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Histopathological changes induced in an animal model by potentially pathogenic *Enterococcus faecalis* strains recovered from ready-to-eat food outlets in Osun State, Nigeria

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Abstract: Enterococci have been implicated as an emerging important cause of several diseases and multiple antibiotic resistance. However, there is little information about the prevalence of pathogenic and/or antibiotic-resistant *Enterococcus faecalis* in ready-to-eat foods in Nigeria. Here we report the pathogenic potential of three selected antibiotic-resistant *E. faecalis* strains isolated from food canteens and food outlets with different virulence determinant genes, including EFC 12 (with *gel*$^+$, *esp*$^+$, *cylA*$^+$, and *asa1*$^+$), EFT 148 (with *gel*$^+$, *ace*$^+$, and *asa1*$^+$), and EFS 18 (with *esp*$^+$ and *cylA*$^+$) in an animal model. Enterococcemia, hematological parameters, and histopathological changes in organ tissues were examined in experimental animals. The results showed differences in enterococcemia and hematological parameters between the control group and experimental animal group. Enterococcemia was observed for 7 days, and the animal group infected with EFC 12 showed the highest growth rate, followed by EFT 148, with the lowest growth rate seen in the EFS 18-infected group. White blood cell count, packed cell volume, and platelets were significantly reduced ($P<0.05$) in the experimental animals compared with the controls. White blood cells decreased drastically during the study period in rats challenged with EFC 12 (from 7,800 to 6,120 per mm^3) but levels remained higher in the control group (from 9,228 to 9,306 per mm^3). Histopathological changes included areas of pronounced hemorrhage, necrosis, and distortion in liver tissues, which were more marked in rats infected with EFC 12, followed by EFT 148, then EFS 18. The results of this study suggest the presence of potentially pathogenic *E. faecalis* strains in food canteens and food outlets; hence, there is a need for strict adherence to good hygiene practices in the study area owing to the epidemiological significance of foods.

Keywords: pathogenic potentials, antibiotic resistance, *Enterococcus faecalis*, food canteen

Introduction

Enterococci are important bacteria in foods essentially due to their involvement in spoilage, fermentation, and use as probiotics in humans and slaughter animals.[1] Certain specific enterococcal strains have been used as probiotic adjunct cultures in the production of cheddar cheese owing to their ability to improve microbial balance in the intestine.[2,3] However, reports show that the safety of food samples containing enterococci remains a challenge that requires careful consideration and attention.[2,4] Enterococci are important pathogens that cause various infections in humans and other vertebrate animals.[5–7] There are also reports of infections in livestock and poultry in veterinary practice.[8,9]

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Some *Enterococcus* spp. are resistant to antibiotics and possess virulence factors such as adhesins, invasins, pili, and hemolysin,[8] and several of these virulence factors have been described.[3] Eaton and Gasson[10] showed that gelatinase (*gel*) genes may be silent and the phenotype may be negative, even though a *gel* gene is present. Antibiotic-resistant enterococci, especially vancomycin-resistant enterococci, are also increasingly reported.[11,12] Within the genus *Enterococcus*, *E. faecalis* is an important part of the normal intestinal flora in humans and animals. It is the second regular bacterium, next to *Escherichia coli* as an ecological agent.[13,14] *E. faecalis* is ranked second or third in frequency among bacteria isolated from hospitalized patients.[15]

The resistance of enterococci to pasteurization temperatures and their ability to adapt to different substrates and environmental conditions indicate that the organisms can be found in food products manufactured from raw materials (milk or meat) and in heat-treated food products. This means essentially that this bacterium could withstand the usual conditions of food production. In addition, it could contaminate finished products during food processing. Enterococci can become an important part of the fermented food microbiota, especially in fermented cheeses and meats. The findings of Hayakawa et al[16] suggesting the presence of a "non-hospital" pool of vancomycin-resistant *E. faecalis* are very worrying.

