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COMMON LEAF BASED MICROBES AND THEIR ROLE IN NATURE AND HUMAN HOST

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ABSTRACT

Raw vegetables are nutritious but can sometimes pose a threat to public health. They have become the cause of food borne illness in many countries. Detection of multidrug resistant microorganisms from market samples implies implementation of better hygienic measures for safe and healthy food. Leafy vegetables are often eaten as salad which do not require any rigorous treatment and so is considered as a high-risk food.

The aim of the present study was to isolate and identify bacteria from cabbage bought from a local market and analyze their antibiotic sensitivity. Bacteria present in cabbage were identified to be Hafnia sp., Enterobacter sp., Acinetobacter sp., Bacillus sp., Klebsiella sp. and Citrobacter sp. Majority of these bacteria are known as causal agents of various gastrointestinal diseases. Further their effect on shelf life of cabbage was checked which showed reduction in shelf life to half compared to control. One of the bacterial isolates suspected to be Bacillus sp. was found to be multidrug resistant.

Keywords: foodborne illness, multidrug resistant microorganisms, leafy vegetables, gastrointestinal diseases, shelf life.

1. INTRODUCTION

1.1. Background

Salads and fruits are popular among health conscious people. Leafy fresh vegetables and fruits are often not exposed to vigorous cleaning which can pose a health risk. Fresh leafy vegetables are related to outbreaks causing illness. If taken average, outbreaks associated to leafy vegetables are larger than other types of food (Herman et al., 2015). A study carried out in Nigeria showed the presence of *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella*, *Staphylococcus aureus* in market vegetable samples (Faisal Muhammad et al., 2017).

Humans are also exposed to antibiotic resistant microorganisms via raw vegetables. Vegetables are revealed to be rare but potential carriers of extended-spectrum beta-lactamase-producing Enterobacteriaceae, carbapenem resistant *Pseudomonas aeruginosa*, vancomycin resistant

enterococci and linezolid-resistant staphylococci (Hölzel et al., 2018). A study conducted by Hasan et al. (2011) showed the presence of *Bacillus cereus*, *Salmonella* and *E. coli* O157:H7 in raw vegetables. These isolates exhibited resistance to a wide range of antibiotics. This indicates the need of implementation of proper hygienic measures. Harmless bacteria can also enter the human intestine due to consumption of raw vegetables which can pass their plasmids to pathogenic bacteria enabling them to survive the changing environment in the intestine and cause diseases.

Food spoilage is a metabolic process which makes food undesirable for consumption. Spoilage of fruits and vegetables occur at the time of harvesting, transportation, storage, marketing and processing. Microbial spoilage of food can be caused due to the presence of fungi, bacteria, yeast and moulds. It is estimated that 36% of vegetable spoilage is caused by soft rot bacteria. Common pathogens causing this rot are *Alternaria*, *Botrytis*, *Erwinia*, *Pseudomonas* etc. (Rawat, 2015)

1.2. Objectives

Isolation and characterization of microorganisms from leaf of fresh cabbage, check their sensitivity to different antibiotics and their effect on shelf life of cabbage.

2. MATERIALS AND METHODS:

2.1. Collection of vegetable:

We have selected cabbage (*Brassica oleracea*) leaves as our test sample. The left overs of cabbage leaves were collected from the local market area of Bandel. The leaves were collected with sterile forceps into a sterile zip-lock bag.

2.2. Processing of vegetable:

The collected leaf samples in the zip-lock bag was opened into the Laminar air flow. The leaf was placed on a sterile petri-plate. With a sterile scalpel the leaves were cut into smaller pieces. 1g of the leaf was measured on a weighing machine. This 1g leaf sample was placed in 10ml of sterile distilled water. This becomes the stock. This stock was serially diluted up to 10^{-3}. From each dilutions 100µl was plated on different agar plates.

2.3. Media used for the isolation of bacteria from samples:

Nutrient media, Eosin Methylene Blue Agar, Chromogenic Agar Media, MacConkey agar**,** Blood agar, Brilliant Green Agar medium.

