ANTIBIOTIC PROFILE OF YEAST-LIKE FUNGI RECOVERED FROM EXPIRED BEVERAGES SOLD IN THE MARKET

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***Abstract*: The change in stored drinking water quality in containers calls for concern due to possible build up of biofilm by bacteria which find their way into water. The study aimed at determining the biofilm and virulent properties of associated bacteria. Thus, thirty water samples from some homes were collected, stored and analyzed using spread and streaking techniques to isolate characteristic bacterial colonies on standard media. A total of 29 bacterial isolates belonging to five genera were identified molecularly. The bacteria isolates namely: *Bacillus flexus, Chryseobacterium aquifrigidense, Providencia stuartii, Alcaligene faecalis* and *Providencia rustigianii* were obtained. The bacterial isolates with their percentage virulent potential are as follow; *Bacillus flexus* 100%, *Chryseobacterium aquifrigidense*16.6% *Providencia stuartii* 33%, *Alcaligene faecalis* 66% and *Providencia rustigianii* 33.3%. All bacterial isolates developed/expressed biofim formation except *Chryseobacterium aquifrigidense*. Biofilm formation in the interior surface wall of drinking water storage container was also dependent on the nature and type of storage vessel or container used in the home. The virulence factors as exhibited by these potential pathogens have vital role in invading underlying or immune compromised individuals. Thus, the effect in the change of stored drinking water in containers can be mitigated when individuals maintain good personal hygiene and boost their immune system in order to put off the potential of opportunistic pathogens recovered in this study.**

***Key Words:* Biofilm Formation, Bacterial Virulent Properties, Stored Drinking Water, Home.**

I. INTRODUCTON

The act of storing water has existed for a very long time ago and sometimes it is very demanding. It involves sourcing and keeping the water in a special place until it is needed (Duru *et al.,* 2013). It also entails preserving the water from contamination while in the home before consumption. The preservation of water from being contaminated is one way water-borne diseases can be eliminated or reduced in the homes (Clasen *et al.*, 2006). Several reasons have been advanced for storing water used for different purposes including for drinking purpose in the homes (GRSN, 2016). One major reason is the non-availability of water or water facility in the home. In the home, water can be stored in bottles, buckets, cans, basins and tanks.

About a decade ago, water microbiologists began to develop interest in the significance of biofilm incidence on water stored inside some containers (LeChevallier, 2007). Also, the growth of coliform bacteria in water has agitated and encouraged the concern for identification of biofilms (General Laboratory Technology, 2007). Ordinarily, a film is a thin layer of something usually on the surface of something else. Such thin layer may be of dust or oily substance or soot or living substance. Visually, biofilm is a thin layer of living substance on the surface of thing material including water and such living substance is produced by microorganisms specifically bacteria. Biofilm is made up of proteins, lipids, DNA, and extracellular polymeric substances (Freeman *et al*., 1989). Thus, they are collections of organic substances held together and forming a thin layer on some surfaces including water (Sadekuzzaman *et al.,* 2015). Biofilm on the surface of water has implication for the quality of that water. For instance it may cause objectionable taste and odour of the water and can in effect aid corrosion within some water distribution systems including pipes (Kovocs and Knipers, 2011). More often than not, corrosion is common in lead based water distribution system and pipes as well as in materials. Biofilm formation in water that is stored for drinking was reported by LeChevallier (2007). A biofilm formation is due to the long period of time the bacteria survives in the water. Basically, high heterotrophic plate counts may be indicative of biofilm formation in a water distribution system or pipes as reported by General Laboratory Technology (2007). One notable nature of biofilm as described by LeChevallier (2007) is that the population of bacteria increases when it is present, but when it is absent the population of bacteria is minimal. Thus, biofilm accelerates the growth bacteria soon after it develops. Biofilm-borne bacteria are protected from desiccation, antibiotics and a host's body's immune system in the environment it finds itself. In the course of bacteria forming a biofilm, (i) the bacteria first gets attached to a surface, (ii) then forms more colonies and (iii) begins to grow, mature and cover more areas of the surface of the host (LeChevallier, 2007).

