Research article **Phytopharmacology Research Journal (PRJ) Open Access**

Received:11 September 2025 | Revised: 25 June 2025 | Accepted: 26 November 2025 | Published online : 1 December 2025 | ISSN : 2959-9687

Exploring Antibacterial Potential of Secondary Metabolites of *Bacillus* Species against Gram Negative Bacteria Isolated from Junk Foods in Peshawar, Khyber Pakhtunkhwa, Pakistan

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Funding information
Not Applicable

Abstract

The increasing consumption of unhygienic fast food has raised serious health concerns due to its association with bacterial food borne diseases. The present study aimed to determine the antibacterial effect of secondary metabolites of *Bacillus* species against multi-drug-resistant Gram-negative bacteria isolated from the junk foods in Peshawar. A total of 100 samples were collected from various junk foods such as pizza, burger, and shawarma in district Peshawar. Specifically, 33 samples were from pizzas, 33 from burgers and 34 from shawarmas. Three bacterial species, i.e., *E. coli*, *Salmonella* and *Shigella* were identified. Secondary metabolites were produced using the shake flask fermentation method. After an incubation period of 14 days, secondary metabolite extraction was carried out. According to the CLSI 2020 guidelines, antibiotic susceptibility testing was performed using the Kirby-Bauer disc diffusion method on Muller Hinton agar medium. The most contaminated type of junk food was shawarma (39.9%). *E. coli* exhibited the highest resistance to tetracycline (89.1%) followed by amoxicillin (59.4%). Cefuroxime sodium (96.1%), followed by tetracycline (92.3%) and azithromycin (65.3%) were resistant to *Salmonella*. Similarly, cefuroxime sodium (84.2%) followed by ampicillin (84.3%) and streptomycin (63.2%) showed resistance to *Shigella*. During this study, it was observed that Sulphamethoxazole/Trimethoprim showed the strongest activity against *E. coli* at (31 mm) while Azithromycin (29 mm) was most effective against *Shigella*. In conclusion, the present study indicated that the junk foods in district Peshawar had unsatisfactory level of contamination with *E. coli*, *Salmonella* and *Shigella.* These findings highlight the need to improve sanitation practices and personal hygiene to reduce the risk of contamination from junk foods.

***Key words*:** *Bacillus* spp; secondary metabolites; antibiotic resistance; *E. coli; Salmonella; Shigella.*

Introduction

Food is very much essential for the sustaining of life of all living beings. Processed, canned and packaged foods, as well as 'high-calorie' meals served in restaurants and cafeterias, include burgers, pizzas, fried chicken, potato fries, sausages, salty snacks, desserts, chocolates carbonated drinks and beverages [1].

Junk foods refer to fast food which are easy to make and easy to consume. HFSS (High fat, sugar, or salt) is another name for junk food [2]. People all around the world enjoy eating junk food; hence the number of restaurants that serve fast food is expanding by the day. The United States of America, Canada, Britain, Australia, Japan and Sweden, among others are the countries with the highest consumption of junk food worldwide [3].

In today's world economy, junk food is a worldwide concern. It is an integral part of life in both industrialized and developing countries, producing an alarming an increase in obesity [4]. Nowadays, healthy and hygienic nutritious food has been replaced by new dietary preferences fast food [5]. People who eat fast food once in a week are 20% more likely to acquire coronary heart disease. [6]. College-aged adolescents consume fast food both frequently and on a regular basis,

which may lead to an increase in the risk of obesity and related health concerns, resulting in greater health care costs [7].

Regular consumption of junk food raises the risk of obesity and chronic conditions such as type 2 diabetes, heart disease, nonalcoholic fatty liver disease, several types of cancer and tooth decay. According to a WHO estimate, eating too much junk food causes 40,000 deaths worldwide each year [8].

The incidence of food-borne illness is rising in developing nations due to a lack of public awareness, inadequate knowledge of food safety and poor personal hygiene habits [9]. Many harmful microbes, including fungi and bacteria, remain dangerous to the population. The majority of bacteria found in foods are antibiotic-resistant, making it very hard to control them. In certain cases, they have reached a point where they can actually kill a person [10].

E. coli is a gram-negative, rod shaped facultative anaerobic, bacteria that is the primary source of many human disorders [11]. It is a diverse pathogenic species that spreads easily in the environment and can be hazardous to the health of human beings [12]. *Salmonella* is a facultative anaerobic, flagellated, gram-negative bacterium belonging to the family of *Enterobacteriaceae*. It is one of the food-borne pathogens that cause diarrhea all over the world [13].

