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Prevalence of Thermo-Tolerant Anaerobic Bacteria in Unpackaged Spices: A Bacteriological Assessment

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Abstract: Spices are used all over the world for culinary purpose. Pakistanis have strong preference for them. Warm and humid condition is favorable for their growth. For this reason spices are easily contaminated by pathogenic microorganisms. Experimentations were designed to screen anaerobic bacterial presence, and to subsequently study the antibiotic sensitivity and thermo-tolerance potentials of isolated bacteria. A total of 120 spices samples were collected from fifteen different local retail markets of Karachi city. Out of which, 87.5% of the samples were found contaminated with the obligate anaerobic spore formers *Clostridial spp*. Results indicated that the highest load 22 (18%) of anaerobic bacterial strains were found in unpackaged black pepper powder. Metronidazole was found as the most effective antibiotic as it effectively killed all the anaerobic spp. Out of 145 bacterial isolates subjected to high temperature (90°C) for 0-60 mins, 49% (66) isolates were found to be resistant. 93% (125 out of 145) bacterial isolates withstood high temperature after 10 minutes exposure thus determining their survivability. To sum up, this study revealed the presence of temperature and antibiotic resistant bacteria in common spices, which poses a great health risk to the consumers.

Keywords: Spices, Anaerobes, *Clostridial spp*, Temperature Tolerance, Foodborne Illnesses

1. Introduction

Spices are one of the major food commodities which are natural products or their blends that are taken from the roots, seeds, bark, leaves, flowers, fruits, rhizomes, stigmas, styles and / or the entire plant tops. These are widely used in various forms i.e. fresh, ripe, dried, broken and powdered [1-3] for imparting aroma, taste, zest, color and pungency to various national and international cuisines. Thus, are essential ingredient of the culinary art worldwide [1, 3, 4-6].

Nonetheless these important agricultural commodities are grown and harvested in warm and humid conditions. Subsequent to harvesting, spices are often sun-dried either in open field or tarfelt road in most of the spice growing countries including Pakistan. This significantly contributes in their contamination with different microorganisms [1, 3, 7–9] Additionally, transportation of these spices to the local markets further increases the risk of microbial contamination due to the exposure with air, dust, waste water and even human and animal excreta [5, 8]. In view of the fact that these spices are sold in local markets without prior treatment to reduce the microbial load. Therefore, spices may contain an elevated number of pathogenic bacteria, bacterial spores, molds and yeasts [1, 5, 10]. To the extent of bacterial presence, total heterotrophs, *Bacillus cereus*, *Clostridium perfringens*, *Escherichia coli*, *Salmonella Spp*, *Shigella Spp*, *Pseudomonas aeruginosa*, *Aeromonas Spp* and *Staphylococcus aureus* has been reported in various spices [8, 11-16]. In consequence, contaminated spices are reported to be significantly attributed to various foodborne illnesses and alimentary intoxications [8, 17].

Traditionally, Pakistani cuisine is a blend of various spices that are cooked for quite an extensive time period. Therefore, it is a point of concern that prolonged heating may eliminate most of the microorganisms however; presence of anaerobic bacteria and their spores cannot be kept unnoticed. Besides, ability of anaerobic bacteria and their spores to survive in high temperatures is a well-established fact. Moreover, it is

significant to mention herein that most of the scientific community has focused on aerobic bacteria and *Clostridium perfringens*. Conversely, present study solely focuses on studying the anaerobic bacteria. Therefore, keeping all facts in view, present study was designed to screen the presence of anaerobic bacteria in the local spices that serve as common and essential ingredient of the Pakistani cuisine. The study was further continued to access the thermo-tolerance potential of the isolated strains.

2. Materials and Methods

2.1. Study Area

Karachi is the largest and most populated city of Pakistan. Karachi is a metropolis city which is the sixth-most populated city in the world. The city inhabits people from various parts of the country with different ethnicities, thus, converting it into a multicultural city. Topographically, Karachi sits on the coastline of Sindh province in southern Pakistan along a natural harbor on the Arabian Sea. Its longitudinal and latitudinal coordinates are 24º51'38.9″N and 67º0'37.4″E respectively. The city has arid climate with low annual average rain (approximately 250mm). Summer season is dominant, persistent with an elevated humidity. People in Karachi consume variety of spices in their cuisine, making it famous for its unique taste.

