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Natural Micro Flora on Edible Raw Vegetables and Fruits

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A B S T R A C T

Association of lactic acid bacteria (LAB) on the edible plant surfaces is well known. These are beneficial organisms, to exploit their properties *i.e.* association of LAB on surfaces of raw edible fruits and vegetables a study was conducted. Six samples *viz*., guava, amla, radish, carrot, coriander and fenugreek were chosen for research studies. LAB were isolated by standard plate technique. Spoilage bacteria and molds were isolated from same spoiled fruits and vegetables by standard plate count method. Spoilage moulds *viz*., *Penicillium* sp., *Fusarium* sp., *Cephalosporium* sp., *Rhizopus* sp., *Aspergillus niger* and *Aspergillus flavus*. 30 LAB isolates were isolated. The highest LAB population was present on carrot's surface *i.e.* (214 x 10^3 CFU/g) followed by coriander (98.66 x 10^3 CFU/g) and guava (48.08 x10^3 CFU/g).

Introduction

Fruits and vegetables supply vitamins, minerals, dietary fibers that function as anti-oxidants, phytoestrogens and anti-inflammatory agents. Vegetables are rich in vitamin A, vitamin C, fiber, folate and potassium. Folate helps in the formation of red blood cells. The healthiest choices are fresh fruits as fruits are naturally low in fat, sodium and calories, and rich in potassium, fiber, vitamin C and folate. Fiber in fruit helps to protect against heart disease and lower cholesterol. Raw fruits and vegetables harbor some of the microorganisms on their surfaces which may be friends and also foes. It is important to know the natural micro flora on these edible raw fruits and vegetables, which in turns to help us to know the native micro flora may be beneficial to human health or neutral or causes deterioration of these fruits and vegetables.

So keeping in mind the importance of above factors the objectives of this study was designed to enumerate the friendly bacteria which in turn beneficial for human health that are to isolate and to characterize the native lactic acid bacteria from edible plant surfaces. Other microorganisms are foes means, microorganisms which are responsible for spoilage. Based on this other objective was

designed to isolate the spoilage microorganisms from the fruits and vegetables. The edible raw fruits and vegetables were chosen based on the consumption pattern of people living in Karnataka state, India. They are fruits such as amla and guava, vegetables such as coriander leaves, fenugreek leaves, radish and carrot.

Materials and Methods

Sample collection

Different samples of raw fruits and vegetables were collected for isolation of LAB from different locations *viz*., horticulture garden, market and food outlets at locality of yelahanka in Bangalore district, Karnataka state. Samples included amla, guava, radish, carrot, fenugreek leaves and coriander leaves.

Isolation and purification of lactic acid bacteria

Fruits and vegetables were gently washed. The peels of fruits and vegetables were peeled; 10g of sample was weighed and added to 100ml sterile water blank. It was subjected to serial dilution up to 10^{-7} dilutions. Lactic acid bacteria (LAB) were isolated by standard plate count technique using MRS medium. (De Man, Rogosa and Sharpe, 1960) (Fig. 1).

Total thirty isolates were isolated, five isolates from each sample. The colony morphology resembling to LAB were isolated, transferred to MRS broth and incubated at room temperature for four days. A loop of LAB culture from MRS broth was streaked on MRS agar medium (Fig. 2). The inoculated Petri plates were incubated at room temperature for four days and observed for single isolated colony. The single colony isolated was streaked on MRS slants for preservation. LAB cultures were maintained on MRS slants and sub culturing was done after six months.

Identification of lactic acid bacteria

The bacterial isolates were examined for colony morphology (Becking, 1974) included pigment, margin, elevation, position in the agar medium.

Cell morphology of the isolated LAB isolates was studied by simple staining and identified through various biochemical tests such as Gram staining (Harrigan and McCance, 1998), Catalase test (Balazevic and Ederes, 1975), Motility test (Tittsler and Sandholzer, 1936), Endospore staining (Murray *et al.*, 1994), Acid and gas production (Seeley and Vandemark, 1970, Hydrolysis of gelatin (Ewing, 1966), Starch hydrolysis (Yazdanparast, 1993), Lipid hydrolysis (Aneja, 2012), Exopolysaccharide production (Paulo *et al.*, 2012).

Isolation and purification of spoilage molds and bacteria

The same spoiled fruits and vegetables were selected and infected parts were removed by cutting and it was added to 100ml sterile distilled water. It was further diluted up to 10^{-7} dilution.

