Evaluation of the microbial load and antibiotic resistance profile of Clostridium perfringens isolates from local corn flour for infants sold at markets in the city of Daloa (Haut-Sassandra, Côte d'Ivoire)

Abstract :

The World Health Organization (WHO) recommends the introduction of adequate complementary foods from the age of six months. However, infant corn porridges, often produced in an artisanal way, present significant health risks. This study aims to assess the impact of Clostridium perfringens-contaminated cornflour on infant health in Daloa. A total of 26 cornmeal samples were collected from vendors in several markets in Daloa. Samples were grown on TSN medium and incubated at 45°C for 48 hours to enumerate vegetative and spore-forming organisms. Biochemical characteristics such as color, glucose fermentation, lecithin fermentation and gas production were carried out. An antibiogram was performed on extracted isolates to assess antibiotic resistance. The results of the study revealed a preponderance of vegetative forms of Clostridium perfringens in the flours analyzed, with a 100% compliance rate, indicating recent contamination. Antibiotic susceptibility testing showed multi-resistance of the strains to the antibiotics used, posing a serious public health problem. These results underline the fact that the production and packaging techniques used for artisanal cornflour do not guarantee the safety of infants. The presence of Clostridium perfringens in cornmeal constitutes a serious public health problem.

1. INTRODUCTION

In underdeveloped countries, and particularly in Côte d'Ivoire, the problem of infant and young child nutrition is a serious public health concern [1]. Malnutrition accounts for 33% of child mortality, representing an estimated 128,354 deaths of children under five per year [2]. Inappropriate feeding practices, particularly after the age of 6 months, when breast milk is either insufficient or no longer sufficient to meet the growing need for nutrients for their development, lead to high rates of child malnutrition. What's more, the premature introduction of inadequate foods at different stages of a child's growth predisposes him or her to

malnutrition, with serious repercussions on his or her adult life [3]. For this reason, [4] recommends that infants receive nutritionally adequate and safe complementary foods from the age of 6 months, while continuing to breastfeed until the age of 2 years or more. Cereals are therefore the raw materials most commonly used to make complementary foods for infants and young children.

Corn represents a diverse group of cultivated grasses, and is the world's most widely grown cereal for use in animal and human nutrition. The main chemical component of corn kernels is starch. Other carbohydrates are simple sugars in the form of glucose, sucrose and fructose. Because of its functional properties as a thickener, binder, humectant, gelling agent, sweetener, anti-crystallizer, colorant and acidifier, corn starch in particular is used in the manufacture of many food products [5].

Maize is an indispensable resource with many uses. When consumed on a large scale, infant porridges made from fermented maize grain must not contain germs capable of causing food poisoning. However, the production channels for local, artisanal infant cereal flours result in inadequate hygiene practices [6]. Most of these microbes can impair the quality or safety of food products.

The main germs causing food-borne illnesses are Staphylococcus aureus, Salmonella, Campylobacter, Yersinia enterocolitica, Clostridium perfringens and Bacillus cereus [7]. In sub-Saharan Africa, a number of countries have experienced food poisoning outbreaks. In November 2008, food poisoning caused by porridge resulted in seventeen deaths in the town of Bongouanou [8]. In 2019, C. perfringens enterotoxins caused two (2) cases of food poisoning. Approximately 36 patients with symptoms of watery diarrhoea were recorded [9]. Clostridium perfringens is a special case because it is ingested alive in vegetative form to trigger the disease. However, when they lack the nutrients to enable them to grow optimally, they resist in the form of spores [10]. The strength of spore-forming strains, relying on their toxicity and firmness in food, poses a considerable risk to consumers [11]. Infection with C. perfringens can cause severe diarrhoea and often severe abdominal cramps. Symptoms of vomiting or fever are rare in most cases. The patient usually recovers rapidly within two or three days of infection. Dehydration may occur in the elderly, immunocompromised or young children following infection with C. perfcringens [12]. Antibiotics have proved indispensable in the fight against diseases caused by by bacteria that can affect humans and animals. Antibiotics have been the subject of major therapeutic discoveries for human health. Their use has helped to reduce mortality and morbidity rates worldwide for many years. Unfortunately,

antibiotic misuse and overuse have led to resistance in certain microbial strains. Almost all rural and urban mothers feed their children porridges made from locally-produced flours [13]. The sanitary quality of the various locally-produced infant flours is important, as they are used during the period of dietary diversification [14]. However, it has to be said that no studies have been carried out on the locally-produced baby food consumed by infants in the town of Daloa. Indeed, the hygienic conditions under which these infant meals are produced can encourage the development of certain bacteria. As a result, the consumption of this food in Daloa's markets could lead to infections. The general objective of the study is to assess the impact of home-made corn flour contaminated with Clostridium perfringens on infant health. Specifically, it will aim to :

