. The growth of a large number of other bacterial species in this medium is inhibited by the incorporation of acriflavine hydrochloride, lithium chloride, and the antibiotics ceftazidime and polymyxin B. The aesculin incorporated in this medium can be hydrolysed by *Listeria* spp., resulting in the formation of aesculetin. The aesculetin reacts with the ferric ammonium citrate present in the medium, which results in a black discoloration. In addition, the incorporated mannitol can be fermented by some bacterial species other than *Listeria* spp., resulting in an acidic alteration of the pH. In order to make this acidification visible, phenol red is added to the medium, which serves as a pH indicator with a colour range from yellow to red (acid-alkaline). Colonies of *L. monocytogenes* appear grey-greenish, are surrounded with a black zone, and could have a black centre on PALCAM agar plates. In this study pre-poured Petri dishes with PALCAM Agar - K031P090KP (bioTRADING, The Netherlands) were used.

Tryptone soya yeast extract agar

Tryptone soya yeast extract agar (TSYEA) was used to subculture and store bacterial colonies which were suspected to be either *L. monocytogenes*, or other *Listeria* spp.. In this study Agar Bacteriological (Agar No.1) - LP0011 (Oxoid Ltd, UK), Tryptone Soya Broth - CM0129 (Oxoid Ltd, UK), and Yeast Extract - 212750 (BD Bacto™, USA) were used to prepare TSYEA. The dehydrated media were suspended in distilled water (Agar No.1: 15 g/L; Tryptone Soya Broth: 30 g/L; Yeast Extract: 6 g/L), which was brought to the boil in order to achieve complete dissolution. The solution was sterilized at 121°C for 15 minutes and after cooling down to 50°C, it was poured into sterile Petri dishes. The plates were left to solidify and afterwards they were stored upside-down in the fridge (± 3°C) until use.

Figure 13 Materials used for the detection of *Listeria monocytogenes* and other *Listeria* species. (From left to right: UVM-broth, Fraser broth, COMPASS© Listeria agar, PALCAM agar, and tryptone soya yeast extract agar (TSYEA).)

Hydrogen peroxide solution

Hydrogen peroxide solution was used for the catalase test, one of the tests used for the confirmation of bacterial isolates which were suspected to be either *L. monocytogenes*, or other *Listeria* spp.. In this study Hydrogen peroxide 30% - 1.07209 (Merck, Germany) was used.

Gram staining reagents

A number of reagents was used to perform a Gram staining, which was used for the confirmation of bacterial isolates which were suspected to be either *L. monocytogenes*, or other *Listeria* spp.. The reagents used in this study were: a crystal violet solution, a Lugol's solution, an iodine-ethanol solution, and a fuchsine solution.

Peptone salt solution

A 0.1% peptone salt solution was used to make a suspension of a bacterial isolate which was suspected to be *Listeria* spp., in order to perform a Gram staining. In this study Maximum Recovery Diluent - CM0733 (Oxoid Ltd, UK) was used.

Tryptone soya yeast extract broth

Tryptone soya yeast extract broth (TSYEB) was used to subculture bacterial isolates which were suspected to be either *Listeria monocytogenes*, or other *Listeria* spp., in order to perform the confirmatory motility test by hanging drop preparation. In this study Tryptone Soya Broth - CM0129 (Oxoid Ltd, UK) and Yeast Extract - 212750 (BD Bacto™, USA) were used to prepare TSYEB. The dehydrated media were suspended in distilled water (Tryptone Soya Broth: 30 g/L; Yeast Extract: 6 g/L), which was brought to the boil in order to achieve complete dissolution. The solution was dispensed into test tubes (10 mL), which subsequently were sterilized at 121°C for 15 minutes. After cooling down, the filled test tubes were stored in the fridge (± 3°C) until use.

Sheep blood agar

Agar supplemented with sheep blood was used for both testing for β-haemolysis, and the CAMP test, two of the tests used for the confirmation of bacterial isolates which were suspected to be *L. monocytogenes*. In this study pre-poured Petri dishes with Blood Agar +Sheep Blood - K004P090KP (bioTRADING, The Netherlands) were used.

Figure 14 Some of the materials used for the confirmation of presumptive *Listeria monocytogenes* colonies. (From left to right: tryptone soya yeast extract broth (TSYEB), phenol red broth, and a sheep blood agar plate.)

Phenol red broth

Phenol red broth was used for the carbohydrate fermentation test, one of the tests used for the confirmation of bacterial isolates which were suspected to be *L. monocytogenes*. This medium contains phenol red, which serves as a pH indicator with a colour range from yellow to red (acid-alkaline). In this study Phenol-red broth - 1.10987 (Merck, Germany) was used. The dehydrated medium was suspended in distilled water (15 g/L) and after complete dissolution, the solution was dispensed into test tubes (5 mL). Subsequently, Durham tubes were inserted upside down in the filled test tubes in order to be able to detect possible gas production after bacterial culturing. The filled test tubes were sterilized at 121°C for 15 minutes and after cooling down, they were stored in the fridge (± 3°C) until use.

Carbohydrate disks

Rhamnose and xylose disks were used for the carbohydrate fermentation test, one of the tests used for the confirmation of bacterial isolates which were suspected to be *L. monocytogenes*. Before bacterial culturing, either a rhamnose, or a xylose disk was added to a test tube filled with phenol red broth. In this way, the supplemented broth could be used to detect an acidification as a result of the fermentation of one of these two sugars. In this study both Rhamnose Disks - 93999 (Sigma-Aldrich, USA), and Xylose Disks - 07411 (Sigma-Aldrich, USA) were used.

5.2 Procedure

In this study the samples were analysed for the presence of *Listeria monocytogenes* and other *Listeria* spp. according to ISO 11290-1:1996 Microbiology of food and animal feeding stuffs - Horizontal method for the detection and enumeration of *Listeria monocytogenes* - Part 1: Detection method (ISO, 1996), with two modifications. For the primary enrichment UVM *Listeria* enrichment broth was used instead of Half Fraser Broth and for the selective isolation COMPASS® *Listeria* agar was used instead of ALOA™ OCLA (ISO) agar. The procedure can roughly be divided into two phases, starting with sample enrichment and selective isolation of the bacteria from the samples. Subsequently, presumptive *Listeria* spp. colonies were confirmed using six confirmatory tests, which were the catalase test, a gram stain, the motility test, the haemolysis test, the carbohydrate utilization test and the CAMP test. The first three confirmatory tests were used to determine whether a presumptive colony was a *Listeria* spp. and when this was the case, the other three tests were used to determine whether it was *Listeria monocytogenes*.

5.2.1 Enrichment and isolation

Enrichment

- Obtain a 25 g sample from the product with a sterile spoon and transfer it into a sterile blender bag.
- Add 225 mL of UVM *Listeria* enrichment broth and homogenize the sample by hand for 90 seconds.
- Incubate the sample suspension for 24±2 hours at 30°C.
- Add 0.1 mL of the enriched sample suspension to a test tube filled with 10 mL of Fraser broth and mix by vortexing.
- Incubate the Fraser broth suspension for 48±2 hours at 37°C, also incubate the primary enriched UVM suspension for an additional 24±2 hours at 30°C.

