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# ISOLATION, IDENTIFICATION & ANTIBIOTIC SENSITIVITY OF BACTERIA OBTAINED FROM COMPARATIVE MICROBIAL ANALYSIS OF REFRIGERATED AND NON-REFRIGERATED FRUIT CONTAINING SOFT DRINKS FROM CHENNAI, TAMIL NADU, INDIA.

**V. Pushpa Rani[1*], E. Saravanan[2], A. Anitha Nancy[3], K. Shanmuga Priya[4] and G. Meena Kumari[5]**

1-5 P.G. & Research Department of Advanced Zoology & Biotechnology, Loyola College, Chennai-600 034. Tamil Nadu, India.

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***Correspondence for Author**
**V. Pushpa Rani**
1-5 P.G. & Research Department of Advanced Zoology & Biotechnology, Loyola College, Chennai-600 034. Tamil Nadu, India.

## ABSTRACT

The present study deals with comparative analysis between refrigerated and non-refrigerated fruit containing soft drinks for quantitative microbial analysis. The number of individual colonies on an agar plate was counted using by Colony Counter for both refrigerated and non-refrigerated. Based on the Morphological and Cultural characteristics, the bacteria was identified and isolated. The Bacterial isolates were analysed for Gram staining characterization and their motility and various biochemical tests such as Indole Test, Methyl Red Test, Triple Sugar Iron Agar and Citrate Utilization Test were performed by serial dilution of well-isolated colony from soft drinks. Based on biochemical analysis (IMViC), the bacteria isolated were found to be E.coli, *Staphylococcus, Proteus sp, Pseudomonas sp* and *Bacillus* from the refrigerated and non-refrigerated fruit containing soft drinks. The current investigation was extended to determine the antibiotic sensitivity whether the tested bacterial species is sensitive or resistant to the tested antibiotics. The bacteria which present in the fruit containing soft drinks were more sensitive and few shows resistance towards the antibiotics such as Ciprofloxacin, Amoxicillin Clavulanate, Tetracycline, Penicillin-G and Azithromycin.

**KEYWORDS:** E.coli, *Staphylococcus, Proteus sp, Pseudomonas sp* and *Bacillus.*

## INTRODUCTION

The soft drinks sector is one of the fastest growing, most innovative and rapidly changing areas of the food and drink industry. Many micro-organisms are found in soft drinks as environmental or raw material contaminants, but relatively few can grow within the acidic or low oxygen environment. Yeasts are the most significant group of micro-organisms associated with the spoilage of soft drinks and fruit juices (Jayalakshmi *et al*., 2011). Soft drinks are enormously popular beverages consisting primarily of carbonated water, sugar and flavorings. As flavored carbonated beverages gained popularity manufactures struggled to find an appropriate name for the drinks. The most appealing name however was "soft drink" adapted in the hopes that would ultimately supplant the hard liquor market (Pindi *et al*., 2012). Soft drinks bottled at low temperatures have high values of the water activity, which allow microbial growth; the pH, the sugar content and the addition of preservatives prevent the microorganism's growth in soft drinks (Stiles *et al*., 2001). Bacteria that have been associated with spoilage in the soft drink industry include *Acetobacter, Alicyclobacillus, Bacillus, Clostridium, Gluconobacter, Lactobacillus, Leuconostoc, Saccharobacter, Zymobacter* and *Zymomonas*. Many microorganisms are found in soft drinks as environmental or raw materials contaminants, but relatively can grow within the acidic and low oxygen environment (Pindi *et al*., 2012). Centrimide-nalidixic acid agar (CNA) and Malachite green enrichment broth (MGB) used for the enumeration of *Ps. aeruginosa* (Shubert and Blum, 1974; Cianand, 1981) were isolated to assess the occurrence of atypical colonies, as well as to identify other soft drinks bacteria capable of growth on these media. Rapid assessment of microbial presence and viability in food productions is of paramount importance for the food industry, daily compelled with the control of positive and negative microorganisms (Donnelly *et al*., 1988; McClelland and Pinder, 1994). Protection of all types of drinks from hazardous microbial contaminants is a global issue. Various gastrointestinal illnesses are the most common consequences of consuming contaminated drinks (Frobisher *et al*., 1974). Good manufacturing practices (;/GMPs) are not always followed in the food and beverage industries, particularly in developing countries and this can result in wide variations in the microbial quality of products, such as bottled water and other drinks (Hara *et al*., 2005; Venieri *et al*., 2006).

