AUTO AGGREGATION AND BIOFILM FORMATION IN MICROBES ASSOCIATED WITH EXTERNAL AND INTERNAL SURFACES OF *PERIPLANETA AMERICANA (cockroach)*

U. Rafi, Asmat, S.S. Bokhari, Y. Roheela and A.W. Qurashi

Department of Biology, Lahore Garrison University, Sector-C, Av-4, phase VI, DHA Lahore, Pakistan
Corresponding author's email: uzmazeeshan@lgu.edu.pk

ABSTRACT: *Periplaneta americana* (cockroach) are the carriers of all sorts of pathogens and pose great public health risks. Present study was aimed to check the response of bacteria isolated from external surface (CXA2 and CXA3) and rectum part (CIA1, CIA2, CIA3) of *Periplaneta americana* (captured from kitchen sewerage) towards biofilm formation and auto aggregation. Both these parameters are the prerequisite characteristics for establishing pathogenicity of any bacteria. Bacterial isolates showed diverse morphology and biochemical characteristics. Biofilm at shaking and non-shaking conditions was quantified and found highest at non-shaking conditions in CIA3 as compared to rest of bacterial isolates. Among all isolates, auto aggregation in CIA3 showed 143 % while 196 % in CXA2 as compared to rest of bacterial isolates at 3rd hour (at 37°C) of culture incubation. Results showed that biofilm and cell aggregation of microbes helped them to colonize abiotic surfaces that result in bacterial infection. Therefore, cleaning practices should be the cohesive part of domestic routines to remove the carrier pests i.e., cockroaches as well as bacteria.

Key words: Autoaggregation, biofilm formation, cleaning and disinfection, kitchen sewerage and *Periplanata americana,*

(Received 20-08-2017 Accepted 02-06-2018)

INTRODUCTION

Cockroaches are common insects and part of the earth for approximately 270 million years (Chamavit *et al*., 2011). Generally, these are American cockroach (*Periplaneta americana*) and the German cockroach (*Blattella germanica*) as reported by Gonzalez-Domenech *et al*., (2012). They prefer warm, humid and dark environment like kitchens, toilets and garages. They act as the vectors of pathogenic agents like the bacteria and certain protozoan parasites that cause various diseases in humans such as typhoid fever, cholera (Shahraki, 2011) and diarrheal diseases in urban and rural setups by foodborne parasites (Graczyk *et al*., 2005). Bacterial communities in which cells are surrounded by matrix of extracellular polymeric compounds attached to a surface are known as biofilms (Dogsa *et al*., 2013) Bacteria living in biofilms are protected from adverse environmental conditions. Biofilm formation is an important factor in the infection progression of bacterial pathogens (Kumar *et al*., 2017). Various extracellular compounds such as exopolysaccharides (EPSs), lipopolysaccharides (LPSs), flagella, quorum-sensing signals and environment play a vital role in the biofilm development (Bogino *et al*., 2013).

Like many other microorganisms bacteria also have a strong tendency to accumulate or aggregate. This phenomenon is referred to as auto aggregation, and it depends on adhesive interactions amongst bacteria. Auto aggregation can be viewed under microscope by "fluffing" of cells in fluid environments. This fluffing is due to bacterial aggregation and finally settling at the bottom due to sedimentation of the cell clusters under static environments (Sorroche *et al*., 2012). Bacteria that usually propagate in a non-aggregated way or severe growth conditions or slight metabolic activity have been reported to produce aggregative actions (Nicholas and Charles, 2013).

This study was aimed to check the response of bacterial isolates from *Periplaneta americana* towards auto aggregation and biofilm formation for evaluating their colonization potential.

MATERIALS AND METHODS

Cockroaches collection, bacterial isolation and characterization: In the month of March, 2016 *Periplenata americana* (cockroaches) were trapped from house kitchen sewerage of Lahore, Pakistan and brought to laboratory for bacterial isolation.

Briefly, the bacteria from external surface of cockroaches were isolated using sterile swab and spreading sample containing swabs under sterile conditions over Luria-Bertani (LB) agar media (Gerhardt *et al*., 1994). Bacterial growth was obtained after incubation of inoculated media plates for 24 hours at 37°C. Quadrant streaking was used to purify isolated colonies (Cappuccino and Sherman, 2002).

