UJMR, Special Conference Edition, Vol. 9 No. 3. June, 2024, pp.89 - 103

E-ISSN: 2814 – 1822; P-ISSN: 2616 – 0668

https://doi.org/10.47430/ujmr.2493.012

Received: 11th March 2024 **Accepted:** 14th June 2024

Antagonistic effects of *Bacillus* species against bacterial multi-drug resistant (MDR) food-borne pathogens and aflatoxigenic fungi

[1,4]Temilade Ozabor, [1]Deborah Falomo, [1]Elizabeth Taiwo, [1]Oyindamola Alabi, [1]Precious Adediran, [2,3]Femi Ayoade, [4]Ilesanmi Fadahunsi and [1]Janet Olaitan

[1]Department of Microbiology, Osun State University, Osogbo

[2]Department of Biological Sciences, College of Natural Sciences, Redeemer's University, Ede, Osun State, Nigeria

[3]African Centre of Excellence for the Genomics of Infectious Diseases (ACEGID), Redeemer's University, Ede, Osun State, Nigeria

[4]Food Microbiology and Biotechnology Unit, Department of Microbiology, University of Ibadan

*Correspondence author: praise.ozabor@uniosun.edu.ng, +2347035235241

Abstract

This study was designed to investigate the antagonistic pattern of Bacillus species against MDR bacterial food-borne pathogens and aflatoxigenic fungi and evaluate their technological properties. Morphological and biochemical characterizations were done using standard methods. Production of cell-free metabolites, agar well diffusion, optimization of Bacillus growth rates, and enzymatic assays were also carried out using standard techniques, while aflatoxin quantification and qualification were done using high-performance thin-layer chromatography (HP-TLC). Results revealed that B. subtilis OKOI7.12ia had the highest inhibitory activity against S. enteritidis ATCC 13875 (27mm), while B. paralicheniformis had the least inhibitory activity against A. niger (7mm). B. subtilis OKOI7.12ia also had the highest growth rate at 30°C, followed by B. subtilis IPOI3.12ia and B. paralicheniformis OKAO4.12ia. However, there was no significant difference in the growth rates of B. subtilis IPOI3.12ia at 30°C and 40°C ($p < 0.05$). Furthermore, B. subtilis OKOI7.12ia and B. subtilis IPOI3.12ia had the highest growth rate at pH 8, while a lower growth rate was observed at pH6 ($p < 0.05$) in all five Bacillus sp. In addition, B. subtilis OKOI7.12ia and B. subtilis IPOI5.10ia had the highest growth rates using glucose and galactose as carbon sources, respectively. Growth in nitrogen sources showed that B. subtilis OKOI7.12ia had the highest growth rate, while B. subtilis IPOI5.10ia and B. subtilis OGOA10.7ii growths were not significantly different at $p < 0.05$. More so, B. subtilis IPOI3.12i had the least growth in peptone. In addition, B. subtilis OKOI7.12ia also produced the highest amounts of protease, amylase, and lipase enzymes, while B. subtilis IPOI3.12ia produced the least. Therefore, from the results obtained in this study, it can be concluded that B. subtilis OKOI7.12ia can be employed as a potential starter culture for producing microbiologically safe foods.

Keywords: Enzymes, spectrophotometer, antimicrobial metabolites, agar well diffusion, high-performance thin-layer chromatography

INTRODUCTION

The literature has documented Bacillus species as a spore-forming Gram-positive, rod-shaped, aerobic organism with diverse inherent antimicrobial peptides such as antibiotics and bacteriocins (Riffat *et al.*, 2020). Due to their endospore formation, *Bacillus* can be found in several places, such as aquatic environments, foods, soil, rocks, and gastrointestinal tracts of humans and animals. Literature has also reported that they can be found in extreme environments such as those found in high pH (*B. alcalophilus*), high temperature (*B. thermophilus*), and high salt concentration (*B. halodurans*) (War and Joshi, 2014; Jooste *et al.*, 2019). The antimicrobial peptides produced are either ribosomally or non-ribosomally synthesized. Examples include fengycin, iturin, and surfactin. These AMPs have been widely reported to be used for various beneficial purposes in the food, pharmaceutical, medical, biotechnological, and agriculture-based industries (Beladjal *et al.*, 2018; Caulier *et al.*, 2019).

According to the reports of Christie and Setlow (2020), *Bacillus* can produce numerous enzymes and metabolites that can be harnessed into

useful products in various industries (Eijlander *et al.* 2011). Food spoilage and pathogenic bacteria have been documented to be threats to food quality and safety. Pathogens such as *Enterococcus faecalis, Streptococcus pyogenes, Staphylococcus aureus, Escherichia coli Clostridium botulinum,* and *Listeria monocytogenes* have been reported to cause inflammatory, respiratory, systemic and intestinal infections when ingested with food (Todorov *et al.*, 2015; Suganthi *et al.*, 2015). As the use of chemical preservatives has been reported to be highly detrimental to animal and human health, the focus has been shifted to the use of safe microbial metabolites/peptides with functional properties that can be used for the production and preservation of foods (Ramesh *et al.*, 2014; Yost *et al.*, 2014; Zhao *et al.*, 2016).

Literatures have documented some members of the *Bacillus* species to be recognized as GRAS (generally regarded as safe) such as: *B. subtilis*, *B. licheniformis* and *B. polymyxa* due to their safe use in the food and pharmaceutical industries based to their safety assessment records, short fermentation time, high growth rates, high secretion of antimicrobial peptides into the fermenting medium and usage as supplements in human foods and animal feeds (Benitez *et al.*, 2010; Abriouel *et al.*, 2011; Cutting, 2011; Chopra *et al.*, 2015 and Eishaghabee, 2017). In addition, other non-food grade species have also been well documented to be used in agriculture, such as *B. thuringiensis* and *B. siamensis*, which are known to produce secondary inhibitory metabolites used for the production of insecticides and destruction of fungal plant pathogens such as *Rhizoctonia solani* and *Botrytis cinerea* respectively (Slonczewski and Foster, 2011; Jeong *et al.*, 2012). According to the reports of Ryan and Ray (2014), many *Bacillus* species produce industrially important enzymes such as barnase, amylase, protease, and BamH1 restriction enzymes. Thioldisulphide oxidoreductase in *B. subtilis* is key for the secretion of disulfide-bond-containing proteins (Schallney *et al.*, 2014).

Antimicrobial metabolites such as bacteriocins and bacteriocin-like substances are peptides secreted by microorganisms, including the *Bacillus* species, to act as self-defense (Abriouel *et al.*, 2011; Hashemizadeh *et al.*, 2011). Due to the high demand for minimally processed foods with very little or no chemical preservatives, the search for natural antimicrobials is increasing (Cotter *et al.*, 2013). GRAS *Bacillus* metabolites are generally safe and stable with therapeutic potentials as antimicrobials (Kaskoniene *et al.*, 2017; Noda *et al.*, 2018). In recent times, attention has also been drawn to the ability of *Bacillus* metabolites to inhibit the growth of multi-drug antibiotic-resistant organisms such as Methicillin-resistant *Staphylococcus aureus* (MRSA), Penicillin-resistant *Staphylococcus pneumoniae* (PRSP), *Mycobacterium tuberculosis, Pseudomonas aeruginosa, Escherichia coli* 0157: H7. (Sivaranjani *et al.*, 2019; Du *et al.*, 2020; Simon *et al.*, 2020 and Huang *et al.*, 2021). Furthermore, the inhibitory capacity of *Bacillus* metabolites against cancerous cells has also been documented (Kaur *et al.*, 2015; Javed *et al.*, 2020). Therefore, this study was designed to investigate the antagonistic effects of food-grade *Bacillus* species against typed multi-drug resistant food-borne bacterial pathogens and aflatoxin-producing fungi as well as evaluate their technological potentials of enzyme production to use them as potential starter cultures during fermentation processes for the production of microbiologically safe foods.