2.2. Sample Collection

A total of 120 spices samples were collected from fifteen different local retail markets of Karachi city (Figure 1). Each vendor was selected for the collection of eight (n=8) different spices samples (Table 1) on weekly basis for the period of six months (September, 2016 to February, 2017). Approximately 50g of the unpacked sample of each spice was separately collected in sterile sample collection bag. Collected samples were then transported to the laboratory for immediate microbiological processing.

Table 1. *Description of Collected Spices Samples.*

Spices Type	Local Name	Scientific Name	No. of samples collected
Red Pepper	La'al mirch	*Capsicum annuum*	15
Turmeric	Haldi	*Curcuma longa*	15
Cumin	Zeera	*Cumin cyminum*	15
Coriander	Dhanya	*Coriandrum sativum*	15
Whole Spices	Garam masala	NA*	15
Black Pepper	Kali mirch	*Piper nigrum*	15
Curry Powder	Salan masala	*Murraya koenigii*	15
Spice Mix	Chaat masala	NA*	15

*NA= Not available.

2.3. Media & Chemicals

Unless otherwise stated, all the bacteriological media, anaerobic jars, anaerogen sachets and antibiotic discs were purchased from Oxoid, Basingstoke, Hampshire, UK. Chemicals used for the identification of anaerobic bacteria were obtained from Merck, Darmstadt, Germany.

2.4. Microbiological Detection of Anaerobic Bacteria

Representative 10g portion of each of species samples was aseptically weighed and transferred into 100mL of sterile Buffered Peptone Water (BPW) and vortexed for two minutes.

Later, these samples were carefully swirled few times to ensure a homogenous mixture followed by the incubation at 37°C for 24 hours. Presence of anaerobic bacteria was determined by inoculating an aliquot of 10µL BPW pre-enriched samples on the Reinforced Clostridial Agar (RCA) plates in triplicates. These inoculated plates were then incubated at 37°C under anaerobic conditions obtained by using anaerogen sachets in an anaerobic jar. All plates were initially incubated for 24 hours; incubation was then continued for 48 hours for late growers. Observations were initially made at 24 hours and then after 48 hours. Anaerobes were identified on the basis of colonial and cellular morphology and standard biochemical tests. These include catalase and lecithinase production, motility and nitrate reduction, gelatin and starch hydrolysis, hemolysis test and reverse CAMP test [3].

2.5. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was done on Muller-Hinton agar using disk diffusion technique according to Kirby–Bauer method [18]. Antimicrobial agents tested were: Ceftazidime (30µg), Cefoxitin (30µg), Metronidazoles (50µg), Chloramphenicol (30µg), Nalidixic Acid (30µg), Amoycillin/Clavulanic Acid (30µg), Tetracycline (30µg), Ciprofloxacin (30µg), Ampicillin (10µg), Kanamycin (30µg), Gentamicin (10µg), Cefotaxime (30µg), Cefepime (30µg) and Streptomycin (10µg). After 18 h incubation at 37°C, the size of the zone of inhibition was measured and interpreted by comparing with the standard antibiotic sensitivity chart to determine their resistance patterns.

2.6. Thermo - Tolerance Testing

In order to determine the effect of temperature, anaerobic bacterial strains were separately grown overnight in nutrient broth at 37°C. Later, each bacterial strain was placed in the water bath at the temperature of 90°C for 45 min. Then, initially at 0 minutes exposure followed by an interval of 15 minutes an aliquot of 10 µL of bacterial suspension was inoculated at Nutrient Agar plates. These inoculated plates

were then incubated at 37°C under anaerobic conditions as mentioned earlier. Next, the plates were observed for the development of bacterial colonies. All the experiments mentioned herein were run in triplicate.

