Drinking water must be free from pathogenic bacteria as this could be a transmission vehicle for plethora of opportunistic and pathogenic strains. The main purpose of the present research was to evaluate the survival and risk of the biofilm associated bacteria (BAB) in the drinking water microcosms and distribution systems. Therefore the first objective set was to analyze the types of drinking water which is of high risk to the consumer in terms of BAB. For the selection of the drinking water under severe threat of biofilms, a preliminary work has been done in five different types of drinking water (Chapter 2). They included well water, municipal tap water, harvested rain water, a popular brand of packaged drinking water (PDW) and a local brand of PDW. The occurrence of extended survival of BAB, with multiple antibiotic resistance (MAR) and beta haemolytic properties were detected from all the microcosms. However, the PDW microcosms were observed to be the high risk category among the different drinking water based on the survival and virulence of BAB followed by the municipal tap water.

The work then focused on the survey of bacteriological quality of PDW (Chapter 3) since this is a commodity which is consumed without prior treatment. Moreover, a complete bacteriological survey incorporating the BAB attached to the inner surface of the PDW bottles is rare. A total of 219 packaged drinking water samples belonging to 43 different brands commercialized in the markets were randomly collected from the retail outlets of various districts of Kerala, South India. The major findings of this chapter are:-
The percentage occurrence of heterotrophic plate count (HPC) of bacteria within the bulk water and biofilms in the PDW samples commercialized in the markets of Kerala were 90.41% and 87.67% respectively.

- Percentage occurrence of TC, FC and FS were 83.11%, 14.61% and 31.05% respectively.
- HPC in packaged drinking water ranged from 0-300000 cfu/mL while HPC of biofilms in cfu/cm² ranged from 0- 2250000.
- The TC, FC and FS ranged from 0-1100, 0-350 and 0-59 per 100mL respectively.
- Regardless of the limits set by Bureau of Indian Standards (BIS), 78.60% and 95.52% of the samples exceeded the HPC limits in the bulk water and biofilms.
- Outrageously 90.55% of the samples exceeded the coliform limits set by BIS.
- Remarkably only 6.97% of the samples exceeded the limit in terms of faecal coliforms.
- The study clearly indicates that the bacteriological quality of PDW sold in Kerala is unsafe for human consumption
- The study confirmed the importance of the assessment of BAB in routine sampling.

The results indicated the necessity of stringent quality standards by Government authorities which should be enforced immediately to receive safe supply of drinking water in the markets of India, as it is a human right. Moreover reducing the expiration period is an utmost need, since it increases the chances of pathogen survival in the biofilms.
Consumers of Packaged Drinking Water (PDW) ingest countless “heterotrophic bacteria” without being aware of the health risk associated. And so, the work then concentrated on the assessment of virulent features of 201 BAB isolated from the PDW commercialized in the markets of Kerala (Chapter 4). The main objective of this chapter was to check whether there is a risk in consuming innumerable HPC associated with the biofilms of the PDW. In order to achieve the objective, organisms were tested for resistance to 16 antibiotics, 8 extracellular enzymes production, and beta haemolysis.

The main findings of the chapter are:

- Out of the isolated biofilm associated heterotrophic bacteria, 70.65% were resistant to at least one antibiotic tested and only 29.35% of the BAB was susceptible to all the 16 antibiotics tested.
- Nevertheless, 51.24% and 26.87% of biofilm associated heterotrophic bacteria were resistant to two and three antibiotics respectively.
- The study witnessed MAR index with 15.92%, 11.94%, 10.95%, 9.95%, 8.96%, 5.975, 3.98% and 2.98% of biofilm associated heterotrophic bacteria being resistant up to four, five, six, seven, eight, nine, ten and eleven antibiotics respectively.
- The study revealed 48 patterns of antibiotic resistance profile prevalent among the isolated biofilm associated bacterial strains.
- The biofilm associated heterotrophic bacteria displayed significantly high resistance to ampicillin, penicillin and nalidixic acid.
- The least resistance was towards ciprofloxacin and amikacin.
- Only lower resistance was exhibited towards gentamicin (6.97%), tetracycline (5.97%), chloramphenicol (5.97%), amikacin (1.00%), ciprofloxacin (0%).
- The study categorized 41.29% of the strains to be beta haemolytic.
The percentage of enzyme production observed is in the order: coagulase (43.78%) > DNase (32.84%) ≥ proteinase (32.84%) > lecithinase (30.35%) > lipase (15.92%) > gelatinase (12.94%) > pyocyanin (2.99%) ≥ flourescein (2.99%).

