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Microbial Profiles and Patterns of Antibiotic Susceptibility of Bacterial Isolates from Edible Sprouts of Flaxseeds (*Linum usitatissimum L.*)

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Abstract

Flaxseed (*Linum usitatissimum L.*) is a plant-based food that provides healthy fat, antioxidants, and fiber. It is referred to as a "functional food" by certain individuals, which suggests that someone can eat it to improve their health. A study on sprouted flaxseed produced in the laboratory revealed the presence of microbial flora, namely bacteria and molds, which is of concern for the health-conscious public. A total of 39 flaxseed sprout samples were cultured to evaluate microbial profiles and to determine antibiotic susceptibility patterns by the Kirby-Bauer Disc diffusion method. Bacterial isolates were tested only against 11 antibiotics, viz. Amoxycillin, Gentamicin, Ciprofloxacin**,** Colistin, Meropenem, Cefixime, Azithromycin, Mecillinam, Cotrimoxazole, Cefaclor, and Moxifloxacin. 39 (100%) of the 39 flaxseed sprout samples cultured tested positive for bacteria and molds. Among them, positive cultures, single bacterial growth, multiple bacterial growth, and molds were 33 (84.6%), 6 (15.4%) and 39 (100%) respectively. The most predominant organisms were *Serratia marcescens*, comprising 15(33.3%), followed by *Citrobacter freundii* 10(22.2%), *Pseudomonas aeruginosa* 9(20.0%), and other bacterial isolates were *Enterobacter cloacae complex*, *Morganella morganii,* and *Aeromonas hydrophila*, whose frequencies were 6(13.3%), 4(8.9%) and 1(2.2%) respectively. All of the bacterial isolates were 100% sensitive to Ciprofloxacin, Gentamicin, Mecillinam, and Moxifloxacin, and only two antibiotics were shown 100% resistance in Amoxicillin and Cefixime. Based on the results, it is suggested that consumers should pay attention to producing with hygienic techniques and cooking properly to avoid foodborne diseases from flaxseed sprouts.

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Introduction

Flaxseed (*Linum usitatissimum L.*) sprouts have greatly increased in recent years due in part to the claimed nutritional advantages. The seeds are a good source of fat, fiber, and protein. The omega-3 fatty acid i.e., alpha-linolenic acid, which makes up 60% of the seeds' total fatty acid composition, is one of the plant kingdom's greatest of fatty acid (Bayra *et al.*, 2010 & Nitrayová *et al.*, 2014). In a variety of formats, flaxseeds are frequently eaten raw and either by themselves or as a component in other raw or cooked foods. Now a day, flaxseed sprouts are a new type of ready-to-eat raw food product (Aliani *et al.,* 2012; Byars, 2015; Inglett, 2013; Khouryieh and Aramouni 2012).

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A number of microbiological risks are associated with ingestion of raw sprouts. Firstly, throughout growth, harvest, storage, and transit, there is a chance that the seeds could become infected with bacterial pathogens from a variety of sources (Beuchat *et al.,* 2013 & Finn *et al.,* 2013). Low-moisture conditions, like those on seeds, can allow the protracted survival of bacterial pathogens for up to 2 years even though they are not favorable for growth (Beuchat *et al.,* 2013 & Van der Linden *et al.,* 2013).

The initial concentrations of bacteria can be magnified by as much as 4 orders of magnitude in the warm, moist conditions necessary for seed germination and sprouting (Charkowski *et al.,* 2002; Fu *et al.,*, 2008 & Jaquette *et al.,* 1996).

Despite sprouted flax seeds' rising popularity, there isn't any information available about their physicochemical and bacteriological characteristics. Here, we provide the first study documenting the bacteriological profiles of flax seed sprouts samples naturally contaminated with microbial flora and antibiotic susceptibility profiles from edible sprouts of flaxseeds.

Materials and Methods

Study Place

The present study was carried out in the department of Microbiology at Khwaja Yunus Ali University, Enayetpur, Sirajganj, Bangladesh.

