Original Research Article

COMPARATIVE ANALYSIS OF BACTERIAL FLORA OF VEGETABLES COLLECTED DIRECTLY FROM SUPERMARKET AND VEGETABLE MARKET

Abstract

To prevent food borne diseases, microbiological control is very important in the food industry. Therefore, the present investigation was undertaken for microbiological quality assessment of fresh vegetables that were collected from several regions of Chandigarh. Our purpose was to determine the bacterial load and safety of vegetables present in market. Vegetable samples (tomato and capsicum) collected from two different sites i.e. vegetables market and supermarket, were analyzed for their bacteriological contaminants following different procedures. The maximum bacterial count present on vegetable market compare to super market. The micro flora of vegetable samples was dominated by *Staphylococcus* and *Bacillus*. *Bacillus* and *Staphylococcus* were detected in tomato and Capsicum respectively. *Bacillus* and *staphylococcus* were included in most abundant species and opportunistic pathogens which may raise concern to improve the microbial quality of fresh vegetables. Our study demonstrated that the vegetables, sold in vegetable market, were found with higher microbial load that may represent a risk for human health. Hence, for many microorganisms, vegetables may act as a reservoir from where they will be colonized and infect host.

Keywords

Microorganism, Identification, Biochemical test, Microbial load, Vegetables.

Introduction

Microbiology is the study of microorganism those which are so small that they cannot be seen with the naked eyes and observed only by microscope. Microbiology and all organisms include microbes that are divided into prokaryotes and eukaryotes. Prokaryotes are always single cell and DNA is not enclosed by a membrane, whereas eukaryotes are multi-cellular organisms where DNA is closed by a membrane. Robot Hooke observes first microbes in fruiting bodies of moulds. Leuwenhoek is father of microbiology as he experimented with the microbiology in 1670. Scientific microbiology was developed in 19th century through the Louis Pasteur and Robert Koch work. Microbiology includes bacteria, archaea, virus, fungi, protozoa and algae. There are present several pathogenic microbes that have found their way into fresh vegetables and fruits which are a great source of human healthy diet (Balali et al, 2020). Spoilage is the process in which fruits and vegetables is contaminated and any change which reduce a product and unacceptable for human consumption. Some spoilage organisms such as

yeast target the fruits and vegetables due to their low pH and bacteria can grow on the fruits and vegetables surface. Human infection outbreak is associated with consumption of raw vegetables (Beuchat et al, 2022). Bacteria spoilage is the cause of tissue softening as pectin are degraded. Micro flora is dominated by spoilage bacteria such as *Bacillus cereus, Salmonella, E.*coli, and *Clostridium, Staphylococcus* etc (Tambekar et al, 2006). Food poisoning is the major problem in case when it was not stored properly. The commonly found bacteria that causes food poisoning are *Salmonella, Staphylococcus aureus, E.coil* etc. Common bacteria are present on the surface of vegetable and fruits like *Staphylococcus, Bacillus, E.coli, Clostridium, and Agrobacterium.*

Nutrients are necessary for growth of the microbes. The substrate on which bacterial culture is done is known as medium which provide nutrition and pH to the organism. There are different type of culture nutrient media such as a NAM, PDA, and YMA etc. On the basis of consistency, there were used two types of media i.e. solid media and liquid media. Solid media were used for studying the cloning character of the bacteria and liquid media for the profuse growth e.g. blood culture**.** On the basis of chemical composition two types of media were used i.e. routine laboratory media and synthetic media. Autoclave, dry heat and radiation methods were used as common laboratory sterilization method.

Here are mentioned some bacterial community associated with surface of vegetables. Fresh apples, peppers, tomatoes and capsicum are known to harbor large bacterial populations. We were started to explore the diversity of these communities. Human pathogens can be associated with fruits and vegetables examples *Bacillus, Staphylococcus, E.coli, and Salmonella*. Many of the microbes found on the kitchen surface appear to come from produce source. Fruits and vegetable contamination occur at any stage from the production to consumption. The presence of *Staphylococcus* in retail food has been reported in China (Shubo et al, 2015).

Materials and Methods

Location of samples

Bacteria sample was carried out from the vegetable market sector 40 Chandigarh and supermarket SCF4 sector 9D Chandigarh. *Bacillus c*ollected from surface of tomato was collected from the vegetable market and *Staphylococcus* falling microorganism were collected from surface of a capsicum was collected from supermarket.