However, 32.34% does not produce any enzymes while the rest 67.66% of the strains produced at least one extracellular enzyme tested.

Capability to produce multiple enzymes was exhibited by 48.76% of the strains.

However, 51.24% of the isolates were not capable of producing multiple (2 or more) enzymes.

Meanwhile, 29.35% and 15.42% of the strains produced more than 3 enzymes and 4 enzymes respectively. However, only 6.47% and 1% of the BAB produced more than 5 and 6 enzymes respectively.

Only 1.99% of the isolated biofilm associated strains were capable of producing all the eight enzymes tested.

Surprisingly, 80.72% of beta haemolytic strains were multiple extracellular enzyme producers.

Totally, 8.46% of the BAB isolated from 219 packaged drinking water samples belonging to 43 brands were categorized to high risk category with all the three features including MAR, multiple enzyme profile (MEP), and ability to lyse the erythrocytes.

The chapter specifically confirmed that the so called natural aqueous bacteria or HPC bacteria in the biofilms of PDW poses high virulence features. The present work points towards the fact that HPC associated with biofilms in the PDW bottles are of high risk to the consumers in terms of the high production of extracellular enzymes and MAR Index.
Hence biofilm formation is a feature, which is considered notorious in terms of virulence associated with bacteria, the study endeavored to quantify and classify these strains into various groups based on their ability to produce biofilms (Chapter 5). Though the HPC analyzed for virulence were already isolated from PDW, Chapter 5 analyzed the independent biofilm formation potential of 25 selected BAB with virulent features. Moreover, the selected biofilm associated bacterial strains with either 2 combinations of virulence features described in the previous chapters along with the biofilm formation potential via MTP assay were identified to the molecular level.

- Only 4% of isolates from the 36 hour batch and 8% of isolates from 78 hour batch were found to be weak biofilm producers.
- Interestingly, at 150 hours none of the strains were weak, as they were either moderate or strong.
- In short, 96%, 92% and 100% respectively from 36, 78 and 150 hours were found to be biofilm producers, either strong or moderate.
- However, 20%, 32% and 36% were found to be moderate biofilm producers at 36, 78 and 150 hours respectively, which appear to be in the increasing order. Finally 76%, 60% and 64% of the BAB were found to be strong biofilm producers at 36, 78 and 150 hours respectively.
- Overall, there was significant difference (p<0.05) in the biofilm formation potential of BAB at different time periods, with 150 hours being significantly superior (p<0.05) in the biofilm production.
- The results show that as time increases all the members of the group turned out to be either strong or moderate biofilm producers. Interestingly none of the selected BAB was non-biofilm producer, and only 3 organisms were detected as weak producers altogether.
CRA plate shows 19 strains (76%) of the BAB selected to be biofilm/slime producers.

MTP assay was found to be more accurate than CRA assay. However, Specificity and positive predictive values (PPV) were 100% with the 36 hour of biofilm growth.

As per the sequences, the organisms with virulence features identified are: *Salmonella enterica* sub spp. Enterica, *E. coli* strain K12, *Acinetobacter baumannii*, *Bacillus safensis* NBRC 100820, *Bacillus flexus* strain NBRC 1S715, *Bacillus safensis* strain F0, *Bacillus subtilis* sub sp. spizizenii strain ATCC 663, *Bacillus tequilensis* strain 10b, *Pseudomonas aeruginosa* PAO1, *Bacillus eiseniae* strain A1-2, *Bacillus subtilis* JCM 1465, *Bacillus aerius* strain 24K, *Bacillus anthracis* str Ames. Multiple organisms were detected from the same species. However, *Bacillus* was the dominating genera with virulence features in the PDW samples.