The soft drinks sector is one of the fastest growing, most innovative and rapidly changing areas of the food and drink industry. So, the present study deals with the comparison of microbial analysis between refrigerated and non-refrigerated fruit containing soft drink

samples from Chennai, India. The study extended for isolation and identification of organisms to determine the antibiotic sensitivity whether the tested bacterial species is sensitive or resistant to the tested antibiotics.

## MATERIALS AND METHODS

### Sample Collection & Procedure

Total of Five soft drinks containing fruits intact containers (plastic bottles) of both non-refrigerated and refrigerated were obtained from the local markets during the month of December in Chennai. Based on the consumer demand, 5 types of juices namely 2 different brands of orange flavored (carbonated), 2 different brands of mango flavoured (non-carbonated) and 1sample of mixed-fruits (100ml each) were selected for microbial analysis (Mahale *et al*., 2008). The Samples was named as Sample-1 (S1), Sample-2 (S2), Sample-3 (S3), Sample-4 (S4) and Sample-5 (S5). The samples of both refrigerated and non-refrigerated conditioned were processed to identify the number of organisms by the spread plate technique.

### Isolation and identification of bacteria

One ml of packed juice sample was taken into $10^{-1}$ tube containing 9 ml of saline. From this, 1 ml of sample was serially diluted $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$. Then 0.1 ml was taken from each tube and inoculated into nutrient agar plates and spread using L –rod. the inoculated Plates were incubated at 37$^{o}$C for 24 hours. Colony morphology was observed and the isolated colonies were inoculated into nutrient broth and incubated at 37$^{o}$C for 24 hours. This test sample was further used to identify the morphological and biochemical characteristics of the organisms.

### Enumeration of Bacterial isolates

The number of individual colonies on an agar plate were counted using Colony Counter.

Number of CFU/ml of packed juices = Average number of colonies per plate/vol. of sample x dilution factor. Morphological characterization studies, such as Gram staining, Motility Test i.e. Hanging Drop method is carried out (Sand *et al*., 1976).

### Biochemical Reactions

After the morphological characterization studies, the test samples were further identified by using biochemical reactions by doing IMViC. The organisms can be identified by IMViC (Indole, Methyl red, Voges Proskauer and citrate utilization test) and TSI confirmed by doing

tests such as Starch hydrolysis, Catalase test, TSI test, Urease test etc.

## ANTI-BIOTIC SENSITIVITY

Antibiotic susceptibility were determine by the agar diffusion technique on Mueller-Hinton agar (Kirby-Bauer NCCLS modified disc diffusion technique) by using 5 antibiotic discs (Biotec Lab, United Kingdom) Ciprofloxacin (CP), Amoxicillin Clavulanate (AU), Tetracycline (Te), Penicillin-G (PG), Azithromycin (AZ). The test organisms streaked on the plates were allowed to dry for 5 minutes. Then antibiotic discs were placed on the surface of the agar by sterilized forceps and gently pressed the discs onto the surface of the agar using flame sterilized forceps or inoculation loop. Then inoculated plates were carefully inverted and incubated for 24 hours at 37°C. After incubation, metric ruler was used to measure the diameter of the zone of inhibition for each antibiotic used. The measurement obtained from the individual antibiotics were compared with the standard table to determine the sensitivity zone, whether the tested bacterial species is sensitive or resistant to the tested antibiotic (Tagoe *et al.*, 2011).

## RESULTS AND DISCUSSION

In the present study, comparative analysis of five refrigerated soft drinks with non-refrigerated soft drinks of same, namely two numbers of orange flavoured (carbonated), two numbers of mango flavoured (non-carbonated) and one sample of mixed-fruits (non-carbonated) named as Sample 1 (S1), Sample 2 (S2), Sample 3 (S3), Sample 4 (S4) and Sample 5 (S5) were collected from the local markets during the month of December in Chennai, India. The colony forming unit (CFU/ml) was noted for each samples for different dilutions of $10^{-3}$, $10^{-4}$ and $10^{-5}$ in Table-1 & Plate no. 1. The number of organisms present in sample decreases when the dilution factor increases. By serial dilution technique, colony forming unit per ml were also studied. This study proves that there was difference between CFU/ml between refrigerated and non-refrigerated fruit containing soft drinks.