P. aeruginosa is a common rod-shaped, Gram-negative, aerobic, facultative anaerobic bacterium. Inadequate cleanliness contributes to the spread of *P. aeruginosa* [14]. *P. aeruginosa* causes infections in the urinary tract, respiratory system, GI tract, bacteremia bone and joint. *Klebsiella* species is ubiquitous in nature and is an important pathogen of humans and animals. *K. pneumoniae* is the most opportunistic pathogen mainly affecting the hospitalized immunocompromised patients and accounts for urinary tract, respiratory tract, blood and wound infection.

Secondary metabolites (SMs) are small, biologically active chemical compounds with unusual structures. Some of these include atypical sugar, hydroxamic acids, nucleosides, beta-lactam rings and cyclic peptides. Numerous antibiotics, chemotherapeutic medications, immune suppressants and other medications are derived from the secondary metabolites of bacteria [15]. *Bacillus* spp are also known to produce bioactive secondary metabolites including fatty acids, macro lactones and polypeptides and they have a broad range of biological capabilities, including antibacterial, anticancer and anti-algal properties [17]. The current study aims to investigate the antibacterial potential of secondary metabolites of *Bacillus* species against multi-drug-resistant gram-negative bacteria isolated from the junk foods in Peshawar Khyber Pakhtunkhwa, Pakistan.

Materials and Methods

Study Design

It was a lab-based experimental investigation, and it was carried out at the Microbiology Research Laboratory of the Abasyn University Peshawar.

Inclusion and exclusion criteria

In this study, the fresh junk foods such as pizza, burger and shawarma were included while the other junk foods like sandwich, fried chips, fried chicken etc were excluded. Spoiled and non-healthy junk foods were excluded.

Bacterial isolation from junk foods

In the proposed study, 100 samples were collected from various junk foods such as pizza, burger and shawarma aseptically. All the samples were immediately transported to Microbiology Research Laboratory, Abasyn University Peshawar, Pakistan for further processing.

Culturing and sub culturing

All the samples were cultured on Nutrient agar media. Sub culturing of the sample was done on MacConkey media. For sub culturing, a small portion of inoculum was transferred to a fresh culture medium using a loop to pick up a bacterial colony. After 24 hrs, many colonies were found on the plates. The samples were streaked on sterile agar plates and were incubated for 24 hrs at 37˚C [18].

Isolation of *Bacillus* species for secondary metabolites production

Different regions of District Peshawar were selected for sampling. The soil samples were collected from 8 different sites at random using clean, dry sterile, polythene bags with the help of sterile spatula. The microorganisms were isolated by the method of serial dilution on nutrient agar medium [19].

The nutrient agar media was used to isolate *Bacillus* species. Samples (100 µl) of each dilution as added to sterile plate using sterile plate under aseptic condition. After that, media was poured in sterile Petri plates under aseptic condition (Laminar flow cabinet/hood). The plates were incubated at 37°C for 24 hrs. For further purification of *Bacillus* species, sub culturing was carried out on nutrient agar medium. For

sub culturing, a small portion of inoculum was transferred to a fresh culture medium using sterile inoculating loop to pick up a bacterial colony. After 24 hrs, many colonies were found on the plates. The samples were streaked on sterile agar plates and were incubated for 24 hrs at 37˚C [20].

Identification

The identification of microorganisms was performed by using morphological and biochemical tests including, triple sugar iron, urease, Simon's citrate, indole, oxidase and catalase [21]. For morphological identification, the Gram staining technique was used. On the basis of size, shape and farm, the isolates were identified. Clinical Laboratory Standards Institute (CLSI, 2020) guidelines were followed to ensure accuracy and reliability in the laboratory practices [22]. Molecular identification of isolated species was performed by amplifying the 16S rRNA gene using universal primers obtained from Macrogen Universal primer 785F —5′- GGATTAGATACCCTGGTA 3′ and 907R– 5′-: CCGTCAATTCMTTTRAGTTT-3′

Preliminary screening

The antimicrobial activity of *Bacillus species* was subjected to primary screening against various test organisms (*E. coli, S. aureus* and *K. pneumoniae)* using the cross-streak method on Muller-Hinton Agar. For the determination of antibacterial activities, a modified cross-streak method (MCSM) was used using standard procedure [23].