MATERIALS AND METHODS

Collection and resuscitation of *Bacillus* cultures

Bacillus cultures previously isolated from traditionally fermented condiments, namely *Parkia biglobosa, Ricinus communis, Pentaclethra macrophylla,* and *Prosopis africana,* were obtained from the culture collection center of the Department of Microbiology, University of Ibadan, and resuscitated to confirm their potency. The isolates were resuscitated by streaking on nutrient agar (Liofilchem, Italy) and incubated for 24 hours at 37°C. Each isolate was reinoculated into trypticase soya broth (TSB) and incubated for 24 hours at 37°C. The pure cultures were obtained by repeated streaking on trypticase soya agar (TSA) and stocked on TSA slants at 4°C. Moreso, typed MDR food-borne pathogens were obtained from the culture collection centres of the Nigeria Institute of Medical Research (NIMR), Yaba, Lagos State and Redemers University, Ede, Osun State, Nigeria.

Phenotypic, biochemical, and sugar fermentation tests

Pure *Bacillus* cultures (18-24 hr. old) were subjected to morphological, biochemical, and sugar fermentation tests such as Gram staining, motility, endospore staining, catalase, oxidase, citrate utilization, lysine, gas and hydrogen sulfide production, indole, starch hydrolysis, haemolysis, and carbohydrate utilization as recommended in the Bergey's manual of determinative bacteriology 9th edition (Olaitan *et al.*, 2022).

Production of cell-free metabolites from *Bacillus* cultures

Bacteria isolates were inoculated into 250 ml Erlenmeyer flasks (SSG, UK) containing 200 ml of tryptic soya broth with 1% yeast extract and incubated at room temperature for 48 hr. with intermittent shaking. The broth cultures were filtered to separate the cells. The filtrate was filtered through a 0.22 µm size membrane (Millipore, India), and the crude extracts were stored at 4°C (Fadahunsi *et al.*, 2021).

Isolation and characterization of aflatoxin-producing fungi from groundnut samples

One gram of sample was homogenized in 10 ml of sterile distilled water and serially diluted. One (1) ml of 10^3 and 10^7 dilution factors were inoculated into sterile potato dextrose agar (PDA) plates and incubated at 30 ± 2°C for 5-7 days. The fungi were characterized using the fungi compendium (Alexopoulus). Distinct colonies were identified based on the colony appearance, texture, color, reverse side color, and growth rate (Jonathan *et al.*, 2016).

Aflatoxin quantification using high-performance thin layer chromatography (HPTLC) technique

The presence of aflatoxins in the groundnut samples was quantified at the International Institute for Tropical Agriculture (IITA), Ibadan, using HPTLC (Rheotype Gilson Abimed Model 231). one hundred (100) g of groundnut sample was defatted with N-hexene Soxhlet extractor, and the defatted residue extracted with ethyl acetate (three times, 60 mL/each). The extracts were combined and dried over anhydrous sodium sulfate and filtered. The filtrate was transferred into a glass vial and evaporated under nitrogen steam. The crude extracts were then suspended into 1mL chloroform and placed in a 14 × 0.8 cm column containing 2.5 Kiesel gel 60 and 70/230 silica gel. Aflatoxin quantification for AF B1 and B2; G1 and G2 were done using the Lichrosorb RP-18 column. The result was compared with a standard aflatoxin B1, B2, G1, and G2 curve (Jonathan *et al.*, 2016).

Agar well diffusion assay of *Bacillus* crude metabolites on multi-drug resistant (MDR) food-borne pathogens and aflatoxigenic fungi

The following typed multi-drug bacterial cultures collected from the Nigerian Institute of Medical Research (NIMR) were used as pathogenic indicator organisms: *Escherichia coli* DCM 10974, *Escherichia coli* ATCC 43816, *Salmonella enteritidis* ATCC 13875, *Methicillin-resistant Staphylococcus aureus* GP054 and *Pseudomonas stutzeri* GN029 while two untyped aflatoxin producing fungal cultures isolated from groundnut samples namely: *Aspergillus flavus* GB and *Aspergillus niger* AGM were also used. Gentamycin (30µg) was used as a control. All indicator organisms were reconfirmed by subjecting them to biochemical and sugar fermentation tests using Bergey's Manual of Determinative Bacteriology 9th edition (Olaitan *et al.*, 2022). The fungal isolates were grown on sterile potato dextrose agar (PDA) incubated at 35 °C for 3-5 days. Their growths were characterized using the fungi compendium. The aflatoxins in the groundnut samples were quantified using the high-performance thin layer (HPTLC) technique (Jonathan *et al.*, 2016).

Sterile molten Mueller Hinton agar (20 ml) was dispensed into Petri plates and seeded with 0.2 ml broth culture of 0.5 McFarland turbidity standards (1.5×10^8 cfu/ml). The plates were swirled gently to allow even distribution, and a sterile cork borer was used to make wells of 8mm diameter on the Petri plates. One hundred (100) µl of *Bacillus* crude metabolites was aseptically dispensed into the wells and left on the laboratory bench for 2 hours to allow diffusion. The plates were incubated at 37°C for

24 hr. Zones of inhibition (mm) were measured and recorded (Jadhav *et al.*, 2010; Fadahunsi *et al.*, 2021). Isolates with the highest inhibition zones were selected for the optimization process and enzyme assay.

Optimization processes: effect of temperature, pH, carbon, and nitrogen sources on the growth of *Bacillus* species

Effect of temperature on the growth of *Bacillus* sp.

A loopful of twenty-four (24) hr. colonies of each *Bacillus* isolate was inoculated into 10ml of nutrient broth and incubated at 30°C for 24 hr to observe growth by turbidity. This was measured using a Shimadzu UV-VIS spectrophotometer (model no: 1780). The isolates that grew at 30°C were further subjected to growth at 40°C and 50°C (Panda and Sahu, 2013).

Effect of pH on the growth of *Bacillus* sp.

An aliquot of 0.5 ml of each crude *Bacillus* cell-free supernatant was inoculated into 5 ml nutrient broth at pH 6 and 8, respectively. 0.1 ml of each isolate was collected at T_0=0h and after incubation for 4hrs (T_1), at 37°C. (Unban *et al.*, 2020). The test tubes were adjusted to 0.5 McFarland turbidity standards (1.5×10^8 cfu/ml) and read using a UV-spectrophotometer (Cary 300 Bio; 00-100784) at 600nm.

Effect of carbon and nitrogen sources on the growth of *Bacillus* sp.

To determine the effect of carbon sources on the growth of *Bacillus* sp. metabolites, 1g of glucose, fructose, and galactose was added into 10 ml tryptic soy broth (TSB), while 1.0g of peptone and tryptone were added into 10ml TSB for the nitrogen sources. Each test tube was inoculated with a loopful of supernatant and incubated at 37°C for 18-24 hr (Abo-Amer, 2011). The test tubes were adjusted to 0.5 McFarland turbidity standards (1.5×10^8 cfu/ml) and read using a UV-spectrophotometer (Cary 300 Bio; 00-100784) at 600nm.