The virulent features in bacteria enable it to survive in hostile environment including the human body. Disease such as diarrhea acquires stability in the human body due to

enterotoxin, hemolysins and verocytoxin virulence features produced by *Escherichia coli*, the causative agent of diarrhoea disease. According to Chakraborty and Nishith (2008), the virulent factor produced by *Staphylococcus aureus* is responsible for Staphylococcal food poisoning. In addition, Exfoliative toxin, also a virulent factor produced by *Staphylococcus aureus* is responsible for scalded skin syndrome. Infections such as sore throat, scalet fever, skin lesions, impetigo and bone and joint infections are caused by *Streptococcus pyogenes*, due to the successful expression of some virulent factors such as hyaluronidase, deoxyribonuclease, streptokinase, hemolysins and erythrogenic toxins which the bacteria posses. The consumption of bacterial contaminated water causes varying illnesses amongst people who drink such water, and about 3.4 million deaths annually are reported to be related to it (Okeke, 2018). Outbreaks of several waterborne diseases such as cholera, typhoid, diarrhea and dysentery have been associated with the drinking of water that has been exposed to microorganisms, biotoxins and toxic contaminants (Okeke, 2018). Malama (2015) attribute the rise in waterborne disease in Lusaka to low water table profile which are close to pit latrines. Report has it that cryptosporidium was the leading etiological agent of waterborne disease outbreaks in United States with more than 400 people in Havelock North who came down with gastric illness (Hawthorner, 2018). In Nigeria, 2,300 suspected cases of waterborne diseases were reported in the Mid-August of 2017, killing 44 people in the troubled North-East (Silva, 2017). The study was aimed at identifying and quantifying biofilm bacteria and their virulent features that are able to initiate disease emergence as a result of drinking stored water and thus, recommend measures to ward-off biofilm and virulent bacteria.

II. MATERIALS AND METHODS

Description of Study Area

The study area chosen for this study is a suburb of Port Harcourt City in Port Harcourt Local Government Area of Rivers State, Nigeria. The Diobu community is a commercial district, with a big market, and several other educational and religious institutions. These special features of Diobu community is responsible for the influx of people and goods, resulting to high demand for housing and other social amenities such as water which are not readily available.

Sample Collection

Thirty stored drinking water samples were collected in a 50ml sterile plastic container from some homes in the study area. The water samples collected were observed stored in plastic jerry cans and plastic buckets in the homes. The stored drinking water were mostly kept at the kitchen section of the house. Samples collected were further stored for additional 10 days in the laboratory refrigerator to affirm storage before analysis.

Media Preparation and Microbial Analysis

The spread plate technique as carried out by Wemedo *et al.* (2016) was adopted for the determination of bacteria isolates on Nutrient, MacConkey, TCBS and Salmonella-Shigella media. Water samples were then inoculated on the media with the aid of a sterile (1ml) pipette, and a glass spreader used to spread the inocula over the surface of the media. The media were incubated at a temperature of 37^0C for 24 hours. Bacterial isolates were then recovered and maintained in 10% glycerol.

Molecular Identification of Isolates

The isolates were subjected to molecular polymerase chain reaction techniques as described by Collins and Lyme (1984). In carrying this identification, the isolates were subjected to (i) DNA extraction, as adopted by (ii) DNA quantification (iii) DNA amplification (iv) DNA sequencing

Bacterial Biofilm Production Capacity

The test procedure involved: (i) inoculating the known test bacteria onto a freshly prepared Congo red media, followed by incubation at temperature of 37^oC for 22 - 24 hours. Black colonies with a dry crystalline consistency developed; thus indicated biofilm production while an absence indicated no biofilm formed (Sadekuzzaman *et al.,* 2015).

Interior Surface Biofilm Formation Ability of Storage Containers

In furtherance to establish that storage containers or vessels in the home can encourage the formation of biofilm and thus act as agents of bacterial cross-contamination, the swabbing technique was adopted and applied on the interior wall surface area (2cm x 10cm) of the container and then onto Congo red medium aimed at enumerating and quantifying the bacterial biofilm presence per cm^2. The technique involved swabbing a known internal area of the storage vessel using a sterile swab to get a stock concentration and thereafter the stock undergoes a serial 10 fold dilution. The dilution was then inoculated into a freshly prepared Congo red medium and incubated at a temperature of 37^0C for 18- 24 hours. A biofilm formation was indicated by black colonies while non black colonies showed no presence of biofilm (Freeman *et al.,* 1989). Similarly, swab culture results were expressed in colony forming units using colony counter and result were expressed in CFU/cm^2

Virulent Property Assay

This assay was done to ascertain the ability or capacity of the bacteria to cause disease (Charkraborty and Nishith, 2008). In this work, morphological characters, proteins and enzyme factors were used to determine the virulence of isolated bacteria. In otherwords, the following activities were sorted-out: DNase hydrolytic activity, lipolytic activities, Hemolytic activity, lecithin activity, flagella presence and Catalytic activities, all of which were sorted to implicated the isolates as potential pathogens (Charkraborty and Nishith, 2008).