Secondary metabolites extraction

After preliminary test of the isolates, the isolated bacteria were subjected for secondary metabolites production. 13.1g of nutrient broth powder was added in one liter of distill water. The shake flask fermentation method was used for the production of secondary metabolites. The synthetic medium was used as a production medium. Then the medium was incubated in an orbital shaking incubator using 150 rpm at 35°C for 24 to 72 hrs. After an incubation period of 14 days, secondary metabolite extraction was carried out. About 200-500 µL of 40% HCL was added to the bacterial culture. After that, the culture was blended using a blender. An equal volume of ethyl acetate was added and the culture was mixed for 40 min. This culture slurry was filtered through cheese cloth. After that, the filtered medium was added to a separated funnel and allowed to stand for 10 min that was resulted in two layers. The organic layer containing metabolites of interests was separated from the aqueous phase and was washed. To remove remaining traces of water from the organic layer, anhydrous sodium sulfate (Na2SO4) was added to it. Finally, the isolated metabolites were then concentrated by rotary evaporator at 45°C and 150 rpm [24].

Antibiotic susceptibility testing

According to the CLSI 2020 guidelines, the antibiotic susceptibility testing was performed using the Kirby Bauer disc diffusion method on MHA media. Antibiotics used in this research study were as shown in Table 1. All the cultures were streaked on to sterile MHA plates and were incubated for 24 hrs at 37˚C. Sterilized cotton swab was dipped in solution and excess broth was squeezed at the edge and then was swabbed onto MHA media plates. With the help of sterile forceps, the antibiotic discs were placed aseptically on MHA media plates. After that, the plates were incubated at 37˚C for 24 hrs. Sensitive (S), Intermediate (I) and Resistant ®, results were recorded [25].

Table 1: List of antibiotics used in the study

S. No	Antibiotic disc	Abbreviation	Concentration
1	Ampicillin	AMP	10µg
2	Streptomycin	S	10µg
3	Cefuroxime sodium	CXM	30µg
4	Tetracycline	TE	30µg
5	Azithromycin	AZM	15µg
6	Chloramphenicol	C	30µg
7	Amoxicillin /Clavulanic acid	AMC	30µg
8	Sulphamethoxazole/ Trimethoprim	SXT	25µg

Evaluation of crude extract along with antibiotics

To evaluate the activity of crude extract and antibiotics first the MHA media was prepared. After that, media was sterilized in autoclave at 121°C for 15 min. After sterilization the media was poured in Petri plates in aseptic condition. Following the conventional procedure, the antibiotic disc containing the extract was introduced to the culture, and the corresponding zone of inhibition was measured [26].

Statistical analysis

The data was interpreted using a Microsoft Excel spreadsheet and after that, it was analyzed using SPSS software version 16.0 and Chi-squared test was used for association [27].

Results

Frequency distribution of junk foods

Among 100 junk foods, 34% were shawarmas,

33% were burgers and 33% were pizzas as shown in Table 2.

Table 2: Frequency distribution of junk foods.

S.no	Foods	Frequency	Percentages
1	Shawarma	34	34%
2	Burger	33	33%
3	Pizza	33	33%

A total of 100 samples were collected from different junk foods in district Peshawar consisting of thirty-three samples of each of pizza, burger and thirty-four of shawarma. The samples include fresh foods. Among them 79 samples showed growth on nutrient agar media, while 21 samples showed no growth on nutrient agar media. The bacteria grown over nutrient agar was frequently sub cultured to obtain pure culture of bacteria.

Isolation of microorganisms

Bacterial isolates

In this study, a total of 3 types of bacteria were obtained from junk food samples at microbiology research laboratory. The obtained bacteria were *E. coli*, *Shigella* and *Salmonella*. Further, the obtained bacteria were confirmed by using culture characterizing, gram staining and different biochemical test, as the biochemical reaction results shown in table 4.3. Higher bacterial counts were noted in shawarma samples.

Percentage wise data of isolates of junk foods

According to percentage wise, out of 100 samples 21% samples showed no growth, while 79% samples showed growth on nutrient agar media. The most prevalent bacterial species isolated were *E. coli* followed by *Salmonella* and *Shigella*. Out of these 79 samples a total of 37 (46%) samples were as *E. coli*, 26 (32.9%) were *Salmonella* and 16 (21%) were *Shigella*. The results are summarized in the Table 3 and shown in Figure 1.

Table 3: Percentage wise distribution of isolates of junk foods

S.no	Isolates	Percentage wise data of isolates
1	*E. coli*	46%
2	*Salmonella*	33%
3	*Shigella*	21%
	Total	100%