Bacterial isolation from internal surface of cockroach: The cockroaches were dissected using sterile dissecting tools. The digestive system of cockroach was opened. One gram of the dissected part from the colon portion of the digestive tract was serially diluted and 100 µl suspensions from 10^7 dilutions were used for spreading on LB agar media (Gerhardt *et al*., 1994) separately. Isolates were obtained from plates incubated at 37°C for 24 hours. Selected colonies from internal and external surface of cockroaches were purified by quadrant streaking method (Cappuccino and Sherman, (2002).

Morphological and biochemical characterization of bacterial isolates: Cultural characteristics of isolates from both sources (external and internal surfaces) were analyzed as per Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). Morphological characterization of bacterial isolates was done using staining techniques (*i.e*., simple staining using crystal violet, gram Staining, spore staining and capsule staining) following same procedures as described previously in manual (Cappuccino and Sherman, 2002).

In gram staining, bacterial smear was prepared taken from pure culture, bacteria colony and distilled water was properly mixed until just slightly turbid on dry and clean glass slide using sterile loop or needle. Then it was heats fixed using spirit lamp and allowed to cool at room temperature. Then it was treated by crystal violet solution as primary stain and kept for 1 minute and then it was washed by using distilled water. The slide was flooded with gram's iodine kept for 1 minute and washed. Then put 1 to drops of gram's decolorizer on the smear and kept for 30 seconds and washed properly. Then put counter stain safranin for 60 seconds and rinsed with distilled water and allow to air dry. The slide was then observed under the light microscope at 100X objective lens. For capsule staining, bacterial smear was prepared taken from pure culture, and mixed with a drop of water, a drop of india ink and air dried. This smear was flooded with crystal violet for 1 minute. Slide was washed with water and blot to dry and observed under microscope. For endospore staining, bacterial smear was allowed to dry and then heat-fixed by flooding the slide with malachite green stain on the staining rack. Flooded slide was heat fixed by placing over steam till evaporation of stain. After washing the slide with running water cells were counterstained with aqueous safranine for 1 minute. Finally slide was blot gently and allowed to air dry. Results were observed using immersion oil.

Biochemical characterization of the bacterial isolates were done by starch hydrolysis test, citrate utilization test, litmus milk reaction, methyl red test, voges, proskauer test, catalase test following same procedures as described previously in manual using fresh bacterial culture (Cappuccino and Sherman, 2002).

Starch Hydrolysis test was performed by streaking the starch agar plate with the pure culture. Plates were incubated for 24 hours at 37 °C. Presence of clear zones aroundcolonies were scored as positive results. For Catalase assay, bacterial colony was added to a surface of clean, dry glass slide and a drop of 3 % H_2O_2 was placed on to the slide and mix using sterile toothpick. Rapid evolution of oxygen (within 5-10 second) as evidenced by bubbling indicated positive result. In Citrate assay, fresh cultures are inoculated on a medium containing sodium citrate and incubated at 37°C for 24 hours. Development of blue color showed positive results. Fresh culture was inoculated in MR-VP broth and incubated at temperature 37°C for 3 days. For methyl red test, 2-3 drops of Methyl red was added after incubation, and the color change from yellow to red color was scored as positive result while negative test indicated no color change. While for Voges-Proskauer test, cultured MR-VP broth was supplemented with 0.6 ml of Barritt's reagent A and 0.2 ml of Barritt's reagent B. This was followed by vortexing the tubes for 1 minute and allowed to stand for 1 hour at room temperature. Positive results were interpreted when red coloration appeared on top of the culture and negative results for yellow color. Litmus milk test was performed by inoculating (with fresh culture of test microbe) tube containing litmus milk for 8 hours and change in color to white or pale yellow was recorded as positive result for reduction of litmus milk

Auto aggregation assay: This assay was performed following same method as previously reported by Abdulla *et al*., (2014). Isolates were allowed to grow in LB-broth at 37°C in shaking incubator for 24 hours. Centrifugation at 2000 rpm for 20 minutes was carried out to to harvest the cells from the grown cultures and the harvested cells were washed with phosphate buffer saline (pH 7.2) twice and then kept suspended in phosphate buffered saline. Autoaggregation was determined during 4 hours of incubation at room temperature of 22-25°C using upper suspension. After different time intervals of 1, 2, 3 and 4 hours, 100 µl from upper suspension were transferred to tube with phosphate buffer saline (PBS) and absorbance was measured at 600 nm. Auto aggregation was calculated using the following formula;

$$\% \text{ Auto aggregation} = 1 - (At / A0) \times 100$$

Where At shows absorbance (OD600) determined at final respective time interval (i.e., 1 hour or 2 hour or 3 hour or 4 hour) and A0 is the Initial OD (OD600) observed in the beginning at 0 hour of incubation

Biofilm formation assay: Optical densities of the bacteria were adjusted to 0.5 at 600 nm and bacterial biofilm was determined following same modifications in method reported previously by Qurashi *et al.,* (2012).