Composition of Nutrient agar medium

Bacteria falling on the surface of vegetable were measured by two types of agar media (nutrient agar media and peptone dextrose agar media) and two types of growth media (Mannitol salt agar and Luria Bertani)**.** Nutrient agar media (Table 1) was used for the bacterial growth count. It can grow a variety of bacteria types and fungi. Peptone dextrose agar was a general media for fungal isolation from plant sample (Table 2). Here organic

compounds was used as a source of energy. Yeast extract peptone dextrose was used as a growth medium to grow Yeast. Mannitol salt agar media were used for selective and differential growth medium in laboratory (Table 3). It contains high salt concentration. The identification of staphylococcus aureus clinical isolates requires a battery of test and costly in resources limited setting. Luria Bertani (LB) is a nutritionally rich medium for culturing the bacteria (Table 4). LB media allow the plating of bacterial culture and growth of colonies.

Table 1 Nutrient medium

Chemical name	g/l
Peptone	5
Beef extract	3
Sodium chloride	5
pH	5.5
Agar	15

Table 2 Peptone dextrose agar

Chemical name	g/l
Potato starch	4
Dextrose	20
Agar	15
pH	7.5

Table 3 Mannitol salt agar media

Chemical name	g/l
Peptone	10
Beef extract	1.00
Sodium chloride	75.0
D-Mannitol	10.00
Phenol Red	0.025

pH	7.3
Agar	15

Table 4 Luria Bertani (LB)

Chemical name	g/l
Tryptone	0.25
Yeast extract	0.126
Sodium chloride	0.125
pH	7.5

Identification of bacteria

Identification of bacteria was done by different physical and chemical methods.

A. Physical methods

1. Shape of bacterial cell

Antonivan Leeuwenhoek first show microbes through a microscopes in 1670 and differentiate it on basis of their shape and size. Basic shapes of bacteria are Coccus (spherical), Bacillus (rod shape), and Spiral (twisted).

2. **Gram staining**

Hans Christian's gram developed the gram stained technique in 1884. It is a common technique that were used to differentiate two large bacterial group based on their cell wall (Coico et al, 2005). The gram staining procedure distinguished between gram negative and gram positive groups by coloring the cell violet or red. Violet color appears due to presence of thick layer of peptidoglycan in bacterial cell wall which retains the crystal violet. This technique is useful for bacterial sample contamination assessment and observation of morphological features of bacteria that were isolated from mixed cultures (Moyes et al, 2009).

B. **Biochemical test**

1. Catalase test

Catalase is enzymes that detoxify hydrogen peroxide into molecular oxygen and water (Gagnon et al, 1959). A Catalase test is positive if bubbles are observed after adding hydrogen peroxide. It should appear as if simple water was dropped on the smear. Catalase test is used for gram positive bacteria and can utilize to distinguish Staphylococcus and Micrococcus.

2. Citrate test

Citrate test is used to determine the bacterial ability to use sodium citrate as the source of carbon (Table 5). The citrate test is possible for those organisms which are capable of fermenting citrate. Inoculate a tube having citrate medium with bacteria or it is also possible to streak a deep inoculation into "Simmons citrate tube" and incubate it. Positive test was seen when a particular color change was

observed in Simmons citrate tube and negative test was observed in case of no color change. The citrate test was used to distinguish between *Bacillus*, *Citrobacter*, *E.coil* and *Staphylococcus*.

Table 5 Citrate test

Chemical name	g/l
Sodium chloride	5
Sodium citrate	2
Ammonium dihydrogen phosphate	1
Dipotassium phosphate	0.2
Magnesium sulphate	0.2
Bromothymol blue	0.08

3. **Vogues -Proskauer (VP) test**

Ability of bacteria to convert glucose to acetoin creates a red/purple color near top of tube. Bacteria producing a negative Voges-Proskauer result exhibit no color change (Hasan et al, 2015). When potassium hydroxide is added into acetoin then acetoin is converted to diacetyl that later reacts with alpha-naphtol and make pink color compound (Table 6). This test depends on the digestion of glucose to acetyl methyl- carbinol. Color changes to pink was observed in positive test and no color change was seen in negative test.