The 10^{-2} and 10^{-3} diluents were used for isolation of spoilage molds, 10^{-6} and 10^{-7} diluents were used for isolation of spoilage bacteria. (Chaudhary and Dhaka, 2016) purification of spoilage molds and bacteria was done on PDA medium. The molds were purified by selecting an isolated mold colony plug with the help of cork borer and placed on PDA medium.

Spoilage bacteria were purified by selecting a single colony from streaked plate and streaked again on nutrient agar slants.

The cultures were preserved and sub cultured once in 3 months.

Characterization of spoilage molds and bacteria

Identification of spoilage molds (Leck, 1999)

A loop of fungal mycelia was transferred on clean slide consisting of water drop. Cotton blue (2 drops) was added to it and was covered with cover slip. The slide was observed for characteristics fruiting bodies of mold, arrangement of conidia.

Characterization of spoilage bacteria

Spoilage bacteria were examined for morphological characteristics such as colony pigment, margin, elevation and position in the agar medium. Cell morphology was also studied by simple staining.

Various biochemical test were performed such as Gram staining, catalase test, endospore staining, gelatin hydrolysis, starch hydrolysis, lipid hydrolysis Citrate utilization test (Vaughn *et al.,* 1950), Casein hydrolysis (Aneja, 2012), Oxidase test (Tarrand and Groschel, 1982) Methyl red and Voges-Proskauer tests (Clarke and kirner, 1941), H_2S production (Aneja, 2012), Indole production test (Aneja, 2012), Urease test (Seeliger, 1956) in order to identify them upto generic level.

Results and Discussion

Enumeration of lactic Acid (LA) bacteria was carried out by standard plate count method using De Man, Rogosa and Sharpe (MRS) medium. Results related to enumeration are presented in figure 3. LAB population was the highest in carrot (214.0x 10^3 cfu/g), followed by coriander (98.66 x 10^3 cfu/g), guava (48.08 x 10^3 cfu/g), fenugreek (38.1 x 10^3 cfu/g), amla (34.9 x 10^3 cfu/g) and the least was observed in radish (22.7 x 10^3 cfu/g).

Our results generally demonstrated that the lactic acid bacterial population was highest on the surfaces of carrot. Enumeration of LAB were done from raw edible fruits and vegetables where the same work was already done by Jalali *et al.*, they also notified that higher densities of LAB were found on carrots tomato, soybeans, radish and lettuces.

Identification of lactic acid bacterial isolates

LAB isolates were characterized morphologically and biochemically for identification up to generic level. LAB colonies were white colored with varied sizes like medium, small and bold (Table 1). It was found that majority of LAB colonies were round and submerged, the results were in congruent with Ali *et al.*, Biochemical characterizations of LAB isolates were also carried out (Table 2). In Gram staining test, all the 30 isolates were Gram positive. Some isolates were rods and some were cocci. These isolates were tested for catalase enzyme production and motility. All the isolates were non-motile and catalase negative. The LAB isolates were tested for lipid hydrolysis, clear transparent zone around the streaked colony indicates positive for the test. LAB isolates (27) could hydrolyze lipid. Isolates were subjected to gelatin hydrolysis test. Liquefied nutrient gelatin stabs were recorded as positive for this test and stabs with solidified gelatin were recorded as negative. In present study, all the nutrient gelatin stabs were not liquefied indicating that all the 30 isolates were negative for gelatin liquefaction. Further, these results were in concurrent with Pal *et al.*, (2005).

The starch hydrolysis was also carried out as one of the biochemical test the results are in conformation with findings of Olympia *et al.*, (1995), they showed that several strains of LAB could hydrolyze starch such as

Streptococcus equines, *S. bovis*, *Lactobacillus amylovorous* and *Lactobacillus plantarum.* Exopolysaccharide production was also one of the biochemical tests done in order to know the capacity of the isolates to produce exopolysaccharide. Precipitation formation in absolute alcohol indicates exopolysaccharide production. Out of 30 isolates 27 isolates shown positive results to this test. Similar results were reported by Welman and Maddox (2003). LAB such as *Streptococcus thermophilus*, *Lactobacillus helviticus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* produced Exopolysaccharides. They also worked on enhancement of EPS production in these organisms. Results of all the above tests done indicated that isolates belonged to LAB genera.

