Microbial Diversity in Oral Microbiome of Postpartum Females

By

Bibi Khadija

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Department of Microbiology

Faculty of Biological Sciences

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A thesis submitted in the partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Microbiology

By

Bibi Khadija

Department of Microbiology

Faculty of Biological Sciences

Quaid-i-Azam University,

Islamabad, Pakistan

2020

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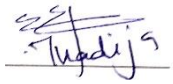
Student / Author Signature: 

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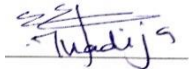
Student Name: **Ms. Bibi Khadija** Signature: 

Examination Committee:

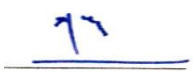
a) External Examiner 1: Signature: 

Prof. Dr. Syed Habib Ali Bokhari
Head Department of Bioscience
COMSATS University, Park Road, Islamabad.

b) External Examiner 2: Signature: 

Dr. Hussnain A Janjua
Associate Professor
ASAB, National University of Sciences and Technology (NUST)
Campus H-12, Islamabad

Supervisor Name: **Prof. Dr. Rani Faryal** Signature: 

Co. Supervisor Name: **Dr. Shahzad Hussain** Signature: 
NIH, Islamabad

Name of HOD: **Prof. Dr. Aamer Ali Shah** Signature:_____

Dedicated to my beloved parents

my sisters

and

brothers

For their endless prayers, support

and encouragement

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List of Abbreviations

Abbreviations	Description
α	Alpha
ATCC	American Type Culture Collection
AIDS	Acquired Immune Deficiency Syndrome
API	Analytical profile index
APOs	Adverse pregnancy outcomes
β	Beta
BMI	Body Mass Index
BC	Bray-Curtis
BHI	Brain heart infusion
BMI	Body Mass Index
BP	Blood pressure
BSA	Bovine serum albumin
°C	Degree Celsius
cKP	Classical *K. pneumoniae*
CLSI	Clinical and Laboratory Standards Institute
CRA	Congo red assay
CSP	Competence-stimulating peptide
CV	Crystal violet
CVD	Cardiovascular disease
CoNS	Coagulase-negative *staphylococcus*
dex	Dextranase
DMFT	Decayed, missing, filled teeth
DMFS	Decayed, missed, filled surfaces
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EMB	Eosin methylene blue
EPS	Extracellular polymeric matrix/substance
FTB	Full term birth
γ	Gamma
GMM	Glucose Methylene Blue Muller Hinton (GMM)

Gtfs	Glucosyl transferases
h	Hour
HGT	Horizontal gene transfer
HIV	Human immunodeficiency virus
HCl	Hydrochloric acid
HGP	Human Genome Project
HCHO	High carbohydrate
HMP	Human Microbiome Project
hvKP	hypervirulent *K. pneumoniae*
I	Intermediate
Ica	Intercellular adhesion operon
IL	Interleukin
ITS	Internal transcribed spacer
Kg	Kilogram
L	Liter
LCHF	Low carbohydrate high fat
LMIC	Low and middle-income countries
LWB	Low weight birth
LPS	Lipopolysaccharides
M	Molar
min	Minutes
mL	Milliliter
MHA	Muller Hinton agar
MRSA	Methicillin-resistant *S. aureus*
MS	Mutans streptococci
MSA	Mannitol salt agar
MTP	Microtiter plate
µg	Microgram
µL	Microliter
µm	Micrometer
mM	Millimolar
mm	Millimeter

N	Normal
NA	Nutrient agar
NaCl	Sodium chloride
nm	Nanometer
NaOH	Sodium hydroxide
NB	Nutrient broth
NGS	Next generation sequencing
NMDS	Nonmetric multidimensional
OD	Optical density
ODc	Cut off optical density
OR	Odd Ratio
OTU	Operational taxonomic unit
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PCOA	Principal Coordinates analysis
PCHO	Periodized carbohydrate
PGE2	Prostaglandin E2
PIA	Polysaccharide intracellular adhesion
PL	Phospholipases
PLWB	Preterm low weight birth
PPROM	Preterm premature rupture of membranes
%	Percentage
psi	Pounds per Square Inch
PTB	Preterm birth
QAU	Quaid-i-Azam University
QS	Quorum sensing
R	Resistant
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal ribonucleic acid
S	Sensitive
SB	Sucrose-Bacitracin
SD	Standard Deviation

SDB	Sabouraud Dextrose Broth
SDS	Sodium Dodecyl sulphate
spp.	Species
TBE	Tris Borate EDTA buffer
TE	Tris EDTA buffer
TNF-α	Tumor Necrosis factor-alpha
TSI	Triple sugar iron
UTI	Urinary Tract Infections
UV	Ultraviolet
USA	United States of America
WHO	World Health Organization
w/v	Weight/Volume

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Abstract

Pregnancy is a state, which predisposes the female body to various reversible changes including a shift in oral microbial diversity which can be pathogenic. In the present study, salivary culturable and unculturable microbial diversity in early postpartum phase was investigated and compared with nonpregnant females diversity by using standard microbiological methods and by targeting V4 region of 16*S rRNA* and *ITS* gene. The association of microbial diversity with oral health status and adverse pregnancy outcomes (APOs) was also assessed. Unstimulated saliva samples were collected from 267 postpartum and 54 nonpregnant healthy females. Oral health problems were present in 47.2% postpartum females. Frequency of low weight birth (LWB) was 22.5%, preterm birth (PTB) 21.7% and preeclampsia was 11.6%. PTB and LWB were not associated with oral disorders. However, preeclampsia had an association with gingivitis (P=0.01).

By using culture based methods, various Gram positive and negative species were identified in saliva. *Streptococcus mutans* were significantly more prevalent (93.2%) in saliva samples of postpartum females, while 53.5% were also culture positive for *Streptococcus sobrinus*. Colonization of *S. mutans* was significantly high among females having gingivitis (P=0.007), dental caries (P=0.006), low brushing frequency, high sugary liquid intake, and in those giving birth to a baby of low weight. Colonization of *S. sobrinus* was significant among females having low brushing frequency and high sugary liquid intake. Its colonization varied with gestational period and preeclampsia. In the postpartum group, 65.1% females were culture positive for *Staphylococcus* species [*Staphylococcus aureus* (n=100) *Staphylococcus epidermidis* (n=78) and *Staphylococcus saprophyticus* (n=32)]. Postpartum females showed significantly higher *S. epidermidis* colonization (P=0.005). *Staphylococcus* species colonization increased approximately one to two-fold risk for oral disorders and APOs. For identification of biofilm former isolates, phenotypic [Congo red assay (CRA) and microtiter plate assay (MTP)] and genotypic methods were used. By CRA method, 15.7% of these isolates showed biofilm forming ability and 40.9% by microtiter plate (MTP) method. By genotypic method of biofilm detection, 64% and 48.3% isolates (*S. aureus* and *S. epidermidis*) had presence of *Ica* A and *Ica* D gene, respectively. *S. aureus* was highly resistant to penicillin, quinolones, antibiotics of class cephalosporin and fluoroquinolones, erythromycin and gentamycin. *S. epidermidis* and *S.*

saprophyticus showed high resistance against antibiotics of class penicillin and cephalosporin.

Streptococcus species were detected in 27.3% postpartum females. Prevalence of *Streptococcus* species was significantly raised in postpartum group (P=0.001) compared to nonpregnant females. Among postpartum group, 12.7% females were culture positive for *Lactobacilli*, 10.4% for *Neisseria meningitides*, 6.3% for *Klebsiella pneumoniae*, 5.6% for *Enterobacter* species and 2.6% for *Escherichia coli*. By MTP method 100% *E. coli*, 80% isolates of *Enterobacter* species and 58.8% *K. pneumoniae* displayed biofilm forming ability. *K. pneumoniae* and *E. coli* showed high resistance against most of the tested antibiotics. Only detected fungal specie by culture based method was *Candida*, isolated from 55% postpartum (P<0.001) and 22.2% nonpregnant females. *Candida* colonization showed one to three-fold risk with oral health and different obstetric factors. *Candida* isolates from postpartum females expressed various virulence factors such as high esterase, phospholipase, biofilm forming activity and also displayed antifungal resistance.

Unculturable bacterial and fungal diversity was also studied among postpartum and nonpregnant healthy female. In total 16 bacterial phyla and 156 genera were observed in all saliva samples tested. Decrease in alpha diversity and high Bray-Curtis dissimilarity was seen in salivary microbiome of postpartum female having oral health issues with preterm low weight birth (PLWB) compared to females with full term birth (FTB). Female with oral health problems and who gave FTB showed predominance of genera *Streptococcus* followed by *Yersinia, Haemophilus, Neisseria, Fusobacterium, Gemella, Prevotella* and *Aggregatibacter*, while female with PLWB was also dominated by *Streptococcus*, followed by *Gemella, Prevotella, Rothia, Veillonella, Haemophilus, Neisseria* and *Granulicatella*. Upon fungal diversity analysis, 55 genera and 92 species were detected in all samples. Postpartum female having oral health issues with PLWB, showed reduced richness, evenness with elevated levels of *Saccharomyces, Candida, Hyphodontia* and *Malassezia* compared to those females having FTB.

In present work, dual-species biofilm assays for different combinations of *Candida* and bacterial species were developed as dual-specie biofilm model to study behavior of dual species as consortium in oral niche that can lead to pathogenesis. Analysis showed that

all three *Candida* species with each other and with *E. coli* in dual-specie biofilms showed antagonistic behavior. Biofilm biomass production was raised for *C. albicans* and *C. glabrata* dual-specie biofilm with *Staphylococcus* species except with clinical isolate of *S. aureus* and in *C. glabrata-S. epidermidis* dual-species biofilm. *Candida* species with clinical isolate of *K. pneumoniae* in dual-species biofilm assay showed increased in biofilm biomass after 48 h of incubation except for *C. glabrata-K. pneumoniae* dual-species biofilms. Dual-specie assay showed that in consortium various species change their behavior and display different virulence factors. In consortium, species can be antagonistic or synergistic with other species.

In conclusion, findings from present work showed that pregnancy with or without oral health issues is associated with culturable and unculturable oral microbial diversity change. This change in diversity is more towards pathogenic culturable multi-drug resistant isolates with strong biofilms forming ability and enhanced expression of virulence factors. In addition to commonly known fungal and bacterial pathogens, less frequently studied microbes with regard to oral pathologies such as *C. glabrata* and *S. epidermidis* also emerged as oral pathogens, their prevalence was high in females with oral disorders and APOs. These isolates also showed either synergistic or antagonistic behavior with each other in consortia when studied in mono and dual-species biofilm model. Females with oral disorders and APOs also showed decrease in species richness and evenness, suggesting the possible role of changing microbial diversity with oral disorders leading to induction of APOs. Overall, this study contributes to literature on existing culturable and unculturable microbial diversity and existence of pathogenic potential for its possible role in APOs. Based on present study findings, it can be easily recommended that there is need for development of guidelines for health practitioners to provide awareness to the females on importance of keeping good oral health and routine dental checkup during pregnancy.

PUBLICATION

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CHAPTER 1: GENERAL INTRODUCTION

Oral microbiota is highly dynamic in nature, and contains microorganisms with pathogenic potential when environmental conditions are favourable (Cobb *et al*., 2017). It is a second most complex community after colon, comprising of over 1000 different microbial species which exist in the state of equilibrium with host immune system. It is composed of both allochthonous (transient) and autochthonous (stable) species at a given time. Microbes can colonize both soft (mucosa) and hard (teeth) surfaces of oral cavity as biofilms, in which they showed intra as well as inter-species collaboration and antagonistic associations to maintain ecological stability. Majority of the oral flora is unculturable. Oral specie level identification is not enough for understanding the host-microbes interaction in health and adverse state (disease), also there is genetic heterogeneity of resident microbes and constant strong influence by environment. Commensal oral bacteria play role in maintaining local oral and systemic health by preventing the overgrowth of opportunistic pathogens (colonization resistance). Microbes play diverse role depending upon the oral cavity's condition and due to effect of external stimuli. For example, disruption of oral bacteria by antimicrobials leads to the increased risk for *Candida* and *Staphylococcus aureus* infections, both are commonly isolated from oral cavity but became pathogenic when over grow. Inhibition of *Streptococcus salivarius* strain K12 leads to the development of periodontitis because its produces bacteriocin that inhibit the growth of periodontopathogens (Zaura *et al*., 2009; Chen *et al*., 2010; Wade, 2013; Kilian *et al*., 2016; Kumar, 2017).

Oral microbes are commonly associated with two important oral disorders, which are periodontal disease and dental caries (Wade, 2013). Oral healthy environment can change to pathogen rich ecosystem, for example if there is extensive use of antibiotics, reduce host defence, reduce oxygen tension promoting thickness of biofilms, nutritional, metabolic and structural changes. Inflammatory immune response initiated against such colonization lead to destruction of gingival and tooth surfaces, which can ultimately lead to periodontal disease development and dental caries. Microbes in oral cavity can spread according to "focal infection theory", which states that bacteria, their toxins and by-products can enter the systemic circulation from localized asymptomatic lesion and contribute to the production of various systemic disorders such as pulmonary disorders, cardiovascular disorders and adverse

pregnancy outcomes (APOs) (Baig *et al*., 2013; Jacob and Nath, 2014; Walker *et al*., 2014; Kumar, 2017)

Gingivitis and periodontitis are forms of periodontal diseases. Gingivitis is the infection of gums due to increased growth of microbial pathogens (Kinane and Bouchard, 2008). Initially, gingivitis is characterized by swelling and redness of gums. Later, gingival bleeding is mostly seen during brushing of teeth, but tooth structure remains intact despite of irritation of gums. Gingivitis must be treated at its early stage, because it can progress to advance stage of a disease called periodontitis. In this stage, small spaces known as periodontal pockets are formed between gums and teeth due to removal of inner layers of gums and bones from teeth. These pockets are the source of disease as they get infected with microbes (Pihlstrom *et al*., 2005; Jared and Boggess, 2008; Han, 2011).

Periodontal diseases are caused by Gram negative anaerobic bacteria that grow in the subgingival area. These include *Porphyromonas gingivalis*, *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans*, *Campylobacter rectus, Prevotella intermedia, Streptococcus intermedius* and *Treponema* species. It is well established now that these organisms are also isolated from healthy oral cavity. Moreover, a study reporting absence of *P. gingivalis* from half of the studied patients with aggressive periodontitis is also available (Curtis *et al*., 2011; Pozhitkov *et al*., 2015). The bacterial count in disease condition is increased up to ten times more than those associated with healthy state (Lovegrove, 2003). In addition to periodontal pathogenic microorganisms, response by host immune system also exacerbates the situation causing severe inflammation and damage of tissues that surrounds and supports the teeth (Kornman, 2008; Silva *et al*., 2015).

Besides anaerobic bacteria, other factors contributing to pathogenesis of periodontal diseases include smoking, diabetes, immunocompromised state such as acquired immunodeficiency syndrome and chemotherapy, increased intake of sugary food and hormonal changes (Piscoya *et al*., 2012; Vogt *et al*., 2012). Pregnancy and pregnancy related hormonal changes influence the periodontal tissues e.g. elevated estrogen and progesterone production in pregnancy increases blood flow towards gingival tissue and decrease immune response thus facilitating in periodontal disease development. Various studies report the prevalence of gingivitis in pregnant females ranges between

35% to 100% (Dhaliwal *et al*., 2013; Patil *et al*., 2013; Wu *et al*., 2013; Onigbinde *et al*., 2014).

Several studies are available in literature which showed role of periodontal diseases in induction of APOs (Sanz and Kornman, 2013). Periodontal bacteria are proposed to induce APOs either by initiating intrauterine immune response by enhancing production of several inflammatory chemokines and cytokines or by directly entering the foetal circulation via crossing placental barrier or by direct action of lipopolysaccharides (LPS) on the foetus. Studies are available, which showed the presence of oral commensals such as *Streptococcus, Fusobacterium, Prevotella, Neisseria,* and *Porphyromonas* in human and murine placenta. Close similarities were found between maternal oral and placental microbial diversity, indicating the presence of low grade bacteraemia, which worsen in case of periodontitis and lead to APOs (Mcgaw, 2002; Sampaio-Maia *et al*, 2016; Gomez and Nelson, 2017; Kruse *et al*., 2018; Kumar, 2017).

Previously, concept of sterile womb was common, and the presence of microbe in placenta was considered as indication of infection. But now it is evident from various studies that microbiome acquisition starts during intrauterine phase of foetal development. Placenta harbours a low abundance and non-pathogenic commensal microbiota, composed of five main phyla including Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes, and Fusobacteria (Aagaard *et al*., 2016; Underwood and Sohn, 2017). Currently, most of the studies available are based on vaginal samples investigation in which link between changing microbial diversity and pregnancy outcomes is asserted. These studies suggest that majority of intrauterine infections are originated by the infectious agent ascending from genital tract to placenta. But an increasing number of studies reported that these agents are mostly commensal to maternal oral cavity (Fardini *et al*., 2010; Mysorekar and Cao, 2014; Corwin *et al*., 2017; Neuman and Koren, 2017). Pregnancy compromised the immune status of females, which can increase risk for development of oral disorders (Robinson and Schmerman, 2015). Other possible risk factors for oral disorders in these females include oral acidic environment, hormonal shift, cravings for carbohydrate rich diet and limited oral health care (AL-Sultani, 2013).

Limited studies are available that confirms the increased risk of dental caries in pregnancy (Alneamy, 2018). Change in composition of saliva, recurrent gastric reflexes, and poor oral health are the main triggering factors that increases the risk of caries during gestational period (Siddiqui *et al*, 2018). It is one of the most prevalent oral disorder and human remain susceptible to develop dental carries throughout their life. In case of excessive sugar intake, saliva loses its buffering capacity and decrease in pH lead to the tooth destruction. These conditions favour the colonization of aciduric bacteria like *Streptococcus mutans* and species of lactobacilli. *Bifidobacterium, Scardovia* and *Propionibacterium* species have also found to be associated with dental caries. *S. salivarius* act antagonistically to these cariogenic bacteria by production of ammonia from arginine to maintain homeostasis (Duran-Oinedo and Frias-Lopez, 2015; He *et al*, 2015).

Pregnancy outcomes are of great concern in health care settings worldwide, most importantly in less developed regions. Both economically established and under-developed parts of the world are at a risk of facing APOs. Despite being a physiologically healthy process, pregnancy has possible risk of experiencing adverse complications such as PTB, LWB, still birth, preeclampsia and abortion. The pregnancy related complications adversely affect both mother and child's health and can also be a risk factor for the development of chronic diseases in future (Sanz and Kornman, 2013).

PTB and LBW are major perinatal problems across the world and their incidence is increasing with the passage of time. After pneumonia, PTB is the second most common cause of death in infants of age less than five years and its rate is still increasing in many countries (Chang *et al*., 2014). Asian and sub-Saharan African countries are responsible for more than 60% of PTB. The World Health Organization (WHO), has reported deaths of about 1.1 million infants each year from problems associated with PTB. Despite advances in the field of medicine, APOs especially PTB and LBW are frequently associated with prenatal mortality and morbidity (Blencowe *et al*., 2013; Satterfield *et al*., 2016).

Several risk factors responsible for causing preterm low birth weight (PLBW) have been identified such as maternal related infections either intrauterine or extrauterine, gestational diabetes, socioeconomic factors, smoking, poor maternal health, stress and

drug use. But fundamental mechanism responsible for severe pregnancy related outcomes is not known (Alijahan *et al*., 2014; Padhi *et al*., 2015). During infection of uterus, microorganisms and their products activate innate immune system, as immune system recognize the pattern recognition receptors. This stimulate the release of pro-inflammatory mediators including interleukin (IL)-1β, IL-8, IL-1, IL-6 and tumour necrosis factor (TNF)-α. These cytokines and chemokines stimulate production of prostaglandins (PGE-2) and enzymes for extracellular degradation like matrix metalloproteinases (MMPs). As a consequence, contraction starts in the muscles of uterus and extracellular matrix of membranes surrounding foetus also degrades, which lead to preterm premature rupture of membranes (PPROM) that ultimately results in preterm delivery (Agrawal and Hirsch, 2013).

As PLWB is becoming a major issue, more emphasis is given to the maternal health care and focus of attention is on maternal oral health during pregnancy (Boggess and Edelstein, 2006). The association between PTB, periodontal diseases and microflora of oral cavity has been investigated in many studies (Offenbacher *et al*., 2001; Lin *et al*., 2003; Xiong *et al*, 2007; Polyzos *et al*., 2010; Han, 2011; Jacob and Nath, 2014). Moreover, with reference to 'focal infection theory', infections of oral cavity are correlated to birth outcomes (Pizzo *et al*., 2010).

Most of the previous studies focusing on pregnancy-associated oral microbial diversity and it's association with oral health problems leading to APOs, were culture or PCR-based identification of selected anaerobic periodontopathogenic microbes in oral cavity or placenta. Limited data is available in the literature related to aerobic culturable resident microflora and oral microbiome in pregnant females especially in early postpartum phase. Until now, no literature is available on the identification and assessment of salivary culturable and unculturable microbial diversity in early postpartum females with oral health issues and its possible association with pregnancy outcomes. The present study was carried out with the focus on use of culture-based identification method for oral culturable and unculturable microflora by high throughput sequencing platform to find the salivary microbial diversity in early postpartum females.

HYPOTHESIS

Throughout pregnancy, female body undergoes various physiological and hormonal changes including change in oral microbiota. This change in oral flora can be pathogenic especially at the end of pregnancy and lead to the development of various oral disorders which could affect pregnancy outcomes.

AIM AND OBJECTIVES

The present study was carried out with the aim to determine the culturable and unculturable bacterial and fungal species in oral microbiome of postpartum females in Pakistani population. Objectives of the study were;

1. To study demographic, oral health and obstetric characteristics of postpartum females
2. To study Mutans streptococci in postpartum females as indicator of oral health status
3. To study culturable and unculturable oral bacterial diversity and its pathogenic potential in postpartum females
4. To study culturable and unculturable oral fungal diversity and its pathogenic potential in postpartum females
5. To study fungal-bacterial interaction in dual-species biofilm for pathogenesis

CHAPTER 2: LITERATURE REVIEW

Pregnancy is a phase of complex physiological, biological and molecular changes in women's life. These changes can lead to either normal or APOs depending upon foeto-maternal general health status (Muwazi *et al*., 2014; Dunlop *et al*., 2015). In developed countries, mostly females are less prone to have pregnancy related complication during and after gestation period. There is awareness about the importance of maintaining good maternal health and its impact on pregnancy outcomes among these females. Whereas in developing countries, gradual rise in foeto-maternal death is becoming important along with pregnancy related complications such as PTB, LWB and preeclampsia. These females encounter several challenges especially poor nutrition and lack of medical health facilities due to high cost of treatment, limited number of hospitals, doctors and paramedical staff especially in rural areas with inadequate treatment facilities, insufficient lab facilities and lack of instruction for patients to locate OPDs, wards and labs in hospitals (Kramer, 2003). Maintaining good oral health during pregnancy is also important, because it is a portal of entry for microbes and is considered as a key factor for affecting the general health of the expecting mothers (Anil *et al*., 2015). Pathogenic microbes and their metabolic by-products produced in oral disorders can cross oral mucosal barrier and cause infections at a places in close vicinity or remote from the oral cavity (Barnett, 2006). During pregnancy periodontal disorders, gingival hyperplasia, gum lesions, dental caries and salivary changes are commonly seen, that are linked with several systemic infections such as APOs (Tarsitano and Rollings, 1993; Robinson and Schmerman, 2015).

2.1- Periodontal diseases

Periodontal disorders are responsible for causing inflammation of the teeth surrounding and supporting tissues (Jared and Boggess, 2008). These including gingiva, alveolar bone, and periodontal ligament, all these structures are collectively called periodontium. Gram negative bacteria either directly or indirectly by production of their toxins contribute to disease pathogenesis (Hoogmoed *et al*., 2008; Muwazi *et al*., 2014). In gingivitis, microbial flora shifted from commensal *Streptococcus* species to *Actinomycetes, Eikenella, Prevotella, Capnocytophaga, Fusobacterium* and *Campylobacter* (Zaura *et al*., 2009). These species penetrate deep

in tissues surrounding the teeth and cause destruction of alveolar bone, gums, periodontal ligament and cementum resulting in a tooth loss (Pihlstrom *et al*., 2005; Ferguson *et al*., 2007). Gingivitis most commonly affect the population, whereas periodontitis also affects approximately 10-15% of the total population (Preshaw *et al*., 2012).

Dramatic shift in symbiotic microbial community can leads to disease state from healthy one. Health associated oral microbial community is mainly composed of genera *Actinomyces* and *Streptococcus*, while in dysbiosis it is dominated by anaerobic genera belonging to phyla Proteobacteria, Bacteroidetes, Firmicutes, Spirochetes, and Synergistetes. Periodontitis associated bacteria mainly reside in gingival crevice. In addition to dysbiosis, host genotype, stress, diet, and behaviour also play role in progression to periodontitis. Various cytokines and their receptors like IL-1β, IL-6, TNF, FcγIIA receptor, C5, WNT5A and CD14 are suggested to be involved in disease development and progression (Hajishengallis, 2015).

Next generation sequencing (NGS), reveals that there are also a range of microbes other than traditionally considered pathogens, which are associated with periodontitis including *Treponema forsythia* and *Treponema denticola*, species of *Bacteroidetes, Saphenum, Eubacterium, Porphyromonas endodontalis, Peptostreptococcus* species, *Porphyromonas denticola*, *Parvimonasmicra*, *Dialiste*r species, *Desulfobulbus* species, *Filifactoralocis,* and *Synergistetes* species. Whereas *Streptococcus, Abiotrophia, Veillonella, Campylobacter, Gemella, Capnocytophaga*, and *Neisseria* which are normally considered as beneficial bacteria. A surprising result were seen in a study on aggressive periodontitis, where *Selenomonas* was predominant genera in subgingival plaque but *A. actinomycetemcomitans* were not detected, which was previously believed to be the main periodontopathogenic bacteria (He *et al*., 2014).

In periodontal inflammation, dental plaque bacteria invade gingival tissue and enter systemic circulation. Resulting bacteraemia and increased levels of inflammatory mediators in circulation leads to inflammation also in other parts of the body. Studies reports that there are elevation of serum C-reactive protein and IL-6 levels in patients with periodontitis (Arimatsu *et al*., 2014; Tonetti *et al*., 2018). Periodontal disease is mainly explored for its bacterial dysbiosis, however, few studies also documented role of yeast in such conditions. Yeast is the normal oral flora and is frequently detected in

different oral sites (Raja *et al*., 2010; Canabaro *et al*., 2013) Despite polymicrobial nature of periodontal disorders, role of yeast in periodontal disease has not gain much attention, it is still underappreciated and less studied (Machado *et al*., 2010).

2.1.1- Pregnancy as a risk factor for periodontal diseases

Pregnancy predisposes approximately 60-70% of the females to develop gingivitis in gestational period (Dommisch *et al*., 2015; Maybodi *et al*., 2015). Alterations in levels of two hormones i.e. estrogen and progesterone are the main causative factors for gingival inflammation. These hormones also produced at high level in pregnancy and are seen to affect healing process and promote development of periodontal disease by increased gingival vascularization and decreasing immunity (Apoorva and Suchetha, 2010). Periodontopathogenic *Prevotella* species showed increased growth in response to secretion of these reproductive hormones (Baskaradoss *et al*., 2012; Alchalabi *et al*., 2013). Gingivitis commonly effects females in 2nd month of pregnancy and severity progresses towards the end of gestational period of gestation (Gajendra and Kumar, 2004; Pirie *et al*., 2007).

Improper hygienic conditions and compromised oral health leading to the progression of gingivitis to periodontitis, which can result in the loss of tooth supporting structure. Approximately 30% of the females during pregnancy suffer from periodontitis (Newnham *et al*., 2009; Amini and Casamassimo, 2010). Alterations of minerals levels in tooth supporting structures lead to teeth loss, which is called tooth mobility (Hunter and Hunter, 1997). This loss can also occurs because of effects of increased progesterone and estrogen levels on periodontium (Boggess and Edelstein, 2006). Hormonal alterations in combination with bacteria and local irritants can also cause gums lesions called pregnancy granulomas in 0%-9.6% females (Laine, 2002; Torgerson *et al*., 2006; Silk *et al*., 2008). These lesions are erythromatous and smooth in nature. Initially these lesions are produced on gingiva but later also affects soft palate, buccal mucosal surfaces and tongue. Pregnancy related tumours are more prevalent during first trimester (Sills *et al*., 1996). Frequent vomiting induced decrease in oral pH that can also cause tooth erosion, which is another periodontal problem (Giglio *et al*., 2009).

2.1.2- Prevalence of periodontal diseases

Occurrence of periodontitis varied between different countries and even among inhabitants of same country. Reduction in the rates of periodontal diseases over the time are reported in adults from developed countries, however, condition is still prevalent in developing countries due to poor oral hygiene (Petersen and Ogawa, 2005).

Dental health status is assessed by determining the Decayed Missing Filled Teeth (DMFT) index as recommended by WHO. A National Health Survey of Pakistan in 2008, reported high DMFT index scores in females as compared to men of age ranging between 24-30 years. High value of the indicator was associated with poor dental hygiene (Mobeen *et al*., 2008). Shahzad *et al*., (2015) conducted a study in which reported prevalence percentage of periodontitis of around 18.2% among females in comparison to males. After gingivitis and dental caries, among periodontal disorders, periodontitis is found to be the third most common disease affecting population. High prevalence of gingivitis in females as compared to males was also reported from Islamic International Dental Hospital (Pakistan), where gingivitis and periodontitis prevalence was present in 69% and 31% of the study subjects, respectively (Ali *et al*., 2012).

2.2- Dental caries

Dental caries is the commonly seen dental lesion, human are susceptible to it throughout their life. It not only causes tooth decay but also cause periapical and pulp infections. *S. mutans* is commonly studied as etiological agent of dental caries but studies have also shown other genera like *Streptococcus, Actinomyces, Veillonella, Granulicatella, Thiomonas, Leptotrichia, Prevotella* and *Bifidobacterium* to be associated with severe early childhood caries. In adults with caries, plaque predominantly contain members of genera *Streptococcus, Actinomycetes, Lactobacillus, Veillonella* and *Propionibacterium.* In elders, genera *Atopobium, Pseudoramibacter, Olsenella, Selenomonas,* and *Propionibacterium,* which are involved in the initiation and progression of root caries (He *et al*., 2015). Another study reported that *Rothia aeria, Veillonella parvula,* and *Rothia dentocariosa* ratios may be used as indicators of bad oral hygiene status and caries risk in population

(Mashima *et al*., 2019). Al-hebshi *et al*., (2018), found a strongest association between caries and species of *Prevotella, Veillonella*, also yet unnamed *Actinomyces*, and *Atopobium* in children.

Mature dental biofilm communities commonly harbour *S. mutans*, which under favourable condition become pathogenic (Kreth *et al*., 2005). These cariogenic microbes cause dental caries by acid production from fermentation of dietary carbohydrates and formation of branched extracellular polysaccharides (glucans), which help in trapping acidic metabolite in matrix. pH reduction due to acid production lead to initiation of dental caries development, which is later involved in the dissolution of tooth enamel and dentine followed by formation of cavities (Tagg and Dierksen, 2003). Demineralization occur approximately at pH of 5.5. Caries development depends upon bacterial profile, tooth susceptibility, quality and quantity of saliva and time duration available for dietary carbohydrates fermentation by bacteria (Pannu *et al*., 2013).

In addition to commonly associated cariogenic bacteria and other bacteria detected by sequencing, studies are available based on culture based methods for identification of less frequently detected and studied microbial species associated with dental caries. Altayyar *et al.,* (2015), conducted a study to identify aerobic bacteria from dental plaque to decipher their role in the etiology of gingivitis and dental caries. In total 110 species were isolated, out of which 84.5% were Gram-positive consisting of *Streptococcus* species 39%, *Lactobacillus* 24.5%, *Staphylococcus* 21% and 15.5% Gram-negative bacteria including *E. coli* 6.4%, *Enterobacter* 5.5% and *Proteus* 3.6%.

Recently, *Candida* species are also identified as potential cariogenic candidate as these are isolated from caries lesions of children and adults in high frequency. Like mutans streptococci (MS), *Candida* is also acidogenic and aciduric as it produces mainly pyruvic acid and acetic acid. Additionally their ability to adhere different surfaces by their biofilm development, production of large number of proteinases (facilitates collagenolysis) and invasion of dentinal tubular region makes *Candida* a potent cariogenic pathogen (Fakhruddin *et al*., 2018).

During the last three decades, prevalence of dental caries has decreased in industrialized countries but in young population especially in children rates are still high. In developing countries, majority of population consume more free sugar and

carbohydrates, which are fermentable along with non-availability of fluoride, there is still increasing caries prevalence (Pannu *et al*., 2013).

2.2.1- Pregnancy as a risk factor for dental caries

Dental caries commonly affects females during reproductive age. Pregnant females are at high risk for tooth decay due to increased acidity in oral cavity, frequent vomiting, recurrent gastric reflux, dietary changes and poor oral hygienic practices (Berkowitz, 2006). Acidic environment produced by these bacteria rapidly cause hydroxyapatite crystals demineralization from tooth enamel and induced proteolytic breakdown of tooth hard tissue structures (Hicks *et al*., 2003; García-Godoy and Hicks, 2008; Marsh, 2012). Loss of calcium phosphate and carbonates from tooth ultimately lead to creation of caries cavity. This process can be sometimes reversed by tooth remineralization with calcium, phosphate and fluoride (Marsh *et al*., 2011). Salivary components including levels of calcium ion, phosphate, fluoride and salivary antibacterial components play important role in initiation, progression and inhibition of caries (Kidd and Fejerskov, 2004; García-Godoy and Hicks, 2008).