Collection of Sample

Flax seed samples were purchased from local market from two different retail shops name Krishi market, Dhaka and local market of Enayetpur, Sirajganj, Bangladesh.

Seed Sprouting

50 g of sample seeds were placed in a plastic basket containing a plastic net and then saturated with tap water. The seeds were incubated at room temperature (25^0C) for 5 days.

Fig. 1. Flaxseeds.

Fig. 2. Flaxseed sprouting.

Fig. 3. Flaxseed sprouts.

Microbial Analysis of Seed Sprouts

10.0 g of flaxseed sprouts were suspended in 90 ml of sterile distilled water after 5 days of sprouting and then 10 fold serial dilutions were made after the flasks were shaken for 15 minutes on a rotary shaker. Plates containing Plate Count Agar, MacConkey Agar, and Chromogenic agar were distributed with aliquots of 0.1 ml of the relevant dilutions, and the plates were then incubated at 37^0C for 24 hours. On SDA (Sabouraud Dextrose Agar) growth plates for fungus, additional aliquots of 0.1 ml of suitable dilutions were applied, and they were cultured at 26^0C for 48 hours. After incubation, the plates were examined for colony growth and features. Following a census of the various colonies that appeared on the plates, the number of bacteria found on each sprout was estimated.

$$STANDARD\ FORMULA = \frac{Colony\ count\ on\ agar\ plate}{Total\ dilution\ of\ tube\ X\ Amount\ plated}$$

Fig. 4. Flaxseeds sprouts dilution.

Fig. 5. TVC on PCA plate.

Fig. 6. Bacterial isolates on Chromogenic agar.

Fig. 7. Molds growth on SDA.

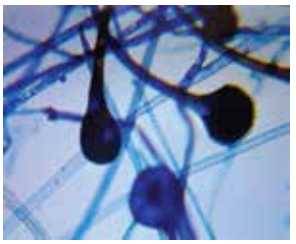

Fig. 8. Molds undermicroscope.

Cultural Identifiers

The colonies' color, texture, contour, opacity, pigmentation, and other characteristics were examined, and any variations found across the three media were tallied.

Identification of Morphological Features

The colonies were chosen and prepared for the gram staining method to distinguish between gram positive and gram negative bacteria as well as the configuration of the cells.

Media Preparation

Culture media (Biomaxima, Poland) were prepared according to manufacturer instructions, namely Plate Count Agar, MacConkey Agar, Chromogenic Agar, and Sabouraud Dextrose Agar, which were sterilized by autoclaving at 121°C under 15psi for 15 minutes.

Identification of Bacterial Isolates

For identification of the bacterial pathogens, Gram's staining was performed to characterize the bacterial pathogens and then certain biochemical tests were conducted for further identification.

In-Vitro Drug Susceptibility Analysis

Antimicrobial susceptibility testing was carried out in order to select which antimicrobial agent to use

against a particular strain of bacteria due to the introduction of numerous antibiotic resistant types of bacteria. The disc diffusion method was used to assess antibiotic susceptibility in accordance with accepted microbiological practices. In this procedure, Muller-Hinton Agar plates were inoculated with a standard suspension of the bacteria to be tested (0.5 turbidity, McFarland standard). Filter paper discs soaked with predetermined antimicrobial agent concentrations were applied on the surface of the agar and overnight incubation at 37^0C was performed. After incubation, the susceptibility was calculated and the zone of bacterial growth inhibition around each disc was measured (CLSI, 2021). The following chemotherapeutic antibacterial drugs were employed as discs in this study: Amoxycillin (30 mcg/disc), Azithromycin (15 mcg/disc), Ciprofloxacin (5 mcg/disc), (10 mcg/disc), Cotrimoxazole (25 mcg/disc), Cefaclor (30 mcg/disc), Cefixime (5 mcg/disc), Colistin (10 mcg/disc), Gentamicin (10 mcg/disc), Meropenem (10 mcg/disc), Mecillinam (10 mcg/disc), Moxifloxacin (5 mcg/disc).