These tested samples were further used to identify the morphological and cultural characteristics. So, the bacterial isolates were analyzed for Gram character (Plate-2) and their motility and various biochemical tests were performed by inoculating small portion of well-isolated colony into a series of media and performed Indole Test, Methyl Red Test, Voges Proskauer Test, Triple Sugar Iron Agar (TSI) (Table-2).

In this current investigation, the bacterial isolates were identified as *Proteus sp, Staphylococcus sp, Bacillus, E. coli* and *Pseudomonas sp* in Sample1, Sample 2, Sample 3, Sample 4 and Sample 5. Many micro-organisms found in soft drinks environmental and raw materials contaminant, but relatively can grow within the acid and low oxygen environment.

The isolated organisms from the fruit containing soft drink samples were further tested for antibiotic sensitivity and the bacteria which are sensitive to the antibiotic was found by a clear ring or zone of inhibition shown in plate.no.3 and the zone of inhibition of antibiotics for isolated organism was listed in the table 3. Based on the Standard Zone Size Interpretation Chart (CLSI, 2012), the interpretative result was shown in the table 4.

Ciprofloxacin shown more sensitivity towards *Pseudomonas sp*, *Staphylococcus sp, Bacillus, Proteus sp* in higher hierarchy and *E. coli* observed to be intermediate for this antibiotic. Amoxicillin Clavulanate was revealed its sensitivity towards Bacillus, *Proteus sp, E. coli* bacterial strains, reacted intermediately for *Staphylococcus sp* and established resistance towards *Pseudomonas sp*. Tetracycline was not found to be resistance towards any of these organisms and shown sensitivity for *Bacillus, Pseudomonas sp* and *E. coli*; intermediate for *Proteus sp, Staphylococcus sp*. Penicillin-G shows resistant towards *Bacillus, Pseudomonas sp, Proteus sp* and *Staphylococcus sp*; intermediate level in *E. coli*. Azithromycin shows more sensitive towards *Bacillus, Proteus sp, Staphylococcus sp* and *E. coli*; more resistive towards *Pseudomonas sp*.

The bacterial isolates from different soft drinks samples in my investigation shown, various degree of resistance. The emergence of bacteria resistant to most of the commonly used antibiotics is of considerable medical significance because of the public health implications, and there are several reports on the incidence of bacterial resistance among bacterial isolates obtained from food materials (Khan and Malik, 2001).