Antibiotic-resistant enterococci are widespread in food, including meat, dairy products, and ready-to-eat foods, as well as within enterococcal strains used as probiotics.[17,18] Acquired resistance traits for a number of antibiotics in enterococci from food origin have been reported throughout Europe,[17] while the role of antibiotic-resistant enterococci, especially vancomycin-resistant enterococci, has been emphasized, implicating food as a possible natural reservoir in the dissemination of antibiotic resistance traits in the environment.[18]

The role of enterococci in disease has consistently raised suspicion with regard to the safety of these organisms for use in foods and as probiotics. There has been a degree of suspicion regarding a link between the use of some antibiotics in livestock and humans becoming colonized by antibiotic-resistant enterococci via the food chain.[19,20] However, information about the prevalence of pathogenic *E. faecalis* from food origin, especially ready-to-eat foods, remains scanty, particularly in the study area. Therefore, the present study examined the pathogenic potential of selected antibiotic-resistant *E. faecalis* with certain virulence factors isolated from food canteens and outlets in Osun State, Nigeria.

Materials and methods

Bacterial isolates

Three *E. faecalis* strains recovered from canteen samples (food, plates, and palms of food handlers) previously identified by a biochemical protocol and confirmed by polymerase chain reaction using 16S primers[21,22] were obtained from the Department of Microbiology, Ekiti State University, Ado-Ekiti, Nigeria. Susceptibility testing of the isolates as well as their virulence potential (Table 1) had been determined and are reported elsewhere.[23]

Sources of experimental animals

Male albino rats of the Wistar strain and aged 28 days were sourced from the animal house, University of Ibadan, Nigeria, and divided into four (one control and three experimental) study groups, with five rats in each group.

Preparation of inoculum for enterococcal challenge test

Overnight culture of each of the isolates grown in peptone water was harvested by centrifugation at 14,636 × *g* for 15 minutes at 26°C. The bacterial pellet was suspended in 2 mL of sterile normal saline, adjusted to a final concentration of 10^9 cfu/mL, diluted to 5×10^5/mL per 100 µL, and fed to the animals orally with a 28 gauge, 0.5 inch intubator. This bacterial suspension has been reported to be optimal for inducing a reproducible, longer-term, non-acute infection with morbidity and mortality in lower-order mammals.[24] The population of bacterial cells fed into each rat was confirmed by the viable plate count technique. The animals were observed daily for morbidity and mortality, and any rat exhibiting noticeable changes, such as a scruffy coat or lethargy, was sacrificed. Infection experiment was repeated to confirm results.

Determination of enterococcemia and hematological changes

Three groups of albino rats were fed orally with enterococcal strains (EFC 12, EFS 18, and EFT 148) that possess different virulence determinant genes through an intubator as described by Singh et al[25] while the control group was fed with the same volume of sterile normal saline. After challenge, enterococcemia was determined in blood samples.

Blood was collected from each animal's tail to investigate enterococcemia as follows. Two drops of blood were placed in a test tube containing 1 mL of sterile peptone water and allowed to stand for 20 minutes. The sample was then plated on

Table 1 Antibiotic-resistance phenotypes and virulence determinant genes in selected *Enterococcus faecalis*

Organisms	Antibiotic-resistant phenotypes												Virulence determinant genes			
	AMX	TET	COT	ERY	CLX	GEN	CLO	AUG	VAN	LEV	CIP	SPA	NOR	PER	*Gel*	*Esp*	*CylA*	*Asa1*	*Ace*
EFT 148	R	R	R	R	R	R	S	R	R	S	R	S	R	R	+	–	–	+	+
EFS 18	S	R	R	R	R	R	R	R	R	S	R	R	R	R	+	–	+	–	–
EFC 12	S	R	R	R	R	R	R	R	R	S	R	S	R	R	+	+	+	+	–

Abbreviations: EFT, fast-food canteen; EFS, school canteen; EFC, *Bukataria*; AMX, amoxicillin; TET, tetracycline; COT, cotrimoxazole; ERY, erythromycin; CLX, cloxacillin; GEN, gentamicin; CLO, chloramphenicol; AUG, amoxicillin/clavulanic acid; VAN, vancomycin; LEV, levofloxacin; CIP, ciprofloxacin; SPA, sparfloxacin; NOR, norfloxacin; PER, perfloxacin ; R, resistant; s, susceptible.

Bile Aesculin Agar (Oxoid, Basingstoke, UK) and incubated at 37°C for 24 hours. The blood samples were also smeared and stained on grease-free glass slides and viewed under a microscope (AmScope, Irvine, CA, USA) for the presence of bacteria with a cocci shape typical of enterococci.

