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Occurrence of *Clostridium perfringens* in sausages sold in Meknes city, Morocco

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Abstract
In Morocco, the consumption of meat products has experienced a sharp increase in recent years despite the presence of pathogenic bacteria due to hygiene failure. The present study was designed to determine the prevalence of *Clostridium perfringens* in sausages sold in Meknes city (Morocco) and to study the different factors affecting it contamination with this bacterium. To this end, 156 samples of sausages were taken in various shopping sites during one year from March 2014 to February 2015. The microbiological analysis was carried out using the specific medium for isolation and identification of *C. perfringens*. ANOVA test was used for Statistical analysis ($p< 0.05$). The results of this study showed the presence of *C. perfringens* in 77.56% (121 of 156) samples, with 88.88% (32 of 36) in street vendors, 79.16% (19 of 24) in a weekly market, 70.83% (51 of 72) in butchery and 62.5% (15 of 24) in a supermarket. The average rate was 2.42 Log CFU/g, with a minimum value of 0 CFU/g recorded in several outlets and a maximum value of 6.05 Log CFU/g recorded in butchery. This study reveals that the contamination rate of sausages with *C. perfringens* is related to the sausages origin, retail sites and seasonal variations related to temperature increase.
Keywords: *Clostridium perfringens*, Food contamination, Food safety, Morocco, Sausages.

Introduction

Food-borne diseases have a major public health impact. In Morocco, 630 cases were reported between 2001 and 2006 (Aoued *et al*., 2010), while *C. perfringens* was responsible for over than 28% of the cases (Belomaria *et al*., 2007). This bacterium is a major cause of food-borne diseases, usually associated with consumption of insalubrious meat goods (Miki *et al.*, 2008). Some strains are able to produce and release the enterotoxin in the gastrointestinal tract, causing nausea, abdominal pain, and diarrhea (McClane *et al*., 2006; McClane and Robertson, 2013).

As known, *C. perfringens* is a Gram-positive bacterium, anaerobic, immobile; it forms the heat resistant endospores and to multiply rapidly in order to produce cytotoxic enterotoxin (CPE) (Brynestad and Granum, 2002).

C. perfringens is divided into five types, A, B, C, D, and E, based on the synthesis of four major lethal toxins: alpha, beta, epsilon, and iota (Petit *et al*., 1999). *C. perfringens* type A is a common cause of food-borne diseases worldwide (Labbe, 1990), it is responsible for Gas gangrene characterized by myonecrosis and gas production and also it causes necrotic enteritis (Granum, 1990; Brynestad and Granum, 2002; Immerseel *et al*., 2004).

The symptoms appear usually after 6 to 24 hours of ingesting the contaminated food (Maslanka *et al*., 1999).

However, *C. perfringens* types B, C, D, and E are associated with dysentery in the young of many animal species, hemorrhagic enterotoxaemia (struck) in sheep and cattle, pulpy kidney disease in sheep and sudden death with dysentery in calves and lambs, respectively (Manteca *et al*., 2002; Uzal and Songer, 2008).

As with other Moroccan cities, Meknes (northwestern of Morocco) has many sites for preparation and sale of sausages.

These sausages are made in poor hygienic conditions and exposed for sale at an ambient temperature usually around 20°C (Benkerroum *et al*., 2003); which favors its contamination by pathogenic bacteria (Ed-dra *et al*., 2017a).

Furthermore, the economic status of Morocco is highly dependent on the agricultural sector with a focus on the domestic local demand, such pathogens can have a dangerous potential for agribusiness, food balance, and on the socio-economic sector.

The aims of this study were to determine the occurrence of *C. perfringens* in sausages, to study the effect of season, retail sites and origin of the raw material on the contamination rate by *C. perfringens*.