Selective isolation

- Inoculate both the primary enriched (i.e. UVM), and the secondary enriched (i.e. Fraser broth) sample suspension onto both a COMPASS® *Listeria* agar plate, and a PALCAM agar plate. Do this by streaking with a sterile inoculation loop in order to dilute the applied sample, resulting in the obtaining of isolated bacterial colonies.
- Incubate both agar plates for 24±3 hours at 37°C. (When necessary (e.g. when the colonies are too small to judge and/or subculture) the plates can be incubated for an additional 24±3 hours.)
- Read the agar plates for the presence of morphological characteristic bacterial colonies.

  Due to the use of certain substances in the medium, *Listeria* spp. colonies will appear blue-greenish on the COMPASS® *Listeria* agar plates. In addition, *L. monocytogenes* colonies will be surrounded by an untransparent zone. On the PALCAM agar plates *L. monocytogenes* colonies will appear grey-greenish, possibly with a black centre, and will be surrounded with a black zone.
- Transfer a maximum of six presumptive *Listeria monocytogenes* colonies (i.e. max. three presumptive colonies from COMPASS® *Listeria* agar and max. three from PALCAM agar) with a sterile inoculation loop onto a TSYEA plate and strike them on one quarter of this plate (i.e. max. four presumptive colonies per TSYEA plate).
- Incubate the TSYEA plate for 18-24 hours at 37°C.
- Store the TSYEA plate in the fridge (± 3°C) until the confirmation of the bacterial isolates.

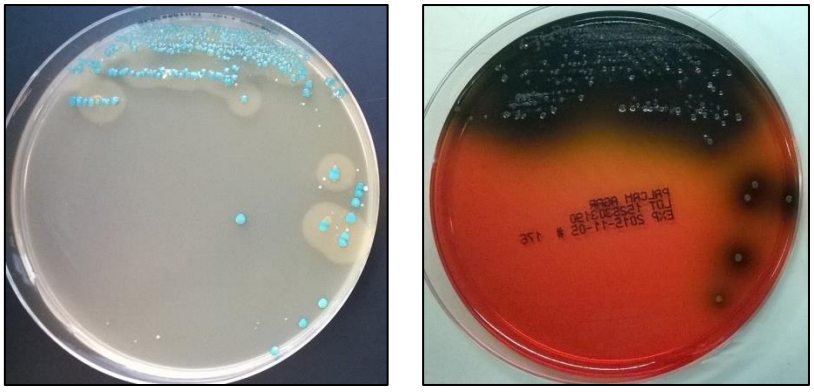

Figure 15 A COMPASS® *Listeria* (left) and a PALCAM (right) agar plate inoculated with an enriched sample suspension, after incubation for 24 hours at 37°C. On the COMPASS® *Listeria* agar plate *L. monocytogenes* colonies appear blue-greenish and are surrounded with an untransparent zone. On the PALCAM agar plate *L. monocytogenes* colonies appear grey-greenish and are surrounded with a black zone.

5.2.2 Confirmation

Catalase test

- Put a drop of hydrogen peroxide solution on a sterile microscopic slide.
- Touch the presumptive *Listeria* spp. isolate on the TSYEA plate with a sterile inoculation loop and subsequently, smear the sample into the drop of hydrogen peroxide solution.

The immediate production of gas bubbles, in some cases visible as froth, is regarded as a positive reaction. In case of a negative reaction no gas bubbles are visible. Since *Listeria* spp. possess catalase activity, these bacterial species are able to immediately decompose hydrogen peroxide into water and oxygen. Therefore, the presence of *Listeria* spp. in the culture results in a positive reaction.

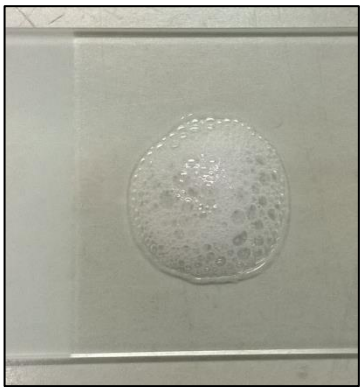

Figure 16 A positive catalase test (as for *Listeria* spp.), immediate gas production visible as froth.

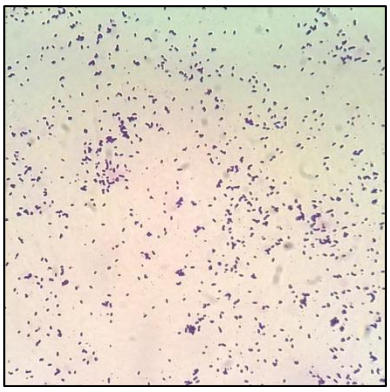

Figure 17 A gram staining (viewed microscopically) in which *Listeria* spp. are visible as Gram-positive (i.e. purple-coloured), short rod-shaped bacteria.

Gram staining

- Put a drop of peptone salt solution on a sterile microscopic slide.
- Touch the presumptive *Listeria* spp. isolate on the TSYEA plate with a sterile inoculation loop and subsequently, emulsify the sample in the drop of peptone salt solution.
- Spread the suspension on half of the microscopic slide and leave it to air-dry.
- Carefully pass the microscopic slide through the flame of a Bunsen burner three times in order to heat fix the sample.
- Dip the slide in a crystal violet solution for 45-60 seconds and subsequently, gently wash it with distilled water.
- Dip the slide in a Lugol's solution for 60 seconds and subsequently, gently wash it with distilled water.
- Dip the slide in an iodine-ethanol solution for 45 seconds and subsequently, gently wash it with distilled water.

- Finally, dip the slide in a fuchsine solution for 90 seconds and subsequently, gently wash it with distilled water.
- Leave the microscopic slide to air-dry.
- Examine the Gram stain microscopically.

When viewed microscopically, *Listeria* spp. are visible as Gram-positive (i.e. purple-coloured), short rod-shaped bacteria.

Motility test
- Transfer some material of the presumptive *Listeria* spp. isolate into a test tube filled with 10 mL of TSYEB with a sterile inoculation loop and emulsify it by vortexing.
- Incubate the test tube for 24±2 hours at 30°C.
- Put a little amount of Vaseline on the corners of a clean coverslip using an inoculation loop.
- Put a drop of the bacterial culture in the centre of this coverslip using a sterile inoculation loop.
- Place a clean concavity slide (with its concavity down) over the drop on the coverslip in such a way that the Vaseline attaches the coverslip to the slide around its concavity.
- Carefully turn the slide around in a fast motion, so the coverslip is on top.
- Examine the drop microscopically (with a closed diaphragm).

When viewed microscopically, *Listeria* spp. are visible as short rod-shaped bacteria which exhibit a distinctive tumbling movement.

Haemolysis test
- Touch the presumptive *L. monocytogenes* isolate on the TSYEA plate with a sterile inoculation needle and subsequently, stab this needle into a sheep blood agar plate.
- Repeat these actions for a control *L. monocytogenes* strain (i.e. a positive control) on the same sheep blood agar plate.
- Incubate the agar plate for 24±2 hours at 37°C.
- Read the agar plate for the presence of haemolytic zones and compare the presumptive *L. monocytogenes* isolate with the positive control strain.