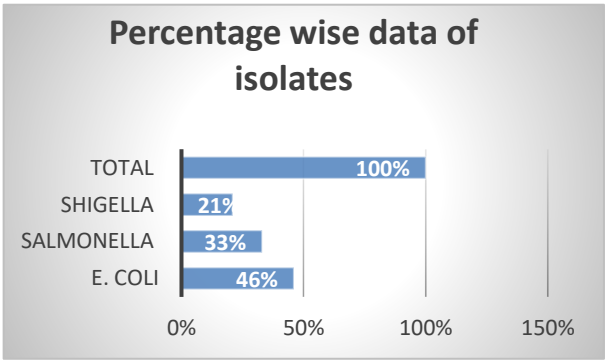

Fig 1: Percentage wise data of isolates of junk foods

Out of 100 samples, 79% samples showed growth on nutrient agar media. Out of these 79, a total of 37(46%) samples were as *E. coli*. Out of 37, 14(37.8%) were collected from pizzas, 12(32.4%) were from burgers and 11 (29.8%) were from shawarmas. Out of 79%, a total of 26 (32.9%) were reported as *Salmonella* in which 14(53.7%) were from shawarmas, 7(27%) were from burgers and 5 (19.3%) were from pizzas. Similarly, out of 79%, a total of 16 (21%) were recorded as *Shigella* in which 6 (37.5%) were collected from burgers, 4(25%) were from pizzas and 6 (37.5%) were from shawarmas as shown in Figure 2.

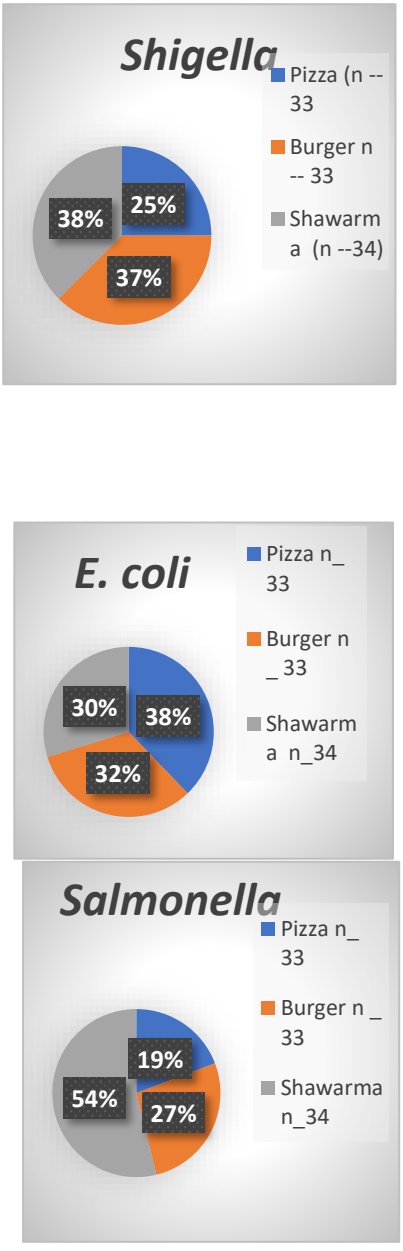

Fig 2: Percentage wise distribution of *E. coli*, *Salmonella* & *Shigella*

Biochemical and molecular tests for identification of isolated organisms of junk foods

Biochemical tests were done in accordance to standard procedure. *E. coli* is a gram-negative rod bacterium, Triple sugar iron was positive for *E. coli*, the gas production in the butt indicates fermentation of sugar. Indole and catalase were positive for *E. coli* while oxidase, citrate and urease were negative for *E. coli*. Likewise, triple sugar iron, catalase and citrate test were positive for *Salmonella* while oxidase and urease were recorded negative for *Salmonella*. Similarly, citrate, oxidase and urease were showed negative results for *Shigella* while catalase triple sugar iron and indole were showed positive results against *Shigella* as shown in the Table 4.

Table 4: Biochemical tests for identification of isolated organisms of junk foods

S.no	Bacteria Isolated	Gram stain	Indole	Urease	Sugar Tripper	Citrate	Oxidase	Catalase
1	*E. coli*	–	+	–	Acid Gas production	–	–	+
2	*Shigella*	–	+	–	+	–	–	+
3	*Salmonella*	–	–	–	Gas + Acid	+	–	+

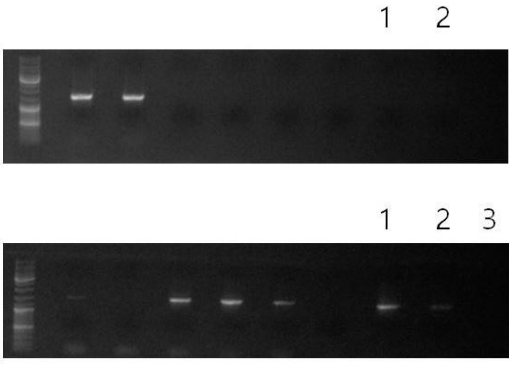

Figure 3: Molecular identification of isolated species using 16S rRNA gene

Isolation of bacteria from different sites

Total eight soil samples were collected from different sites of agriculture and hospital lawn. The isolated samples were identified on the basis of biochemical and morphological tests. The isolated species B1 B6 B7 B8 were rod-shaped Gram-positive bacteria as studied under compound microscope while remaining isolates were gram negative bacteria. Different biochemical test was performed for the identification of isolated organisms from soil to know about the biochemical characteristics of antibiotic