2.3- Systemic disorders associated with oral local infections

Oral bacteria are associated with number of non-oral disorders such as ischemic stroke, cardiovascular diseases, cancer, bacterial endocarditis, paediatric Crohn's disease, pneumoniae (Krishnan *et al*., 2017), diabetes type 2 and Obesity (Lu *et al*., 2019). Studies are available that linked role of oral hygiene and oral flora associated inflammation with various cancers like pancreatic cancer and colorectal cancer (Fan *et al*., 2017). Likewise, in cases with periodontitis chronic inflammation is seen which predisposes individuals to develop cardiovascular disease (CVD). Oral pathogens contribute to CVD by deregulation of immune response, progressive inflammation and endothelial cell surface disruption. Poor oral hygienic conditions also showed association with infective endocarditis and atherosclerosis leading to stroke and myocardial infarction due to bacteraemia (Slocum *et al*., 2016; Samaranayake and Matsubara, 2017). Several anaerobic oral species such as *A. actinomycetemcomitans* and *S. constellatus* have been found to predispose individual to develop pneumonia (Krishnan *et al*., 2017). In Alzheimer's disease, periodontal associated inflammation is now recognized for their possible role as key player for the disease pathology (Shoemark and Allen, 2015).

2.3.1- Role of periodontitis in induction of APOs

Pregnant females are more susceptible to develop periodontal diseases (Saini *et al*., 2009). Role of periodontal disorders in induction of PTB, LWB and other pregnancy complications like preeclampsia, gestational diabetes mellitus and neonatal mortality was first reported by Offenbacher *et al*., (1996). PTB is induced by activation of innate immune response against maternal periodontal infections. Immune system together with matrix degrading enzymes caused PPROM and can ultimately induce preterm delivery (Anil *et al*., 2015).

2.3.1.1- Epidemiology and causes of PTB

PLBW is one of the leading cause of increased new born mortality and morbidity affecting babies worldwide. PTB is the birth of the baby before 37 weeks of gestation, while LWB is the birth of a baby with weight less than 2500 g at the time of delivery may be due to term or PTB (Lux, 2007; Chang *et al*., 2014). Globally, every one out of ten babies is born prematurely every year (Blencowe *et al*., 2012). PTB is the second leading cause of neonatal death after pneumonia. These infants are more susceptible to suffer from respiratory, neuro-developmental complications, gastrointestinal disorders, ear infections, asthma and congenital anomalies (Mcgaw, 2002; Oliver and Lamont, 2013). PTB can occur because of spontaneous preterm labour, PPROM, eclampsia, preeclampsia and due to foeto-maternal infections or inflammations (Staneva *et al*., 2015). In PPROM, amniotic membranes containing foetus rapture before 37 weeks of gestation (Mercer *et al*., 2006). Normally in full term birth (FTB), membrane rapture is mediated by enzymatic activity and by apoptosis but infection associated elevated cytokines are also associated with PPROM inducing PTB (Behrman and Butler, 2007).

Etiology of PTB is multifactorial, however exact mechanism for PTB induction is still unknown. Commonly known causes for PTB includes vaginal bleeding, cervical insufficiency, uterus damage due to inflammation, urinary tract infections (UTIs), nutrient and oxygen deficiency for foetus in placenta, and stress induced hormonal imbalance. In addition to these factors, demographic and behavioural characteristics also play role in PTB such as age at the time of pregnancy (<17 years to >35 years), smoking, alcohol and drugs use, low socio-economic status, ethnicity, multiple births,

poor prenatal care, maternal systemic disorders and nutritional deficiency or malnutrition (Menon *et al*., 2011). Studies are available that showed the role of ethnicity with PTB as well (Patel *et al*., 2004; Ananth and Vintzileos, 2006;)

PTB risk is high in black women (African-American) compared to white (American) with rates range between 16-18% and 5-9%, respectively (Varner and Esplin, 2005; Kistka *et al*., 2007). High rates of LWB are reported from South Asian countries including sub-continent females (George *et al*., 2009). Females from under-developed countries having low educational status, who experience stress and anxiety are more likely to suffer PTB (Smith *et al*., 2007). Low BMI, nutritional insufficiency such as low concentrations of iron and zinc plus systemic inflammatory response along with smoking can induced spontaneous PTB (Bermudez *et al*., 2002; Goldenberg *et al*., 2008). Intrauterine and extrauterine infections are both important causes of PTB and accounting for 25-40% of the cases (Lahra and Jeffery, 2004). Intrauterine infection can originate in foetus, within foetal membranes, between foetal membranes and uterus, amniotic fluid and in umbilical cord (Goldenberg *et al*., 2000; Kemp, 2014). Bacterial vaginosis, vaginal candidiasis, maternal systemic infections by rubella, pneumonia, malaria, encephalitis and pyelonephritis are the common conditions associated with pathophysiology of PTB (Romero *et al*., 2006; Sharma and Thapa, 2007; Sobel, 2007).

2.3.1.2- Pathogenesis of periodontal infection induced PTB

Exact mechanism of periodontal induced PTB is still unclear, however, there are different concepts that explaining the pathophysiology of periodontal diseases leading to PTB. In case of maternal infections inflammatory mediators and cytokines raised abnormally and can induced labour preterm delivery. These mediators are also present in normal pregnancy, but their levels raise gradually towards the end of pregnancy which induces a normal full term delivery (Cruz *et al*., 2009). How levels of these inflammatory molecules rise to pathogenic levels for PTB induction is still unclear, however, few proposed mechanisms are either by direct transfer of microorganism to the foeto-placental unit, or by action of mediators or by action of the microbial lipopolysaccharide induced inflammatory response (Lux, 2007). Oral microbes or their by-products spread through hematogenous route and cause activation of maternal immune response. Peripheral blood monocytes get activated and initiate production

of cytokines such as IL-1β, IL-6, TNF-α, PGE2 and MMP (Madianos *et al*., 2013). Which in turn cause increase in levels of serum TNF-α, decrease production of IL-10 in liver and uterus while in amniotic fluid increase in the level of PGE2 and TNF- α. All these responses together induce uterine muscles contraction and foetal membrane extracellular matrix degradation by MMPs, that results in PTB (Lin *et al*., 2003).

2.3.1.3- Periodontal disease linkage to PLWB

The numbers of studies are available in literature, which showed link between periodontitis and PLWB (Offenbacher *et al*., 1996; Mitchell-lewis *et al*., 2001; Kotz *et al*., 2009; Pozo *et al*., 2016). Periodontal infections predispose seven-fold risk for PTB in females. These females also have high levels of periodontal pathogens in oral cavity. A study reported high (42.7%) prevalence of periodontitis in females with PLWB (Cruz *et al*., 2009). Significant association between periodontitis and PLWB was also reported by Mokeem *et al*., (2004) in postpartum females. In study, where postpartum females with PLWB were compared with females with FTB. Moreover, chronic periodontitis can also adversely affect the maternal haemoglobin levels resulting in anaemia that ultimately affects the foetal development (Kothiwale *et al*., 2014).

Dörtbudak *et al*., (2005), reported the presence of elevated concentrations of PGE2, IL-1β and IL- 8 in the amniotic fluid of pregnant females suffering from periodontal infections. Role of inflammatory mediators raised by dental infections in PTB induction is also studied by using animal models. One such study found increased concentration of inflammatory cytokines at the infections site and in amniotic fluid of pregnant hamsters which compromised the development of foetus (Bobetsis *et al*., 2006). However, there are studies which showed no significant association between periodontitis and PLWB (Mumghamba and Manji, 2007; Michalowicz *et al*., 2009; Srinivas *et al*., 2009; Abati *et al*., 2013).

2.4- Oral microbiome

Before the completion of Human Genome Project (HGP), researchers estimated that there are approximately 100,000 genes in human body. But the findings from HGP were surprising, that human body only harbours approximately 20,000 protein coding

genes (Turnbaugh *et al*., 2007). But now studies of microbiome showed that human also harbours trillions of microorganisms that either lived inside or outside the human body, their number is of the same order of magnitude as the human cells. Collectively all these microbes are called human microbiome. Human microbiome contains genomes from all the major domains of life including bacteria, fungi, archaea, protists and viruses. Genomes of these microbes provides the human body with the traits, that human did not evolve on their own. They help their human host in nutrient absorption, modulation of immune system and protection against pathogenic microbes. Human cells along with their microbial associates, genes from human genome and from microbiome, human metabolic characteristics influenced by human and microbial traits, all these features collectively are present in the human are called "super-organism" or "holobiont" (Blaser & Dominguez-bello, 2016; Vogtmann and Goedert, 2016; Davenport *et al*., 2017; Ferretti *et al*., 2018).

Human body provides various ecological niches for microbial colonization including skin, oral cavity, gastrointestinal, urogenital and respiratory tract. Most of the human microbiome studies are focused on gut with fewer number of studies exploring vaginal, oral and skin microbiome. Microbial composition of each niche is distinct and preferential. Microbiome diversity can change rapidly, but community features remain stable for years. To avoid innate immune response against these commensal microbes, microbial sensing is tightly regulated to ensure symbiotic relationship between host and microorganisms. In the absence of innate immune recognition of commensals, there is a loss of host-microbe mutualistic association, which consequently leads to dysbiosis and health consequences. Therefore, crosstalk between innate immunity and microorganisms is important to ensure homeostatic balance between health and disease (Blekhman *et al*., 2015; Thaiss *et al.*, 2016; Lloyd-price *et al.*, 2017). Whenever, there is dysbiosis these organisms can lead to many diseases like inflammatory bowel disease such as Crohn's disease, necrotizing enterocolitis, type1 and 2 diabetes, different type of cancers and asthma (Ahn *et al*., 2012; Ferretti *et al*., 2018).

Early microbiome research was mainly focused on defining "core microbiome", present in all healthy individuals, and it was hypothesized that absence of which was considered as source of dysbiosis and associated with pathology, however, studies showed significant inter-individual variations. Even at shared taxa level, greater

differences in magnitude of abundance is seen between healthy individuals. Thus, characterizing a core microbiome is an ideal set of microbes is practically not feasible (Lloyd-price *et al*., 2016). Only a small number of human microbial world has been yet isolated, very little is known about the species that are unculturable. However, with the advances in metagenomics, NGS approaches, expanding computational and bioinformatics tools, now it become possible to directly explore this "microbial dark matter" including unculturable and yet not detected microbes, but still understanding about their functions is poor (Kuleshov *et al*., 2016; Takahashi, 2015; Solden *et al*., 2016; Vogtmann and Goedert, 2016). Most recent studies from comparative genomics showed significant genomic difference in the strains of the same species among different individuals, which can lead to pathogenicity. Metagenomics showed little individuality at specie level, but at sub-specie level high individual specificity is seen, making it problematic to study bacterial stability over time (Mukherjee *et al*., 2018).

Oral microbiome is the second most diverse microbial community comprising both of culturable and unculturable microbiota. Predominant oral bacteria belong to genus *Streptococci, Lactobacilli, Staphylococci* and *Corynebacterium.* These microbes mostly live as biofilms which develop on the teeth, tongue, gingival sulcus, and buccal mucosa. Whenever there is a local or systemic infections, excessive medicines intake modifies this microbial pattern and unusual microorganisms begin to prevail and some normal microbes such as *Staphylococcus* became pathogenic (Dewhirst *et al*., 2010).

Predominant fungal genera in healthy oral mycobiome mainly includes *Candida, Aureobasidium, Cladosporium, Aspergillus, Fusarium, Saccharomycetales* and *Cryptococcus* (Ghannoum *et al*., 2010). *Candida* species are a group of opportunistic pathogens and are part of normal flora of mouth. In healthy individuals, they do not cause any harm but in compromised host, infection can occur (Kraneveld *et al*., 2012). Their isolation frequency is low in healthy individuals (Bharathi *et al*., 2012). *Candida* is usually found at buccal mucosa, dorsum and lateral side of tongue, surface of tooth, hard and soft palate and denture bearing surfaces. Their colonization on different mouth surfaces is facilitated by low salivary flow rate and pH, diet rich in carbohydrates, loss of epithelial layer and several proteins such as agglutinin and integrin-like proteins that help in the development of biofilm structures (Cannon and Chaffin, 1999; Siar *et al*., 2003)

2.4.1- Staphylococcal species

Despite frequent isolation of *Staphylococcus* species from the human oral cavity and its pathogenic potential, little attention is paid to oral carriage of these isolates in health and disease (Smith *et al*., 2001). In health and immunocompetent state, they exist as normal flora but can cause infections in an immunocompromised condition. They possess several types of virulence factor which are involved in pathogenesis of many disorders including pneumonia, endocarditis and septicaemia (Loberto *et al*., 2004). *S. aureus* is responsible for causing serious life-threatening infections by entering systematic circulations, lower respiratory tract, skin and soft tissues. *S. aureus* can attach and gain access to tissues by secretion of several toxins and exoproteins such as lipoteichoic acid, toxic shock syndrome toxin-I and staphylococcal enterotoxin A and B. In addition to these factors, *S. aureus* also express other pathogenic factors like leukocidin, Protein A and capsular polysaccharide (Pauli *et al.,* 2014).

Oral cavity also showed high carriage rate for coagulase negative staphylococcal species (CoNS) (Rams *et al*., 1990; Loberto *et al*., 2003). *S. epidermidis* is frequently associated with nosocomial, medical implant devices and catheters associated infections. It is emerging as a potential pathogen because of its biofilm forming ability (Cafiso *et al*., 2004). Both *S. aureus* and *S. epidermidis* possess *Ica* gene operon, required for the polysaccharide adhesion molecules production. Its production is predominant under *in vivo* condition as compared to *in vitro*. *Ica* operon show phase variation. Alteration of virulence genes regulation is achieved by altering activity of regulatory proteins, local genomic re-arrangements, transcriptional and translational modulation by strand slippage mechanisms (O'Gara, 2007).

Bueris *et al*., (2005) checked the oral incidence of *S. aureus* in three healthy Brazilian families and found high level of colonization of *S. aureus* (56.2%) and 25.9% of the individuals had methicillin-resistant *S. aureus* (MRSA) in their oral cavity. In another study, high salivary carriage rate of *S. aureus* (43%) was seen in children (Petti *et al*., 2014). Cuesta *et al*., (2010) conducted a study on periodontal patients from Argentina and found 69.5% patients were positive for Staphylococcal species in oral cavity with 15.8% prevalence of *S. aureus*. Ohara-Nemoto *et al*., (2009), studied salivary colonization of *Staphylococci* in 56 healthy adults from Japan. *Staphylococcus* species

were detected in 83.9% of the cases and bacterial count was 10^2-10^4 CFU/mL. *S. aureus* was the most prevalent species found in 46.4 % cases, followed by 41.1% of *S. epidermidis*, and the frequency of other species was 1.8 to 12.5 %.

2.4.2- Mutans streptococci (MS)

S. mutans and *S. sobrinus* (MS) are identified as primary cariogenic bacteria (Varenne *et al*., 2004). Dental caries develop as a result of microbial imbalance in dental plaque biofilms (Marsh and Percival, 2006). Some studies have indicated the role of sucrose and glucosyl transferases (*Gtfs*) in biofilm formation by *S. mutans*. *Gtfs* synthesize glucan on the tooth surface, which provide enhanced binding sites for MS (Hayacibara *et al*., 2004). Both *S. mutans* and *S. sobrinus* are known for *Gtfs* production (Song *et al*., 2006). *S. mutans* produced three genetically distinct *Gtfs*, which are involved in production of structurally distinct glucan from sucrose. Each enzyme plays distinct role in dental plaque development. *Gtfs* can also bind many other oral bacteria with each other (Ooshima *et al*., 2001; Mattos-graner *et al*., 2004; Koo *et al*., 2010). Elevated levels of insoluble glucans in plaque also significantly reduced the levels of inorganic ions especially calcium, phosphorous and fluorine in the matrix (Cury *et al*., 2000). In addition to *Gtfs*, extracellular dextranase (*dex*) produce by both MS also influenced nature and amount of glucan in plaque (Igarashi *et al*., 1995; Otsuka *et al*., 2015). Dextranase catalyses the breakdown of glucans by cleaving 1–6 linkages within the dextran chain (Walker *et al*., 1981; Hayacibara *et al*., 2004).

Most studies available in literature are based on isolation of MS from children reporting prevalence rate ranging from 35.4%-85.9% for *S. mutans* and 14.3%-78.9% for *S. Sobrinus* (Franco *et al*., 2007; Okada *et al*., 2012; Sánchez-acedo *et al*., 2013). A study carried on outpatients from hospital of Japan showed 78.7% and 83.5% prevalence for *S. mutans* and *S. sobrinus* in oral cavity, respectively (Oda *et al*., 2015). An Indian study, reported a significantly high prevalence of *S. mutans* (92%) in females compared to male (82%) (Pannu *et al*., 2013).

2.4.3- *Candida* Species

Candida species normally exist as commensals but are also potent opportunistic especially in an immunocompromised host. *Candida* is one of the leading cause of

nosocomial infections in United States (US) (Pfaller and Diekema, 2007; Pfaller and Diekema, 2010). *C. albicans* is the most important member of this genus causing both superficial and systemic infections (Calderone and Fonzi, 2001). *C. glabrata* is also an emerging opportunistic pathogen due to use of immunosuppressive therapy, broad-spectrum antibiotics and indwelling medical devices (Rodrigues *et al*., 2014). *Candida* species express various virulence factors including phenotypic switching, adhesins mediated surface attachment, biofilm forming ability and expression of various hydrolytic enzymes (Silva *et al*., 2012).

To establish infections, first step for the colonization of *Candida* species is adherence to host surfaces which is followed by biofilm development (Ramage *et al*., 2006). *Candida* species can form antibiotic resistant biofilms on both abiotic and biotic surfaces (Verstrepen and Klis, 2006). Biofilm formation by *Candida* is associated with high risk for morbidity and mortality (Kumamoto, 2002). *C. albicans* express more biofilms forming ability as compared to other *Candida* species (Fanning and Mitchell, 2012). Type of fungal specie, strain, environmental conditions and host immune response influence pathogenesis of *Candida* biofilms (Jain *et al*., 2007).

After establishing on host surface, for dissemination *Candida* species secrete hydrolytic enzymes including phospholipases, proteinases, esterases and hemolysins (Mayer *et al*., 2013). Esterase is a lipolytic enzyme that hydrolyses the ester bonds of lipid components of host's cell such as monoacylglycerols, di-and tri acylglycerols plus of phospholipids that facilitate pathogens to adhere, penetrate and invade host cell (Koga-Ito *et al*., 2006). Phospholipases are secreted by various species of *Candida* genus and act by hydrolysis of membrane phospholipids. The four types of phospholipases present in *C. albicans* include PLA, PLB, PLC, and PLD (Pawar *et al*., 2015). Such lipases promote oral epithelial cell adhesion, invasion and also affects different signalling cascades (Nasution, 2013). *Candida* species exhibit 90% of phospholipase activity due to PLB (Moris *et al*., 2008). Most of the *Candida* species produce aspartyle proteinase, which cleaves the host cell proteins and also cause damage to host immune cells (Schaller *et al*., 2005).

Candida commonly isolated from dental plaque associated with destruction of periodontal pockets especially in immunocompromised individuals (Lamster *et al*., 1998; Jabra-rizk *et al*., 2001; Song and White, 2003). A study showed 17%

prevalence of *Candida* in subgingival area of periodontitis patients from Norway (Reynaud *et al*., 2001). *C. albicans* has been identified in the subgingival samples in both healthy and in those individuals suffering from periodontal disorders (Nejad *et al*., 2011). *C. albicans* is isolated from patients of chronic periodontitis with prevalence ranging from 7.1% to 19.6% (Slots *et al*., 1988; Reynaud *et al*., 2001). Females are commonly affected by *Candida* species compared to men (Lyon *et al*., 2006; Dudko and Kurnatowska, 2007), because of enhanced mucosal inflammation (Figueiral *et al*., 2007; Urzua *et al*., 2008).

2.4.5- Factors affecting the oral microbial diversity:

Our understandings of changing microbial composition and its effects on human phenotypes is still a work in progress. During past few decades, ancient microbiota showed significant change in comparison to modern cosmopolitan microbial diversity. This shift is due to changing lifestyle, dietary habits, antimicrobial use, environmental changes and due to genetic factors (Dagli *et al*., 2016; Demmitt *et al*., 2017). Human microbiome is composed of both core and variable microbiome. Variable microbiome is individual specific affected by various host related and environmental factors (Turnbaugh *et al*., 2007).

2.4.5.1- Diet

Diet's impact on oral microbial diversity is not well characterized. But few studies which are available in literature, showed that diet can influences oral microbiota. In a study, effect of three commonly used dietary pattern by elite endurance athletes during intense training on oral microbiota was checked. Three dietary patterns include; a high carbohydrate (HCHO) diet, a periodized carbohydrate (PCHO) diet and ketogenic low carbohydrate high fat (LCHF) diet. Baseline level saliva samples were collected before treatment and after three-week period of treatment. Alpha diversity was reduced in all diet-training interventions after treatment compared to baseline level. Consumption of LCHF showed most dramatic change in diversity with reduction in *Haemophilus, Neisseria*, and *Prevotella* species, and coincident increased relative abundance of *Streptococcus* species (Murtaza *et al*., 2019).

Dietary intake and energy are correlated with composition of oral microbiota. A significantly positive correlation is reported between genera of *Clostridia* class (e.g.

Selenomonas, Johnsonella, Ruminococcaceae and *Veillonella*) with total energy, fat and protein intake, while negative correlation with carbohydrates and total intake of fibre (e.g. *Peptostreptococcus* and *Catonella*). Total fibre intake also had positive correlation with genus *Prevotella*. Further some bacterial taxa were found to be positively correlated with vegetable-rich diet (genus *Prevotella*) or with diet which are rich in protein/fat with class *Clostridia* (Cattaneo *et al*., 2019).

Alcohol intake can affect the oral microbiota by three possible mechanisms; 1) directly cytotoxic to bacteria, 2) destruction of interaction between bacteria and saliva, 3) by ethanol breakdown products are formed which act as substrate for metabolism. A study conducted by Fan *et al*., (2018), showed that microbial diversity changes in oral wash samples between alcohol drinkers and non-alcohol drinkers, with decreased abundance of *Lactobacilli* in heavy alcohol drinkers. This group also showed enrichment of certain genera including *Actinomyces, Leptotrichia, Cardiobacterium,* and *Neisseria*. Excessive alcohol use with co-use of tobacco was also associated with reduction in specie richness and abundance of *Neisseria, Aggregatibacter* and *Fusobacteria* in oral biofilms developed on mucosal surfaces (Signoretto *et al*., 2010).

Alcohol consumption predisposes risk for cancers of upper aero-digestive tract including oral cavity, throat, oesophagus and voice box. Alcohol is metabolized to ethanol and subsequently to acetaldehyde by oral bacteria including *Neisseria* species, *R. mucilaginosa, P. histicola* and *S. mitis*. These bacteria possess alcohol dehydrogenase enzyme which metabolize aldehyde. Acetaldehyde is toxic and mutagenic, hence cause DNA mutations. In addition to oral bacteria, oral *Candida* species can also convert ethanol to acetaldehyde and lead to oral cancer development (Moritani *et al*., 2015; Yokoyama *et al*., 2018).

Betel nut chewing is the main cause of oral cancers in Asian and Pacific countries. After tobacco, alcohol and caffeine, it is the fourth commonly used addictive substance. It mainly contains polyphenols and alkaloids. Regular use of betel nut can cause inflammation and epithelial cell damage in oral cavity. Betel nut users often have bad oral hygiene and develop periodontitis. Long term betel nut chewers show significantly low bacterial diversity, and reduced *Streptococcus* and *Parascardovia.*

Significantly raised level of *S. infantis* was seen in current betel nut chewers (Hernandez *et al*., 2017).

2.4.5.2- Use of antimicrobials

Actinobacteria, Firmicutes, Bacteroidetes and Proteobacteria are the most commonly influenced phyla by antimicrobials. The most commonly affected genera by antibiotics use are; *Enterococcus, Clostridium, Ruminococcus, Lactobacillus, Streptococcus, Faecalibacterium, Prevotella, Bacteroides, Eubacterium, Blautia, Escherichia* and *Bifidobacterium* (Rojo *et al*., 2017).

In oral cavity, microbes live in the form of mixed species consortia, in which there is an increased risk for horizontal gene transfer (HGT). Oral bacteria are constantly exposed to natural antimicrobial agents present in our diet. These antimicrobials include plant-based oils and flavonoids, agents used for personal hygiene including triclosan, chlorhexidine and toothpaste. Oral intake of clinically relevant antibiotics are also a major source for antimicrobial resistance through HGT as microbial diversity dynamics changes leads to different inter/intra species interactions. Metagenomic studies also revealed presence of reservoir of antibiotic resistant genes on plasmids and transposons in resident bacteria (Shaw *et al*., 2017).

Different toothpastes are used to enhance antimicrobial defence mechanisms of saliva. As these different toothpastes have different compositions, hence they impart different effects on microbiome. In Zendium^{TM} toothpaste contain triple enzyme system; aminoglycosidase, lactoperoxidase and glucose oxidase. These enzymes generate hypothiocyanite ion and hydrogen peroxide, a natural antimicrobial. Use of Zendium^{TM} increased the levels of salivary lysozymes, which reduce pathogenic flora but commensal increase. Another study conducted on effects of toothpaste containing enzymes and proteins showed that its use was significantly associated with increase in the level of 12 gingival health associated taxa's and decrease in 10 periodontitis associated taxa's (Adams *et al*., 2017; Pedersen *et al*., 2019).

A study, conducted on intake of an antiseptic mouth wash in healthy individuals, showed that it can cause disruption of several beneficial bacteria, which are involved in nitrate-nitrite-nitric oxide pathway. In these individuals, levels of nitrite in oral and plasma reduced with increased risk for blood pressure (BP) (Petersson *et al*., 2009).

2.4.5.3- Cigarette smoking

Cigarette smoke contain numerous toxicants, which come in direct contact with oral bacteria. Such toxicants have antibiotic effect on oral ecology and causes deprivation of oxygen supply. Smoking causes the loss of several beneficial oral bacterial, which enhance colonization of pathogens and ultimately disease development like periodontitis. Smokers showed difference in their microbial composition, with lower abundance of Proteobacteria compared to non-smokers. Moreover, they also show depletion of genera *Capnocytophaga*, *Leptotrichia* and *Peptostreptococcus*, and enrichment of *Streptococcus* and *Atopobium* (Wu *et al*., 2016). Another study reported that smoking is responsible for causing 33% reduction in Betaproteobacteria (particularly *Neisseria*) and 23% increase in relative count of *Veillonellaceae* family members (Kato *et al*., 2017).

2.4.5.4- Host genetic factors

Most genetic based studies are available for gut microbiota and understanding about role of genetic variation in establishing oral microbiome is still poor. In a study conducted on 752 twin pairs, a significantly lower beta diversity of monozygotic twins than unrelated or dizygotic twins was detected, and oral microbiota was seen to be relatively stable over a period of time in related individuals (Demmitt *et al*., 2017). But despite of having high genetic similarity, response to different environmental and biological stresses is individual specific (Filoche *et al*., 2010). Host genetic variation in immunity related pathway are found to be in correlation with microbiota. It is also found in the studies that host genes that are associated with microflora are enriched in genes linked with complex diseases (Blekhman *et al*., 2015). Depending upon an individual genotype, there is a possibility particular genotype allow certain pathogenic bacteria to colonize and prevent colonization of beneficial bacteria. A study showed that about 20% of the individual did not develop dental caries despite of receiving high frequency of cariogenic foods, moreover siblings and parents of these individuals also showed low dental caries prevalence, supporting the concept of genetic resistance to caries (Philip *et al*., 2018).

2.4.5.5- Industrial revolution

With industrial revolution, oral microflora became dominated with cariogenic bacteria and showed decrease in microbial diversity. Reason behind this shift is increased in intake of refined carbohydrate food. Decreasing diversity with industrial revolution, make the population more susceptible to disorders (Dagli *et al*., 2016; Adler *et al*., 2017).

2.4.5.6- Mode of delivery

Mode of delivery also effect the microbial composition. After C-section, oral microbial flora of infant delivered by vaginal route and C-section was resemble to mother vaginal and skin microflora, respectively. Infants delivered vaginally showed presence of more bacterial taxa (Dominguez-bello *et al*., 2016; Nuriel-ohayon *et al*., 2016).

2.4.5.7- Use of cell phone

Up till now, no study has been conducted on effect of radiation exposure from mobile phone use on oral microflora, but there is a possibility that it can indirectly influence oral microbial composition. Mobile phone use is found to alter the quality and quantity of saliva and environment of oral cavity (Dagli *et al*., 2016). Studies are available that showed that mobile phone use is associated with increasing flow rate of saliva, decreasing concentration of protein and increasing parotid gland volume (Goldwein and Aframian, 2010; Bhargava *et al*., 2012). However, another study showed reduction in salivary flow, amylase and albumin activity, and protein concentration in mobile users (Hamzany *et al*., 2012).

2.4.6- Benefits of commensal microflora to human host:

It is proved by several studies that bacterial colonization started before the birth of the baby. Jimenez *et al*., (2008), in their study inoculated a mouse during pregnancy with genetically labelled strain of *Enterococcus faecium*, and later labelled bacteria were isolated from meconium of the offspring delivered by C-section. Compared to adult, meconium showed lower diversity (Nuriel-ohayon *et al*., 2016). Notable increase in oral microbial diversity is seen from 0-3 years of life (Shaw *et al*., 2017). Human

microbial communities play critical role in various human metabolic, physiological and immunological processes (Zaura *et al*., 2014).

2.4.6.1- Colonization resistance and its role in mucosal defence

Colonization resistance is one of the most important role played by human microbiota. Oral bacteria compete for binding sites on mucosal, dental and prosthetic surfaces, as a result salivary pellicle became saturated with oral resident bacteria and prevent pathogens colonization. These resident bacterial communities also effectively compete for nutrients and growth factors like mucin and transferrin along with their degradation as poly-species. These residents create the unfavourable conditions for exogenous bacteria to grow in oral cavity like pH change, redox potential, oxygen tension and production of several inhibitory molecules like bacteriocins. Resident microbes also showed phenomenon of co-aggregation with either same (homotypic) species or with different (heterotypic) species, which reduced the survival of single non-coaggregated microbes (Marsh *et al*., 2016; Samaranayake and Matsubara, 2017).

Commensal microbiota maintained a balance environment to prevent the overgrowth of opportunistic resident microbes, that are already present in health compatible environment (Kumar and Mason, 2015). *S. salivarius* is the classical example of oral bacteria, which maintain homeostasis and prevent spread of pathogens in oral cavity by producing bacteriocin like inhibitory substance and other mechanisms. It acts agonistically against *S. pyogenes* (Upton *et al*., 2001; Wescombe *et al*., 2012). *S. salivarius* inhibit *S mutans* GS-5 strain biofilm formation in oral cavity by inhibiting competence-stimulating peptide (CSP) system required for *S. mutans* biofilm formation (Ogawa et al., 2011). Inhibition of same *S. mutans* strains by CSP signalling system inhibition, was also studied with *S. gordonii* (Wang and Kuramitsu, 2005).

S. mutans show antagonistic association with *S. sanguinis* in oral biofilms. Children with early and high colonization with *S. sanguinis* showed delayed colonization by *S. mutans*. Both exist in a "competitive exclusion" manner in oral cavity (Kreth *et al*., 2005). Resident oral lactobacilli are proved to be effective in reducing *P. gingivalis* and *P. intermedia* in 82% and 65% of the cases respectively (Meurman and Stamatova, 2007). In a study, conducted on testing the reduction of periodontopathogens by using antagonistic bacteria including*: Streptococcus crista, S.*

sanguinis, S. salivarius, S. mitis, H. parainfluenzae and *Actinomyces naeslundii* strains showed that all except for *S. crista,* inhibit adhesion by *P. gingivalis* on pre-adherent surfaces with antagonistic strain. Reduction in adherence of *P. intermedia* by *S. mitis* was also seen (Hoogmoed *et al*., 2008).

2.4.6.2- Role of oral microbiota in regulating metabolic processes

Oral bacteria also play role in different protective metabolic processes. Acid production from carbohydrates breakdown by bacterial glycolysis, creates acidic environment in oral cavity that promote tooth demineralization. However, under normal condition, phase of demineralization is followed by alkalization by various mechanisms including acid diffusion from biofilms, salivary buffering capacity by bicarbonates and peptides, and by metabolism of urea and arginine by bacteria, that promotes remineralization. Urea is hydrolysed to ammonia by ureases produced by *S salivarius,* oral haemophilli and *A. naeslundii*. Arginine is hydrolysed by arginine deaminase to ammonia, ornithine and carbon dioxide by *S. gordonii, S. rattus, S. parasanguis S. sanguis,* some *Lactobacilli* and Spirochetes (Burne and Marquis, 2000).

Oral bacteria especially present on tongue are involved in nitrate reduction to nitrite by bacterial nitrate reductases. Oral cavity harbours more than 300 bacterial species which produces nitrate reductases and denitrifying enzymes such as *S. salivarius, S. bovis, S. mitis, S. aureus, S. epidermidis, Veillonella* species, *Corynebacterium* species and *Nocardia* species (Lundberg *et al*., 2004; Hezel and Weitzberg, 2015). Dental damage causing bacteria like *S. mutans, L. casei* and *A. naeslundii* are susceptible to nitrite and if they produce acid in such nitrite rich environment then these are self-destroyed (Mendez *et al*., 1999). Nitric oxide which is produced by nitrate reduction is associated with growth inhibitory effect on *F. nucleatum, P. gingivalis* and *E. corrodens* (Allaker *et al*., 2001).

2.4.6.3- Microflora as regulator of immune system

Several commensal microbes have been identified for their role in modulating host immune response. *S. salivarius* is known for influencing inflammatory response associated with periodontopathogens and enteric pathogens. *S. salivarius* strain K12 is involved in downregulating nuclear factor-κB activation suggesting its anti-

inflammatory role (Kaci *et al*., 2011). *S. salivarius* modulate epithelial cell immune responses. It changes the expression of several host genes, especially of those involved in innate immune response, epithelial cell homeostasis and function, cell development and migration, cytoskeletal remoulding, and signalling pathways (Cosseau *et al*., 2008).

2.4.7- Oral microbiome dysbiosis; a cause of local and systemic health issues

Oral microbiota of healthy individuals is different from that of individuals with disorders. Healthy oral cavity mainly harbours *Streptococcus, Gemella, Veillonella, Actinomyces, Rothia, Granulicatella, Corynebacterium, Prevotella, Capnocytophaga, Porphyromonas, Fusobacterium, Haemophilus, Lactobacterium, Treponema, Neisseria, Peptostreptococcus, Leptotrichia, Eubacteria, Eikenella, Propionibacterium* and *Staphylococcus* (Aas *et al*., 2005; Bik *et al*., 2010). Oral health depends upon ecological balance in which oral microbe maintain communalistic association within microbial consortia and mutualistic association with their host. Commensals microbes' flourishes in oral cavity without harming their co-habitants and play role in establishing biodiversity. However, there are several mechanisms through which pathological shifts occur in oral cavity including; 1) change in microbe-microbe and microbe-host interactions, 2); an increase in relative abundance, 3) virulence factors acquisitions (Figure 2.1). Oral microbial dysbiosis and pathological shifts are associated with several local and systemic disorders (Zarco *et al*., 2012).