Statistical Methods

Using the Excel 2010 program, descriptive statistics (mean and standard deviation) were computed (Microsoft, Redmond, WA)**.** When necessary, Excel 2010 estimated significant differences ($P< 0.05$) between means using a two-tailed t-test under the assumption of equal variance.

Results and Discussion

A total of 39 flaxseed sprouts were studied for microbial profiles and antibiotics responsiveness of bacterial isolates from edible sprouts of flaxseeds (*Linum usitatissimum L.*) were investigated from August 2020 to July 2021 in the Department of Microbiology at Khwaja Yunus Ali University. In this study, the assessment of the microbial quality of sprouts samples was evaluated. The lowest and highest level of TVC of bacterial population were $2X10^7$CFU/g (7.3 log 10), and $2.3X10^9$CFU/g (9.4 log 10). Moreover, it was analyzed in molds, the lowest and highest level of total viable counts (TVC) was $1X10^3$ CFU/g (3.0 log 10) and $2x10^4$ CFU/g (4.3 log 10) (Table 1); similar to earlier studies (Peles *et al*., 2012 & Abedin *et al.,* 2022). The morphological characteristics of newly produced flaxseed sprouts' bacterial isolates were tabulated (Table-2).

Table 1. Total viable microbial profiles counted from flaxseeds (*Linum usitatissimum* L.) sprouts.

Samples ID	Bacterial Total Viable count			Molds Total Viable count		
	Total Bacteria colonies	TVC (CFU/g)	TVC (Log10)	Total molds colonies	TVC of Molds	TVC (CFU/g)
1	180	$1.8x10^9$	9.3	3	$3x10^3$	3.5
2	120	$1.2X10^9$	9.1	1	$1X10^3$	3.0
3	130	$1.3X10^9$	9.1	5	$5X10^3$	3.7
4	90	$9X10^8$	8.9	6	$6X10^3$	3.8
5	90	$9X10^8$	8.9	9	$9X10^3$	3.9
6	110	$1.1X10^9$	9.0	12	$1.2X10^3$	3.1
7	130	$1.3X10^9$	9.1	12	$1.2X10^3$	3.1
8	120	$1.2X10^9$	9.1	9	$9X10^3$	3.9
9	50	$5X10^8$	*8.7*	6	$6X10^3$	3.8
10	70	$7X10^8$	8.8	4	$4X10^3$	3.6
11	60	$6X10^7$	7.8	5	$5X10^3$	3.7
12	50	$5X10^7$	*7.7*	4	$4X10^3$	3.6
13	230	$2.3X10^9$	9.4	5	$5X10^3$	3.7
14	80	$8X10^7$	7.9	5	$5X10^3$	3.7

Continued

Samples ID	Bacterial Total Viable count			Molds Total Viable count		
	Total Bacteria colonies	TVC (CFU/g)	TVC (Log10)	Total molds colonies	TVC of Molds	TVC (CFU/g)
15	130	$1.3X10^8$	8.1	3	$3x10^3$	3.5
16	70	$7X10^7$	7.8	4	$4X10^3$	3.6
17	40	$4X10^7$	7.6	2	$2X10^3$	3.3
18	60	$6X10^7$	7.8	1	$1X10^3$	3.0
19	30	$3X10^7$	7.5	5	$5X10^3$	3.7
20	20	$2X10^7$	7.3	1	$1X10^3$	3.0
21	30	$3X10^7$	7.5	5	$5X10^3$	3.7
22	30	$3X10^7$	7.5	2	$2X10^3$	3.3
23	40	$4X10^7$	7.6	7	$7X10^3$	3.8
24	40	$4X10^7$	7.6	3	$3x10^3$	3.5
25	30	$3X10^7$	7.5	3	$3x10^3$	3.5
26	30	$3X10^7$	7.5	2	$2X10^3$	3.3
27	30	$3X10^7$	7.5	4	$4X10^3$	3.6
28	30	$3X10^7$	7.5	5	$5X10^3$	3.7
29	20	$2X10^7$	7.3	5	$5X10^3$	3.7
30	30	$3X10^7$	7.5	7	$7X10^3$	3.8
31	110	$1.1X10^8$	8.0	15	$1.5X10^4$	4.2
32	170	$1.7x10^8$	8.2	5	$5X10^3$	3.7
33	40	$4X10^7$	7.6	10	$1X10^4$	4.0
34	80	$8X10^7$	7.9	8	$8X10^3$	3.9
35	190	$1.9X10^8$	8.2	15	$1.5X10^4$	4.2
36	70	$7X10^8$	8.8	15	$1.5X10^4$	4.2
37	130	$1.3X10^8$	8.1	5	$5X10^3$	3.7
38	110	$1.1X10^8$	8.0	6	$6X10^3$	3.8
39	110	$1.1X10^8$	8.0	20	$2X10^4$	4.3
40	120	$1.2X10^8$	8.1	10	$1X10^4$	4.0

