

Phenotypic characterization and antibiotic resistance of *Bacillus cereus* isolated from local infant corn flour sold in Daloa markets (Côte d'Ivoire).

ABSTRACT

Obtaining corn flour as a complementary food for infants is done by artisanal methods by women, which could compromise their hygienic quality. The objective of this work was to determine the presence of *Bacillus cereus* in corn flour intended for infant consumption. Ninety samples of corn flour were collected from vendors in the markets of Lobia, Orly, Abattoir du grand marché in Daloa (Côte d'Ivoire). These samples were cultured on Mossel agar and incubated at 30°C for 24 hours. Lecithinase-positive colonies, a total of 150, were extracted. Biochemical tests were then carried out, including Gram staining, catalase test, glucose and mannitol fermentation, followed by motility analysis and Simmons citrate utilization. Confirmation of the isolates with the MALDITOF test was carried out. An antibiogram was performed to assess the resistance of *Bacillus cereus* strains to the various selected antibiotics. A total of 85 strains, or 56.67%, were identified as *Bacillus cereus*. Of the remaining 65 isolates, 44 strains, or 29.33%, were *Bacillus subtilis* ssp. *subtilis*, and 21 strains, or 14.00%, were *Bacillus velezensis*. The antibiogram revealed multiple resistances ranging from 2 to 9 antibiotics depending on the strain. It is worth noting high resistance to beta-lactams, with high rates (60%) for imipenene and 53.33% for amoxicillin + clavulanic acid (Amc). The uncontrolled use of antibiotics in the food environment poses a serious public health problem because it causes multiple resistance in *Bacillus cereus* strains.

Keywords: *Bacillus cereus*; infants; corn flour; lecithinase, *Bacillus velezensis*

1. INTRODUCTION

According to the WHO, all mothers worldwide should exclusively give their infants breast milk for the first six months to ensure proper development, growth, and optimal health (Keita & Sangho, 2018). From the age of six months, breast milk alone is no longer sufficient to meet infants' growing nutrient needs. Infants then require other food sources to ensure their proper

development. This is the period of infant dietary diversification. Infant dietary diversification preferably begins with solid foods in liquid or semi-liquid form, such as porridge, juice, and often purees, due to the lack of strong dental apparatus and the fragility of the digestive tract (Maillier *et al.*, 2019). Cereals, particularly corn, millet, and soy, are therefore the raw materials frequently used for the manufacture of complementary foods for infants and young children. Maize (*Zea mays* L.) is one of the most essential cereals used in human nutrition. At 41%, maize is the world's leading cultivated cereal, with a total production exceeding that of wheat, which is 40%, and rice, which is also estimated at 9% (Droh *et al.*, 2022). African maize production is estimated at over 70 million tonnes for an area of approximately 34 million hectares. Maize accounts for almost half of Africa's calorie and protein intake, and this commodity is a source of livelihood for over 300 million people in sub-Saharan Africa (Harold & Tabo, 2015). Côte d'Ivoire is among the top 20 maize-producing countries in sub-Saharan Africa (O'Neil *et al.*, 2022). In Côte d'Ivoire, maize is the staple food for many Ivorian populations. Its production increased from 661,285 tonnes in 2013 to 1,025,000 tonnes in 2017 (Siene *et al.*, 2020). This plant is cultivated in a large part of the regions of Côte d'Ivoire. These are the regions of Poro, Hambol, Folon, Kabadougou, and Worodougou in the north, but also Bagoué, Haut Sassandra, and Beré. The finished products of this plant are essential in infant nutrition (Tshite & Ndianabo, 2015). Depending on the needs, maize is used in several forms, namely in flour for industry, drilling, paper industry, etc. Furthermore, due to low purchasing power in underdeveloped countries, many families do not have access to infant flour produced in industries (Gbogouri *et al.*, 2019). In Côte d'Ivoire, mothers process cereals locally using their own technological knowledge or obtain them commercially (public markets, etc.) to feed their young children (Abro *et al.*, 2015, Kouton *et al.*, 2017; Nago *et al.*, 2018). Unfortunately, like many local products that lack suitable industrial procedures, corn flour is preferentially manufactured using artisanal or semi-artisanal processes in unsanitary conditions by millers with particularly low levels of education (N'goran *et al.*, 2017). This lack of hygiene can lead to the contamination of infant flours by numerous pathogenic microorganisms such as bacteria. Among these microorganisms, spore-forming bacteria such as *Bacillus cereus* are the most abundant in corn-based foods (Gdoura *et al.*, 2018). Recent work by N'guessan *et al.* (2019) reported a 31% prevalence of *Bacillus cereus* isolated from infant corn flour. Also, 0.61% of cases of acute bacterial diarrhea in infants were caused by *B. cereus*. *Bacillus cereus* is responsible for two types of symptoms characterized by diarrheal syndrome and emetic-like syndrome (Tuipulotu *et al.*, 2021). Furthermore, strains can resist certain antibiotics, making it difficult to treat the foodborne infections they cause. In recent years, there has been an increase in foodborne