Replicates of test tubes were incubated under shaking and non-shaking conditions at 37°C. After 6 days of incubation, growth of both cultured sets was measured at 600 nm. Cultures were discarded from test tubes and empty test tubes were filled up with 0.01 % crystal violet up to the side wall mark. After 20 minutes crystal violet was discarded and filled up the test tubes with 70 % ethanol for 5-10 minutes, then vortexed and optical densities were recorded at 570 nm for biofilm formation.

Statistical analysis: All the experimental data was analyzed statistically by calculating the mean values of replicates and measuring the standard errors of the means (Steel and Torrie, 1981). The error bars are shown in each figure. Correlation between autoaggregation and biofilm formation was checked using SPSS software (p=5% probability level).

RESULTS AND DISCUSSION

Cultural and biochemical characterization of isolates: Five bacterial isolates from cockroach; CIA1, CIA2, CIA3, CXA2 and CXA3 were characterized using its colony morphology. Isolates CIA2, CIA3 and CXA2 showed rounded margins of colonies but CIA1 and CXA3 showed colonies with irregular margins (Table-1). When colonies were evaluated for colony coloration, all of the isolates showed off white coloration as colony characteristics. Isolate CIA1, CIA2 and CXA2 showed rough margins while CIA3 showed entire margins, flat elevation and CXA3 showed raised elevation (Table-1). Simple staining using crystal violet revealed that all strains were rods. Among these isolates gram negative rods were dominant (80 %) bacteria however, isolate CIA1 was gram positive. (Table-2). In many previous reports, gram negative bacterial rods isolated from the arthropods belonged to the following genera: *Klebsiella* spp, *Escherichia* spp, *Enterobacter* spp, *Citrobacter* spp, *Proteus* spp, *Seratia* spp, *Pseudomonas* spp, *Acinetobacter* spp (Mikulak *et al*., 2013). Biochemical characterization of bacterial strains was done using various tests. Clear zone formation of isolates CIA1, CIA3 and CXA3 in starch hydrolysis test was observed as positive results while CIA2 and CXA2 showed negative results for starch hydrolysis. Litmus test for all isolates was positive. Isolates CIA1, CIA3 and CXA2 were MR positive while the isolates CIA2 and CXA3 were MR test negative. Isolates CIA1 and CIA3 were negative while CIA2, CXA2 and CXA3 were positive for Voges-Proskauer analysis. All the isolates showed positive results for catalase test except CXA3 that which showed catalase negative results (Table-3).

For bacterial identification flagellar arrangement, shape and staining were the significant morphological parameters (Ramos *et al*., 2016). Cockroaches transport several pathogenic as well as non-pathogenic bacteria. Numerous bacterial species have been isolated from cockroaches in which *S.aureus*, *Pseud. aeruginosa*, *Strep. spp* and *Escherichia* are familiar one. Cockroaches serve as mechanical vector in spreading *Cryptosporidium parvum*, *Salmonella* and *Shigella* that are the also the cause of various diarrheal ailments in humans (Bala and Sule, 2012). Different biochemical and morphological similiarity was detected with those microbes associated on the surface of cockroaches (Menasria *et al*., 2014). Data of the present study is in line with previous findings (Haghi *et al*., 2014) showing that cockroaches were the carrier of different gram positive and gram negative bacteria. This showed the potential of cockroaches (existing in kitchen premises) in the transfer of pathogenic bacteria and is of great concern to public health. Previous reports also pointed out the role of cockroaches (from hospitals) in the transmission of infection from contaminated source (Brooks *et al*., 2010).

