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Original Research

Isolation and Characterization of Facultative Anaerobes Involved in Canned Food Spoilage and Examining the Effect of Different Preservatives

Irum Maqbool[1] | Rumesha Rafiq[2] | Azka Asif[3*]

[1]Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan
[2]Department of Life Sciences, University of Management and Technology, Lahore, Pakistan
[3]School of Cancer & Pharmaceutical Sciences, Institute of Pharmaceutical Science, King's College London, United Kingdom

***Correspondence**
Azka Asif
Email: azka.asif@kcl.ac.uk

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ABSTRACT

Spoilage of canned food is a major issue worldwide. The major cause of spoilage of food in cans is the bacterial contamination that happens due to mishandling, damage, or improper packaging treatment. To avoid spoilage and increase the shelf-life of canned food, various food preservatives are used. However, it is ideal to use specific preservatives for specific foods. There is a need to check the types of organisms causing the food spoilage and the preservatives that can be used against them. The current study focuses on the isolation of facultative anaerobic bacterial species which are specifically involved in spoilage of canned food items, their characterization and effectiveness of different food preservatives against them. For this propose samples were taken from expired peas, meat, and pineapple cans. Each sample was cultured on Acid agar, Mannitol salt agar, DTPB agar, and EMB agar. The bacterial colonies were purified and characterized morphologically and biochemically. The effectiveness of food preservatives such as ascorbic acid, sodium benzoate, sodium propionic acid and sodium nitrite was analyzed on the isolated strains. Selected isolates were identified through 16S rRNA gene sequencing. Facultative anaerobes belonging to the genera *Leucobacter and Exiguobacterium* were identified in the expired food cans. Among the tested food preservatives, ascorbic acid was proved highly effective as it inhibited the growth of these organisms. Facultative anaerobes play an important role in canned food spoilage and by utilizing specific food preservatives these microbes can be minimized.

1. INTRODUCTION

Spoilage of food items is a multifarious mechanism, that makes the food undesirable for consumer as the result of physical or chemical changes, as well as microbial activities (Petruzzi *et al.*, 2017). According to an estimation, around 25% of the worldwide food products deteriorate due to microbes (Bondi *et al.*, 2014). Owing to microbial spoilage, large amount of food is rotten despite of current day preservation practices (Gram *et al.*, 2002; Rawat, 2015). Heat, light, air, humidity are the basic factors of detrimental variations arising in processed food items that promotes the growth of bacteria (Sahu and Bala, 2017; Preetha and Narayanan, 2020), which then triggers chronic as well as acute diseases in humans (Ünüvar, 2018). Changes in the texture, odour, pH, and gas production in canned food depicts its spoilage (André *et al.*, 2013). Mostly, non-spore forming mesophilic aerobes and spore forming bacteria are involved in the deterioration of canned food. Moreover, some thermophilic spore formers like *Desulfotomaculum* sp. and *Thermoanaero* sp. are engaged in gas production in cans, whereas *Bacillus* and *Geobacillus* sp. are involved in flat sour can spoilage. Gram positive bacteria, facultative or strict anaerobes, have also been reported in canned food spoilage, such as *Clostridium* sp. (Barth *et al.*, 2009; Abdullahi *et al.*, 2019). Mesophilic putrefactive anaerobes are important in spoilage of all the meat and marine products (Cambron, 1938; Stumbo, 2013).

Commercially, tin has been used for over a hundred years as a strong practice for packaging food. Canned packaging of foods has given rise to an industry worth USD 89 billion around the globe (Blunden and Wallace, 2003). The confirmation of canned food spoilage can be easily detected by the appearance of cans such as having flipper in appearance it's an initial phase of swell, others involved springer, swell, flat sour, hydrogen swell, buckles, and leakers (Evancho *et al.*, 2009). Moreover, food items from their manufacturing sites are now frequently traded in far-off areas of the world and therefore need extended shelf-lives (Abdullahi *et al.*, 2019). International trade of processed food products flourished by the development of cold delivery chain, however, just refrigeration cannot guarantee the safety level. So, common conventional preservatives are normally used as safe anti-microbial and anti-fungal additives (Banwart, 2012).