Table 6 Vogues -Proskauer (VP) test

Chemical name	g/l
Peptone	2
Dextrose	2
Potassium phosphate	2
Ph	6.1

4. **Oxidase test**

This test is used to determine the presence of bacterial enzyme cytochrome oxidase (Tarrand et al, 1982). Cytochrome are iron containing hemoproteins that transfer electrons to oxygen to form water in aerobic respiration. Cytochrome oxidase is a transmembrane protein complex, also present in the mitochondrial cytoplasmic membrane. Use a loop and pick the isolated colony from the fresh bacterial plate and rub it into filter paper after it add 2 drop of oxidase reagent on the organism smear and observe for color change. In Positive test, there was dark purple color and no color in case of negative test.

5. **Urease test**

Urease test identifies that organism that is capable of hydrolyzing urea to produce ammonia and carbon dioxide (Brink et al, 2019). Some bacteria have the enzyme that convert urea to ammonia and carbon dioxide which form ammonium carbonate in the presence of water. Urease test is a biochemical test that

detect the alkaline fermentation of urine with the resultant production (Table7). Rapids positive organism turns the entire medium pink within 24 hours. Negative organism produces no color change or yellow as a react of acidic production.

Table 7 Urease test

Chemical name	g/l
Peptone	1
Dextrose	1
Sodium chloride	5
Potassium phosphate	2
Phenol red	0.01
Urea	20
Agar	15
pH	6.7

6. Methyl red test

Many bacteria have ability to utilize glucose and converted into a stable acidic acid like acetic acids, formic acid or lactic acid, as the end product. Methyl red changes the color immediately and help to predict rather it's basic or acidic. Bacteria metabolize the glucose in pyruvic acid and later pyruvic acid metabolize through the mixed acid pathway to produce stable acids. The acid production is different from species to species and depends on the enzymatic pathway present in the bacteria. Red color and yellow color or colorless appear in case of positive and negative test respectively (Table 8) (In Atlasal oral microbiology).

Table 8 Methyl red test

Chemical name	g/l
Peptone	2
Dextrose	2
Potassium phosphate	2
pH	6.1

7. Nitrate reduction test

Nitrate test is used to determine the presence and absence of nitrate in the solution (Table 9). Nitrate test differentiate those bacteria that have ability to produce nitrate reductase enzymes. This enzyme hydrolyze nitrate to nitrite and nitrite further degrade into various nitrogen product like nitrogen oxides, nitrous oxides and ammonia. It can be performed by adding sulfanilic acid that will react with the nitrous acid and produce diazotized sulfanilic acid. This diazotized sulfanilic acid react with alpha-naphthylamine and form a red color compound in positive test. While no color was detected in case of negative test (Tlaieakhon et al, 2018).

Table 9 Nitrate reduction test

Chemical name	g/l
Peptone	5
Beef extract	3
Potassium nitrate	1
Sodium chloride	30
pH	7

8. Carbohydrate test

The microbes utilize carbohydrate to produce energy in form of ATP (Table 10). Degradation of the monosaccharide, disaccharides and polysaccharides by microorganism under anaerobic condition is carried out in the fermentation. Its end product can either be organic acids like formic, lactic, or organic acids. The fermentation medium contain carbohydrate with PH indicator. After fermentation, organic acid product turns into yellow color with the reduction of PH.

A) Preparation of carbohydrates fermentation broth

Dissolve sodium chloride, trypticase and phenol red in 100 ml of distilled water and transfer into flasks. Add 0.5-1% of carbohydrate into the flask. Sterilize in autoclave at 115 degree Celsius for 10 min. The overheating may result in breakdown of molecule and browning of sugar. Transfer it into the fermentation tubes or screw- capped tubes and labeled it.

B) Bacterial culture inoculation into fermentation medium tube

Incubate fermentation tube for 18 hours at 37 degree Celsius and observe it for acids and gas production. When the medium changes to yellow color, it means organisms are able to ferment carbohydrate. If it retains red color, means organism do not utilize the carbohydrate (Weldezgina et al, 2016).