DNaseAssay

The DNase test was done to determine the ability of the bacterial isolates to hydrolyze deoxiribonuclase acid and utilize it as a source of carbon and energy for growth. In sorting this feature, the bacterial isolates were streaked on a freshly prepared (DNase) medium and incubated at 37°C for 24 hours. After incubation growth colonies were observed, and the plates were then `flooded with diluted hydrochloric acid (HCL). DNase positive cultures showed a distinct clear zone around the streak while negative cultures showed no clear zone. The test procedure was strictly carried out as described by Buchanan (2006).

Lipase Test

The investigation was done to know if the bacterial isolates can breakdown the lipids of its host. The bacterial isolates were streaked onto a freshly prepared Lipid agar medium and incubated for 24 hours at 37°C. After incubation and growth was observed, a blue spirit indicator chemical was used to flood the medium, and then observed within seconds. Light blue opaque medium, signified a positive result, while a negative result showed no lightblue opaque medium.

LecithinaseAssay

The enzyme assay was carried out as prescribed by Bharadway and Prakasam (2016). The isolates were streaked onto a freshly prepared Egg Yolk Agar medium and then incubated at 37°C. After incubation for 24 hours, positive result showed precipitation (Lecithin breakdown) around the streak of bacteria while negative result showed no precipitation.

Haemolytic Activity

Hemolytic activity was determined by streaking the isolates onto freshly prepared blood agar medium as described by Sagar (2015), and incubated at 37°C for 24 - 48 hours. Positive cultures showed a distinct clear zone around the streak showing the ability of the bacteria to produce toxin while negative result revealed no clear zone around the streak.

Catalase Activity

The procedure employed to express this activity in bacteria as adopted by Sagar (2015) involved transferring a small fraction of the bacterial isolate onto a clean microscope slide and adding a loopful of hydrogen peroxide. After a few seconds, where gas bubble ensued it indicated a positive result while the absence of gas bubble indicated negative result.

Motility Test

The procedure for motility test as done by Charkraborty and Nishith (2008) required the preparation of emulsified nutrient medium, followed by pouring into a test tube and then with the aid of a sterile needle inoculate the isolate in the medium by piercing. The tube was then incubated for 24 hours at 37°C. A diffused growth spread within the tube and not along the stab line indicated motility/virulence of the bacterial isolate.

III. RESULTS

Molecular Characterization

A total of twenty nine (29) isolates was obtained and five (5) genera on molecular investigation of their 16S rRNA revealed a similar match and evolutionary distance during the mega BLAST search for highly similar sequence from the NCBI data base showing the genera in figure 1 as: *Bacillus flexus, Chryseobacterium aquifrigidense* strain C3, *Alcaligene faecalis* strain OSBR60, *Providencia stuartii* strain MPIUTIRB3 and *Providencia rustigianii* JCM3954 with accession numbers MN611429, MN294685, MN036406, MN611442 and LC420104 respectively.