- Evaluate the sanitary quality of locally-produced and artisanal corn flour sold in Daloa markets contaminated by Clostridium perfringens ;

- Study the antimicrobial resistance profile of identified C. perfringens isolates.

2. MATERIALS AND METHODS

2.1. Sampling

A total of twenty-six (26) maize flour samples were collected for this study. These included ten (10) samples from the Lobia market, eight (08) samples from the Grand marché, four (4) samples from the Abattoir market, and four (4) samples from the Orly market.

2.2. Analysis techniques

2.2.1. Inoculation, enumeration and incubation

For a quantity of 10 g of each sample weighed, 90 mL of Buffered Peptone Water broth is added. The mixture was homogenized for 1 min to obtain the stock suspension, which is left on the bench for 30 min at room temperature to multiply the microorganisms. Decimal dilutions are made [15].

To detect and enumerate spore-forming Clostridium perfringens, 10 mL of the mother suspension obtained is transferred to a sterile test tube. The tube is then treated in a water bath at 80°C for 10 minutes and cooled in water containing ice. This treatment creates a thermal shock which causes the spores to explode. Approximately 1 ml of the treated solution is introduced into another tube containing 9 ml of sterile distilled water for decimal dilutions. 1 mL of treated inoculum and 1 mL of untreated inoculum are respectively inoculated into 15

mL of TSN agar previously prepared, poured and sterilized in tubes. The inoculated tubes are incubated at 45°C for 48 h to detect and count Clostridium perfringens. Biochemical characteristics such as color, glucose fermentation, mobility and gas production are read. A candle is lit to rapidly consume excess oxygen while generating CO2, and the jar is then hermetically sealed and incubated at 37°C for 24 to 48 hours.

2.2.2. Extraction and identification of presumptive isolates

Approximately three presumptive Clostridium perfringens isolates are selected and extracted using a platinum loop. These are the black colonies introduced into Heart Brain broth. A 3 cm layer of kerosene is poured over the surface of the broth to maintain anaerobiosis. Isolation is carried out on nutrient agar. Colonies selected from the various Petri dishes were transferred to the nutrient agar and placed in an anaerobic jar. A candle was lit to rapidly consume excess oxygen while generating CO2, and the jar was then hermetically sealed and incubated at 37°C for 24 hours. These 24-hour culture colonies are used to characterize biochemistry, which took into account catalase assay, morphological characteristics, mobility and lecithinase and lipase production.

2.2. 3.. Determination of antibiotic resistance

Antibiotic susceptibility testing is carried out on Mueller-Hinton agar using the classic agar diffusion method. A suspension equivalent to the McFarland 0.5 standard (~108 CFU/mL) is prepared from an 18 to 24-hour pure culture on nutrient agar, by emulsifying 2-3 colonies picked with a sterile Pasteur pipette in a test tube containing 10 mL sterile saline solution (0.9% NaCl), diluted 1:1000. Mueller-Hinton agar was poured into Petri dishes and dried for 30 min at 45°C. Inoculation was carried out by flooding with inoculum diluted 1 :1000. The plates The inoculated cells were resealed and dried at room temperature on the bench for 15 min, then incubated at 37°C for 18 to 24 hours in an anaerobic jar. The zones of inhibition, including the diameter of the antibiotic, were measured using a caliper, and the diameters were interpreted according to the instructions of the Comité de l'Antibiogramme de la Société Française de Microbiologie [16].

2.3. Statistical analysis

The results obtained were analyzed using Excel 2016 software for descriptive analyses and STATISTICA 7.1 software. For the one-factor analysis of variance (ANOVA) at the threshold of $\alpha = 0.05$. Tukey's HSD test was recommended for calculating and classifying means.