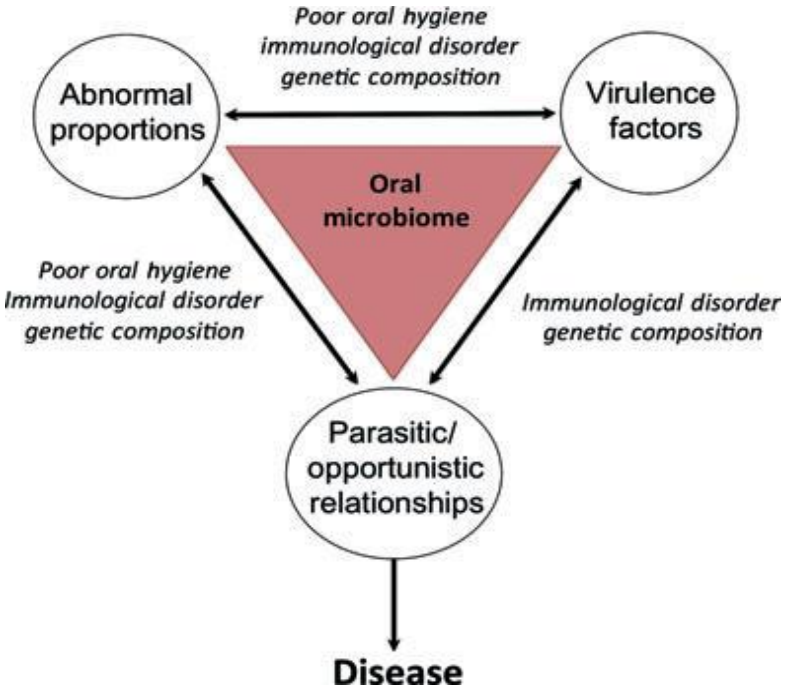

Figure 2.1: Cycle of disease-causing ecological shifts in oral microbiota. To start this cycle major contributor are; 1) Poor oral hygienic practices, 2) immunological diseases, and 3) certain genetic compositions/genotypes

CHAPTER 3: DEMOGRAPHIC, ORAL HEALTH AND OBSTETRIC CHARACTERISTICS OF POSTPARTUM FEMALES

3.1- INTRODUCTION

Pregnancy is a unique and complex phase, during which female body experiences several changes, among which prominent shift is seen in physiological body features and hormonal levels (Cao *et al*., 2014). Levels of progesterone and estrogen dramatically raise during pregnancy, contributing to ecological imbalance in oral cavity, which can ultimately lead to pregnancy associated gingivitis. If such changes persist and proper hygiene is not maintained, then it worsen towards the end of pregnancy and can progress to develop periodontitis and caries lesions (Gupta and Siddiqui, 2014). Effect of these contributing factors is further exacerbated by frequent vomiting, changing eating and drinking habits with more cravings towards sugary diet (Ramazani *et al*., 2014).

For ensuring good foeto-maternal health during pregnancy, maintaining good oral health of the expecting mother is important (George *et al*., 2013). Local inflammatory response in oral cavity against pathological shifts in oral ecology and flora during pregnancy, can contribute to various systematic disorders even APOs (Gaszynska *et al*., 2015). APOs are undesirable, such commonly seen APOs are PTB, LWB and preeclampsia (Sukkarwalla *et al*., 2015). Low and middle-income countries (LMIC) have been reported to have high prevalence of APOs (Harjunmaa *et al*., 2015).

Females during pregnancy are mostly unaware about the importance of their oral health impact on their baby health (Imran *et al*., 2015). Despite having oral health issues during pregnancy, mostly females are reluctant to take treatments because they are under impression that taking orthodontic treatments can be harmful to developing baby, as a consequence maternal oral health get compromised (Ibrahim and Ghandour, 2012). On the contrary, it has been proved that dental treatment during pregnancy is not only safe (Thomas *et al*., 2008), but also helpful in improving maternal oral health and preventing the placental transmission of harmful microbes plus their products to developing baby (George *et al*., 2013). In Pakistan, no oral health guidelines are available to physicians to guide females during their routine visits. Therefore, it is the need of time to develop health strategies at national level to educate the females about the safe oral health measures that can be helpful in reducing

maternal hard and soft tissues infection, which might have impact on babies health (Rashid *et al*., 2016).

In Pakistan, majority of females give little importance to oral health, oral health issues are mostly left untreated either due to less awareness about oral health, unavailability of health facilities and due to socioeconomic pressure (Imran *et al*., 2015). Only few reports related to prevalence of oral disorders especially in pregnancy are available from Pakistani population (Baig *et al*., 2013). WHO reports approximately 18% prevalence of dental disorders in all Pakistani population (Amin and Tarar, 2016). Data related to oral disorders and impact on pregnancy outcomes in Pakistani females is limited, so there is a dire need to determine the prevalence and its associations with various demographic and obstetric characteristics. In present work, association of various demographic features and oral health status with obstetric factors was assessed for postpartum females after first day of delivery. These findings will be proved helpful in formulating oral health strategies that will help in reducing APOs, which are currently unavailable.

3.2- MATERIAL AND METHODS

3.2.1- Study design, settings and data collection

The present study was a hospital-based retrospective case-control study, approved from ethical review boards of Capital Hospital (Islamabad), Holy Family Hospital-Rawalpindi Medical University (Rawalpindi), and Quaid-i-Azam University (Islamabad) ethical committees (QAU-BRC). Written informed consent was obtained from all females (Appendix A). Postpartum females attending Gynaecology Department of Capital Hospital, Islamabad and Holy Family Hospital, Rawalpindi, from January 2016 to March 2018 were included along with healthy nonpregnant females as control from general population. Structured questionnaire was used for data collection (Appendix B), from healthy nonpregnant and postpartum females after first day of delivery. Patient's medical history was also used for collecting general health related data. Dental health was assessed by the qualified dentists. All postpartum females having oral health issues were included. Gingivitis was confirmed in females having mean periodontal pocket depth of 3-4 mm, gums bleeding with flossing and brushing, swollen red gums, no tooth mobility and bone loss by qualified

dentist. In females for dental caries status, data related to decayed, missing, filled teeth/filled surfaces (DMFT and DMFS) were also collected.

3.2.2- Criteria for inclusion and exclusion

3.2.2.1- Inclusion criteria

All those postpartum females were included in the study having age range of 18-40 years. These females were either having healthy oral cavity or were suffering from periodontal disorders (periodontitis, gingivitis) and dental caries. Nonpregnant females of same age range having healthy oral cavity were included as controls.

3.2.2.2- Exclusion criteria

Postpartum females suffering from systemic, infectious and metabolic illness other than periodontal disease and dental caries were excluded. Females having antibiotics intake during last three months of gestation were also excluded from the study.

3.2.3- Data variables of questionnaire

The questionnaire comprised of 3 sections: (1) Demographic data of postpartum females: including age, height, weight, socio-economic status (low, middle and high), residential area, education and employment status; (2) Oral health status and practices: including bleeding gums, pain in gums, tooth erosion, tooth mobility, orthodontic treatment during pregnancy, brushing frequency, intake of juices and drinks (frequently; daily intake, and not frequently; 1-2 times a week), intake of tea and coffee, antibiotic treatments during pregnancy, smoking, intake of snuff (Naswar), hookah (Tobacco pipe of Eastern origin), and betelnut (Chalian); (3) Obstetric factors: including history of previous pregnancies, gestation period, baby weight, presence of preeclampsia, blood pressure (BP) at the time of delivery, pregnancy depression and hypertension.

3.2.4- Statistical data analysis

For statistical analysis, IBM SPSS (version 21) was used. Mean and standard deviation (SD) was measured for descriptive data, frequencies and percentages were calculated for categorical data. For risk measurements odds ratio (OR) was calculated. Chi-square (χ^2) test and Fisher's exact test (where count was less than 5), were used to

determine significant associations between different variables, where $P<0.05$ was considered as level of significance.

3.3- RESULTS

3.3.1- Demographic features of postpartum females and healthy control group

In present study, 267 postpartum females with mean age 27.84±4.876 years and 54 healthy control females with mean age 25.90±4.27 years were included. Majority of these females belonged to age group of 21-30 years, to middle class, living in urban areas. Most of the postpartum females were illiterate (24.7%) and had high BMI (47.2% cases) (Table 3.1).

3.3.2- Oral health status and practices of postpartum females and healthy control group

In control group none of the female was suffering from oral health issues. In postpartum group, 47.2% females were suffering from different type of oral health problems. Periodontitis was not diagnosed in any of the postpartum female, however, 27.3% were suffering from gingivitis and 28.1% from dental caries. Co-occurrence of both disorders (gingivitis and dental caries) was present in 8.2% postpartum females. Among postpartum females group, 41.66% (5/12) females of age range 16-20 years, 37.5% (33/88) of age range 21-25 years, 46.6% (41/88) of age range 26-30 years, 56.25% (36/64) of age range 31-35 years and 73.4% (11/15) of age range 36-40 years were suffering from different periodontal and dental disorders. Frequency of brushing was low among majority of these females, 60.7% brushed once daily and none of them were taking other oral hygienic measures such as use of antiseptic mouth wash. High intake frequency of juices/drinks (44.2%) and tea/coffee was seen among the postpartum females. Mostly postpartum females consumed tea or coffee 1-2 times a day with overall consumption was among 74.2% of the cases. Smoking, snuff, hookah, and betalnut intake was uncommon as only one postpartum female was addicted to betelnut intake (Table 3.2).

3.3.3- Obstetric data of postpartum females

Mostly females were multiparous (70.8 % cases) and common mode of delivery was caesarean (65.5%). LWB and PTB prevalence was high in theses females with 22.5%

and 21.7% cases, respectively. Preeclampsia was common APOs and was present in 11.6% of the cases. At the time of delivery, most of the females had normal blood pressure (71.5%). Pregnancy related hypertension and depression was uncommon as it was seen in 2.2% and 3.7% of the cases, respectively (Table 3.3). Despite having oral health issues, only 16.9% of these females had undergone orthodontic treatment during the period of pregnancy.

Table 3.1: Demographic characteristics of postpartum females (*n*=267) and healthy control group (*n*=54)

Factors		**Postpartum females** ***n (%)***	**Healthy control females** ***n (%)***
Age (years)	16-20	12 (4.50)	03 (5.5)
	21-25	88 (33.0)	21 (39.0)
	26-30	88 (33.0)	23 (42.6)
	31-35	64 (24.0)	04 (7.40)
	36-40	15 (5.60)	03 (5.5)
Residential area	Rural	118 (44.2)	05 (9.3)
	Urban	149 (55.8)	49 (90.7)
Education level	No	66 (24.7)	03 (5.5)
	Primary	39 (14.6)	02 (3.7)
	Middle	32 (12.0)	00 (0.00)
	Metric	27 (10.1)	02 (3.7)
	Intermediate	65 (24.3)	01 (1.9)
	Graduation	34 (12.7)	09 (16.7)
	Higher level	04 (1.50)	37 (68.5)
Socio-economic status	Low	64 (24.0)	07 (12.9)
	Middle	203 (76.0)	42 (77.8)
	High	00 (0.00)	05 (9.3)
Body mass index (BMI)	Normal	140 (52.4)	36 (66.7)
	Overweight	126 (47.2)	05 (9.3)
	Underweight	01 (0.40)	13 (24.0)
Total		267 (100)	54 (100)

Table 3.2: Oral health status and practices of postpartum females (*n*=267) and healthy control group (*n*=54)

Factors		**Postpartum females** ***n (%)***	**Healthy control females** ***n (%)***
Oral health problems	Present	126 (47.2)	00 (0.00)
Gingivitis	Present	73 (27.3)	00 (0.00)
Dental caries	Present	75 (28.1)	00 (0.00)
Dental caries + Gingivitis	Present	22 (8.20)	00 (0.00)
Brushing frequency/day	1 time	162 (60.7)	42 (77.8)
	2 times	98 (36.7)	11 (20.3)
	3 times	03 (1.10)	01 (1.9)
	No brushing	04 (1.50)	00 (0.00)
Intake of drinks and juices	Frequently	118 (44.2)	27 (50.0)
	Not frequently	88 (22.8)	20 (37.0)
	No intake	61 (33.0)	07 (13.0)
Tea and coffee intake	1-2 times daily	198 (74.2)	24 (44.5)
	3-4 times daily	40 (15.0)	23 (42.6)
	> 4 times daily	02 (0.70)	04 (7.40)
	No intake	27 (10.1)	03 (5.5)
Smoking, Snuff, Hookah, and Betelnut intake	Yes	01 (0.40)	00 (0.00)
	No	266 (99.6)	54 (100)
Total		276 (100)	54 (100)

Table 3.3: Frequency distribution of postpartum females according to obstetric factors (*n*=267)

Factors		**Frequency** ***n*** **(%)**
Parity	Nulliparous	78 (29.2)
	Multiparous	189 (70.8)
Mode of delivery	Vaginal	92 (34.5)
	Caesarean	175 (65.5)
Gestational age	PTB	58 (21.72)
	FTB	209 (78.28)
Baby weight (Kg)	< 2.5	60 (22.5)
	2.5-4.0	203 (76.0)
	> 4.0	04 (1.50)
Preeclampsia	Present	31 (11.6)
BP at the time of delivery	High	48 (18.0)
	Low	28 (10.5)
	Normal	191 (71.5)
Pregnancy depression	Present	10 (3.70)
Hypertension	Present	06 (2.20)
Orthodontic treatment during pregnancy	Yes	45 (16.9)
	No	222 (83.1)
Total		267 (100)

3.3.4- Associations of obstetric factors and oral health practices with oral disorders

The participants with preeclampsia showed significant association with oral health problems (P=0.040, χ^2=4.224), and also females with less brushing frequency (P=0.044, χ^2=8.056). However, other APOs were not associated with oral health problems (gestational age; P=0.281, baby weight; P=0.924). There were no associations of APOs with pregnancy related depression (P=0.079), intake of juices/drinks (P=0.505), and with tea/coffee intake consumption per day (P=0.231). Approximately one-fold increase in development of PTB and pregnancy associated depression among females with oral health issues was found (Table 3.4a).

Similarly, preeclampsia was also common in females suffering from gingivitis (P=0.018, χ^2=5.607), and in those postpartum females having low brushing frequency (P=0.004, χ^2=13.29). Preeclampsia interestingly showed no association with other APOs and oral health related factors. Presence of gingivitis increased approximately two-fold risk for having PTB and preeclampsia, and also its risk was increased with decreasing brushing frequency and increasing tea/coffee intake (Table 3.4b).

Significantly high prevalence of dental caries was seen in patients having low brushing frequency (P=0.024, χ^2=9.367) and high intake of tea/coffee (P=0.032, χ^2=9.367). No association was seen between dental caries with any other obstetric and oral health related factor studied. However, odds showed risk for having APOs in females with dental caries. Likewise, gingivitis, risk for dental caries was increased with decreasing brushing frequency and increasing sugary liquid intake (Table 3.4c).

3.3.5- Associations between different obstetric factors

LBW was common in postpartum females suffering from preeclampsia (P=0.003) and with gestational hypertension (P<0.001). It was not significantly associated with postpartum females having pregnancy depression. Gestational age of baby did not show any associations with preeclampsia, hypertension and depression (Table 3.5).

Table 3.4a: Association of obstetric factors, oral and general health practices with oral health problems

Factors		**Oral health problems**		***P*-value (χ^2)**	**OR (95% Cl)**
		No ***n* (%)**	**Yes** ***n* (%)**		
Gestational age	FTB PTB	114(42.70) 27(10.11)	95(35.58) 31(11.61)	0.281 (1.164)	1.37(0.76-2.46)
Baby weight (Kg)	< 2.5 2.5-4 > 4	33(12.36) 106(39.7) 02(0.75)	27(10.12) 97(36.32) 02(0.75)	0.924 (0.157)	- 0.89(0.50-1.59) 0.91(0.12-6.6)
Preeclampsia	Yes No	11(4.12) 130(48.68)	20(7.50) 106(39.7)	0.040 (4.224)	0.44(0.20-0.97)
Pregnancy depression	Yes No	08(03.0) 133(49.81)	02(0.75) 124(46.44)	0.079 (3.082)	1.01(0.70-1.47)
Frequency of brushing/day	No 1 time 2 times 3 times	00(0.00) 94(35.20) 46(17.22) 01(0.38)	04(1.50) 68(25.47) 52(19.48) 02(0.75)	0.044 (8.056)	- - 0.63(0.38-1.06) 0.56 (0.04-6.4)
Tea and Coffee intake	No 1-2 times daily 3-4 times daily > 4 times daily	13(4.86) 111(41.56) 15(5.62) 02(0.76)	13(4.86) 88(32.96) 19(7.12) 06(2.26)	0.231 (4.30)	- 0.79(0.34-1.79) 1.59(0.76-3.32) 2.36(0.41-13.4)
Intake of juices/drinks	No intake Not frequently Frequently	34(12.73) 42(15.73) 65(24.34)	27(10.12) 46(17.22) 53(19.86)	0.505 (1.367)	- 1.37(0.71-2.6) 0.74(0.42-1.29)

Table 3.4b: Association of obstetric factors, oral and general health practices with gingivitis

Factors		**Gingivitis**		***P*-value (χ^2)**	**OR (95% Cl)**
		No ***n* (%)**	**Yes** ***n* (%)**		
Gestational age	FTB	157(58.8)	52(19.48)	0.087 (2.932)	1.71(0.92-3.1)
	PTB	37(13.86)	21(7.86)		
Baby weight (Kg)	< 2.5	45(16.86)	15(5.62)	0.401 (1.825)	-
	2.5-4	145(54.3)	58(21.72)		0.83(0.43-1.6)
	> 4	04(1.50)	00(0.00)		-
Preeclampsia	Yes	17(6.36)	14(5.24)	0.018 (5.607)	2.47(1.14-5.31)
	No	177(66.3)	59(22.1)		
Pregnancy depression	Yes	09(3.38)	01(0.38)	0.209 (1.573)	0.28(0.03-2.29)
	No	185(69.28)	72(26.96)		
Frequency of brushing/day	No	00(0.00)	04(1.50)	0.004 (13.29)	-
	1 time	120(44.94)	42(15.73)		1.02(0.57-1.81)
	2 times	73(27.34)	25(9.36)		0.17(0.01-1.97)
	3 times	01(0.38)	02(0.75)		-
Tea and coffee intake	No	19(7.14)	07(3.00)	0.724 (1.323)	-
	1-2 times daily	145(53.40)	54(20.22)		1.01(0.40-2.53)
	3-4 times daily	23(8.70)	11(4.14)		1.28(0.58-2.81)
	> 4 times daily	07(3.00)	01(0.40)		0.29(0.03-2.73)
Intake of juices/drinks	No intake	47(17.60)	14(5.24)	0.216 (3.064)	-
	Not frequently	58(21.72)	30(11.24)		1.73(0.82-3.64)
	Frequently	89(33.34)	29(10.86)		0.63(0.34-1.15)

Table 3.4c: Association of obstetric factors, oral and general health practices with dental caries

Factors		**Dental caries**		***P*-value (χ^2)**	**OR (95% Cl)**
		No ***n* (%)**	**Yes** ***n* (%)**		
Gestational age	FTB	151(56.56)	58(21.72)	0.815	1.07(0.56-2.05)
	PTB	41(15.36)	17(6.36)	(0.055)	
Baby weight (Kg)	< 2.5	42(15.73)	18(6.74)	0.560	-
	2.5-4	148(55.44)	55(20.59)	(1.159)	1.15(0.61-2.17)
	> 4	02(0.75)	02(0.75)		0.37(0.05-2.70)
Preeclampsia	Yes	19(7.11)	12(4.50)	0.162	1.73(0.79-3.77)
	No	173(64.80)	63(23.59)	(1.958)	
Pregnancy depression	Yes	08(3.0)	02(0.75)	0.561	0.63(0.13-3.03)
	No	184(68.91)	73(27.34)	(0.337)	
Frequency of brushing/day	No	01(0.38)	03(1.12)	0.024	-
	1 time	124(46.44)	38(14.23)	(9.367)	9.78(0.98-96.8)
	2 times	64(23.98)	34(12.73)		0.57(0.33-1.00)
	3 times	03(1.12)	00(0.00)		-
Tea and coffee intake	No	19(7.14)	07(2.62)	0.032	-
	1-2 times daily	150(56.17)	49(18.35)	(9.367)	0.88(0.35-2.23)
	3-4 times daily	20(7.49)	14(5.24)		2.41(1.00-4.56)
	> 4 times daily	03(1.12)	05(1.87)		2.38(0.48-11.6)
Intake of juices/drinks	No intake	45(16.86)	16(6.0)	0.870	-
	Not frequently	64(23.98)	24(8.98)	(0.278)	1.05(0.50-2.20)
	Frequently	83(31.08)	35(13.10)		1.12(0.60-2.07)

Table 3.5: Association of LWB and PTB with other obstetric factors

Factors		**Baby weight (kg)**			***P*-value (χ^2 value)**	**Gestational Age**		***P*-value (χ^2 value)**
		< 2.5	**2.5-4**	**> 4**		**FTB**	**PTB**	
Preeclampsia	No	48	186	02	0.003	185	51	0.902
	Yes	12	17	02	(11.931)	24	07	(0.015)
Gestational	No	57	202	02	<0.001	205	56	0.485
hypertension	Yes	03	01	02	(46.438)	04	02	(0.487)
Pregnancy	No	59	194	04	0.565	200	57	0.360
Depression	Yes	01	09	00	(1.141)	09	01	(0.840)

3.4- DISCUSION

Hormonal changes during pregnancy and suboptimal oral health practices can lead to poor oral health conditions, ultimately resulting in the development of oral hard tissues and mucosal infections (Gaffar *et al*., 2016). In present work, a high percentage (47.2%) of females were having different oral health issues (gingivitis and dental caries), but only 16.9% of these females underwent orthodontic treatments during pregnancy. Despite having oral health problems during pregnancy, they were not taking any antibiotic treatment because of their misunderstanding that such treatment can harm their baby's health. Data from this study of Pakistani population is same as exists in highly developed countries even United State of America (USA), where several national level health policies for improving oral health during pregnancy exist, but only 44.7% females consult the physician for their dental health during their gestational phase. This number is even lower among Australian females which is about 30-36% females consulting dentist (George *et al*., 2013) . In Sudan, 90% of the females do not consult dentist because they either consider that during pregnancy having a dental problem is a normal sign or taking treatments will be harmful for both mother and foetus (Ibrahim and Ghandour, 2012). In Pakistan, only few studies are available related to female visiting dentists during pregnancy. A study conducted by Sukkarwalla *et al*., (2015), reported that only 13% pregnant females visit dentist for treatment purpose. Females avoid taking dental treatments due to time constrain (24%), anxiety to treatments (21%) and cost of treatment (20%) also.

Almost all females brushed daily but with varying frequency, brushing once was predominant (60.7%), which is in line with the previous study from Karachi Pakistan where almost similar brushing frequency rate was reported (Mohiuddin *et al*., 2015). Females from this study with low brushing frequency (Once a day) commonly suffered from different oral health problems *(P*=0.044) and were predominantly affected by gingivitis (*P*=0.004) and caries lesions (*P*=0.024). Among such affected females, sugary tea and coffee drinkers showed significantly high prevalence of dental caries (*P*=0.032). The reason might be that during pregnancy, these females had changed dietary habits more cravings to sugary food intake. Such availability of high sugar content can shift the ecological balance in oral cavity and lead to colonization of cariogenic flora.

Pakistan is sixth most populous country in the world. It is ranked second in terms of stillbirths and third in terms of new born mortality (Ghaffar *et al*., 2015). Estimated rate of premature birth is about 15 million per year, of which approximately 1.1 million babies die after birth. About 5-18% of PTB cases are seen commonly across 184 countries. In developing countries every year, this level is increasing alarmingly. Cases from South Asian countries, Sub-Sahara Africa and some cases from developed countries like USA account for 60% of PTB (Satterfield *et al*., 2016). In Europe and Africa, estimated rate of PTB is about 5% and 18%, respectively. Like other Asian countries, PTB incidence is also unfortunately increasing with 748,100 cases seen annually in Pakistani population. Pakistan, in term of PTB is also ranked 4th after India, China and Nigeria. Studies related to PTB incidence in Pakistani population are limited (Hanif *et al*., 2017). Present work recorded relatively high (21.72%) PTB rate compared to the previous studies from different regions of Pakistan. Shaikh *et al*., (2011), reported 15.7% rate and in another meta-analysis this rate was about 18.89% (Hanif *et al*., 2017). The reason for this difference in rate might be due to difference in geographic location in Pakistan. As most of the Pakistani population is living in rural areas, where common practice is delivery at home and even in some urban areas, most babies are born at home. It is believed that PTB and LWB cases from Pakistan are still unreported.

Like incidence of PTB, LWB prevalence is also very high in Pakistan, like other LMICs. Annually, about 15% babies, born with low weight worldwide. Of these low weight babies, globally about 17% die before the age of 5 years (Khan *et al*., 2015).

WHO in 2016 conducted a survey and reported 32% cases of LWB out of all child births in Pakistani population (WHO, 2016). A study from Lahore (Pakistan), showed that about 19% baby born have low weight (Najmi, 2000). Higher rate of LWB compared to the present finding was reported by WHO, but, lower LWB rate was reported by Najmi in 2000. These differences in rate of LWB could be due the reason that these previous studies are carried out from different region (urban and rural). To get the clear estimate of PTB and LWB increasing burden, there is a need to conduct a comprehensive survey across Pakistan.

Prevalence of preeclampsia was high (11.6%) in the present studied females and was significantly associated with LWB. Preeclampsia and hypertension are the common pregnancy related complications, which affect about 2-8% of the females in gestation period (Connealy *et al*., 2014) and especially in developing poor countries causing about 60,000 maternal deaths per year (Sheikh *et al*., 2015). It is also associated with increasing incidence of foetal morbidity, mortality (Walker *et al*., 2014), intrauterine growth restriction and PTB (Shamsi *et al*., 2010). A survey by WHO in LMICs, showed about 4% prevalence of preeclampsia and it was significant in causing maternal and perinatal death, PTB and LWB. This WHO report showed that among Asian countries, India, showed prevalence rate of 4.60% and about 2.80% in China (Bilano *et al*., 2014). Similarly, high rate of preeclampsia was recorded in present study subjects compared to previous reports, it was high among multiparous with high BMI.

Among these studied postpartum females' oral disorders (gingivitis and dental caries) were prevalent. It could be the due to craving towards sugary diet and frequent vomiting, which might be also responsible for disturbing oral flora and oral health. In addition, these females also had high intake of juices and tea. Initially, dental caries was considered as disease of developed countries, affecting 60-90% of children and elders. But now it has become a major health problem especially in developing countries, because of the poor oral hygienic measures and diet (Mohiuddin *et al*., 2015). It is even the most prevalent disease among Pakistani children (Kamran *et al*., 2017).

WHO reported about 31% prevalence of periodontitis in overall Pakistani population (Amin and Tarar, 2016). Umer *et al*., (2016), conducted a study in Sargodha

(Pakistan), reported 14.5 % prevalence of gingivitis and 45.9% prevalence of dental caries in children. However, there is no data available on the prevalence of dental issues in Pakistani postpartum females. Compared to present study high rates of dental disorders is found in other countries such as 63.1% cases among pregnant females from Malawi. Of which 27.8 % females had deep caries lesions, 23.5 % periapical infections and 31.9% diagnosed with periodontitis (Harjunmaa *et al*., 2015). Variation in geographical location, race, ethnicity and age could be the reason for differences in prevalence rate among different regions of the world. Risk of dental disorders increased with age, with incidence rate ranging from 36%-67% (Ali *et al*., 2016). About 65 million adults from USA, of over age of 30 years are suffer from different periodontal disorders. Geographical area also affects the periodontal health status by affecting 5-70% of the population worldwide (Vanterpool *et al*., 2016). In present study, in comparison to previous studies the prevalence of oral disorder was low. However, increasing trend was seen in disease occurrence with increasing age as 41.66% in young females (16-20 years) to 73.4% cases among females of age range 36-40 years. Periodontal diseases usually progress to more severe form with increasing age. However, in present study majority of the females were of age 21-35 years, that's might be the reason for low prevalence of periodontal disorders among present study subjects.

Literature showing periodontal disease and APOs associations are available, however, until now no conclusive evidence is available (Azofeifa *et al*., 2014). One out of every five females during pregnancy, suffers from periodontitis and is at the risk of developing APOs, where calculated risk of ten-fold of PTB in females with moderate to severe cases of periodontitis compared to periodontitis-free females (Offenbacher *et al*., 2001). In present study, oral health problems did not show any association with PTB and LWB, however, they were significantly associated with preeclampsia. Females with APOs also showed low prevalence of dental caries. Presence of different oral disorders also increased approximately one to two-fold risk for PTB and preeclampsia. This risk was lesser than as reported by Offenbacher *et al*., it was because in present study females were either suffering from gingivitis or dental caries but not with periodontitis, which is a more aggressive form of periodontal disorders.

In conclusion, present study provides the overview of dental health status and practices followed by females during pregnancy from Rawalpindi and Islamabad

(Pakistan). These postpartum females despite suffering from oral health problems did not seek dental treatment. Brushing frequency was associated with oral health problems. No direct association was observed for APOs like PTB and LWB, but other APOs like preeclampsia was associated among the females having oral disorders. Preeclamptic females delivered LWB, might be possible indirect relationship need to be further investigated. There is a need for conducting such studies at national level to get the clear estimates of oral health issues during pregnancy and its association with pregnancy related complications. Present study data cannot be considered as representative of all population as it was conducted in only two hospitals, of two cities of Pakistan. There is also a dire need to investigate this issue in rural area of Pakistan which represent majority of the Pakistani population with lack of awareness for dental hygiene and its possible adverse outcomes. However, findings from the present work are significant for initiating oral health awareness strategies, to provide the pregnant females knowledge of maintaining their good oral health and its positive effects on their baby's health.

CHAPTER 4: STUDY OF MUTANS STREPTOCOCCI IN POSTPARTUM FEMALES AS INDICATOR OF ORAL HEALTH STATUS

4.1- INTRODUCTION

Mutans streptococci (MS) is a subgroup of Viridans group of *Streptococci*, which is a member of human oral flora**.** MS group due to its cariogenic properties are of great oral health concern. Most important cariogenic members of this group are *S. mutans* and *Streptococcus sobrinus*. *S. mutans* is the most abundant specie isolated from caries lesions (Okada *et al*., 2012; Ravikumar *et al*., 2018; Villhauer *et al*., 2017; Leathers *et al*., 2018), while *S. sobrinus* is less frequently isolated (10-14% of the cases). Tooth demineralization is the characteristic feature of dental caries, which results from acid production from sugars present in diet by acidophilic and aciduric bacteria (Johansson *et al*., 2015; Choi *et al*., 2016; Sales *et al*., 2018). One of the way to predict development of caries is isolation of MS from oral cavity (Saravia *et al*., 2011). Poor oral hygiene, sources of microbial contaminations, structure of tooth, genetics and lifestyle are other etiological agents that predisposes the individual to have increased risk for colonization of cariogenic microorganisms and development of dental caries (Mokhtari *et al*., 2019).

During pregnancy maintaining good oral hygiene and health is important for maintaining good general body health of both mother and their baby (Shamsi *at al*., 2013). Unfortunately, it is a common practice that expecting mother avoid taking oral health care, even when they have some dental health issues due to misunderstanding of its negative effects (Acharya *et al*., 2018; Singh, 2018). Pregnancy predisposes the females to develop periodontal disease and caries lesions, its frequency is high in pregnant females compared to nonpregnant state (Balan *et al*., 2018). Presence of persistent and sub-clinical oral infections during pregnancy can cause induction of inflammatory response, which ultimately can lead to the induction of premature labour and baby birth before 37 weeks of gestation. In pregnant females inflammatory response along with presence of oral Streptococcal and periodontopathogenic species in dental plaques and amniotic fluid of mothers indicated that there is association between maternal oral health and APOs (Mendes *et al*., 2018; Vieira *et al*., 2019).

In pregnancy, gingivitis is one of the common periodontal disorder affecting approximately 30-100% of the pregnant females. This condition starts in second

month of gestation and progressively worsen towards the end of gestation, also in early postpartum phase and later returns to normal condition in the late postpartum phase, if proper oral hygiene is maintained (Ramos-e-silva *et al*., 2016). Plaque if not regularly removed induce gingivitis as symbiotic Gram-positive bacteria of healthy plaque matures and allowed the colonization of pathogenic Gram-negative rods, which cause further development of gingivitis and can even ultimately lead to periodontitis (Hasan *et al*., 2017). Limited number of studies are available that confirmed that dental caries can develop during pregnancy (Alneamy, 2018), such studies report prevalence range of 41% to 99.9% (Vasiliauskiene, 2003; Azofeifa *et al*., 2016). Due to compositional changes in saliva, poor oral health and gastric reflexes risk of caries increase in pregnancy. Depending upon the time duration required for initiation and progression of caries lesion that can ultimately lead to tooth loss make it unlikely for a complete course of dental caries development and tooth loss to occur in a short time frame of pregnancy (Siddiqui *et al*., 2018).

Mostly *S. mutans* and *S. sobrinus* are studied for their pathogenic role in causing childhood caries (Okada *et al*., 2012; Saraithong *et al*., 2015; Fragkou *et al*., 2016). However, these species are rarely studied for their role in causing dental caries and periodontal disease during pregnancy and postpartum period. Few studies are available in literature for identifying the prevalence of *S. mutans* during pregnancy but association has not been yet identified with APOs (Kamate *et al*., 2017). No data is available in literature regarding prevalence of *S. sobrinus* in oral cavity during pregnancy or postpartum phase and its possible association with caries development and APOs. In addition to this, role of both MS species in gingivitis development during pregnancy and postpartum phase have not been yet evaluated. The present study was designed to study salivary colonization of *S. mutans* and *S. sobrinus* in early postpartum phase females along with healthy nonpregnant females to assess their associations with gingivitis, dental caries and APOs.

