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Prevalence and critical antibiotic-resistance traits of *Bacillus cereus* and *Staphylococcus aureus* isolated from raw and ready-to-eat meat products.

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ABSTRACT

The study aimed to compare *Bacillus cereus* (*B. cereus*) and *Staphylococcus aureus (S. aureus*) prevalence and antimicrobial resistance profiles in raw and ready-to-eat meat products (RTEM). One hundred samples of raw hamburger, sausage, RTEM hawawshi, and kofta were tested using standard culture and automated VITEK2 methods. PCR was used to detect resistance genetic components. The overall prevalence of *S. aureus* and *B. cereus* was 20%, and 14%, respectively. Staphylococcus aureus and *B. cereus were* isolated at similarly high rates (60.0% and 73.7%, respectively) from raw meat products. Five percent (1/20) of the *B. cereus* isolates detected in raw burger samples were multidrug-resistant (MDR), whereas 25 % of *S. aureus* were MDR, with three resistance patterns. Neither the *mcr*1 nor the *van*A genes were found in the *B. cereus*. While *nor*A was found in four isolates, both bla_{TEM} and bla_{CTX} were found in three. The bla_{SHV} was found only in three raw-derived isolates, two of which also shared bla_{TEM}, bla_{CTX}, and *nor*A or bla_{TEM} and *nor*A. The data revealed that all of the MDR *S. aureus* isolates tested positive for *mec*A but not *van*A genes. Such pathogens in RTE meat with genes confer resistance to key antibiotics, endangering public health and hastening the emergence of superbugs.

1. INTRODUCTION

Staphylococcus aureus is accountable for a substantial portion of the global burden of foodborne illness in both developed and developing countries (Ou et al., 2017). Staphylococcus aureus is intimately related to key human foods such as dairy, beef, and chicken and is thus seen as a potential vehicle of *S. aureus* transmission from farm into human households (Thapaliya et al., 2017). Meat has a lot of protein, which gives *S. aureus* the amino acids and low-molecular-weight peptides needs to not only thrive but also grow and produce enterotoxins (Ou et al., 2017). Unfortunately, estimates of the global burden of *S. aureus* and *Bacillus cereus* (*B. cereus*) disease are still incomplete because only incidence data from high-income subregions could be retrieved (WHO, 2015). The lowest and highest incidences of *B. cereus* intoxication were 7.9-58.3 per 100,000 based on data from the United States of America (5.2-49.4) and the Netherlands (11.5-67.2), respectively. The median incidence of *B. cereus* intoxication was 21.4 per 100,000 and was documented in England (WHO, 2015).

Antimicrobial resistance (AMR), according to the World Health Organization, is a major global concern that has already reached harmful levels in various regions of the world (WHO, 2022). Antimicrobial resistance already imposes a significant economic and social strain. It is estimated that it causes 700,000 deaths worldwide (O'Neill, 2016). It is anticipated that in the absence of proactive solutions to slow the rise of drug resistance now, humanity will lose 10 million lives per year and a total of 100 trillion USD in economic output by 2050 (O'Neill, 2016). With 27.3 fatalities per 100,000, the developing world, particularly western Sub-Saharan Africa, has the highest rate of all-age deaths caused by resistance (Murray et al., 2022).

Improved surveillance systems and the tight deployment of a monitoring and evaluation framework are among the most important steps for efficient AMR control and containment worldwide (Aenishaenslin et al., 2019). However, in low- and middle-income countries, inter-sectoral coordination among One Health stakeholders is undeveloped. More importantly, ready-to-eat foods such as beef burgers, hot dog sandwiches (Wang et al., 2013; Mahros et al., 2021), and cooked meat products (Hazards and Panel, 2016; Yu et al., 2020) have been linked to higher outbreaks and contamination rates of priority pathogens such as Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Bacillus cereus* (*B. cereus*). Therefore, the objective of this study was to determine the prevalence of the foodborne *S. aureus*, and *B. cereus* in raw burger and sausage and ready-to-eat meat products including Hawawshi and Kofta. Antibiotic resistance risks associated with isolated pathogens were also estimated.