2.7. Statistical Analysis

Data obtained was analyzed through calculation of mean, proportions, percentages and graphs and tables were prepared for data visualization.

3. Results and Discussion

3.1. Collection and Potability of Spices

To screen the presence of anaerobic bacteria, a total 120 spice samples were collected from different local markets of Karachi (Figure 1). The map for sampling sites was drawn using ArcGIS tools. Out of these 12.5% of the samples yielded no anaerobic bacterial strain and were therefore found satisfactory. Conversely, 87.5% of the samples were found contaminated with the obligate anaerobic spore formers *Clostridial spp* (Figure 2).

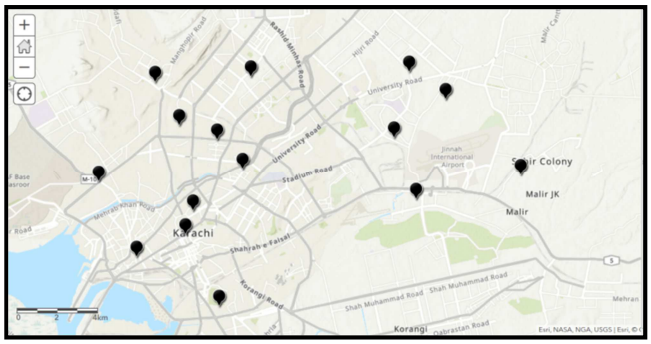

Figure 1. *Location map of Karachi city for spices sample collection.*

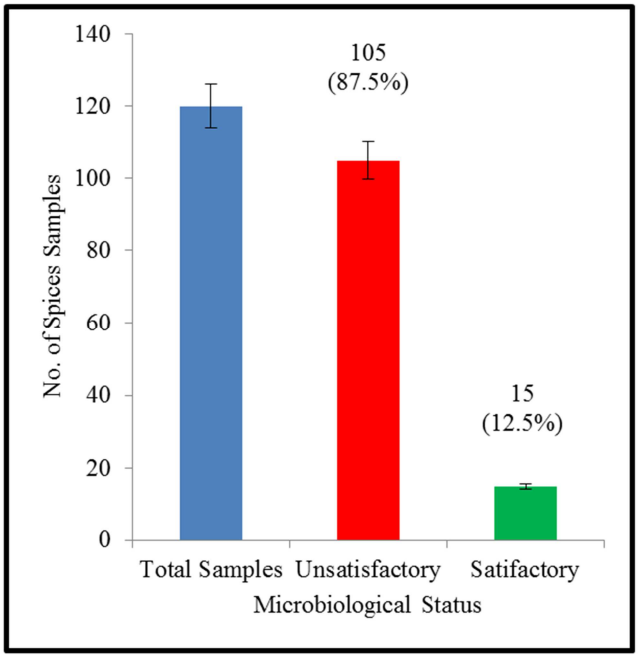

Figure 2. *Microbiological Status of the collected spices samples.*

According to The International Standard Organization (ISO) spices are defined as "vegetable products or mixtures thereof, free from extraneous matter, used for flavoring, seasoning and imparting aroma in foods" [37]. A growing number of Microbiological publications on spices suggest their importance as i) They exhibit antimicrobial activity and potentially aid in preservation; ii) They may contain elevated numbers of aerobic microorganisms i.e. bacteria and fungi that may cause spoilage or sometimes illness when introduced into food; iii) They can support mold growth if improperly dried or allowed to become moist in storage, leading to spoilage and sometimes mycotoxin production [3, 19 – 31]. Contrary to the scientific community, present study was designed with special emphasis on the screening of anaerobic bacteria from the unpackaged spices (in powdered form) sold in the city.