Enzyme assay of *Bacillus* species

Production of protease: The spot method was used to determine protease production by the selected *Bacillus* species. Nutrient agar was supplemented with 10% (v/v) skimmed milk and autoclaved at 121°C for 15 minutes. The *Bacillus* cultures were spotted on the sterile medium and incubated at 37°C for 24 hrs. The appearance of transparent halos around the spots indicates the presence of protease enzyme (Chantawannakul *et al.*, 2002)

Production of amylase

Nutrient agar was supplemented with 2% (w/v) potato starch and autoclaved at 121°C for 15 minutes. The *Bacillus* cultures were spotted on the sterile medium and incubated at 37°C for 24 hrs. After incubation, the Petri plates were sprayed with Lugol's iodine and kept on the laboratory bench for 15 minutes. Clear halos around the spotted area indicate the presence of amylase (Savadogo *et al.*, 2011).

Production of Lipase

Nutrient agar was supplemented with 3% (v/v) *Cocos nucifera* (coconut) oil and autoclaved at 121°C for 15 minutes. The *Bacillus* cultures were spotted on the sterile medium and incubated at 37°C for 24 hrs. Clear halos around the spotted area indicate the presence of lipase (Dahiya *et al.*, 2011).

Statistical analysis

Values are presented as means ± standard error of duplicate values.

RESULTS

The phenotypic characterization of *Bacillus* species is summarized in Table 1. The shape, size, consistency, color, opacity, elevation, surface, and edge of the resuscitated isolates ranged from irregular, circular, filamentous; 0.2-.0.5 mm; friable, viscoid, butyrous; white, cream; opaque, translucent; flat, raised, convex; rough, dull, smooth; lobate, entire, rhizoid and fimbriate respectively.

In Table 2 below, the biochemical and sugar fermentation tests are documented. All the *Bacillus* isolates were Gram-positive rods,

E-ISSN: 2814 – 1822; P-ISSN: 2616 – 0668

catalase-positive and oxidase-negative. The hemolysis test on blood agar showed alpha and beta hemolysis only. About 98% of all the *Bacillus* were able to ferment glucose, lactose, fructose, maltose, and D-mannose with gas production.

Table 1: Phenotypic characterization of *Bacillus* species

S/N	Isolates Code	Shape	Size (mm)	Consistency	Color	Opacity	Elevation	Surface	Edge
1	OO14.7iib	irregular	0.4	friable	white	opaque	flat	rough	lobate
2	OKO17.12ia	Circular	0.2	viscoid	white	opaque	flat	dull	fimbriate
3	IPOI3.12ia	Circular	0.3	friable	cream	opaque	flat	smooth	entire
4	OO13.10i	irregular	0.3	butyrous	cream	opaque	flat	smooth	Lobate
5	OOI1.7i	irregular	0.3	friable	white	opaque	convex	rough	fimbriate
6	OGOA10.7iia	irregular	0.4	friable	cream	translucent	raised	dull	fimbriate
7	IPOI3.12ia	filamentous	0.4	viscoid	white	opaque	convex	rough	Rhizoid
8	OO13.10i	filamentous	0.4	viscoid	white	opaque	convex	rough	Rhizoid
9	OKAO4.12ia	Circular	0.4	butyrous	white	opaque	convex	rough	Entire
10	OGOA7.12ia	irregular	0.4	friable	cream	translucent	raised	smooth	Entire
11	IPOI5.10ia	irregular	0.5	friable	white	opaque	raised	dull	Entire
12	0015.10i	irregular	0.4	friable	white	translucent	convex	smooth	Entire
13	OGOA6.7iia	Circular	0.3	friable	white	opaque	raised	dull	circular
14	OGOA8.7iia	Circular	0.5	friable	white	opaque	raised	smooth	circular
15	OOI2.10iic	irregular	0.2	butyrous	cream	translucent	raised	smooth	irregular
16	OKOI7.12ia	Circular	0.2	friable	white	opaque	flat	smooth	fimbriate
17	OO13.107	irregular	0.2	butyrous	white	opaque	raised	raised	irregular
18	OOI1.7i	Circular	0.2	friable	cream	translucent	convex	raised	circular
19	OGOA7.12ia	Circular	0.2	butyrous	cream	translucent	raised	smooth	circular
20	OGOA4.7i	irregular	0.4	friable	white	opaque	raised	smooth	irregular

E-ISSN: 2814 – 1822; P-ISSN: 2616 – 0668

Table 2: Biochemical and sugar fermentation tests of *Bacillus* species

S/N	Isolate Code	Gram Reaction	Catalase Test	Hemolysis Test	Endospore Staining	Oxidase	Glucose	Lactose	Fructose	Maltose	D-Mannose
1	OO14.7iib	GPR	+	α	+	-	F+ G+	F+ G+	F+ G+	F+ G+	F+ G+
2	OKO17.12ia	GPR	+	α	+	-	F+ G+	F+ G+	F+ G+	F- G-	F+ G+
3	IPOI3.12ia	GPR	+	ß	+	-	F+ G+	F+ G+	F+ G+	F+ G+	F+ G+
4	OO13.10i	GPR	+	ß	+	-	F+ G+	F+ G+	F+ G+	F+ G+	F+ G+
5	OOI1.7i	GPR	+	α	+	-	F+G+	F+ G-	F+ G+	F+ G+	F+ G+
6	OGOA10.7iia	GPR	+	α	+	-	F+ G+	F+ G+	F+ G+	F+ G+	F+ G+
7	IPOI3.12ia	GPR	+	ß	+	-	F+ G+	F+ G-	F+ G+	F+ G+	F+ G+
8	OO13.10i	GPR	+	ß	+	-	F+ G+	F+ G+	F+ G+	F+ G+	F+ G+
9	OKAO4.12ia	GPR	+	α	+	-	F+ G+	F+ G+	F+ G+	F+ G+	F+ G+
10	OGOA7.12ia	GPR	+	ß	+	-	F+ G+	F+ G-	F+ G+	F+ G+	F+ G+
11	IPOI5.10ia	GPR	+	ß	+	-	F+ G+	F+ G+	F+ G+	F+ G+	F+ G+
12	0015.10i	GPR	+	ß	+	-	F+ G+	F+ G+	F+ G+	F+ G+	F+ G+
13	OGOA6.7iia	GPR	+	α	+	-	F+ G+	F+ G-	F+ G+	F+ G+	F+ G+
14	OGOA8.7iia	GPR	+	α	+	-	F+G+	F+ G-	F+ G+	F+ G+	F+ G+
15	OOI2.10iic	GPR	+	ß	+	-	F+ G-	F+ G-	F+ G-	F+ G-	F+ G-
16	OKOI7.12ia	GPR	+	α	+	-	F+ G+	F+ G+	F+ G+	F+ G+	F+ G+
17	OO13.107	GPR	+	α	+	-	F+ G+	F+ G+	F+ G+	F+ G+	F+ G+
18	OOI1.7i	GPR	+	α	+	-	F+ G+	F+ G+	F+ G+	F+ G+	F+ G+
19	OGOA7.12ia	GPR	+	ß	+	-	F+ G+	F+ G+	F+ G+	F+ G+	F+ G+
20	OGOA4.7i	GPR	+	ß	+	-	F+ G+	F+ G+	F+ G+	F+ G+	F+ G+

Key: GPR= Gram positive rod; ß= Beta hemolysis; α= Alpha hemolysis; F+; G+= positive fermentation and gas production; F-; G-= negative fermentation and gas production

The inhibitory activities of the *Bacillus* cell-free supernatants against indicator organisms were done using the agar well diffusion method, and the results are documented in Figure 2. Five typed bacterial cultures, namely: *E. coli* DCM 10974, *E. coli* ATCC 43816, *S enteritidis* ATCC

13875, *Methicillin-resistant Staphylococcus aureus* NTCC/GP054 and *P. stutzeri* NTCC/GN029 and two confirmed aflatoxin producing fungal cultures were also used, namely: *Aspergillus flavus* GB and *Aspergillus niger* AGM.

