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Incidence and contamination level of *Clostridium perfringens* in meat and meat products sold in Sakarya province of Turkey

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ABSTRACT

Totally 101 meat and meat product samples obtained from local markets and restaurants were analyzed for incidence and contamination level of *Clostridium perfringens*. The typical colonies grown anaerobically on Tryptose Sulfite Cycloserine Agar supplemented with 4-Methyliumbelliferyl (MUP) were confirmed by biochemical tests. Forty-eight of the samples (47.5%) were contaminated with *C. perfringens*. The highest incidence of the pathogen was determined in uncooked meatball samples (72.2%) followed by ground beef samples (61.3%). The incidence of *C. perfringens* in chicken meat, cooked meat döner, cooked chicken döner and emulsified meat product samples were 33.3, 33.3, 28.6 and 16.7%, respectively. Thirteen out of 101 samples (12.9%) yielded typical colonies on TSC-MUP Agar, but could not be confirmed as *C. perfringens.* Average contamination levels in sample groups ranged from 8.3 to 1.5×10^2 cfu/g, with the highest ground beef and the lowest chicken meat.

Keywords: *Clostridium perfringens*, Incidence, Meat, Meat products, Ground beef, Meatball

Introduction

Clostridium perfringens is an anaerobic, non-motile, sulfite reducing, spore-forming, Gram-positive and rod-shaped bacterium (Brynestad and Granum, 2002; Garcia et al., 2019). Spores are usually located sub-terminally and formed only in specially formulated culture media (Juneja et al., 2010). It was isolated in 1892 and named as *Bacillus aerogenes* and then *as Clostridium welchii* (Garcia and Heredia, 2011). It can grow at temperatures from 15°C to 50°C with an optimum at 45°C for most strains (Brynestad and Granum, 2002). *C. perfringens* is very common in nature and can be isolated from soil, dust, gastrointestinal systems of human and animals, on surfaces of vegetables as well as other raw and processed foods (Juneja et al., 2010). Although it has an anaerobic nature, it can grow at E_h values of +350 mV and can reduce its environment to less than -400 mV (Garcia and Heredia, 2011).

Acute diarrhea and severe abdominal pain are observed in 8-24 h following the digestion of the food harbored high number vegetative cells of *C. perfringens*. Vomiting and fever are very rare. Generally the patient recovers in 24-48 h. Death is rare and particularly seen in elderly patients (Labbe and Juneja, 2017). *C. perfringens* is classified as A, B, C, D and E according to toxin type. The type A is related food poisoning, meanwhile can cause gaseous gangrene and septicemia (Brown, 2000; McClane et al., 2012). In the European Union, *C. pefringens* caused 124 out of 160 total outbreaks occurred in 2014 (EFSA-ECDC, 2015). It is estimated that *C. perfringens* poisoning is more common since the mild cases are not reported (Juneja et al., 2010). Considering the relatively mild symptoms, the under-reported cases have been estimated as 10-fold (Mead et al., 1999). Foodborne poisoning associated with *C. perfringens* is due to improper handling and preparation of foods. A recent report revealed that improper stored and inadequate reheated meat could result in large *C. perfringens* outbreak (Mellou et al. 2019). The high number of vegetative cells (>10^6 cfu/g) multiplied in food exposed to elevated temperatures are ingested and then food poisoning occurs (Garcia and Heredia, 2011).

C. perfringens contaminated meat and meat products via fecal contamination of carcasses or contamination from other ingredients such as spices, post-processing contamination is possible as well (Juneja et al., 2010; McClane et al., 2012; EFSA-ECDC, 2015). Although *C. perfringens* can be isolated from different types of foods, it is mostly isolated from meat and meat products. Because meat and meat products are good sources of thirteen amino acids which cannot be produced by *C. perfringens* and needed for growth of this pathogen (Andersson et al., 1995). The previous studies have shown than *C. perfringens* incidence ranged from 7 to 96% in meat and meat products including ground beef (Başkaya et al., 2004; Kamber et al., 2007), chicken meat (Çakmak et al., 2006; Yıldırım et al., 2015), beef and sheep meats (Guran et al., 2014), cured raw meat products (Taormina et al., 2003), emulsified meat products (Elmalı et al., 2005) and cooked döner (Vazgecer et al., 2004). As far as we have known there is not any survey study in the literature on incidence of *C. perfringens* in meat and meat products sold in Sakarya, Turkey. Therefore, considering the survey studies may provide a better understanding the risk for foodborne pathogens, it was aimed in this study to determine the incidence and contamination level of *C. perfringens* in meat and meat products sold in Sakarya province of Turkey.