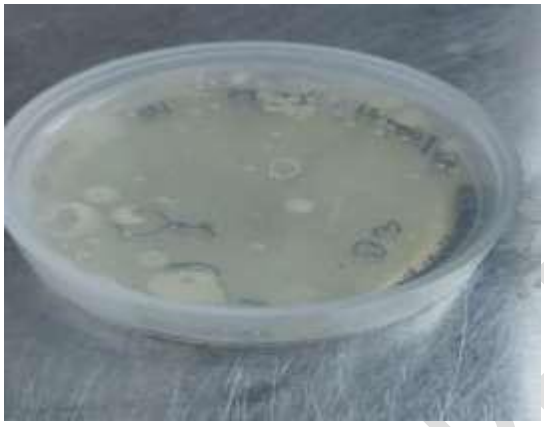
Table 10 Carbohydrates test

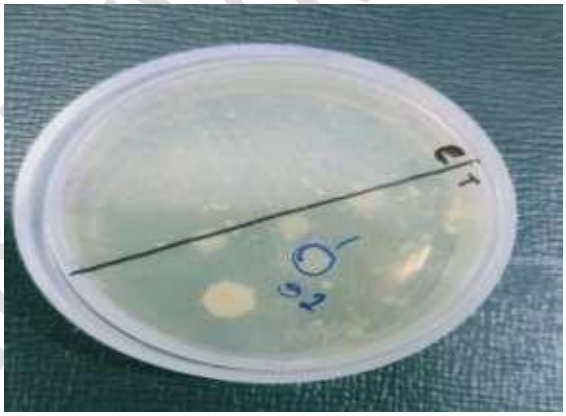
Chemical name	g/l
Peptone	5
Beef extract	3
Sodium chloride	5
pH	6.8

Result

Our experimented petri plates showed the colonies of bacteria that were present on the surface of vegetables. Vegetable Market had high number of bacterial presence as compared to supermarket area. The sector 40D vegetable markets had 20 bacterial colonies on NAM media & the Supermarket area had 15 bacterial colonies (Fig. 1). We found *Bacillus* bacteria on tomato that were taken from the vegetable market and *Staphylococcus* bacteria was observed on the surface of capsicum annum of supermarket. Confirmation of *Bacillus* and *Staphylococcus* bacteria was done by different type of tests as described below.

FIG.1 Bacterial colonies appear on Petri plates





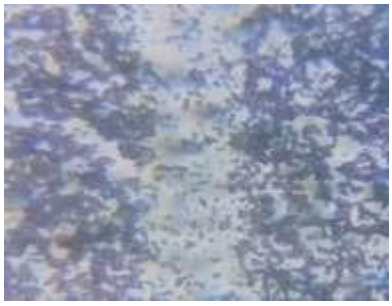
Bacillus **bacteria collected from vegetable market (tomato)**

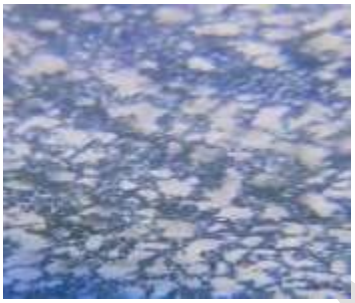
Staphylococcus bacteria collected from supermarket (Capsicum)

1. **Gram staining**

   The bacteria react to the Gram stain and gram stain is purple colored. After the stain combine with bacteria, the bacteria will stay purple or turn pink. In our experiment, both vegetables market and supermarket samples containing *bacillus* and *staphylococcus* respectively were found positive with purple color (Fig. 2).

Fig.2 Gram staining experiments





Sample A (Bacillus) **Sample B (Staphylococcus)**

2. **Catalase Test**

   The Catalase test is used to differentiate bacteria. The bacteria produce enzyme Catalase that by using the oxygen, protects themselves from the toxic by-products of oxygen metabolism. Both Bacillus and staphylococcus sample bubbles confirm the bacteria as Catalase positive (Fig. 3).

Fig.3 Catalase test

Sample A (*Bacillus*) **Sample B (*Staphylococcus*)**

3. **Citrate test**

   When an organism use citrate then it shows positive reaction but not necessarily as the sole carbon source. The pH turns the pH indicator from green to blue. The blue color appears which indicate the test is positive. Both *Bacillus* and *Staphylococcus* sample were found positive as shown in fig 4a. The observed color change is from green to blue.

FIG.4 (a) Citrate test

Sample A (*Bacillus)* ***Sample B (Staphylococcus)***

4. **Nitrate test**

When nitrate is present in the medium, then red color will be produced. In our experiment *Bacillus* and *Staphylococcus* culture don't show any color change so both sample were found negative (Fig 4b).