Table 2. Morphological features of bacterial isolates from freshly produced flaxseeds sprouts.

SL	Media	Colony characteristics	Staining properties
01.	Plate count Agar Medium	Round, pin headed whitish	Gram-negative
02.	MacConkey Agar Medium	Dark pink, flat, small, circular	Gram-negative
		Light pink with centered, gummy, dome shaped, circular	Gram-negative
03.	HiCrome Universal Differential Medium	Colorless (greenish pigment may be observed)	Gram-negative
		Blue color colonies	Gram-negative
		Brown color colonies	Gram-negative
		Blue green halo color colonies	Gram-negative
		Pink pigment diffused into the medium, creating a wide, pink cloudy appearance around the colonies.	Gram-negative
		Green-blue to metallic blue	Gram-negative
04.	Sabouraud Dextrose Agar (SDA)	Filamentous fungi	-

The total aerobic bacteria (TAB), namely *S. marcescens* and *C. freundii* were significantly higher frequencies that were compliant with other studies (Kim *et al.,* 2009). In this study, the culture positive bacterial growths were confirmed by Gram staining technique and biochemical parameters (Table 3). The majority of sprouts samples were contaminated with a single bacterium that was 84.6% and only 15.4% were double bacterial strains contaminations (Fig. 9). Among the both isolates, *S. marcescens* 15 (33.3%) was highest prevalence.

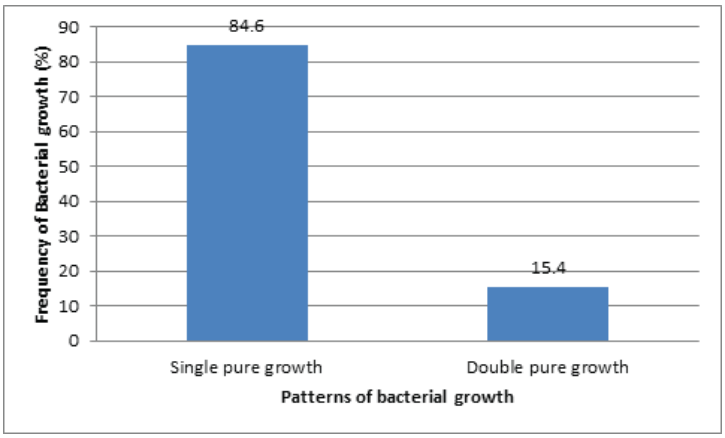

Fig. 9. Patterns of bacterial single and double pure growth on solid culture media.

In this study; 15 (33.3%) of flaxseed sprouts bacteria were late lactose fomenters (LLF) of *S. marcescens* grown in red-pigmented colonies in culture media, lactose fermenters (LF); such as *C. freundii* 10 (22.2%), and *E. cloacae* complex 6(13.3%); which fermented lactose to produce acidic environments that appeared as pink colonies; and the non-lactose fermenters (NLF); namely *P. aeruginosa* 9 (20.0%) produced normally colorless colonies and rest of NLF were *M. morganii)* 4(8.9%), and *A. hydrophila* 1(2.2%) (Fig.10). According to epidemiological investigations; isolated microbial profiles from bean sprouts that resembled these studies (Park and Sanders, 1990; Abedin *et al*., 2022).