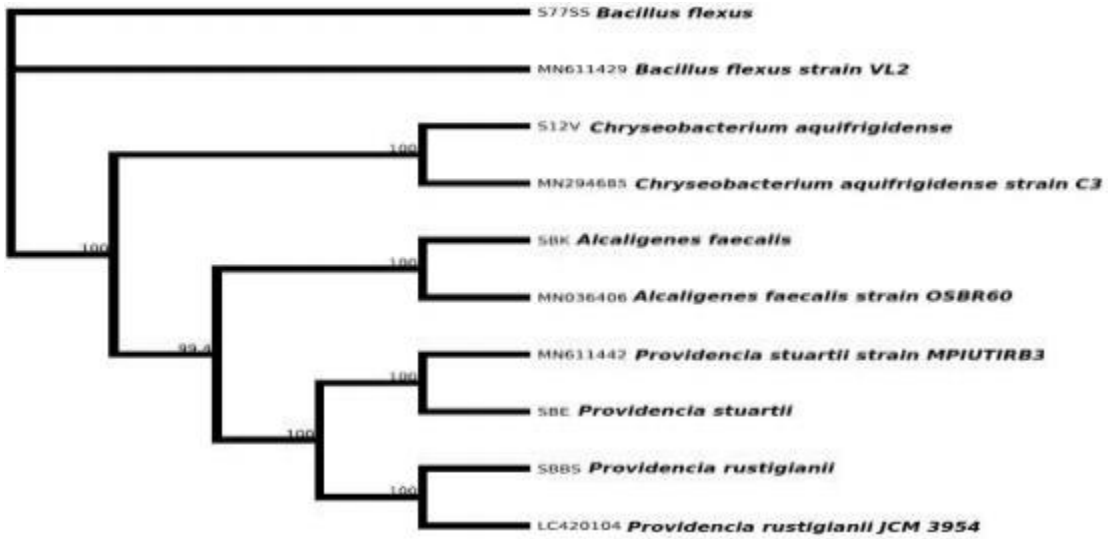

Figure: 1: Phylogenetic Tree (Characterization)

Biofilm Formation by Bacteria Isolatedfrom the Water Samples

The result in Table 1, shows that bacterial biofilm forming capacities of the bacterial isolates from the water samples. Consequently, amongst the bacteria investigated, *Chryseobacterium aquifrigidense* did not form biofilm.

The percentage of bacteria isolates with biofilm forming capacity in the drinking water samples were as follows: *Bacillus flexus* 34.5%, *Chryseobacterium aquifrigidense* 0%, *Providencia stuartii* 20.7%, *Alcaligene faecalis* 31% and *Providencia rustigianii* 10.3%.

Table 1 Biofilm Formation by the Bacteria Isolates

	Bacteria	Number of Bacteria	Biofilm Positive Bacteria	Biofilm Negative Bacteria	%Biofilm Forming Capacity
1	*Bacillusflexus*	10	10	0	34.5
2	*Chryseobacterium aquifrigidense*	1	0	1	0
3	*Providencia stuartii*	6	6	0	20.7
4	*Alcaligenefaecalis*	9	9	0	31
5	*Providencia rustigianii*	3	3	0	10.3

Detection and Density of Biofilm Bacteria on the Interior Wall Surface of Water Containers in the Homes

Table 2 shows the swab results of investigation into biofilm presence on the interior wall surface of containers used for drinking

The results showed that most cells or colonies were actively growing on the interior surface wall of the containers and the biofilm formation culminated into a biofilm presence of 53.3% in the containers. However, biofilm were not observed in 46.7% of the interior wall-surface of the containers.

Thus, the bacterial biofilm forming colonies on the interior wall surface of the sample containers recorded a swab per area range of 0 to 3.5 x 10^2 CFU/cm^2 for non and actively growing cells respectively.

Table 2 Detection and Density of Biofilm Bacteria on the Interior Wall Surface of Water Containers in the Home

S/No. Storage Container	Area of Swab 2x10 (cm^2)	Biofilm Presence/ Absence	Counts Per Swab (CFU)	Swab Per Area(CFU/cm^2)
SC1	20	ND	0	0
SC2	20	D	4 x10^3	2 x10^2
SC3	20	ND	0	0
SC4	20	ND	0	0
SC5	20	ND	0	0
SC6	20	D	5 x10^3	2.5 x10^2
SC7	20	ND	0	0
SC8	20	D	1 x10^3	5 x 10
SC9	20	ND	0	0
SC10	20	D	1 x10^3	5 x 10
SC11	20	ND	0	0
SC12	20	D	2 x10^3	1 x 10^2
SC13	20	ND	0	0
SC14	20	D	1 x10^3	5 x 10
SC15	20	ND	0	0
SC16	20	D	2 x10^3	1 x 10^2
SC17	20	D	4 x10^3	2 x10^2
SC18	20	ND	0	0
SC19	20	D	1 x10^3	5 x 10
SC20	20	D	3 x 10^3	1.5 x 10^2
SC21	20	D	7 x 10^3	3.5 x 10^2
SC22	20	D	6 x 10^3	3 x 10^2
SC23	20	D	4 x10^3	2 x10^2
SC24	20	D	1 x10^3	5 x 10
SC25	20	ND	0	0
SC26	20	D	5 x10^3	2.5 x10^2
SC27	20	ND	0	0
SC28	20	ND	0	0
SC29	20	ND	0	0
SC30	20	D	5 x10^3	2.5 x10^2
% Biofilm Present	-	53.3	-	-
% Biofilm Absent	-	46.7	-	-

Key:

D= Biofilm detected, ND =Biofilm not detected, % = percentage, SC = Storage container, cm^2 = centimeter cube, CFU= Coliform Forming Unit