Food preservatives can be utilized alone or in combination of other approaches to inhibit the growth of microbes. Mostly, anti-microbial food additives like sodium nitrate, sulfites, disodium EDTA, spices, vinegar, common salt, sugar, ascorbic acid, salt of benzoic acid, salt of sorbic acid and propionic acid and its salts act as well-known preservatives at any phase of processing, manufacturing, storage and transportation of food to avoid any spoilage (Shee *et al.*, 2010). Sugar and salts produce high osmotic pressure causing the plasmolysis of microbial cells, inhibiting their growth. Enzymatic browning is inhibited by salt and hence also act as excellent antioxidant. Moreover, salts enhances the food flavour, tint and taste in processed food items (Seetaramaiah *et al.*, 2011; Rawat, 2015). Acids deliver a characteristic taste in processed foods and beverages. They control the formation of pectin-gel as well as kills bacteria by maintaining low pH which can damage their protein, DNA, enzymatic activities, and extracellular membrane (Ben Braïek and Smaoui, 2021).

The basic aim of this study was to isolate facultative anaerobic bacteria responsible for spoilage of food cans and analyze the effectiveness of preservatives on these bacteria. This could help in the identification of specific preservatives targeting a certain group of bacteria.

2. MATERIALS AND METHODS

2.1 Sample Collection

For isolation of facultative anaerobes involved in the spoilage of canned food products, expired and spoiled pineapple, peas, and meat food cans were procured. These cans were additionally incubated at 55°C for 7 to 14 days.

2.2 Media Preparation and Culture

Four types of culture media i.e., acid agar, mannitol salt agar, dextrose tryptone purple bromocresol agar, and eosin methylene blue agar were used for the isolation of facultative anaerobes from the spoiled food cans. Culture media were prepared according to the methods described by Atlas, 2010 with some minor modifications. 25 uL of the serial dilutions (10^{-3} and 10^{-4}) of the samples was platted on these culture media plates aseptically, which were then incubated in anaerobic jar using anaerobic sachet (Oxoid) at 37℃ for 7 days.

2.3 Bacterial Characterization

For morphological identification, different bacterial colonies were selected from each sample grown on different culture media. The colonies were purified through repeated quadrant streaking. These colonies were characterized based on colony morphology including size, shape, configuration, margins, elevation, texture, colour, pigmentation and transparency. Gram staining was used for detection of cellular morphology of isolated strains and to check whether they are gram positive or negative. Different biochemical tests were also performed.

2.4 Effectiveness of Chemical Preservative

Four types of chemical preservatives i.e., ascorbic acid, sodium benzoate, sodium propionic acid and sodium nitrite were used to check their effectiveness on the selected facultative anaerobes. Stock solutions were prepared, and filter sterilized the solution. The solutions were stored at low temperature. Luria Bertani (LB) broth tubes were prepared and autoclaved. As 40 mM of ascorbic acid is known to be effective, all preservative solutions were examined at 40 mM concentration. Preservative stock solutions were added in the LB-broth tubes aseptically to achieve a final concentration of 40 mM. The tubes were inoculated with 25 µL of overnight cultures of selected bacterial strains aseptically. The test tubes were incubated anaerobically overnight at 37℃. Results were noted by observing growth in test tubes.

2.5 Preservatives Efficacy by Well Plate Method

Effectiveness of preservatives was also determined via well plate method. Firstly, the LB-agar media plates were prepared. The bacteria were inoculated on these plates using cotton swab. Four wells were made on each plate at equal distance from the center using disinfected pasture pipette. 40 µL of each preservative solution was poured separately in the wells. The plates were incubated at 37℃ for 24 hours. After incubation, plates were observed for the presence of zones of inhibition.

2.6 16S rRNA Gene Sequencing

Genomic DNA was isolated from selected bacterial strains using GeneJet Genomic DNA isolation Kit following manufacturer's instructions and stored at -20℃. DNA yield was confirmed using agarose gel electrophoresis. 16S rRNA gene was amplified through polymerase chain reaction (PCR) using the universal primers 27F and 1492R. The amplicons were detected using agarose gel electrophoresis. The amplicons were sequenced from Macrogen (South Korea) through Sanger sequencing technology. The sequences were classified using NCBI BLAST. Phylogenetic relationship of the isolates was visualized through construction of phylogenetic tree using nearest homologues from the BLAST results. Neighbour joining Phylogenetic tree was made in Mega 7.0 using 500 bootstrap value as a measure of confidence.

3. RESULTS

3.1 Isolation and Characterization

The expired food cans which were further spoiled by facultative anaerobes, showed the growth of bacterial colonies on plates of mannitol salt agar, acid agar, dextrose tryptone purple bromocresol agar and eosin Methylene blue agar under anaerobic conditions. On all type of agars plates, different number of colonies were observed. The CFU g^{-1} calculation of the plates showed varying number of viable bacteria on these media

(Table 1). Selected colonies had different colony morphologies from each other. MM-2, MM-3, PM-8, PM-4, 1,2,3,4,5 and 6 were Gram positive cocci whereas PM-5 and PM-1 were Gram +ve rods. Isolates MM-2, MM-3, PM-8, PM-4, PM-5 and PM-1 from mannitol salt agar gave catalase test positive, while Isolates 1,2,3,4,5 and 6 from DTBP agar gave negative result as no bubble formation occurred. In case of oxidase test, isolates 1,2,3,4,5,6, PM-5, and PM-1 showed negative test whereas MM-2, MM-3, PM-8 and PM-4 were oxidase positive.