2. MATERIAL AND METHODS

2.1. Ethical approval

All methods used in this study were approved by the Benha University, Faculty of Veterinary Medicine's Institutional Animal Care and Use Committee Research Ethics number (BUFVTM50-06-23).

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2.2. Sample collection.
A total of 100 random samples of four meat products (25 of each) were purchased from various butchers and restaurants in Benha City, Egypt. Burger and sausage were among the raw meat categories, while Hawawshi and kofta were included in the RTE category.

2.3. *Isolation and identification*
2.3.1. *Isolation and identification of S. aureus*
The ISO 6888-2 method was used to isolate *S. aureus* on Baird Parker agar plates (ISO, 1999). The cultures were identified separately using the Gram-positive identification (GPI card) of the automated VITEK2 system (compact model, bioMérieux).

2.3.2. *Isolation and identification of Bacillus cereus*
The horizontal ISO 7932:2004 method was used to isolate putative *B. cereus* cells. After enrichment on brain heart infusion broth (BHIB) containing polymyxin (100 U/ml) at 37°C for 24-48 hours, the Shinagawa (1990) method for isolation of *B. cereus* on Polymyxin-pyruvate-Egg yolk-Mannitol-Bromothymol blue agar (PEMBA) media was applied simultaneously. Finally, the colonies were identified independently using the automated VITEK2 system's Bacillus identification card (BCL) (compact model, bioMérieux) (Halket et al., 2010).

2.4. Disk diffusion antimicrobial susceptibility testing.
The Kirby-Bauer disc diffusion method was used to test antimicrobial susceptibility as fully outlined in our prior research (Sabeq et al., 2022). Pathogens were evaluated for phenotypic resistance to nine regularly used antibiotics that are both vital and critical in Egypt's veterinary and medical sectors. The five antibiotic classes included beta-lactams such as ampicillin (AMP, 30 µg) and penicillin (PCN,10 IU); aminoglycosides such as gentamicin (GEN,10 µg), kanamycin (KAN, 5 µg) and neomycin (NEO, 30 µg); fluoroquinolones such as ciprofloxacin (CIP, 5 µg) and enrofloxacin (ENR, 5 µg); macrolide such as erythromycin (ERY, 15 µg); and third-generation cephalosporin that involved ceftriaxone (CTR, 30 µg).

2.5. *Molecular characterization of targeted pathogens for antimicrobial resistance*
PCR was applied to assess confirmed isolates for the presence of critical resistance genes including bla_{TEM} (Colom et al., 2003), bla_{CTX} (Mohamudha Parveen et al., 2012), bla_{SHV} (Rankin et al., 2005), *mecA* (Stegger et al., 2012), *norA* (Sierra, 2000), *mcr1* (Liu et al., 2016) , and *vanA* (Dutka-Malen et al., 1995) under comparable published conditions, as fully outlined in our prior research (Sabeq et al., 2022).

Table S1. PCR primers and conditions for *Bacillus cereus* and *Staphylococcus aureus*, isolated from raw and ready-to-eat meat products, gene amplification.

Target gene	Primer	Sequences (5' to 3')	Amplicon size (bp)	Annealing Temperature	Reference
bla_{TEM}	Forward	ATCAGCAATAAACCAGC0	516	55˚C	(Colom et al., 2003)
	Reverse	CCCCGAAGAACGTTTTC			
bla_{CTX}	Forward	CGC TTT GCC ATG TGC AGC ACC	307	54˚C	(Mohamudha Parveen et al., 2012)
	Reverse	GCT CAG TAC GAT CGA GCC			
bla_{SHV}	Forward	GGTTATTCTTATTTGTCGCTTCTT	1233	54˚C	(Rankin et al., 2005)
	Reverse	TACGTTACGCCACCTGGCTA			
norA	Forward	TTCACCAAGC CATCAAAAAG	704	60˚C	(Sierra, 2000)
	Reverse	GCACATCAAA TAACGCACCT			
mcr1	Forward	CGGTCAGTCCGTTTGTTC	305	60˚C	(Liu et al., 2016)
	Reverse	CTTGGTCGGTCTGTAGGG			
	Reverse	TGCTTGACCACTTTTATCAGC			
*van*A	Forward	GGGAAAACGACAATTGC	732	55˚C	(Dutka-Malen et al., 1995)
	Reverse	GTACAATGCGGCCGTTA			