Plates 1 and 2 show the growth of *A. flavus* on PDA. *A. flavus* is usually identified with its characteristic green color on PDA.

Figure 1 shows the presence of aflatoxin B1 and B2 in the sampled groundnuts.

Figure 2 presents the inhibitory activity of five *Bacillus* sp. against food-borne indicator organisms. *B. subtilis* OKOI7.12ia had the highest inhibitory activity against *S. enteritidis* ATCC 13875, while *B. paralicheniformis* had the least inhibitory activity against *A. niger*. *B. subtilis* OKO17.12ia showed significant antibacterial and antifungal inhibitory activities than the control antibiotics (gentamycin).

B. subtilis OKOI7.12ia had the highest metabolite growth rate at 30°C, followed by *B. subtilis* IPOI3.12ia and *B. paralicheniformis* OKAO4.12ia. However, there is no significant difference in the growth rate of *B. subtilis* IPOI3.12ia at 30°C and 40°C ($p < 0.05$), as presented in Figure 3.

Figure 3 represents the metabolite growth rate of the five *Bacillus* sp. used in this study. *B. subtilis* OKOI7.12ia and *B. subtilis* IPOI3.12ia had the highest metabolites growth rate at pH 8, while a lower growth rate was observed at pH 6 at $p < 0.05$.

From Figure 4, *Bacillus* metabolite growth rates were observed using different carbon and nitrogen sources. *B. subtilis* OKOI7.12ia and *B. subtilis* IPOI5.10ia had the highest growth rate using glucose and galactose as carbon sources, respectively. For growth in nitrogen sources, *B. subtilis* OKOI7.12ia had the highest growth rate, while *B. subtilis* IPOI5.10ia and *B. subtilis* OGOA10.7ii are not significantly different at $p < 0.05$. however, *B. subtilis* IPOI3.12ia had the least growth in peptone.

Table 4 documents the Bacillus species' production of three (3) enzymes. The clear zones around the spot area on the appropriate growth medium indicate the enzyme's presence. Protease, amylase, and lipase were assayed for in the *Bacillus* sp. *B. subtilis* OKOI7.12ia produced the highest amount of protease (25), amylase (27) and lipase (16). However, *B. subtilis* IPOI3.12ia produced only amylase.

Plate 1: *A. flavus* GB

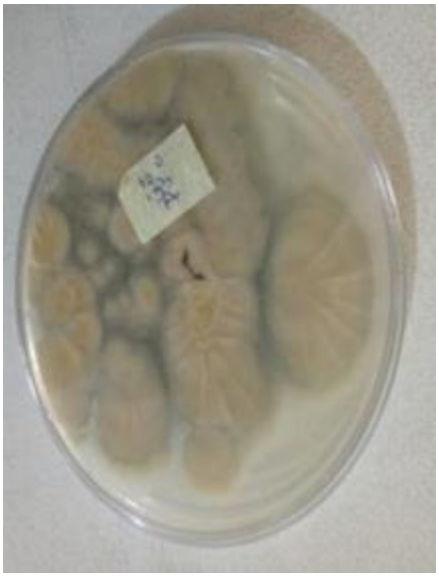

Plate 2: *A. flavus* GB (Reverse side)

E-ISSN: 2814 – 1822; P-ISSN: 2616 – 0668

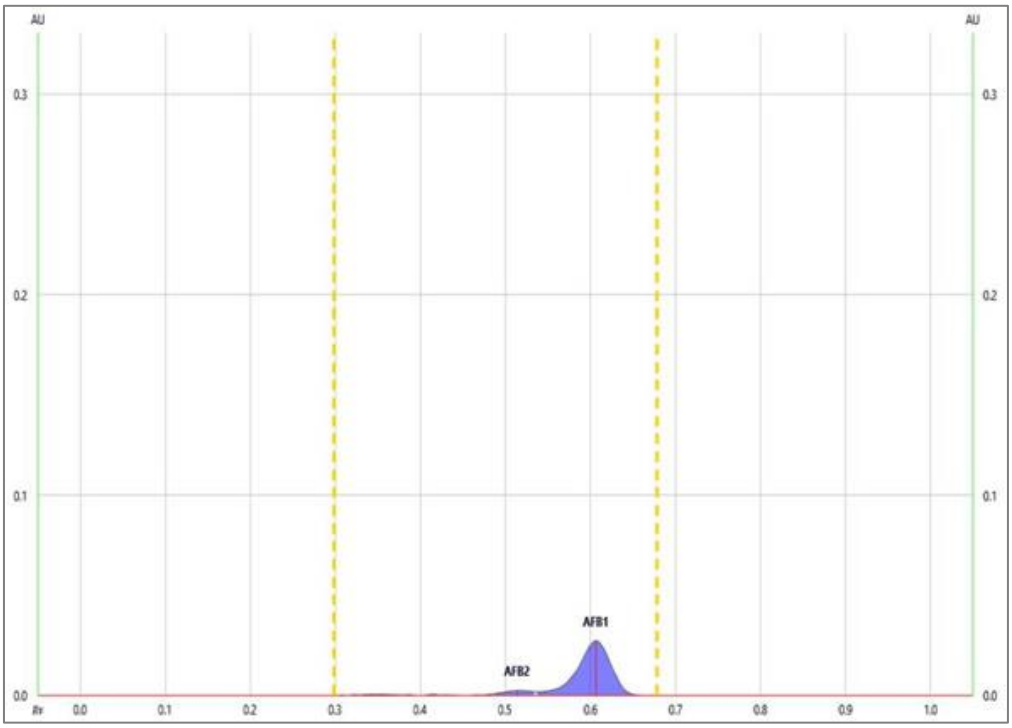

Figure 1: Chromatogram showing aflatoxins in groundnut sample

Table 3: Aflatoxin quantification

Sample type	Aflatoxin concentrations (ppb)			
Groundnut (milled)	B1 23	B2 3	G1 0	G2 0

From the analyzed groundnut samples, the concentrations of aflatoxin B1 and B2 are 23 and 3 ppb, respectively