Since *L. monocytogenes* is able to lyse red blood cells completely, a small clear and light zone is visible around colonies of this bacterial species cultured on sheep blood agar.

Carbohydrate utilization test
- Use a sterile inoculation loop to transfer some material of the presumptive *L. monocytogenes* isolate into both a test tube filled with 5 mL of phenol red broth supplemented with a rhamnose disk, and a test tube filled with 5 mL of phenol red broth supplemented with a xylose disk and emulsify by vortexing.
- Incubate the test tubes for a maximum of 48 hours at 37°C.
- Examine the test tubes for a discoloration from red to yellow after 18-24 hours and again after 48 hours (since reversal of the discoloration is possible, the tubes should be frequently examined).

A yellow discoloration of the red-coloured broth due to the fermentation of the incorporated sugar, is regarded as a positive reaction. In case of a negative reaction the colour of the broth remains red during all the time. Since *L. monocytogenes* is able to ferment rhamnose, but not to ferment xylose, the presence of this bacterial species in the culture results in a positive reaction in the rhamnose-supplemented test tube and in a negative reaction in the xylose-supplemented test tube.

CAMP test
- Streak a *Rhodococcus equi* strain in a straight line onto the left side of a sheep blood agar plate with a sterile inoculation loop and repeat this action for a *Staphylococcus aureus* strain on the right side of the plate.
- Use a sterile inoculation loop to streak some material of the presumptive *L. monocytogenes* isolate in a straight line between the *R. equi* and *S. aureus* streaks (keep a distance of 1-2 mm) on the sheep blood agar plate.
- Repeat this action for a control *L. monocytogenes* strain (i.e. a positive control) on the same sheep blood agar plate.
- Incubate the agar plate for 18 hours at 37°C.
- Read the agar plate for the presence of haemolytic zones and compare the presumptive *L. monocytogenes* isolate with the positive control strain.

Since the interaction of *L. monocytogenes* with *S. aureus* results in synergistic haemolysis, a wider haemolytic zone is visible around the streak of *L. monocytogenes* in the proximity of the *S. aureus* streak. A widening of the haemolytic zone around the *L. monocytogenes* streak is not visible in the proximity of the *R. equi* streak, because the interaction of these two bacterial species does not result in synergistic haemolysis.

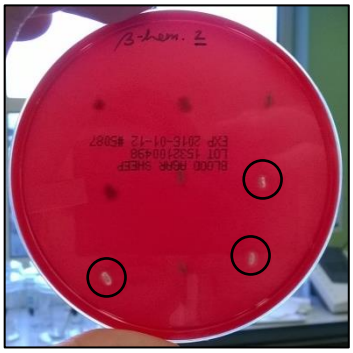

Figure 18 A sheep blood agar plate used for a haemolysis test. A small clear and light zone is visible around the encircled colonies. Therefore, those colonies are confirmed to be *Listeria monocytogenes.*

Figure 19 A positive rhamnose utilization test (as for *Listeria monocytogenes*; left) and a negative xylose utilization test (as for *L. monocytogenes*; right). A yellow discoloration of the red-coloured broth is regarded as a positive reaction.

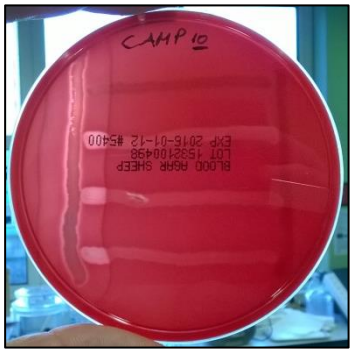

Figure 20 A sheep blood agar plate used for a CAMP test. A wider haemolytic zone is visible around the bottom three streaks, in the proximity of the *S. aureus* streak (left) and not in the proximity of the *R. equi* streak (right). Therefore, those streaks are confirmed to be *Listeria monocytogenes.*

6 Detection of *Salmonella* species (1,5,8-10,26-38)

In this study the samples were analysed for the presence of *Salmonella* spp., a Gram-negative, rod-shaped, and usually motile bacterial species belonging to the family of Enterobacteriaceae. Salmonellae are able to cause gastroenteritis, septicemic disease, and other organ infections in both humans and animals. Common sources of infection are contaminated foods and water, but *Salmonella* spp. can also be widespread in the environment. In addition, direct transmission from animals to humans and the other way around have been mentioned. This is not surprising since many *Salmonella*-serotypes are known to be able to infect a wide range of animal species including humans, hence it can be considered to be a zoonotic pathogen.

6.1 Materials

Buffered peptone water

Buffered peptone water (BPW) was used for both preparing a suspension of the solid sample, and the subsequent pre-enrichment. In this study Buffered Peptone Water - BK018HA (Biokar Diagnostics, France) was used. The dehydrated medium was suspended in distilled water (25.5 g/L) and after complete dissolution, the solution was dispensed into bottles (225 mL). The filled bottles were sterilized at 121°C for 15 minutes and after cooling down they were stored in the fridge (± 3°C) until use.

Rappaport Vassiliadis soya peptone broth

Rappaport Vassiliadis soya peptone (RVS) broth was used for the selective enrichment of the pre-enriched sample suspension. The growth of a large number of bacterial species other than *Salmonella* spp. is inhibited by several characteristics of this medium, including a relatively low pH value, a relatively high osmotic pressure, a relatively low nutritional value, and the presence of malachite green. In this study Rappaport-Vassiliadis Soya Peptone Broth - CM0866 (Oxoid Ltd, UK) was used. The dehydrated medium was suspended in distilled water (26.75 g/L), which was gently heated in order to achieve complete dissolution. The solution was dispensed into test tubes (10 mL), which were subsequently sterilized at 115°C for 15 minutes. After cooling down, the filled test tubes were stored in the fridge (± 3°C) until use.

Muller-Kauffmann tetrathionate novobiocin broth

Muller-Kauffmann tetrathionate novobiocin (MKTTn) broth was used for the selective enrichment of the pre-enriched sample suspension. Both the ox bile, and the brilliant green present in this medium support its selectivity by inhibiting the growth of gram-positive and a large number of gram-negative bacteria, other than *Salmonella* spp.. In addition, the antibiotic novobiocin supports the suppression of *Proteus* spp.. In this study prefilled 10 mL tubes of Muller-Kauffmann Tetrathionate-Novobiocin Broth - TV5065E (Oxoid Ltd, UK) were used.

Brilliant green agar

Brilliant green agar (BGA) was used to isolate *Salmonella* spp. from the enriched suspensions. This medium contains brilliant green, which inhibits the growth of gram-positive and a large number of gram-negative bacteria, other than *Salmonella* spp.. It also contains phenol red, which serves as a pH indicator with a colour range from yellow to red (acid-alkaline). Since *Salmonella* spp. do not form acid from lactose and sucrose, two sugars present in this medium, the colonies of this bacterial species appear pink-reddish on BGA plates. In this study Brilliant Green Agar (Modified) - CM0329 (Oxoid Ltd, UK) was used. The dehydrated medium was suspended in distilled water (52 g/L), which was gently heated up to almost boiling temperature in order to achieve complete dissolution. After cooling down to 50°C and mixing, the solution was poured into sterile Petri dishes. The plates were left to solidify, afterwards they were turned upside-down and left overnight at room temperature to check for contamination (i.e. bacterial growth). Subsequently, they were stored in the fridge (± 3°C) until use.