producing species. Citrate utilization test was positive for B1 & B2. Catalase test was also positive for all isolates. While urease biochemical test was negative for B2 and positive for remaining isolates of *Bacillus.* Methyl red test was positive for both species of *Bacillus* while Voges Proskauer test was negative for all isolates. Oxidase and coagulase test were negative for all isolates as shown in Table 5.

Table 5: Biochemical test for isolated organisms of soil

S. no	Isolated bacteria	Gram	Catalase	Indole	Urease	Oxidase	Methyl	VP	Coagulase	Citrate
1	*B. subtilis*	+	+	–	–	–	+	–	–	+
2	*B. cereus*	+	+	–	+	–	+	–	–	+

Antibiotic susceptibility testing

The activity of the test organisms was further examined against different antibiotics such as tetracycline, ampicillin, azithromycin, chloramphenicol, cefuroxime sodium, streptomycin, amoxicillin and sulphamethoxazole/Trimethoprim via a spread plate method while their ability to inhibit bacteria was measured in ZI. *E. coli* was sensitive to chloramphenicol (67.6%) and sulphamethoxazole (56.7%), but resistant to amoxicillin (59.4%), tetracycline (89.1%), azithromycin (70.2%) and streptomycin (78.4%). *E. coli* was resistant to the majority of antibiotics. *Salmonella* resistance was found in cefuroxime sodium (96.1%), tetracycline (92.3%), amoxicillin (30.7%), and azithromycin (65.3%). While sensitive to sulphamethoxazole (65.5%) and chloramphenicol (73.1%). Likewise, sulphamethoxazole (92.7%), followed by chloramphenicol (84.2%) and azithromycin (98.4%) were sensitive to *Shigella*, but cefuroxime sodium (84.2%), ampicillin (84.3%), streptomycin (63.2%) and amoxicillin (52.6%) shown resistance to *Shigella.*

Antibacterial activity of *Bacillus* spp

The crude extract of *Bacillus* spp was screened against the bacterial species isolated from the junk foods. The agar well diffusion method was used for the screening of antibacterial activities [28]. For this purpose, MHA media was prepared, autoclaved and poured in sterile Petri plates under LFH. The pathogenic bacterial species selected were *E. coli*, *Salmonella* and *Shigella*. These species were cultured on media and then each specie was streaked uniformly in MHA plates with sterile cotton swab. Then three wells were made in agar plates. The sample was prepared by dissolving 30 g of crude extract in 100 ml of DMSO. Samples was taken in different concentrations i.e 50, 100µl and transferred in each well. After that, the Petri dishes were left for few minutes until the sample soaks in the media. The agar plates were kept in incubator at 37˚C for 24 hrs. Then results were noted. The zone of inhibition produced by extract was measured. Maximum zone of inhibition was observed at concentration of 100µl against *E. coli* (25 mm) followed by *Salmonella* (22 mm) and *Shigella* (19 mm) results are shown in Table 6 and Figure 4.

Table 6: Antibacterial activity of crud extract of *Bacillus* at 50 & 100µl concentration

S. no	Isolated bacteria	Negative Control	Positive control Chloramphenicol	Concentration 50µl	Concentration 100µl
1	*E. coli*	0	24mm	16mm	25mm
2	*Salmonella*	0	27mm	20mm	22mm
3	*Shigella*	0	23mm	17mm	19mm

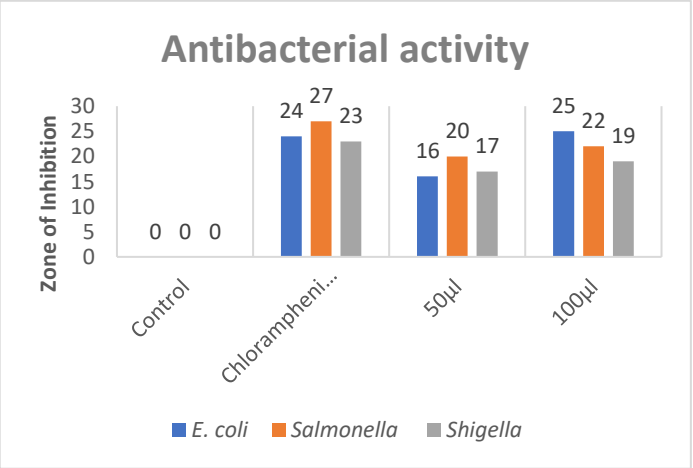

Fig 4: Antibacterial activity of crude extract of *Bacillus* species against pathogenic bacteria at 50 & 100µl concentration