2.4. Microbiological analysis and biochemical tests:

IMViC test, Catalase test, Gram staining.

2.5. Antimicrobial susceptibility test:

The disk diffusion susceptibility method is simple and practical and has been well-standardized. The test was performed by spreading bacterial inoculum of organism 1,2,3,4,5 and 6 to the surface of Mueller-Hinton agar plate. Up to 10 commercially-prepared, fixed concentration, paper antibiotic disks were placed on the inoculated agar surface. Plates were then incubated for 24 h at 35°C prior to determination of results. The zones of growth inhibition around each of the antibiotic disks were measured. The diameter of the zone is related to the susceptibility of the isolates and to the diffusion rate of the drug through the agar medium.

2.6. Effect on shelf life of vegetable:

To check the shelf life of cabbage by the particular isolated organisms, the freshly market bought cabbage leaves were first cut into pieces and was washed properly and kept under UV exposure for a long time. The cabbage leaves were then put into the sterilized zip-lock bags. Each bag was marked as org.1, 2, 3, 4, 5, 6, mixed and control and so were the organisms inoculated respectively. The zip-lock cabbage bags were kept under room temperature. It was analyzed and weighed for two weeks.

3. RESULTS:

3.1. Identification and characterization:

Six isolates were collected from cabbage sample. These isolates were identified by gram staining and biochemical tests (table no. 1). They were grown in different types of media for further characterization. Organism 1, 2, 3, 4, 5 and 6 were suspected to be *Hafnia* sp., *Enterobacter* sp., *Acinetobacter* sp., *Bacillus* sp., *Klebsiella* sp. and *Citrobacter* sp. respectively.

3.2. Antibiotic sensitivity test:

The antibiotic sensitivity pattern of the isolates was determined by disc diffusion method (table no. 2). Organism 4 was resistant to each antibiotic tested against it. Organism 3 was found resistant to ampicillin as well as linezolid and organism 2 was resistant to ciprofloxacin only.

3.3. Effect on shelf life of cabbage:

Effect of each isolate and their consortium on shelf life of cabbage with respect to the control (inoculated with only sterile distilled water) was analysed. Deterioration in the quality of cabbage was comparatively more in case of consortium of the isolates. Shelf life of cabbage was approximately 10 days for control whereas it was reduced to 5 days in case of consortium of isolates. Organism 1 and 4 contributed more towards the decrease in shelf life of cabbage. (table no. 3).

4. DISCUSSION:

Micro-organisms present in fresh vegetables & fruits are introduced by irrigation water, field run off water, micro-flora of the storage place, individuals handling it, marketplace environment. The type & characteristics of the microflora reflects the hygiene status of its farm &market. Importance of this study lies in the proper upgradation of hygiene status & irrigation practices to prevent occurrence of pathogenic microorganisms if found (Harding *et al.*, 2017).

A cabbage was purchased from Bandel, West Bengal for its microbial characterization & identification. Cabbage is widely cultivated & highly consumed by the masses in West Bengal. Microbial assessment of cabbage is highly significant because of its use in salads & sandwich which does not involve any cooking reducing / destroying any sort of micro-organisms present. (Erickson, 2010)

The bacteria present in cabbage are suspected to be *Hafnia* sp., *Enterobacter* sp., *Acinetobacter* sp., *Bacillus* sp., *Klebsiella* sp., *Citrobacter* sp. (Plews *et al*. 1985, Prashanth *et al*. 2000, Gundogan *et al*. 2011, Baral *et al*. 2018). This data raises questions about health status since *Hafnia* spp. are known to cause intestinal & extra-intestinal infection and *Klebsiella* sp. is associated with

gastrointestinal diseases such as crohn's disease, ulcerative colitis as well as colorectal cancer (Kaur *et al*., 2018). *Citrobacter* sp. can be isolated from blood, pleural space, or abdominal cavity which strongly suggests that the upper gastrointestinal (or biliary) tract is the site of significant pathologic disease (Lew *et al*., 1984). *Enterobacter* sp. is mainly found related to nosocomial infections and *Acinetobacter* causes nosocomial & healthcare associated infection while some members of the *Bacillus* genus are reported to be the cause of diarrhea (Wong *et al*. 2017, Guentzel *et al*. 1996, *www.foodsafety.gov*).