Virulent Features of Bacteria Isolated

Table 3 shows the result of the analyses of virulent potentials of bacteria isolates from the water samples. All the isolates of *Bacillus flexus* in water samples expressed the potential to be virulent to their host's body. The virulent features expressed by the isolates were lipase activity, lecithinase features, motility features, catalase production and DNase production. Observation showed all of the *Chryseobacterium aquifrigidense* isolates were not motile, could not express lecithinase and DNase activity and in addition were unable to digest red blood cell but were successful in catalase activity. *Providencia stuartii* isolates did not express lipase production, lecithinase activity, catalase production and DNase but were able to express motility and hemolytic activities. Motility, hemolytic, catalase production and DNase activity were elaborated by all *Alcaligenes faecalis*. However, *Alcaligenes faecalis* failed to elaborate lipase, lecithinase and ureolytic

activities. *Providencia rustigianii* assay for virulence analysis expressed heamolytic activity and motility while lipase, lecithinase, catalase and DNase productions were not expressed. The virulence potential percentage of *Alcaligenes faecalis* and *Bacillus flexus* isolates were 66.6% and 100% respectively. *Providencia stuartii and Providencia rustigianii* isolates had 33.3% each, and *Chryseobacterium aquifrigidense* isolates with 16%.

Table 3 Virulent Properties of the Bacteria Isolates

S/n	Bacteria	No. of Isolates	Lipolytic Activity n(%)	Lecith n(%)	Hemol n (%)	Motility n (%)	Catalytic Activity n (%)	DNAse n (%)	Bacterial Virulent Potential(%)
1	*B.flexus*	10	10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)	100
2	*C. aquifr*	1	1 (0%)	1 (0%)	1 (0%)	1 (0%)	1 (100%)	1 (0%)	16.6
3	*P. stuartii*	6	6 (0%)	6 (0%)	6 (100%)	6 (100%)	6 (0%)	6 (0%)	33.3
4	*A. Faecalis*	9	9 (0%)	9 (0%)	9 (100%)	9 (100%)	9 (100%)	9 (100%)	66.6
5	*P. rustigianii*	3	3 (0%)	3 (0%)	3 (100%)	3 (100%)	3 (0%)	3 (0%)	33.3

Key:

n (%)= Number of occurrence / Percentage Positive, *B. flexus= Bacillus flexus, C. aquifr = Chryseobacterium aquifrigidense, P. stuartii = Providencia stuartii, A.faecalis= Alcaligenefaecalis,P. rustigianii= Providencia rustigianii,* Lecith = Lecithinase, Hemol = Hemolysin

IV. DISCUSSION

On the formation of biofilm, all isolates of *Bacillus flexus, Alcaligenes faecalis, Providencia stuartii* and *Providencia rustigianii* were able to form biofilm due to their possession of adherent structures such as flagellate that aid motility to receptor sites (substratum). Thus, their ability to adhere to a substratum enabled them to grow on surfaces through interaction.

The capacity of *Bacillus flexus*, *Alcaligenes faecalis, Providencia stuartii* and *Providencia rustigianii* to produce biofilm quite agreed with report by Sravan (2014), that isolates in water truly lived in a biofilm community where they protected them self against harsh environmental conditions or harmful chemicals that would have destroyed them. *Chryseobacterium aquifrigidense* inability to form biofilm maybe due to the absence of flagella:::::: Flagella has been reported to function as adhesins during bacterial invasion of a host cell; an action which signifies its virulence. However, the presence of flagella in *Chryseobacterium aquifrigidense* would have enabled motility to receptor sites on a substratum (Suzina *et al.,* 2011) thereby forming biofilm. This result thus agreed with research carried out by Seong *et al.* (2008), where they classified *Chryseobacterium aquifrigidense* as a non-colonizer. The strategy, for which these bacteria form biofilm enables them increase and hence, deteriorate the water quality through generation of taste and change in colour of the drinking water.

The result in which 53.3% of the storage containers had biofilm formed on their interior surface walls strongly suggests that the storage vessels provided the attachment or receptor sites for bacterial biofilm formation and growth (Taylor and Collins, 1999). However, the results in which 46.7% of the storage vessel had no biofilm formed on their interior surface walls suggests the lack of attachment or receptor sites (Taylor and Collins, 1999) to harbor the bacterial isolates and allow it form biofilm. This, thus decrease the persistence of the bacteria in biofilm (Taylor and Collins, 1999). Similarly, in a study carried out by Duru *etal.* (2013), on the effect of different storage vessel on water quality, they reported that some vessels encouraged the growth of microbes than others. Duru *et al.* (2013) also reported on the bioload of biofilm in vessels which revealed that calabash vessels had a higher biofilm load than a clay pot vessel, followed by metal vessel, plastic and glass vessel.