Xylose lysine desoxycholate agar

Xylose lysine desoxycholate (XLD) agar was used for the isolation of *Salmonella* spp. from the enriched suspensions. This medium contains phenol red, which serves as a pH indicator with a colour range from yellow to red (acid-alkaline). The xylose incorporated in this medium is readily fermented by *Salmonella* spp. which would alter the pH to acidic, however the lysine is decarboxylated by these bacterial species which results in a net alkaline alteration of the pH. Furthermore, *Salmonella* spp. are able to produce hydrogen sulphide which results in the precipitation of ferric ammonium citrate from the sodium thiosulphate-ferric ammonium citrate complexes present in this medium, visible as a black discoloration. Therefore, *Salmonella* spp. colonies appear reddish and have a black centre on XLD agar plates. The growth of coliforms is inhibited by the sodium desoxycholate in this medium and on top of that these bacteria are not able to appear reddish on XLD agar, since they do ferment both the sucrose, and the lactose present in this medium, which results in an acidic pH. In this study X.L.D. Agar - CM0469 (Oxoid Ltd, UK) was used. The dehydrated medium was suspended in distilled water (53 g/L), which was gently heated up to boiling temperature for up to 30 minutes and with frequent mixing (every 10 minutes). The solution was cooled down in a 50°C water bath for up to 30 minutes and subsequently poured into sterile Petri dishes. The plates were left to solidify, afterwards they were turned upside-down and left overnight at room temperature to check for contamination (i.e. bacterial growth). Subsequently, they were stored in the fridge (± 3°C) until use.

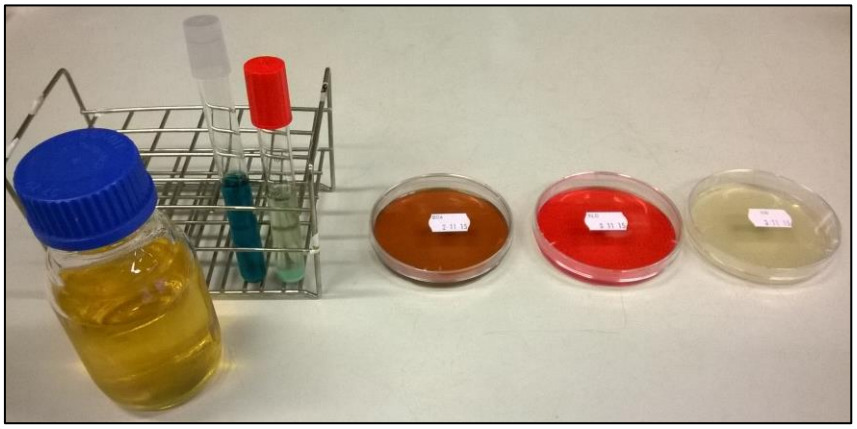

Figure 21 Materials used for the detection and enumeration of *Salmonella* species. (From left to right: buffered peptone water (BPW), Rappaport Vassiliadis (RV) broth, Muller-Kauffmann tetrathionate-novobiocin (MKTTn) broth, brilliant green agar (BGA), xylose lysine deoxycholate (XLD) agar, and nutrient agar (NA).)

Nutrient agar

Nutrient agar (NA) was used to subculture and store bacterial colonies which were suspected to be *Salmonella* spp.. In this study Nutrient Agar - CM0003 (Oxoid Ltd, UK) was used. The dehydrated medium was suspended in distilled water (28 g/L), which was brought to the boil for complete dissolution. Next, it was sterilized at 121°C for 15 minutes and after cooling down to 50°C, the solution was poured into sterile Petri dishes. The plates were left to solidify, afterwards they were turned upside-down and left overnight at room temperature to check for contamination (i.e. bacterial growth). Subsequently, they were stored in the fridge (± 3°C) until use.

Triple sugar iron agar

Triple sugar iron (TSI) agar slants were used for the sugar fermentation test, one of the tests used for the confirmation of bacterial isolates which were suspected to be *Salmonella* spp.. TSI agar contains glucose, lactose, and sucrose and can therefore be used for the fermentation test of these three sugars. It also contains phenol red, which serves as a pH indicator with a colour range from yellow to red (acid-alkaline). In addition, sodium thiosulphate-ferric ammonium complexes, which serve as an indicator for the formation of hydrogen sulphide by black discoloration as a result of the precipitation of ferric ammonium citrate, are present in this medium. In this study prefilled tubes with Triple Sugar Iron Slang Agar - TV5074D (Oxoid Ltd, UK) were used.

Urea agar

Urea agar slants were used for the urease test, one of the tests used for the confirmation of bacterial isolates which were suspected to be *Salmonella* spp.. The hydrolysis of the urea in this medium results in the production of ammonia, which is an alkaline substance. The medium also contains phenol red, which serves as a pH indicator with a colour range from yellow to red (acid-alkaline). In this study prefilled tubes with Urea Agar pH 6.8 - U011.86.0008 (Tritium Microbiologie, The Netherlands) were used.

Lysine decarboxylase broth

Lysine decarboxylase (LDC) broth was used for the lysine decarboxylase test, one of the tests used for the confirmation of bacterial isolates which were suspected to be *Salmonella* spp.. The fermentation of glucose in this medium results in an acidic alteration of the pH, however the decarboxylation of l-lysine results in the formation of the amine cadaverine and the release of CO_2, an alkaline reaction. The medium also contains bromocresol purple, which serves as a pH indicator with a colour range from yellow to purple (acid-alkaline). In this study prefilled 6 mL tubes of Lysine Decarboxylase Broth (Taylor) - TV5028N (Oxoid Ltd, UK) were used.

β-Galactosidase reagent

β-Galactosidase reagent was used for the β-galactosidase test, one of the tests for the confirmation of bacterial isolates which were suspected to be *Salmonella* spp.. Some bacterial species possess the enzyme β-galactosidase, which can break a β-galactoside bond. *o*-Nitrophenyl-β-galactoside (ONPG), present in this reagent, can therefore be hydrolysed by such bacterial species, which results in the release of both galactose, and the yellow coloured *o*-nitrophenol. In this study ONPG - 34055 (Thermo Scientific, USA), Sodium Dihydrogen Phosphate Monohydrate - K300.1 (Carl Roth, Germany), and Sodium hydroxide - 1.06498 (Merck, Germany) were used to prepare this reagent. First, 6.9 g of NaH_2PO_4 was suspended in 45 mL of distilled water and after adjusting its pH to seven with a NaOH-solution (10 mol/L), distilled water was added up to a total of 50 mL. This solution served as a buffer solution in the reagent. Subsequently, 15 mL of distilled water was heated up to 50°C and 80 mg of ONPG was suspended in it. After cooling down, 5 mL of the buffer solution was added to the ONPG-solution and it was sterilized at 121°C for 15 minutes.