4.2- MATERIAL AND METHODS

4.2.1- Study design, settings and data collection

Previously described in chapter 3 (section 3.2).

4.2.2- Study population and sampling technique

Saliva samples from 267 postpartum females admitted in Gynaecology Department of Capital Hospital, Islamabad and Holy Family Hospital, Rawalpindi were collected post one day of delivery. In addition, to postpartum females, demographic data and saliva samples was also collected from 54 non-pregnant healthy females from general population. For sample collection passive drooling method was used (https://salimetrics.com/wp-content/uploads/2018/02/passive-drool-saliva-collection-instructions.pdf). Unstimulated saliva sample (3.0 to 4.0 mL) was collected in a sterile plastic sterile container having air-tight screw cap. Sample collection was done between 9:00 a.m. and 10:00 a.m. in the morning. Females were advised, not to eat or drink any food item and medicine for at least an hour before sample collection. After collection, containers were sealed and properly labelled with sample code numbers and transported to laboratory in ice box and processed within 3 h in Molecular Medicine Laboratory, Microbiology Department, QAU, Islamabad.

4.2.3- MS isolation and identification

4.2.3.1- MS isolation on Modified Sucrose-Bacitracin agar (SB-20M)

For isolation of MS, SB-20M media was used. This media was prepared by adding bacto-casitone 15.0 g, yeast extract (Oxoid, England) 5.0 g, L-cysteine 0.2 g, Sodium sulfite 0.1 g, Sodium acetate 20.0 g, coarse granular cane sugar 200 g and technical agar 15.0 g in distilled water (1000 mL). Media was mixed well and autoclaved at 121˚C for 15 min (15 psi). After autoclaving, media was cooled (50˚C), bacitracin 0.2 units/mL was added, and media was poured in petri plates under sterile conditions (Saravia *et al*., 2013). An aliquot of 0.1 mL saliva sample was inoculated on media plate, wrapped in paraffin film and incubated in a candle jar for 48-72 h at 37˚C, anaerobically. On SB-20M media, *S. mutans* and *S. sobrinus* produced characteristic colonies. Addition of coarse granular cane sugar in place of sucrose makes the media differential and selective. *S. mutans* produced transparent, crystalline, hard, firmly surface adhering crushed glass like colonies with polysaccharide drop on their surface on SB-20M media. Whereas, *S. sobrinus* produce characteristic circular, opaque, milky white colour colonies with whitish halo around them on media plates. For purification, colonies of *S. mutans* were cultured in 5.0 mL of thioglycolate broth, while for *S. sobrinus* purification SB-20M media was used. For preparation of

thioglycolate broth, 29.75 g media was dissolved in 1000 mL distilled water, autoclaved at 121˚C for 15 min (15 psi). Media (5.0 mL) was dispensed in screw cap sterile glass tubes. Bacteria was inoculated and incubate anaerobically for 48 h in a candle jar. Purification was done by inoculating *S. mutans* broth culture on SB-20M media.

4.2.3.2- Stereomicroscopy

Culture plates of MS were used for visualization of colony morphology under 20X magnification of stereoscopic/dissecting microscope.

4.2.3.3- Gram staining and biochemical testing

4.2.3.3.1- Gram staining

From a pure culture, an isolated single colony was picked, a smear was prepared on a clean glass slide and then fixed by heating. Next, slide was flooded for 1 min with primary stain, crystal violet (CV). Rinsed slide with water to remove excess stain and then flooded for 30-60 sec with mordant (Gram's Iodine). Washing with water was done again. Decolorization was done by using 95% ethanol, it was left on slide for 30 sec. Again, washing with water was done and counter stain safranin was flooded on slide for 45 sec. After washing with water, slide was air dried and microscopically examined for Gram-reaction and cell morphology under 100X lens (Micros-Austria).

4.2.3.3.2- Catalase test

Catalase enzyme activity exhibited by bacteria was determined by using catalase test. This enzyme cause hydrogen peroxide (H_2O_2) catabolism to molecular oxygen and water. This test differentiates between *Streptococcus* and *Staphylococcus* species. For this test, a small drop of 3% H_2O_2 was placed on a glass slide and mixed with a single pure bacterial colony by using sterile toothpick. Catalase activity was confirmed by active bubbles production within few seconds.

4.2.3.3.3- Arginine di-hydrolase test

Arginine di-hydrolase test was performed to determine the ability of isolates to hydrolyse arginine amino acid as a carbon source with the help of enzyme arginine di-hydrolase. For arginine di-hydrolase test, broth was prepared, which was composed of

yeast extract 2.5 g, K_2HPO_4 1.0 g, tryptone 2.5 g, L- Arginine monohydrochloride 1.5 g, and 500 mL distilled water. Media was autoclaved at 121˚C for 15 min (15 psi). Media was added to glass test tubes and inoculated with *S. mutans* fresh cultures and it was later incubated at 37˚C for 48 h, anaerobically. After incubation, Nessler's reagent (0.1 mL) was added to each tube and the conversion of media greenish yellow colour to orange indicated positive results.

4.2.3.3.4- Sugar fermentation test

Ability of MS to ferment sorbitol was also determined. For this test, broth was prepared which had yeast extract (Oxoid, England) 2.5 g, tryptone (Oxoid, England) 0.5 g, and distilled water 500 mL. A litmus blue solution (10% w/v) and sorbitol sugar (10% w/v), was added to the media for making a final volume of litmus blue 0.4 % and sugar 1.0 %. Media (5.0 mL) was poured in sterile screw caped test tubes, inoculated with S*treptococci* fresh cultures and incubated, anaerobically at 37˚C for 48 h. Red colour appearance indicated positive results, while persistence of blue colour is an indicator of negative results.

4.2.3.4- Molecular identification of MS

4.2.3.4.1- DNA Extraction

For DNA extraction, phenol chloroform method was used, it was a two days protocol. Composition of different chemicals used for DNA extraction is given in Appendix C1 and C2. Isolates were grown in 1000 µL thioglycolate broth for 48 h at 37˚C, anaerobically.

Day 1:-

1. Centrifuged overnight culture for 5 min at 7000 rpm.
2. Supernatant was discarded after centrifugation.
3. Pellet was resuspended in 280 µL of Tris HCl (200 mM) and 280 µL EDTA (10mM).
4. Lysozyme (µL) was added and mixed gently.
5. After mixing it was incubated for 30 min at 37^{o}C.
6. After incubation, 40 µL of 20% SDS and 4 µL Proteinase K was added and then incubation in water bath was done at 54^{o}C for 1 h.

7. After 1 h, 400 µL phenol: isoamyl: chloroform in a ratio of 25:24:1 was added and centrifuged for half hour at 7000 rpm. As a result, 3 layers will be appeared.
8. Upper layer was separated in separate eppendorf and 200 µL NaCl (200mM) and 600 µL isopropanol was added.
9. It was incubated overnight in freezer at -20^{o}C.

Day 2:-

1. Overnight frozen eppendorf were thawed and centrifugation was done for 15 min at 10,000 rpm.
2. Supernatant was discarded after centrifugation and pellet was washed, 1 mL of 70% chilled ethanol was added.
3. Centrifugation was done again for 5 min at 10,000 rpm and after discarding supernatant, pellet was air dried.
4. At the end, to dissolve DNA present in the pellet, 100 µL of TE buffer was added.

4.2.3.4.2- Agarose gel electrophoresis

For confirmation of DNA isolation, 1 % agarose gel was used. For preparation of 1% agarose gel, agarose 0.8 g was added in 80 mL of 1X TBE buffer. Ethidium bromide 3.0 µL was added after cooling of gel. It is used for staining DNA after loading. For loading DNA, it was mixed with 2-3 µL of bromophenol blue dye and loaded into wells of the gel. Gel electrophoresis for DNA products, were performed for 30 min at 110 volts. The gel was observed under gel documentation system (SYGENE). Composition of different chemicals used for agarose gel electrophoresis is given in Appendix C1 and C2.

4.2.3.4.3- Identification of *Streptococci* by PCR amplification of *dex*A and *Gtf* I gene

For molecular detection of *S. mutans,* PCR amplification of *dex*A gene and for *S. sobrinus Gtf* I gene was achieved by using a pair of primers (Fermentas, Germany) as shown in Table 4.1. PCR amplification conditions were optimized at various annealing temperatures and time duration. A reaction mixture of 10 µL was prepared (Table 4.2). Amplification cycle was completed in Biometra thermal cycler (Table 4.3 and 4.4). For visualization and quantification of PCR products, 2 % agarose gel was

prepared. For 2 % gel preparation, agarose 1.6 g was added in 80 mL of 1X TBE buffer and completely dissolved by heating. Ethidium bromide 0.3 µL was added after cooling of gel. It was used for staining DNA after loading. For loading, PCR products were mixed with 2-3 µL of bromophenol blue dye and loaded into wells of the gel. Gel electrophoresis for PCR products, was performed for 40 min at 110 volts. After electrophoresis, gel was visualized by using Gel documentation system (SYGENE).

Table 4.1: Sequence of primer used for amplification of *S. mutans dex*A and *S. sobrinus Gtf* I gene

Gene	**Primer sequence 5' → 3'**	**Product size (bp)**
*dex*A	Forward 5'-TAT GCT GCTATT GGA GGT TC-3' Reverse 5'-AAG GTT AC CAA TTG AAT CG-3'	1271
Gtf I	Forward 5'-GATAACTACCTGACAGCTGACT-3' Reverse 5'-AAGCT GCCTTAAGGTAATCACT-3'	712

4.2.4- Haemolysis test

To check haemolytic activity of *Streptococcus* isolates, blood agar was used. Blood agar is a rich medium as it is supplemented with fresh 5 % sheep blood. Blood agar was prepared by adding blood agar base 40.0 g in 1000 mL of distilled water and later autoclaved for 15 min at 121˚C (15 psi). Media was cooled after autoclaving (45˚C to 50˚C) and 5 % sheep blood was added, aseptically. Plates were inoculated with 24 h old cultures and incubation was done at 37°C for 24-48 h, aerobically. The culture that shows a dark colour or discoloration of the medium in the area of growth determines α-haemolysis. While Cultures showing clear zones around colonies and under growth presents β haemolysis. Colonies with no change in media are confirmed as γ haemolytic.

4.2.5- Statistical analysis

Described previously in chapter 3 (section 3.2.3).

Table 4.2: PCR reaction mixture composition for *dex*A and *Gtf* I gene amplification

Reagents	**Volume (µL)**
10x Taq buffer	1.0
25mM $MgCl_2$	0.8
dNTPs	0.4
Forward Primer	0.5
Reverse Primer	0.5
Taq Polymerase	0.2
PCR H_2O	5.6
DNA	1.0
Total volume	10.0

Table 4.3: Optimized conditions of PCR for amplification of *dex*A gene

Conditions	**Temperature (˚C)**	**Duration (min)**	**Cycles**
Initial denaturation	95.0	02	01
Final denaturation	95.0	01	36
Annealing	55.0	01	
Extension	72.0	01	
Final extension	72.0	05	01

Table 4.4: Optimized conditions of PCR for amplification of *Gtf* I gene

Conditions	**Temperature (˚C)**	**Duration**	**Cycles**
Initial denaturation	95.0	02 min	01
Final denaturation	95.0	30 sec	35
Annealing	59.0	30 sec	
Extension	72.0	01 min	
Final extension	72.0	05 min	01

4.3- RESULTS

4.3.1- Clinicodemographic features of postpartum females

Described previously in chapter 3 (section 3.3).

4.3.2- Isolation and identification of MS

4.3.2.1- Stereomicroscopy of isolated MS on modified SB-20 media

Due to the composition of SB-20 media, all isolated *S. mutans* and *S. sobrinus* produced colonies with unique morphology on modified SB-20 media which was confirmed by stereomicroscopy (Figure 4.1). Colonies of *S. mutans* appeared to be transparent, crystalline, having a granular surface like crushed glass and some colonies had a polysaccharide drop on their surface, while *S. sobrinus* colonies appeared as circular, opaque, milky white having whitish halo around them. Among postpartum females, 249/267 saliva samples were culture positive, hence were suspected as *S. mutans* and 143/267 were suspected as *S. sobrinus*, which were further confirmed by biochemical tests. In nonpregnant group, out of 54 saliva samples 23 were culture positive, which were suspected to be *S. mutans* and 28 for *S. sobrinus*.

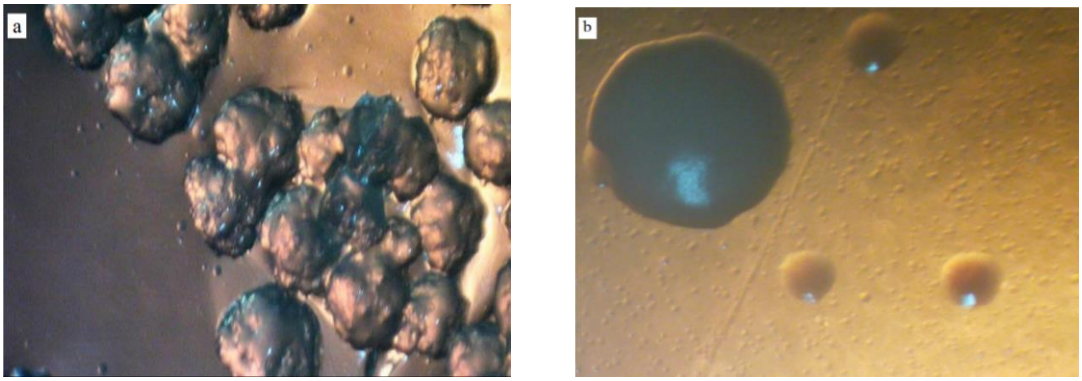

Figure 4.1: Colony morphology of MS on SB-20M media under stereomicroscope 20X magnification. (a) morphology of *S. mutans*-BK261; colonies appeared transparent, crystalline, having a granular surface like crushed glass (b) morphology of *S. sobrinus*-BK262; colonies appeared as circular, opaque, milky white having whitish halo around them

4.3.2.2- Biochemical confirmatory tests and molecular characterization of MS

All isolates were examined for Gram reaction under microscope at 40X and 100X. All suspected *S. mutans* and *S. sobrinus* showed Gram-positive reaction, and on

microscopy cocci appeared in chain forms. All isolates gave negative catalase test. A negative Arginine di-hydrolase test was used as confirmatory test for *S. mutans*. All. *S. mutans* isolates (*n*=249) gave negative arginine di-hydrolase test because they do not possess arginine hydrolysing enzyme arginine di-hydrolase. Raffinose fermentation test was also used as confirmatory test for *S. sobrinus*. Suspected *S. sobrinus* isolates (*n*=143) gave negative raffinose fermentation test because it cannot utilize raffinose as substrate. PCR amplification of *dex*A gene and *Gtf* I gene were used for final confirmation of the isolates. All suspected *S. mutans* isolates showed amplification of *dex*A gene and all *S. sobrinus* amplify *Gtf* I gene.

4.3.2.3- Comparative colonization of MS in postpartum and nonpregnant females

In present study, out of 267 postpartum females saliva samples, 92.26% were positive for *S. mutans*, while *S. Sobrinus* positive cultures were 53.5%. Among nonpregnant group, 42.60% were culture positive for *S. mutans* colonization and 51.85% for colonization of *S. sobrinus*. In comparison to nonpregnant females, significantly high colonization of *S. mutans* was found in postpartum group (*P*<0.0001), and there was also an approximately nineteen-fold increased risk for colonization of *S. mutans* in postpartum females (OR=18.64). Postpartum females showed one-fold increased risk for oral colonization of *S. sobrinus* (Table 4.5).

Table 4.5: *S. mutans* and *S. sobrinus* colonization in postpartum (*n*=267) and nonpregnant females (*n*=54)

Isolates		**Postpartum females *n* (%)**	**Nonpregnant females *n* (%)**	***P*-value (χ^2)**	**OR (95%Cl)**
S. mutans	Present	249(93.26)	23(42.60)	<0.0001 (89.14)	18.64 (9.06-38.34)
	Absent	18(6.74)	31(57.40)		
S. sobrinus	Present	143(53.5)	28(51.85)	0.818 (0.05)	1.07 (0.59-1.92)
	Absent	124(46.44)	26(48.14)		

4.3.3- Haemolytic reaction

All *S. mutans* were γ-haemolytic. Among postpartum females, out of 143 *S. sobrinus* isolates 138 (96.50%) were α-haemolytic, 04 (2.79%) isolates γ-haemolytic and 01

(0.69%) isolate gave β-haemolytic activity. Among nonpregnant females out of 28 *S. sobrinus* 27 (96.42%) showed α-haemolysis and one isolate showed β-haemolytic activity.

4.3.4- Associations of isolated salivary *S. mutans* with oral disorders, dietary habits and obstetric factors

Gingivitis and dental caries were significantly associated with *S. mutans* colonization in postpartum group (P=0.007 and P=0.006, respectively). Increased risk was seen for gingivitis and dental caries development in females with *S. mutans* colonization (OR=15.40 and OR=16.00, respectively). Decreasing brushing frequency was significantly affecting the *S. mutans* colonization in oral cavity (P<0.001) of these females with approximately two-fold increased risk for *S. mutans* colonization. *S. mutans* was significantly raised in postpartum females having high juices/drinks intake (P=0.01), and its colonization risk was high in females with increased tea/coffee intake (from OR=0.40 to OR=7.80). Mothers giving birth to low weight babies had significantly high (P<0.001) colonization of *S. mutans* (Table 4.6).

4.3.5- Associations of isolated *S. sobrinus* with oral disorders, dietary habits and obstetric factors

Although, *S. sobrinus* colonization was not significantly high in postpartum females with oral disorders, however, it showed approximately one-fold increased risk for development of gingivitis. Its colonization was also significantly changed in females with low brushing frequency (P=0.025) and high sugary liquid intake (juices/drinks intake P=0.008 and tea/coffee intake P=0.01). Like *S. mutans*, *S. sobrinus* also showed increased risk for colonization in postpartum females with increased intake of sugary fluids. Among APOs, its colonization showed significant association with term (P=0.03) and preeclampsia (P<0.001) (Table 4.7).

Table 4.6: Associations of isolated *S. mutans* with oral disorders, dietary habits and obstetric factors among postpartum females (*n*=267)

Factors		***S. mutans***		**P-value (χ^2)**	**OR (95% Cl)**
		Case positive *n* (%)	**Case negative *n* (%)**		
Gingivitis	Present	73(27.34)	00(0.00)	0.007	15.40
	Absent	176(65.92)	18(6.74)	(7.26)	(0.916-259.06)
Dental caries	Present	75(28.08)	00(0.00)	0.006	16.00
	Absent	174(65.16)	18(6.74)	(7.54)	(0.95-269.11)
Brushing frequency/ Day	No	04(1.50)	00(0.00)	<0.001	-
	1 time	155(58.06)	07(2.62)	(43.61)	2.30(0.11-46.8)
	2 times	90(33.70)	08(3.00)		0.50(0.17-1.44)
	3 times	00(0.00)	03(1.12)		0.01(0.0006-0.28)
Intake of juices/drinks	No	61(22.84)	00(0.00)	0.01	-
	Not frequently	83(31.10)	05(1.88)	(7.99)	1.12(0.006-2.27)
	Frequently	105(39.32)	13(4.86)		0.48(0.16-1.41)
Tea and coffee intake	No	26 (9.74)	01(0.38)	0.21	-
	1-2 times daily	181(67.80)	17(6.36)	(4.50)	0.40(0.05-3.20)
	3-4 times daily	40(14.98)	00(0.00)		7.80(0.46-132.57)
	>4 times daily	02(0.74)	00(0.00)		-
Gestational period	PTB	53(19.86)	05(1.88)	0.51	0.70
	FTB	196(73.40)	13(4.86)	(0.41)	(0.23-2.06)
Baby weight (Kg)	<2.5	50(18.72)	10(3.74)	<0.001	-
	2.5-4	197(73.80)	06(2.24)	(25.9)	0.15(0.05-0.43)
	>4	02(0.75)	02(0.75)		0.03(0.003-0.25)
Preeclampsia	Present	28(10.48)	03(1.12)	0.48	0.63(0.17-2.32)
	Absent	221(82.77)	15(5.61)	(0.48)	

Table 4.7: Associations of isolated *S. sobrinus* with oral disorders, dietary habits and obstetric factors among postpartum females (*n*=267)

Factors		***S. sobrinus***		***P*-value (χ^2)**	**OR (95% Cl)**
		Case positive *n* (%)	**Case negative *n* (%)**		
Gingivitis	Present	40(14.98)	33(12.36)	0.80	1.07
	Absent	103(38.56)	91(34.10)	(0.06)	(0.62-1.83)
Dental caries	Present	37(13.86)	38(14.24)	0.38	0.79
	Absent	106(39.70)	86(32.20)	(0.748)	(0.46-1.34)
Brushing frequency/day	No	03(1.12)	01(0.38)	0.025	-
	1 time	96(35.96)	66(24.72)	(9.27)	0.48(0.04-4.73)
	2 times	44(16.48)	54(20.22)		0.56(3.37-0.92)
	3 times	00(0.00)	03(1.12)		0.17(0.008-3.47)
Intake of juices/drinks	No	31(11.61)	30(11.24)	0.008	-
	Not frequently	37(13.85)	51(19.10)	(9.61)	0.70(0.36-1.35)
	Frequently	75 (28.10)	43(16.10)		2.40(1.36-4.23)
Tea and coffee intake	No	21(7.86)	06(2.24)	0.01	-
	1-2 times daily	96(35.96)	102(38.20)	(10.82)	0.26(0.10-0.69)
	3-4 times daily	24(9.00)	16(6.00)		1.59(0.79-3.18)
	>4 times daily	02(0.74)	00(0.00)		3.36(0.15-74.74)
Gestational period	PTB	24 (9.00)	34(12.74)	0.03	0.53
	FTB	119(44.56)	90(33.70)	(4.41)	(0.29-0.96)
Baby weight (Kg)	<2.5	26(9.74)	34(12.74)	0.189	-
	2.5-4.0	115(43.08)	88(32.96)	(3.32)	1.58(0.32-1.04)
	>4	02(0.74)	02(0.74)		1.30(0.18-9.46)
Preeclampsia	Present	05(1.88)	26(9.74)	<0.001	0.13
	Absent	138(51.68)	98(36.70)	(19.75)	(0.05-0.36)

4.4- DISCUSION

Dental health of the expecting mother deteriorates with each pregnancy, and the condition became worse with increasing number of pregnancies as multiparous females showed more tooth decay in comparison to the females having one child. Literature is available that showed association of pregnancy with tooth loss and development of periodontal diseases (Morelli *et al*., 2005; Kateeb and Momany, 2018). Oral health status during pregnancy can be used as an indicator of possible pregnancy outcomes (Silk *et al*., 2008). In addition to PTB and LWB, these outcomes also include high risk of caries development in infants (Krauer and Krauer, 1977; Ito, 2000). In present study, colonization of caries causing pathogens *S. mutans* and *S. sobrinus* in postpartum females (post one day of delivery) were studied to determine the oral health status of these females. Furthermore, presence of MS was also used to assess the association of cariogenic bacterial presence with oral disorders and pregnancy outcomes.

Postpartum females studied in the present work showed significantly high (93.2%) prevalence of *S. mutans* compared to non-pregnant group (42.6%). In the past, MS colonization and oral disorders association have been only seen in children (Ito, 2000). No study is available in Pakistani population on the prevalence of MS in postpartum females, even pregnant females are rarely investigated. However, a study from neighbouring country India reported significantly high *S. mutans* colonization in oral cavity of females of postpartum phase compared to nonpregnant females (Kamate *et al*., 2017). In present work, similar trend was seen in postpartum phase with high *S. mutans* colonization ($P<0.0001$) and approximately nineteen-fold increased risk. However, another study from India, reported non-significant results for salivary colonization of *S. mutans* during pregnancy compared to non-pregnant group. In this study, stimulated saliva was used to determine colonization with low sample size of 25 pregnant and 25 non-pregnant female (Singh and Shah, 2017). The discrepancy in the results from the study conducted in India, could be due to the method of sample collection they employed. Composition of saliva including salivary microflora is affected by the method of collection. As in current study, samples of unstimulated saliva were collected which are known to contain more microbial diversity as representative of different oral surfaces of oral cavity. In stimulated saliva samples different chewing materials are given to increased saliva production, which can

interfere with normal salivary composition and oral flora. Moreover, their sample count was also very low (*n*=25) to 267 unstimulated saliva samples collected from postpartum females admitted in Capital Hospital (Islamabad) and Holy family hospital (Rawalpindi), as there are higher number of participating females, this data generate might be more comprehensive and significant.

Beside high MS colonization in these females, a significantly higher salivary *S. mutans* prevalence was found in postpartum females suffering from dental caries (*P*=0.006) and gingivitis (*P*=0.007). MS colonization was also seen to increase the risk for development of these oral disorders. Similar to our finding, it is now becoming well established fact from several reports that acidic environment during pregnancy promotes the growth of these cariogenic microbes and increased risk for tooth demineralization (Silk *et al*., 2008). Hence, presence of MS in saliva can be used as an indicator for predicting caries risk in an individual (Martinez-Mier and Zandona, 2013). The current work strengthens the previous findings on MS prevalence.

Like present work significant results for association between salivary *S. mutans* carriage with dental caries was also reported by a study conducted in Greece but on children (Fragkou *et al*., 2016). Unlike present work, a study conducted by Scalioni *et al*., (2017), on Brazilian children and aldoscents diagnosed with Down syndrome showed no association of *S. mutans* (*P*=0.09) colonization in saliva with caries incidence, this variation might be due to difference in age and presence of genetic disorder. In present work, all postpartum females suffering from gingivitis and dental caries were positive for *S. mutans* colonization, which is an indication of role of MS in initiation or progression of dental disorders. This can be due to hormone mediated inflammatory response associated with pregnancy. This response act as a key player for increasing severity of gingival inflammation. These inflamed sites provide the environment, which increased the risk for colonization of pathogenic periodontal disease and dental caries causing agents. Severity of this inflammatory response becomes high as the pregnancy progresses towards the end (Geisinger *et al*., 2014; Hassan *et al*., 2018). In current work saliva samples were collected in early phase of postpartum period, where oral health status could be used as representative of late phase of pregnancy. Therefore, in early postpartum period, effect of inflammation and hormonal shift is still similar to late pregnancy period/third trimester.

S. sobrinus colonization was comparatively higher in postpartum group of the present study to the nonpregnant females (53.5% and 51.85%, respectively). Although, its colonization was not significant in females with oral disorders, still it posed one-fold risk for development of gingivitis. After *S. mutans*, *S. sobrinus* is also considered as an important cariogenic pathogen (Nurelhuda *et al*., 2010). There is a dearth of data in literature related to the salivary carriage of *S. sobrinus* during pregnancy or postpartum phase. Like *S. mutans*, mostly studies which are available are based on role of *S. sobrinus* in causing dental caries in children. Comparable results to this work were reported from a study conducted on Romanian and Swedish aldoscents. This study reported 85.7% prevalence of *S. mutans* and 50% prevalence of *S. sobrinus* among Romanian aldoscents. However, in Swedish population, *S. mutans* was prevalent in 50% of the cases while none of the sample was positive for *S. sobrinus*. Differences between these two population was due to the good hygienic measures practiced by Swedish population (Johansson *et al*., 2015). Here, postpartum females with poor oral hygiene were seen to be more prone to oral MS colonization in comparison to females with higher brushing frequency and who consumed high sugary liquid. Cariogenic bacteria breakdown sugar present in diet and creates the environment in which these bacteria flourish rapidly. Overgrowth of these bacteria ultimately leads to the development of different oral pathologies (Marla *et al*., 2018).

Among postpartum females having pregnancy related complications, *S. mutans* colonization was significantly high ($P<0.001$) in mothers who delivered babies with low weight, while *S. sobrinus* showed significant associations with gestation term ($P=0.03$) and preeclampsia ($P<0.001$). Until now, no such studies are available in literature which identifying the relationship between colonization of oral MS with APOs. Data is available that showed an association between oral periodontopathogens with APOs. These bacteria either directly by crossing mucosal barrier or by the action of their metabolic products, predisposes females to pregnancy related complications. Pregnancy promotes the gingival inflammation and plaque development (Lux and Lavigne, 2004). As *S. mutans* and *S. sobrinus* are commonly found in oral biofilms and *Streptococcus* species are also isolated from placenta, there is a possibility of their direct or indirect pathogenic role in induction of pregnancy complication. Moreover, oral conditions and inflammatory response during pregnancy also promote overgrowth of these bacteria.

In summary, the findings from the present work provides a new set of information about the salivary MS carriage in the postpartum phase, as no such studies are available in the literature. In postpartum phase especially during early phase, females are more susceptible for colonization of cariogenic microbes. This is might be due to alteration in hormonal levels, physiological changes in the body affecting oral health, recurrent vomiting, acidic gastric reflexes and dietary patterns. *S. mutans* colonization was seen significantly high in postpartum females especially in those suffering from dental caries, gingivitis and in those delivered babies with low weight. *S. sobrinus* isolation was comparatively high in postpartum females compared to nonpregnant group and had significant association with APOs. Colonization of MS was significantly raised with poor oral hygiene and with increased intake of sugary diet. Studies regarding the pathogenic potential of *S. mutans* in developing dental caries during pregnancy and postpartum phase are rare, with none of these are based on exploring its role in causing gingivitis and APOs. Until now no study is available related to determine the association between *S. sobrinus* colonization with oral disorders leading to APOs. Findings from the present work will be prove helpful in conducting future studies based on investigating the pathogenic potential of cariogenic bacteria in causing oral disorders and also in exploring their possible role in induction of APOs.

CHAPTER 5: STUDY OF CULTURABLE AND UNCULTURABLE ORAL BACTERIAL DIVERSITY AND ITS PATHOGENIC POTENTIAL IN POSTPARTUM FEMALES

5.1- INTRODUCTION

Human oral cavity harbours unique microbial ecosystem, made up of over 700 distinct species of bacteria (Digiulio *et al*., 2015; Cobb *et al*., 2017). Balance in this microbial ecosystem is important for maintaining local and systematic health of an individual (Verma *et al*., 2018). In health, symbiotic association is maintained between oral microbiota and their host. However, any alteration in these health associated communities can create dysbiosis, leading to the development of oral disorders such as periodontal disorders and caries lesions (Adams *et al*., 2017; Cobb *et al*., 2017). Both host genetics and environmental factors play important role in establishing these microbial communities (Paropkari *et al*., 2016).

By NGS analysis of 16*S rRNA* gene from nine different sites in oral cavity of over 200 healthy individuals in HMP, it was seen that various oral sites were colonized by 13-19 different bacterial phyla and 185-322 genera (Zaura *et al*., 2014). Among these major bacterial phyla, Firmicutes, Proteobacteria, Bacteroidetes, Spirochaetes, Actinobacteria, and Fusobacteria were present (Sampaio-Maia *et al*., 2016; Idris *et al*., 2017; Verma *et al*., 2018). Salivary microbiota comprised of different bacteria, which are shed from different oral surfaces (Belstrøm *et al*., 2017), which belong to phyla Actinobacteria, Firmicutes, Bacteroides, Proteobacteria, Fusobacteria, Spirochaetes, and TM7 (He *et al*., 2014). Although different individual shared similar salivary microflora, there was an inter-individual differences in composition of salivary microbial diversity especially at low taxonomic level (Sampaio-Maia *et al*., 2016; Pelzer *et al*., 2017). Composition of salivary flora vary in patients with different disorders like periodontitis, dental caries, oral squamous cell carcinoma and pancreatic cancer as compared to healthy population, as mostly flora is specific in such conditions. Salivary microbiota can be also used as biomarkers for disease diagnosis (He *et al*., 2014).

Saliva is constantly washing oral cavity and it has a strong effect on ecology of mouth. It maintains pH between 6.75-7.25, which is best for the growth of many organisms (Zaura *et al*., 2009). Saliva also effects microbial colonization by effecting

microbial adsorption to oral surfaces, act as a source of nutrients for microbes, covers bacterial antigens thus making them more host like. It also enables clearance of organisms by swallowing and preventing attachment plus growth of exogenous microbes (Batabyal *et al*., 2012). Co-existing microbial communities in mouth include microbes with different growth requirements like microaerophilic, facultative, capnophilic and obligatory anaerobic conditions (Avila *et al*., 2009).

Oral microbes specifically colonize different ecological niche. Oral microbiota is highly diverse, which is comprised of bacterial genera's especially *Streptococci, Lactobacilli, Staphylococci and Corynebacterium* (Dewhirst *et al*., 2010). It is not clear whether other non-commensal and transient bacteria can efficiently grow in these oral microbial communities (Thurnheer and Belibasakis, 2015). Previously, members of Enterobacteriaceae were regarded as transient colonizers of oral cavity, however, literature showed that *E. coli* is prevalent in oral cavity particularly in case of antibiotic therapy and immunocompromised conditions (Bandara *et al*., 2009).

Changing environmental conditions such as hormonal levels, diet and smoking can affects microbial composition and ultimately can lead to inflammation of soft oral tissues and tooth decay. Beside other conditions, pregnancy is one of such state during which almost every part of female body is disturbed including oral health. Oral microbial diversity is altered due to hormonal shift (progesterone and estrogen), immune and metabolic changes during pregnancy. Interplay of various above-mentioned factors with the microbial diversity is bidirectional in nature. Microbes respond to and regulate hormonal levels, modulate immune system and metabolic processes, which can in turn influences bacterial growth. During pregnancy, microbial load increases and can results in periodontal and dental problems. Various studies found a degree of link between oral polymicrobial disorders and pregnancy outcomes, but its molecular mechanism is still unclear (Gonzales-marin *et al*., 2012; Han and Wang, 2013; Borgo *et al*., 2014). Shift of oral microflora in periodontal diseases and its possible effect on PTB gained attention after work of Offenbacher *et al*., (1996) which was a case control study of pregnant females with oral health issues. However, up to now no conclusive evidence is available to support association of oral microbial dysbiosis with APOs as different studies report conflicting results (Yang *et al*., 2015; Vinturache *et al*., 2016; Tettamanti *et al*., 2017; Wagle *et al*., 2018).