2.6. *Safety assessment of studied meat products*
All raw and ready-to-eat (RTE) meat products were tested for safety in accordance with Egyptian Organization of Standardization (EOS) safety standards, ES:1973/2005 frozen balls (Kofta) specifications (EOS, 2005a), ES:1972/2005 frozen sausage specifications (EOS, 2005b), ES:1688/2005 specified for frozen burger (EOS, 2005c), ES: 1694/ 2005 minced meat specification (EOS, 2005d), ES: 4334:2004 fresh meat specification (EOS, 2004) and ES:2911/2005 frozen poultry sausage specifications (EOS, 2005e), to determine their safety for human consumption.
2.7. *Statistics*
Statistical analysis was performed using SPSS Statistics 20 (SPSS Inc., USA). The collected results from various sources were computed using descriptive statistics such as frequency, percentage, and/or proportion.

3. RESULTS

Overall, 25% of the studied samples were contaminated with *S. aureus* (11/25), *B. cereus* (5/25) or both (9/25) of which 60% (15/25) originated from raw products (Table 1). Contamination levels in the raw products (2 to 3.37 $\log_{10}$ CFU/gm) were higher than in RTEM samples (2 to 2.3 $\log_{10}$ CFU/gm), where five samples produced levels greater than 3 $\log_{10}$ CFU/gm. Bacillus cereus contamination varied from 2 to 3.46 $\log_{10}$ CFU/gm in raw products, with six samples exceeding 3 $\log_{10}$ CFU/gm, and from 2 to 3.15 $\log_{10}$ CFU/gm in RTEM samples. According to EOS guidelines, the maximum permissible limit of *S. aureus* in raw minced meat (EOS, 2005d) or examined raw products (EOS, 2005b, 2005e, 2005c, 2005a) is two $\log_{10}$ CFU/gm. As a result, all (11) samples above the two $\log_{10}$ CFU/gm limit are classified as unsuitable for human consumption. There were no explicit authorized EOS requirements for *B. cereus* in the examined RTEM products, although one sample would be unfit for consumption based on European (British (Health Protection Agency, 2009) and French (NSWFA, 2009) standards that permit no more than three $\log_{10}$ CFU/g of *B. cereus*.
The Kirby-Bauer disc diffusion antimicrobial susceptibility test for *S. aureus* revealed resistance rates ranging from 5 to 100% of the nine antibiotics tested. Five, ten, and fifteen percent of the *Staph. aureus* isolates were resistant to enrofloxacin, ciprofloxacin, and ceftriaxone, respectively, whereas forty and sixty-five percent of the isolates were resistant to beta-lactams ampicillin and penicillin, respectively. The aminoglycoside gentamicin generated the greatest antimicrobial effect to on *S. aureus*, leading to 35% resistance, while the entire isolates were completely resistant to kanamycin and 90% resistant to neomycin. Erythromycin resistance was found in 65% of *S. aureus* isolates. Gentamicin, fluoroquinolones, and ceftriaxone inhibited the growth of all *B. cereus* isolates, while 36 and 21% showed intermediate resistance to ciprofloxacin and enrofloxacin,

respectively. *Bacillus cereus* isolates produced the highest resistance rates to the beta-lactam antibiotics ampicillin (85.7%) and penicillin (100%), while exhibiting low resistance to erythromycin (7.1 %), kanamycin, and neomycin (14.3%).

Bacterial isolates that showed resistance to at least three different classes of antimicrobial drugs were deemed multidrug-resistant (MDR). Only one *B. cereus* isolated from raw burger was MDR to three different antibiotic classes, whereas 25% (three raw and two RTEM) of *S. aureus* isolates were MDR, with three resistance patterns ranging from three to five different antibiotic classes. The range of multiple antibiotic resistance index (MAR) values for raw-derived *S. aureus* isolates (0.33-0.89) was broader and higher than for RTEM (0.22-0.67). Similarly, raw-derived *B. cereus* isolates had wider and higher MAR index values (0.11-0.44) than RTEM-driven isolates (0.22-0.33) (Fig. 1).

Table 1. Incidences of the targeted pathogen isolated from raw and ready-to-eat (RTE) meat products (*n*=50).