Organism 4 exhibited multidrug resistance by giving no inhibition zone surrounding the antibiotic discs used as assessed by Kirby Bauer method. The other micro-organisms showed its resistance to varying degrees. Organism showed resistance against ciprofloxacin (table 2).

The isolated bacteria are inoculated in sterilized cabbage leaves kept at room temperature to determine its spoilage activity. Gradual reduction in leaves' mass occurred with changes in appearance over the weeks. This was done to determine the shelf life of cabbage if infected by the isolated bacteria.

Table no.1: Identification and characterization

S.no.	BIOCHEMICAL TESTS AND MICROBIOLOGICAL ANALYSIS	Bacterial isolate number					
		Organism 1	Organism 2	Organism 3	Organism 4	Organism 5	Organism 6
1.	METHYL RED	+	-	+	-	-	+
2.	VOGES PROSKAUER	-	-	-	-	+	-
3.	INDOLE PRODUCTION	-	-	-	-	-	-
4.	CITRATE UTILIZATION	+ (after 48 hours)	+	+	+	+	+
5.	CATALASE	+	+	+	+	+	+
6.	GRAM CHARACHTER	- Short rod	- Short rod	- Short rod	+ Medium rod	- Short rod	- Short rod
S.no.	GROWTH CHARACTERISTICS ON DIFFERENT MEDIA	Bacterial isolate number					
		Organism 1	Organism 2	Organism 3	Organism 4	Organism 5	Organism 6
1.	EMB AGAR	Poor growth (purple tinge)	Purple colonies	Purple colonies	-	Purple colonies	Purple colonies
2.	MACCONKEY AGAR	Orange-red colonies (slow fermenter)	Orange-red colonies (slow fermenter)	Light pink colonies	-	Pink colonies	Reddish colonies (slow fermenter)
3.	CHROME AGAR	Poor growth (purple tinge)	Poor growth (blue tinge)	Cream white colonies	-	Purple colonies	Bluish green colonies
4.	BLOOD AGAR	White colonies	Opaque greyish colonies	Grey-white colonies (poor haemolysis)	-	Grey-white colonies (poor haemolysis)	Grey-white colonies (poor haemolysis)
BACTERIA SUSPECTED		*Hafnia* sp.	*Enterobacter* sp.	*Acinetobacter* sp.	*Bacillus* sp.	*Klebsiella* sp.	*Citrobacter* sp.

- negative test + positive test

Table no.2: Antimicrobial sensitivity

S.no.	ANTIMICROBIAL AGENT	DISK CODE	DISK POTENCY (mcg)	INHIBITION ZONE DIAMETER (in cm)					
				Organism 1	Organism 2	Organism 3	Organism 4	Organism 5	Organism 6
1.	AMPICILLIN	AM	10	2.3	1.1	0	0	0.8	2.5
2.	NALIDIXIC ACID	NA	30	-	-	-	-	1.7	1.4
3.	LINEZOLID	LZ	30	2.5	1.2	0	0	0.9	1.1
4.	CIPROFLOXACIN	RC	5	0	2.8	1.1	0	2.2	2.2
5.	NITROFURANTOIN	FD	300	1.2	0.9	1	0	0.8	1.5
6.	MEROPENEM	MP	10	-	3	2.3	-	1.3	1.8
7.	IMPENEM	IM	10	2.7	2	1.2	0	1.4	2.3
8.	TETRACYCLINE	TE	30	2	1	1.3	0	1.6	1.3
9.	STREPTOMYCIN	SM	10	0.9	2.5	2.9	0	2.3	2.2
10.	AMOXY/CLAV	AG	30	2.1	1.6	1	0	1.6	2.5
11.	CHLORAMPHENICOL	CH	30	2	-	-	0	-	-