Saline solution

A 0.85% saline solution was used for the β-galactosidase test, one of the tests for the confirmation of bacterial isolates which were suspected to be *Salmonella* spp.. This solution was used to make a suspension of a bacterial colony. In this study Sodium chloride - 1.06404 (Merck, Germany) was used to make the solution. The salt was suspended in distilled water (8.5 g/L) and after complete dissolution, it was sterilized at 121°C for 20 minutes.

Toluene

Toluene was used for the β-galactosidase test, one of the tests for the confirmation of bacterial isolates which were suspected to be *Salmonella* spp.. This substance promotes the possible release of the enzyme β-galactosidase from the bacterium. In this study Toluene - 244511 (Sigma-Aldrich, USA) was used.

Methyl-red Voges-Proskauer broth

Methyl-red Voges-Proskauer (MR-VP) broth was used for the Voges-Proskauer test, one of the tests used for the confirmation of bacterial isolates which were suspected to be *Salmonella* spp.. Some bacterial species are able to produce acetyl methyl carbinol after the fermentation of glucose, which is present in this medium. Acetyl methyl carbinol is oxidized to diacetyl in the presence of oxygen, after which the diacetyl reacts with a protein incorporated in the peptone present in this medium to produce a pink-reddish colour. In this study Methyl-red Voges-Proskauer Broth - 1.05712 (Merck, Germany) was used. The dehydrated medium was suspended in distilled water (17 g/L) and after complete dissolution, the solution was dispensed into test tubes (5 mL). The filled test tubes were sterilized at 121°C for 15 minutes and after cooling down they were stored in the fridge (± 3°C) until use.

α-Naphthol solution

An α-naphthol solution was used for the Voges-Proskauer test, one of the tests used for the confirmation of bacterial isolates which were suspected to be *Salmonella* spp.. α-Naphthol serves to intensify the colour formed by the reaction of diacetyl and therefore increases the sensitivity of the test. In this study 5% Alpha Naphthol (VP A) - R21200 (Remel, USA) was used. Before use the dehydrated medium in the vial had to be reconstituted by adding 12 mL of 95% ethanol.

Potassium hydroxide solution

Potassium hydroxide (KOH) solution was used for the Voges-Proskauer test, one of the tests for the confirmation of bacterial isolates which were suspected to be *Salmonella* spp.. The addition of KOH accelerates the oxidation of acetyl methyl carbinol to diacetyl by providing in oxygen. In this study Potassium hydroxide VP2 - 70422 (bioMérieux, France) was used.

Tryptone water

Tryptone water (TW) was used for the indole test, one of the tests used for the confirmation of bacterial isolates which were suspected to be *Salmonella* spp.. The tryptophan present in this medium can be converted into indole by certain bacterial species. In this study Tryptone Water - CM0087 (Oxoid Ltd, UK) was used. The dehydrated medium was suspended in distilled water (15 g/L) and after complete dissolution, the solution was dispensed into test tubes (5 mL). The filled test tubes were sterilized at 121°C for 15 minutes and after cooling down they were stored in the fridge (± 3°C) until use.

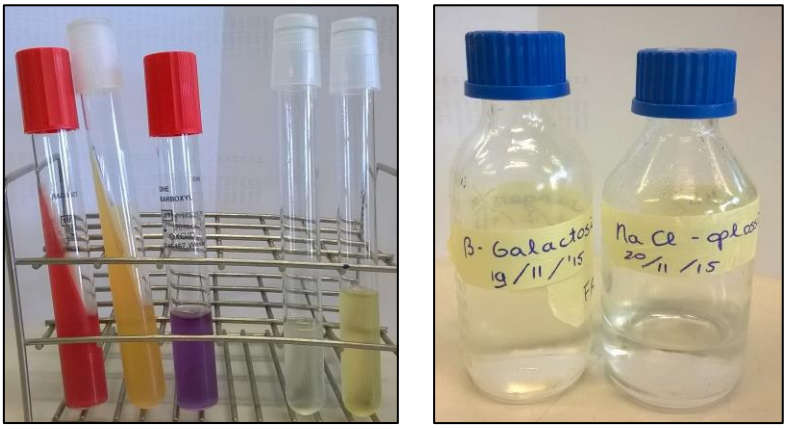

Figure 22 Materials used for the confirmation of presumptive *Salmonella* species. (From left to right: triple sugar iron (TSI) agar, urea agar, lysine decarboxylase (LDC) broth, tryptone water (TW), methyl-red Voges-Proskauer (MR-VP) broth, β-Galactosidase reagent, and a saline solution (0.85%).)

Kovac's reagent

Kovac's reagent was used for the detection of indole during the indole test, one of the tests used for the confirmation of bacterial isolates which were suspected to be *Salmonella* spp.. This reagent contains p-aminobenzaldehyde, which can react with tryptophan resulting in the formation of a pink-red complex. In this study Kovac's Reagent for indoles - 60983 (Sigma-Aldrich, USA) was used.

Salmonella-antiserum

Salmonella-antiserum was used for the serological confirmation of bacterial isolates which were suspected to be *Salmonella* spp.. Serological testing was performed for the presence of somatic (O) antigens (group A-S) only. In this study both Salmonella agglutination serum Polyvalent O A-S - PL.6002 (Pro-Lab Diagnostics, Canada), and Agglutinating serum Salmonella Polyvalent-O Groups A-S - ZC02 (Remel, UK) were used.

6.2 Procedure

In this study the samples were analysed for the presence of *Salmonella* spp. according to ISO 6579:2002 Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp. (ISO, 2002), with one modification. The presumptive *Salmonella* spp. colonies were only serologically confirmed if their biochemical confirmation was positive and they were only analysed for the presence of O-antigens. Analysis for the presence of Vi-antigens and/or H-antigens was not performed in this study. The procedure can roughly be divided into two phases, starting with sample enrichment and selective isolation of the bacteria from the samples. Subsequently, presumptive *Salmonella* spp. colonies were confirmed using seven confirmatory tests, which were the Triple Sugar Iron test, the urease test, the lysine decarboxylase test, the β-galactosidase test, the Voges-Proskauer test, the indole test, and serological confirmation.

6.2.1 Enrichment and isolation

Enrichment

- Obtain a 25 g sample from the product with a sterile spoon and transfer it into a sterile blender bag.
- Add 225 mL of BPW and homogenize the sample by hand for 90 seconds.
- Incubate the sample suspension for 18±2 hours at 37°C.
- Add 0.1 mL of the pre-enriched sample suspension to a test tube filled with 10 mL of RVS broth and mix by vortexing.
- Add 1 mL of the pre-enriched sample suspension to a test tube filled with 10 mL of MKTTn broth and mix by vortexing.
- Incubate the RVS broth suspension for 24±3 hours in a 41.5°C water bath, also incubate the MKTTn broth for 24±3 hours at 37°C.