LAB may be classified as homofermentative or heterofermentative based on their byproducts of sugar fermentation. A test was conducted to know acid and gas production. Change in color from purple to pink indicated positive for acid production test. Production of air bubble in Durham's tube indicated positive results for gas production. Out of thirty isolates, twenty-two were homofermentative and eight isolates were heterofermentative. Results are tabulated in Table 3.

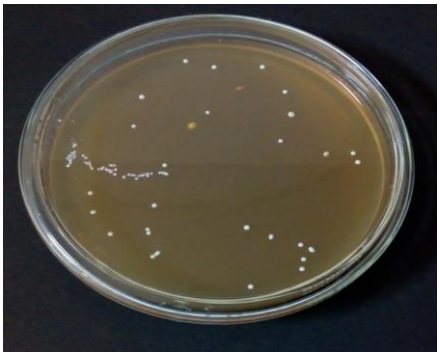


Fig.1: Lactic acid bacterial colonies

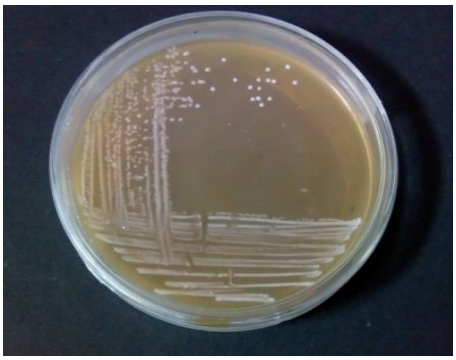
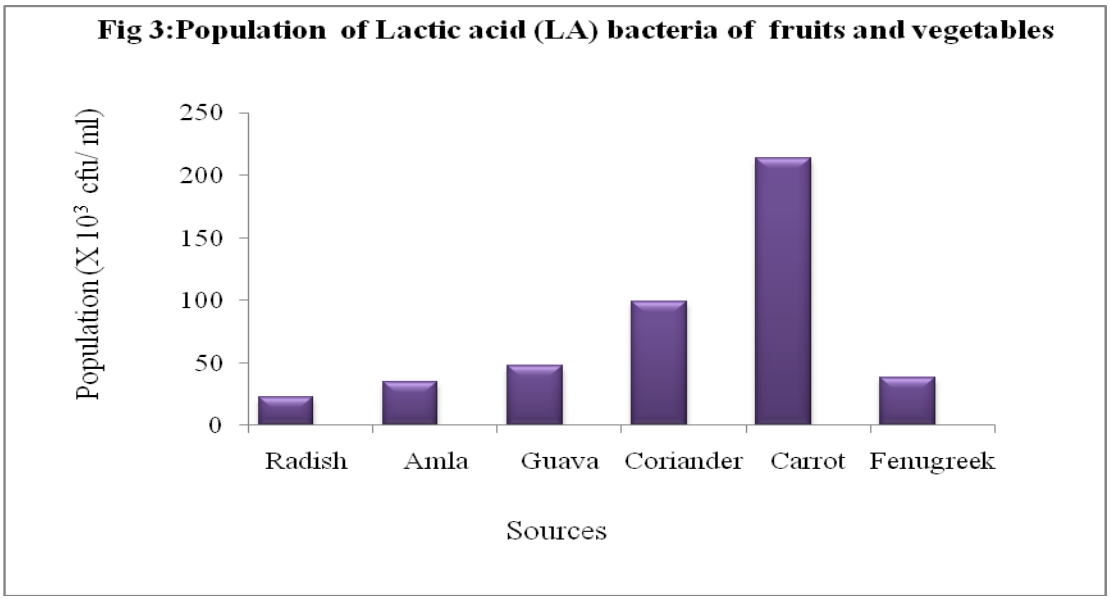