3. Results

3.1 Average microbial load of corn samples analyzed

Microbiological analyses revealed the presence of Clostridium perfringens in corn meal collected from certain markets. These microorganisms were present with loads ranging from 0.0±0.00 to 16.81±0.64 for vegetative strains and from 0.0±0.00 to 1.36±0.64 for spore-forming strains. Samples E3 and E5 had the highest loads for vegetative forms, with an average of 16.81±0.64 for E3 and 15.45±1.28 for E5. For spore-forms, the highest loads were found in samples E26, E3 and E4, with a load of 1.36±0.64. Virtually all the corn flour samples analyzed in the course of this work did not show loads in excess of the criterion defined by the current standard, which is 102 cfu/g of flour. Statistical analysis shows that there is a significant difference at $p>0.05$ between the charge mean microbial load of sample E2 (5.45±1.28b) and the mean microbial loads of the other samples analyzed for vegetative forms. On the other hand, the analysis revealed no significant difference at $p>0.05$ between the average microbial loads of all samples analyzed for spore-forming fungi (Table I).

Table I : Average microbial load of maize samples analysed

Samples	Vegetative form	Sporulated form
E1	0,00±0,00^{c}	0,00± 0,00^{a}
E2	5,45±1,28^{b}	0,00± 0,00^{a}
E3	16,81±0,64^{a}	1,36±0,64^{a}
E4	1,81±1,28^{c}	1,36±0,64^{a}
E5	15,45±1,28^{a}	0,00± 0,00^{a}
E6	1,81±0,00^{c}	0,00± 0,00^{a}
E7	0,45±0,64^{c}	0,00± 0,00^{a}
E8	1,36±0,64^{c}	0,00± 0,00^{a}
E9	0,45±0,64^{c}	0,45±0,64^{a}
E10	0,45±0,64^{c}	0,00± 0,00^{a}
E11	0,00±0,00^{c}	0,00± 0,00^{a}
E12	0,00±0,00^{c}	0,00± 0,00^{a}

E13	0,00±0,00^{c}	0,00± 0,00^{a}
E14	0,00±0,00^{c}	0,00± 0,00^{a}
E15	0,90±0,00^{c}	0,90±1,28^{a}
E16	0,00±0,00^{c}	0,00± 0,00^{a}
E17	0,00±0,00^{c}	0,00± 0,00^{a}
E18	0,45±0,63^{c}	0,00± 0,00^{a}
E19	1,36±0,64^{c}	0,00± 0,00^{a}
E20	0,00±0,00^{c}	0,00± 0,00^{a}
E21	0,00±0,00^{c}	0,00± 0,00^{a}
E22	0,00±0,00^{c}	0,00± 0,00^{a}
E23	0,45±0,64^{c}	0,00± 0,00^{a}
E24	1,00±1,41^{c}	0,00± 0,00^{a}
E25	1,36±0,64^{c}	0,45±0,64^{a}
E26	0,00±0,00^{c}	1,36±0,64^{a}
Normative criteria		**10^{2} cfu/g**

Mean microbial loads with the same letters in the same column are not significantly different at p>0.05

3.2 Evolution of the different forms of C. perfringens in the corn flour sold

Microbiological analyses revealed that vegetative forms persisted in all samples except E26. In this sample, only spore-forms were present, with an average load of 1.36±0.64, and the germs in vegetative form of Clostridium perfringens had all been transformed into spore-forms (Figure 1).