Selective isolation

- Inoculate both selectively enriched sample suspensions (i.e. both the RVS, and the MKTTn broth) onto both a BGA plate, and a XLD agar plate. Do this by streaking with a sterile inoculation loop in order to dilute the applied sample, resulting in the obtaining of isolated bacterial colonies.
- Incubate both agar plates for 24±3 hours at 37°C.
- Read the agar plates for the presence of morphological characteristic bacterial colonies.

  Due to the use of certain substances in the medium, *Salmonella* spp. colonies will appear pink-reddish on the BGA agar plates. On the XLD agar plates they will appear reddish and will have a black centre.
- Transfer a maximum of six presumptive *Salmonella* spp. colonies (i.e. max. three presumptive colonies from BGA and max. three from XLD agar) with a sterile inoculation loop onto a NA plate and strike them on one quarter of this plate (i.e. max. four presumptive colonies per NA plate).
- Incubate the NA plate for 24±3 hours at 37°C.
- Store the NA plate in the fridge (± 3°C) until the confirmation of the bacterial isolates.

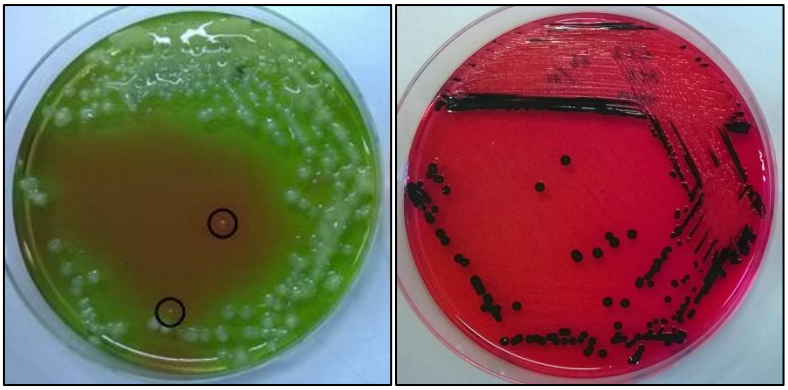

Figure 23 A brilliant green agar (BGA; left) and a xylose lysine desoxycholate (XLD) agar **(right) plate inoculated with an enriched sample suspension, after incubation for 24 hours at 37°C. On the BGA plate *Salmonella* colonies appear pink-reddish (encircled) and on the XLD agar plate *Salmonella* colonies appear reddish and have a black centre.**

6.2.2 Confirmation

Triple Sugar Iron test

- Take some material of a presumptive *Salmonella* spp. isolate from the NA plate using a sterile inoculation loop and inoculate a tube filled with a TSI agar slant by smearing its slope and stabbing its butt.
- Incubate the TSI agar slant tube for 48 hours at 35°C.
- Examine the tube for discolorations and for cracks and/or air bubbles (i.e. gas formation) in the agar after 18 hours and again after 48 hours.

The presence of certain bacterial species results in a typical reaction in the TSI agar slant. A discoloration from red to yellow is caused by the fermentation of the sugars incorporated in this medium, however a reversal of this discoloration occurs when only glucose is fermented. In addition, the fermentation of glucose results in the formation of gas. The production of hydrogen sulphide results in a black discoloration of the medium. *Salmonella* spp. are able to ferment glucose, but not to ferment lactose and sucrose. Moreover, they are able to produce hydrogen sulphide. Therefore, the presence of this bacterial species in the culture results in the formation of cracks and/or air bubbles in the agar and a yellow-coloured butt of the agar slant, whereas the slope remains red. However, the yellow colour can be masked by the simultaneous blackening of the medium.

Urease test

- Use a sterile inoculation loop to take some material of a presumptive *Salmonella* spp. isolate from the NA plate and inoculate a tube filled with a urea agar slant by smearing its slope.
- Incubate the urea agar slant tube for 18 hours at 35°C.
- Examine the agar slant for a discoloration from orange to pink after 5 hours and again after 18 hours.

A pink discoloration of the orange agar slant due to the hydrolysis of the incorporated urea, is regarded as a positive reaction. In case of a negative reaction the colour of the agar slant remains orange-yellow. Since *Salmonella* spp. do not possess urease activity, these bacterial species are not able to catalyse the hydrolysis of the urea in this medium. Therefore, the presence of *Salmonella* spp. in the culture results in a negative reaction.

Lysine decarboxylase test

- Use a sterile inoculation loop to transfer some material of the presumptive *Salmonella* spp. isolate into a test tube filled with 6 mL of lysine decarboxylase broth and emulsify by vortexing.
- Incubate the test tube for 24 hours at 35°C.
- Examine the medium for a discoloration from purple to yellow.

A yellow discoloration of the purple broth due to the fermentation of the incorporated glucose, is regarded as a negative reaction. In case of a positive reaction this discoloration is reversed by the decarboxylation of the incorporated l-lysine and so the medium is purple-coloured. Since *Salmonella* spp. possess lysine decarboxylase activity, these bacterial species are able to decarboxylate l-lysine. Therefore, the presence of *Salmonella* spp. in the culture results in a positive reaction.

Figure 24 **A negative (left) and two positive (as for *Salmonella* spp.; right) Triple Sugar Iron tests. The fermentation of glucose results in the formation of cracks and/or air bubbles and a yellow discoloration of the agar, whereas the production of hydrogen sulphide results in a black discoloration of the medium.**

Figure 25 **A negative (as for *Salmonella* spp.; left) and a positive (right) urease test. The test is regarded as negative, when the agar remains orange-yellow coloured after incubation.**

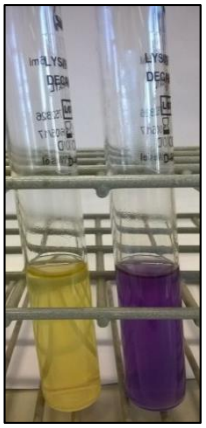

Figure 26 **A negative (left) and a positive (as for *Salmonella* spp.; right) lysine decarboxylase test. A reversal of the yellow discoloration back to purple is regarded as a positive reaction.**

β-galactosidase test

- Transfer some material of the presumptive *Salmonella* spp. isolate into a test tube filled with 0.25 mL of saline solution (0.85%) with a sterile inoculation loop and emulsify by vortexing.
- Add one drop of toluene to the test tube using a sterile syringe and mix the suspension thoroughly.
- Incubate the test tube for 5 minutes in a 37°C water bath.
- Add 0.25 mL of β-galactosidase reagent to the suspension and mix by shaking.
- Incubate the test tube for a maximum of 24±2 hours in a 37°C water bath.
- Examine the suspension for a yellow discoloration after 30 minutes and again after every few hours.

A yellow discoloration of the suspension due to the hydrolysis of the incorporated ONPG, is regarded as a positive reaction. In case of a negative reaction a discoloration does not occur and the suspension remains colourless. Since *Salmonella* spp. do not possess β-galactosidase activity, these bacterial species are not able to hydrolyse the ONPG present in the suspension. Therefore, the presence of *Salmonella* spp. in the culture results in a negative reaction.