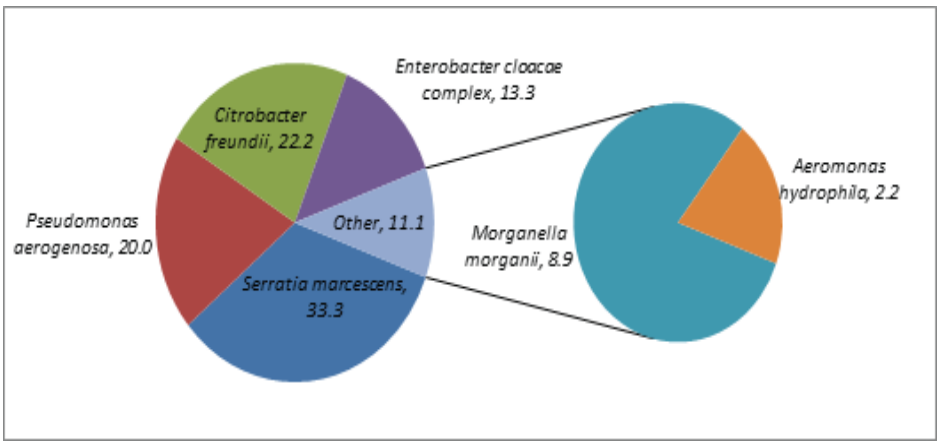

Fig. 10. Total bacterial isolates from flaxseed sprouts

Table 3. Results of biochemical tests of the isolated bacterial species from sprouts samples.

Bacterial Isolates	Gram Reaction	KIA				MIU			Oxidase	S.citrate	Catalase
		Slant	Butt	Gas	H_2S	Mot	Indole	Urease			
S. marcescens.	G-ve	R/Y	Y	±	-	+	-	±	-	+	+
E. cloacae complex	G-ve	Y	Y	+	-	+	-	-	-	+	+
P. aeruginosa	G-ve	R	R	-	-	+	-	±	+	+	+
C. freundii	G-ve	R/Y	Y	+	±	+	+	±	-	+	+
M. morganii	G-ve	R	Y	±	-	+	+	+	-	-	+
A. hydrophila	G-ve	R	Y	+	-	+	+	-	+	+	+

Note: G-ve= Gram negative, KIA = Kligler's Iron Agar *test,* MIU=Motility indole urease test, (+) =Positive; (-) =Negative reaction; (±)=Variable; R=Red (Alkaline reaction);Y=Yellow (Acid reaction); H_2S=Hydrogen sulphide; Cat=Catalase test, Mot=Motility test.

In this experiment, 45 bacterial isolates were tested for susceptibility to 11 antibiotics belonging to 10 antibiotic classes using the disk diffusion method according to CLSI-2021 recommendations. All of the bacterial isolates were 100% sensitive to only four antibiotics; namely Gentamicin, Ciprofloxacin; Mecillinam and Moxifloxacin; and only two antibiotics were 100% resistant name as Amoxycillin and Cefixime.

In this study, the most frequent bacterial isolates of *S. marcescens* were shown to be highly sensitive (100%) to Azithromycin, Ciprofloxacin, Gentamicin, Mecillinam, and Moxifloxacin. Amoxicillin and cefixime were shown to be 100% resistant, but Cefaclor and Cotrimoxazole were demonstrated as 73.3% and 20.0% resistant (Fig. 11). The second most common isolates of *P. aeruginosa* were shown to be highly sensitive (100%) to Ciprofloxacin, gentamicin, mecillinam, and moxifloxacin and moderately sensitive to Cotrimoxazole (66.7%), Colistin (77.8%) and Meropenem (88.9%). Amoxycillin and Cefixime were shown to be 100% resistant, but Cefaclor, Azithromycin, and Cotrimoxazole were demonstrated as 66.7%, 33.3%, and 33.3% resistant (Fig. 12). The significant levels of sensitivity from regularly utilized antibiotics are similar with Abedin *et al.* (2022).