Results of the study revealed that 105 spices samples showed the presence of *Clostridial spp*. These results can possibly be explained by the fact that unpacked spices are commonly sold in bulk in plastic shoppers and are continually exposed to the environmental conditions (air, sun and humidity). Furthermore, they are handled with bare hands and the shoppers usually left unclosed. Since Clostridia are known spore formers therefore the aforementioned conditions create a favorable environment for their survival. Consequently, these spices serve as the principle source for inclusion of large volume of these bacteria into various foods, where they can germinate and multiply to the infective and toxic levels. This may lead to the foodborne illnesses in the consumers.

3.2. Anaerobic Bacteria Isolation and Identification

In this experiment, spices samples were evaluated for the presence of spore formers obligate anaerobic bacteria. Results indicated that the highest load 22 (18%) of anaerobic bacterial strains were found in unpackaged black pepper powder. Further analysis of the results indicated that each red pepper and whole spice samples yielded 20 (16.7%) isolates of spore forming anaerobes respectively. It is significant to mention herein that 18 (15%) anaerobes each were found in turmeric samples. Conversely, coriander and cumin samples yielded 17 (14%) anaerobic bacterial spp. Furthermore, samples of spice mix and curry powder revealed 16 (13.3%) and 15 (12.5%) strains of the obligate anaerobic bacteria (Figure 3). Nonetheless, such high numbers of obligate anaerobic spore former bacteria significantly highlight the extreme health concern for the consumers.

It was interesting to note that all the spice samples included in the present study revealed the presence of *Clostridial spp*. In Pakistan, these spices are most commonly used both as in cooked and raw form. Therefore, presence of anaerobic bacteria in them may have an important role in increasing the bacterial and / or bacterial spore load in the food. Results of the present study are in accordance with other reports [1, 3, 5, 7-9, 31-33].

The genus Clostridium comprise of a number of spore forming, obligate anaerobic, rod shaped bacilli. Among these *Clostridium tetani*, *Clostridium botulinum* and *Clostridium perfringens* are known for the production of medically important toxins of neurological interest [34]. Presence of

Clostridial spp and / or their spores in the commonly used food commodities is a serious health concern.

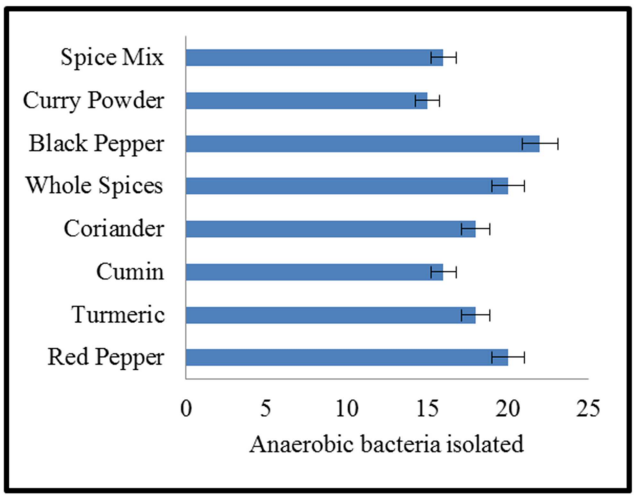

Figure 3. *Pictorial representation of the total anaerobic bacterial strains isolated from spices samples.*

3.3. Antimicrobial Susceptibility Profile

This experiment was conducted to evaluate the antibiotic sensitivity potentials of anaerobic bacterial isolates, results of which are summarized in Figure 4. This analysis indicated that, 91 (62.76%) isolates were found resistant against Ceftazidime whereas 54 (37.25%) showed sensitivity. Cefoxitin was found effective against 90 (62.06%) isolates however, 55 (37.93%) of the strains found resistant against this antibiotic. Metronidazole was found as the most effective antibiotic as it effectively killed all the anaerobic spp. In contrast, Chloramphenicol was found ineffective against 38 (26.2%) strains, whereas 47 (32.41%) strains showed resistance against Nalidixic Acid. Amoycillin/Clavulanic Acid and Tetracycline showed inhibitory activity against 64 (44.13%) and 58 (40%) of spore formers anaerobes respectively.