Pathogen	Raw (n=50)					RTE (n=50)					Total (n=100)	
	Burger[1] (n=25)		Sausage[1] (n=25)		Subtotal[1] (n=50)	Hawawshi (n=25)		Kofta[1] (n=25)		Subtotal[1] (n=50)		
	No.	%[1]	No.	%	%	No.	%	No.	%	%	No.	%
B. cereus	6	24	4	16	20	2	8	2	8	8	14	14
S. aureus	5	20	7	28	24	4	16	4	16	16	20	20

[1] The incidence was determined per product by dividing positive samples by 25, and the category subtotal was obtained by dividing positive samples of either raw or RTE products by 50. [2] ND, not detected.

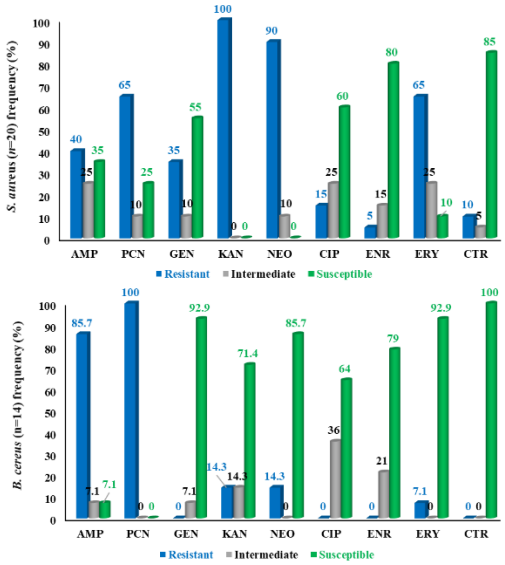

Figure 1 The antimicrobial susceptibility profile of *Bacillus cereus* and *Staphylococcus aureus* isolated from raw and ready-to-eat (RTE) meat products using a disc diffusion test. *B. cereus, Bacillus cereus; S. aureus, Staphylococcus aureus;* AMP, ampicillin (30 µg); PCN, penicillin (10 IU); GEN, gentamicin (10 µg); KAN, kanamycin (5 µg); NEO, neomycin (30 µg); CIP, ciprofloxacin (5 µg); ENR, enrofloxacin (5 µg); ERY, erythromycin (15 µg); CTR, ceftriaxone (30 µg).

The five MDR *S. aureus* isolates were genetically characterized for two genes, *mecA* and *vanA*, which are critical for distinguishing MRSA and vancomycin-resistant *Staphylococcus aureus* (VRSA). While, five *B. cereus* isolates, including one MDR, tested positive for *bla*$_{TEM}$, *bla*$_{CTX}$, *bla*$_{SHV}$, *mcr*1, *nor*A, and *van*A (Table 2). The data revealed that all ~~of~~ the MDR *S. aureus* isolates tested positive for *mec*A but not *van*A genes. Fortunately, neither the *mcr1* nor the *van*A genes were found in the *B. cereus* isolates. *Nor*A was identified in four of the *B. cereus* isolates, but *bla*$_{TEM}$ and *bla*$_{CTX}$ were identified in three isolates (3/5), with *bla*$_{TEM}$ being more common in raw-derived isolates (2/3) and *bla*$_{CTX}$ being ~~more~~ common in RTEM-derived isolates (2/3). The *bla*$_{SHV}$ was determined only in three raw-derived isolates (3/5), two of which also co-expressed *bla*$_{TEM}$, *bla*$_{CTX}$, and *nor*A or *bla*$_{TEM}$ and *nor*A.

Table 2 Antibiogram and multidrug resistance profiles of *B. cereus* and *S. aureus* isolated from raw and ready-to-eat meat products.