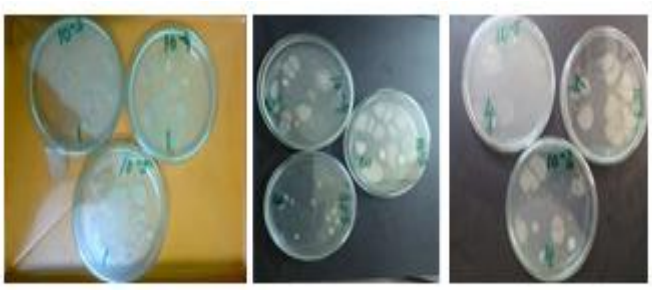

**Plate. 1 Culture Plate Containing Bacterial Colonies.**

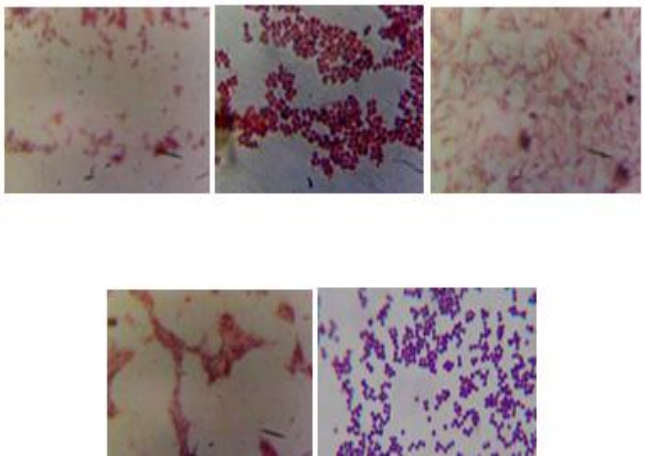

**Plate. 2 Gram Staining.**

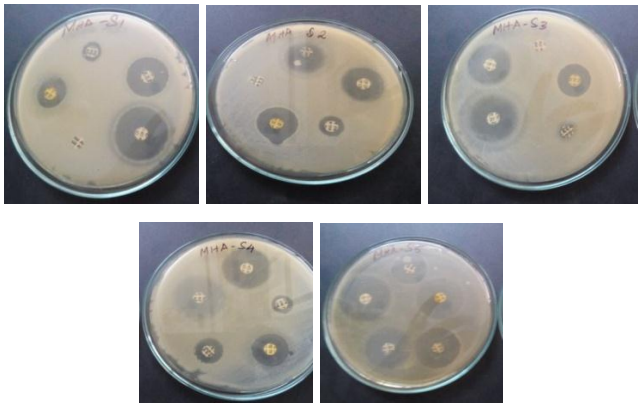

**Plate. 3 Antibiotic Sensitivity for Isolates obtained from fruit containing different soft drinks.**

**Table-1 Comparison between refrigerated and non-refrigerated soft drinks.**

| S.NO | Sample | Type | Dilution | CFU/ml | Gram staining |
|---|---|---|---|---|---|
| 1 | Sample1 (S1) | Refrigerated | $10^{-3}$ | TNTC* | - |
| 2 | | | $10^{-4}$ | $95x10^{5}$ | - |
| 3 | | | $10^{-5}$ | $71x10^{6}$ | - |
| 4 | | Non-refrigerated | $10^{-3}$ | $71x10^{4}$ | - |
| 5 | | | $10^{-4}$ | $61x10^{5}$ | - |
| 6 | | | $10^{-5}$ | $53x10^{6}$ | - |
| 7 | Sample2 (S2) | Refrigerated | $10^{-3}$ | $200x10^{4}$ | + |
| 8 | | | $10^{-4}$ | $130x10^{5}$ | + |
| 9 | | | $10^{-5}$ | $48x10^{6}$ | + |
| 10 | | Non-refrigerated | $10^{-3}$ | TNTC* | + |
| 11 | | | $10^{-4}$ | TNTC* | + |
| 12 | | | $10^{-5}$ | TNTC* | + |
| 13 | Sample3 (S3) | Refrigerated | $10^{-3}$ | TNTC* | + |
| 14 | | | $10^{-4}$ | TNTC* | + |
| 15 | | | $10^{-5}$ | TNTC* | + |
| 16 | | Non-refrigerated | $10^{-3}$ | $100x10^{4}$ | + |

| | | | | | |
|---|---|---|---|---|---|
| 17 | | | $10^{-4}$ | $87x10^{5}$ | + |
| 18 | | | $10^{-5}$ | $70\ x10^{6}$ | + |
| 19 | Sample4 (S4) | Refrigerated | $10^{-3}$ | $24x10^{4}$ | - |
| 20 | | | $10^{-4}$ | $21x10^{5}$ | - |
| 21 | | | $10^{-5}$ | $20\ x10^{6}$ | - |
| 22 | | Non-refrigerated | $10^{-3}$ | TNTC* | - |
| 23 | | | $10^{-4}$ | TNTC* | - |
| 24 | | | $10^{-5}$ | $63x10^{6}$ | - |
| 25 | Sample5 (S5) | Refrigerated | $10^{-3}$ | $113x10^{4}$ | - |
| 26 | | | $10^{-4}$ | TNTC* | - |
| 27 | | | $10^{-5}$ | TNTC* | - |
| 28 | | Non-refrigerated | $10^{-3}$ | TNTC* | - |
| 29 | | | $10^{-4}$ | TNTC* | - |
| 30 | | | $10^{-5}$ | TNTC* | - |