pathogens resistant to various antimicrobials, posing serious threats to public health. Studies and reports have indicated that some strains of *Bacillus cereus* isolated from different foods are resistant to a wide range of antimicrobials such as ampicillin, penicillin, streptomycin, tetracycline, trimethoprim and ceftriaxone (Aklilu *et al.*, 2016; Yibar *et al.*, 2017). The overall objective of this study is to determine the presence of *Bacillus cereus* in corn flour intended for infant consumption.

2. MATERIALS AND METHODS

2.1. Sampling

A total of ninety (90) samples of potash-free corn flour were collected from four markets for this study. These were Lobia Market, Orly Market, Abattoir Market, and Grand Marché Market.

2.2. Analysis of the Collected Samples

For the preparation of the stock suspension, 10 g of corn flour from each sample was weighed and then added to 90 ml of previously prepared Buffered Peptone Water in a flask. The mixture was homogenized for 1 minute to obtain the stock suspension. This suspension was left on the bench at room temperature for 30 minutes for the microorganisms to multiply. Approximately 1 ml of the contents of each stock suspension is added to nine ml of sterile distilled water to make decimal dilutions (NF EN ISO 6887-1, 2017).

2.2.1. Heat Treatment of Spores

The spores are treated in test tubes using the previously prepared stock suspension. A 10 ml sample is withdrawn and added to the test tube. The tubes are then placed in a water bath at 80°C for 10 minutes and then instantly cooled in an ice-filled water bath. Approximately 1 ml of the tube contents is added to 9 ml of sterile distilled water for decimal dilutions.

2.2.2. Inoculation and Incubation

Approximately 0.1 ml of each ten-fold dilution is placed in a Petri dish containing 20 ml of prepared and poured agar. The 0.1 ml is spread over the surface of the agar using a sterile spreader. The inoculated plates are incubated for 24 hours at 30°C to detect spore-forming forms and vegetative cells of *Bacillus cereus*.

2.2.3. Extraction and Purification of Presumptive *Bacillus cereus* Isolates

After 24 hours of incubation, three presumptive *Bacillus cereus* colonies were selected per plate. A total of 150 presumptive *B. cereus* isolates were extracted. Colonies with opaque borders were extracted using a platinum loop and then introduced into Brain Heart Broth for

further work. From the Brain Heart Broth, the colonies were subcultured onto nutrient agar and incubated at 37°C for 24 hours for isolation and purification. These isolates were purified for further work..

2.2.4. Identification of presumptive isolates of *Bacillus cereus* isolated from flours

It started with the catalase test, followed by the stained smear, then the anaerobic fermentation of glucose on Kligler Hajna medium was carried out. Mannitol mobility medium allowed to observe the utilization of mannitol and the mobility of the isolates. The use of Simmons citrate as the sole carbon source by the presumptive isolates of *Bacillus cereus* was observed. Identification with the MALDI-TOF technique was also carried out.