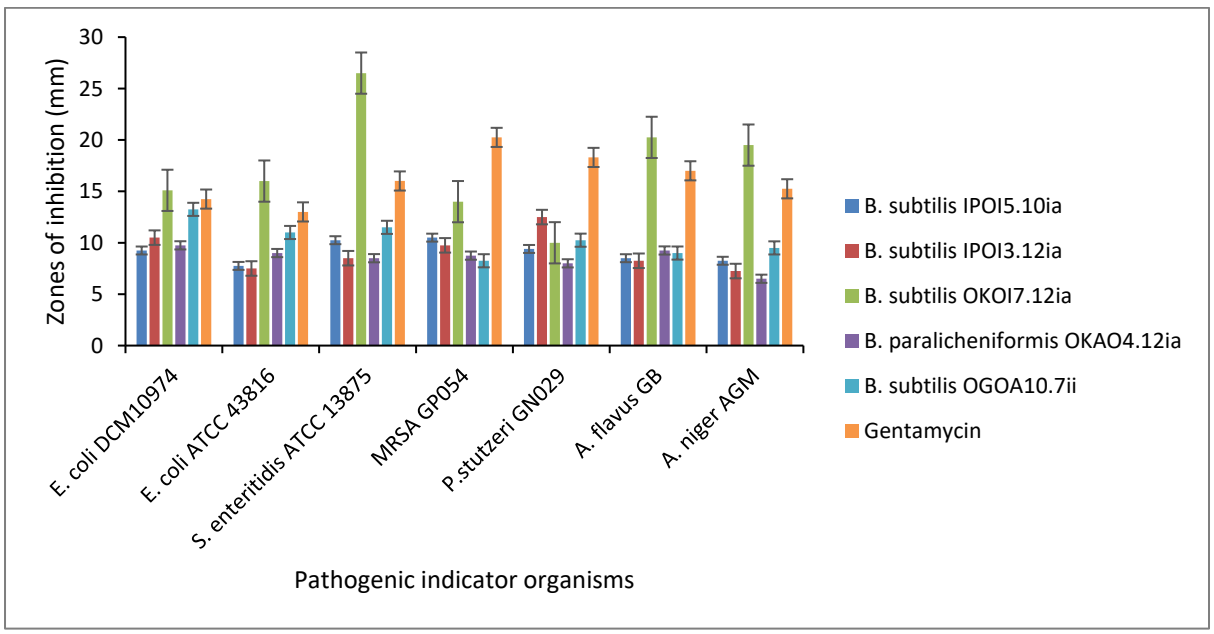

Figure 2: Inhibitory activities of *Bacillus* sp. against food-borne indicator organisms
Values are presented as means± standard error of duplicate values.

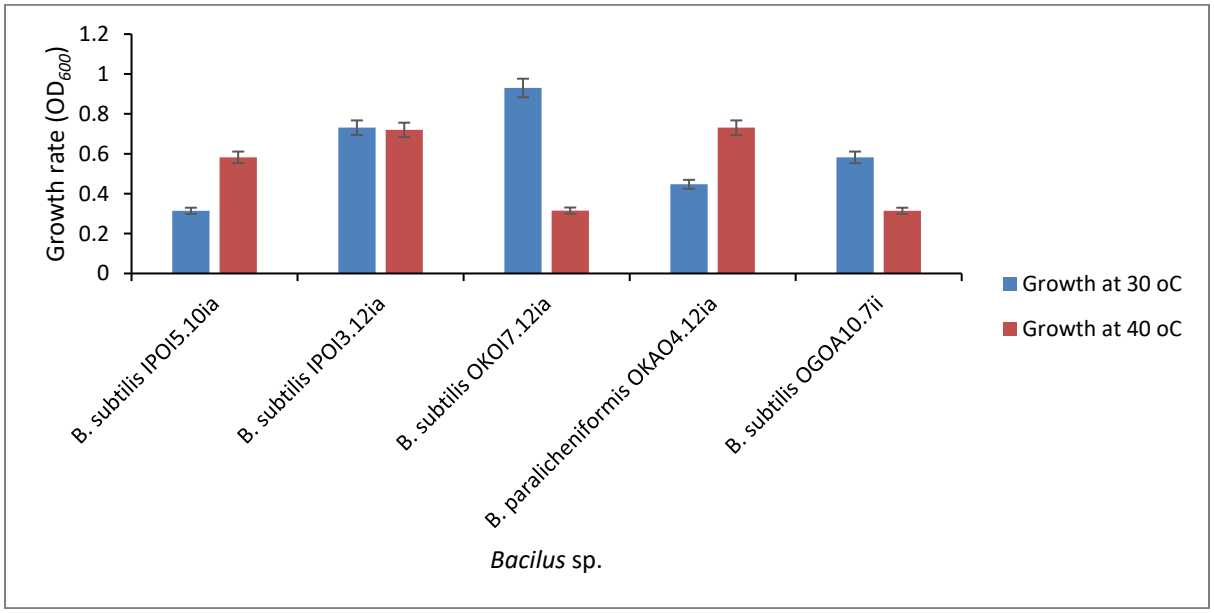

Figure 3: *Bacillus* sp. growth at 30ºC and 40ºC
Values are presented as means± standard error of duplicate values.

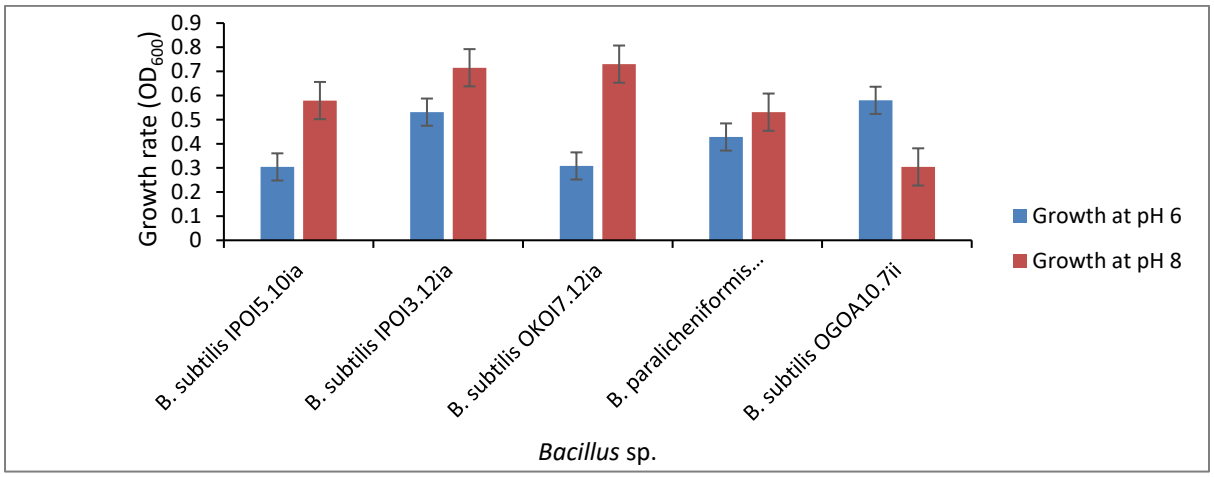

Figure 4: Growth of *Bacillus* sp. at pH 6 and 8
Values are presented as means± standard error of duplicate values.

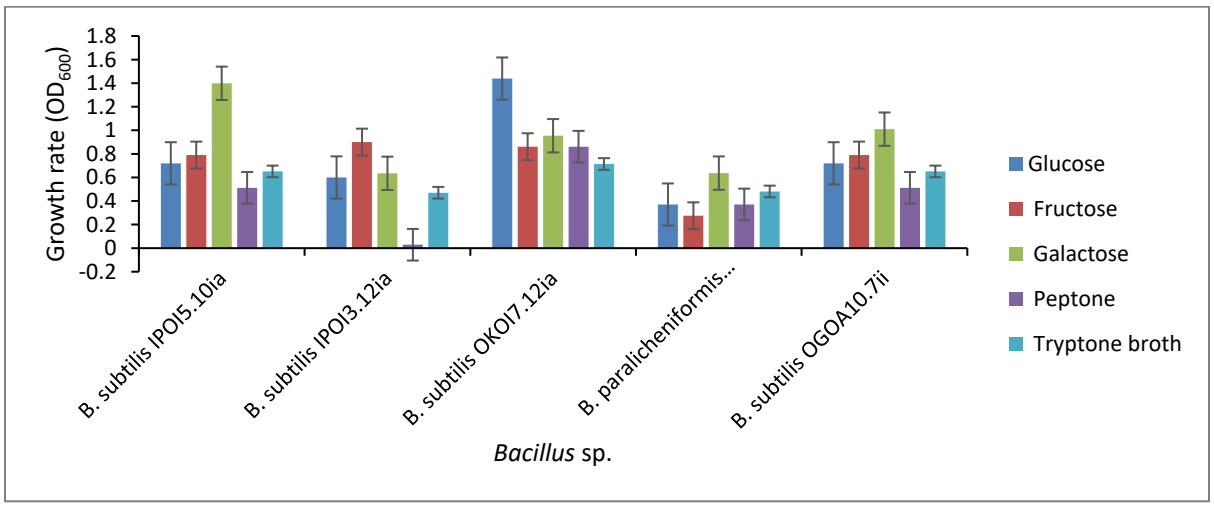

Figure 5: Growth of *Bacillus* sp. in different carbon and nitrogen sources
Values are presented as means± standard error of duplicate values.