Pathogen/Serotypes (ID)	Origin	Resistance	
		Phenotypes	Genes[1]
B. cereus[2]	Burger	AMP, PCN, NEO, ERY	*bla*$_{TEM}$, *bla*$_{SHV}$, *norA*
	Burger	AMP, PCN	*bla*$_{SHV}$
	Sausage	AMP, PCN, KAN, NEO	*bla*$_{TEM}$, *bla*$_{CTX}$, *bla*$_{SHV}$, *norA*
	Hawawshi	AMP, PCN	*bla*$_{CTX}$, *norA*
	Kofta	AMP, PCN, KAN	*bla*$_{TEM}$, *bla*$_{CTX}$, *norA*
S. aureus[2]	Burger	AMP, PCN, GEN, KAN, NEO, CIP, ERY	*mecA*
	Sausage	AMP, PCN, GEN, KAN, NEO, CIP, ERY, CTR	*mecA*
	Sausage	AMP, PCN, GEN, NEO, KAN, NEO, CIP, ERY, CTR	*mecA*
	Hawawshi	AMP, PCN, GEN, KAN, NEO, ERY	*mecA*
	Kofta	AMP, PCN, GEN, KAN, ERY	*mecA*

[1] *VanA* and *mcr1* genes testing revealed that none of the isolates were resistant to vancomycin nor colistin. [2] *B. cereus, Bacillus cereus*; *Staph. aureus, Staphylococcus aureus* [3] AMP, ampicillin (30 µg); PCN, penicillin (10 IU); GEN, gentamicin (10 µg); KAN, kanamycin (5 µg); NEO, neomycin (30 µg); CIP, ciprofloxacin (5 µg); ENR, enrofloxacin (5 µg); ERY, erythromycin (15 µg); CTR, ceftriaxone (30 µg).

4. DISCUSSION

Surveillance is one of the principles of infectious disease management, but it has long been underappreciated and insufficiently funded in the fight against AMR (O'Neill, 2016). Therefore, the study compared the incidence of *S. aureus* and *B. cereus* in raw and ready-to-eat meat products and described the associated risks of antibiotic resistance.

Food-borne *B. cereus* causes emetic and diarrheal syndromes, two separate illnesses. Cereulide, a small-molecular weight toxin produced in food, causes nausea and vomiting (Agata et al., 1995), whereas enterotoxins, produced in the intestinal tract, cause diarrhea (Granum, 1997). In this investigation, *B. cereus* was detected in 14% of samples and the recovery rate was higher in raw foods than in retail RTEM (Table 1). Fortunately, foodborne *B. cereus* illness is strongly correlated with their load. Following guidelines from different countries and regions, their dangerous threshold ranged between more than four and five $\log_{10}$ CFU/g, and the permissible limit shall be not more than three $\log_{10}$ CFU/g (Health Protection Agency, 2009; NSWFA, 2009). EFSA has established no further official hygienic processes or food safety guidelines for living cells, spores, or toxins of *B. cereus* in products, except dried infant formulae and dried dietary foods for medical uses intended for infants under six months of age. France, on the other hand, suggested national rules and reference levels (two $\log_{10}$ CFU/g) for *B. cereus* in cooked meat-based products, ready-to-eat meals, fish, and cold-served preserved meats (AFSSA, 2008). *B. cereus* was detected in 18.3% of meat products and 6.6% of raw meat from six Japanese prefectures. Higher *B. cereus* levels of two to four

log_{10} CFU/g were discovered in meat samples containing additives, implying that additives are the primary source of contamination (Konuma et al., 1988). Furthermore, ambient pollution, inadequate food temperature processing, and incorrect cleaning of food production equipment and preparation surfaces are among the frequent risk factors that contribute to the spread of *B. cereus* foodborne intoxications (Yu et al., 2020). Unfortunately, *B. cereus* spores are viscous, highly heat and drought-resistant, and ready-to-eat (RTE) foods are not commonly sterilized by heat treatment before consumption, posing major food safety concerns (Yu et al., 2020). Five *B. cereus* isolates, including one MDR, tested positive for *bla*$_{TEM}$, *bla*$_{CTX}$, *bla*$_{SHV}$, *mcr*1, *nor*A, and *van*A (Table 2). Fortunately, neither the *mcr1* nor the *van*A genes were found in the *B. cereus* isolates. *Nor*A was identified in four of the *B. cereus* isolates, but *bla*$_{TEM}$ and *bla*$_{CTX}$ were identified in three isolates, with *bla*$_{TEM}$ being Overuse of antimicrobial agents in a variety of industries, including animal medicine, and poor infection control have accelerated the rate at which resistance develops and spreads through relevant food products, resulting in the emergence of superbugs such as MRSA and VRSA, which has become a growing concern in recent years (O'Neill, 2016). Only one *B. cereus* isolated from raw burger was MDR to three different antibiotic classes, whereas 25% (three raw and two RTEM) of *S. aureus* isolates were MDR, with three resistance patterns ranging from three to five different antibiotic classes.