Hematological changes were examined after the 1st, 2nd, and 3rd week of infection. Packed cell volume, red blood cells, white blood cells (WBCs), platelets, and hemoglobin were determined using the methods described by Cheesbrough.[26]

Histopathological changes were examined in the experimental animals following sacrifice 3 weeks after challenge. The methods of Gentry-Weeks et al[27] were used to collect, process, and examine the samples. Pathological changes in the samples were observed by microscopy (AmScope) with a camera attachment.

Statistical analyses

The hematological data were subjected to analysis of variance using Statistical Package for Social Sciences version 17 software (SPSS Inc., Chicago, IL, USA).

Results and discussion

Growth of the test organisms was monitored in the experimental animals to investigate enterococcemia. The three strains of *E. faecalis* used were EFC 12 with four virulence genes (*gel*$^+$, *esp*$^+$, *cylA*$^+$, and *asa1*$^+$), EFT 148 with three virulence genes (*gel*$^+$, *ace*$^+$, and *asa1*$^+$) and EFS 18 with two virulence genes (*esp*$^+$ and *cylA*$^+$). With time, there was a reduction in the enterococcal load in all experimental groups, with a sharp decrease from the onset of the experiment to day 7. EFC 148 had the smallest and EFC 12 the highest enterococcal load by day 7 (Figure 1).

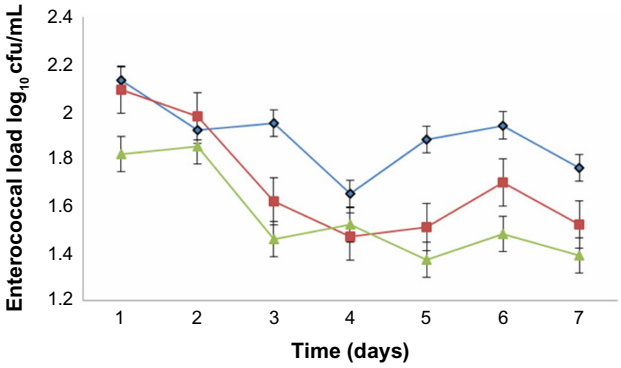

Figure 1 Development of enterococcemia in albino rats fed orally with *Enterococcus faecalis* strains with different virulence determinant genes. (◆) EFC 12 with *gel*$^+$, *esp*$^+$, *cylA*$^+$, and *asa1*$^+$ genes, (■) EFT 148 with *gel*$^+$, *ace*$^+$, and *asa1*$^+$ genes and (▲) EFS 18 with *gel*$^-$, *esp*$^+$, and *cylA*$^+$ genes.

The hematological findings indicated a significant ($P<0.05$) reduction in WBC count, packed cell volume, and platelets in the experimental animals when compared with the controls. Over the period of the study, WBC levels decreased markedly in rats challenged with EFC 12 (from 7,800 to 6,120 per mm^3) but remained higher in control groups (from 9,228 to 9,306 per mm^3). There was a reduction in platelet count in the experimental group compared with the control group. The WBC count, which was markedly lower in animals challenged with *gel*$^+$ *E. faecalis* may be due to the cytotoxic, tissue-destructive, bone marrow depressive, or inhibitory effects of the gelatinase.[3,24,28] Further, in the EFT 148-infected and EFS 18-infected groups, WBC counts were lower (7,110–7,012 and 7,610–7,060 per mm^3, respectively) but this was not up to that obtained in the *gel*$^+$ EFC 12 group (Table 2). Increased WBC counts have been associated with microbial infections, leukemia, and tissue necrosis.[26] Animals in the group infected with EFC 12 had the lowest packed cell volume, hemoglobin concentration, and red blood cell counts (34.01%, 10.03 g/dL, and $3.12\times10^6/mm^3$, respectively). Hemolysins can inhibit leukocyte function[29] and cause lysis of erythrocytes that may provide iron and nutrients in tissue while low packed cell volume value has been attributed to anemia.[30] A decreased red blood cell count indicates destruction of circulating erythrocytes or impairment of blood-forming (erythropoietic) centers in rats.[31]