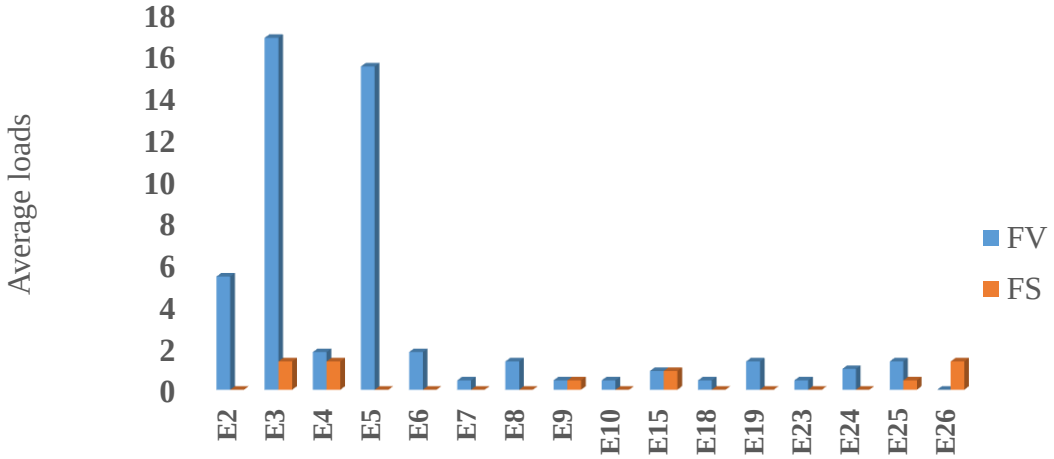

Figure 1 : Level of C. perfringens contamination in cornmeal samples

3.3. Antibiotic resistance of C. perfringens isolates

3.3.1. Resistance of C.perfringens isolates to beta-lactams

Resistance to beta-lactam antibiotics is variable. The highest resistance was recorded for amoxicillin + clavulanic acid, with a rate of 83.86%. Resistance rates for cefoxitin and ampicillin were 45.45% and 63.64% respectively (Figure 2).

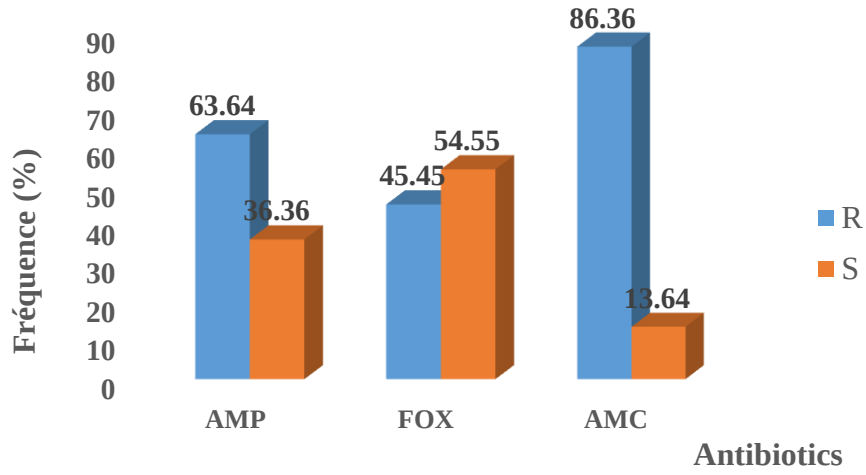

Figure 2 : beta-lactam resistance of C. perfringens isolated from corn flour

3.3.2. Resistance of C. perfringens isolates to other antibiotics

Resistance in C. perfringens isolates varies from one antibiotic to another. Resistance to amoxicillin + clavulanic acid is highest at 86.36%, and lowest at 13.63% for gentamicin. In addition, four antibiotics have resistance levels above 50%. These are Doxycycline, Erythromycin, Ampicillin and Ciprofloxacin, with resistance rates of 81.82%, 72.73%, 63.64% and 54.55% respectively. Some isolates were resistant to cefoxitin (45.45%), chloramphenicol (36.36%) and tetracycline (31.82%) (Figure 3).