Staphylococcus aureus was ranked second among the top six bacteria responsible for resistance-related death. Methicillin-resistant *Staphylococcus aureus* (MRSA) continues to be a significant global public health concern due to the severity of the symptoms associated with infections. It increased from 2% in 1974 to 64% in 2004 and was only linked to hospital-acquired infections (Ou et al., 2017), but numerous studies have shown that foodborne MRSA outbreaks have occurred (Rhee and Woo, 2010). In 2019, methicillin-resistant *S. aureus* caused over 100,000 AMR fatalities (Courtenay et al., 2019). Staphylococcus aureus incidence was higher in raw (24%, 12/50) than in RTE (16%, 8/50) meat products and the total was 20% (Table 1). Twelve samples (11 raw and one RTEM) failed to meet the EOS requirements for *S. aureus*, which states that the maximum permitted limit in minced meat generally or investigated unprocessed products is 100 CFU/gm. The five MDR *S. aureus* isolates were genetically characterized for two genes, *mecA*, and *vanA*, which are critical for distinguishing MRSA and vancomycin-resistant *S. aureus* (VRSA) (Table 2). The data revealed that all the MDR *S. aureus* tested positive for *mecA* but not *vanA* genes. MRSA strains share the *mecA* gene, which produces low-affinity penicillin-binding protein (PBP2) and promotes resistance to all subclasses of β-lactam antibiotics (Schnellmann et al., 2006). In Mansoura, Egypt, RTE beef burgers and hot dog sandwiches (225) had a higher coagulase-positive *S. aureus* incidence of 83.1%, 22.6% of them were identified as MRSA and expressed *mecA* genes with 75.2% of them being MDR (Mahros et al., 2021). In the USA, 78 out of 1032 *S. aureus* isolated from retail meats from eight states tested positive for MRSA. Of them, 10.4% (107/1032) were MDR, including 37.2% (29/78) of the MRSA isolates (Ge et al., 2017). The overall pooled prevalence rate of *S. aureus* was 29.2% (22.8 to 35.9%), according to a meta-analysis of the global prevalence rates of various raw meat products published in research up to June 2016. It was also significantly more prevalent in samples taken from retail sources than from slaughterhouses and processing plants (Ou et al., 2017). Thus, unsanitary meat handling, pre-, and post-slaughter in processing facilities are considered a major source of contamination, and staphylococcal food poisoning is frequently associated with excessively manually handled food (Ou et al., 2017).

more common in raw-derived isolates and *bla*$_{CTX}$ being more common in RTEM-derived isolates. The *bla*$_{SHV}$ was determined only in three raw-derived isolates, two of which also co-expressed *bla*$_{TEM}$, *bla*$_{CTX}$, and *nor*A or *bla*$_{TEM}$ and *nor*A. In contrast to the single MDR, *B. cereus* reported here, an earlier Chinese screening classified all *B. cereus*, isolated from 34% of cooked meat samples, as MDR. However, the low MDR phenotypes could be attributed to the low number of antibiotics tested here. However, both study isolates had significant resistance rates to the majority of β-lactam antibiotics, which was linked to their ability to produce β-lactamases (Yu et al., 2020). Furthermore, current multi-genetic resistance components in *B. cereus* isolates confirm previous reports that ESBL-encoding genes frequently co-circulate with genes encoding resistance to other critical antibiotics (Yu et al., 2020).

5. CONCLUSIONS

The current prevalence of *S. aureus* and *B. cereus* may be lower than in previous studies from Egypt and other countries, and thankfully, genetic resistance components such as *mcr1* and the *vanA* of last resort antibiotics weren't identified. However, the presence of these pathogens in RTE meat products with genetic elements including *mecA* that confer resistance to highly critical antibiotics, such as β-lactam antibiotics, co-circulating with other resistance genes, endangers public health and speeds up the rate at which resistance develops and spreads, and the emergence of superbugs.

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