Measurements of auto aggregation of the bacterial isolates have been reported to be an important feature because it facilitates the adhesion of bacteria to epithelial cells (auto-aggregation) (Armas *et al*., 2017). Bacterial auto aggregation response was recorded after one hour of suspension incubation and the results showed that after first hour, auto aggregation started increasing however; the extreme increase was recorded afterwards. The maximum auto aggregation was recorded after 3 hours in isolates CIA2, CIA3 and CXA2 while after 2 hours in CIA1 and after 4 hours of culture incubation in CXA3 (Fig. 1). Bacterial adhesion and auto aggregation thought to be associated with surface proteins, that can also enhance the cell hydrophobicity but impart hydrophilic characteristics are imparted by polysaccharides molecules (Mora *et al*., 2015). Bacterial auto aggregation has been stated to show significant interaction towards bacterial adhesion (Pan *et al*., 2017) and microbial aggregation in normal atmosphere (Rosenberg, 2017). Auto-aggregation and adhesion have been reported to result in aggregate creation within cells showing genetic similiarity (Khan *et al*., 2017) that might favour the cells towards biofilm formation. Presence of auto aggregation and development of biofilm in pest associated microbes showed features of great significance because by inhibiting these abilities of bacteria the diseases associated with pathogens harboured by cockroaches can be controlled.

Bacteria commonly associated with common pests i.e., cockroaches can be carriers of different diseases and also cause food pathogenicity. So the trend of bacterial strains towards biofilm formation can be significant in this regard. To test this, biofilm formation of bacterial isolates was evaluated at different physical conditions like shaking and non-shaking. It was observed that at non-shaking conditions, isolates have better biofilm than the strains that were incubated at shaking

condition (Fig-2). Bacterial biofilm grown at non shaking conditions showed a positive correlation with bacterial aggregation at 4 hours of culture incubation (r=0.90; p=0.05), however, contrary to this biofilm development at shaking conditions was positively correlated to bacterial aggregation after 1st hours of culture incubation(r=0.81; p=0.05). These results are in line with previous findings where the biofilm formation at non-shaking condition favored bacterial adherence of pathogenic bacterium *Listeria monocytogenes* from food processing plants (dos Reis-Teixeira *et al*., 2017). The increasing flow rate can cause detachment and erosion of biofilm portions showing decreased biofilm formation in shaking as compared to non-shaking conditions (Gomes *et al*., 2017). Results of present investigation are in line with the previously reported findings Cai and Arias, (2017) showed that the bacterial pathogens in fish farms had colonizing potential surfaces and may use biofilm as reservoirs. A state of dynamic equilibrium is attained by biofilm at the shaking conditions for numerous reasons such as accumulation of noxious metabolic by-products (Pande, 2015), shear forces (Paul *et al.,* 2012) and deficiency of nutrients (Boelee *et al.,* 2012).

It is important to consider that microbial communities strive for existence utilizing appropriate conditions of moisture, nutrients and temperature. Utilization of carriers pests as a conveyance is important to approach suitable growth conditions. So interruption of this conveyance using cost effective sanitary measures and cleaning practices can save million dollars. This is especially important for food industries. Regular cleaning practices, removal of organic and inorganic residues in kitchen reservoirs and use of disinfectants can be effective in removing these pests which harbors bacteria as well. At this point it is not possible to point out that what genetic attributes are further contributing towards biofilm formation and aggregation of bacterial communities associated with cockroaches, however, in an effort to survive, these parameters (biofilm formation and aggregation) enable the bacteria to cause pathogenicity by successful colonization of living surfaces. Detailed genetic analysis of other factors will further unravel the contributing factors of disease dispersal associated with these pests and associated microbes.

Table-1: Colony characteristics of different isolates from internal and external surface of cockroaches.

Isolates	Shape	Coloration	Edge	Elevation
CIA1	Irregular	Off white	Entire	Rough
CIA2	Round	Off white	Entire	Rough
CIA3	Round	Off white	Entire	Flat
CXA2	Round	Off white	Entire	Rough
CXA3	Round	Off white	Entire	Raised

Table-2: Morphological characterizations of bacterial isolates from internal and external surfaces of cockroaches.