Several studies have reported the colonization of *Staphylococcus* in different oral sites including saliva, where it resides as commensal, also in dental plaque and periodontal pockets indicating its possible role in the etiology of periodontal diseases and dental caries (Al-Tayyar *et al*., 2015; Nejabat *et al*., 2015). Among the members of this family, *S. aureus* is a commonly known human pathogen causing infections of skin and mucosa (Bueris *et al*., 2005), which are increasing day by day. This increase is because of rise in MRSA, specifically community-associated-MRSA (Chen *et al*., 2009). Many species of genus *Staphylococcus* especially *S. aureus* is responsible for causing many diseases by producing a different enzymes, toxins and also invasion of host cells by hemolysis (Kuroda *et al*., 2001). Another important virulence factor for *Staphylococci* is production of biofilm (Archer *et al*., 2011). *S. epidermidis* was the first species reported to be involved in biofilm formation; but afterwards same ability was found in *S. aureus* and other *Staphylococcal* species (Fey and Olson, 2010). *S. aureus* and *S. epidermidis* both have the intercellular adhesion operon (*Ica*), which might have role in biofilm forming ability. This operon is controlled by *Ica*R gene. *Ica* operon comprised four genes; *Ica*A, *Ica*D, *Ica*B and *Ica*D genes (Cue *et al*., 2012). This *Ica*ADBC gene cluster encodes for a polysaccharide intracellular adhesion (PIA) molecules, suggesting a possible role of this operon in *staphylococci* slime production, especially in Coagulase-negative *Staphylococcus* species (CoNS) (Pokrovskaya *et al*., 2013).

Previously most of the studies, which aimed to find the pregnancy associated microbial diversity and its association with oral problems and APOs, were based on culture or PCR-based identification of periodontopathogens or cariogenic microbes in maternal oral cavity or placenta. Most of these microbes are anaerobes. Data regarding prevalence of other culturable bacterial species in the oral cavity among pregnant is rare and none in postpartum females. There is a need to decipher the role of these species in periodontal diseases and pregnancy outcomes. Moreover, up to the knowledge of the author, no literature is available on the identification and assessment of salivary microbiome in postpartum female with oral health issues and its possible association with pregnancy outcomes. The present study was aimed to use culture dependent and independent methods to explore salivary microbial diversity and its pathogenic potential in postpartum phase and it's if any association with oral health issues and APOs.

5.2- MATERIAL AND METHODS

5.2.1- Study design and settings

Described previously in chapter 3 (section 3.2).

5.2.2- Study population and sampling technique

Described previously in chapter 4 (section 4.2.2).

5.2.3- Part I: Study of oral bacteria by culture dependent methods

5.2.3.1- Isolation of culturable bacteria from saliva samples

In laboratory, Nutrient agar plates (Oxoid, England) were inoculated with saliva samples (0.1 mL) and under aerobic condition incubated for 24-48 h at 37ºC. For preparation of NA medium, 28.0 g of NA was dissolved in 1000 mL distilled water and autoclave for 15 min at 121º C (15 psi). Media was cooled after autoclaving and poured in petri plate under sterile conditions. After incubation, depending upon varying colony morphology, by using sterile inoculating loop, different colonies were picked and were streaked separately to obtain pure cultures on plates. All these plates were incubated for 24-48 h at 37ºC, aerobically. The pure cultures obtained were then examined for morphology by using Gram staining technique.

5.2.3.2- Gram Staining

Described previously in chapter 4 (section 4.2.3.3.1).

5.2.3.3- Identification of *Staphylococcus* species

5.2.3.3.1- Catalase test

Described previously in chapter 4 (section 4.2.3.3.2).

5.2.3.3.2- Growth on Mannitol Salt Agar (MSA)

MSA is used for differentiating between mannitol fermenting *S. aureus* from other *Staphylococci*. Media contain mannitol and phenol red (pH indicator), which detects the acid production from fermentation of mannitol by *S. aureus*. For MSA (Oxoid, England) media preparation, 111 g of MSA was dissolved in 1000 mL distilled water and autoclaved for 15 min at 121º C (15 psi). Media was cooled after autoclaving and

poured under sterile conditions in petri plates. Single colony was picked from pure culture and streaked on the MSA plates and aerobically incubated for 24-48 h at 37ºC. Appearance of yellow colour growth showed presence of *S. aureus* while all other species of *Staphylococci* produce pink colonies.

5.2.3.3.3- Tube coagulase test

It is used as confirmatory test for *S. aureus,* which is used for detection of coagulase enzyme production. For this test, bacteria were allowed to grow in 500 µL of nutrient broth (Oxoid, England) in test tube at 37ºC for 24 h. NB was prepared by adding 13.0 g of media in 1000 mL of distilled water and autoclaved at 121ºC for 15 min (15 psi). After incubation, 2-3 drops of plasma were added in test tube and again incubated for about 4-5 h. Presence of coagulation after incubation confirmed the presence of *S. aureus*.

5.2.3.3.4- DNase test

This test is used to examine the bacterial ability to hydrolyse DNA as a carbon and energy source for growth. For this test DNase agar is used, it is a pale green colour media containing DNA-methyl green complex. Methyl green is positively charged which binds DNA which is negatively charged. If organism produce DNase enzyme, it causes DNA breakdown into small fragments. Fragments of DNA cannot bind methyl green and as a result colour of media fades and colourless zone appear around the colony.

DNase test was used for final confirmation of *S. aureus*. For media preparation, 39.0 g of DNase agar (Oxoid, England) was dissolved in 1000 mL of water and autoclaved for 15 min at 121ºC (15 psi). After autoclaving, media was cooled and poured in petri plate under sterile conditions. The samples were then inoculated on the plates. For inoculation heavy inoculum was used and a line of 3-4 cm was drawn on DNase agar plate. Then plates were incubated at 37°C for 18-24 h. After incubation, plates were flooded with 1N HCl. Plates were left to stand for a few min to allow the reagent to absorb into the plate. Then excess HCl was removed and examined against a dark background within 5 min.

5.2.3.3.5- Novobiocin differential test

For identification and differentiation between S. *epidermidis* and *S. saprophyticus,* novobiocin disk sensitivity test was performed. Isolates that showed sensitivity to novobiocin are *S. epidermidis* while resistant organisms are *S. saprophyticus.* Selected isolates of *Staphylococcus* were grown on NA for 24 h at 37ºC. Suspension of pure colony was prepared in normal saline (0.85% NaCl) to achieve turbidity equivalent to 0.5 McFarland. To obtain confluent growth, Mueller Hinton agar (Oxoid, England) plate was inoculated with a sterile swab and then 5µg novobiocin disc (Oxoid, England) were applied on the MHA surface. MHA was prepared by dissolving 38.0 g of media in 1000 mL distilled water and autoclaved at 121ºC for 15 min (15 psi). Zone of inhibition were interpreted according to recommendation of Clinical and Laboratory Standards Institute-2018-M100 guideline (CLSI, 2018). A zone of inhibition >16 mm showed that isolate is sensitive while a zone of inhibition ≤16 mm show that isolate is resistant to novobiocin.

5.2.3.3.6- Molecular characterization of *Staphylococcus* species:

5.2.3.3.6.1- Bacterial DNA extraction

For DNA extraction, phenol chloroform method was used of two days protocol. Isolates were grown in 1000 µL NB for 24 h at 37˚C, aerobically. Remaining procedure is described previously in chapter 4 (section 4.2.3.4.1).

5.2.3.3.6.2- Agarose gel electrophoresis

Described previously in chapter 4 (section 4.2.3.4.2).

5.2.3.3.6.3- PCR amplification of *nuc* gene for identification of *S. aureus*

For detection and confirmation of *S. aureus*, *nuc* primers (Fermentas, Germany) were used. PCR (Biometra) was optimized at various annealing temperatures and time. Primer sequence, and reaction mixture composition are given in Table 5.1 and 5.2, respectively. Thermocycler amplification conditions are given in Table 5.3.

Table 5.1 Sequence of primer for amplification of *nuc* gene of *S. aureus*

Gene	**Primer sequence 5' → 3'**	**Product size (bp)**
nuc	Forward 5'-GCGTTGATGGTGATACGGTT-3' Reverse 5'-AGCCAAGCCTTGACCAACTAAAGG-3'	279

Table 5.2 Composition of PCR reaction mixture for amplification of *nuc* gene of *S. aureus*

Reagents	**Volume (µL)**
10x Taq buffer	1.0
25mM $MgCl_2$	0.8
dNTPs	0.4
Forward Primer	0.5
Reverse Primer	0.5
Taq Polymerase	0.2
PCR H_2O	5.6
DNA	1.0
Total volume	10.0

Table 5.3: Optimized conditions of PCR for amplification of *nuc* gene of *S. aureus*

Conditions	**Temperature (˚C)**	**Duration (min)**	**Cycles**
Initial denaturation	94.0	05	01
Final denaturation	94.0	01	33
Annealing	56.0	01	
Extension	72.0	01	
Final extension	72.0	07	01

5.2.3.4- Identification of Enterobacteriaceae members

5.2.3.4.1- Growth of bacteria on MacConkey agar

This media contains CV and bile salts, which make it selective for Gram-negative bacteria. It inhibits the growth of most of the Gram-positive bacteria. It differentiates between Gram-negative lactose fermenting and non-lactose fermenting bacteria. All identified Gram-negative rods were identified for their lactose fermentation ability by culturing on MacConkey agar for 24 h at 37˚C, aerobically. This media contains lactose monohydrate as a source of carbohydrate, peptone as source of minerals and vitamins, and pH indicator neutral red to detect acid production from lactose fermentation by changing colour to pink**.** MacConkey agar was prepared by suspending 52.0 g of MacConkey agar (Oxoid, England) in 1000 mL of distilled water and then autoclaved for 15 min at 121°C (15psi) and poured in petri plates. Lactose fermenters produce pink colour colonies on MacConkey agar.

5.2.3.4.2- Growth on eosin methylene blue (EMB) agar

EMB is a differential and selective media for the growth of Gram-negative bacteria. It is composed of eosin and methylene blue dyes, which inhibit the growth of most of the Gram-positive bacteria. Lactose fermenting Gram-negative bacteria lower the pH of the media by acid production, which increase the colonies capacity to absorb the dye, as a result colony appear purple black in colour. *E. coli* produce purple colonies having green metallic sheen on media due to the metachromatic properties of the fermentation product. On EMB agar all Gram-negative rods were cultured for 24 h at 37˚C aerobically. To prepare EMB agar plates, 37.5 g of agar (Oxoid, England) was suspend in 1000 mL of distilled water and then autoclaved for 15 min at 121°C (15psi). Cool, mix and then media was poured in petri plates

5.2.3.4.3- Citrate utilization test

To differentiate between members of Enterobacteriaceae citrate utilization test was performed. This test detects their ability to utilize citrate as a carbon source. Simmon citrate medium is used for this purpose. Citrate utilization causes pH change which is detected by bromothymol blue indicator. To prepare this media, 23.0 g of Simmon citrate agar (Oxoid, England) was suspended in 1000 mL of distilled water and autoclaved for 15 min at 121°C (15 psi) and then 5.0 mL of media was poured in

sterile test tube and then cooled in slanted position. For streaking tube, a single colony was streak back and forth on slant. Incubation was done for 24 h at 37 °C. Colour change from green to blue indicated positive results.

5.2.3.4.4- Motility test

Motility was checked by using soft agar stabbing on tube method, for this media was prepared by adding pancreatic digest of gelatin 10.0 g, NaCl 5.0 g, beef extract 3.0 g and agar 4.0 g dissolved in 1000 mL of distilled water and autoclaved for 15 min at 121ºC (15 psi). After autoclaving, 5.0 mL was poured in a sterile test tube and allowed to cool. After picking an isolated colony, media was inoculated by stabbing the medium at the centre to depth of half inch. Incubation was done at 37ºC for 24 h. Presence of growth spreading from the line of inoculation, was reported as positive test. A growth confined to stab line indicated negative motility test.

5.2.3.4.5- Triple sugar iron (TSI) test

TSI is a differential media used for identification of Enterobacteriaceae members that can ferment carbohydrates and produce gas. TSI contain glucose, sucrose and lactose as a carbon source and phenol red pH indicator, which detect acid production by sugar fermentation. TSI (Oxoid, England) was prepared by dissolving 65.0 g in 1000 mL of distilled water and autoclaved for 15 min at 121˚C (15 psi). After autoclaving TSI media (5.0 mL) was poured in test tube and cooled in a slanted position. A single colony was picked and stabbed on butt deeply and slant surface was streaked. Incubated for 18-24 h at 37˚C, aerobically.

5.2.3.4.5.1- Results interpretation

1. Lactose (or sucrose) fermentation produce large amount of acid, which turn indicator yellow both in slant and butt. Those organisms that produces gases produce bubbles or cracks in media.

2. Glucose fermentation causes butt to turn yellow, and slant appear red.

3. In case, when none of the sugar is fermented, both slant and butt appear red.

4. In case of H_2S production, black colouration of media occurs due to ferrous sulfide production.

5.2.3.4.6- API kit

Remel RapID ONE system was used for confirmation of Enterobacteriaceae members according to manufacturer's guidelines. This system was composed of RapID ONE panel and RapID ONE reagents. RapID one panel was a plastic disposable tray containing different substrate, degradation of which by inoculating organisms was then used for the identification. These substrates include urea, ornithine, arginine, aliphatic thiol, lysine, sugar aldehyde, fatty acid ester, ρ-nitrophenyl-β, sorbitol, D-glucuronide, ρ-nitrophenyl-β,D-glucoside, σ-nitrophenyl-β,D-galactoside, ρ-nitrophenyl-β,D-xyloside, malonate, ρ-nitrophenyl-n-acetyl-β,D-glucosaminide, proline-β-naphthylamide, pyrrolidonyl-β-naphthylamide, γ-glutamyl-β-naphthylamide, tryptophane and adonitol.

5.2.3.4- Other biochemical tests for isolated bacterial species

5.2.3.4.1- Oxidase test

It is used for differentiating between lactose fermenting and non-lactose fermenting bacteria, and for identification of Cytochrome c oxidase producers, an enzyme involved in electron transport chain. This enzyme can oxidase tetramethyl-p-phenylenediamine reagent to purple colour indophenols product. To this test, filter paper was soaked in tetramethyl-p-phenylenediamine dihydrochloride. A single pure colony of test organism was smeared on filter paper. Positive test was indicated by a colour change within 10-30 sec. Members of Enterobacteriaceae gives negative oxidase test while *Pseudomonas aeruginosa* gives positive oxidase test. All catalase positive Gram-negative cocci suspected to be a member of *Neisseria* family gives positive oxidase test and were further confirmed by sugar fermentation test. All catalase positive Gram-positive rods were also subjected to oxidase test to confirm them as *Lactobacilli* species.

5.2.3.4.2- Carbohydrates fermentation test for identification of *Neisseria* species

To differentiate between suspected *Neisseria* species e.g. *Neisseria meningitides* and *Neisseria gonorrhoeae*, maltose and glucose fermentation test was performed. Both *Neisseria* species can ferment glucose, but maltose is only fermented by *N. meningitides*. Phenol red carbohydrate broth was prepared for the test by adding:

Trypticase 10.0 g, Beef extract 1.0, NaCl 5.0 g, phenol red pH indicator 0.018 g and carbohydrate source (glucose and maltose) 10.0 g was added in 1000 mL distilled water and dissolved completely. Add 5.0 mL broth in sterile test tube and insert durham tubes. Autoclave at 121˚C for 15 min for glucose and 3 min for maltose. Inoculate tube with microorganism and then incubated for 24 h at 37˚C. Positive test is indicated by acid production in which liquid turns yellow while in case of negative results media remains red. A positive gas production was indicated by a small bubble in inverted Durham's tube while in case of negative result no bubble will be formed.

5.2.3.5- Detection of Virulence factors of isolated bacteria

5.2.3.5.1- Haemolysis test

Described previously in chapter 4 (section 4.2.4).

5.2.3.5.2- String test

String test determine Hyper-mucoviscosity of suspected *K. pneumoniae* isolates. Freshly grown pure isolated colonies of *K. pneumoniae* on MacConkey agar plates were touched by sterile inoculating loop and slightly raised to check their ability to form string. The string greater than 5.0 mm in length is an indication of positive hyper-mucoviscosity.

5.2.3.5.3- Detection of biofilm forming ability

5.2.3.5.3.1- Congo red assay (CRA) for screening of biofilm formation

To detect biofilm producing isolates Congo red assay (CRA) was used. CRA media was prepared by supplementing brain heart infusion (Oxoid, England) broth (37.0 g/L) with sucrose 50.0 g/L, technical agar (no. 1) 10.0 g/L and 0.8 g/L of Congo red dye. Congo red dye solution and BHI media was prepared separately and autoclaved for 15 min at 121˚C (15 psi). After autoclaving media was cooled and Congo red dye solution was added to BHI supplemented media. It was poured into sterile petri plates, freshly cultured colonies were streaked on it and incubation was done for 24-48 h at 37˚C, aerobically. After incubation, plates were checked for appearance of coloured colonies. Black crusty colonies were produced by biofilm formers, while pink or red colour colonies were produced by non-biofilm producing isolates.

5.2.3.5.3.2- Biofilm formation screening by microtiter plate method (MTP)

In this assay, 24 h old stock cultures were inoculated on the test tubes containing NB containing 10% w/v glucose and incubated for 24 h at 37°C. Next day, 96-well microtiter plate was inoculated with 198 µL of NB and 2.0 µL of each culture. All isolates were inoculated in triplicates. Three sterile nutrient broth containing wells without culture were designated as negative control. The plate was placed inside incubator for 24 h at 37°C, after covering with lid and sealing with parafilm.

After incubation, without disrupting the established biofilms, media was removed carefully. Washing of each well was done thrice with 200-300 µL of phosphate buffer saline of pH 7.2 (Appendix C3) to remove non-adherent cells. Then 95% ethanol was added to the wells for fixation of adhered cells and plate was incubated for 30 min at room temperature. Then the adherent cells were stained with 200 µL of 1% w/v CV stain (Appendix C4) and again incubated at room temperature for 15 min. Deionized water was used to rinse the wells of microtiter plate to remove any excess stain. After washing, air dried the adherent cell and to solubilize the CV stain treated with 33% acetic acid (Appendix C5) at room temperature (15 min). Optical density (OD) was measured at wavelength 630 nm for each well by using spectrophotometer (Multiskan Go, Thermoscientific). Cut off optical density (ODc) was calculated and was used for comparison with mean ODs of test isolate. Biofilm farming ability was categorized by the method as described previously (Hassan *et al*., 2011; Dhanasekaran *et al*., 2014) (Table 5.4). ODc calculation was done by following formulae:

$$ODc = \text{Mean OD of control} + (3 \times \text{S.D of control})$$

5.2.3.5.3.3- Detection of biofilm forming genes in Staphylococcal isolates

For the detection of biofilm forming genes in Staphylococcal isolates (*S. aureus* and *S. epidermidis*), *Ica* operon genes were amplified by specific primers (Fermentas, Germany) (Table 5.5). A reaction mixture of 10.0 µL was prepared (Table 5.6). Amplification cycle was completed in Biometra thermal cycler (Table 5.7). For visualization and quantification of PCR products, 2% agarose gel was prepared.

Table 5.4: Classification criteria of biofilm forming ability by MTP method

OD values	**Biofilm Formers**
OD ≤ ODc	Negative
ODc< OD ≤ 2 x ODc	Weak
2 x ODc< OD ≤ 4 x ODc	Moderate
4 x ODc< OD	Strong

Table 5.5: Primer sequences used for amplification of *Ica* operon genes of *S. aureus* and *S. epidermidis*

Primers	**Sequence 5' → 3'**	**Product size (bp)**
IcaA	Forward 5'-ACACTTGCTGGCGCAGTCAA-3'	188
	Reverse 5'-TCTGGAACCAACATCCAACA-3'	
IcaB	Forward 5'-AGAATCGTGAAGTATAGAAAATT-3'	880
	Reverse 5'-TCTAATCTTTTTCATGGAATCCGT-3'	
IcaC	Forward 5'-ATGGGACGGATTCCATGAAAAAGA-3'	1056
	Reverse 5'-TAATAAGCATTAATGTTCAATT-3'	
IcaD	Forward 5'-ATGGTCAAGCCCAGACAGAG-3'	198
	Reverse 5'-AGTATTTTCAATGTTTAAAGCAA-3'	

Table 5.6: PCR reaction mixture composition for amplification of *Ica* operon genes of *staphylococcus* species

Reagents	**Volume (µL)**
Master mix	05
Forward primer	0.2
Reverse primer	0.2
PCR water	3.6
Template DNA	1.0
Total volume	10.0

Table 5.7: PCR amplification conditions for *Ica* operon genes of *Staphylococcus* species

Steps	**Temperature (˚C)**	**Time duration**	**Cycles**
Initial Denaturation	95.0	05 min	01
Denaturation	95.0	45 sec	35
Annealing	52.0 for *Ica*A and *Ica*B genes 39.0 for *Ica*C gene 42.0 for *Ica*D gene	45 sec	
Extension	72.0	45 sec	
Final Extension	72.0	07 min	01

5.2.3.5.4- Antibacterial susceptibility testing

Antibacterial activity was checked by Kirby-Bauer disc diffusion method according to the recommendation of CLSI guidelines. For testing methicillin susceptibility of *Staphylococcus*, cefoxitin FOX30 disks (Oxoid, England) 30 µg were used. All bacteria were tested for antimicrobial activity against different antibiotics. For this, bacterial suspensions were made in 1.0 mL normal saline, by mixing isolated colony from 18-24 h old cultures. Suspensions were prepared according to 0.5 McFarland standard. Suspensions were inoculated on MHA plate by using sterile swab. Different antibiotics were placed 24 mm apart on MHA plates and incubation was done for 24 h at 37˚C, aerobically. After overnight incubation results were interpreted as recommended by CLSI-2018-M100 (CLSI, 2018) as shown in Table 5.8.

5.2.3.5.5- Statistical analysis

Described previously in chapter 3 (section 3.2.3).

Table 5.8: List of antibiotics with break points of inhibition recommended by CLSI-2018-M100 (CLSI, 2018)

Class	**Antibiotics**	**S (mm)**	**I (mm)**	**R (mm)**
Aminoglycosides	Tobramycin 30µg (Oxoid, England)	≥15	13-14	≤12
	Amikacin 30µg (Oxoid, England)	≥17	15-16	≤14
	Gentamicin 10µg (Oxoid, England)	≥15	13-14	≤12
Quinolones	Nalidixic acid 30µg (Oxoid, England)	≥19	14-18	≤13
Fluoroquinolones	Ciprofloxacin 5µg (Oxoid, England)	≥21	16-20	≤15
	Ofloxacin 5µg (Oxoid, England)	≥16	13-15	≤12
Cephalosporins	Ceftriaxone 30µg (Oxoid, England)	≥27	25-26	≤24
	Cefoxitin 30µg (Oxoid, England)	≥22	-	≤21
	Cefotaxime 30µg (Oxoid, England)	≥22	-	≤21
Sulfonamides	Sulphamethox/Trimethoprim 25µg (Oxoid, England)	≥16	11-15	≤10
Tetracycline	Tetracycline 30µg (Oxoid, England)	≥15	12-14	≤11
Penicillin	Penicillin 10 units (Oxoid, England)	≥29	-	≤ 8
	Ampicillin 10µg (Oxoid, England)	≥17	14-16	≤13
Macrolides	Erythromycin 15µg (Oxoid, England)	≥23	-	≤13
Lincosamide	Clindamycin 10µg (Oxoid, England)	≥16	11-15	≤10
Nitrofurantoin	Nitrofurantoin 300µg (Oxoid, England)	≥17	15-16	≤14
Carbapenem	Imipenem 10µg (Oxoid, England)	≥16	14-15	≤13
Chloramphenicol	Chloramphenicol 30µg (Oxoid, England)	≥16	13-15	≤12
Linezolid	Linezolid 30µg (Oxoid, England)	≥21	-	≤20

S: Sensitive; I: Intermediate; R: Resistant

5.2.4- Part II. Study of bacterial diversity by culture independent method

5.2.4.1- Study population and sample collection

Among postpartum group, three female saliva samples, while one from nonpregnant group was processed for identification of salivary microbiome by amplification of V4 region of 16*S rRNA*. Subjects were divided into 2 groups: 1st group comprised of two postpartum female having dental caries and gingivitis, one of them delivered PLWB (BK2) and 2nd one had FTB with normal weight baby (BK1), while the 2nd group comprised of healthy postpartum female without dental issues having FTB and normal weight baby (BKC1) along with healthy nonpregnant female (BKC2).

5.2.4.2- Microbial DNA extraction from saliva and sequencing

DNA extraction was carried out in two-day protocol as described previously (http://www.openwetware.org/wiki/Gill:S._aureus_genomic_DNA_isolation), with few modifications. After DNA extraction, it was quantified via A260/A280 ratio by using nanodrop spectrophotometer (Titertek Berthhod, Germany). PCR amplification of V4 region of 16*S rRNA* gene was carried out by using primers 515F/806R (Table 5.9), having barcode on forward primer in 30 cycle PCR. PCR amplification conditions are mentioned in Table 5.10. After amplification, 2 % agarose gel was used for products visualization. Multiple PCR samples in equal concentrations, based on their molecular weight, were pooled together and purified by using calibrated Ampure, XP beads. DNA library was then prepared from this pooled and purified PCR product, by using Illumina TruSeq DNA library preparation protocol. Illumina MiSeq platform was used for sequencing and it was performed at MR DNA (www.mrdnala.com, Shallowater, TX, USA) according to manufacturer's guideline. Raw data sequences have been submitted in Sequence Read Archive (SRA) and was assigned an accession number PRJNA505914.

Table 5.9: Primer sequences used for amplification of bacterial V4 region of 16*S rRNA* gene

Primers	**Sequence 5' → 3'**
515F	5'GTGCCAGCMGCCGCGGTAA-3'
806R	5'GGACTACHVHHHTWTCTAAT-3'

Table 5.10: PCR thermocycler conditions for amplification of bacterial V4 region of 16*S rRNA* gene

Steps	**Temperature (˚C)**	**Time duration**	**Cycles**
Initial Denaturation	94.0	03 min	01
Denaturation	94.0	30 sec	28
Annealing	53.0	40 sec	
Extension	72.0	01 min	
Final Extension	72.0	05 min	01

5.3- RESULTS

5.3.1- Part I. Isolation of aerobic culturable bacteria from saliva samples

Saliva samples of postpartum females and healthy controls were inoculated on NA for 24 h at 37ºC. After incubation, colonies with different colony morphology were picked, streaked on NA and incubated for 24 h at 37 ºC. Out of 267 samples, 384 isolates and among nonpregnant females, out of 54 samples 69 isolates obtained, purified and subjected to Gram stain for further identification.

5.3.2- Gram Staining

All isolates were Gram stained and out of 384 isolates, 283 (73.69%) were Gram-positive cocci, 39 (10.15%) Gram-negative rods, 34 (8.85%) Gram-positive rods and 28 (7.29%) Gram-negative cocci. Among nonpregnant group, 69 isolates were cultured, out of which 42 (60.86%) were confirmed as Gram-positive cocci, 10 (14.49%) Gram-negative cocci, 11 (15.94%) Gram-negative rods and 06 (8.69%) Gram-positive rods. All of these isolates were further proceeded for identification.

5.3.3- Identification of Gram-positive cocci

5.3.3.1- Catalase test

All Gram positive 283 isolates were subjected to catalase test. Out of 283 isolates, 210 (74.20%) isolates were catalase positive (*Staphylococcus*) while 73 (25.80%) were catalase negative (*Streptococcus*). Out of 267 samples, 174 samples were culture positive for *Staphylococcus* species. Thirty-six samples produced more than one type

of *Staphylococcus* species. Among nonpregnant group, 32 samples were positive for *Staphylococcus* species from which 38 (90.47%) isolates were cultured. Four isolates were confirmed as *Streptococcus* species (9.52%) from nonpregnant group.

5.3.3.2- Growth on MSA

All catalase positive staphylococcal species were grown on plates of MSA. Out of 210 isolates, 100 (47.62%) isolates showed yellow growth on MSA, an indication of possible *S. aureus* strains, but it was further confirmed by coagulase and DNase test. Among nonpregnant group out of 38 isolates, 29 (76.31%) isolates produced yellow growth on MSA and 09 (23.68%) pink colonies.

5.3.3.3- Coagulase test

All suspected *S. aureus* isolates (*n*=100) gave positive coagulase test.

5.3.3.4- DNase test

For final confirmation of the suspected isolates of coagulase positive *S. aureus*, DNase test was performed (Figure 5.1). DNase positive isolates were confirmed as *S. aureus* (*n*=100).

5.3.3.5- Novobiocin disc sensitivity testing

For identification and differentiation between *S. epidermidis* and *S. saprophyticus* novobiocin disk test was performed for 110 CoNS isolates (Figure 5.2). Seventy-eight (37.14%) isolates were identified as *S. epidermidis* as these were novobiocin sensitive while 32 (15.24%) isolates were identified as *S. saprophyticus* (Novobiocin resistant). Among nonpregnant group, 07 (16.66%) isolates were confirmed as *S. epidermidis* and 02 (4.76%) as *S. saprophyticus*.

5.3.3.6- *S. aureus* identification by *nuc* gene amplification

For molecular detection of *S. aureus* from other *Staphylococcu*s species, *nuc* gene amplification was done. After PCR amplification, all suspected *S. aureus* isolates produced a 279 bp PCR product.

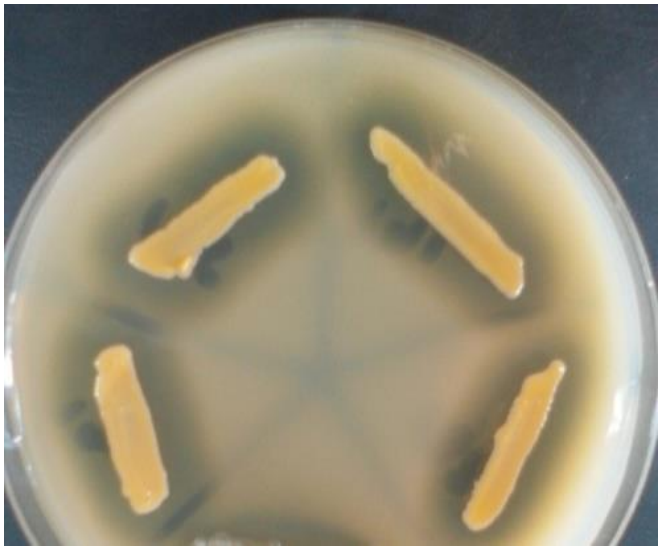

Figure 5.1: Positive DNase test for *S. aureus*-BK21, BK67, BK89 and BK245 isolate from saliva samples, producing colourless zone around the colony

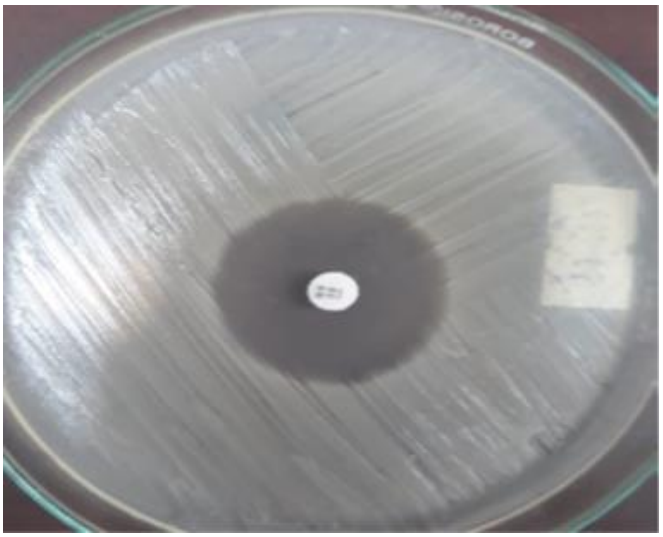

Figure 5.2: Novobiocin differential test for *S. epidermidis*-BK226 isolate from saliva. *S. epidermidis* showed sensitivity while *S. saprophyticus* showed resistance to Novobiocin

5.3.3.7- Comparative colonization of various Gram-positive cocci among postpartum females

Out of 267 samples, 174 (65.16%) samples were positive for *Staphylococcus* species while among nonpregnant group 59.25%. Approximately one-fold increased colonization risk for *Staphylococcus* species in oral cavity of postpartum group was seen in comparison to nonpregnant females (OR=1.28). Prevalence of *S. aureus* was comparatively high in nonpregnant group (42.59%) compared to postpartum (32.58%). *S. epidermidis* presence was significantly raised in postpartum (*P*=0.005) and approximately three-fold more risk was seen for colonization of *S. epidermidis* and *S. saprophyticus* among postpartum group. *Streptococcus* isolates also showed significantly raised prevalence in postpartum females in comparison to nonpregnant females with approximately five-fold increased risk for colonization (Table 5.11)

Table 5.11: Colonization of Gram-positive cocci in oral cavity of postpartum females compared to nonpregnant females

Isolates		**Postpartum females *n* (%)**	**Nonpregnant females *n* (%)**	***P*-value (χ^2)**	**OR (95%Cl)**
Staphylococcus species	Present	174 (65.16)	32 (59.25)	0.408 (0.682)	1.28 (0.70-2.34)
	Absent	93 (34.83)	22 (40.74)		
S. aureus	Present	87 (32.58)	23 (42.59)	0.157 (1.99)	0.65 (0.35-1.18)
	Absent	180 (67.41)	31 (57.40)		
S. epidermidis	Present	78(29.22)	06 (11.12)	0.005 (7.61)	3.34 (1.37-8.12)
	Absent	189(70.78)	48 (88.88)		
S. saprophyticus	Present	32 (11.98)	02 (3.70)	0.071 (3.25)	3.54 (0.82-15.24)
	Absent	235 (88.02)	52 (96.29)		
Streptococcus species	Present	73(27.34)	04(7.84)	0.001 (9.78)	4.70 (1.64-13.48)
	Absent	194(72.65)	50(92.59)		

5.3.3.8- Detection of Virulence factors in oral *Staphylococcus* isolates

5.3.3.8.1- Haemolysis test

Haemolytic activity was checked for *S. aureus* oral isolates. Among postpartum females, out of 100 isolates, 30 gave α, 35 γ and 35 β haemolytic reaction. Among nonpregnant group *S. aureus* isolates, 14/29 (48.27%) produced β hemolysis, 10/29 (34.48%) γ haemolytic and 5/29 (17.24%) were α haemolysis producers.