3.2 Identification of Facultative Anaerobes

For the molecular identification of the selected isolates, 16S rRNA gene sequencing was performed. Isolate PM-1 and PM-5 showed maximum homology with *Exiguobacterium profundum* (percentage identity 100%) and *Leucobacter tardus* (percentage identity 99.93%). The classification was also confirmed through phylogenetic tree (Figure 1).

Table 1. CFU g^{-1} of different food samples on different culture media

Culture Media	Meat (CFU g^{-1})	Peas (CFU g^{-1})	Pineapple (CFU g^{-1})
Mannitol salt agar	4.8×10^4	6×10^3	2.4×10^3
Acid agar	4.98×10^4	1.0×10^4	2×10^3
EMB agar	1×10^3	1.4×10^3	2×10^3
DTBP agar	3.6×10^4	4.2×10^4	1.4×10^4

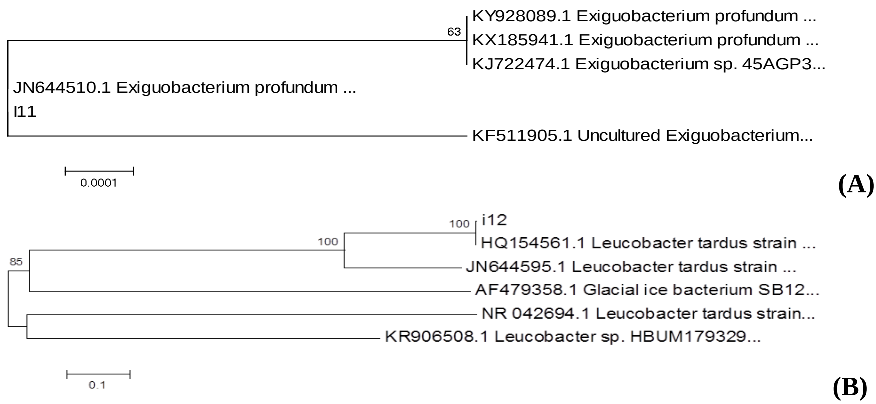

Figure 1. (A) Neighbour-Joining Phylogenetic tree of bacterial isolate PM-1 (B) Neighbour-Joining Phylogenetic tree of bacterial isolate PM-5

Table 2. Effect of different preservatives on growth of the bacterial strains

Bacterial strain	Ascorbic acid	Propionic acid	Sodium benzoate	Sodium nitrate
PM-1	No growth	Growth	No growth	No growth
MM-3	No growth	Growth	No growth	No growth
6	No growth	Growth	Growth	No growth
MM-2	No growth	Growth	Growth	Growth
PM-5	No growth	Growth	No growth	No growth

3.3 Effect of Preservatives

The effect of ascorbic acid, sodium benzoate, sodium propionic acid and sodium nitrite were used to check on the growth of different bacterial strains. Ascorbic acid proved highly effective against all the five strains compared to other preservatives which gave variable results as shown in Table 2. In agar well diffusion method, the growth of all the strains was inhibited by ascorbic acid showing zones of inhibition of different diameters. Sodium benzoate gave zone of inhibition with 2 isolates. No zones were observed in the case of sodium nitrite, sodium propionic acid (Table 3, Figure 2).

Table 3. Zones of inhibition by ascorbic acid and sodium benzoate

Isolates	Ascorbic acid (cm)	Sodium benzoate (cm)
PM-1	1.8 $\pm$ 0.2	0 $\pm$ 0
MM-3	1.8 $\pm$ 0.3	1.4 $\pm$ 0.2
6	1.3 $\pm$ 0.4	0 $\pm$ 0
MM-2	1.1 $\pm$ 0.2	0 $\pm$ 0
PM-5	1.7 $\pm$ 0.3	0 $\pm$ 0