- not tested

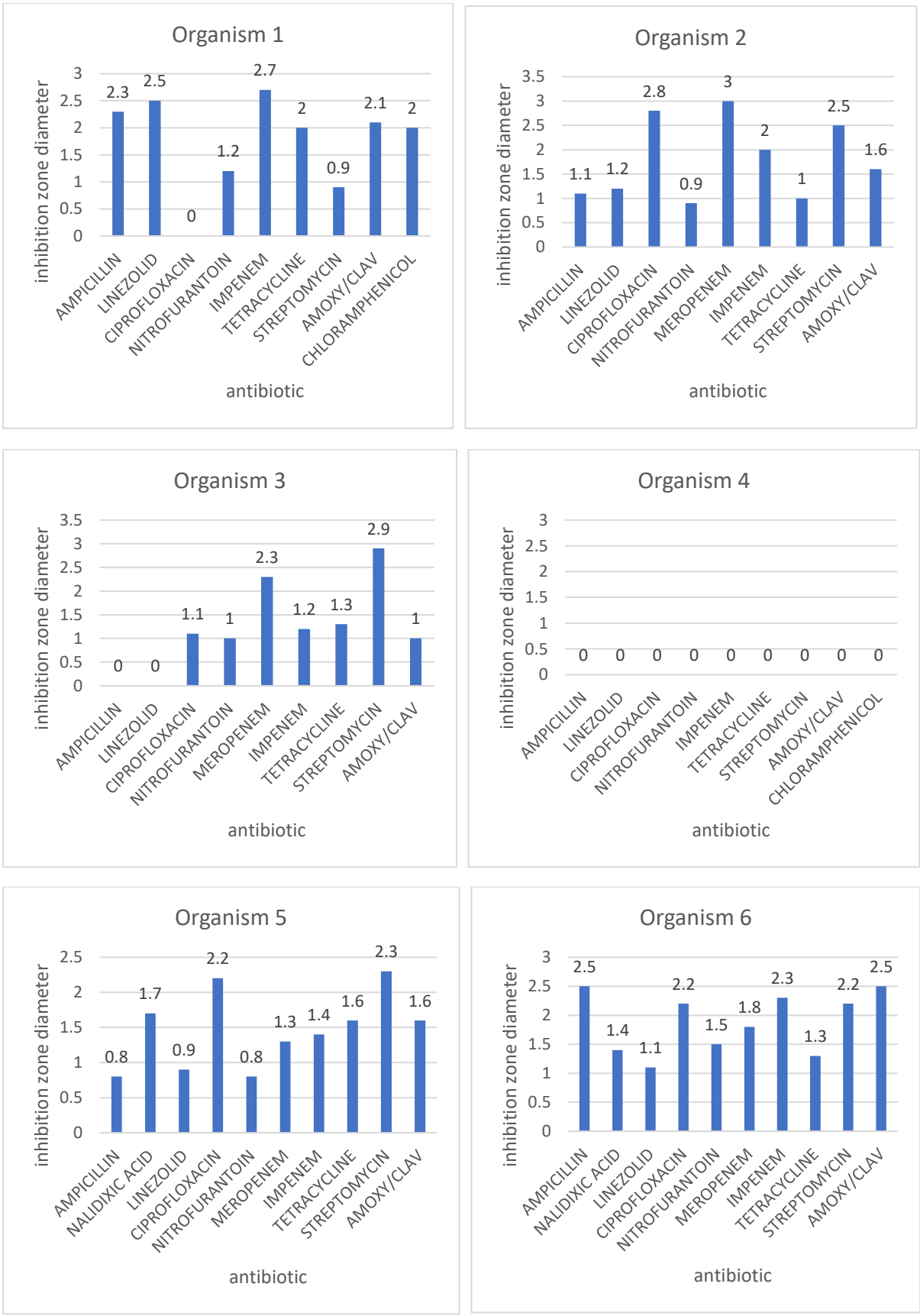

Fig.1. inhibition zone diameter (in cm) for the 6 isolates with different antibiotics.