The current chapter indubitably confirmed the biofilm formation potential in bacteria associated with virulence in the inner surfaces of PDW microcosms, which was totally neglected. The detection of pathogens like *Salmonella enterica*, *Bacillus anthracis*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* urges the necessity of stringent laws and careful manufacturing procedures for packaged water, which is relied by the natives and tourists alike. The present work also suggest the Bureau of Indian Standards (BIS) and International Bottled Water Association (IBWA), to include the biofilm assays to the routine water analysis, since a pathogen protected in the inner films of the bottle is of high risk to the consumers.

The final part of the study was concentrated on the survival of *E. coli* and *S. enterica* in the MDWDS’s (Chapter 6). Water quality deterioration due
to the microbial biofilm formation in pipes is a major issue which is unable to be monitored properly through routine assessment. DWDS’s are recognized to be a successful haven for many pathogenic bacterial contaminants those exists viable and being protected from the harsh conditions including shear stress, temperature and chlorine. For this purpose an indicator and pathogenic bacteria were inoculated separately in the laboratory scale model drinking water distribution systems (MDWDS’s). The experiment was run for a period of seven weeks and the biofilm formation was closely monitored through assessing the survival of the bacteria in the system through HPC using selective media. The *E. coli* and *S. enterica* used in this chapter were originally isolate from the biofilms of PDW samples and the organisms were confirmed for the MAR, Haemolysis and MEP. The 16S rRNA sequences of these potential strains with virulence features screened out in the present chapter along with other BAB with virulent features were, submitted to NCBI Genbank. The major findings for this chapter are:

- Both the organisms survived well in the biofilms of the DWDS’s irrespective of the chlorination status, though the growth of *Salmonella enterica* in the biofilms were significantly lower (P<0.01) when compared to the *E. coli*.
- Both the organisms were significantly higher (P<0.01) in the biofilms than their counter parts in the bulk water.
- The study witnessed drastic and steep reduction of biofilm counts, which was due to the dispersion of the biofilm cells of the monocultures due to the less structural integrity, which may be due to the delicate biofilms in the oligotrophic DWDS’s.
- The growth of the both the organisms in the MDWDS’s were significantly different from first to the final week.
The growth was significantly superior in the non-chlorinated tanks, though the more stable and balanced survival was observed in the chlorinated tanks.

- *E. coli* was found to be well flourished in both the biofilm and bulk water of non-chlorinated and chlorinated DWDS’s, whereas *Salmonella* ceased its growth in the bulk water after 5 weeks of inoculation, switching completely to the biofilm mode of life. The adaptive response of *S. enterica* towards chlorine stress, thereby resorting to biofilm is to be considered as a risk factor, as this is definitely a threat to the public.

- Although chlorination in the miniature MDWDS’s could eradicate *S. enterica* from the bulk water, it couldn’t eradicate the biofilm phenotypes attached to the biofilm test plugs in cooperated

The chapter concludes pointing out the importance of revising routine monitoring techniques expanding the analysis to the attached reservoirs of pathogens. The study suggests that proper management of the DWDS’s with regular/uninterrupted and adequate chlorination right from the initial stages preventing the entrance of a pathogen may help with the problem. Moreover, aged distribution pipelines should be replaced and monitored properly. Despite the water being chlorinated or non-chlorinated, it is advised to the public, especially the immunocompromised population including the children and elderly, in a developing country like India, to consume water only after boiling since the chlorination process employed cannot be ensured for the safety of pathogen free water.

The present study analyzed the risk associated with the survival of BAB bacteria in various drinking water microcosms, particularly concentrated on PDW and distribution systems. Today, ready to use
commodities like PDW comes with various claims of treatments like reverse osmosis, UV filtration, membrane filtration etc. But the study proved that the PDW commercialized in the markets of Kerala are unfit for consumption, not only because of the free floating planktonic bacteria in the water, but also the biofilm associated life which is attached to the inner surface of the bottles. Moreover, these BAB can be a threat due to the various pathogenic features it exhibits. The study also points out the risk of a biofilm associated pathogen being introduced in a drinking water distribution system, since it has proved the survival of the protected pathogen in the film for many weeks, periodically shedding the same into bulk water at times. The work also highlights the risks of thick biofilms inside our municipal, public and household drinking water systems which can harbor a lot of opportunistic and obligate pathogens that can be accidently introduced, but can survive for weeks even in the chlorinated conditions.

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Survival and Risk Assessment of Biofilm Associated Bacteria in Drinking Water Microcosms and Distribution Systems