2.3. Carrying out the antibiogram of the identified isolates

Pure 24-hour cultures of *B. cereus* are used for the preparation of inocula. A suspension is made in a hemolysis tube by bubbling pure 24- to 48-hour colonies obtained on nutrient agar into 2 mL of sterile physiological saline. Once well homogenized, the suspension is read with a Densimat by adjusting it until an opacity of 0.5 McFarland is obtained. The final inoculum of the species is obtained by adding 100 µl of this suspension to 10 mL of sterile physiological saline placed in a screw-top test tube. This final suspension containing approximately 10^8 CFU/ml is the inoculum used for the antibiogram (Bauer *et al.*, 1966). To do this, a previously prepared volume of inoculum (10 mL) is poured onto the surface of the prepared Muller Hinton agar and poured at a rate of 20 mL per dish. After a contact time of 5 minutes, the excess inoculum is removed with a micropipette. Using a sterile swab, the excess inoculum is removed by pressing on the edges of the dish by tilting it 60°C. The dish is dried at 37°C for 3 min. The antibiotic discs are applied aseptically with forceps to the surface of the agar. The reading was carried out taking into account the measurements of the growth inhibition zones around the disc (CA-SFM, 2023).

3. RESULTATS

3.1. Presumed *Bacillus cereus* isolated from corn flour

Macroscopic and microscopic identification carried out on 150 presumed isolates of *B. cereus* revealed that after further identification with the MALDITOF test, there were 85 strains of *Bacillus cereus*, either a rate of 56.67%, 44 strains of *Bacillus subtilis ssp subtilis*, either a rate of 29.33% and 21 strains of *Bacillus velezensis*, either a rate of 14.00% (Figure 1).

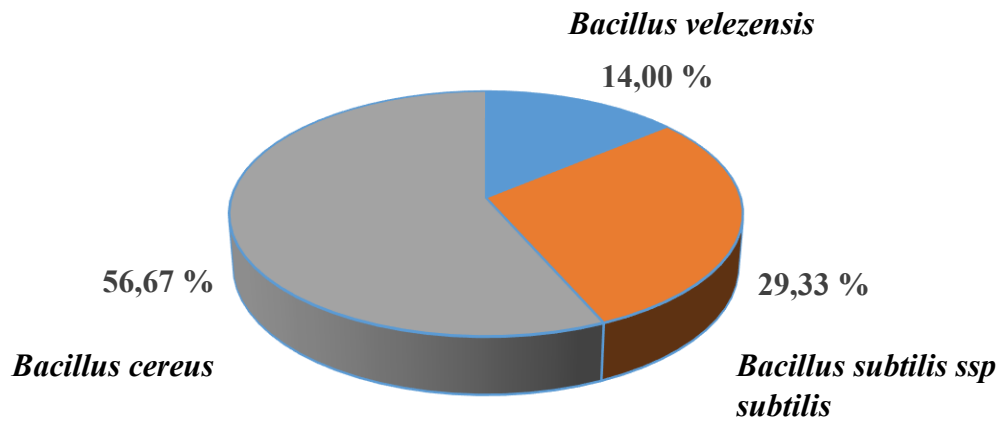
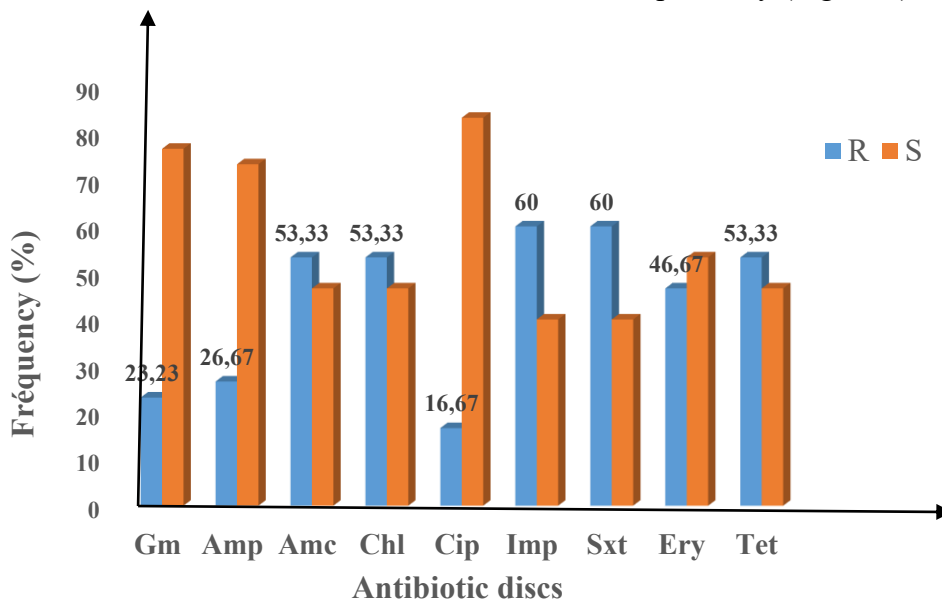