Fig. 3: Pure culture colonies of LAB



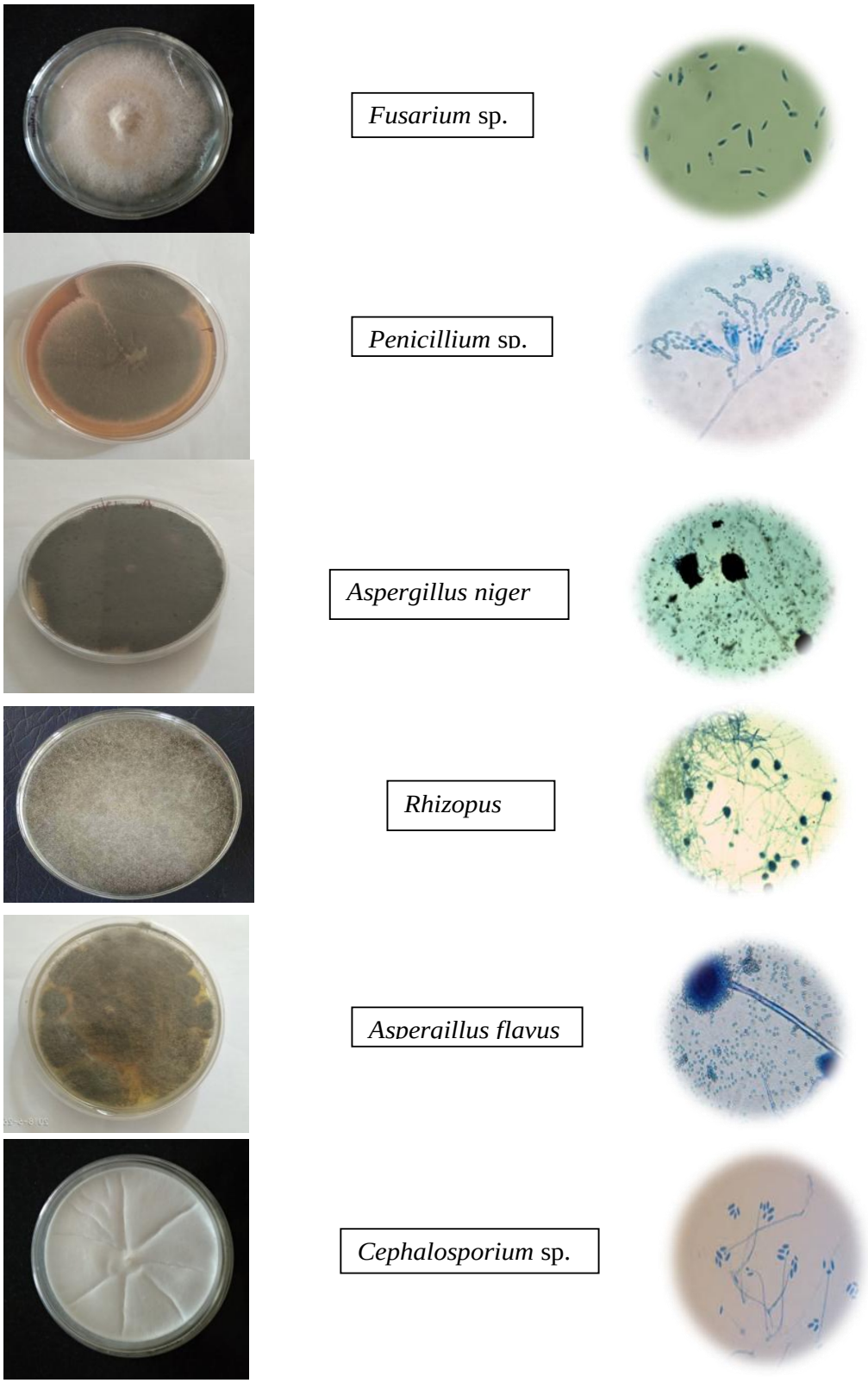


Fig.4: Spoilage molds isolated and their microscopic view

Table.1 Colony characteristics of LAB isolates of fruits and vegetables

Sl. No.	Source	Isolates	Color	Size	Shape	Position
1.	Radish	R-1	White	Medium	Round	Submerged
		R-2	White	Bold	Oval	Submerged
		R-3	White	Medium	Round	Submerged
		R-4	White	Small	Round	Submerged
		R-5	White	Small	Round	Surface
2.	Carrot	Ct-1	White	Medium	Round	Submerged
		Ct-2	White	Small	Round	Submerged
		Ct-3	White	Bold	Round	Submerged
		Ct-4	White	Small	Irregular	Submerged
		Ct-5	White	Medium	Round	Surface
3.	Guava	G-1	White	Medium	Round	Submerged
		G-2	White	Small	Round	Surface
		G-3	White	Small	Round	Submerged
		G-4	White	Bold	Round	Surface
		G-5	White	Medium	Irregular	Submerged
4.	Amla	Am-1	White	Bold	Round	Surface
		Am-2	White	Small	Round	Submerged
		Am-3	White	Medium	Round	Surface
		Am-4	White	Small	Round	Submerged
		Am-5	White	Medium	Round	Submerged
5.	Coriander	Co-1	White	Small	Round	Submerged
		Co-2	White	Medium	Round	Submerged
		Co-3	White	Bold	Irregular	Submerged
		Co-4	White	Bold	Surface	Surface
		Co-5	White	Bold	Oval	Submerged
6.	Fenugreek	Fg-1	White	Small	Round	Surface
		Fg-2	White	Small	Round	Submerged
		Fg-3	White	Small	Round	Surface
		Fg-4	White	Bold	Round	Surface
		Fg-5	White	Bold	Oval	Submerged