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Materials and Methods

Samples Collection

A total of 156 samples of sausages collected as follow: 60 of turkey sausages, 60 of beef sausages and 36 of artisanal sausages "Merguez". Collected from different shopping sites: butchery, street vendors, supermarket, and Souk (refers to a weekly market that combines the population of the small villages around Meknes city). The sampling frequency was 13 samples per month. The collection was carried out during one year from March 2014 to February 2015. The collected amount was about 40 grams of sausage per sample. The samples are transferred in a cooler to the laboratory of Microbiology at the Faculty of Science Meknes.

Microbiological identification and confirmation

25 grams of sausage were mixed with 225 mL of buffered peptone water (Oxoid), afterward, the mixture was ground in a Masticator (Stomacher 400 Circulator, Seward) for 1 min at 260 rotation per minute (RTM). The decimal dilutions were prepared. Then, the enumeration of *C. perfringens* was carried out in TSC medium (Tryptone Sulfite Cycloserine Agar, Biokar) supplemented with D-cycloserine (Biokar) and incubated under anaerobic conditions at 37°C for 24 to 48 hours (ISO 7937, 2004). The confirmation of *C. perfringens* was carried out using biochemical and Gram staining tests as follow: the suspect colonies that have a black color were inoculated in the Thioglycollate Broth with Resazurin (Biokar) and incubated at 44°C for 24 hours in the anaerobic conditions. 1 mL of the positive tests was transferred into Lactose-Sulfite broth (Biokar) and incubated at 44°C for 24 hours. The positive strains were also confirmed by Gram staining which appear as bacilli with violet coloration (Gram-positive).

Statistical analysis

A two-way analysis between groups was conducted to explore the impact of sites and seasons sampling on the contamination rate for both bovine and turkey sausages with *C. perfringens*. The analysis was executed using sampling sites (Butchery, weekly market, and supermarket) and season's sampling as the independent variable, whereas the contamination rates were used as the dependent variable. Cohen's effect sizes criteria were applied, where *r* of 0.1 has a "small" effect size, *r* of 0.3 represents "medium" effect size and *r* of 0.5 has a "large" effect size (Cohen, 1988). The artisanal sausages "Merguez" are sold only in street vendors, for this reason, we have realized a one-way ANOVA to explore the impact of seasons on the contamination rate of artisanal sausages "Merguez". Contamination rates served as the continuous dependent variable, whereas Season (e.g. winter, autumn, spring, and summer) served as the categorical independent variable. The statistical analyses were performed with an alpha value 0.05 using SPSS V21 for windows.

Results

Prevalence of C. perfringens in sausage samples

The bacteriological analysis shows the presence of *C. perfringens* in 77.56% (121 of 156) of analyzed samples, with 88.88% (32 of 36) in street vendors, 79.16% (19 of 24) in weekly market, 70.83% (51 of 72) in butchery and 62.5% (15 of 24) in Supermarket (Table 1).

Artisanal sausages "Merguez" are most contaminated with 88.88% (32 of 36), followed by beef sausages 75% (45 of 60) and turkey sausages 73.33% (44 of 60) (Table 2).

The average rate is about 2.42 Log CFU/g, with a minimum value of 0 CFU/g recorded in several outlets and a maximum value of 6.05 Log CFU/g recorded in butchery.

Table 1. Effect of sampling sites on sausages contamination with *C. perfringens.*

Sampling sites	Number of samples		
	Analyzes	Positives	%
Butchery	72	51	70.83%
Supermarket	24	15	62.5%
Street vendors	36	32	88.88%
Weekly market	24	19	79.16%

Table 2. Effect of raw material origin on sausages contamination with *C. perfringens.*

Origin	Number of samples		
	Analyzes	Positives	%
Turkey sausage	60	44	73.33%
Beef sausage	60	45	75%
Artisanal sausages "Merguez"	36	32	88.88%

The impact of sampling sites and the seasons on contamination rate of beef sausages

The interaction effect between the sites and seasons was not statistically significant {F (6, 48)= 0.92, p= 0.49}. Whereas, there was a statistically significant main effect for sites sampling {F (2, 42)= 7.47, p= 0.002} and also for seasons sampling {F (3, 48)= 8.23, p< 0.001}. The medium effect size was found with partial eta squared equal to 0.24 and 0.34 for both sites and seasons sampling. Post-hoc comparison through the use of Tukey HSD test indicates that the mean contamination rate for butchery site (M=2.64, SD=2) was significantly higher from the supermarket site (M=1.04, SD= 0.9). Simultaneously, the mean contamination rate for the weekly market (M=2.99, SD=1.72) was significantly higher than the supermarket site (M=1.04, SD= 0.9) (Fig. 1).