It is worth mentioning here that Ciprofloxacin and Ampicillin were found effective against 29 (20%) and 65 (44.28%) of strains respectively. Further analysis of the results revealed that Kanamycin and Gentamicin showed activity against 43 (29.66%) and 46 (31.72%) bacterial strains simultaneously. In contrast, the inhibitory effects of Cefotaxime, Cefepime and Streptomycin were found as 71 (48.96%), 68 (46.89%) and 83 (57.24%). Thus, these results highlight that Metronidazole may serve as drug of choice against the infections caused by isolated bacterial pathogen in the present study.

Results of the present study can be explained by the fact that anaerobic bacteria are to some extent sensitive to few antibiotics. However, their spores are supposed to be remarkably resistant against most of the antimicrobials. Our results are further supported by the findings of Lins et al., 2018. Moreover, *Clostridial spp* are able to transform from a vegetative cell into a dormant spore, which significantly increases their resistance against harsh environmental conditions and treatments like UV light, detergents, heat treatment, lack of nutrients and desiccation [33, 35].

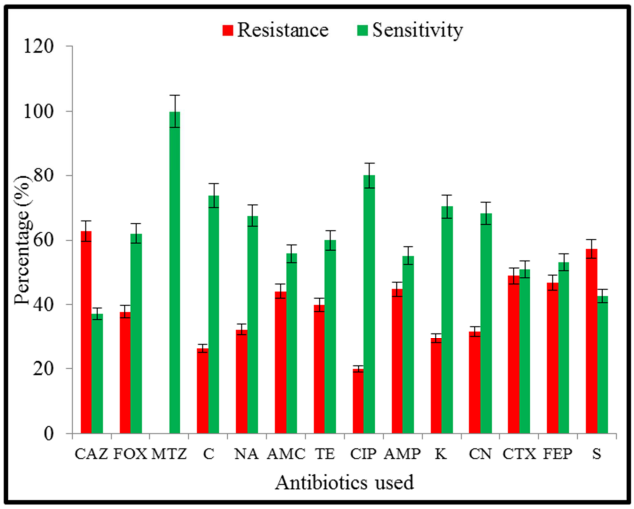

Figure 4. *Antibiotic sensitivity profile of anaerobic bacteria.*

3.4. Thermo – Tolerance Profile

This set of experiment was conducted to evaluate the thermo-tolerance ability of isolated anaerobes at high temperature *i.e.* 90°C. A total of 145 bacterial isolates were subjected to high temperature (90°C) for different time intervals of 0-60 mins. The results of the study revealed that 49% (66) isolates out of 145 found to be resistant to elevated temperature when exposed up to 60 minutes (Figure 5). Survivability of bacterial isolates was found to be significantly dependent on the length of exposure time to high temperature (90°C). 93% (125 out of 145) bacterial isolates withstood high temperature after exposure interval of 10 minutes. 79% (107 out of 145) isolates survived at 90°C after being exposed for 20 minutes. Thermo-tolerance of anaerobic bacteria was subsequently observed as; 65% (88 isolates out of 145), 56% (76 isolates out of 145), 50% (68 isolates out of 145), and 49% (66 isolates out of 145) at 30, 40, 50, and 60 minutes exposure time (Figure 6).

Results of the aforementioned experiments warranted us to screen the effect of heat on the isolated *Clostridial spp*. Analysis of the data revealed that 49% of the isolates survived the high temperature. Results of the present study can possibly explained by the fact that spores and / or intact living strains of *Clostridia spp* are able to persist under harsh condition, and thus, are predestined as a high risk hazards in the food category dried spices and herbs. These results can be further supported by the fact that once ingested the vegetative cells of *Clostridium perfringens* synthesize CPE during sporulation which is subsequently released into the host intestine by the lysis of the mother cell. CPEs are the pore-forming enterotoxin that mainly depends on the dose of intoxication, an apoptotic cell and oncosis cell death pathway is triggered that leads to the membrane permeability alterations and DNA cleavage [33, 36].