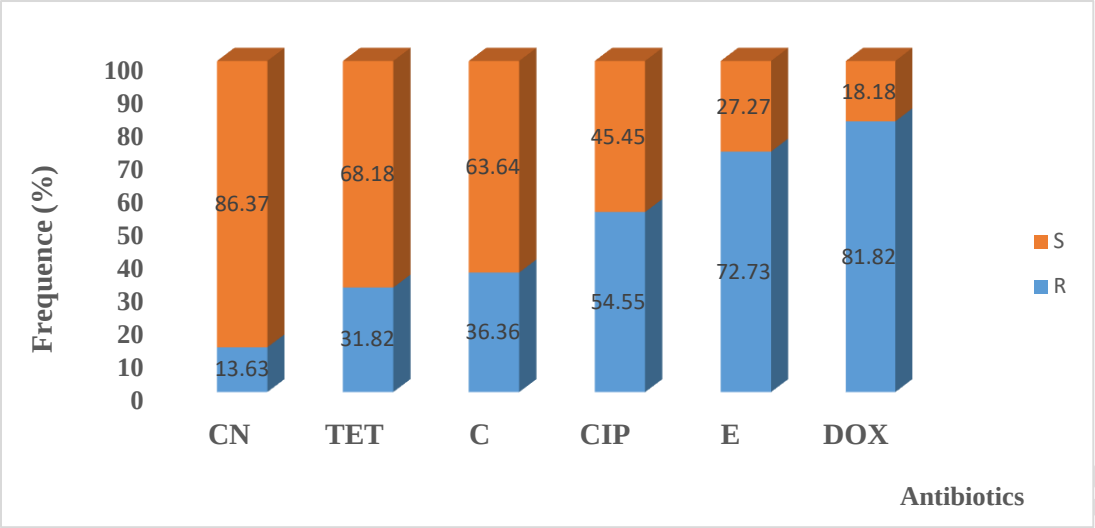

Figure 3 : Antibiotic resistance of Clostridium perfringens strains

3.3.3. Multiresistance of Clostridium perfringens isolates

The isolates studied were multi-resistant. Isolates obtained from millet flour were resistant to two, three, four, five, six, seven and eight antibiotics. Some isolates were resistant to two and four antibiotics at a rate of 9.09% each, while others were resistant to seven and eight antibiotics at a rate of 4.54%. Resistance to three antibiotics was also observed in isolates with a rate of 13.63%. Multi-resistance was observed in isolates resistant to five and six antibiogram molecules at rates of 31.81% and 27.27% respectively (Figure 4).

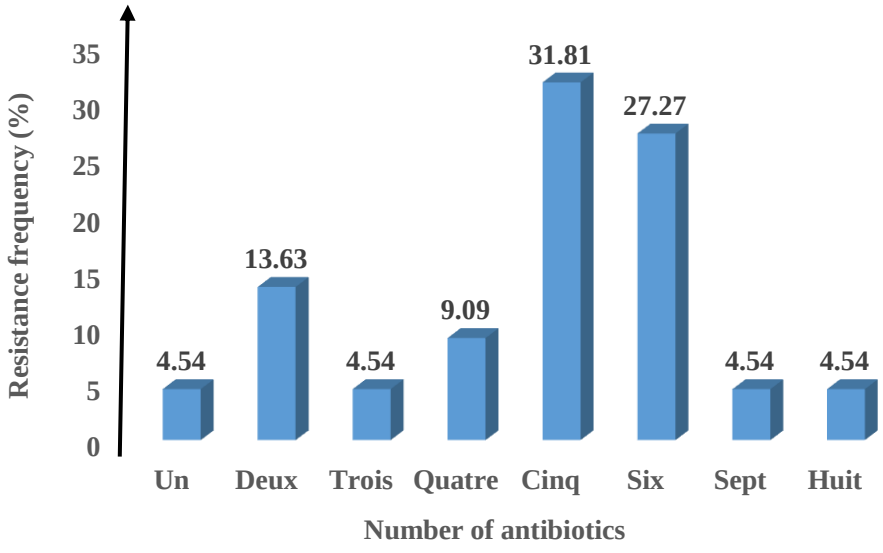

Figure 4 : Isolate resistance frequencies as a function of number of antibiotics

3.3.4. Diversity of C. perfringens isolated from maize meal

The diversity of C. perfringens isolates was assessed through resistance to antibiotic molecules. Taking into account this observed resistance, 17 profiles were established, ranging from c1 to c16. Apart from profile c4, with a single isolate (LoMi2) resistant to one antibiotic, multiple resistances were observed in the other profiles. These were profile c8, c9 and c12, with isolates GmMi6, GmMi7 and GmMi11 resistant to 2 antibiotic molecules, and profile c17, with isolate LoMi6 resistant to 3 molecules. Resistance to 4 molecules is also noted in isolates LoMi5, GmMi14 of profile c10 and c16, and resistance to 5 and 6 molecules respectively in isolates GmMi4, LoMi3, LoMi4, GmMi8, GmMi9, GmMi10 and LoMi9 of profile c5 with isolates GmMi1, GmMi2, GmMi3, LoMi1, GmMi12, and LoMi7 of profile c6. As for profiles c7 and c8, the isolates are multi-resistant to 7 and 8 antibiotics at a time. These are respectively isolates GmMi5 and GmMi13 (Table II).