5.3.3.8.2- CRA for screening of biofilm production

CRA was performed for all *Staphylococcus* species. Out of 210 isolates from postpartum females, 22 % isolates of *S. aureus* showed positive results for biofilm formation i.e. black, dry crystalline colonies (Figure 5.3), 09 (11.53%) isolates of *S. epidermidis* were also positive for biofilm forming activity. Among other *Staphylococcus* species, two *S. saprophyticus* isolates were biofilm producers. Among nonpregnant females 11 (37.93%) *S. aureus* and 03 (42.85%) *S. epidermidis* isolates exhibited biofilm forming activity, while both *S. saprophyticus* isolates showed non-biofilm forming ability (Table 5.12).

5.3.3.8.3- MTP assay for assessing biofilm formatting ability

Among all isolates, 86 (40.95%) *Staphylococcus* isolates expressed biofilm forming ability by MTP method. Out of 86 biofilm forming isolates of *Staphylococci,* only one (1.16%) isolate was strong, 03 (3.48%) moderate and 82 (95.34%) were weak biofilm formers. Greater biofilm forming ability was shown by *S. aureus* isolates (47%) followed by *S. epidermidis* (35.89%) and *S. saprophyticus* (34.37%). Among isolates from nonpregnant females, 21 (55.26%) expressed biofilm forming ability of which seven isolates of *S. aureus*, three of *S. epidermidis* and only one of *S. saprophyticus* had biofilm forming activity (Table 5.12).

5.3.3.8.4- Detection of *Ica* operon (*Ica*ADBC) genes in Staphylococcal isolates

The genotypic detection of biofilm formation in Staphylococcal isolates (*S. aureus* and *S. epidermidis*) was done by using a set of primers. Among *Staphylococcal* isolates, 114 (64.04%) showed presence of *Ica*A gene and 86 (48.31%) were positive for *Ica*D gene. Prevalence of *Ica*B and *Ica*C was low as it was detected in 32.02% and 23.03% cases, respectively. Among nonpregnant females, presence of *Ica* operon

genes was high in isolates of *S. aureus* compared to postpartum females except for *Ica*A gene. Among *S. epidermidis* isolates *Ica*A was detected in 14.28% of the isolates, *Ica*D and *Ica*B in 71.42% isolates and *Ica*C was absent in all isolates (Figure 5.4, Table 5.13).

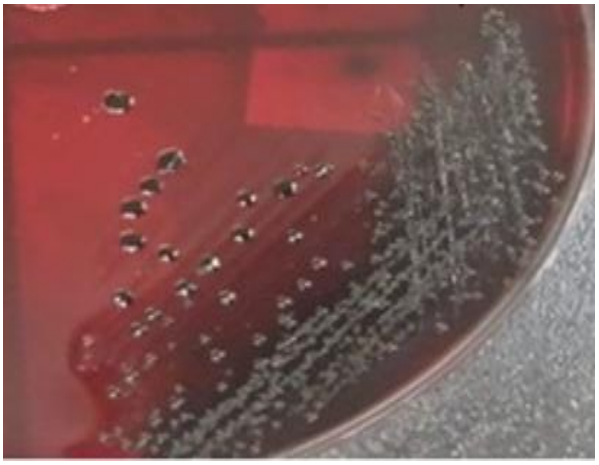

Figure 5.3: Biofilm forming ability shown by *S. aureus*-BK21 on CRA; Black crystalline colonies produced showing strong biofilm forming ability

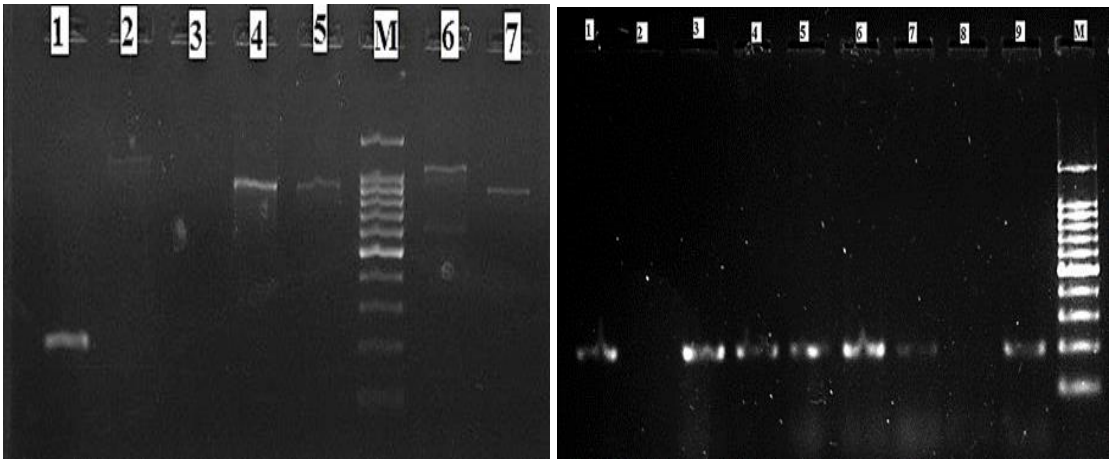

Figure 5.4: PCR amplification products of *Ica* operon gene. (a) Lane M: 100 bp molecular-size DNA ladder, Lane 1; *Ica*A gene of product size 188 bp, Lane 4,5,7: *Ica*B gene of 880 bp size, Lane 6, *IcaC* gene product of 1056 bp (b) PCR amplification product of *Ica*D gene. Lane M: 100 bp molecular-size DNA ladder, Lane 1, 3,4,5,6,7,9; *Ica*D gene of product size 198bp

Table 5.12: Comparative analysis of biofilm forming ability of *Staphylococcus* species by CRA and MTP method

***Staphylococcus* species**		**CRA**		**MTP**	
		Postpartum females *n* (%)	**Non-pregnant females *n* (%)**	**Postpartum females *n* (%)**	**Non-pregnant females *n* (%)**
S. aureus	Biofilm former	22(22)	11(37.93)	47(47)	17(58.62)
	Non-biofilm former	78 (78)	18(62.06)	53(53)	12(41.37)
S. epidermidis	Biofilm former	09(11.53)	03(42.85)	28(35.89)	03(42.85)
	Non-biofilm former	69(88.46)	04(57.1)	50(64.10)	04(57.1)
S. saprophyticus	Biofilm former	02(6.25)	00(0.00)	11(34.37)	01(50)
	Non-biofilm former	30(93.75)	02(100)	21(66.62)	01(50)

Table 5.13: Detection of *Ica* operon genes in various *Staphylococcus* isolates from postpartum and nonpregnant females

***Ica* operon genes**		***S. aureus***		***S. epidermidis***	
		Postpartum females *n* (%)	**Nonpregnant females *n* (%)**	**Postpartum females *n* (%)**	**Nonpregnant females *n* (%)**
*Ica*A	Present	73(73)	13(44.82)	41(52.56)	01(14.28)
	Absent	27(27)	16(55.17)	37(47.43)	06(85.72)
*Ica*D	Present	57(57)	22(75.86)	29(37.18)	05(71.42)
	Absent	43(43)	07(24.13)	49(62.82)	02(28.58)
*Ica*B	Present	22(22)	10(34.48)	35(44.88)	02(71.42)
	Absent	78(78)	19(65.51)	43(55.12)	05(28.58)
*Ica*C	Present	18(18)	05(17.25)	23(29.48)	00(0.00)
	Absent	82(82)	24(82.75)	55(70.51)	07(100)

5.3.3.8.3- Antibiotic sensitivity testing

S. aureus isolates were tested against 17 antibiotics. Among cephalosporin group, 60% of the isolates showed methicillin sensitivity. Resistance was high against cefotaxime (62%). Sensitivity was very low against penicillin class with 93% and 63% resistance against penicillin and ampicillin, respectively. High resistance was also seen against quinolones (65%), antibiotics of class fluoroquinolones (45%), erythromycin (47%) and gentamycin (35%). Low resistance was seen against amikacin (5%), chloramphenicol (8%), nitrofurantoin (10%), sulfonamides (15%), linezolid (15%), clindamycin (20%) and tetracycline (25%). *S. epidermidis* was tested against ten antibiotics, in which all isolates showed complete sensitivity to tetracycline, ciprofloxacin and gentamicin but chloramphenicol resistance was in 10.25% isolates. High resistance was seen against antibiotics of class penicillin (89.74% against penicillin and 44.87% against ampicillin) and cephalosporins (66.67% against ceftriaxone, 60.26% against cefoxitin and 55.12% against cefotaxime). Against erythromycin comparatively low resistance was seen (33.33%) in the isolates. *S. saprophyticus* was tested against eight antibiotics. Complete resistant to cefotaxime and penicillin was shown by all isolates. Against cefoxitin resistance was high as 65.62%. None of the isolate was resistant to chloramphenicol and nitrofurantoin. Comparatively low resistance was seen against ampicillin, erythromycin and nalidixic acid where 34.38% of the isolates being resistant to these antibiotics (Table 5.14a and 5.14b).

Table 5.14a: Antibiotic susceptibility pattern of *Staphylococcus* species isolated from postpartum females

Class	**Antibiotics**		***S. aureus* n (%)**	***S. epidermidis* n (%)**	***S. saprophyticus* n (%)**
Cephalosporins	Cefoxitin (FOX30)	R	40 (40)	47(60.26)	21(65.62)
		S	60 (60)	31(39.74)	11(34.38)
	Cefotaxime (CTX30)	R	62(62)	43(55.12)	32(100)
		S	38(38)	35(44.88)	00(0.00)
	Ceftriaxone (CRO30)	R	25(25)	52(66.67)	-
		S	70(70)	26(33.33)	-
		I	05(5)	0(0.00)	-
Penicillin	Penicillin (P10U)	R	93(93)	70(89.74)	32(100)
		S	07(7)	08(10.26)	00(0.00)
	Ampicillin (AMP10)	R	63(63)	35(44.87)	11(34.38)
		S	22(22)	35(44.87)	21(65.62)
		I	15(15)	08(10.26)	0(0.00)
Lincosamide	Clindamycin (DA10)	R	20(20)	-	-
		S	80(80)	-	-
Macrolides	Erythromycin (E15)	R	47(47)	26(33.33)	11(34.38)
		S	22(22)	44(56.41)	00(0.00)
		I	31(31)	08(10.26)	21(65.62)
Tetracycline	Tetracycline (TE30)	R	25(25)	00(0.00)	-
		S	60(60)	78(100)	-
		I	15(15)	00(0.00)	-
Linezolid	Linezolid (LZD10)	R	15(15)	-	-
		S	85(85)	-	-
Fluoroquinolones	Ofloxacin (OFX5)	R	45(45)	-	-
		S	45(45)	-	-
		I	10(10)	-	-
	Ciprofloxacin (CIPS5)	R	45(45)	00(0.00)	-
		S	55(55)	78(100)	-
		I	00(0.00)	00(0.00)	-
Chloramphenicol	Chloramphenicol (C30)	R	08(8)	08(10.25)	00(0.00)
		S	69(69)	52(66.68)	11(34.38)
		I	23(23)	18(23.07)	21(65.62)
Nitrofurantoin	Nitrofurantoin (F300)	R	10(10)	-	00(0.00)
		S	90(90)	-	32(100)
Sulfonamides	Sulphamethox/ Trimethoprim (SXT25)	R	15(15)	-	-
		S	85(85)	-	-
		I	00(0.00)	-	-

Table 5.14b: Antibiotic susceptibility pattern of *Staphylococcus* species isolated from postpartum females

Class	**Antibiotics**		***S. aureus* *n* (%)**	***S. epidermidis* *n* (%)**	***S. saprophyticus* *n* (%)**
Quinolones	Nalidixic acid (NA30)	R	65(65)	-	11(34.38)
		S	25(25)	-	21(65.62)
		I	10(10)	-	00(0.00)
Aminoglycosides	Amikacin (AK30)	R	05(5)	-	-
		S	80(80)	-	-
		I	15(15)	-	-
	Gentamicin (CN10)	R	35(35)	00(0.00)	-
		S	65(65)	78(100)	-
		I	00(0.00)	00(0.00)	-

R: Resistant, S: sensitive, I: Intermediate

5.3.3.8.4- Association of *Staphylococcus* species colonization with oral health factors, obstetric factors and with liquid intake among postpartum females

Colonization of *Staphylococcus* species was not significantly associated with any of the oral health and obstetric factors, except for *S. saprophyticus* which showed significant association with increased juices/drinks intake (*P*=0.01). *S. aureus* colonization increased approximately among one-fold risk with oral disorders. Its colonization was also seen to be increased in females with high juice/drinks intake (OR=0.87 to OR=1.44) (Table 5.15). The presence of *S. epidermidis* in saliva showed approximately one-fold risk for gingivitis and its colonization was increased with lower brushing frequency (Table 5.16). Its colonization was also increased in females with increasing tea/coffee intake. Among postpartum females, *S. saprophyticus* colonization increased one to two-fold risk for oral disorders and APOs (Table 5.17). Its colonization was also increased with increasing juices/drinks intake (OR=1.67 to OR=2.65).

Table 5.15: Association of *S. aureus* colonization with oral health factors, obstetric factors and with liquid intake among postpartum females (*n*=267)

Factors		***S. aureus***		**P-value (χ^2)**	**OR (95% Cl)**
		Case positive *n* (%)	**Case negative *n* (%)**		
Gingivitis	Present	25(9.36)	48(17.98)	0.72	1.10
	Absent	62(23.22)	132(49.44)	(0.12)	(0.62-1.96)
Dental caries	Present	28(10.48)	47(17.60)	0.30	1.34
	Absent	59(22.10)	133(49.82)	(1.07)	(0.76-2.34)
Brushing frequency/ Day	No	00(0.00)	04(1.50)	0.07	-
	1 time	46(17.30)	116(43.46)	(6.98)	
	2 times	41(15.00)	57(21.36)		
	3 times	01(0.40)	02(0.80)		
Intake of juices/drinks	No	19(7.11)	42(15.73)	0.45	-
	Not frequently	25(9.36)	63(23.59)	(1.55)	0.87(0.43-1.78)
	Frequently	43(16.10)	75(28.08)		1.44(0.79-2.62)
Tea and coffee intake	No	09(3.37)	18(6.74)	0.51	-
	1-2 times daily	68(25.46)	130(48.68)	(2.30)	1.04(0.44-2.45)
	3-4 times daily	10(3.70)	30(11.23)		0.63(0.29-1.38)
	>4 times daily	00(0.00)	02(1.17)		
Gestational period	PTB	16(5.60)	42(16.00)	0.35	0.74
	FTB	71(26.60)	138(51.80)	(0.84)	(0.38-1.40)
Baby weight (Kg)	<2.5	11(4.12)	49(18.36)	<0.001	-
	2.5-4.0	72(26.96)	131(49.06)	(14.59)	
	>4	04(1.50)	00(0.00)		
Preeclampsia	Present	09(3.37)	22(8.23)	0.65	0.82
	Absent	78(29.21)	158(59.17)	(0.20)	(0.36-1.88)

Table 5.16: Association of *S. epidermidis* colonization with oral health factors, obstetric factors and with liquid intake among postpartum females (*n*=267)

Factors		***S. epidermidis***		**P-value (χ^2)**	**OR (95% Cl)**
		Case positive *n* (%)	**Case negative *n* (%)**		
Gingivitis	Present	23(8.62)	50(18.72)	0.61	1.16
	Absent	55(20.59)	139(52.05)	(0.25)	(0.64-2.08)
Dental caries	Present	21(7.86)	54(20.22)	0.78	0.92
	Absent	57(21.35)	135(50.57)	(0.07)	(0.50-1.66)
Brushing frequency/day	No	00(0.00)	04(1.50)	0.56	-
	1 time	50(18.72)	112(41.94)	(2.02)	-
	2 times	27(10.12)	71(26.60)		1.17(0.67-2.04)
	3 times	01(0.37)	02(0.74)		0.76(0.06-8.73)
Intake of juices/drinks	No	11(4.12)	50(18.72)	0.08	-
	Not frequently	28(10.48)	60(22.48)	(4.81)	2.12(0.96-4.68)
	Frequently	39(14.60)	79(29.60)		1.05(0.58-1.90)
Tea and coffee intake	No	07(2.62)	20(7.50)	0.85	-
	1-2 times daily	57(21.34)	141(52.80)	(0.78)	1.15(0.46-2.88)
	3-4 times daily	13(4.86)	27(10.12)		1.19(0.57-2.47)
	>4 times daily	01(0.38)	01(0.38)		2.07(0.12-35.8)
Gestational period	PTB	13(4.86)	45(16.85)	0.19	0.64
	FTB	65(24.34)	144(53.95)	(1.65)	(0.32-1.26)
Baby weight (Kg)	<2.5	13(4.86)	47(17.60)	0.33	-
	2.5-4.0	64(23.98)	139(52.06)	(2.21)	0.60(0.30-1.18)
	>4	01(0.38)	03(1.12)		1.38(0.14-13.5)
Preeclampsia	Present	09(3.38)	22(8.24)	0.98	0.99
	Absent	69(25.84)	167(62.54)	(0.001)	(0.43-2.25)

Table 4.17: Association of *S. saprophyticus* colonization with oral health factors, obstetric factors and with liquid intake among postpartum females (*n*=267)

Factors		***S. saprophyticus***		**P-value (χ^2)**	**OR (95% Cl)**
		Case positive *n* (%)	**Case negative *n* (%)**		
Gingivitis	Present	09(3.37)	64(23.97)	0.91	1.04
	Absent	23(8.61)	171(64.04)	(0.01)	(0.45-4.09)
Dental caries	Present	13(4.86)	62(23.22)	0.09	1.90
	Absent	19(7.11)	173(64.79)	(2.82)	(0.89-3.84)
Brushing frequency/day	No	00(0.00)	04(1.49)		-
	1 time	25(9.36)	137(51.31)	0.17	
	2 times	07(2.62)	91(34.08)	(4.95)	
	3 times	00(0.00)	03(1.12)		
Intake of juices/drinks	No	03(1.12)	58(21.72)	0.01	-
	Not frequently	07(2.62)	81(30.33)	(9.20)	1.67(0.41-6.73)
	Frequently	22(8.23)	96(35.95)		2.65(1.07-6.52)
Tea and coffee intake	No	02(0.74)	25(9.36)	0.82	-
	1-2 times daily	25(9.36)	173(64.79)	(0.89)	1.80(0.40-8.09)
	3-4 times daily	05(1.87)	35(13.10)		0.98(0.35-2.75)
	>4 times daily	00(0.00)	02(0.74)		-
Gestational period	PTB	09(3.37)	49(18.35)	0.34	1.48
	FTB	23(8.61)	186(69.66)	(0.87)	(0.64-3.41)
Baby weight (Kg)	<2.5	06(2.24)	54(20.22)	0.05	-
	2.5-4.0	24(8.98)	179(67.04)	(5.70)	0.82(0.32-2.13)
	>4	02(0.74)	02(0.74)		0.13(0.01-0.99)
Preeclampsia	Present	05(1.87)	26(9.73)	0.44	1.48
	Absent	27(10.11)	209(78.27)	(0.57)	(0.52-4.20)

5.3.4- Isolation and identification of Gram-negative and positive rods and Gram-negative cocci from postpartum and nonpregnant females

5.3.4.1- Isolation and identification of Gram-negative rods

5.3.4.1.1- Growth on MacConkey agar

All of the isolated Gram-negative rods from postpartum females, produced pink colour colonies on MacConkey agar indicating that all isolates were lactose fermenters, while among nonpregnant female group, out of 11 Gram-negative isolates seven were lactose fermenters.

5.3.4.2- Oxidase test

Among postpartum group, all Gram-negative rods gave negative oxidase test while among nonpregnant group out of 11 isolates there were four non-lactose fermenter having positive oxidase test, hence were suspected to be *P. aeruginosa*.

5.3.4.3- Growth on EMB

Among postpartum female group, seven isolates produced purple colour colonies with green metallic sheen while 17 isolates produced dark purple mucoid colonies and 15 produced light purple colonies. Isolates with dark purple colour and green metallic sheen were identified as *E. coli*. Among nonpregnant females, one isolate produced dark purple colonies with green metallic sheen, four produced mucoid dark purple colonies and two produced light purple colonies. Four oxidase negative isolate produced colourless colonies.

5.3.4.4- Citrate utilization test

Among postpartum group, seven suspected *E. coli* gave negative result for citrate test, while remaining isolates were citrate positive. Among nonpregnant group one suspected *E. coli* isolate was citrate negative while all remaining isolates gave positive reaction.

5.3.4.5- Triple sugar iron test (TSI)

In postpartum group and nonpregnant females all suspected isolates of *E. coli, K. pneumoniae* and *Enterobacter* species produced acidic slant and acidic butt (A/A)

with positive gas production while four isolates suspected to be *P. aeruginosa* produced alkaline slant and alkaline butt (K/K) with no gas and H_2S production.

5.3.4.6- Motility assay

All suspected *E. coli* isolate, *Enterobacter* species and *P. aeruginosa* isolates gave positive motility test and *K. pneumonia* were negative for motility test.

5.3.4.7- API kit for Enterobacteriaceae

Remel RapID$_{TM}$ ONE system was used for confirmation of suspected Enterobacteriaceae members. Among postpartum group out of 39 isolates, seven were *E. coli*, 17 *K. pneumoniae* and 15 belonged to *Enterobacter* group while among nonpregnant females, out of 11 isolates one was confirmed as *E. coli*, four as *K. pneumoniae* and two as *Enterobacter* species.

5.3.4.2- Identification of Gram-positive rods and Gram-negative cocci

5.3.4.2.1- Catalase test

All Gram-negative cocci and rods from postpartum females and nonpregnant females gave positive catalase test, indicating that they can be members of *Lactobacilli* and *Neisseria* family, but further confirmation was done by other biochemical tests.

5.3.4.2.2- Oxidase test

All Gram-positive rods (*n*=34) were oxidase positive and were confirmed as member of *Lactobacilli* species. Gram negative cocci with positive catalase were suspected to be member of *Neisseria* family as these were also oxidase positive.

5.3.4.2.3- Carbohydrates fermentation test for identification of *Neisseria* species

Gram-negative cocci were subjected to carbohydrate utilization test, and all (*n*=28) fermented both glucose and maltose indicating that all isolates were *N. meningitidis*.

5.3.4.3- Comparative colonization of Gram-negative, Gram-positive rods and Gram-negative cocci isolated from postpartum females

Out of 267 saliva samples from postpartum group, 17 (6.36%) were positive for *K. pneumoniae*, 15 (5.61%) *Enterobacter* species, seven (2.62%) *E. coli*, 34 (12.73%)

Lactobacilli and 28 (10.48%) for *N. meningitides*. Among nonpregnant group, four (7.84%) samples were positive for *K. pneumoniae*, two (3.93%) *Enterobacter* species, one (1.96%) for *E. coli*, five (9.80%) *Lactobacilli* and 10 (19.60%) were positive for *N. meningitides*. No significant association was seen for colonization of these bacteria in postpartum females' group in comparison to nonpregnant group, however, colonization risk was approximately 1.5-fold of *Enterobacter* species, *E. coli* and *Lactobacilli* species in postpartum females (Table 5.18).

5.3.4.4- Screening of virulence factors

5.3.4.4.1- String test for *K. pneumonia*

String test was carried out for only *K. pneumonia* isolates to identified hypervirulent *K. pneumonia.* In this method isolated colony of *K. pneumoniae* was touched with sterile wire loop and withdrawn slowly. Bacteria forming a 'string' of at least 5.0 mm were positive (Figure 5.5). Among postpartum females, 7/17 isolates of *K. pneumoniae* were string test positive while among nonpregnant females only two, hence were designated as hypervirulent *K. pneumoniae* (hvKP) and rest two classical *K. pneumoniae* (cKP).

5.3.4.4.2- Screening of biofilm formation by CRA

All *K. pneumoniae* and *E. coli* isolates gave negative biofilm forming ability on CRA, while only two (13.34%) isolates of *Enterobacter* species produced black crystalline colonies on CRA plate. Among Gram positive rods, dark black dry colonies were produced by six isolates (17.64%). Among Gram negative cocci, only nine (32.14%) isolates were biofilm former (Table 5.17).

5.3.4.4.3- MTP assay for assessing biofilm forming ability

In MTP assay of all these isolates, none of the isolate was identified as having strong or moderate biofilm forming ability. Out of 39 Gram negative rods, 10 were non-biofilm formers while all the remaining isolates showed weak biofilm forming activity. All *E. coli* isolates showed weak biofilm forming ability by MTP method. Twelve of *Enterobacter* species and 10 of *K. pneumoniae* isolates were confirmed as weak biofilm formers. Among Gram positive rods, 12 were non-biofilm formers

while all remaining 64.70% were confirmed as weak biofilm formers. Eleven (39.28%) Gram negative cocci expressed weak biofilm forming ability (Table 5.19).

Table 5.18: Colonization of Enterobacteriaceae members, *Lactobacilli* species and *N. meningitides* in postpartum females compared to nonpregnant females

Isolates		**Postpartum female *n* (%)**	**Nonpregnant female *n* (%)**	***P*-value (χ^2)**	**OR (95%Cl)**
K. pneumoniae	Yes	17 (6.36)	04 (7.40)	0.777 (0.07)	0.85 (0.27-2.63)
	No	250 (93.63)	50 (92.59)		
Enterobacter species	Yes	15 (5.61)	02 (3.70)	0.566 (0.32)	1.54 (0.34-6.97)
	No	252(94.38)	52(96.29)		
E. coli	Yes	07(2.62)	01(1.85)	0.740 (0.10)	1.42 (0.17-11.84)
	No	260(97.37)	53(98.14)		
Lactobacilli species	Yes	34(12.73)	05(9.25)	0.475 (0.50)	1.43 (0.53-3.84)
	No	233(87.26)	49(90.74)		
N. meningitides	Yes	28(10.48)	10(18.52)	0.095 (2.77)	0.51 (0.23-1.13)
	No	239(89.51)	44(80.48)		

Fig 5.5: Positive string test (string greater than 5.0 mm in length) for *K. pneumonia*-BK167. Freshly grown pure isolated colonies of *K. pneumoniae* on MacConkey agar plates were touched by sterile inoculating loop and slightly raised for hyper-mucoviscosity

5.3.4.5- Antibiotic susceptibility testing

K. pneumoniae and *E. coli* isolates were tested against 18 antibiotics. Isolates of *K. pneumoniae* showed complete resistance against most of the antibiotics like erythromycin and penicillin. Comparatively high resistance was also seen against ampicillin, clindamycin, tobramycin, tetracycline (88.24% against each), cephalosporins (82.36% against cefotaxime and 47.06% against ceftriaxone), linezolid (64.70%), nitrofurantoin (52.94%), chloramphenicol and nalidixic acid. The most effective drugs against isolates were imipenem and amikacin (100% sensitivity), Sulphamethox/Trimethoprim (88.24% sensitivity), fluoroquinolones (11.76% resistance against ofloxacin and 17.64% against ciprofloxacin) and gentamicin (29.42% resistance). All *E. coli* isolates were 100% resistant to ceftriaxone, penicillin, clindamycin, erythromycin, tetracycline and linezolid. But 71 % isolates were resistant to nalidixic acid, nitrofurantoin and ciprofloxacin. Most effective drugs against *E. coli* were imipenem, amikacin, tobramycin and Sulphamethox/Trimethoprim (100% sensitivity). Also 71 % sensitivity was seen against gentamicin, chloramphenicol, ofloxacin and ampicillin. *Enterobacter* species were tested against ten antibiotics. Their sensitivity against most of the antibiotics was 100% like the antibiotics of class aminoglycosides and carbapenems followed by 86.66% against chloramphenicol, 80% against ofloxacin, 66.67% against cefotaxime, 46.66% against each ceftriaxone and ampicillin (Table 5.20a and 5.20b).

Table 5.19: Biofilm forming ability shown by members of Enterobacteriaceae, *Lactobacilli* and *N. meningitides* by CRA and MTP methods

Isolates		**CRA**	**MTP**
		Postpartum females *n* (%)	**Postpartum females *n* (%)**
k. pneumoniae	Biofilm former	00(0.00)	10(58.82)
	Non-biofilm former	17(100)	07(41.18)
E. coli	Biofilm former	00(0.00)	07(100)
	Non-biofilm former	07(100)	00(0.00)
Enterobacter species	Biofilm former	02(13.34)	12(80.00)
	Non-biofilm former	13(86.66)	03(20.00)
Lactobacilli Species	Biofilm former	06(17.64)	22(64.70)
	Non-biofilm former	28(82.35)	12(35.29)
N. meningitides	Biofilm former	09(32.14)	11(39.28)
	Non-biofilm former	19(67.86)	17(60.72)

Table 5.20a: Antibiotic sensitivity pattern of Enterobacteriaceae members isolated from saliva of postpartum females

Class	**Antibiotics**		***K. pneumonia* *n* (%)**	***E. coli* *n* (%)**	***Enterobacter* species *n* (%)**
Cephalosporins	Cefotaxime (CTX30)	R	14(82.36)	02(29.0)	05(33.33)
		S	03(17.64)	05(71.0)	10(66.67)
	Ceftriaxone (CRO30)	R	08(47.06)	07(100)	08(53.34)
		S	09(52.94)	00(0.00)	07(46.66)
		I	00(0.00)	00(0.00)	00(0.00)
Penicillin	Penicillin (P10U)	R	17(100)	07(100)	-
		S	00(0.00)	00(0.00)	-
	Ampicillin (AMP10)	R	15(88.24)	02(29.0)	06(40.00)
		S	02(11.76)	00(0.00)	07(46.66)
		I	00(0.00)	05(71.0)	02(13.34)
Lincosamide	Clindamycin (DA10)	R	15(88.24)	07(100)	-
		S	02(11.76)	00(0.00)	-
Macrolides	Erythromycin (E15)	R	17(100)	07(100)	-
		S	00(0.00)	00(0.00)	-
		I	00(0.00)	00(0.00)	-

Table 5.20b: Antibiotic sensitivity pattern of Enterobacteriaceae members isolated from saliva of postpartum females

Class	**Antibiotics**		***K. pneumonia* *n* (%)**	***E. coli* *n* (%)**	***Enterobacter* species *n* (%)**
Tetracycline	Tetracycline (TE30)	R	15(88.24)	07(100)	03(20.00)
		S	02(11.76)	00(0.00)	12(80.00)
		I	00(0.00)	00(0.00)	00(0.00)
Linezolid	Linezolid (LZD10)	R	11(64.70)	07(100)	-
		S	06(35.30)	00(0.00)	-
Fluoroquinolones	Ofloxacin (OFX5)	R	02(11.76)	00(0.00)	00(0.00)
		S	15(88.24)	05(71.0)	12(80.00)
		I	00(0.00)	02(29.0)	03(20.00)
	Ciprofloxacin (CIPS5)	R	03(17.64)	05(71.0)	-
		S	11(64.70)	00(0.00)	-
		I	03(17.64)	02(29.0)	-
Chloramphenicol	Chloramphenicol (C30)	R	08(47.06)	02(29.0)	02(13.34)
		S	09(52.94)	05(71.0)	13(86.66)
		I	00(0.00)	00(0.00)	00(0.00)
Nitrofurantoin	Nitrofurantoin (F300)	R	09(52.94)	05(71.0)	-
		S	08(47.06)	02(29.0)	-
Sulfonamides	Sulphamethox/ Trimethoprim (SXT25)	R	02(11.76)	00(0.00)	-
		S	15(88.24)	07(100)	-
		I	00(0.00)	00(0.00)	-
Quinolones	Nalidixic acid (NA30)	R	08(47.06)	05(71.0)	-
		S	09(52.94)	02(29.0)	-
		I	00(0.00)	00(0.00)	-
Aminoglycosides	Tobramycin (TOB30)	R	15(88.24)	00(0.00)	00(0.00)
		S	02(11.76)	07(100)	15(100)
	Amikacin (AK30)	R	00(0.00)	00(0.00)	00(0.00)
		S	17(100)	07(100)	15(100)
		I	00(0.00)	00(0.00)	00(0.00)
	Gentamicin (CN10)	R	05(29.42)	02(29.0)	00(0.00)
		S	12(70.58)	05(71.0)	15(100)
Carbapenems	Imipenem IMI10	R	00(0.00)	00(0.00)	00(0.00)
		S	17(100)	07(100)	15(100)

S: Sensitive; I: Intermediate; R: Resistant

5.3.2- Part II: Study of microbial diversity by culture independent methods

5.3.2.2- Sequence characteristics

From four saliva samples, 6,467,93 reads were obtained. 1st sample (BK1) had count of 1,349,91, sample 2 (BK2) 1,783,28, sample 3 (BKC1) 1,563,84, and sample 4 (BKC2) had total count of 1,770,90. In total detected OTUs were 527. After data trimming, filtering and normalization, predominantly detected OTUs with count ≥2 were 420.