Materials and Methods

Materials

Totally 101 samples including ground beef (31), chicken meat (27), meatball (18), cooked meat döner (12), cooked chicken döner (7) and emulsified meat products (6) were collected from 57 different butcher shops, markets and fast food restaurants between April 2013 and February 2014 in Sakarya province of Turkey. Samples were transferred to laboratory in cooled conditions and kept in refrigerator until analysis.

Preparation of Samples for Analyses and C. perfringens Enumeration

C. perfringens enumeration and confirmation was performed according to the method described by Rhodehamel and Harmon (1998). Aseptically 25 g sample was transferred into a stomacher bag with 225 mL sterile peptone water (1% peptone) and homogenized (BagMixer® 400, Interscience Co., Saint Nom, France) for two minutes at low speed. Serial dilutions were prepared using peptone water. The pour plating technique was used for enumeration of *C. perfringens*. One mL-portions from 10^{-1} and 10^{-2} dilutions were transferred to sterile petri plates and Tryptose Sulfite Cycloserine (TSC) Agar (Merck, Darmstadt, Germany) containing MUP (4-Methylumbelliferyl phosphate) was poured and mixed well. After solidifying, the plates were overlaid with an additional 10 mL TSC Agar. The plates were incubated anaerobically (Anaerocult A; Merck, Darmstadt, Germany) at 37°C for 24 h. Following incubation plates were examined under 366 nm ultraviolet light (366 nm UV Lamp; Merck Darmstadt, Germany) and the black colonies with blue florescence were counted as *C. perfringens*.

Morphological and Biochemical Confirmation

The typical colonies grown on TSC Agar were picked and inoculated in Thioglycolate Broth (Merck, Darmstadt, Germany). Tubes were incubated anaerobically at 37°C for 24 h. The Thioglycolate Broth cultures of the isolates were maintained at -20°C by adding 15% glycerol. Gram-positive and sporeforming isolates were subjected to biochemical tests. *C. perfringens* was confirmed by motility-nitrate and lactose-gelatin tests (Anonymous, 1999). The active cultures of suspected isolates were stab-inoculated into Motility-Nitrate Medium (Sigma-Aldrich, St. Louis, MO, USA) and incubated anaerobically at 37°C for 24 h. The cultures grown only along the stab line in Motility-Nitrate Medium were considered non-motile, while those grown away from the stab line were considered motile. Red color formation after addition of nitrite-detection reagent (Sigma-Aldrich, St. Louis, MO, USA) showed the reduction of nitrate. If no red color observed within 15 min, zinc dust was added and color of the medium was checked after 10 min. The Lactose-Gelatin Medium (Sigma-Aldrich, St. Louis, MO, USA) tubes inoculated with active cultures were incubated anaerobically at 37°C for 24 h. The gas blisters and converting the color from red to yellow were the signs of lactose fermentation. To detect gelatinase activity, the tubes were kept at 5°C for 1 h and checked for liquefaction. In case solidification occurred, the tubes were incubated at 37°C for additional 24 h. The cultures in liquefied tubes were considered gelatinase positive.

Results and Discussion

Totally 101 samples, including ground beef, chicken meat, uncooked meatball, cooked meat döner, cooked chicken döner and emulsified meat products, were analyzed for incidence and contamination level of *C. perfringens*. Table 1 depicts incidence of the pathogen in the samples. Out of 101 samples, 48 (47.5%) were positive for *C. perfringens*. Although thirteen samples (4 ground beef, 3 uncooked meatballs, 3 chicken meat, and one emulsified meat product) yielded typical colonies on TSC Agar, these colonies could not be confirmed as *C. perfringens* by biochemical tests. This result is not surprising considering the specificity of the culture medium used in this study. Fischer at al. (2012) have mentioned that the specificity of TSC-MUP Agar was 74.5%. In other words this culture medium yielded 24.5% false negative results and non-perfringens *Clostridium* species were isolated frequently. The highest incidence of the pathogen was in uncooked meatball samples (72.2%) followed by ground beef samples (61.3%). The previous studies have shown that incidence of *C. perfringens* and/or sulfite reducing bacteria in beef meat or ground beef is ranged from 18 to 96%. *C. perfringens* incidence was reported as 18% in ground beef samples sold in small butcher shops and local markets (Kamber et al., 2007). Başkaya et al. (2004) determined sulfite reducing anaerobic bacteria in 74% of the ground beef samples. Guran et al. (2014) reported that 96% of beef meat samples were contaminated with *C. perfringens*.