Figure 1: Prevalence of *Bacillus* species isolated from corn flours

3.2. Resistance of *Bacillus cereus* strains to antibiotics

Resistance among *B. cereus* strains is highly variable. Indeed, the strains were resistant to Imipenem (Imp) and Trimethoprim-sulfametoxazole (Sxt) at a rate of 60%. As for Tetracycline (Tet), Chloramphenicol (Chl), and Amoxicillin + clavulanic acid (Amc), a resistance rate of 53.33% each was noted. As for erythromycin (Ery), a rate of 46.67% was recorded. Ampicillin (Amp) recorded a resistance of 26.67%, while Gentamicin (Gm) and Ciprofloxacin (Cip) recorded resistance rates of 23.23% and 16.67%, respectively (Figure 2).



R: Resistant and S: Susceptible

Figure 2: Antibiotic resistance of *Bacillus cereus* isolated from corn flour

3.3. Resistance of *Bacillus cereus* strains to beta-lactams

The resistance observed at the beta-lactam level is highly variable. The highest resistance was observed at the Imipenem (Imp) molecule level, with a rate of 60%, and the lowest at the Ampicillin (Amp) level, with a rate of 26.67%. As for Amoxicillin + clavulanic acid (Amc), a resistance rate of 53.33% was recorded (Figure 3)