The impact of sampling sites and the seasons on contamination rate of Turkey sausages

The interaction effect between sites and seasons for turkey sausages was not statistically significant {F (6, 48)= 0.61, p= 0.72}.

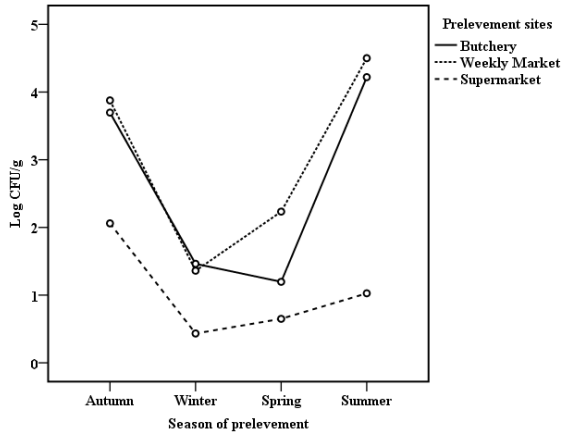

Fig. 1. Contamination average rate of beef sausages by season's sampling.

There was a statistically significant main effect for both sites sampling {F (2, 48)= 4.54, *p*= 0.01} and seasons sampling {F (3, 48)=7.02, *p*= 0.001}; with a small to medium effect size (*r* = 0.16 and *r* = 0.3 for sites and seasons sampling; respectively). Post-hoc comparison by Tukey HSD test shows that the mean score contamination rate for the butchery site (M= 2.11, SD=1.54) was significantly higher than the supermarket site (M=0.95, SD=0.97), whereas an inconclusive higher contamination rate result was found for weekly market (M=2.13, SD=1.51) and supermarket site (M=0.95, SD=0.97) with a *p-value* of 0.51 (Fig. 2).

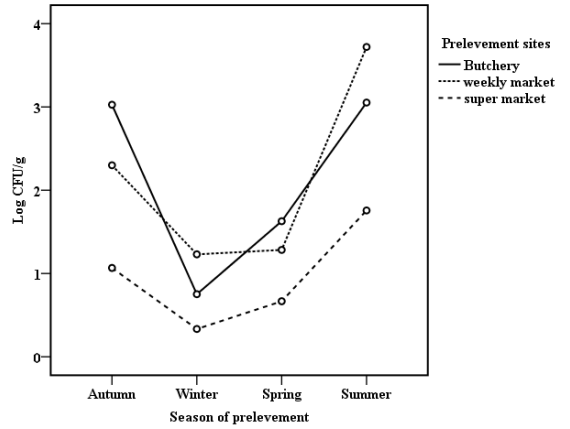

Fig. 2. Contamination average rate of turkey sausages by season's sampling

The impact of seasons on contamination rate of artisanal sausage "Merguez"

Samples were analyzed according to seasons, the four seasons were included (Fig. 3). Levene's test for homogeneity did not reach a significant value supporting that the assumption of homogeneity was not violated. There was a statistically significant difference at $p< 0.05$ level for the four seasons {F (3, 32)= 10.1, $p< 0.001$}.

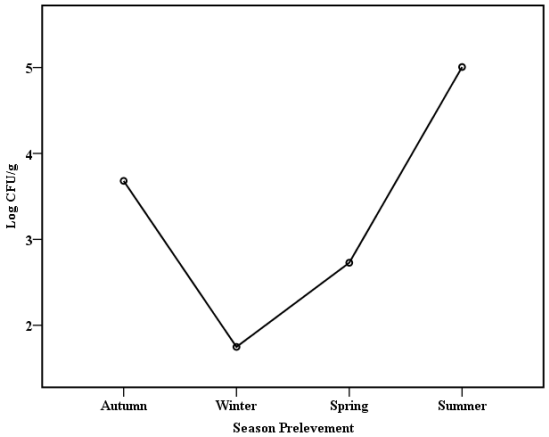

Fig. 3. Contamination average rate of artisanal sausages by season's sampling.