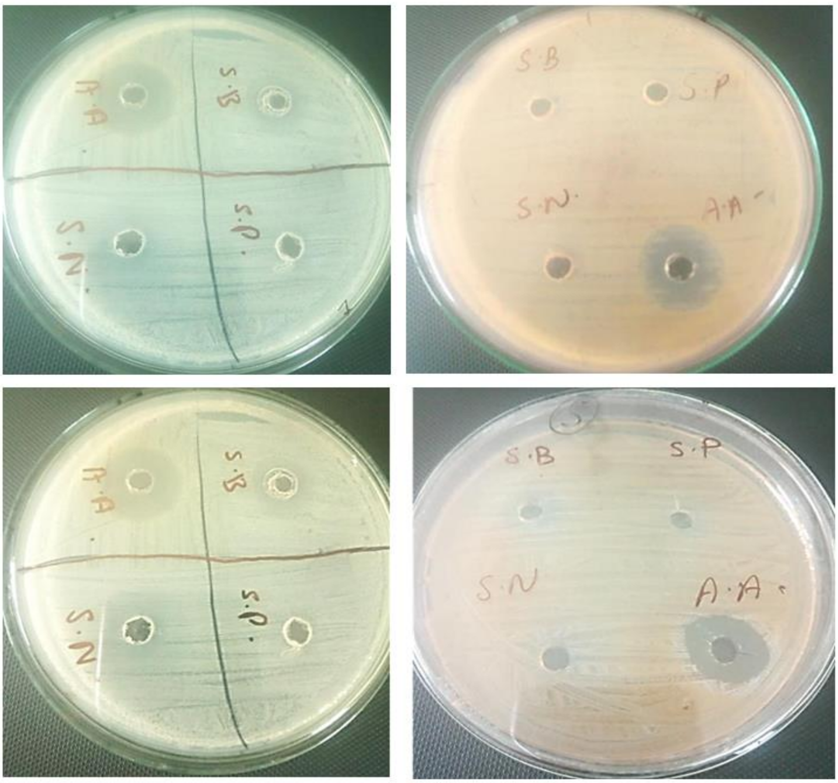

Figure 2. Zones of inhibition shown by ascorbic acid, sodium benzoate, sodium propionic acid and sodium nitrite against different strains

4. DISCUSSION

Processed food items can increase the foreign exportation of a country and have a great impact on the food industry and the economy. However, microorganisms can cause spoilage of the canned foods, making them undesirable and unfit for consumption (Lima Tribst *et al.*, 2009). Thermophilic facultative anaerobes have a crucial role in spoilage of canned food products. In consequence of any leakage, punctures, mishandling and improper heat treatment or pasteurization, a diverse variety of facultative anaerobes can grow in the canned food items, which is the major concern of public health (Carlier *et al.*, 2006). In this study, a number of facultative anaerobes were recovered, and two of them were identified as *Exiguobacterium* and *Leucobacter* species. To control the growth of gram-positive facultative anaerobes and other foodborne microbes in processed food containers, food agencies develop strict strategies and guidelines. One of these is the addition of preservatives like ascorbic acid, sodium benzoate, sodium nitrate and sodium propionic acid to increase shelf-life of the canned products. Preservatives cause oxidative stress results in inhibition of the bacterial growth (Stanojevic *et al.*, 2009; Mousavi *et al.*, 2019). In our study, when the isolated bacterial strains were exposed to a specific concentration of these preservatives, varied growth patterns were observed including no growth at all in certain cases. Ascorbic acid proved the most efficient preservatives against all the facultative anaerobes isolated in this study. A variety of other antimicrobial compounds have also been used against such bacteria (Schuetz, 2014; Rawat, 2015). Recently, researchers have been working to discover novel natural preservatives to stop the spoilage of food by microbes. Plant based essential oils have shown potential of food preservation. Recent studies on *Cymbopogon citratus* essential oil suggest that it can be used as food preservative and shows excellent anti-microbial efficacy (Ekpenyong and Akpan, 2017). Moreover, vanillin, cinnamon, and variety of herbs such as thyme and oregano have also repressive action against microbes involved in spoiled food (Martínez-Graciá *et al.*, 2015). Addition of such preservatives along with maintaining storage conditions (such low temperature) can reduce bacterial growth further and can improve shelf-life of the product (Abdullahi *et al.*, 2019). Moreover, recent advancements such as MAP (modified atmosphere packaging) can also be used in such combinations. In MAP, food is covered in a gas atmosphere that usually contains an inert gas such as N_2, and an antimicrobially active gas such as CO_2, along with low oxygen content (Petruzzi *et al.*, 2017). Good hygiene practices in industries and proper handling of the food containers before or during storage and transportation, along with addition of specific preservatives and combination of other such strategies as described above, can play an important role in preventing microbial spoilage of the canned food products.

5. CONCLUSION

The results of this study confirm the involvement of facultative anaerobic bacteria in spoilage of food cans. Ascorbic acid is one of most efficient food preservatives that shows anti-bacterial activity against a variety of facultative anaerobic bacteria.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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