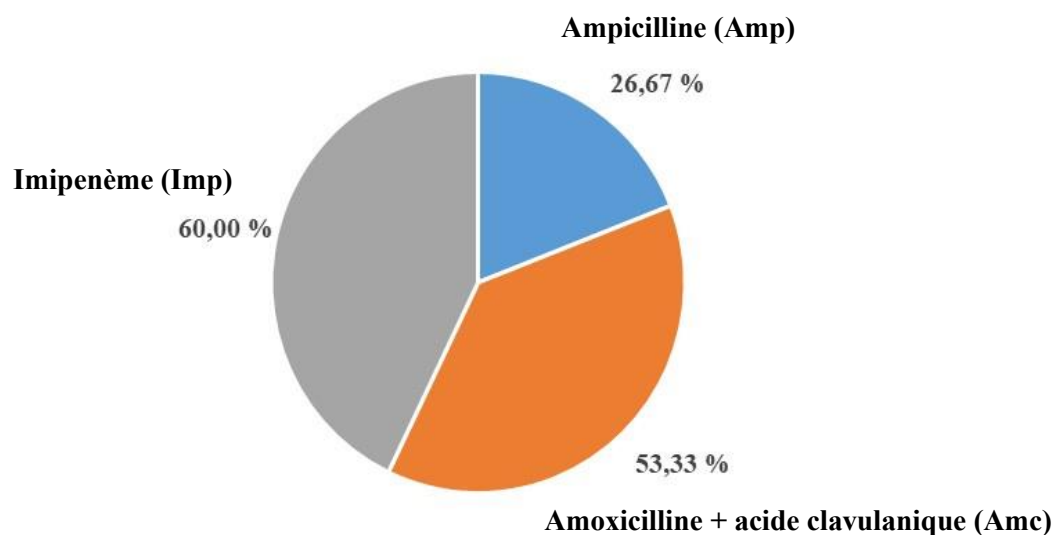


Figure 3: Beta-lactam resistance of *B. cereus* isolated from corn flour

3.4. Multiple resistance of *Bacillus cereus* strains to antibiotics

It is worth noting a multi-resistance (beyond 2 molecules) of *Bacillus cereus* isolates from corn flour from the different markets of the city of Daloa. Thus, 59 isolates recorded resistance to more than 2 antibiotic molecules. Furthermore, with a rate of 10.17%, 6 isolates are resistant to both two (2) and three (3) antibiotics. Similarly, eight (8) isolates are resistant to five (5) antibiotics with a rate of 13.56%. About twenty-four (24) isolates are resistant to six (6) antibiotics with a rate of 40.68, followed by 12 isolates resistant to (7) antibiotics with a rate of 20.34%. Three isolates are resistant to the nine antibiotic molecules used in this study with a rate of 05.08% (Table 1).

Table 1: Distribution of resistance of *B. cereus* strains according to the number of antibiotics

NUMBER OF ANTIBIOTICS	NUMBER OF ISOLATES	FREQUENCY (%)
TWO (2)	6	10.17
TREE (3)	6	10.17
FIVE (5)	8	13.56
SIX (6)	24	40.68
SEVEN (7)	12	20.34
NINE(9)	3	05.08
TOTAL	59	100

3.5. Diversity of multidrug-resistant *Bacillus cereus* isolated from corn flour

The diversity of multidrug-resistant *Bacillus cereus* was assessed by analyzing the antibiogram profile. The antibiogram revealed 15 resistance profiles numbered B1 to B15. The origin of the isolates and the molecules to which they are resistant are listed in the table below (Table 2).

Table 2: resistance profiles of *Bacillus cereus* strains to antibiotics

PROFILES	ANTIBIOTIC MOLECULES	ISOLATES CONCERNED
B1	Chl-Ipm	LoMa17, LoMa40, GdMa18
B2	Gm-Ipm	OrMa25, OrMa45, GdMa21
B3	Chl-Ipm-Sxt	OrMa8, AbMa14, LoMa9
B4	Chl-Sxt-Ery	GdMa33, OrMa11, AbMa35
B5	Amc-Chl-Ipm-Sxt-Tet	AbMa32, AbMa22
B6	Amp-Ipm-Sxt-Ery-Tet	LoMa22, GdMa17, GdMa7
B7	Gm-Amp-Amc-Ipm-Sxt	GdMa5, LoMa19, AbMa44
B8	Amc-Chl-Ipm-Sxt-Ery-Tet	GdMa13, GdMa39, LoMa9, LoMa21, AbMa13, AbMa3
B9	Gm-Amp-Amc-Sxt-Ery-Tet	LoMa14, OrMa15, AbMa8, AbMa42, GdMa15
B10	Amp-Amc-Chl-Ipm-Ery-Tet	LoMa4, LoMa6, OrMa7, AbMa34 GdMa38, GdMa10, OrMa3
B11	Amp-Chl-Ipm-Sxt-Ery-Tet	OrMa20, LoMa11, GdMa25 GdMa22, AbMa17, OrMa24
B12	Amp-Amc-Chl-Cip- Sxt-Ery-Tet	AbMa15, GdMa15, GdMa1, LoMa2
B13	Amp-Amc-Chl- Ipm-Sxt-Ery-Tet	OrMa31, LoMa24, GdMa3 AbMa12
B14	Amc-Chl- Cip- Ipm-Sxt-Ery-Tet	AbMa10, OrMa13, GdMa5, GdMa19
B15	Gm-Amp-Amc- Chl-Cip- Ipm-Sxt-Ery-Tet	AbMa30, GdMa12, LoMa16

Imipenem (Imp), Ampicillin (Amp), Amoxicillin + clavulanic acid (Amc), Trimethoprim-sulfametoxazole (Sxt), Tetracycline (Tet), chloramphenicol (Chl), Erythromycin (Ery), Ampicillin (Amp), Gentamicin (Gm), Ciprofloxacin (Cip), Lo; Lobia, Gd; Grand, Ab: Abattoir, Or: Orly, Ma; Market