Even though the statistical significance was reached, the actual difference between contamination rates among the groups was medium. The effect size was calculated, using eta squared, revealing a value of 0.5. Post-hoc comparisons using the Tukey HSD test indicated that the contamination rate means score for winter (M=1.74; SD=1.27) was significantly lower than autumn (M=3.68; SD=1.68) and summer (M=5; SD= 0.82), respectively.

In the other hand, the contamination rate means score in autumn (M=3.68; SD=1.68) also differ significantly and was lower than summer (M=5; SD= 0.82). No other significant results were found among other seasons combination.

Discussion

The bacteriological analysis shows the presence of *C. perfringens* in 77.56% (121 of 156) of analyzed samples; this result is comparable to that found in Kingdom of Saudi Arabia (78.9%) (Alkheraije, 2013), U.S.A (69.6%) (Cooper *et al*., 2013), Turkey (70%) (Çakmak *et al*., 2006), and Japan (70%) (Miki *et al*., 2008). Moreover, this prevalence is superior to that registered in Nigeria (3.5%) (Tizhe *et al*., 2015) and India (8.25%) (Gurmu *et al*., 2013). A study in Casablanca (Morocco) on fermentation sausage showed the absence of this bacterium in all samples analyzed (Malti and Amarouch, 2008).

C. perfringens is a normal microflora of the intestinal tract of animals, the contamination of carcass from the intestinal contents is almost inevitable (McClane *et al*., 2006). During the preparation of the raw material, the animals are slaughtered in poor hygienic conditions, with the use of the same instruments for the removal of intestines and meat cutting, which increases the chances of contamination of carcasses by feces (Chaiba and Rhazi Filali, 2011).

Results comparison of different studied sausages types shows that artisanal sausages "Merguez" collected from street vendors have a significantly higher

contamination rate than turkey sausages and beef sausages. This difference could be attributed to the poor conditions of hygiene during the manufacturing chain and the failure to respect the cold chain during transportation and the sale of products. The difference in composition of sausages can be influencing also their hygienic quality and the survival of some pathogenic bacteria (Benkerroum *et al*., 2003; Ed-dra *et al*., 2017b).

However, the exhibition of these products in contact with dust and the poor hygienic conditions of sales in street vendors promote their contamination by pathogenic bacteria (Rane, 2011).

The ability of *C. perfringens* to grow between 15°C and 50°C with an optimum of 45°C (Gurmu *et al*., 2013), promotes its multiplication during the summer and autumn season, especially for a city like Meknes where the temperatures are usually above the 40°C in summer. The spore germination in small numbers can lead to the multiplication and production of toxins causing a serious food poisoning or multiple infectious diseases such as gangrene and necrotic enteritis food-borne (Petit *et al*., 1999; Brynestad and Granum, 2002). Therefore, the sausages can be converted into a food-borne disease source, if the optimal hygienic conditions of production are not set or if the cold chain supplies management is not applied throughout all the production process (Kamber *et al*., 2007).

Conclusion

The bacteriological analysis showed that 77.56% of sausages samples marketed in Meknes city are contaminated with *C. perfringens*, which reflects the neglect of hygiene practices throughout the manufacturing chain, storage, transport, and distribution of these products.

Besides, the critical points of sausages contamination are concise in the origin of raw material, storage temperature, hygiene of preparation places, and the seasonal variations. So, it is advisable to implement awareness programs on hygiene practices and contamination risk factors for encouraging the manufacturers to respect the cold chain and Critical Control Points (HACCP) all along the chain of manufacture and distribution.

Conflict of interest

The authors declare that there is no conflict of interest.

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