The swabbing process of the interior surface wall of water container or vessels as engaged on in this study was in agreement with the European Union Act of 1990, legislation on environmental monitoring of contact materials or surfaces or premises. It was done in order to examine, determine and ensure the cleanliness of vessels/containers interior wall surfaces which are found to be vehicles of contamination of water and food substance (PHE, 2017). Based on bacterial biofilm efficiency it is established by Morin *et al*. (1997) that the maximum bacterial biofilm densities could range from 10^5 to 10^8 cells/cm^2. However the report of the investigation on interior wall surface which accounted between the range of 0 to 3.5 x 10^2 CFU/cm^2 for non and actively growing cells respectively thus indicating a moderate microbial adherence to interior wall surfaces of the containers (Morin *et al.*,1997). Conclusively, the success of *Bacillus flexus*, *Alcaligenes faecalis Providencia stuartii* and *Providencia rustigianii* to produce biofilm is based on their associated diseases on the gastro intestinal tract, were the gastro intestinal tract provides a huge surface area for biofilm construction (Taylor and Collins, 1999). Thus, the epithelium of the gastro intestinal tract to which bacteria attach is altered and often damaged (Taylor and Collins, 1999). A significance effect for which,

biofilm construction in the body evades immune system and presents a hostile community for antibiotics to function.

Hemolytic activities expressed by *Bacillus flexus, Alcaligenes faecalis, Providencia stuartii* and *Providencia rustigianii,* are suggestive of the capacity to produce hydrogen peroxide by these cells which in turn enabled the cells to digest red blood cells (Nagababu *et al*., 2003). However, viable cells of *Chryseobacterium aquifrigidense* have no capacity of producing hydrogen peroxide, so cannot heamolize red blood cell (Nagababu *et al*., 2003). The inability of *Chryseobacterium aquifrigidense* to produce deoxyribonucleases is suggestive of the fact that the cell nuclei are unreactive. DNase is an extracellular enzyme that is secreted outside a bacterial cell membrane. The enzyme when expressed is indicative of a virulent property of bacteria, which enables it to cause disease. The presence of deoxyribonucleases allows the cell escape from neutrophil extracellular traps (Haas 2014). The catalytic property of *Chryseobacterium aquifrigidense, Bacillus flexus* and *Alcaligenes faecalis* is suggestive of possession of catalase enzymes, which the bacteria can use to exert its virulent ability when phagocytes produce hydrogen peroxide to poison the invading bacteria. The expression of lipolytic and lecithinase characters by *Bacillus flexus* only is based on the fact that the bacteria can break down Lecithin and lipase which the bacterium possesses is able to derive carbon as its source of energy for growth and survival (Bharadway and Prakasam, 2016) enabling it to act on the host lipids and membranes (Bharadway and Prakasam, 2016). The increased potential of *Bacillus flexus* to cause disease justifies it as a pathogen in this study, despite the study carried out by Bharadway and Prakasam, (2016) where they reported that *Bacillus flexus* have not received adequate clinical attention, though only implicated with opportunistic infection in immune compromised persons. *Chryseobacterium aquifrigidense* which had the least virulent capacity due to its expression of catalytic properties, quite agreed with studies by Seong *et al*. (2008) where they considered it pathogenic but not significant enough to be virulent.

V. CONCLUSION AND RECOMMENDATIONS

The study concludes that *Providencia rustigianii, Alcaligenes faecalis, Providencia stuartii* and *Bacillus flexus* isolated from the stored drinking water samples were able to form biofilm except *Chryseobacterium aquifrigidense* isolates, whose inability reduces its persistence or prevalence in the water stored for drinking in the homes. Although, biofilm formation in the interior surface-wall of drinking water storage container or vessel is dependent on the nature and type of storage vessel or container used in the home.

All bacteria isolated during this study were able to express at least a virulent property. The study recommends, that inhabitants of homes should always maintain good storage drinking water hygiene practices such as washing, cleaning or steam flushing their drinking water storage containers or vessels before initial use and refilling in order to check bacterial occurrence and eliminate biofilm formation incidences respectively. The study further recommends that inhabitants should withdraw from acts and habits capable of lowering their body immune system in order to discourage the potentialities of opportunistic pathogens that were isolated in this study.

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