5.3.2.3- Taxonomic analysis

A total of 16 phyla, 25 classes, 43 orders, 84 families, 156 genera and 282 species were observed in all four samples. Subject BK1 had 16 phyla, dominated by Proteobacteria (47.06%) followed by Firmicutes (31.55%), Bacteroidetes (9.16%), Fusobacteria (6.57%), Actinobacteria (3.91%), Spirochaetes (1.3%), Tenericutes (0.34%), Cyanobacteria (0.062%), Planctomycetes (0.026%), Acidobacteria (0.0098%), Synergistetes (0.0065%), Deferribacteres (0.0065%), Deinococcous thermus (0.0065%), Gemmatimonadetes (0.004%) and Candidatus saccharibacteria (0.0008%). Subject BK2 had 12 phyla, dominated by Firmicutes (64.24%) followed by Proteobacteria (12.03%), Bacteroidetes (10.86%), Actinobacteria (10.11%), Fusobacteria (2.55%), Spirochaetes (0.14%), Tenericutes (0.03%), Acidobacteria (0.003%), Candidatus saccharibacteria (0.0008%), Synergistetes (0.0019%), Deferribacteres (0.0019%) and Cyanobacteria (0.0013%). In case of BKC1, there were 12 phyla, which was again dominated by Firmicutes (50.28%) followed by Bacteroidetes (21.69%), Proteobacteria (11.15%), Actinobacteria (9.66%), Fusobacteria (6.41%), Cyanobacteria (0.57%), Spirochaetes (0.157%), Tenericutes (0.021%), Candidatus saccharibacteria (0.012%), Synergistetes (0.011%), Acidobacteria (0.0014%) and Deferribacteres (0.00072%). Subject BKC2 had 13 phyla, dominated by Firmicutes (41.29%) followed by Proteobacteria (35.07%), Bacteroidetes (7.50%), Fusobacteria (7.40%), Actinobacteria (7.10%), Spirochaetes (1.46%), Tenericutes (0.049%), Acidobacteria (0.046%), Synergistetes (0.031%), Deferribacteres (0.022%), Cyanobacteria (0.018%), Candidatus saccharibacteria (0.004%), and Deinococcus thermus (0.0006%) (Figure 5.6a)

In these samples, abundance of major genera was recorded. In BK1 major genera were: *Streptococcus* (20.91%) followed by *Yersinia* (20.76%), *Haemophilus* (5.90%), *Neisseria* (5.90%), *Fusobacterium* (4.58%), *Gemella* (3.90%), *Prevotella* (3.64%), *Aggregatibacter* (3.14%), *Rothia* (2.54%), *Agrobacterium* (2.49%), *Veillonella* (2.35%), *Porphyromonas* (2.29%), *Leptotrichia* (1.92%), *Chryseobacterium* (1.74%), *Pandoraea* (1.607%), *Enterobacter* (1.604%) and *Treponema* (1.23%). In BK2, most abundant genera were different compared to BK1. In this saliva sample, dominant genera were: *Streptococcus* (40.75%), followed by *Gemella* (12.47%), *Prevotella* (9.69%), *Rothia* (6.55%), *Veillonella* (4.29%), *Haemophilus* (4.24%), *Neisseria* (4.086%), *Granulicatella* (4.082%), *Actinomyces* (2.911%), *Fusobacterium* (1.79%), *Campylobacter* (0.91%), *Pseudomonas* (0.81%), *Leptotrichia* (0.74%), *Porphyromonas* (0.69%) and *Aggregatibacter* (0.66%). BKC1 had predominant following genera; *Streptococcus* (30.04%) followed by *Prevotella* (13.35%), *Gemella* (6.61%), *Granulicatella* (4.51%), *Rothia* (4.09%), *Actinomyces* (3.68%), *Fusobacterium* (3.36%), *Porphyromonas* (3.26%), *Neisseria* (3.07%), *Leptotrichia* (2.98%), *Chryseobacterium* (2.76%), *Lautropia* (2.66%), *Abiotrophia* (2.20%), *Yersinia* (1.57%), *Haemophilus* (1.56%), *Veillonella* (1.43%) and *Aggregatibacter* (0.444%). In BKC2, most abundant genera were: *Streptococcus* (26.05%), followed by *Neisseria* (21.64%), *Haemophilus* (8.05%), *Fusobacterium* (5.88%), *Rothia* (5.67%), *Gemella* (4.66%), *Granulicatella* (4.16%), *Prevotella* (3.45%), *Porphyromonas* (3.43%) and *Aggregatibacter* (2.98%) (Figure 5.6b).

At species level, BK1 showed predominant species which were: *Yersinia pseudotuberculosis* (20.76%) followed by *S. sanguinis* (16.40%), *H. parainfluenzae* (5.90%), *Neisseria subflava* (4.13%), *Gemella sanguinis* (3.90%), *F. nucleatum* (3.59%), *S. salivarius* (3.28%), *Agrobacterium tumefaciens* (2.49%), *Aggregatibacter segnis* (2.39%), *Veillonella dispar* (1.86%), *P. histicola* (1.81%), *Chryseobacterium moechotypicola* (1.74%), *R. dentocariosa* (1.61%), *Pandora easputorum* (1.60%), *Enterobacter hormaechei* (1.60%) and *P. endodontalis* (1.44%). In BK2 major identified species were *S. sanguinis* (34.07%), *G. sanguinis* (12.45%), *P. histicola* (6.83%), *H. parainfluenzae* (4.24%), *V. dispar* (4.07%), *N. subflava* (3.53%), *S. salivarius* (3.53%), *Streptococcus* spp. (2.23%), *Fusobacterium* spp. (1.01%), *F. nucleatum* (0.63%), *Porphyromonas* spp. (0.63%), *R. dentocariosa* (0.53%), *A. segnis* (0.5%) and *Prevotella* spp. (0.55%). In BKC1 predominant species were *Y.*

pseudotuberculosis (1.57%), *S. sanguinis* (25.71%), *P. histicola* (10.31%), *G. sanguinis* (6.61%), *Porphyromonas* spp. (3.20%), *A. segnis* (2.75%), *N. subflava* (2.20%), *C. moechotypicola* (1.74%), *Lautropia* spp. (1.74%), *F. nucleatum* (1.62%), *S. salivarius* (1.60%), *H. parainfluenzae* (1.56%), *Fusobacterium* spp. (1.35%) and *V. dispar* (1.25%). In BKC2 major identified species were *S. sanguinis* (23.26%), *N. subflava* (20.43%), *H. parainfluenzae* (8.05%), *R. mucilaginosa* (5.16%), *G. sanguinis* (4.65%), *Gemella paradiacens* (2.584%), *A. segnis* (2.583%), *Fusobacterium* spp. (2.47%) and *Porphyromonas* spp. (2.40%).

5.3.3.4- Community profiling

5.3.3.4.1- Alpha diversity

To measure alpha diversity observed, Shannon, Simpson and Chao 1 diversity index were calculated to determine the richness and evenness of the samples, and statistical analysis was performed by Mann-Whitney/Kruskal-Wallis method. Results from these analyses showed greater diversity in females with FTB compared to PLWB (Table 5.21). Shannon index for BK1, BKC1 and BKC2 saliva samples showed relatively high richness and evenness compared to BK2 sample, which had PLWB. Although both BK1 and BK2 postpartum females were suffering from gingivitis and dental carries, but BK1 delivered a normal weight full term baby. Results of Simpson diversity index also showed greater diversity in BK1, BKC1 and BKC2 compared to BK2. However statistical analysis showed insignificant results between these samples.

5.3.3.4.2- Beta diversity

Bray-Curtis dissimilarity showed greater dissimilarity between BK1 and BK2 sample (BC=0.55) compared to BK1 and BKC1 (BC=0.54), BK1 and BKC2 (BC=0.52), and BKC1 and BKC2 (BC=0.43). In Principal Coordinates analysis (PCoA) and Nonmetric multidimensional (NMDS) scaling by using Bray-Curtis, clustered samples at distance and results were also non-significant. PERMANOVA statistical analysis value was for F-Value: 0.63916; R-squared: 0.56108; p-value < 0.833, Stress = 0 (Figure 5.7).

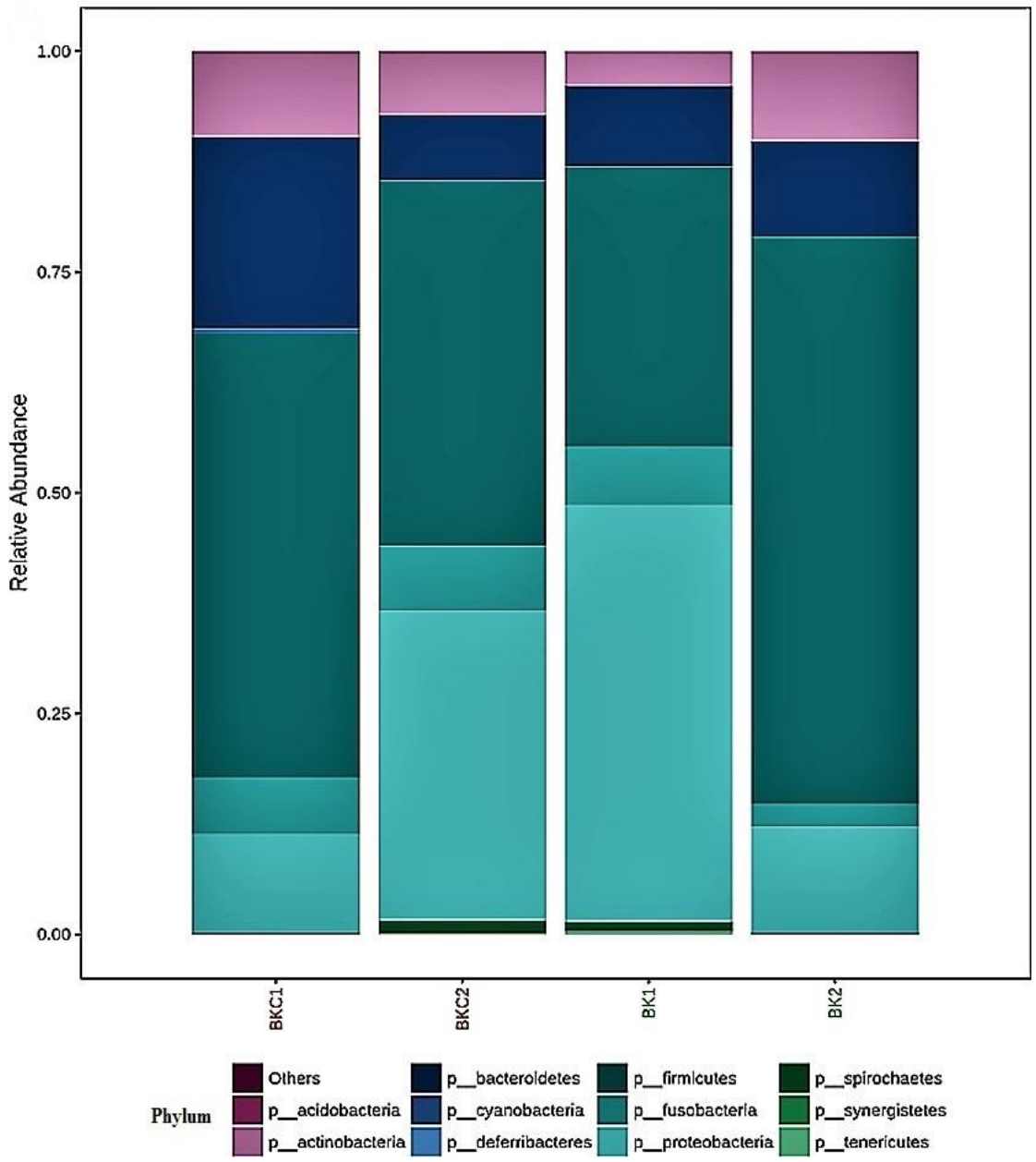

Figure 5.6a: Relative abundance of predominant salivary bacterial taxa in postpartum and nonpregnant females at phylum level: Subject BK1 had dominated phyla Proteobacteria followed by Firmicutes, Bacteroidetes, Fusobacteria and Actinobacteria, subject BK2 had dominated phyla Firmicutes followed by Proteobacteria, Bacteroidetes, Actinobacteria and Fusobacteria, subject BKC1was dominated by Firmicutes followed by Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria, subject BKC2 had dominated phyla Firmicutes followed by Proteobacteria, Bacteroidetes, Fusobacteria and Actinobacteria

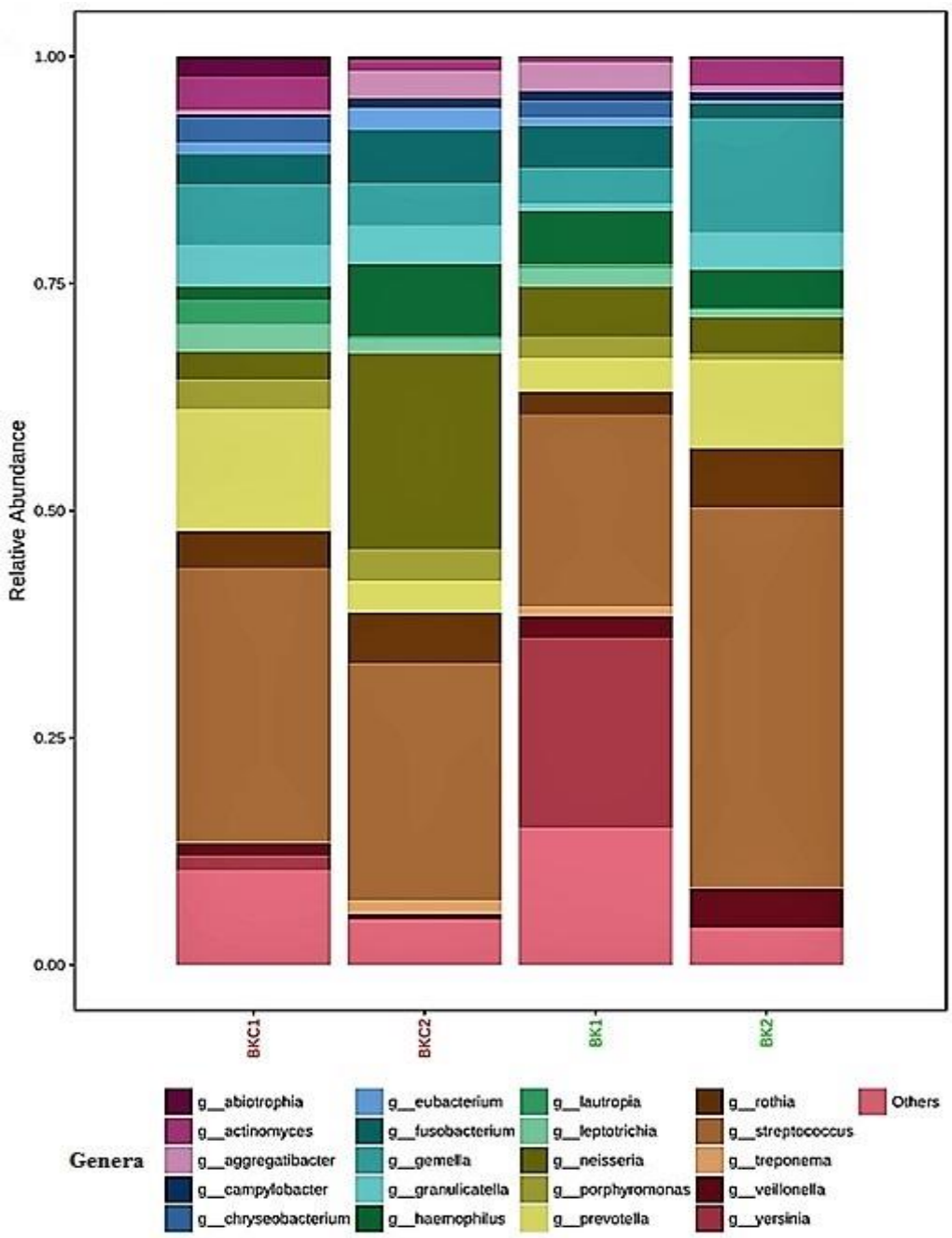

Figure 5.6b: Relative abundance of predominant salivary bacterial taxa in postpartum and nonpregnant females at genera level; In BK1 major genera were: *Streptococcus* followed by *Yersinia*, *Haemophilus*, *Neisseria*, *Fusobacterium*, *Gemella*, *Prevotella*, *Aggregatibacter* and *Rothia*, In BK2: *Streptococcus* followed by *Gemella*, *Prevotella*, *Rothia*, *Veillonella*, *Haemophilus*, *Neisseria* and *Granulicatella*, In BKC1: *Streptococcus* followed by *Prevotella*, *Gemella*, *Granulicatella*, *Rothia*, *Actinomyces*, *Fusobacterium*, *Porphyromonas* and *Neisseria*, In BKC2: *Streptococcus* followed by *Neisseria*, *Haemophilus*, *Fusobacterium*, *Rothia*, *Gemella*, *Granulicatella* and *Prevotella*

Table 5.21: Alpha diversity indices for salivary microbiome of postpartum females and nonpregnant female

Sample ID	Observed	Simpson's Index	Shannon Index	Chao 1 index
BK1	356	0.916	3.50	385.06
BK2	365	0.858	3.05	389.66
BKC1	373	0.914	3.61	388.75
BKC2	370	0.907	3.38	386.73

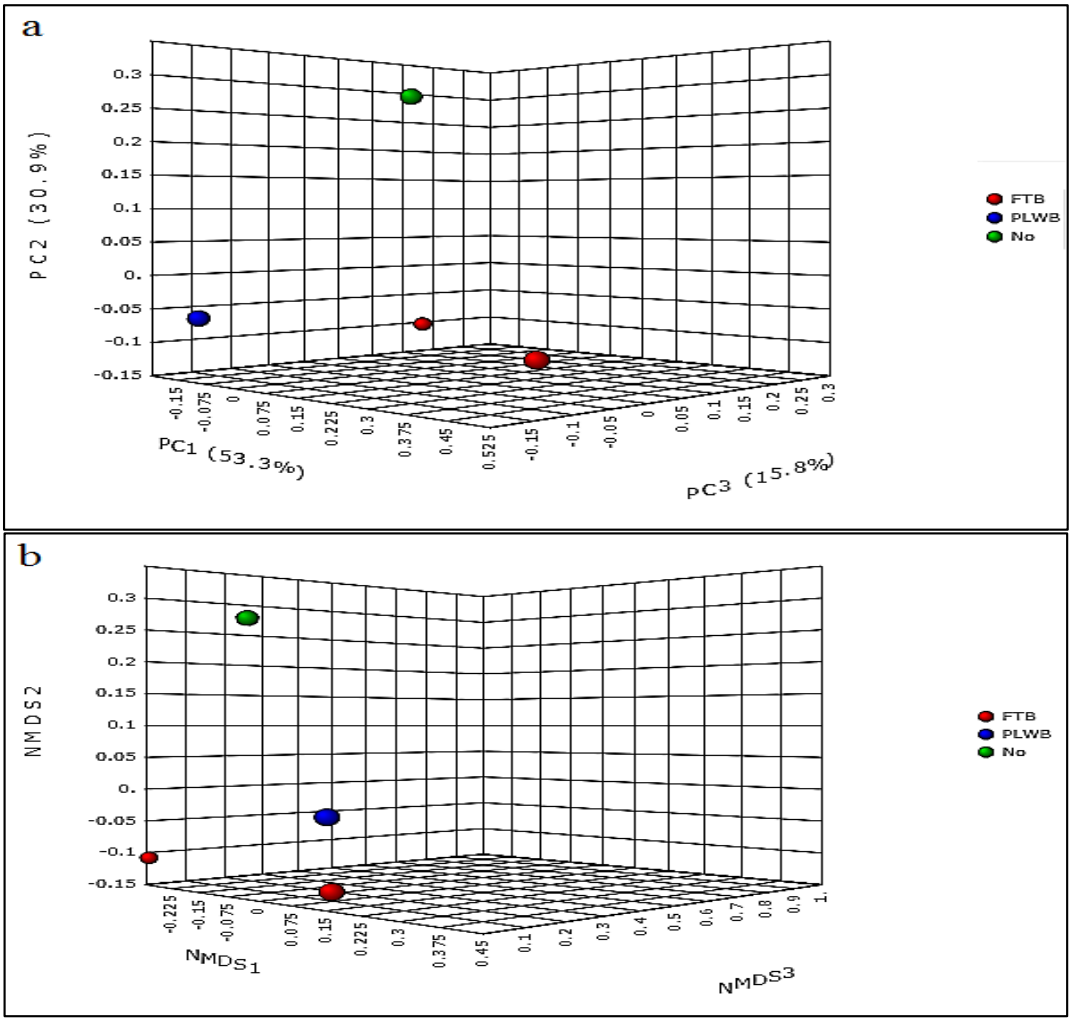

Figure 5.7: PCoA and NMDS analysis of OTU based clustering of microbial communities in postpartum and nonpregnant females (a) PCOA score for Axis 1, Axis 2 and Axis 3 for samples were: BK1; 0.32853, -0.10786 and -0.00555, BK2; -0.17592, -0.06453 and -0.16313, BKC1; -0.1771, -0.09854 and 0.1538, BKC2; 0.024483, 0.27092 and 0.014878 (b) NMDS score for MDS1 and MDS2 for samples were: BK1; -0.28845 and -0.10708, BK2; 0.15326 and -0.02368, BKC1; 0.16319 and -0.12344, BKC2; -0.02799 and 0.2542

5.3.3.5- Core microbiome analysis

At genera level, 85 out of 156 genera were shared between all 4 female saliva samples (Figure 4.29A). Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria and Actinobacteria were the top taxa shared by all females. At genera level *Streptococcus, Veillonelle, Prevotella, Neisseria, Gamella, Rothia, Haemophilus, Fusobacterium, Porphyromonas, Granulicatella, Leptotrichia, Campylobacter, Actniomyces, Aggregatibacter, Lautropia,* and *Eubacterium* were top genera (Figure 5.8).

5.3.3.6- Correlation analysis

Strong positive correlation was seen between deferribacteres with Acidobacteria, Firmicutes with Actinobacteria, Fusobacteria with Spirochaetes, Proteobacteria with Tenericutes (Figure 5.9). Similarly, strong positive correlation was also seen between most of the detected genera.

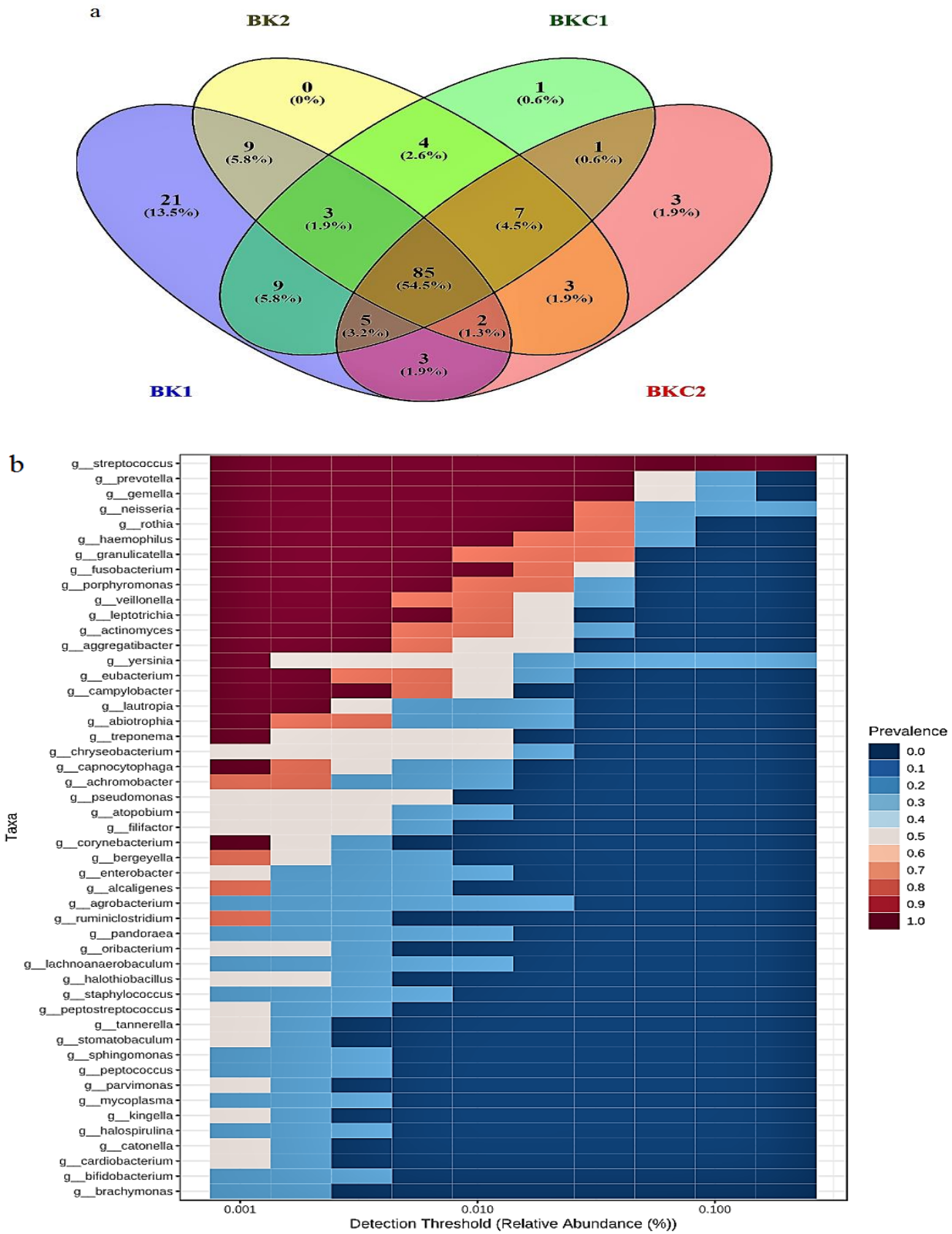

Figure 5.8: Core microbiome analysis of saliva samples from postpartum females at genus level. (a) Percentage abundance of core genera with all saliva samples sharing 54.5% of detected genera (b) Heatmap clustering for core microbiome with prevalence ranging from 0 to 1. Taxas with prevalence less than 20% and relative abundance of less than 0.2 % were removed for analysis

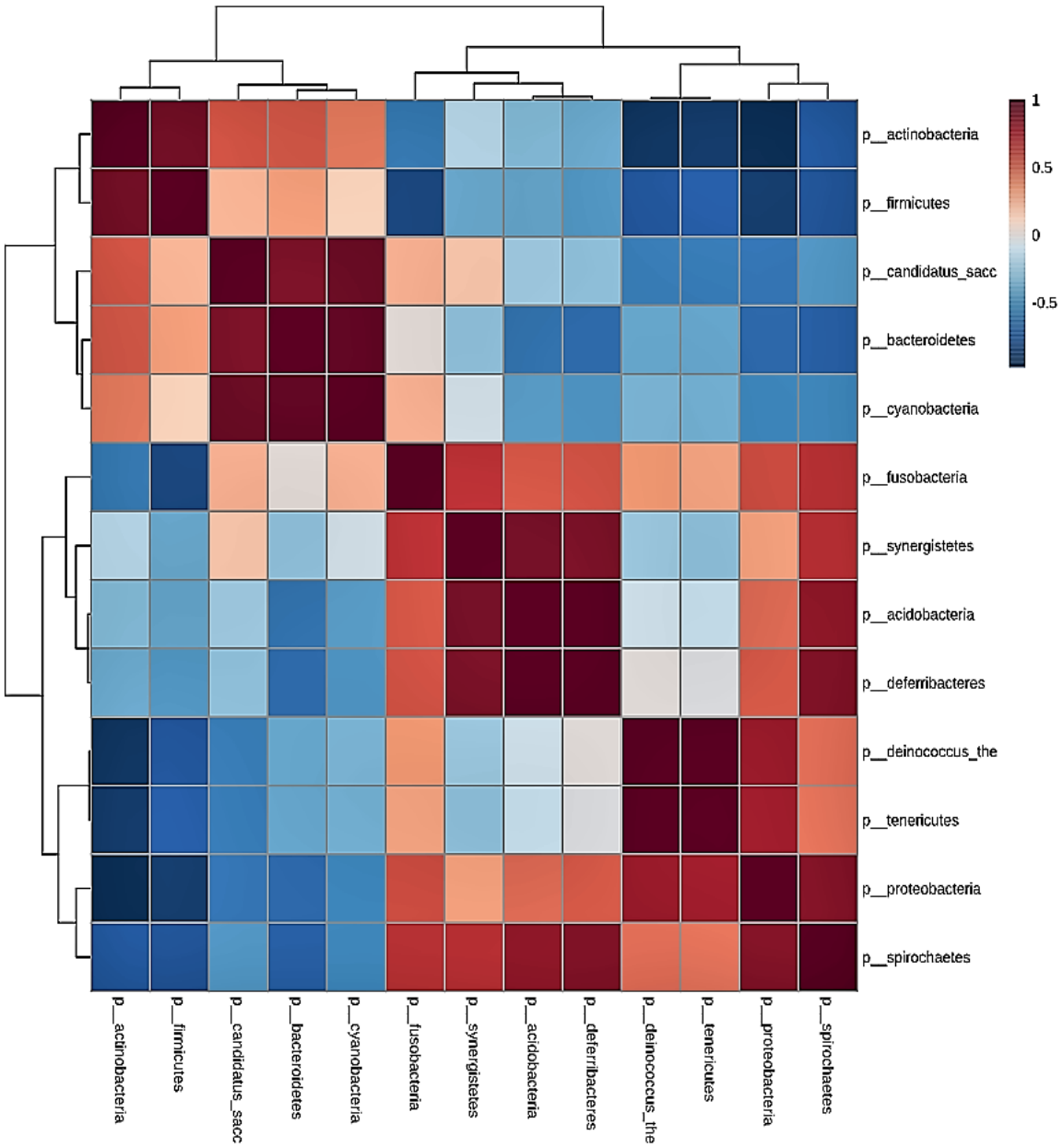

Figure 5.9: Correlation analysis of the oral microbiome by Spearman's correlation analysis a genus level. The heat map scale ranges from positively correlated, 1, to negatively correlated 1.

5.4- DISCUSSION

Oral cavity is comprised of an open, diverse and dynamic system, which is heterogeneous in nature harbouring both exo and endogenous microbial species. Nowadays, different oral cavity sites are primarily explored for finding the specific microbiota and its possible role in the development of oral disorders. Typically, members of *S. viridians* are considered as the inhabitants of healthy oral cavity. These are the earlier colonizers of developing oral biofilms, which create environment that favours the growth of multispecies biofilms. These multilayer biofilms maintain the oral homeostasis, however, several abiotic and biotic factors can influence this balance and lead to the overgrowth of specific endogenous opportunistic bacterial species (Zawadzki *et al*., 2016; Zawadzki *et al*., 2017) . *P. gingivalis, F. nucleatum, P. intermedia, T. denticola*, *T. forsythia,* and *A. actinomycetemcomitans* are the commonly known pathogens associated with pregnancy gingivitis and periodontitis, while *S. mutans* and *Lactobacilli* species are known for causing caries (Shankargouda *et al*., 2013). However, in some periodontitis patients isolation of atypical periodontal pathogens may be found in high numbers including *S. aureus, S. epidermidis, Enterococcus faecalis, Eubacterium* and *Streptococcus* species (Abbas *et al*., 2017).

In present study, oral microbiota was analysed by culture dependent and independent methods in postpartum females and was compared with healthy nonpregnant females to decipher role of culturable bacteria and microbiome in oral disorders with APOs. In the current study, differences in species prevalence between both groups of females were found. The focus of the study was on detecting the aerobic culturable flora of oral cavity followed by microbiome. For isolation of microbes, saliva samples were collected by passive drooling method which is an easy, gentle and non-invasive method. Saliva also contains microflora representative of all oral surfaces including plaque, subgingival and mucosal area (Lira-junior *et al*., 2018).

Frequently detected species in both healthy and postpartum females belonged to *Staphylococcus*, *Streptococcus*, Enterobacteriaceae, *Neisseria* and *Lactobacilli* group. Prevalent species were *S. aureus, S. epidermidis, S. saprophyticus, E. coli and K. pneumoniae*, cultured from these females, but most of these bacteria are commonly known for their associations with respiratory pathologies rather than oral infections (Daniluk *et al*., 2006).

Staphylococcus species were the most prevalent in oral cavity of postpartum (65.16%) compared to nonpregnant females, followed by *Streptococcus* (27.34% in postpartum females). These species (*S. epidermidis* and *Streptococcus* species) were significantly raised in saliva of postpartum females (P=0.005 and P=0.001, respectively). However, despite higher colonization risk, it was not statistically significantly high in females with oral health issues and APOs. Mainly earlier studies on *Staphylococcus* in pregnancy are based on their isolation from vagina, data is lacking about its oral carriage in pregnancy or postpartum phase and its impact on maternal oral health and pregnancy outcomes. *Staphylococcus* species were believed as transient oral flora. It was present in both type of females strengthens the finding that it is a common resident of healthy oral cavity especially in saliva, tongue and supragingival plaque. Its oral carriage is reported from 61.36%-94.0% in oral cavity, which is in line with our work on Pakistani females where its carriage rate was 65.16% (Jackson *et al*., 1999; Loberto *et al*., 2004; Ohara-Nemoto *et al*., 2008). *S. epidermidis* followed by *S. aureus, S. hominis, S. warneri, S. haemolyticus, S. saprophyticus, S. capitis, S xylosus* and *S. simulans* are the commonly isolated species from oral cavity (Smith *et al*., 2001), in contrast to this in postpartum females, *S. aureus* was most common followed by *S. epidermidis* and *S. saprophyticus*.

Despite having pathogenic potential for nosocomial infection, *Staphylococcus* species are not frequently studied for their role in oral disorders. Its colonization in oral cavity increase with increased antibiotic use and in case of immunocompromised conditions. A study conducted on periodontitis patients reported 61.36% prevalence of *Staphylococci* species in mouth rinse samples with most frequently isolated specie was *S. epidermidis* (27.27%) followed by *S. aureus* (25%). In same study significantly high isolation of *Staphylococcus* species was detected in males compared to females with high prevalence in age group of 51-60 years and in 31-40 years, however least number of cases were in age group 25-30 years (Loberto *et al*., 2004). Comparatively high prevalence of *Staphylococcus* species in present work was found in postpartum females, in which *S. aureus* was 32.58% in postpartum and 42.59% in nonpregnant females in comparison to *S. epidermidis* which was 29.22% in postpartum and 11.12% in nonpregnant females. The isolation rate of *S. epidermidis* and *S. saprophyticus* differed in both groups showed clearly shift in Staphylococcal species diversity and there was also age dependent change in the species composition

especially more pronounced in postpartum females of age group 21-30 years. Smith *et al*., (2001), also reported 4-46% isolation frequency of *S. aureus* from saliva source of healthy individuals with 0-70% for CoNS. In present work, a higher CoNS isolation frequency was found in saliva of postpartum females compared to healthy saliva samples. It could be due to changes in hormone and high intake of sugary drinks by these postpartum females which might have favoured the increase in CoNS.