Gelatinase has been associated with virulence determinants in animal models, and its ability to hydrolyze gelatin, collagen, and certain bioactive peptides suggests that it participates in the initiation and propagation of the inflammatory process in infections involving gelatinase-producing organisms such as *E. faecalis*.[9,32,33] In our study, the gelatinase gene was expressed in two (EFC 12 and EFT 148) of the three *E. faecalis* strains examined. These two strains caused significant enterococcemia in the experimental animals. The gelatin-hydrolyzing activity has been suggested to be different from that of caseinase activity.[34,35] Sedgley et al[36] reported that the gelatinase gene (*gelE*) was detected in all endodontic isolates of *E. faecalis* and that two thirds of the isolates expressed gelatinase activity. David et al[33] reported similar findings when investigating clinical isolates. All these studies concluded that evidence of potential virulence determinants was identified in *Enterococcus* spp., specifically production of gelatinase in combination with one or more other virulence determinants. Studies by Hubble et al[37] and Sedgley et al[38] indicated that expression of *gelE* contributed to increased dissemination of *E. faecalis* and was associated with increased in vitro adhesion of *E. faecalis*.

Table 2 Hematological changes during enterococcal infection in rats

Parameters	Treatment group											
	EFC 12			EFT 148			EFS 18			Control		
	W1	W2	W3	W1	W2	W3	W1	W2	W3	W1	W2	W3
WBC (mm^3)	7,800±412.30b	7,830±121.03b	6,120±130.04c	7,110±35.30b	7,220±120.50b	7,012±10.60b	7,610±130.01b	7,635±47.63b	7,060±510.50b	9,228±540.70a	9,240±411.60a	9,306±312.76a
RBC ($10^6/mm^3$)	7.32±0.13bc	6.02±0.04d	3.12±0.26ef	8.13±0.33a	6.60±0.31cd	3.55±0.26e	7.621±0.21ab	6.501±0.04d	3.31±0.21e	2.10±0.30g	2.30±0.31g	2.38±0.40fg
PCV (%)	38.50±0.40bc	35.30±0.56d	34.01±0.54d	37.4±0.46c	34.50±0.50d	34.03±0.45d	37.21±0.42c	35.23±0.53d	35.31±0.55d	37.80±0.38c	39.50±0.56b	44.4±0.64a
Platelets ($10^3/mm^3$)	66.21±1.10bc	54.65±34fg	47.00±1.30h	60.11±3.4cdef	57.40±1.30efg	58.00±3.10defg	64.35±1.21bcd	56.41±4.30efg	52.33±1.4gh	61.14±1.05cde	68.12±2.55ab	73.5±1.05a
Hemoglobin (g/dL)	11.60±0.25a	11.10±0.23ab	10.03±0.47abc	11.30±1.10a	11.20±1.02a	10.54±1.54abc	9.35±0.43abc	8.72±1.35bc	8.14±0.43c	8.32±0.42c	9.20±0.55abc	10.3±0.67abc

Note: Means with the same letter along the row are significantly different ($P<0.05$).
Abbreviations: PCV, packed cell volume; RBC, red blood cells; WBC, white blood cells; W, week.