Bacterial isolates	Simple Staining	Gram staining	Spore staining	Capsule staining
CIA1	Bacillus	Gram +ve	Spore forming	No capsule
CIA2	Bacillus	Gram –ve	Spore forming	No Capsule
CIA3	Bacillus	Gram –ve	Non-spore forming	No capsule
CXA2	Bacillus	Gram –ve	Non-spore forming	No capsule
CXA3	Bacillus	Gram –ve	Spore forming	No capsule

Table-3: Biochemical descriptions of isolates from external (CXA2, CXA3**)and internal surface of cockroaches (**CIA1, CIA2, CIA3**).**

Bacterial isolates	Starch Hydrolysis	Citrate Utilization test	Litmus milk reaction	Methyl red test	Voges-Proskauer test	Catalase test
CIA1	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**+ve**
CIA2	**-ve**	**+ve**	**+ve**	**-ve**	**+ve**	**+ve**
CIA3	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**	**+ve**
CXA2	**-ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**
CXA3	**+ve**	**-ve**	**+ve**	**-ve**	**+ve**	**-ve**

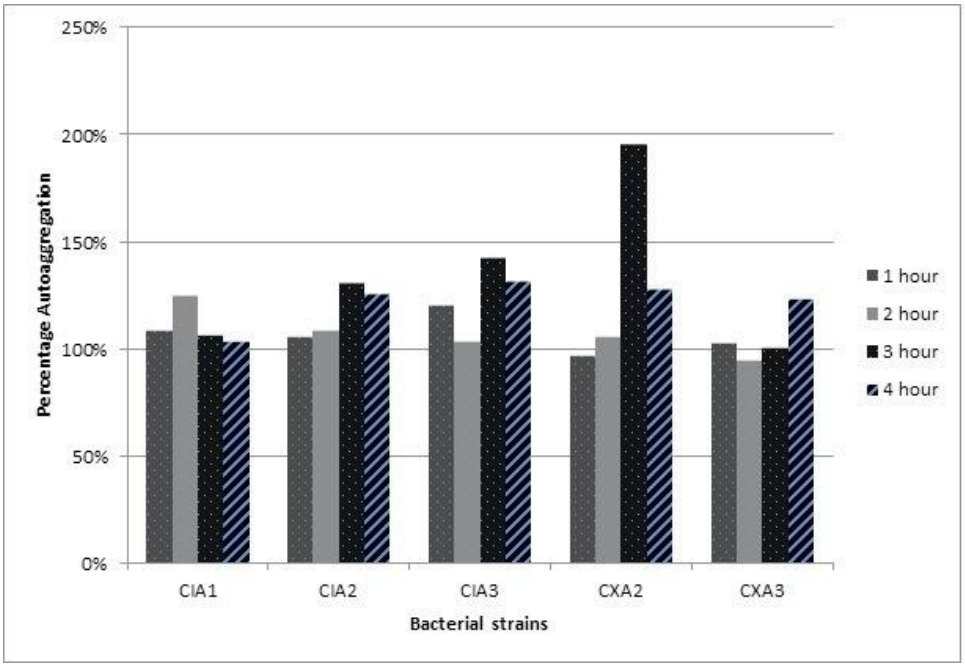

Figure-1: Autoaggregation percentages in five bacterial isolates during incubation in different hours.

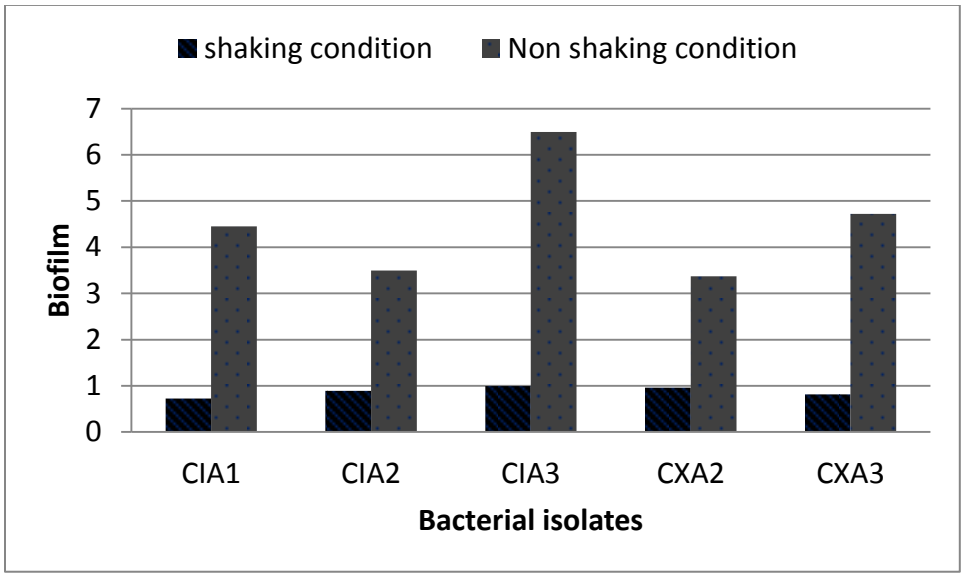

Fig-2: Biofilm values of bacterial isolates in shaking and non-shaking condition.