Evaluation of synergetic effect

The antibiotic disc and the produced secondary metabolites were measured in equal amounts. Following the conventional procedure, the antibiotic disc containing the extract was introduced to the culture, and the corresponding zone of inhibition was measured [29]. The Kirby Bauer disc diffusion method was used to determine the synergistic effect. The study found that the coated Sulphamethoxazole/Trimethoprim revealed the best activity against *E. coli* at (31 mm), followed by chloramphenicol (30 mm), streptomycin (25 mm) and ampicillin (20 mm) as shown in Table 7 and in Figure 5.

Table 7: Synergistic activity of antibiotics against *E coli*

S.no	Antibiotics	*Salmonella* Zone of inhibition (mm)	
		coated	uncoated
1	Ampicillin	13	10
2	Azithromycin	29	7
3	chloramphenicol	35	31
4	Amoxicillin clavulanic acid	11	0
5	streptomycin	19	16
6	Cefuroxime sodium	12	0
7	Tetracycline	25	0
8	Sulphamethoxazole/Trimethoprim	20	15

The antibacterial activity against *Salmonella* was highest for chloramphenicol (35mm), azithromycin (29 mm), tetracycline (25 mm), sulphamethoxazole trimethoprim (20 mm), streptomycin (19 mm) and Ampicillin (13 mm) as shown in Table 8 and in Figure 6.

Table 8: Synergistic activity of antibiotics against *Salmonella*

S. no	Antibiotics	*E coli* Zone of inhibition (mm)	
		coated	uncoated
1	Ampicillin	20	0
2	Azithromycin	19	10
3	chloramphenicol	30	24
4	Amoxicillin clavulanic acid	9	0
5	streptomycin	15	11
6	Cefuroxime sodium	10	0
7	Tetracycline	0	0
8	Sulphamethoxazole/Trimethoprim	31	23