4. DISCUSSION

Of the 150 presumed *Bacillus cereus* isolates subjected to biochemical tests, 56.67% were found to be *Bacillus cereus*. These results are inconsistent with previous work by N'guessan *et al.* (2019) and Yao *et al.* (2016) who found a prevalence of 51% for *Bacillus cereus* bacteria in maize, millet and cassava flours. These results could be explained by the poor drying and storage conditions of the grains before milling. In harvesting areas, mainly rural areas, maize is left to dry on the roadsides on the ground and packaged in bags that have been used several times before. Monitoring bacterial resistance to antimicrobials commonly used in therapy, both in humans and animals, is of paramount importance, in order to implement a strategy to prevent the spread of multi-resistant bacteria. The resistance of *Bacillus cereus* isolates in locally produced corn flour from public markets in the city of Daloa was tested with 9 different antibiotics in order to establish an antibiotic resistance profile. The study of the diversity of *Bacillus cereus* strains showed the existence of resistance, with different proportions depending on the antibiotics used. Indeed, out of 9 (nine) antibiotics tested, *Bacillus cereus* strains show a rate of 60% to trimethoprim-Sulfamethoxazole and β -lactams including imipenem 60%, amoxicillin + clavulanic acid 53.33%, ampicillin records a resistance of 26.67%. These results are in line with recent studies carried out by Kouassi *et al.* (2020, 2021) which presented the resistance of *Bacillus cereus* to trimethoprim-Sulfamethoxazole, β -lactams such as ampicillin, amoxicillin + clavulanic acid. Also other studies carried out by Luna *et al.* (2007); Park *et al.* (2009) and Chon *et al.* (2012) confirmed the results obtained in this study regarding resistance to β -lactams. Furthermore, bacteria of the *B. cereus* group, except *B. anthracis* which declines sensitivity to penicillin, are mostly resistant to trimethoprim and certain antibiotics of the β -lactam family. *Bacillus cereus* is well known for its ability to produce β -lactamase enzymes. Furthermore, they are sensitive to Chloramphenicol, Ciprofloxacin, Erythromycin, Gentamycin, Tetracycline (Luna *et al.*, 2007; Chaves *et al.*, 2011; Chon *et al.*, 2012). In the study carried out on flours, multiple resistance of *B. cereus* isolates to the tested antimicrobials was observed. Thus, several studies carried out by Navaneethan & Effarizah (2021) have shown that *Bacillus cereus* is multi-resistant to several antimicrobials, indicating that almost all of the resistance observed is acquired resistance. Indeed, *Bacillus cereus* has a resistance rate to ciprofloxacin 16.67%, chloramphenicol 53.33%, tetracycline 53.33%, and also an erythromycin rate of 46.67% is noted. As for gentamicin, a resistance rate of 23.23% was highlighted. The results reveal a worrying emergence of strains of *Bacillus cereus* multi-resistant to antibiotics. This resistance is generally associated with mobile genetic elements which play a very important role in the dissemination of several antibiotic resistance genes in isolates (Chen *et*

al., 2022). The high prevalence of these multidrug-resistant strains isolated from corn flour suggests that the most commonly used antibiotic is no longer effective in treating infections caused by *Bacillus cereus*. The antimicrobial resistance patterns of *B. cereus* observed in food are particularly useful in epidemiological studies. Indeed, according to recent work by Zhou *et al.* (2010); Kouamé (2020); Chen *et al.* (2022), a greater proportion of multidrug-resistant patterns among *Bacillus cereus* isolates originate exclusively from food and food-derived products. These multidrug-resistant patterns indicate that antimicrobials are likely being misused or used at sublethal doses in the environment. This has favored the acquisition and spread of these resistances within bacterial populations. This situation highlights the importance of prudent and rational use of antibiotics, in order to limit the emergence and spread of these multi-resistant bacterial strains, which represent a major challenge in terms of public health.

5. CONCLUSION

The different morphological and biochemical characteristics of the *Bacillus cereus* group strains isolated from flours allowed the confirmation of *Bacillus cereus* strains. Multiple resistances were observed during the study. The emerging multidrug resistance of *Bacillus cereus* in food poses a significant challenge for the treatment of infections and requires increased monitoring of antibiotic use in the food environment.

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