Acknowledgements: We are greatly obliged to Dean of Basic Sciences Dr. Abdul Majeed and Head of Biology Department, Dr. Col. Amjad from Lahore Garrison University for encouraging and providing financial research support to investigate this research activity.

REFERENCES

Abdulla, A.A., T.A. Abed and A.M. Saeed (2014). Adhesion, Autoaggregation and Hydrophobicity of Six *Lactobacillus* Strains. Br. Microbiol. Res. J. 4(4): 2231-088.

Armas, F., C. Camperio and C. Marianelli, (2017). *In vitro* assessment of the probiotic potential of *Lactococcus lactis* LMG 7930 against ruminant mastitis-causing pathogens. PLoS ONE 12(1): e0169543. doi:10.1371/journal.pone.0169543.

Bala, A.Y. and H. Sule (2012). Vectorial potential of cockroaches in transmitting parasites of medical importance in Arkilla, Sokoto, Nigeria. Nig. J. Basic Appl. Sci. 20(2): 111-115.

Barbosa, O., C. Ortiz, A. Berenguer-Murcia, R. Torres, R.C. Rodrigues and R. Fernandez-Lafuente (2015). Strategies for the one-step immobilization–purification of enzymes as industrial biocatalysts. Biotechnol. Adv., 33(5): 435-456.

Bogino, P.C., M. M. Oliva., F.G. Sorroche and W. Giordano (2013). The role of bacterial biofilms and surface components in plant-bacterial associations. Int. J. Mol. Sci. 14: 15838-15859.

Boelee, N.C., H. Temmink., M. Janssen., J.C.N. Buisman. and R.H. Wijffels (2011). Nitrogen and phosphorus removal from municipal wastewater effluent using microalgal biofilms. Water Res. *45*(18): 5925-5933.

Brooks, G.F., K.C. Carroll, J.S. Butel, S.A. Morse and M.T.A. Jawetz,. (2010). Melnick and Adelberg's medical microbiology, 25th ed. McGraw Hill.

Cai, W and C.R. Arias (2017). Biofilm formation on aquaculture substrates by selected bacterial fish pathogens. J. Aquat. Anim. Health 29(2): 95-104.

Cappuccino, J.G., and N. Sherman, (2002). Techniques for isolation of pure cultures. Cultural Characteristics of Microorganisms, Microbiology A Laboratory Manual, Pearson Education, 6, 13-23.

Chamavit P., P. Sahaisook and N. Niamnuy (2011). The majority of cockroaches from the Samutprakarn province of Thailand are carriers of parastic organisms. J. Exp. Clin. Med. 10: 218–222.

Dogsa I., M. Brloznik., D. Stopar. and I. Mandic-Mulec (2013). Exopolymer diversity and the role of levan in *Bacillus subtilis* biofilms. PLoS One. 8(4): e62044.

Dos Reis-Teixeira, F.B., Alves, V. F., and E.C.P. de Martinis, (2017). Growth, viability and architecture of biofilms of *Listeria monocytogenes* formed on abiotic surfaces. Braz. J. Microbiol. https://doi.org/10.1016/j.bjm.2017.01.004

Dunne W.M. (2002). Bacterial adhesion: Seen any good biofilms lately? Clin. Microbiol. Rev. 15:155–166.

Garrity, G.M. and J.G. Holt (2001). The road map to the manual. In Bergey's Manual® of Systematic Bacteriology (pp. 119-166). Springer New York.

Gerhardt, P., R.G.E. Murray., W. A. Wood and N.R. Krieg (1994). Methods for general and molecular bacteriology. Washington (DC): ASM Press.

Gomes, L.C., J. Moreira, M.R., Teodósio, J.S., Araújo, J.D.P., Miranda, J.M., Simões, M. and F.J. Mergulhão (2014). 96-well microtiter plates for biofouling simulation in biomedical settings. Biofoul. 30(5): 535-546.