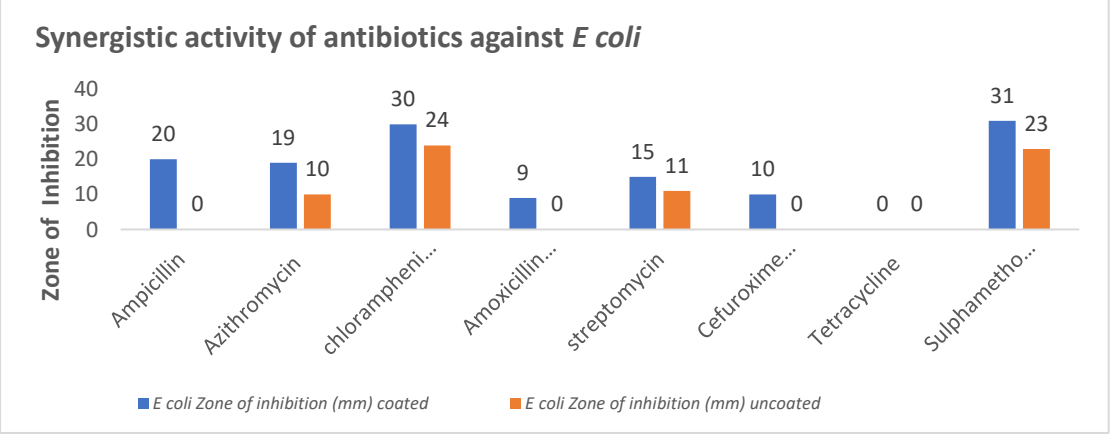

Figure 5: Synergistic activity of antibiotics against *E coli*

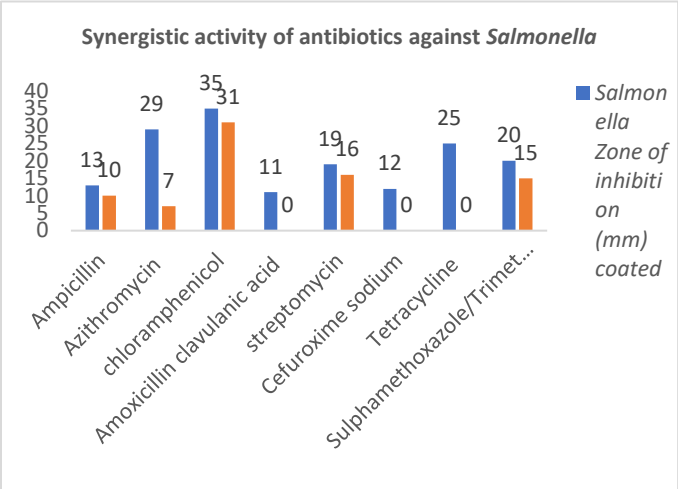

Fig 6: Synergistic activity of antibiotics against *Salmonella*

The activity of antibiotics when combine with extract showed rise in the zone of inhibition such as azithromycin (29 mm), Sulphamethxazole/trimethoprim (23 mm), ampicillin (10 mm), tetracycline (18 mm) and cefuroxime sodium (11 mm) against *Shigella*. Azithromycin (29 mm) showed the highest activity against *Shigella* as shown in Table 9 and Figure 7.

Table 9: Synergistic activity of antibiotics against *Shigella*

S.no	Antibiotics	*Shigella* Zone of inhibition (mm)	
		Coated	uncoated
01	Ampicillin	10	0
02	Azithromycin	29	15
03	chloramphenicol	26	26
04	Amoxicillin clavulanic acid	10	0
05	streptomycin	14	13
06	Cefuroxime sodium	11	0
07	Tetracycline	18	10
08	Sulphamethoxazole/Trimethoprim	23	13

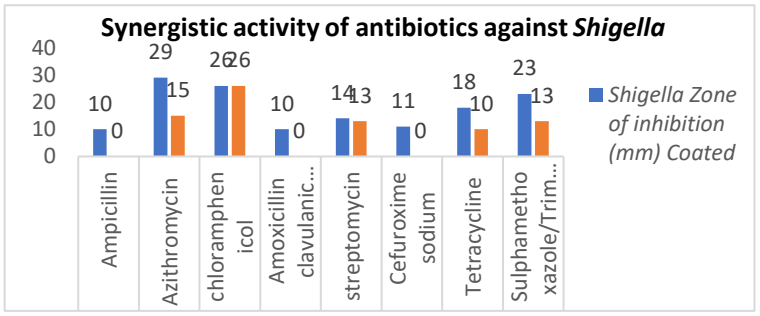

Fig 7: Synergistic activity of antibiotics against *Shigella*

Discussion

The present study was conducted to determine the antibacterial potential of secondary metabolites of *Bacillus* species against gram negative bacteria isolated from junk foods. *Bacillus* species were collected from collected soil samples because soil is the rich source for diverse group of microorganisms. These diverse groups of microorganisms produce secondary metabolites. Similar study was demonstrated [29] that *Bacillus* species are one of the predominant soil bacteria that are able to produce essential secondary metabolites that have antagonistic effects on other microbes. They are Gram-positive, endospore-forming, chemoheterotrophic, aerobic or facultative anaerobic rods usually consisting of flagella for motility.

Current research indicates that two *Bacillus* species, *B. subtilis* and *B. cereus*, were discovered in agricultural and hospital lawn soil. This is consistent with a study [30] that isolated five *Bacillus* species, including *B. megaterium*, *B. velezensis*, *B. aryabhattai*, *B. spizizenii* and *B. subtilis*. All samples showed antimicrobial action against microbes. The antibacterial activity of *B. aryabhattai* and *B. megaterium* was demonstrated against Gram-negative bacteria, whereas that of *B. velezensis* and *B. subtilis* was demonstrated against Gram-positive bacteria. Additionally, *B. spizizenii* demonstrated antibacterial activity against both Gram-positive and Gram-negative bacteria. In a similar way, another study [31] revealed that 30 different *Bacillus* species were identified from Egyptian soil. *B. firmus*, *B. subtilis*, *B. thuringenesis*, and *B. cereus* were the isolated antibiotic-producing *Bacillus* species.

In other similar study carried out by [32] shows that high levels of antibacterial activity were demonstrated by two isolates, GAP2 (*B. subtilis*) and GAP9 (*B. thuringiensis*). *Bacillus* strains that produce secondary metabolites particularly GAP2 and GAP9 isolates are supposed to use for microbes in veterinary care,

agriculture and the food industry.

The results of present study are concluded that as none of the tested junk foods were free of bacteria. Similar study was also conducted [33] in their study showed that in developing countries such as Pakistan, proper hygiene measures are often neglected and food items have been reported to be contaminated with several pathogens.

A similar study [34] stated that many people had experienced various food-borne diseases after consuming street foods. The total mean aerobic count was not under the acceptable microbiological limits at 7.18 ±1.26 CFU/mL. High bacterial counts in completely cooked street food were attributed to poor post handling and personal hygiene of food vendors.