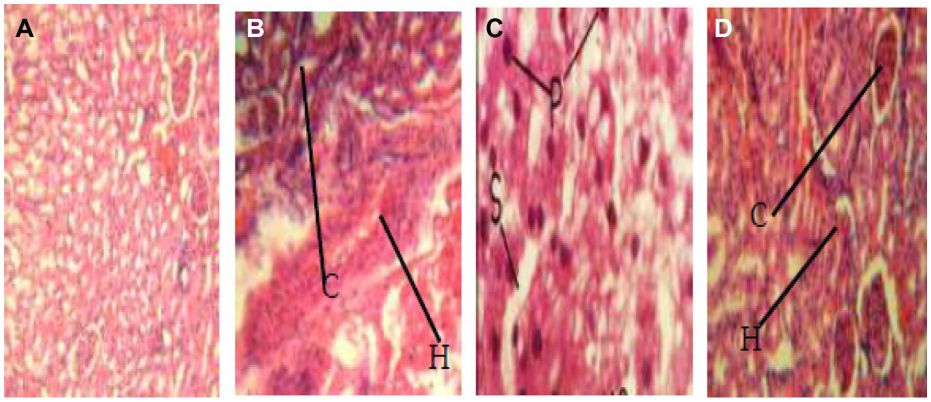

Figure 2 Pathogenic effects of *Enterococcus faecalis* strains on experimental animals (magnification 400×). (**A**) Liver tissue of albino rat (control group) showing normal tissue appearance. (**B**) Liver tissue of albino rat, fed with EFC 12 (having four virulence genes *gel*$^+$, *esp*$^+$, *cylA*$^+$, and *asa1*$^+$) showing pronounced renal corpuscles and areas of inflammatory changes (arrow). (**C**) Liver tissue of albino rat fed with EFT 148 (having three virulence genes *gel*$^+$, *ace*$^+$, and *asa1*$^+$) showing necrosis of hepatic cells with pyknotic nuclei, disorganization of hepatic laminae, and dilation of sinusoids (arrow). (**D**) Liver tissue of albino rats, fed strain of *E. faecalis* strain EFS 18 (having two virulence genes, ie, *gel*$^-$, *esp*$^+$, and *cylA*$^+$) showing well preserved renal corpuscles and less pronounced areas of inflammatory changes (arrow).

In addition, gelatinase is required for efficient biofilm formation and is a major contributor to the pathogenesis of enterococcal endocarditis.[39] Therefore, it is evident that bacterial infection accounts for the WBC reduction seen in the experimental animal groups in our study. Extracellular proteases, gelE and sprE, are two known virulence factors that contribute immensely to the pathogenesis of *E. faecalis*.[33,39,40]

Histopathological investigation showed areas of pronounced hemorrhage, necrosis, and distortion in the liver tissues (Figure 2A–D), which were more marked in rat tissues infected with EFC 12 than in rat tissues infected with EFS 18 and EFT 148. Other tissues examined did not reveal any significant difference compared with control tissue. The combination of cytolysin and *Esp* seems to confer more virulence on EFS 18 than EFT 148. An association between hemolysin production and virulence is well known for bacteria such as streptococci, pneumococci, listeria, clostridia, and some strains or serotypes of *E. coli*.[41–43] Previous research suggests that hemolysin contributes to virulence in experimental and human enterococcal infections. In a rabbit model of endophthalmitis,[44] infections caused by cytolytic *E. faecalis* resulted in 99% loss of retinal function at postoperative day 3, with near total destruction of retinal architecture, compared with loss of 74% in infections caused by non-cytolytic strains, which produced few changes.

A study from Japan reported hemolytic strains in 60% of isolates from adults with systemic infection due to *E. faecalis* in contrast with only 17% of fecal isolates from healthy adults colonized with *E. faecalis*.[24] *Enterococcus* spp. have intrinsic low-level resistance to gentamicin, and some strains can acquire high-level resistance to this drug.[45,46] The finding of Chow et al[24] suggest that enterococcal hemolysin contributes to virulence when associated with aggregation substance in experimental animal endocarditis.

Biofilm formation in *E. faecalis*, although not investigated in the present study, but has been strongly associated with the presence of *esp*, which is a major contributor to pathogenesis.[39,43,47–49] It has been suggested that the presence of *esp* rather than the phenotype (adherence or biofilm formation) is a good marker for identification of strains that are highly adherent to abiotic surfaces. The presence of a pathogenicity island was also not investigated in this study, but it is usually found in *E. faecalis*, and has been reported to contain several virulence determinant genes, which play different roles in the course of infectious disease.[50–52] In the present study, the mortality rate was higher (80%) in the EFC 12-infected animals, while those infected with EFS 18 and EFT 148 had a mortality rate of 60%. This is basically consistent with the findings of Zhou et al.[8] The virulence of the organisms may therefore be attributed to the possible presence of a pathogenicity island as well.

Conclusion

The hematological changes and severe tissue damage observed in this study established pathogenicity in the enterococcal-infected experimental animals. Therefore, we conclude that food canteens and food outlets in the study area are a reservoir for potentially pathogenic antibiotic-resistant *E. faecalis*. Hence, there is a need for improved environmental and personal hygiene in order to ensure good manufacturing practice by food handlers in the area. Also, food canteens and food outlets should be subjected to periodic inspection by the appropriate authorities. Surveillance programs should be put in place to ensure strict compliance. Our study agrees with previous reports on the hazards of ready-to-eat foods

vended to school children.[53] Therefore, it is mandatory that food handlers/vendors be subject to food safety and food hygiene training and that effective national evaluation criteria be developed.[54]

Disclosure

The authors report no conflicts of interest in this work.

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