Table 4: Protease, amylase, and lipase production by *Bacillus* species by measuring clear zones around the growth in mm

Bacillus isolates	protease	amylase	Lipase
B. subtilis IPOI5.10ia	25.00	23.00	16.00
B. subtilis IPOI3.12ia	-	19.00	-
B. subtilis OKOI7.12ia	25.00	27.00	16.00
B. paralicheniformis OKAO4.12ia	23.00	22.00	18.00
B. subtilis OGOA10.7ii	25.00	20.00	15.00

DISCUSSION

Food-grade *Bacillus* species metabolites have been considered safer, cheaper, and biodegradable alternatives to synthetic antimicrobials that can be used as starter cultures during the production of food (Adebo *et al.*, 2017; Fan *et al.*, 2017; Kumariya *et al.*, 2019 and Dabire *et al.*, 2021). Diverse *Bacillus* species such as *B. subtilis, B. paralicheniformis, B. circulans, B. pumilus, B. polymyxa, B. amyloliquefacines, B. sphaericus, B. firmus, B. clausii, B. velezensis*, etc. can be isolated from various food samples such as the Nigerian *iru, ogiri, ugba, okpehe,* and *owoh*; Burkina-Faso *soumbala, maari* and *bikalga*; Benin republic *ikpiru, yanyanku, afintin, sonru, netetou,* and *tayohounta*; Cameroon *mbuja* and Ghana *kantong* (Parkouda *et al.*, 2015; Sieiro *et al.*, 2016; Ademola *et al.*, 2018; Adewumi *et al.*, 2019, Agbobatinkpo *et al.*, 2019 and Owusu-Kwarteng *et al.*, 2020). However, *B. subtilis* has been documented to be the most predominant *Bacillus* species amongst others, which conforms to the reports from this study (Joseph *et al.*, 2013; Aruwa and Olatope, 2015; Dabire *et al.*, 2022). Out of the five (5) *Bacillus* identified in this study, four (4) belong to the *B. subtilis* group, in line with the records of previous researchers mentioned above. The antibacterial and antifungal properties of *Bacillus* species documented in this study shows that *B. subtilis* OKOI7.12ia had the highest antimicrobial activity against indicator food-borne pathogenic bacteria and fungi namely: *E. coli* DCM10974, *E. coli* ATCC 43816, *S. enteritidis* ATCC 13875, MRSA NIMR/GP054, *P.stutzeri* NIMR/GN029, *A. flavus* GB *and A. niger* AGM. This result aligns with the reports of Youcef-Ali *et al.* (2014), who stated that *B. subtilis* had antifungal activity against *Candida albicans*. Oyedele *et al.* (2014) also reported the antifungal inhibitory activities of *B. subtilis* against *A. niger, A. flavus, Fusarium oxysporium,* and *Rhizopus stolonifer* ranged from 8.5-22.5; 12.0-24.5; 9.0-18.0 and 10.0-14.0) respectively. According to the reports of Kadaikunnan *et al.* (2015), the antibacterial antagonistic activity of *B. amyloliquefaciens* against *B. subtilis* (ATCC 7972), *Enterococcus cloacae* (ATCC 29212), *Staphylococcus aureus* (ATCC 25923) and *S. epidermidis* (MTCC 3615) was 20.16±0.28, 19.00±0.5, 22.33±0.57 and 29.83±0.76 respectively while the antifungal inhibitory activities was also documented for: *A. clavatus, A. fumigates, A.niger, A. oryzae, Curvularia lunata, Fusarium oxysporum, Gibberella moniliformis, Humicola grisea, Penicillium chrysogenum* and *P. roqueforti*. Delgadillo *et al.* (2018) also documented the antifungal activity of *B. subtilis* against *Rhizoctonia solani*. Moving forward, Lu *et al.* (2018) have previously reported the antibacterial inhibitory zones by *B. subtilis* against *S. aureus, E. coli, Enterococci* spp., and *Salmonella gallinarum* to be in the range of 7.0-10.00, 7.0-12.00, 7.0-11.00. and 6.50-7.50, respectively.

The result of the optimization process for the optimum production of antimicrobial metabolites at different temperatures and pH, involving the use of different carbon and nitrogen sources, shows that the highest production was observed at 30°C and pH 8, using different carbon and nitrogen sources on the growth of *Bacillus*. Abo-Amer. (2011) documented optimum metabolite growth at 30°C, pH 6.5, 5% lactose, and yeast extract (as carbon and nitrogen sources, respectively), which is a bit different from the results obtained in this study. Delgadillo *et al.* (2018) also reported that optimum *Bacillus* metabolite growth occurs mostly at the stationary phase, which involves temperature and pH ranges

between 15-37°C and 5-8, respectively. Optimum antimicrobial production was also recorded for *B. subtilis* isolated from *P. biglobosa* (*iru*) at 50°C and pH 9 (Oyeleke *et al.*, 2011).

According to the reports of Danilova and Sharipova (2020), *Bacillus subtilis* can produce a diverse of enzymes such as amylases, xylanases, lichenase, β-galactosidase, cellulase, proteases, etc., which is in line with the result of enzymatic assays obtained from this study.

From the enzymatic assays carried out on an enzymatic test medium, *B. subtilis OKOI7.12ia* was observed to show the highest amount of protease (25 mm), amylase (27mm), and lipase (16mm). According to the report of Youcef-Ali *et al.* (2014) reported the production of protease and cellulase but not chitinase by *B. subtilis* and *B. mojavensis* isolated from soils in the arid regions of Algeria showing in mm: 34, 30, and 20, 30 for the production of protease and cellulase by *B. subtilis* and *B. mojavensis* respectfully. In addition, Kadaikunnan *et al.* (2015) documented the production of ornithine decarboxylate by *B. amyloliqufaciens* and *B. subtilis*. In addition, cell-wall degrading and digestive enzymes produced by *Bacillus* isolated from various sources produced different kinds of enzymes, which include amylase, cellulase, protease, lipase, and β-galactosidase (Ananthanarayanan and Dubhashi, 2015). Oyeleke *et al.* (2011) also documented the synthesis of amylase and protease by *B. subtilis* isolated from *P. biglobosa* (*iru*). Furthermore, Dabire *et al.* (2022) reported enzymatic activity of 43.00-60.67mm, 22.59-49.55mm, 20.02-24.57 mm, and 0.00-10.67mm for protease, amylase, lipase and tannase by *Bacillus* species isolated from *soumbala* in Burkina-Faso.

CONCLUSION

From the results obtained in this study, *Bacillus* species, especially the *B. subtilis* group are predominately responsible for the alkaline fermentation of food condiments with promising technological functions such as enzymes and antimicrobial peptides production. The *Bacillus* sp. analyzed in this study showed significant inhibitory activities against food-borne indicator organisms of public health significance. Conclusively, *B. subtilis* OKOI7.12ia was observed to be the most promising *Bacillus* sp that can be used as a potential starter culture for the production of microbiologically safe food products.