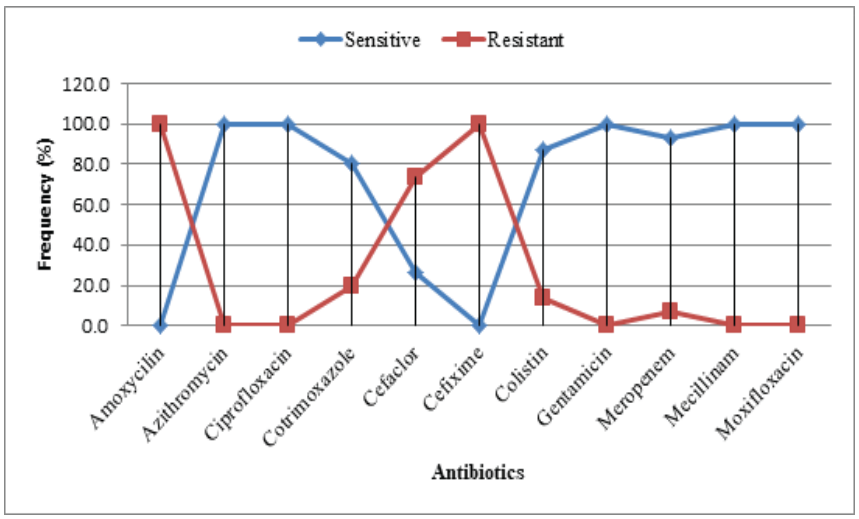

Fig. 11. Antibiotics susceptibility parameters of *S. marcescens*

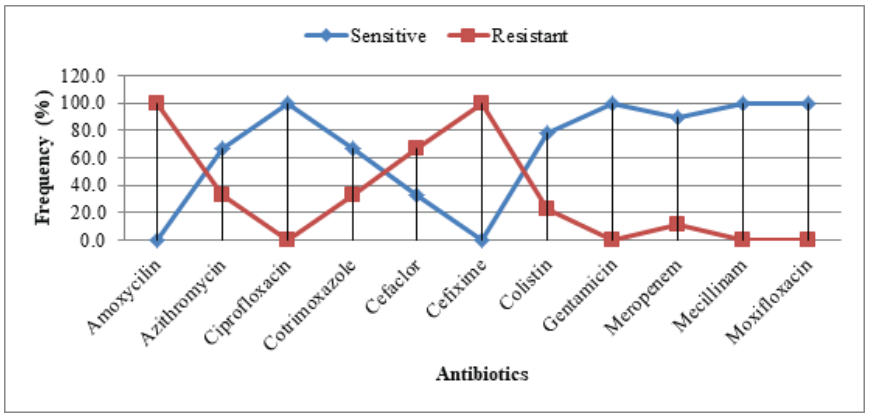

Fig. 12. Antibiotics susceptibility parameters of *P. aeruginosa.*

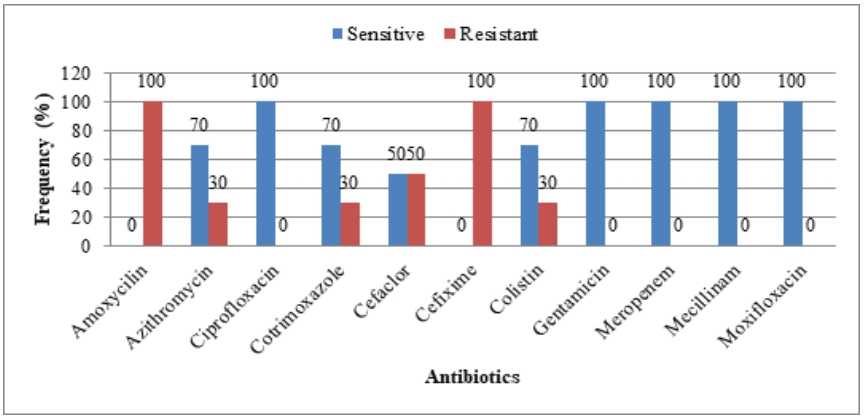

Fig. 13. Antibiotics susceptibility parameters of *C. freundii.*

Table 4. Antibiotics susceptibility parameters of others bacterial isolates.