***CFU above 250-300 are considered TNTC**

+: **positive**, −: **negative**

**Table-2 Biochemical test for fruit containing soft drinks samples.**

| Samples | Indole | Methyl Red | Voges-Proskauser | Citrate | TSI | Identification |
|---|---|---|---|---|---|---|
| **S1** | + | + | - | - | A/A | *Proteus sp* |
| **S2** | Catalase positive | | | | | *Staphylococcus sp* |
| **S3** | - | - | + | + | A/A | *Bacillus* |
| **S4** | + | + | - | - | A/A+G | *E. coli* |
| **S5** | - | - | - | + | K/K | *Pseudomonas sp* |

+: Positive, −: Negative, A/A: Acid-acid, A/A + G: Acid-acid + gas, K/K: Alkaline –alkaline

**Table-3 Antibiotic sensitivity for bacteria in fruit containing soft drinks samples.**

| ANTIBIOTICS | CP | AU | TE | PG | AZ |
|---|---|---|---|---|---|
| **BACTERIA** | **Zone of Inhibition (in mm)** | | | | |
| *Proteus sp* | 23 | 12 | 18 | 15 | 23 |
| *Staphylococcus sp.* | 30 | - | 16 | 10 | 22 |
| *Bacillus* | 27 | 20 | 26 | 24 | 25 |
| *E. coli* | 20 | 12 | 20 | - | 20 |
| *Pseudomonas sp.* | 35 | 11 | 24 | 23 | 08 |

*CP- ciprofloxacin, AU –Amoxicillin Clavulanate, TE – Tetracycline, PG – Penicillin-G, AZ – Azithromycin.

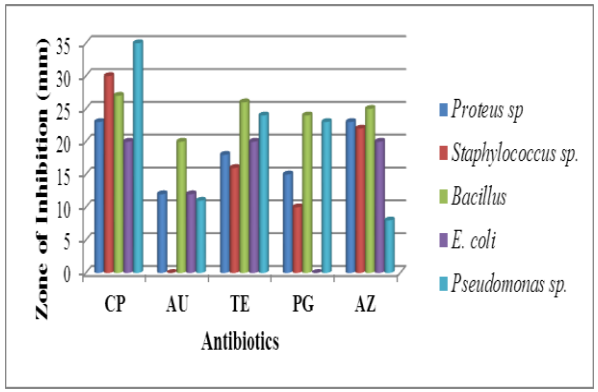


**Figure-1 Comparative antibiotic sensitivity between bacteria in fruit containing soft drink samples.**

**Table-4 Antibiotic sensitivity for bacteria in fruit containing soft drinks samples based on standard zone size interpretation chart.**

| Antibiotics | CP | | AU | | Te | | PG | | AZ | |
|---|---|---|---|---|---|---|---|---|---|---|
| Organism | Zone of Inhibition (in mm) | Interpretative results | Zone of Inhibition (in mm) | Interpretative results | Zone of Inhibition (in mm) | Interpretative results | Zone of Inhibition (in mm) | Interpretative results | Zone of Inhibition (in mm) | Interpretative results |
| ***Proteus sp.*** | 23 | S | 12 | R | 18 | I | 15 | R | 23 | S |
| ***Staphylococcus*** | 30 | S | - | I | 16 | I | 10 | R | 22 | S |
| ***Bacillus*** | 27 | S | 20 | S | 26 | S | 24 | R | 25 | S |
| ***E. coli*** | 20 | I | 12 | R | 20 | S | - | I | 20 | S |
| ***Pseudomonas sp.*** | 35 | S | 11 | R | 24 | S | 23 | R | 8 | R |

*** S – Sensitive**, I – **Intermediate**, **R – Resistant.**

*CP - ciprofloxacin, AU – Amoxicillin Clavulanate, Te – Tetracycline, PG – Penicillin-G, AZ – Azithromycin.

The current study reveals that the soft drinks of both refrigerated and non-refrigerated were more or less similar number of Colony forming unit /ml. So, the refrigeration alone will not eradicate the microbial contamination and the bacteria which present in the fruit containing soft drinks were more sensitive and few shows resistance towards the antibiotics such as Ciprofloxacin, Amoxicillin Clavulanate, Tetracycline, Penicillin-G and Azithromycin. This investigation was ultimately proves that the antibiotic–resistant bacteria may become pandemic through the novel route of spreading, especially in developing countries.

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