The incidence of the pathogen was 72.2% in meatball samples. Meatball is produced mainly from ground beef and/or lamb meat, fat, roasted bread crumbs, salt, onion garlic and various spices. The spices used to prepare meatball may increase the microbial load of product. De Boer et al. (1985) isolated *C. perfringens* spores from 80% of 54 spices and herbs. It is well known that spices are main source of sporeforming pathogenic bacteria including *Bacillus* and *Clostridium* species and under favorable conditions their spores may germinate and multiply (Pafumi, 1986). Therefore, the spices used in meatball recipe may be the reason for high incidence of *C. perfringens* in meatball samples.

Table 1. *C. perfringens* incidence in meat and meat products

Sample type	Sample number	*C. perfringens* confirmed sample number (%)	Sample number with typical colony on TSC Agar but not confirmed (%)	Sample number with no typical colony on TSC Agar (%)
Ground beef	31	19 (61.3)	4 (12.9)	8 (25.8)
Meatball (uncooked)	18	13 (72.2)	3 (16.2)	2 (11.1)
Chicken meat	27	9 (33.3)	5 (18.5)	13 (48.2)
Meat döner (cooked)	12	4 (33.3)	0 (0)	8 (66.7)
Chicken döner (cooked)	7	2 (28.6)	0 (0)	5 (71.4)
Emulsified meat products	6	1 (16.7)	1 (16.7)	4 (66.6)
Total	101	48 (47.5)	13 (12.9)	40 (39.6)

C. perfringens incidence was confirmed only in 9 chicken meat samples (33.3%). The previous studies have shown that the incidence of this pathogen in poultry products may vary from 2.5 to 94% according to product type. Çakmak et al. (2006) detected *C. perfringens* in 70% of frozen raw ground poultry samples while 2.5% of poultry burger samples were contaminated with this pathogen. Yıldırım et al. (2015) reported that 46% of chicken leg and breast meat samples were positive for *C. perfringens.* Shaltout et al. (2017) determined *C. perfringens* in 21.6% of chicken meats samples. Higher incidence levels were reported by Guran and Oksuztepe (2013). These researchers determined that 66-94% of chicken parts were contaminated with *C. perfringens*. Incidence of this pathogen in intestinal tract of broiler chickens can be as high as 95% (Immerseel et al., 2004). Therefore it is not unexpected situation that its incidence in processed meat of poultry is high.

The incidence of *C. perfringens* in cooked beef and chicken döner samples were 33.3 and 28.6%, respectively. The previous studies revealed that the incidence of sulfite reducing Clostridia incidence is low in döner samples. Vazgecer et al. (2004) reported that sulfite reducing Clostridia were determined in the 7% of the cooked döner samples. In contrast, Bostan et al. (2011) did not determined sulfite reducing Clostridia in cooked döner samples. Spores of *C. perfringens* may survive during cooking and then if cooked foods are kept at temperatures between 12°C and 50°C they can germinate and multiply. Moreover an efficient reheating step may be required to kill vegetative cells (Jaloustre et al, 2013). Döner is a very popular meat meal which is prepared by seasoning of meat with spices and then cooking of cone-like shaped meat mass in front of cooking apparatus. Meat mass is rotated slowly during cooking and the cooked surface layer is cut as thin flakes. In this cooking process, the temperature of meat mass may be at ranges that allow survival and growth of spore-forming bacteria including pathogenic species. Considering cooking method, döner may have risk for *C. perfringens.*

C. perfringens was confirmed only in one emulsified meat product sample (16.7%). Apaydın et al. (2003) reported that 10% of bologna-type sausage samples were contaminated with *C. perfringens*. The incidence of the pathogen in emulsified meat products was determined as 22.1% by Elmalı et al. (2005). The spores of *C. perfringens* are heat resistant with 34.2 min D value at 90°C (Byrne et al., 2006). With this in mind, it can be estimated that they can well survive during production of this type of meat products.

The differences between the isolation rates of the current study and the previous ones may be attributed to number of the samples and analysis methods. The method used in this study did not contain any enrichment step. Wen and McClane (2004) reported approximately 50-fold increase in *C. perfringens* recovery when an enrichment procedure is applied using Fluid Thioglycolate medium.