Bacterial Isolates	Gram Reaction	KIA				MIU			Oxidase	S.citrate	Catalase
		Slant	Butt	Gas	H_2S	Mot	Indole	Urease			
S. marcescens.	G-ve	R/Y	Y	±	-	+	-	±	-	+	+
E. cloacae complex	G-ve	Y	Y	+	-	+	-	-	-	+	+
P. aeruginosa	G-ve	R	R	-	-	+	-	±	+	+	+
C. freundii	G-ve	R/Y	Y	+	±	+	+	±	-	+	+
M. morganii	G-ve	R	Y	±	-	+	+	+	-	-	+
A. hydrophila	G-ve	R	Y	+	-	+	+	-	+	+	+

C. freundii is an infrequent but established source of bean sprouts and vegetables related foods and foods products. Five types of antibiotics were 100% sensitive namely Ciprofloxacin, Gentamicin, Meropenam, Mecillinam, and Moxifloxacin and moderately sensitive to Azithromycin (70.0%), Cotrimoxazole (70.0%), Colistin (70.0%) and Cefaclor (50.0%). Moreover, two antibiotics such as Amoxicillin and

Cefixime were 100% resistant (Fig.13). Other bacterial isolates, namely *E. cloacae complex* 6(13.3%), *M. morganii* 4(8.9%), and *A. hydrophila* 1(2.2%), were shown to be highly sensitive (100%) to Gentamicin, Ciprofloxacin, Meropenem, Mecillinam, and Moxifloxacin. Only Amoxycilin was found to be 100% resistant to other bacterial isolates (Table 4). The significant level of resistance from regularly utilized anti-microbials is similar with Abedin *et al.* (2022) and Chauhan *et al.,* (2013).

Note: A disk diffusion test with bacterial isolates from a flaxseed sprouts culture. The diameters of all zones of inhibition are measured and those values translated to categories of S=Susceptible, R=Resistant using the latest tables published by the CLSI-2021.

Conclusions

The microbiological dangers of sprouting flaxseed for human consumption are highlighted in this study. At this moment; it is unknown what causes the batch-to-batch diversity in flax seed sprouts (e.g., seed lot, day-to-day or time-of-day process conditions).The range of physicochemical properties presented here cannot thus be commented on. The appropriateness of these values and if narrower ranges are necessary as target controls for risk mitigation may be clarified by further study. Our findings indicate that indicator organisms may be a more appropriate criterion for this sort of product than specific pathogens, as the Code of Practice for the Hygienic Production of Sprouted Seeds recommends for fresh, fully sprouted products (Canadian Food Inspection Agency. 2007)**.** To determine whether these criteria are appropriate, additional research is needed because the results reported here are based on a very small sample size. The goal of the current study was to use the disk diffusion method in accordance with CLSI standards to assess the antimicrobial susceptibility of isolated bacteria from flaxseed sprouts against 11 antibiotics belonging to 10 antibiotic classes.

i) The isolates were chosen for morphological characterization from various samples on two different types of medium. Other bacterial isolates included *E. cloacae* complex, *M. morganii,* and *A. hydrophila,* and it was discovered that they belonged to *S. marcescens, C. freundii, and P. aeruginosa.*

ii) All of the bacterial isolates showed 100% resistance to Amoxicillin and Cefixime. Because of this, raw sprouts should be eaten with caution regardless of where they come from, i.e., whether they are made at home or bought at a store.

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