Our results showed that higher bacterial counts were noted in shawarma samples as compared to other junk foods. Similar study was also determined by Alharbi *et al*. (2019). Their results indicated that most of junk food samples examined in the study did not meet any bacteriological quality standard as recommended by the New South Wales (NSW) Food Authority to be <5.0 log10 CFU g−1.

This investigation study showed that *E. coli*, *Salmonella* and *Shigella* are prevalent gram-negative bacteria found in junk foods. In this study majority of bacteria that were isolated from junk foods were resistance to amoxicillin, tetracycline and azithromycin while most of them were susceptible to sulphamethoxazole, especially *E. coli* was resistant to the majority of antibiotics.

Our study showed that *E. coli* was significantly resistance to amoxicillin (59.4%) followed by tetracycline (89.1%), azithromycin (89.1%) and streptomycin (78.4%), while susceptible to chloramphenicol (21.6%) and sulphamethoxazole (56.7%). Similar study was conducted by Tan *et al*. (2014). Their results showed that *E. coli* isolates that were resistant to the antibiotics was (85.71%) Penicillin and Chloramphenicol, (57.14%) Sulfamethoxazole-Trimethoprim, Ampicillin and Trimethoprim, (28.57%) Kanamycin and Tetracyline and (14.29%) to ciprofloxacin.

In the current research, *Salmonella* showed (96.1%) resistance to cefuroxime sodium, (92.3%) were resistance to tetracycline, (30.7%) were resistance to amoxicillin and (65.3%) were resistant to azithromycin. While susceptible to chloramphenicol (73.1%) and sulphamethoxazole (65.5%). Similar findings were reported in which Streptomycin, Azithromycin, Gentamicin, Tetracycline and Neomycin were found sensitive for *Salmonella* spp while Vancomycin, Penicillin, Erythromycin, Amoxicillin and Ampicillin were found resistant. In other similar study conducted by Sabuj *et al*. (2018) stated that about 75% of isolates *Salmonella* spp. showed sensitive to gentamycin followed by 87.5% to azithromycin and 75% chloramphenicol respectively and similar results were also observed in *E. coli*

In the current study, 21% of the samples were contaminated with *Shigella* is in line with the study conducted in India in which 66.6% of the ready-to-eat salads were contaminated with *Shigella* spp, which is higher than the current study. Similarly, our research study showed that *Shigella* were resistance to (84.2%) cefuroxime sodium, (84.3%) were resistance to ampicillin, (63.2%) were resistance to streptomycin and (52.6%) were resistance to amoxicillin. While *Shigella* were susceptible to (92.7%) sulphamethoxazole, (84.2%) were susceptible to chloramphenicol (84.2%) and (98.4%) were susceptible to azithromycin is in line with the study conducted of the eleven isolates of *Shigella* spp tested against seven antibiotics, the highest degree of resistance was found against ampicillin (90%). Invariably, all isolates (100%) were susceptible to meropenem and co-trimoxazole whereas 90% were susceptible to ciprofloxacin.

Conclusion

The study was conducted to isolated gram-negative bacteria from junk foods such as pizza, burger and shawarma in District Peshawar. The present study indicated that the junk foods in district Peshawar had unsatisfactory level of contamination with *E. coli*, *Salmonella* and *Shigella.* Most of the samples were found to be contaminated with *E. coli*. The ethyl acetate extract of *Bacillus* spp was tested against bacteria and extract showed significant activity against all bacteria (*E. coli*, *Salmonella* and *Shigella*).

The study found that the coated Sulphamethoxazole/Trimethoprim revealed the best activity against *E. coli* at (31 mm), followed by chloramphenicol (30 mm). *Salmonella* showed largest zone of inhibition against chloramphenicol (32 mm), followed by azithromycin (29 mm). Similarly, Azithromycin (29 mm) showed the highest zone of inhibition against *Shigella*, followed by streptomycin (14 mm) and chloramphenicol (26 mm). Therefore, in order to promote good health, the government must concentrate on conducting seminars about food safety and establishing early preventive measures.

Declarations
Ethics approval:
Not applicable.
Consent to participate
Not applicable.
Consent to publish
Not applicable.
Clinical trial
Not applicable.
Clinical Trial Number
Not applicable.
Conflicts of interest
The authors declare that they have no conflict of interest.
Funding
This work has received no funding.
Data Availability
Data used during the preparation of this manuscript is available within the article.
Authors' contributions
MI, and SK: conceived and designed the project; MI, MY, MAT, SA, DA, TK: analyzed, wrote, revised and proofread the manuscript. All authors contributed to the article and read and approved the final manuscript.
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