Table no. 3: Effect on shelf life

OBSERVATION		Organism 1 (200µl)	Organism 2(200µl)	Organism 3(200µl)	Organism 4(200µl)	Organism 5(200µl)	Organism 6(200µl)	Mixed (30µl each)	Control (200µl)
DAY 1	Fresh weight (g)	8.55	4.40	4.50	7.15	3.21	3.00	6.48	4.20
	Colour change	-	-	-	-	-	-	-	-
DAY 7	Fresh weight (g)	7.97	3.80	3.80	6.47	2.71	2.43	5.73	3.72
	Colour change	Brown (bad odour)	Brownish	brownish	Brown (bad odour)	-	Brownish	Brown (bad odour)	-
DAY 14	Fresh weight (g)	7.50	3.40	3.37	6.00	2.30	2.12	5.05	3.40
	Colour change	Brown (bad odour)	Brown (bad odour)	Brown (bad odour)	Brown (bad odour)	Brown (bad odour)	Brown (bad odour)	Brown (bad odour)	Brownish

- no change

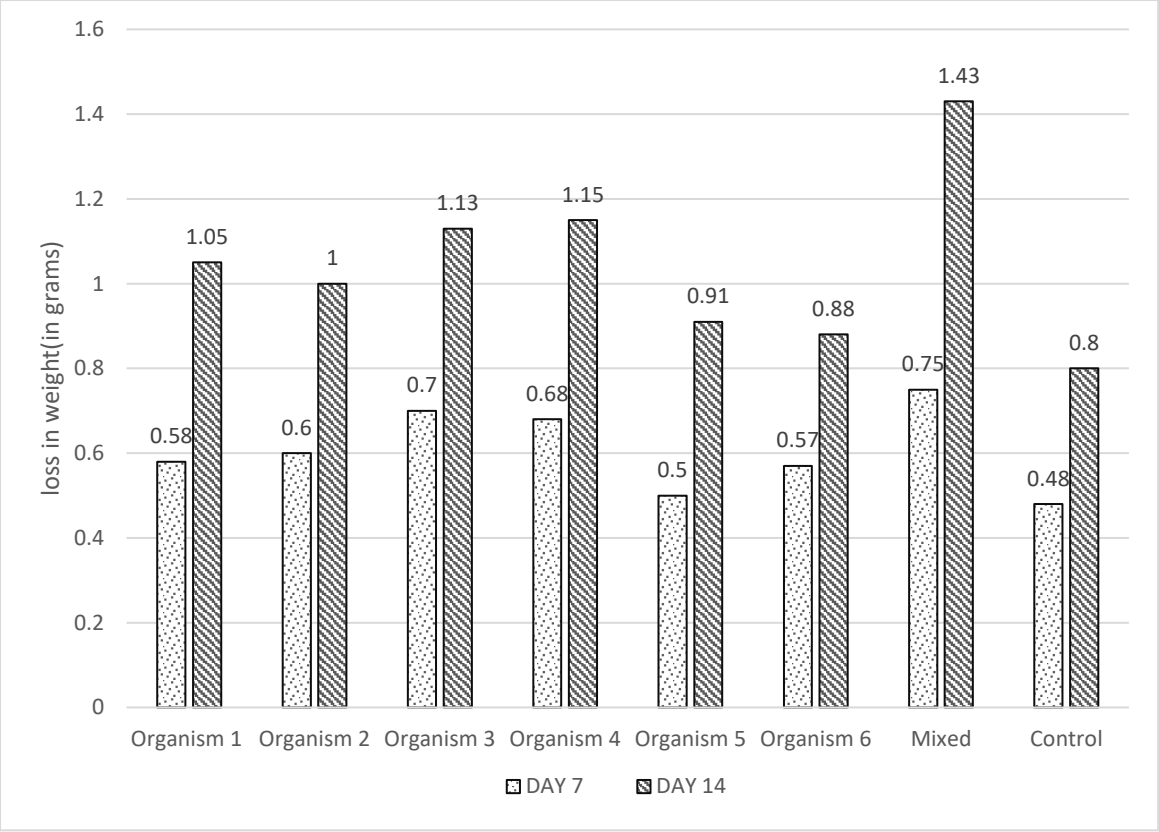

Fig.2. loss in weight of cabbage leaves at day 7 and day 14 w.r.t. the day of inoculation.

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