Table II: Antibiotic resistance profiles of C. perfringens isolates

Profiles	**E**	**C**	**AMC**	**CIP**	**DOX**	**CN**	**AM**	**FOX**	**TET**	**Affected isolates**
c1	R	S	R	S	R	S	R	R	R	GmMi1, GmMi2 GmMi3, GmMi126
c2	R	R	S	R	S	S	R	S	R	GmMi4
c3	R	R	R	R	R	S	R	S	S	LoMi1 6
c4	S	S	R	S	S	S	S	S	S	LoMi2
c5	R	S	R	R	R	S	R	S	S	LoMi3, GmMi8, GmMi10
c6	R	R	R	R	S	S	S	R	S	LoMi4
c7	R	R	R	R	R	S	R	R	S	GmMi5
c8	S	S	S	S	R	S	S	R	S	GmMi6
c9	S	S	S	S	S	S	S	R	R	GmMi7
c10	R	R	R	S	R	S	S	S	S	LoMi5
c11	R	S	R	S	R	S	R	R	S	GmMi9
c12	S	S	R	S	R	S	S	S	S	GmMi11
c13	R	R	R	R	R	R	S	R	R	GmMi13
c14	R	R	R	R	R	R	S	S	S	LoMi7 6
c15	S	R	R	R	S	R	R	S	S	LoMi8
c16	R	S	R	R	S	S	R	S	S	GmMi14
c17	S	S	R	R	S	S	R	S	S	LoMi6

4. Discussion

The study shows that flours from artisanal production sold in public markets in Daloa were contaminated with Clostridium perfringens. This bacterium, which causes food poisoning, can be fatal for children. The presence of Clostridium perfringens is the result of poor hygienic practices during flour production. It should be noted that corn flour is produced using methods based on a shared experience that lacks scientific rigor. The main players are predominantly Malinké (81.40%), a people from northern Côte d'Ivoire where cereal crops predominate [3]. Flour is generally sold by midwives in health centers, but also in markets by women who have no notion of hygiene or good manufacturing practices. These factors constitute a risk, resulting in finished products of unsatisfactory microbiological quality.

The presence of C. perfringens in flours could also be explained by the fact that flours intended for sale are in most cases not covered. Exposure to the open air leads to contact with physical agents such as dust and sand, resulting in rapid contamination with C. perfringens, a ubiquitous, telluric pathogen. In some cases, initially healthy flours become contaminated during the cooking process. The use of soiled utensils and the non-application of hygiene rules during the preparation of porridges can also be a cause of contamination [17].

In addition, the microbial load assessment shows that vegetative forms are more abundant in some flour samples than spore-forming forms, indicating that the flours used for analysis are mostly recent productions, and therefore present recent contamination with C. perfringens. In this type of case, contamination may be due to The recent development of C. perfringens strains does not allow vegetative forms to sporulate, as they are not under stress. Thus, the probability of food poisoning after ingestion of these flours remains low, as the flour samples show a preponderance of vegetative forms. What's more, none of the samples exceeded the current standard of 102 cfu/g of flour. Baking reduces the risk of contamination, but does not eliminate it. However, vegetative forms of C. perfringens are thermolabile and hemolytic, and therefore require heat treatment at 100°C for 10 minutes to inactivate them [17]. Antibiotic susceptibility testing of Clostridium perfringens isolates revealed the existence of resistance, with variable dimensions depending on the antibiotics selected. In fact, 9 (nine) antibiotics were tested on the different strains in order to carry out the antibiogram. These were

ampicillin, amoxicillin + clavulanic acid, cefoxitin, gentamicin, chloramphenicol, ciprofloxacin, erythromycin, tetracycline and doxycycline.