FIG.4 (b) Nitrate test

Sample A (*Bacillus*) **Sample B (*Staphylococcus*)**

5. **Urease method**

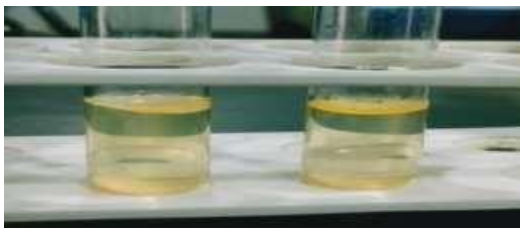
The product of decarboxylation of amino acids is urea. After hydrolysis process, urea produces Carbon dioxide and Ammonia. In our experiment, both *Bacillus* and *Staphylococcus* sample showed positive reaction by developing the intense magenta to bright pink color in 15 min to 24 h (Fig 5).

FIG.5 Urease test

6. **Methyl red test (MR)**

In this test, the test bacteria were grown in a broth medium that contained glucose. As we know, bacteria had ability to utilize glucose with the production of an acid, the color changed was observed from yellow to red in broth culture. *Staphylococcus* has ability to utilize glucose and *Bacillus* does not have ability to utilize glucose so the bacillus showed positive and staphylococcus was with negative result (Fig 6).

Fig.6 Methyl red test (MR)



Sample A (*Bacillus*)

Sample B (*Staphylococcus*)

7. **Voges–Proskauer**

Voges–Proskauer or VP is a test used for detection of acetoin in bacterial broth culture. This test is performed by adding potassium hydroxide and alpha-naphthol to this bacterial inoculated broth. Both samples *bacillus* and *staphylococcus* showed negative reaction i.e. lack of a pink-red color (Fig 7)

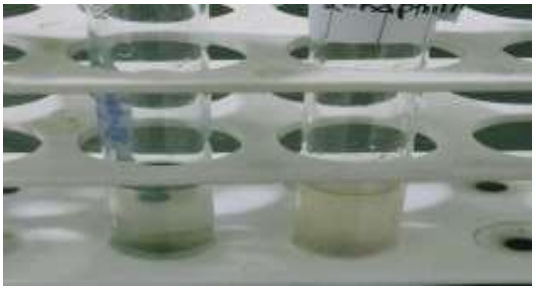


Fig.7 Voges–Proskauer

Sample A (*Bacillus*)

Sample B

(*Staphylococcus*)

8. **Oxidase test**

   The presence of cytochrome oxidase pathway is detected by oxidase test. It catalyzes the transport of electrons between electron donors and a redox dye i.e. tetramethyl-p-phenylene-diamine in bacteria. This dye is reduced to purple color. Here both *Bacillus* and *Staphylococcus* are negative because of no color changes (Fig 8).

Fig.8 Oxidase test

9. **Carbohydrate test**

   During Carbohydrate test, medium changed to yellow color due to production of acid. Microbes produces organic acids after fermentation of carbohydrates. Both Bacillus and Staphylococcus showed the carbohydrates test positive (Fig 9).