CONFLICT OF INTEREST

The authors declared no conflict of interest

REFERENCES

Abo-Amer AE (2011) Optimization of bacteriocin production by Lactobacillus acidophilus AA11, a strain isolated from Egyptian cheese. Annual Microbiology. 61:445-452. **[Crossref]**

Abriouel H, Franz CM, Omar NB (2011) Diver¬sity and applications of Bacillus bacteriocins. FEMS Microbiology Review. 35:201-232. **[Crossref]**

Adebo OA, Njobeh PB, Adebiyi JA, Gbashi S, Phoku JZ, Kayitesi E (2017) Fermented pulse-based food products in developing nations as functional foods and ingredients, in: M.C. Hueda (Ed.), Functional Food: Improve Health through Adequate, Food, Intech, Janeza Trdine, Rijeka. 42(1): 77-100. **[Crossref]**

Ademola OM, Adeyemi TE, Ezeokoli OT, Ayeni KI, Obadina AO, Somorin YM, Omemu AM, Adeleke RA, Nwangburuka CC, Oluwafemi F (2018) Phylogenetic analyses of bacteria associated with the processing of iru and ogiri condiments. Letters in Applied Microbiology. 67 (4):354-62. **[Crossref]**

Adewumi GS, Grover CI, Oguntoyinbo FA (2019) Phylogenetics, Safety and In Vitro Functional Properties of Bacillus Species Isolated from Iru, a Nigerian Fermented Condiment. Microbiology and Biotechnology Letters. 47 (4):498-508. **[Crossref]**

Agbobatinkpo BP, Tossou GM, Adinsi L, Akissoe HN, Hounhouigan DJ (2019) Optimal fermentation parameters for process-ing high quality African locust bean condiments. Journal of Food Science and Technology. 56 (10):4648-57. **[Crossref]**

Aruwa CE, Olatope SOA (2015) Characterization of Bacillus species from convenience foods with conventional and API kit method: A comparative analysis. Journal of Applied Life Sciences International. 3(1): 42-48. **[Crossref]**

Beladjal L, Gheysens T, Clegg JS, Amar M, Mertens J (2018) Survival of dry bacterial spores after very high temperature exposure. Extremophiles: Life Under Extreme Conditions. 22(5):751-759. **[Crossref]**

Benitez LB, Velho RV, Lisboa MP, Medina LF, Brandelli A (2010) Isolation and characterization of antifungal peptides produced by Bacillus amyloliquefaciens LBM5006. Journal of Microbiology. 48:791-797. **[Crossref]**

Caulier S, Nannan C, Gillis A, Licciardi F, Bragard C, Mahillon J (2019) Overview of the antimicrobial compounds produced by members of the Bacillus subtilis group. Frontiers Microbiology. 10(2):302-310. **[Crossref]**

Chantawannakul P, Oncharoen A, Klanbut K, Chukeatirote E, Lumyong S (2002) Characterization of proteases of Bacillus subtilis strain 38 isolated from traditionally fermented soybean in Northern Thailand. Science Asia. 28(3):241. **[Crossref]**

Chopra L, Singh G, Jena K (2015) Sonorensin: a new bacteriocin with potential of an anti-biofilm agent and a food biopreservative. Science Report. 10(5):13412 -13422. **[Crossref]**

Christie G, Setlow P (2020) Bacillus spore germination: Knowns, unknowns and what we need to learn. Cellular Signalling. 74(2):109729. **[Crossref]**

Cotter PD, Ross RP, Hill C (2013) Bacteriocins-a viable alternative to antibiotics? Nature Review in Microbiology. 11(2):95-105. **[Crossref]**

Cutting SM (2011) Bacillus probiotics. Food Microbiology, 28:214-220. **[Crossref]**

Dabire Y, Somda NS, Compaore CS, Mogmenga I, Somda MK, Ouattara AS, Dicko MH, Ugwuanyi JO, Ezeogu LI, Traore AS (2021) Molecular screening of bacteriocin- producing Bacillus spp. isolated from soumbala, a fermented food condiment from Parkia biglobosa seeds. Scientific African. 12(2021): e000836. **[Crossref]**

Dabire Y, Somda NS, Somda MK, Compaore CB, Mogmenga I, Ezeogu LI, Traore AS, Ugwuanyi JO, Dicko MH (2022) Assessment of probiotic and technological properties of Bacillus spp. isolated from Burkinabe soumbala. BMC Microbiology. 22(1): 228-241. **[Crossref]**

Dahiya P, Purkayastha S (2011) Isolation, screening and production of extracellular alkaline lipase from a newly isolated Bacillus sp. PD-12. Journal of Biological Sciences. 11(5):381-7. **[Crossref]**

Danilova L, Sharipova M (2020) The practical potential of Bacilli and their enzymes for industrial production. Frontiers in Microbiology. 11(2): 1782-90. **[Crossref]**

Delgadillo RJ, Valdes-Rodriguez SE, Juarez RA, Portugal VO, Hernandez JLG (2018) Effect of pH and temperature on the growth and antagonistic activity of Bacillus subtilis on Rhizoctonia solani. Mexican Journal of Phytopathology. 36(2): 256-275.

Du H, Zhou L, Lu Z, Bie X, Zhao H, Niu YD, Lu F (2020) Transcriptomic and proteomic389 profiling response of methicillin-resistant Staphylococcus aureus (MRSA) to a novel bacteriocin, 390 plantaricin GZ1-27 and its inhibition of biofilm formation. Applied Microbiology of Biotechnology.104(1):7957-7970. **[Crossref]**

Eijlander RT, Abee T, Kuipers OP (2011) Bacterial spores in food: How phenotypic variability complicates prediction of spore properties and bacterial behavior. Current Opinion in Biotechnology. 22(1):180-186. **[Crossref]**

Elshaghabee FMF, Rokana N, Gulhane RD, Sharma C, Panwar H (2017) Bacillus as potential probiotics: Status, concerns, and future perspectives. Frontiers in Microbiology. 8(1):1490-6. **[Crossref]**

Fadahunsi IF, Olubodun S (2021) Antagonistic pattern of yeast species against some selected food-borne pathogens. Bulletin

of the National Research Centre. 45(1): 34-53. [Crossref]

Fan B, Blom J, Klenk HP, Borriss R (2017) Bacillus amyloliquefaciens, Bacillus velezensis, and Bacillus siamensis form an "operational group B. amyloliquefacien within the B. subtilis species complex. Frontiers in Microbiology. 8 (22): 22-30. [Crossref]

Hashemizadeh Z, Bazargani A, Davarpanah MA (2011) Blood culture contamination in a neonatal intensive care unit in Shiraz, Southwest Central Iran. Medical Principles & Practice. 20(1):133-136. [Crossref]

Huang F, Teng K, Liu Y, Cao Y, Wang T, Ma C, Zhang J, Zhong J (2021) Bacteriocins:400 Potential for human health. Medical Cell Longev. 55(1):882-6. [Crossref]

Jadhav JP, Phugare SS, Dhanve RS, Jadhav SB (2010) Rapid Biodegradation and Decolorization of direct orange 39 (orange TGLL) by an isolated bacterium Pseudomonas aeruginosa strain BCH. Biodegradation. 21(1): 453-463. [Crossref]

Javed F, Ali Z, Iqbal S, Ahmed N, Farooq Z, Masood F, Nawaz M (2020) Production of synbiotic product containing galacto-oligosaccharides and saccharomyces boulardii and evaluation of its in-vitro bifidogenic effect. Journal of Microbiology, Biotechnology and Food Science. 43(2):197-200. [Crossref]

Jeong H, Jeong DE, Kim SH, Song GC, Park SY, Ryu CM (2012) Genome sequence of the plant growth-promoting bacterium Bacillus siamensis KCTC 13613T. Journal of Bacteriology. 194 (15):4148-4149. [Crossref]