Voges-Proskauer test

- Use a sterile inoculation loop to transfer some material of the presumptive *Salmonella* spp. isolate into a test tube filled with 5 mL of MR-VP broth and emulsify by vortexing.
- Incubate the test tube for 24 hours at 37°C.
- Pipet 1 mL of the incubated broth and dispense into another sterile test tube.
- Add 0.6 mL of α-naphthol solution to the test tube and mix by shaking.
- Add 0.2 mL of KOH solution to the test tube and mix well by shaking.
- Examine the mixture for a discoloration from yellow to pink-red after 15 minutes.

A pink-red discoloration of the yellowish solution due to the oxidation of acetyl methyl carbinol, which is present after the fermentation of the glucose incorporated in this medium, is regarded as a positive reaction. In case of a negative reaction the solution does not change colour and remains yellowish. Although *Salmonella* spp. are able to ferment glucose, they do not produce acetyl methyl carbinol during this process. Therefore, the presence of *Salmonella* spp. in the culture results in a negative reaction.

Indole test

- Transfer some material of the presumptive *Salmonella* spp. isolate into a test tube filled with 5 mL of tryptone water with a sterile inoculation loop and emulsify it by vortexing.
- Incubate the test tube for 24-48 hours at 35°C.
- Pipet 0.2-0.5 mL of Kovac's reagent and dispense into the incubated test tube.

The development of a pink-red coloration at the top of the tryptone water within one minute is regarded as a positive reaction. In case of a negative reaction no colour change is visible and the Kovac's reagent at the top of the tryptone water remains yellow. Since *Salmonella* spp. possess tryptophanase activity, these bacterial species are able to split indole from the tryptophane present in the tryptone water. Therefore, the presence of *Salmonella* spp. in the culture results in a positive reaction.

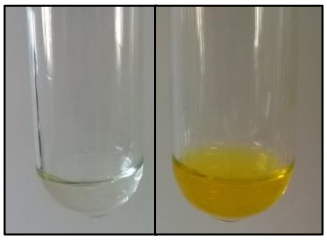

Figure 27 A negative (as for *Salmonella* spp.; left) and a positive (right) β-galactosidase test. The test is regarded as negative, when a yellow discoloration does not occur and the solution remains colourless.

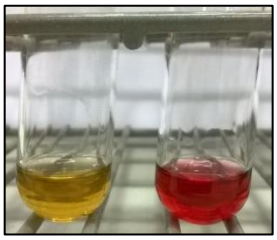

Figure 28 A negative (as for *Salmonella* spp.; left) and a positive (right) Voges-Proskauer test. In case of a negative reaction the solution remains yellow coloured.

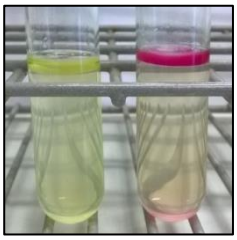

Figure 29 A negative (as for *Salmonella* spp.; left) and a positive (right) indole test. The test is regarded as negative, when the Kovac's reagent remains yellow coloured.

Serological confirmation

- Put one drop of *Salmonella*-antiserum on a sterile microscopic slide.
- Touch the biochemically confirmed *Salmonella* spp. isolate on the NA plate with a sterile inoculation loop and subsequently, emulsify the sample in the drop of *Salmonella*-antiserum.
- Rock the slide for one minute and examine the drop against a dark background.

The antiserum reacts with the somatic (O) antigens of *Salmonella* spp. and therefore, the presence of these bacterial species in the culture results in agglutination.

7 Storage of confirmed bacterial isolates (22)

All confirmed isolates of *E. coli* O157:H7, ESBL-producing *E. coli*, *Listeria* spp., and *Salmonella* spp. were permanently stored deep-frozen in the microbiological data bank of the Institute for Risk Assessment Sciences at Utrecht University. Therefore, subsequent testing of the bacterial strains isolated in this study is possible.

7.1 Materials

Tryptone soya broth

Tryptone soya broth (TSB) was used to subculture the bacterial isolates which were confirmed to be positive for one of the bacterial species of interest, before their permanent storage. In this study Tryptone Soya Broth - CM0129 (Oxoid Ltd, UK) was used. The dehydrated medium was suspended in distilled water (30 g/L), which was brought to the boil in order to achieve complete dissolution. The solution was dispensed into test tubes (10 mL), which subsequently were sterilized at 121°C for 15 minutes. After cooling down, the filled test tubes were stored in the fridge (± 3°C) until use.

Glycerol solution

A 21.25% glycerol solution was used as a supplement to the bacterial suspension for permanent storage. Glycerol was added to prevent freezing damage to the bacterial cell. In this study Glycerol 85% - 1.04094 (Merck, Germany) was used. In order to achieve the right concentration, this substance was suspended in distilled water at a ratio of one to one. (See procedure.)

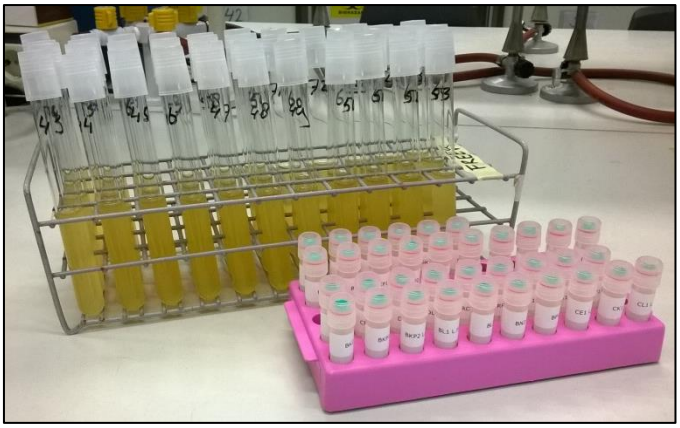

Figure 7 **Materials used for the storage of confirmed bacterial isolates. (From left to right: tryptone soya broth (TSB) and a cryovial filled with a 21,25% glycerol solution.)**

7.2 Procedure

In this study the confirmed bacterial isolates were permanently stored in a -80°C freezer according to the method commonly used at the microbiological laboratory of the Institute for Risk Assessment Sciences at Utrecht University.

- Use a sterile inoculation loop to transfer some material of the confirmed bacterial isolate into a test tube filled with 10 mL of TSB and emulsify it by vortexing.
- Incubate the test tube for 24 hours at 37°C.
- Pipet 0.6 mL of the incubated broth, dispense into a sterilized cryovial filled with glass beads and 0.6 mL of a 42.5% glycerol solution, and mix by vortexing. (After the addition of the broth to the glycerol solution, the preferred glycerol concentration of 21.25% is reached.)
- Put the cryovial in a storage box and store this box in the -80°C freezer.