Fig 9 Carbohydrates test

Fig 9.1 Nutrient Media with carbohydrates for LB

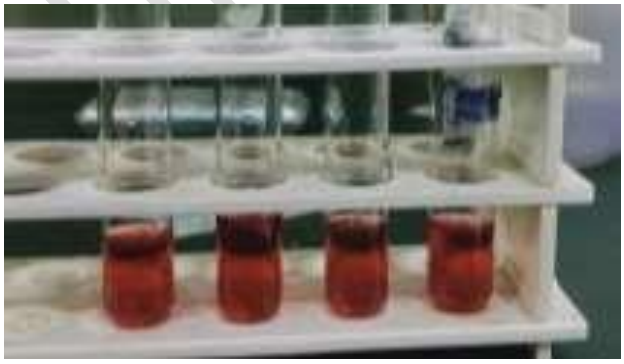


Fig 9.2 Nutrient Media with Carbohydrates for MSA

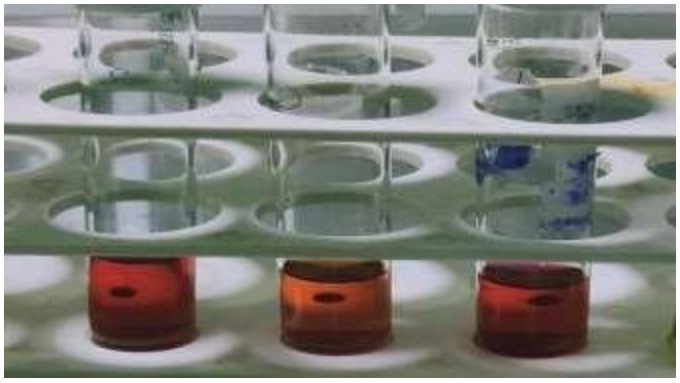
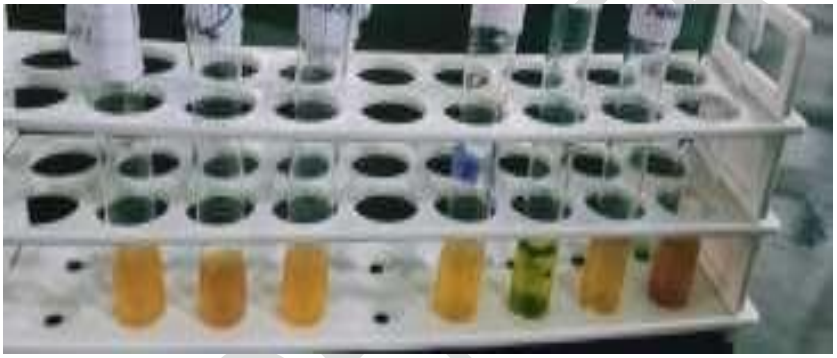


Fig 9.3 After inoculation, color change in sample A and sample B Media



Discussion

Vegetable market area sample contain more bacterial flora compared to the supermarket area as we can see that more contamination was present on the Vegetable market sample. After observing the results of the surface of microbial growth count, we can state that in supermarket area staphylococcus growth count was less i.e. 50 percent as compared to vegetable market where Bacillus count was 90 percent. Further, more experiment was done to identify microbes from environment of different places. Mostly bacteria were found of human and soil

origin. The microbes come from the human are mostly from skin and respiratory tract, were released by sneezing or talking whereas soil microbes were released by dust. The infectious bacteria make us unhealthy by releasing toxin that damage our tissues, such as *Streptococcus*, *Staphylococcus*, *Bacillus*, *and E.coil*. Both *Bacillus* and *Staphylococcus* microbes are gastrointestinal pathogens, *Vibrio cholera* causes cholera, *E.coli* causes cystitis and *Bacillus* pathogen capable to causing disease due to the production of enterotoxin or non ribosomal peptide synthetase toxin. Tambekar and Mundhada in 2006 also reported pathogen detected from fresh vegetables is *bacillus*, *Staphylococcus* and *E.coil*. Several studies reported the presence of pathogenic bacteria in vegetables and fruits but the counts were rarely documented (Olaimat et al, 2012). Some other researchers reported that in 2005, *Staphylococcus aureus* was causal agent of a food poisoning outbreak in Japan in where pickled radish was one of its sources (Kuramoto et al, 2006). It was observed that 50% of all vegetable samples that were collected from 39 major cities in China contained *B. cereus*. The level of contamination was more or less similar as those of previous surveys in other different countries, i.e., 57% in Mexico City, 20–48% in Korea (Chon et al, 2015), and 52% in the southeast of Spain (Flores-Urban et al, 2014). These reports, together with our study, indicate that *Bacillus* and *Staphylococcus* contamination in vegetables is very common. This suggest that consumption of vegetables contaminated with these bacteria is a potential health hazard. Treatment of these bacterial infection also includes good nutrition and intensive care. People who have had closed contact with a person with bacterial disease may need to be treated for the disease. Some precautions required like wash your hands, kitchen utensils, chopping boards and countertops before preparing fruits and vegetables. Clean vegetables and fruits before eating.

Conclusion

The experiment showed the vegetables collected from vegetables market and super market from Chandigarh. The hygienic quality of vegetables and water was seen very poor due to higher mean bacterial counts and it was recorded beyond the standard safe limits. Vegetable micro flora was dominated by *Staphylococcus* and *Bacillus*. The presence of *bacillus* bacteria on vegetable market was in high number than *staphylococcus* bacteria present on the supermarket. Good agricultural practices should be used along with various safety measures during the production of fresh fruits and vegetables. It is better to wash vegetables and stored to them in food grade antibacterial chemicals for a good time to free pathogens and reduces the microbial flora.

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