A resistance rate of 86.36 □ was observed for amoxicillin + clavulanic acid, 63.64 □ for ampicillin and 45.45% for cefoxitin. These antibiotics belong to the β-lactam family, mainly to the penam groups. Studies carried out by [18] show ampicillin resistance at 61.02%, which is lower than in the current study. This resistance is due to the composition of the C. perfringens wall. When β-lactam antibiotics come into contact with the strain, the structure of the outer membrane is modified by the loss of porins, making the wall impermeable to β-lactam antibiotics [19]. The production of β-lactamase is the most frequent mechanism of resistance. All strains secrete a chromosomal β-lactamase encoded by the cepA gene [20]. On the other hand, resistance genes seem to have developed in bacteria as a result of antibiotic overuse [21]. With regard to the aminoglycoside family, gentamicin was tested. The results of this study show that C. perfringens strains have a resistance rate of 13.63%. This low level of resistance indicates that gentamicin has a deleterious effect on C. perfringens. A study by [18] found a gentamicin resistance rate of 35.59 □ which is higher than that recorded in the current study. Given that C. perfringens strains carry plasmids, which often code for virulence-associated proteins [22], gentamicin's bactericidal effect is based primarily on inhibition of protein synthesis by binding to the 30S subunit of bacterial ribosomes, thus altering cell membrane permeability. This leads to progressive rupture of the cell envelope, followed by cell death. Tetracycline and doxycycline have resistance rates of 31.82% and 81.82%, respectively. They both belong to the tetracycline family. Another study on C. Perfringens isolated from raw camel milk reported a tetracycline resistance rate of 37.29% which is higher than the results obtained in this study. These results are comparable to those found by [23], who in his study noted a resistance rate of C. perfringens strains to tetracyclines of between 10 and 76 %. These differences between tetracycline and doxycycline levels are due to the T and P determinants of the Clostridium perfringens PCW3 conjugative plasmid. The two functional tetracycline resistance genes tetA(P) and tetB(P) overlap. The tetA(P) gene encodes a putative 46 kDa transmembrane protein that mediates active tetracycline efflux from the cell, while tetB(P) encodes a putative 72.6 kDa protein that shows significant similarity to proteins from resistance to Tet M type tetracycline. Chloramphenicol with a resistance rate of 36.36%, is part of the phenicol family. These results are superior to those of [24] who noted a resistance of C. perfringens to chloramphenicol of 20%. This resistance to chloramphenicol in C. perfringens may be mediated by the cat P genes which encode

chloramphenicol acetyltransferases. The cat P gene of C. perfringens is located on the Tn 4451 and Tn 4452 transposons.

A resistance of 72.73 % was recorded for erythromycin, belonging to the Macrolide family. These results are lower than those of [25], who during their research carried out in Egypt on necrotic enteritis caused by C. perfringens mentioned a 100% rate of resistance to erythromycin in strains of different species. However, a lower level of C. perfringens resistance to erythromycin reaching 26% of isolates was reported in another study [26]. This strong resistance to erythromycin observed would be due to differences in exposure of C. perfringens to different levels of antibiotic stress in different localities. This could be explained by the presence of other macrolide resistance genes such as MCE (Q) or mce (A). Studies carried out by [27] also show the presence of the macrolide resistance gene (erm B) in 80% of isolates.

Furthermore, ciprofloxacin belonging to the Fluoroquinolones family displays a resistance rate of 54.55%. A study on the evaluation and optimization of the antibiotic resistance profile against Clostridium perfringens from buffaloes and cattle in Pakistan carried out by [28] shows low resistance to ciproflaxin, i.e. 25%. The resistance rate of the study is lower than that obtained in the present study.

5. CONCLUSION

The results showed a predominance of vegetative forms in the flours analyzed, reflecting recent contamination thereof. Likewise, the presence of C. perfringens in flours is a manufacturing defect linked to hygiene, but also to the conditions of sale. The sales environment, often unsanitary, the production, manufacturing, storage and distribution chain are also directly linked to the problem of hygiene not respected by the various players in the sector. The study of the resistance profile of Clostridium perfringens isolates revealed multiple resistance of the isolates to the antibiotics used. Antibiotic resistance in Clostridium perfringens is a worrying public health problem because it makes the treatment of infections more difficult or impossible. It leads to an increased rates of treatment failure, complications and prolonged disease. This resistance could result from overuse, underuse, or inappropriate use during antibiotic treatment.

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