Table.2 Cell morphological characteristics of LAB isolates

Source	Isolates	Shape	Gram reaction	Motility	Catalase test	Lipid hydrolysis	Gelatin liquefaction	Starch hydrolysis	EPS production
Radish	R-1	Rod	+	-	-	+	-	+	+
	R-2	Rod	+	-	-	+	-	+	+
	R-3	Cocci	+	-	-	+	-	+	+
	R-4	Rod	+	-	-	+	-	+	+
	R-5	Cocci	+	-	-	+	-	+	+
Carrot	Ct-1	Rod	+	-	-	+	-	+	+
	Ct-2	Rod	+	-	-	+	-	+	+
	Ct-3	Rod	+	-	-	+	-	+	+
	Ct-4	Cocci	+	-	-	+	-	+	+
	Ct-5	Rod	+	-	-	+	-	+	-
Guava	G-1	Rod	+	-	-	+	-	+	+
	G-2	Cocci	+	-	-	+	-	+	+
	G-3	Rod	+	-	-	+	-	+	+
	G-4	Rod	+	-	-	+	-	+	+
	G-5	Cocci	+	-	-	+	-	+	+
Amla	Am-1	Rod	+	-	-	+	-	+	+
	Am-2	Cocci	+	-	-	+	-	+	-
	Am-3	Rod	+	-	-	+	-	+	+
	Am-4	Cocci	+	-	-	+	-	+	+
	Am-5	Cocci	+	-	-	-	-	-	-
Coriander	Co-1	Cocci	+	-	-	+	-	+	+
	Co-2	Rod	+	-	-	-	-	-	+
	Co-3	Cocci	+	-	-	+	-	+	+
	Co-4	Rod	+	-	-	+	-	+	+
	Co-5	Rod	+	-	-	+	-	+	+
Fenugreek	Fg-1	Cocci	+	-	-	+	-	+	+
	Fg-2	Cocci	+	-	-	+	-	+	+
	Fg-3	Rod	+	-	-	+	-	+	+
	Fg-4	Rod	+	-	+	-	-	-	+
	Fg-5	Rod	+	-	-	+	-	+	+
Reference strain	*L.. plantarum*	Rod	+	-	-	+	-	+	+

Note: Present (+); Absent (-), EPS- Exo polysaccharide production, *L. plantarum- Lactobacillus plantarum.*

Table.3 Acid and Gas production by LAB

Sl. No	Source	Isolates	Acid production	Gas production	Homo fermentative	Hetero fermentative
1.	Radish	R-1	+	-	+	-
		R-2	+	-	+	-
		R-3	+	+	-	+
		R-4	+	+	-	+
		R-5	+	-	+	-
2.	Carrot	Ct-1	+	-	+	-
		Ct-2	+	+	-	+
		Ct-3	+	-	+	-
		Ct-4	+	-	+	-
		Ct-5	+	-	+	-
3.	Guava	G-1	+	-	+	-
		G-2	+	+	-	+
		G-3	+	+	-	+
		G-4	+	-	+	-
		G-5	+	-	+	-
4.	Amla	Am-1	+	-	+	-
		Am-2	+	+	-	+
		Am-3	+	-	+	-
		Am-4	+	-	+	-
		Am-5	+	+	-	+
5.	Coriander	Co-1	+	-	+	-
		Co-2	-	+	+	-
		Co-3	+	-	+	-
		Co-4	+	-	+	-
		Co-5	+	-	+	-
6.	Fenugreek	Fg-1	+	-	+	-
		Fg-2	+	-	+	-
		Fg-3	+	-	+	-
		Fg-4	-	+	+	-
		Fg-5	+	+	-	+
7.	Reference strain	*Lactobacillus plantarum*	+	+	-	+