$$\text{Log Reduction} = \text{Log } (N_0 / N)$$

N_0 = Isolates before treatment
N= Isolates after Treatment

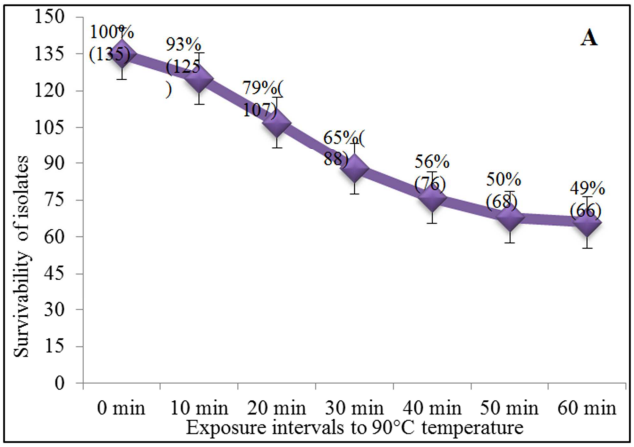

Figure 5. *Thermo – tolerance profile of anaerobes; Survivability.*

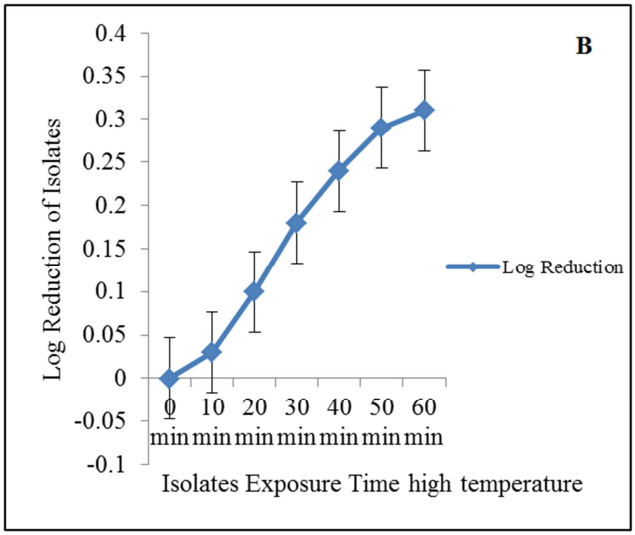

Figure 6. *Thermo – tolerance profile of anaerobes; Log Reduction.*

Conversely, *Clostridium botulinum* and *Clostridium tetani* produce neurotoxins commonly called tetanus and botulinum toxins. They are produced as a single polypeptide of Kb which undergoes post-translational cleavage to form a hevaey (H) chain and a light (L) chain of 100 kDa and 50 KDa respectively linked by a single disulfide bond. The H chain binds to the gangliosides on the plasma membrane of peripheral nerve terminals before internalization via receptor mediated endocytosis. Protonation of the endosome results in the reduction of disulfide bond. The H chain forms a transmembrane pore across the endosome and the L chain then enters the nerve terminal cytosol. The L chains of both the toxins are zinc acitavted proteases. Their targets are a number of specific proteins involved in synaptic vesicle docking – synaptobrevin (also known as VAMP), SNAP – 25 and Syntaxin. Consequently subsequent to the hydrolysis of the selectively target proteins, tetanus toxin results in blockade of neurotransmitter release that leads to the development of flaccid paralysis. On contrary botulinum toxins remains in the nerve terminal and affect both somatic and autonomic nervous systems [34].

4. Conclusion

Present study profiled thermo-tolerant and antibiotic resistant *Clostridia spp* from the powdered form of commonly used unpackaged spices. Consumption of these spices is of utmost concern from the standpoint of health of the consumers. Since *Clostridium tetani*, *Clostridium botulinum* and *Clostridium perfringens* are the principle pathogens of deleterious infections. It is important to mention herein that present study significantly negates the common assumption that cooking of Pakistani cuisine at high temperature kills all bacterial spores. Moreover, black pepper powder and spice mix are generally sprinkled on the cooked food thus consumed as raw. This further increases the risk of development of foodborne infections by these bacteria.

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