Jonathan SG, Adeniyi MA, Asemoloye MD (2016) Fungal biodeterioration, aflatoxin contamination and nutrient value of suya spices. Scientifica. 2016(4): 1-6. [Crossref]

Jooste M, Roets F, Midgley GF (2019) Nitrogen-fixing bacteria and Oxalis-evidence for a vertically inherited bacterial symbiosis. BMC Plant Biology. 19(1): 441-9. [Crossref]

Joseph B, Dhas B, Hena V, Raj J (2013) Bacteriocin from Bacillus subtilis as a novel drug against diabetic foot ulcer bacterial pathogens. Asian Pacific Journal of Tropical Biomedicine. 3(12): 942-946. [Crossref]

Kadaikunnan S, Rejiniemon TS, Khaled JM, Alharbi NS, Mothana R (2015) Antibacterial, antifungal, antioxidant and functional properties of Bacillus amyloliquefaciens. Annals of Clinical Microbiology and Antimicrobials. 14(9): 1-11. [Crossref]

Kaškonienė V, Stankevičius M, Bimbiraitė-Survilienė K (2017) Current state of purifica¬tion, isolation and analysis of bacteriocins produced by lactic acid bacteria. Applied Micro¬biology & Biotechnology.101(2):1323-1335. [Crossref]

Kaur S (2015) Bacteriocins as potential anticancer agents. Frontial Pharmacology, 6:272. [Crossref]

Kumariya R, Garsab AK, Rajputc YS, Soodc SK, Akhtard N, Patele S (2019) Bacteriocins: classification, synthesis, mechanism of action and resistance development infood spoilage causing bacteria. Microbial Pathogens. 128 (2019) 171-177. [Crossref]

Lu Z, Guo W, Liu C (2018) Isolation, identification and characterization of novel Bacillus subtilis. The Journal of Veterinary Medical Science. 80(3): 427-433. [Crossref]

Noda M, Miyauchi R, Danshiitsoodol N, Matoba Y, Kumagai T, Sugiyama M (2018) Expression of genes involved in bacteriocin production and self-resistance in Lactobacillus brevis 174A is mediated by two regulatory proteins. Applied and Environmental Microbiology. 84(3):2707-02717. [Crossref]

Olaitan JO, Ozabor PT, Akinde SB, Oluwajide OO, Oyetunji FT, Arogundade NO (2022) Antibacterial effect of Allium sativum (garlic) and Zingiber officinale (ginger) extracts against antibiotic resistant organisms isolated from chicken

abattoir. Nigerian Journal of Microbiology. 36(1): 5991-6000.

Owusu-Kwarteng J, Parkouda C, Adewumi GA, Ouoba LII, Jespersen L (2020) Technologically relevant Bacillus species and microbial safety of West African traditional alkaline fermented seed condiments. Critical Reviews in Food Science and Nutrition. 62(4): 871-888. **[Crossref]**

Oyedele AO, Ogunbanwo TS (2014) Antifungal activities of Bacillus subtilis isolated from some condiments and soil. African Journal of Microbiology Research. 8(18): 184-1849. **[Crossref]**

Oyeleke SB, Oyewole OA, Egwim EC (2011) Production of protease and amylase from Bacillus subtilis and Aspergillus niger using Parkia biglobosa (African locust beans) as substrate in solid state fermentation. Advances in Life Sciences. 1(2): 49-53. **[Crossref]**

Panda MK, Sahu MK (2013) Isolation and characterization of a thermophilic Bacillus sp. with protease activity isolated from hot spring of Tarabalo, Odisha, India. Iran Journal of Microbiology. 5(2):159-65 (PMID: 23825735).

Parkouda C, Ba F, Ouattara L, Tano-Debrah K, Diawara B (2015) Biochemical changes associated with the fermentation of bao- bab seeds in Maari: An alkaline fermented seeds condiment from western Africa. Journal of Ethnic Foods. 2 (2):58-63. **[Crossref]**

Ramesh A, Sharma SK, Sharma MP, Yadav N, Joshi OP (2014) Inoculation of zinc solubilizing Bacillus aryabhattai strains for improved growth, mobilization and biofortification of zinc in soybean and wheat cultivated in Vertisols of central India. Applied Soil Ecology. 73(1):87-96. **[Crossref]**

Riffat SA, Muhammad A, Mashkoor M, Zafar I (2020) Production and Therapeutic Potential of Bacteriocin produced by indigenous isolates of Bacillus subtilis. Pakistan Journal of Agricultural Science. 57(5):1403-1411

Ryan KJ, Ray CG (2014) Sherris Medical Microbiology. 4th Edition. McGraw Hill. ISBN 978-0-8385-8529-0

Savadogo A, Ilboudo AJ, Gnankiné O, Traoré AS (2011) Numeration and identification of thermotolerant endospore-forming Bacillus from two fermented condiments Bikalga and Soumbala. Advance Environmental Biology. 5(9):2960-2966

Schallmey M, Singh A, Ward OP (2014) Developments in the use of Bacillus species for industrial production. Canadian Journal of Microbiology. 50(1):1-17. **[Crossref]**

Sieiro PA, Lopez MM, Mu D, Kuipers OP (2016) Bacteriocins of lactic acid bacteria: Extending the family. Applied Microbiology and Biotechnology. 100 (7):2939-51. **[Crossref]**

Simons A, Alhanout K, Duval RE (2020) Bacteriocins, antimicrobial peptides from bacterial397 origin: Overview of their biology and their impact against multidrug-resistant bacteria. Microorganisms.98 (8): 630- 639. **[Crossref]**

Sivaranjani M, Leskinen K, Aravindraja C, Saavalainen P, Pandian SK, Skurnik M, Ravi A (2019) Deciphering the antibacterial mode of action of alpha-mangostin on Staphylococcus 394 epidermidis RP62A through an integrated transcriptomic and proteomic approach. Frontiers in Microbiology. 395 (10):1-16. **[Crossref]**

Slonczewski JL, Foster JW, (2011) Microbiology: An Evolving Science. 2nd Edition. Norton.

Suganthi V, Mohanasrinivasan V (2015) Optimization studies for enhanced bacteriocin production by Pediococcus pentosaceus KC692718 using response surface methodology. Journal of Food Science and Technology. 52(2):3773-3783. **[Crossref]**

Todorov SD, Leblanc JG, Franco BD (2012) Evaluation of the probiotic potential and effect of encapsulation on survival for Lactobacillus plantarum ST16Pa isolated from papaya. World Journal of

Microbiology and Biotechnology. 28(1):973-84. **[Crossref]**

Unban K, Kochasee P, Shetty K, Khanongnuc CL (2020) Tannin-tolerant and extracellular tannase producing Bacillus isolated from traditional fermented tea leaves and their probiotic functional properties. Foods. 9(4):1-14. **[Crossref]**

War NF, Joshi S (2014) Epiphytic and endophytic bacteria that promote growth of ethnomedicinal plants in the subtropical forests of Meghalaya, India. Revista de Biología Tropical. 62(4):1295-1308. **[Crossref]**

Yost CK (2014) Biopreservation. Encyclopedia of Meat Science, 76-82. **[Crossref]**

Youcef-Ali M, Chaouche NK, Dehimat L, Bataiche I, Ali MA, Cawoy H. Thonart P (2014) Antifungal activity and bioactive compounds produced by Bacillus mojavensis and Bacillus subtilis. African Journal of Microbiology Research. 8(6): 476-484. **[Crossref]**

Zhao X, Kuipers OP (2016) Identification and classification of known and putative antimicrobial compounds produced by a wide variety of Bacillales species. BMC Genomics. 17(1):882-900. **[Crossref]**