Gonzalez-Domenech, C.M., E. Belda, R. Patino-Navarrete, A. Moya, J. Pereto and A. Latorre (2012). Metabolic stasis in an ancient symbiosis: Genome-scale metabolic networks from two *Blattabacterium cuenoti* strains, primary endosymbionts of cockroaches. BMC Microbiol. 18: 12.

Graczyk, T.K., R. Knight, and L. Tamang (2005). Mechanical transmission of human protozoan parasites by insects. Clin. Microbiol. Rev. 18(1): 128-132.

Haghi, F.M., H. Nikookar, H. Hajati, M.R. Harati, M.M. Shafaroudi, J. Yazdani-Charati and M. Ahanjan (2014). Evaluation of bacterial infection and antibiotic susceptibility of the bacteria isolated from cockroaches in educational hospitals of Mazandaran University of medical sciences. Bull. Env. Pharmacol. Life Sci., Vol 3 (V): 25-28

Holt, J.G., N. R. Krieg., P. H. A. Sneath., J. T. Staley and S.T. Williams (1994). Bergey's manual of determinative bacteriology. 7th ed. Baltimore, USA: William and Wilkin Company.

Khan, M.A., M.M. Altaf and I. Ahmad (2017). Chemical nature of biofilm matrix and its significance. Biofilms in Plant and Soil Health. 151- fnx069.

Kumar, A., A. Alam, M. Rani, N.Z. Ehtesham and S.E. Hasnain (2017). Biofilms: survival and defense strategy for pathogens. Inter. J. Med. Microbiol. 307(8): 481-489.

Menasria, T., F.Moussa, S. El-Hamza, S. Tine, R. Mergi and H. Chenchouni (2014). Bacterial load of German cockroach (*Blattella germanica*) found in hospital environment. Pathog. Glob. Health 108(3): 141-147.

Mikulak, E., A. Gliniewicz, K. Pancer, H. Stypułkowska-Misiurewicz, D. Rabczenko and A. Królasik (2013). Microbiological hazard for patients caused by bacteria present on synantropic arthropods in hospital environment. Microbial. pathogens and strategies for combating them: Science, Technology and Education, 1770-1777.

Mora-Navarro, C., J. Méndez-Vega, M. Caraballo-León, R. Lee, S. Palecek, M. Torres-Lugo and P. Ortiz-Bermúdez (2016). Hydrophobicity of antifungal β-peptides is associated with their cytotoxic Effect on in vitro Human Colon Caco-2 and Liver HepG2 Cells. PloS One, 11(3): e0149271. doi: 10.1128/MMBR.00060-12

Nicholas A.B. and W.B. Charles (2013). The microbiology of malting and brewing. Microbiol. Mol. Biol. Rev. 2: 157-172

P. Mingfang., K.K. Kumaree and N.P. Shah (2017). Physiological changes of surface membrane in

Lactobacillus with prebiotics. J. Food Sci. 82(3): 744-750.

Paul, E., J.C. Ochoa., Y. Pechau, Y. Liu., and A. Liné (2012). Effect of shear stress and growth conditions on detachment and physical properties of biofilms. Water Res. 46(17): 5499-5508.

Pande, S., S. Shitut., L. Freund, M. Westermann, F. Bertels, C. Colesie and C. Kost (2015). Metabolic cross-feeding via intercellular nanotubes among bacteria. Nat. Commu, 6: 6238.

Carla R.A., (2016). Characterizing the fungal and bacterial microflora and concentrations in fitness centres. Indoor Built Environ. 25(6): 872-882.

Rosenberg, M. (2017). Adhesion to hydrocarbons and microbial hydrophobicity—do the MATH. FEMS Microbiol. Lett. 364(10).

Shahraki, G.H., H.M. Noor, J. Rafinejad, M.K. Shahard and Y. Bin-Ibrahim (2011). Field efficacy of siege gel bait in an IPM program on life stages of German cockroach (Blataria, Blattellidae) in a residential building. Asian Biomed. 5(5): 619–624.

Sorroche, F.G. (2012). A positive correlation between bacterial autoaggregation and biofilm formation in native *Sinorhizobium meliloti* isolates from Argentina. Appl. Environ. Microbiol. 78(12): 4092-4101.

Steel, R.G.D and J.H. Torrie (1981). Principle and procedure of statistics. A biometrical approach, Second Ed. Mc Gvaus-Hill Booh Company, New York, US.