Table.4 Spoilage molds isolated from fruits and vegetable

Sl. No.	Source	Spoilage molds	Colony characteristics	Microscopic observation
1.	Radish	*Aspergillus niger*	Black in colour	Globose shaped conidia
2.	Carrot	*Aspergillus flavus*	Yellow to greenish colony	Globose shaped conidia
3.	Guava	*Rhizopus* sp.	Grey color colony later produces black spores	Round sporangium
4.	Amla	*Penicillium* sp.	Initially white colour later turns to dark green	Conidia in long chains brush like head with branched sterigmata
5.	Coriander	*Fusarium* sp.	Wooly, white to pink.	Sickle shaped macroconidia
6.	Fenugreek	*Cephalosporium* sp.	Cottony pure white colony	Conidia held in mass at tip of conidiophore

Table.5 Biochemical characterization of spoilage bacteria isolated from raw fruits and vegetables

Sl. No.	Isolates	RAD	CAR	GUA	AML	COR	FEN
1	Gram staining	-	-	+	+	-	+
2	Shape	Short rods	Cocci	Rods	Cocci	Short rods	Rods
3	H_2S Production	-	-	-	-	-	-
4	Indole production	-	-	-	-	-	-
5	Starch hydrolysis	-	-	+	-	-	-
6	Citrate utilization	+	+	+	-	+	+
7	Nutrient gelatin test	-	+	+	+	-	-
8	Lipid hydrolysis	+	-	+	-	+	-
9	Casein hydrolysis	-	-	+	-	-	-
10	Oxidase test	-	-	-	-	-	-
11	MR	-	+	-	+	-	+
12	VP	+	-	+	+	+	-
13	Catalase test	+	-	+	+	+	-
14	Urease test	-	-	-	+	-	-
15	Endospore staining	-	+	+	-	-	+

Table.6 Assimilation of different carbon sources by spoilage bacterial isolates of fruits and vegetables

Sl. No.	Isolates	Glucose	Sucrose	Lactose
1	RAD	AG	AG	AG
2	GUA	A	A	-
3	AML	A	A	A
4	COR	AG	AG	AG
5	FEN	A	A	-
6	CAR	A	AG	AG

Note:
AG: Acid and gas production
A: Acid production
-: No acid or gas production

Isolation of spoilage molds from fruits and vegetables

Spoilage molds were isolated from each spoiled fruit and vegetable. Six spoilage molds were isolated and their microscopic characteristics are tabulated in table 4. The observations revealed the identity of spoilage molds- *Rhizopus* sp. (guava), *Penicillium* sp., (amla), *Aspergillus niger* (radish), *A. flavus* (carrot), *Fusarium* sp. (coriander) and *Cephalosporium* sp. (fenugreek). The results are in line with Abdullah *et al.*, (2016). They isolated spoilage molds from spoiled fruits. The most common spoilage molds isolated and identified were *Penicillium expansum*, *Colletotrichum musae*, *Aspergillus niger* and *Penicillium glabrum*.

Spoilage bacteria (SB) isolates were characterized morphologically and biochemically up to generic level. Isolates were coded in short forms as CAR isolate (carrot), RAD (radish), FEN (fenugreek), COR (coriander), AML (amla) and GUA (guava). Majority of SB isolates were rods in shape, only two of them were cocci. Isolates were subjected to various biochemical test (Table 5).

Similar for identification of spoilage bacteria certain test were done where, all the above test and results are congruent with Chaudhary and Dhaka (2016) work. They isolated eleven bacterial isolates, caused spoilage in different fruits. Biochemical test such as carbohydrate utilization, indole production, MRVP, citrate utilization, catalase, oxidase, urease production, H_2S production, starch hydrolysis, casein hydrolysis and gelatin liquefaction were done Assimilation of different carbon sources by spoilage bacterial isolates of fruits and vegetables was given in Table 6.

Results lead to thinking that, consumption of raw carrot not only provides β- carotene but also serves as a sink for lactic acid bacteria to render the health benefits. LAB present on various habitats, phyllosphere is one among them. Fruits and vegetables consumption in the form of salads helps in ingestion of live LAB which protects from intestinal pathogens in turn helps in restoring good health. Further, spoilage organisms were isolated from same fruits and vegetables.

Spoilage molds such as *Aspergillus niger*, *A. flavus*, *Rhizopus* sp., *Fusarium* sp., *Penicillium* sp. and *Cephalosporium* sp. were isolated. They were identified based on the conidial structure, arrangement of spores. Spoilage bacteria were also isolated. These informations will help us in preventing these spoilage microorganisms so that it will be

precautionary measure in order to increase its shelf life.

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