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Supplementary file: Table S1 **Complete overview of raw meat-based diets purchased to analyse for the presence of bacterial pathogens**

Brand name	Product name	Composition [1]	Weight (g)	Price (€/kg) [2]	Lot number	Expiry date	Date of purchase
Barfmenu	Kip	Chicken (100%); Meat, Bones, Organs	250	6.76	381 060815.1	06-08-2017	02-10-2015
Barfmenu	Kip	Chicken (100%); Fresh chicken, Fresh chicken bones, Fresh chicken organs	250	8.36	381 020915.1	02-09-2017	02-10-2015
Barfmenu	Konijn	Rabbit (100%); Meat, Bones, Organs	250	9.16	382 181214.1	18-12-2015	02-10-2015
Barfmenu	Konijn	Rabbit (100%); Meat, Bones, Organs	250	9.16	382 090915.1	09-09-2017	02-10-2015
Barfmenu	Lam	Fresh lamb, Fresh lamb bones, Fresh lamb organs, Fresh chicken, Fresh chicken bones	500	6.62	3821a230615.1	23-06-2016	02-10-2015
Barfmenu	Lam	Fresh lamb, Fresh lamb bones, Fresh lamb organs, Fresh chicken, Fresh chicken bones	250	7.56	3822 270815.1	27-08-2017	02-10-2015
Barfmenu	Natuurlijk	Beef, Chicken, Beef organs, Chicken organs, Chicken bones	250	8.36	380 030215.1	03-02-2016	02-10-2015
Barfmenu	Natuurlijk	Beef, Chicken, Beef organs, Chicken organs, Chicken bones	250	6.76	380 090315.1	09-03-2017	02-10-2015
Barfmenu	Paard	Fresh horse meat, Fresh duck bones, Fresh duck organs	1,000	5.00	3812 070715.1	07-07-2016	02-10-2015
Carnibest	Eend en Rijst	Fresh duck meat, Rice, Vegetable oil, Natural vitamins and minerals	500	6.20	31 1	26-07-2016	02-10-2015
Carnibest	Kalkoen en Rijst	Fresh turkey meat, Rice, Vegetable oil, Natural vitamins and minerals	500	6.20	29 1	12-07-2016	02-10-2015
Carnibest	Kip en Rijst	Fresh chicken, Fresh vegetables, Rice, Vegetable oil, Natural vitamins and minerals	500	5.50	28 4	08-07-2016	02-10-2015
Carnibest	Kip en Rijst	Fresh chicken, Fresh vegetables, Rice, Vegetable oil, Natural vitamins and minerals	500	5.50	33 1	09-08-2016	02-10-2015
Carnibest	Lam en Rijst	Fresh lamb meat, Fresh chicken, Fresh vegetables, Rice, Vegetable oil, Natural vitamins and minerals	500	5.50	39 4	23-09-2016	02-10-2015

Brand name	**Product name**	**Composition** [1]	**Weight** (g)	**Price** (€/kg) [2]	**Lot number**	**Expiry date**	**Date of purchase**
Carnibest	Lam en Rijst	Fresh lamb meat, Fresh chicken, Fresh vegetables, Rice, Vegetable oil, Natural vitamins and minerals	500	5.50	34 4	19-08-2016	02-10-2015
Carnibest	Natuurvoer	Fresh beef, Fresh chicken, Fresh vegetables, Rice, Vegetable oil, Natural vitamins and minerals	500	4.70	37 4	09-09-2016	02-10-2015
Carnibest	Natuurvoer	Fresh beef, Fresh chicken, Fresh vegetables, Rice, Vegetable oil, Natural vitamins and minerals	500	4.70	34 1	16-08-2016	02-10-2015
Duck	Beef Complete	Beef (47%), Cartilage (16%), Beef liver (10%), Rice (9%), Beef heart (8%), Carrots (6%)	1,000	4.55	20 11 16 08:57	20-11-2016	02-10-2015
Duck	Beef Liver Chicken Complete	Chicken and chicken carcass (30%), Beef (30%), Beef liver (12%), Beef heart (10%) and beef kidney, Carrots (8%), Rice (6%)	1,000	3.35	06 01 17 15:10	06-01-2017	02-10-2015
Duck	Chicken Single	Chicken and chicken carcass (100%)	1,000	2.75	19 02 17 09:52	19-02-17	02-10-2015
Duck	Lamb & Rice Complete	Lamb tripe (30%), Rice (24%), Lamb liver (9%), Lamb heart (6%), Lamb kidney (2%), Chicken and chicken carcass, Sunflower oil	1,000	4.55	02 12 16 12:04	02-12-2016	02-10-2015
Duck	Tripe-Pens Complete	Beef tripe (50%), Chicken and chicken carcass, Unlocked corn, Wheat flakes and wheat bran	1,000	3.45	24 01 17 09:59	24-01-2017	02-10-2015
Energique	Volwassen Honden	Chicken, Beef, Grains, Oils, Fats, Vegetables	750	5.00	24 06 15 B26 13:59	24-06-2017	02-10-2015
Farm Food	Fresh; Beef complete	Beef, Beef heart, Beef tripe, Beef liver, Sheep fat, Rice, Oils (sunflower oil, linseed oil), Vitamins, Minerals, Trace elements	800	5.24	[3]	17-02-2017	02-10-2015

Brand name	Product name	Composition [1]	Weight (g)	Price (€/kg) [2]	Lot number	Expiry date	Date of purchase
Farm Food	Fresh; Tripe and Heart complete	Beef tripe, Beef heart, Beef, Beef liver, Sheep fat, Rice, Oils (sunflower oil, linseed oil), Vitamins, Minerals, Trace elements	800	4.74	[3]	18-08-2016	02-10-2015
Prins	TotalCare Super Active Complete	Chicken, Beef, Corn, Beetroot, Sunflower oil, Salmon oil	600	5.42	10 03 15 B11 13:04	10-03-2017	02-10-2015
Prins	TotalCare Super Plus Complete	Chicken, Beef, Corn, Beetroot, Sheep fat, Sunflower oil, Salmon oil	600	5.83	21 11 14 B48 12:11	24-11-2016	02-10-2015
Rodi	Complete Rund & Hart	Meat and animal by-products (Beef (96%; Heart (26%))), Minerals	400	2.48	NL90886 0 09-2016 3	09-2016	02-10-2015
Rodi	Lamshart	Meat and animal by-products (Lamb (99%; Heart (70%))), Minerals	400	3.73	NL90886 0 07-2016 2	07-2016	02-10-2015
Rodi	Pens	Meat and animal by-products (Tripe (90%))	400	2.30	NL90886 1 06-2016 6	06-2016	02-10-2015
Rodi	Vlees	Meat and animal by-products (Beef (99%)), Minerals	400	2.30	NL90886 08-2016 6	08-2016	02-10-2015
Smuldier	Hart	Cat. 3 animal by-products (e.g. beef heart)	400	2.88	[3]	28-02-2017	02-10-2015
Smuldier	Kip	Cat. 3 animal by-products (e.g. chicken)	400	1.48	[3]	04-03-2017	02-10-2015
Smuldier	Pens	Cat. 3 animal by-products (e.g. beef tripe)	400	1.55	[3]	03-03-2017	02-10-2015
Smuldier	Vlees	Cat. 3 animal by-products (e.g. beef)	400	1.58	[3]	24-02-2017	02-10-2015

1 The composition of the product as described on the product label.

2 The prices for identical products can change, since products were purchased from different stores.

3 No lot number was given on either the product package, or the product label.