Fluorogenic substrate, 4-methylumbelliferyl phosphate (MUP), added TSC Agar was used in this study for enumeration and isolation of *C. perfringens* from the meat products. MUP is a fluorogenic substrate which is highly specific for *C. perfringens*. MUP is metabolized by *C. perfringens* producing 4-methylumbelliferone which can be detected under a long wave (366 nm) UV light (Adcock and Paint, 2001). Thirteen out of 101 samples yielded typical colonies on TSC Agar supplemented with MUP. That is to say, false-positive result ratio was 12.9%. Similarly, Araujo et al. (2001) reported false-positive results on this culture medium during recovery of *C. perfringens* from groundwater samples. Despite this, the researchers found that the MUP added TSC Agar was superior to the other culture media used for detection of *C. perfringens*.

The contamination levels in the *C. perfringens* confirmed samples are shown in Table 2. Results revealed that 41.6% of the samples harbored *C. perfringens* lower than 10^1 cfu/g. More than half of the confirmed samples (52.1%) contained the pathogen in the range from 10^1 to 10^2 cfu/g. The contamination level of the pathogen was 10^2-10^3 cfu/g only in two meatball samples. The highest level detected in one ground beef sample was 2.4×10^3 cfu/g. The average counts of sample groups were ranged from 8.3 to 1.5×10^2 cfu/g. According to Turkish Food Codex Regulation on Microbiological Criteria (Anonymous, 2011), the counts of sulphite reducing anaerobic bacteria should not exceed 10^4 and 10^3 cfu/g in non-ready-to-eat and ready-to-eat foods, respectively. Based on this knowledge, it can be concluded that the samples analyzed in current study had acceptable levels of *C. perfringens* as a member of sulphite reducing anaerobic bacteria group. On the other hand, these results are generally in consistent with the published data. Kamber et al. (2007) determined levels of *C. perfringens* in ground beef samples obtained from local markets and butcher's shops as 2.75×10^2 and 6.82×10^2 cfu/g, respectively. Apaydın et al. (2003) reported the incidence of *C. perfringens* as 1-1.27 log cfu/g in bologna-style sausages. The mean number of ground poultry samples was determined as 2.6 MPN/g by Çakmak et al. (2006). On the other hand, Yıldırım et al. (2015) have stated that the mean counts of *C. perfringens* in chicken breast and leg meats were 3.21×10^3 and 1.64×10^4 cfu/g, respectively. These levels are higher than both our study and the previous studies.

Table 2. Contamination levels in *C. perfringens* confirmed samples

Sample type	Number of samples for each contamination level				Average (cfu/g)
	$<10^1$	10^1 - 10^2	10^2 – 10^3	$>10^3$	
Ground beef	6	12	0	1	1.5×10^2
Chicken meat	7	2	0	0	8.3
Meatball (uncooked)	5	6	2	0	5.8×10^1
Meat döner (cooked)	2	2	0	0	2.3×10^1
Chicken döner (cooked)	0	2	0	0	2×10^1
Heat processed meat products	0	1	0	0	1.5×10^1
Total	20 (41.6%)	25 (52.1%)	2 (4.2%)	1 (2.1%)	

C. perfringens vegetative cells higher than 10^6 cfu per gram of food are needed to result in food poisoning (Juneja et al., 2010). The numbers of the pathogen in the samples analyzed did not exceed this level. However, to control temperature during cooking and storage is a key factor to avoid *C. perfringens* poisoning. If the cooked food is cooled down slowly, kept at warm temperatures for extended periods and not reheated sufficiently before consumption to destroy vegetative cells, this critical level may be reached. Additionally, cooking may provide more favorable conditions for the growth of *C. perfringens* by increasing anaerobic environment and reducing competing spoilage organisms (Juneja et al., 2010; Kouassi et al. 2014). Moreover, cooking may cause heat shock which triggers the germination of spores (Juneja et al., 2010).

Conclusion

It may be concluded that *C. perfringens* is very common in raw or cooked meat products having regard to its confirmed presence in almost half of samples (47.5 %) analyzed in this study. The highest incidence was in uncooked meatball samples followed by ground beef samples. Although the contamination levels in samples analyzed in this study were lower than that of required for food poisoning (10^6 cfu/g), it seems that presence of this pathogen in meat and meat products is generally unavoidable. All things considered, it should be emphasized that temperature control during processing, transportation and storage is the key factor for prevention growth of *C. perfringens* and thus food poisoning caused by this pathogen. Another key thing to remember is that the temperature and time during cooking and/or reheating should be adequate to kill vegetative cells of *C. perfringens*.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived the conflict of interests.

Ethics committee approval: Author declare that this study does not include any experiments with human or animal subjects.

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Disclosure: -

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