The higher isolation frequency of *S. aureus* (50%) in gingivitis patients was seen in the study of Rams *et al*., (1990). This rate was much lower in present work in which it was 9.36% among females with gingivitis. However, comparable results were reported by Koukos *et al*., (2015) in patients suffering from gingivitis (8%). Another study from Iraq also reported even higher rate of *S. aureus* (46%) in gingivitis patients having orthodontic wires (Abbas *et al*., 2017). Differences in these prevalence rate with present work might be due to the increased *S. aureus* colonization on dental devices which provide the niche for biofilm development by oral bacteria.

A study by Cuesta *et al*., (2010), reported 69.5% prevalence of *Staphylococcus* species in periodontal disease patients of which 15.8% were *S. aureus*. These rates were comparatively high because in our study the only periodontal disorder which was prevalent in postpartum females was gingivitis, none of the female was suffering from periodontitis which is a more severe form of periodontal disorders. However, in study by Cuesta *et al*., they include all periodontal male and female patients, including patients of periodontitis. In this condition oral environment is shifted to pathogen rich ecosystem, immunocompromised the oral health status and make the environment more favourable for *Staphylococcus* colonization.

Comprehensive role of *Staphylococcus* species has not yet been explored in periodontal diseases. However, *S. aureus* can participate in periodontal destruction because they produce various virulence factors that can cause host tissue destruction and evasion of host immune systems. They possess various surface proteins including adhesins and clumping factors for host tissue colonization, invasions for bacterial spreading such as leukocidins and hyaluronidase, antiphagocytic capsule, enhanced survival inside the host due to production of carotenoids and catalase, immune modulation by protein A and coagulase, host membrane and tissue damaging toxins such as hemolysins, enterotoxins A-G, leukotoxin, TSST-1, leucocidin, , and

exfoliative toxin (Reddy *et al*., 2017). In present work, *Staphylococcus* species posed approximately one-fold risk for oral disorders in postpartum females. Their isolation was also increased with increased intake of juices/drinks, suggesting possible role of diet in favouring *Staphylococcus* colonization in oral cavity.

In present study, 10.48% postpartum females suffering from dental caries were positive for *S. aureus* isolation. This rate was lower than reported from a study conducted on caries active supragingival plaque samples (16%), however, colonization was non-significant in subjects of both studies (Hoceini *et al*., 2016). *S. epidermidis* is commonly associated with dental caries. A study from Saudi Arabia reported 16% prevalence of *S. epidermidis* from caries lesions. This rate was comparatively higher than reported in present study (7.86%). In this study, 87% were biofilm formers with 90% isolates showing strong biofilm forming ability, and majority of them were resistant to most of the antibiotics (Divakar *et al*., 2017). Although in comparison to study conducted in Saudi Arabia, lower biofilm activity was reported from *S. epidermidis* isolates of present work, but majority of these were highly resistant to commonly used antibiotics of group penicillin and cephalosporins.

Another study from Brazil reported high prevalence of *Staphylococcus* species (86.7%) in subgingival dental biofilms, but all these isolates were CoNS. In this study, no significant association was seen for *Staphylococcus* isolation with periodontal disease (P=0.78) and with severity of periodontitis (P=0.62). Isolation of *S. epidermidis* was 21.4% and for *S. saprophyticus* it was 2.9% (Santos *et al*., 2014). In present work, a high isolation frequency was found for both *S. epidermidis* and *S. saprophyticus*.

Methicillin resistance was high in isolates of present study from postpartum females with 40% resistance seen in *S. aureus* isolates, 60.26% in *S. epidermidis* and 65.62% in *S. saprophyticus*. This resistance frequency was high from previous reports. Results reported from Brazil in healthy hospital housekeepers from salivary isolation of *S. aureus* (68.32%), showed that 30.23% isolates were MRSA (Cruz *et al*., 2011). A three-year retrospective study of clinical laboratory data in Scotland showed that isolation frequency of *S. aureus* was 19.69% of which 2.38% isolates were MRSA. Isolation frequency was increased with age, with maximum isolation after 70 year of age (Smith *et al*., 2003). Similar work was also conducted by McCormack *et al*.,

(2015), on analysing clinical laboratory data during 1998-2007 on different samples from oral cavity reporting isolation of *S. aureus* from 18% specimens from which only 10% were MRSA. Majority of these resistant isolates were cultured from oral rinse samples (33.33%). MRSA prevalence increased in these postpartum females which is not reported in any earlier work, it could be due to microbial diversity shift to more resistant and biofilm formers as these were detected from saliva samples. Secondly, it might be due to HGT among the multiple species' consortia of biofilm formers on various oral surfaces, which enable the pathogens to thrive and exchange genetic material under biofilm which make these microbes resistant to immune molecules.

Daniluk *et al*., (2006), conducted a study on isolation of oral aerobic bacterial species in denture wearing and non-wearing diabetic and cancer patients. In agreement with the present study, isolation frequency of *Staphylococcus* and *Streptococcus* was also high, but samples sources were different (mucosal and denture swabs) from saliva. Isolation of *Staphylococcus* species was significantly high in denture wearing cancer and diabetic patients ($P<0.05$). Also, from these dentures Gram negative cocci were isolated, but compared to present work in which 10.48% in postpartum and 8.52% in nonpregnant females detected, a high frequency of *Neisseria* Species were detected in denture of cancer (25% in cancer) and diabetic patients (diabetic 57.9%). Also, isolation frequency of *E. coli* (6.3% in cancer and in diabetic15.8 %), and *K. pneumoniae* was high in comparison to present work. However, prevalence of *Enterobacter* species was high as 5.61% postpartum females and 3.70% in nonpregnant females in present study subject compared to its isolation from cancer and diabetic patients. Both cancer and diabetes are conditions where immunity is dampened like postpartum stage, where there is compromised immunity, but higher rate of isolation could be accounted for by the type of sample source which was dentures, which serve as conducive site for colonization and biofilm development.

Another study in HIV patients, also showed high isolation frequency of *S. aureus* (92.4%) and *S. epidermidis* (47%) compared to healthy controls (54% and 61.8%, respectively). Comparable results for *K. pneumoniae* (5.7%) in HIV patients were seen with 16.4% isolation from healthy controls. In present work, isolation frequency of *K. pneumoniae* was also high in control group as compared to postpartum females. About 7.4% HIV patients were positive for *E. coli* and in controls 1.8% were positive.

These rates were higher from findings of present study. *P. aeruginosa* was detected in 1.6% HIV patients vs 1.8% in healthy controls but in contrast there was no isolation for this bacteria in postpartum females (Back-Brito *et al*., 2011). In a study of Brazilian health care workers, saliva samples showed 18.7% colonization of Enterobacteriaceae members, of which 12.5% isolates were *K. pneumoniae* and 6.2% *E. coli. Enterobacter* species account for 34.37% isolates, but lower prevalence was among studied cases in present study (Leao-Vasconcelos *et al*., 2015). The reason for this low prevalence in saliva, could be due to less exposure to exogenous pathogenic flora compare to health care worker who are constantly exposed. These studies report higher prevalence rate for Gram negative bacilli and *Neisseria* species (Gram-negative cocci) compared to results of the present study, this variation could be possibly due to difference in sample collection site and method as dental plaque comprise of layered structured, which provide niche to allow growth of several fastidious microbes that cannot survive antimicrobial effects of saliva.

In present study, biofilm forming ability of all *Staphylococcus* species were also determined by three different methods to determine their pathogenic potential and also compare a better detection method. By CRA method, 15.71% isolates of *Staphylococcus* from postpartum showed biofilm forming activity, of which 66.67% isolates were of *S. aureus* and 27.27% of *S. epidermidis*. By MTP method comparatively large number of isolates expressed this ability (54.65% isolates of *S. aureus* and 32.55% of *S. epidermidis*). For the genotypic detection of biofilm formation in *Staphylococcal* isolates (*S. aureus* and *S. epidermidis*), *Ica* operon genes were amplified. PCR based method proved as better option for biofilm detection, which is already proved to be gold standard method for detection by Rampelotto *et al*., (2018). *Ica* operon genes detection results did not correlate with the results of CRA and MTP method. In comparison to both these methods, a better biofilm forming ability was detected by PCR as most of the isolates were positive for *Ica* genes were negative in either CRA or MTP. Among *Staphylococcus* isolates, 114 (64.04%) showed presence of *Ica* A gene and 86 (48.31%) were positive for *Ica* D gene.

PIA is involved in biofilm production by *S. epidermidis* and *S. aureus* is encoded by *IcaADBC* operon. Among *Ica* operon genes, significant role is played by *IcaA* and *IcaD* in biofilm formation as the *IcaA* gene encodes for enzyme N-

acetylglucosaminyltransferase and *IcaD* increases the expression of this enzyme which leads to the increased capsular polysaccharide production. *IcaC* gene act as a receptor for polysaccharides and *IcaB* gene function is still remains undiscover (Gad *et al*., 2009; Becker *et al*., 2014; Gowrishankar *et al*., 2016).

In comparison to present work, Kord *et al*., (2018), found higher rate of biofilm forming ability by clinical isolates of *S. epidermidis* from different sources by all three methods (24.39% by CRA, 53.65% by MTP and 100% for *Ica*AD genes). El-Mahallawy *et al*., (2009), reported different outcomes when all three methods were used. In contrast to present work they reported a lower rate of *Ica* gene detection in *S. aureus* isolates (50%) and *S. epidermidis* (23.07%) by PCR based method but higher rate of 60% and 30.76% by CRA method for biofilm production, respectively. A study conducted on *S. aureus* isolated from dental caries patients, reported lower biofilm forming ability (50%) compared to present work by both CRA and *Ica*AD gene detection method (Kouidhi *et al*., 2010). Comparable results for detection of *Ica* operon genes to current work, on *S. epidermidis* isolates from orthopaedic implants were reported. However, biofilm forming ability detection was higher by CRA and MTP method compared to isolate of postpartum females (Arciola *et al*., 2006). Such contradictions may be due to multifactorial reasons and can result from the differences in the origin of the specimens, spectrophotometric procedure, CRA composition, incubation conditions and interpretation of the coloured colonies (Kord *et al*., 2018). Differences in results of CRA and MTP method can also be due to high sensitivity of phenotypic biofilm expression to *in vitro* conditions (Shrestha *et al*., 2018).

Biofilm ability was also checked for Enterobacteriaceae members with most of the isolates expressing no ability by CRA method, however, on MTP method 58.82% *K. pneumoniae*, 100% *E. coli* and 80% isolates of *Enterobacter* species were biofilm formers. A study conducted by Hassan *et al*., (2011), reported out of 30 isolates of Gram negative bacteria 46.6% isolates of *E. coli*, 30% of *K. pneumoniae* and 16.6% of *Enterobacter* species showed biofilm forming ability. In another study, out of 100 *E. coli* isolates from urine samples, 40% isolates showed weak biofilm forming ability followed by 37% moderate and 23 % strong activity.

Antibiotic susceptibility profile was also observed in present study, and the results indicated that majority of the bacterial isolates showed resistance to most of the commonly used antibiotics. *S. aureus* showed resistance against penicillin, quinolones, cefotaxime, antibiotics of class fluoroquinolones, erythromycin and gentamycin. Isolates of *K. pneumoniae* showed resistance against most of the antimicrobial drugs including erythromycin, penicillin, clindamycin and tobramycin, tetracycline, cephalosporins, linezolid, chloramphenicol, nitrofurantoin and nalidixic acid. All *E. coli* isolates were resistant to ceftriaxone, penicillin, clindamycin, erythromycin, tetracycline and linezolid. Most effective drugs against Gram negative were imipenem and amikacin (100% sensitivity). In oral cavity, bacteria mostly lived as a part of biofilms. In comparison to planktonic form, biofilm associated bacteria showed more resistant to antibiotics. The reason for less antibiotic susceptibility of these resident attached microbial cells could be reduced penetration of the antibiotic to a complex biofilm matrix containing high bacterial density, low binding ability of antibiotics to biofilm components, and slower growth of cells inside the biofilm matrix (Pinheiro *et al*., 2014; Shrestha *et al*., 2018).

In second part of the study microbial diversity associated with oral disorders and pregnancy outcomes was analysed by sequencing. Gingivitis and dental caries associated microbes were previously commonly identified by PCR and culture-based techniques. These studies were focused on identifying few selected microbes and were unable to provide detailed picture of microbiome associated with these oral conditions. As, oral cavity comprise of a huge and diverse microbiota, identifying individual specie in oral disorder is not enough for establishing treatment strategies. It might lead to treatment failure and can increase chance of antibiotic resistance. It is important to have complete structural and compositional understanding of these microbial consortia in case of health and disease, in deciding causative agents as single specie or consortia and effective treatments. Use of advance NGS and bioinformatics tool make it possible to carry out deep, high resolution characterization of microbial communities residing in different ecological niche (Zawadzki *et al*., 2017; Lin *et al*., 2018).

Oral microbiome in pregnancy and postpartum period is poorly characterized. There is a scarcity of data related to oral microbiome in postpartum female with dental issues and its association with pregnancy outcomes. To our knowledge, it is the first

study on oral microbiome of early phase postpartum females having gingivitis and dental caries, whose saliva sample was collected after first day of delivery and sample were analysed by targeting V4 region. In addition to dental problems, these females were also analysed for association of dental problems with pregnancy outcomes (FTB or PLWB).

To the best of our knowledge the only study related to oral microbiome of late phase of postpartum in female was conducted by Balan *et al*., (2018). They collected the saliva sample and supragingival plaque (SGP) samples from healthy pregnant female during each trimester and in postpartum period, their study showed that during pregnancy, there was shift in oral microbiome to pathogens and it reverts backs to normal in postpartum period (4-6 weeks after childbirth). However, time of saliva sample collection was not specified in postpartum period and also saliva of healthy females without local oral and systematic conditions were used. According to Balan's study, alpha diversity of microbiome in saliva sample of pregnant females did not change dramatically. Predominant phyla during pregnancy were Firmicutes followed by Bacteroidetes and Actinobacteria, in both saliva and SGP samples. In saliva, at genera level, predominant genera were *Prevotella, Streptococcus, Veillonella, Neisseria* and *Terrahaemophilus.* In postpartum condition, level of pathogenic species like *F. nucleatum, P. oris, Vincentii, P. denticola, Dialister invisus, R. dentocariosa, Cornybacterium matruchotti, Seleomonas sputigena, S. anginosus,* and *Kingella oralis* were decreased significantly compared to pregnancy.

In present study, salivary microbiome of postpartum showed similar trend as reported by Balan in pregnant females, with core predominant phyla Firmicutes followed by Proteobacteria, Bacteroidetes, Actinobacteria and Fusobacteria. At genera level in these study subjects, top genera in all females included; *Streptococcus* followed by *Prevotella, Gemella, Rothia, Neisseria, Haemophilus, Veillonella, Granulicatella, Fusobacterium* and *Porphyromonas.* This similarity of postpartum microbiome with pregnancy associated microbiome can be due to the fact, that microbiome return to its normal health associated conditions after few weeks in postpartum period, and in the present study, saliva samples were collected only after first day of delivery, that's why it was more like pregnancy associated microbiome. As the pregnancy proceed towards the end, oral cavity is dominated by pathogenic gingivitis and dental caries associated agents.

Microbial shift during pregnancy and its changes in postpartum period was also analysed by Bieri *et al*., (2013) by using checkerboard DNA-DNA hybridization assay for 74 species in subgingival fluids. All study subjects delivered a baby without complication. In that study, severity of gingivitis and count of some selected bacteria was high at 12 weeks of pregnancy compared to 4-6 weeks of postpartum period. Bleeding on probing was decreased after delivery without any treatment. These changes in gingival health and bacterial count can be due to changing hormonal levels that are associated with pregnancy and which revert to normal after delivery.

Oral microbiome had greater alpha diversity as compared to any other niche in the body, which was apparent after analysis of saliva sample of this study too. Human oral microbiome dataset showed that healthy oral cavity is dominated by Firmicutes (36.7%) followed by Bacteroidetes (17.1%), Proteobacteria (17.1%), Actinobacteria (11.6%), Spirochaetes (7.9%) and Fusobacteria (5.2%), which accounts for 96% of total oral bacteria. At genus level major core microbiome of healthy oral cavity included *Streptococcus, Veillonella, Actinomyces, Porphyromonas, Fusobacterium, Prevotella, Neisseria, Treponema, Lactobacterium, Haemophilus, Capnocytophaga, Eubacteria, Leptotrichia, Eikenella, Staphylococcus, Peptostreptococcus,* and *Propionibacterium* (Dewhirst *et al*., 2010; Shaw *et al*., 2017; Gao *et al*., 2018).

A study conducted by Anukam and Agbakoba, in 2017 on oral microbiome of healthy premenopausal Nigerian female by using MiSeq illumina platform showed that Firmicutes were the most abundant phyla in this group, followed by Proteobacteria, Actniobacteria, Bacteroidetes and Fusobacteria. Most abundant species which were detected included *H. parainflunzae* (80.65%) followed by *H. influenzae* (4.13%), *A. segnis* (2.96%), *Actinobacillus porcinus* (1.64%), *Veillonella* spp. (1.52%), *Lautotropia* spp. *TeTO* (1.36%) and *R. dentocariosa* (0.95%). Although in present study same trend was seen in term of core microbiome phyla and core genera with healthy individuals, however, their abundance was varied between different saliva samples, this change in abundance could be due various factors influencing the pregnancy and one day postpartum actually is not complete reversal to healthy status, hence pregnancy related changes also effected this abundance. Another factor which effected the microbial composition was that these females suffered from dental problems.

Subject BK1 had dental problem and gave FTB which showed more alpha diversity compared to BK2 and was dominated by phyla Proteobacteria followed by Firmicutes, Bacteroidetes, Fusobacteria, Actinobacteria and Spirochaetes. Most abundant genera in BK1 were *Streptococcus* followed by *Yersinia, Haemophilus, Neisseria, Fusobacterium, Gemella, Prevotella, Aggregatibacter* and *Rothia*. Subject BK2 (dental problems plus PLWB) showed least alpha diversity, dominated by Firmicutes followed by Proteobacteria, Bacteroidetes, Actinobacteria, Fusobacteria and Spirochaetes. Common identified genera in BK2 were *Streptococcus,* followed by *Gemella, Prevotella, Rothia, Veillonella, Haemophilus, Neisseria, Granulicatella, Actinomyces* and *Fusobacterium*. Although, both BK1 and BK2 were the cases of dental problems, but their pregnancy outcomes were different. It could be beside other factors due to different diversity pattern and microbial abundance between these two samples. It is also well-known fact that diversity decreases with dental problems, that is why, there might be possibility of link between decreasing bacterial diversity in females with APOs. In addition to alpha diversity, dissimilarity value between BK1 and BK2 was also high. Subject BKC1 who had good oral health and delivered FTB also showed greater diversity compared to BK2 as their diversity index was closer to BK1. This further support the finding that high microbiome diversity closer to healthy microbiome might be helpful in uncomplicated delivery.

By far the most common periodontitis and dental caries associated species detected in APOs cases were *F. nucleatum, P. gingivalis, Bergeyella* spp., *C. rectus, Capnocytophaga* spp. *E. corrodens, Peptostreptococcus microns, P. intermedia, P. nigrescens, R. dentocaiosa, S. mutans, T. forsythia and T. Denticola* (Borgo *et al*., 2014; Cobb *et al*., 2017). Mostly these periodontal or dental caries associated microbes are detected by using traditional cultural or PCR-based methods. In present study, females were suffering from both gingivitis and dental caries but not with periodontitis, hence microbiome had different pattern of microbial colonization compared to periodontitis cases. *S. mutans* is commonly considered as the major agent for causing dental caries, however, work by Belda-Ferre *et al*., (2012), proved that plaque of adult with dental caries showed a high relative abundance of other bacterial species belonging to genera *Streptococcus, Actinomycetes, Propionibacterium, Lactobacillus,* and *Veillonella*. Similar trend was also seen in present study with low

abundance of *S. mutans* in all samples (BK1=0.004%, BK2 and BKC2=0.001%, and BKC1=0.004%) with presence of other gingivitis causing genera.

In summary, by culture dependent method detected species in oral cavity of females mainly belonged to genus *Staphylococcus* and *Streptococcus*. Significantly raised colonization of *S. epidermidis* and *Streptococcus* species were seen in postpartum females. Colonization of cultured bacterial species were not statistically significant in females with oral health issues and APOs. Majority of the isolates were expressing biofilm forming ability and increased antibiotic resistance indicating their enhanced pathogenic potential in case of pregnancy especially in early postpartum phase. Methicillin resistance was also high among *Staphylococcus* isolates. Microbiome analysis showed that female with APOs showed reduced microbial diversity compared to the females delivering full term normal baby, irrespective of the presence or absence of gingivitis and dental caries. Due to small sized study population, conclusive diversity cannot be proposed as there is inter individual variation in microbiome composition. Such work with larger population size is needed to decipher healthy core oral microbiota and to develop strategies to improve the healthy microbiome to avoid any oral and systematic disorders in case of pregnancy. However, as data scarce on culturable and unculturable bacterial diversity in postpartum phase. This work attempts to find its association with oral disorders and its possible association with APOs. The present study will be helpful in designing future studies in pregnancy and postpartum phase for studying their bacterial profile more comprehensively in case of health and disease to establish the core microbiome and to identify possible opportunistic pathogen contributing in pathogenesis or disease development.

CHAPTER 6: STUDY OF CULTURABLE AND UNCULTURABLE ORAL FUNGAL DIVERSITY AND ITS PATHOGENIC POTENTIAL IN POSTPARTUM FEMALES

6.1- INTRODUCTION

Oral cavity harbours interconnected microbial consortia comprising of over 100 fungal species. Oral bacterial diversity and their role is well established in oral health and disease, however, contribution of fungi is still underappreciated. In 2010, Ghannoum *et al*., characterized the oral mycobiome in 20 healthy individuals for the first time by using pyrosequencing and internal transcribed spacer (*ITS*) gene primers. This study revealed greater inter-individual variations among subjects, thus suggested that there is possible role of residing commensals microbes in predisposing host to opportunistic infections (Ghannoum *et al*., 2010; Janus *et al*., 2017; Peters *et al*., 2017; Zakaria *et al*., 2017; Verma *et al*., 2018).

Fungi comprise of a smaller proportion in human microbiota as compared to bacteria (Ward *et al*., 2018). There are several challenges in exploring human fungal diversity which includes, smaller fungal proportion as compared to bacteria in human microbiome (based on CFU <0.1%), many fungal strains are unculturable, difficulty in isolating its genetic material, nomenclature and annotation of fungal genome is also confusing along with availability of quite immature fungal databases with errors. Although under healthy conditions, fungi can exist as commensal in oral cavity and are present in smaller number, but their larger cell size in human microbiota contribute to relatively larger biomass, and also its ability to form hyphae play important role in establishing multi-species biofilms, making species to survive in a harsh environment. Fungi can modulate host immune response and act as a keystones species to affect host microbiota to create dysbiosis, which can develop oral disorders in both immunocompromised and immunocompetent individuals (Bandara *et al*., 2018).

Role of changing fungal diversity in disease was for the first time explored in HIV patients (Hager and Ghannoum, 2018; Klimesova *et al*., 2018). Previously most of the studies were based on understanding pathogenic potential of bacteria in disease progression, neglecting the pathogenic potential of fungi. Fungi is often co-isolated

from periodontal pockets and dental caries lesions with acidogenic bacteria. C*andida albicans* is the most prevalent oral fungal specie. Its presence usually correlate with increasing periodontitis and dental caries severity (Delaney *et al*., 2018). Prevalence of genus *Candida* range from 40-60% of healthy individuals. Imbalance in *Candida* colonization is associated with disturbance in salivary homeostasis and dysbiosis. *Candida* species have pathogenic potential due the presence of various virulence factors which promote its progression towards opportunistic pathogenic behaviour. Pathogenesis produced by *Candida* species depends upon infection type, infection site, duration of infection and host immunity. Currently, most of the studies on *Candida* specie are based on analysing *C. albicans* interaction with oral bacteria in disease development. *C. albicans* promote dental caries development by interacting with *S. oralis.* It also interact with *F. nucleatum* and *P. gingivalis* in evading host immune system and exacerbating periodontal disease (Silva-rocha *et al*., 2014; Ashour *et al*., 2015; Olczak-kowalczyk *et al*., 2015; Baker *et al*., 2017; Kong and Morris, 2017; Barros *et al*., 2018). *Candida* also enhances MMPs expression in endothelial lining of oral mucosa (Claveau *et al*., 2004). Dental caries was normally considered bacterial mediated infection caused by *Streptococcus*, but *Candida* has also been found in initiating tooth decay process and in promoting mucosal infections (Diaz *et al*., 2012).

Periodontal diseases and caries development are mostly considered as bacterial disorders (Bulut *et al*., 2014), therefore, data related to contribution of yeast in causing these disorders is rare. Moreover, studies highlighting the role of bacteria in APOs is available but fungal role is not yet studied in this perspective (Rio *et al*., 2017). Keeping this in view, there is need to analyse the pathogenic potential of fungi and their role in oral disorders for induction of pregnancy related complications (Payne and Bayatibojakhi, 2014). Therefore, the current study was aimed to find the salivary colonization of pathogenic culturable and unculturable fungal species in postpartum females to determine association of this colonization with oral health problems and APOs.

6.2- MATERIAL AND METHODS

6.2.1- Study design, setting and data collection

Described previously in chapter 3 (section 3.2).

6.2.2- Study population and sampling technique

Described in chapter 4 (section 4.2.2).

6.2.3- Part 1: Study of oral fungi by culture dependent methods

6.2.3.1- Isolation of culturable fungi from saliva samples

For culturable fungal species isolation, saliva samples (0.1 mL) were inoculated on Sabouraud dextrose agar (SDA) (Liofilchem, Italy) plates and incubation was done at 37˚C for 24-48 h, aerobically. For SDA media preparation, 65.0 g of SDA was dissolved in 1000 mL distilled water and autoclaved at 121º C and 15 psi for 15 min. After autoclaving, media was cooled and poured in petri plate under sterile conditions. Depending upon colony morphology, different colonies were picked, streaked on SDA plates and incubated for 24-48 h at 37ºC, aerobically. After incubation, pure cultures obtained were then further examined by Gram staining for cell morphology.

6.2.3.2- Identification of *Candida* species

6.2.3.2.1- Gram Staining

Described previously in chapter 4 (section 4.2.3.3.1).

6.2.3.2.2- Germ tube test

For germ test tube, a small portion of suspected *Candida* colonies were picked by sterile loop and inoculated under sterile conditions in a glass test tubes containing 0.5 mL serum for 2-3 h at 37˚C, aerobically. For serum collection, human blood was collected aseptically and at room temperature allowed to clot for 15-30 min. After clotting, it was centrifuged for 15 min at 10,000 rpm. Supernatant layer of serum was then collected and used for germ tube test. After incubation of colonies in serum, a small drop of mixture was placed on clean glass slide, covered with cover slip and then examined for formation of germ tube under a light microscope (Micros-Austria)

by using 10X and 40X objective lenses. *C. albicans* are germ tube positive while *C. dubliniensis* can either be positive or negative for germ tube test. All other *Candida* species are germ tube negative.

6.2.3.2.3- Growth of *Candida* species at 45°C

To differentiation between *C. albicans* and *C. dubliniensis* (both can be germ tube test positive), all germ test tube positive isolates were grown at 45˚C. Only *C. albicans* can survive and grow at this temperature while *C. dubliniensis* cannot grow at 45˚C. To perform this test, freshly cultured isolates were inoculated on SDA plates and incubated at 45˚C under aerobic conditions. Growth was checked daily for 10 days.

6.2.3.2.4- Characterization of *Candida* species using Chromogenic agar

Chromatic Candida agar is a differential and selective media for yeast isolation based on growth colour change. Colonies of freshly cultured *Candida* species were streaked on Chromatic Candida Agar (Licofilchem, Italy) and incubated at 37˚C for 24-48 h, aerobically. For preparation of media, 48.0 g/1000 mL of media was added in distilled water, allowed to completely dissolve by heating in water bath and then poured in petri plates under sterile conditions. After incubation, results were interpreted according to manufacturer's guideline. *C. albicans* produces pale green colonies, *C. tropicalis* produces metallic blue colour colonies, *C. krusei* produces dry pink colonies and *C. glabrata* produces white colour colonies.

6.2.3.2.5- Nitrate reduction test for *Candida* species

This test was used to determine ability of *Candida* species to reduce nitrate to nitrite with the help of enzyme nitrate reductase. For this test freshly, cultured *Candida* isolates were inoculated in test tube containing nitrate broth and incubation was done at 37 °C for 24-48 h under aerobic conditions. Nitrate broth (M439S, HIMEDIA) was prepared by adding 39.0 g of media in 1000 mL of distilled water. Media was completely dissolved, dispensed in test tubes and then autoclaved for 15 min at 121°C (15 psi). Few drops of alpha napthalamine and sulfanilic acid were added to test tube after incubation. Tubes were checked for colour change. Positive test was indicated by red colouration. In case of no colour change, small amount of zinc was added in

nitrate broth. Appearance of red colour indicate negative nitrate reduction test while no change in colour indicates positive reduction test.

6.2.3.2.6- Molecular identification of *Candida* species

6.2.4.2.6.1- DNA extraction of *Candida* species

For DNA extraction, phenol-chloroform method was used. A colony of freshly cultured *Candida* was taken and inoculated in 5.0 mL of Sabouraud dextrose broth (SDB) and incubated under aerobic conditions for 24 h at 37°C. After incubation, centrifugation was done at 10,000 rpm for 10 min to obtain a pellet by using microcentrifuge (HERMLE Labortechnik, Z216MK). Culture pellet was mixed with 700 µL lysis buffer. Lysis buffer was prepared by adding 0.5 M EDTA, 1.0 M Tris-HCl and SDS 10%. Proteinase K (20 mg/mL) was also added. Glass beads of size 0.1 mm (Sigma-Aldrich) weighing 0.4 g were added into mixture, vortexed and incubated for 3 h at 50°C. After incubation, 700 µL of phenol chloroform isoamyl solution was added and centrifuged for 10 min at 10,000 rpm. Aqueous phase was recovered and step of phenol chloroform isoamyl was again repeated. Then to precipitate DNA, to the aqueous phase, 3.0 M sodium acetate in 1/10 volume and 1.0 mL of 99.99% chilled ethanol were added. Later, centrifuged at 10,000 rpm for 10 min at 4°C. Supernatant was removed after centrifugation, and pellet was washed with 400 µL of 70 % ethanol and centrifuged at 10,000 rpm for 10 min at 4°C again. Supernatant was removed after centrifugation, pellet was air dried and mixed with 100 µL of TE buffer and for further analysis stored at -20°C.

6.2.3.2.6.2- Identification of *Candida* species by PCR amplification of *ITS* gene and RFLP

For confirmation of *Candida* species by molecular method, *ITS* of rDNA region were amplified by using pair of primers (Fermentas, Germany) (Table 6.1). PCR (Biometra) conditions for amplification were optimized at various annealing temperatures and time. Reaction mixture composition are given in Table 6.2 and PCR conditions in Table 6.3. After amplification, PCR products were subjected to digestion by 10U *Hpa*II enzyme (Fermentas, Germany) for restriction fragments length polymorphism (RFLP) for 90 min at 37˚C.

6.2.3.2.6.3- Agarose gel electrophoresis

Described previously in chapter 4 (section 4.2.3.4.2).

Table 6.1: Primers sequence used for amplification of Candidal *ITS* gene

Gene	**Primer Sequence 5' → 3'**
ITS	*ITS*1 (Forward) 5'GGT GAA CCT GCG G-3' *ITS*4 (Reverse) 5' TCC TCC GCT TAT TGA TATGC-3'

Table 6.2: PCR reaction mixture composition for amplification of Candidal *ITS* gene

Reagents	**Volume (µL)**
10x Taq buffer	2.5
25mM $MgCl_2$	1.5
dNTPs	0.4
Forward Primer	0.5
Reverse Primer	0.5
Taq Polymerase	0.2
PCR H_2O	16.4
DNA	3.0
Total volume	25.0

Table 6.3: Optimized conditions of PCR for amplification of Candidal *ITS* gene

Conditions	**Temperature (˚C)**	**Duration (min)**	**Cycles**
Initial denaturation	95.0	05	01
Final denaturation	95.0	45	36
Annealing	52.5	01	
Extension	72.0	01	
Final extension	72.0	05	01

6.2.3.2.7 Antifungal susceptibility testing

For antifungal susceptibility testing, Kirby-Bauer disc diffusion method was used as recommended by CLSI M27-A3, 2008 guideline (CLSI, 2008). For this, yeast suspensions were made in 1.0 mL normal saline, by picking isolated colony from 24-48 h old cultures from SDA plates. Suspensions were prepared according to 0.5 McFarland. Suspensions were inoculated on Glucose Methylene Blue Muller Hinton (GMM) plate by using sterile swab. GMM media was prepared by adding 38.0 g MHA, glucose 10.0 g and methylene blue 70 µL in 1000 mL of distilled water and autoclaved at 121ºC for 15 min (15 psi). Lawn was allowed to dry for some time. Three antifungals including amphotericin B 20 µg (Liofilchem, Italy), voriconazole 01 µg (Liofilchem, Italy), and fluconazole 25 µg (Oxoid, England) were placed on the GMM agar plate. All three antifungal discs were placed on prepared lawn on plates at distance of 24 mm apart and incubation was done at 37ºC for 24-48 h. Resistance was reported as inhibition zone was <13mm for fluconazole, <12mm for amphotericin B and ≤12mm for voriconazole (CLSI, 2008).

6.2.3.2.8- Screening of *Candida* species for expression of different virulence factors

6.2.3.2.8.1- Preparation of yeast suspension

For esterase and phospholipase activity, suspensions of *Candida* isolates were prepared. For this, colony was picked from freshly cultured *Candida* on SDA, and was added in PBS, and inoculum size adjusted by comparing with 0.5 McFarland to get final cell density of 1.0×10^7/mL. It was also adjusted to OD of 0.38 at 520 nm, spectrophotometrically (Multiskan Go, Thermoscientific) (Deepa *et al*., 2015).

6.2.3.2.8.1.1- Esterase assay

Tween-80 media was used for determination of esterase activity. Media was prepared by adding peptone 10.0 g, NaCl 5.0 g, $CaCl_2$ 0.1 g and agar 15.0 g in 1000 mL distilled water (pH 6.8). Media was cooled, 5.0 mL Tween-80 was added into it, dissolved completely and poured in sterile petri plates. Yeast suspension (10 µL) was inoculated on plates in duplicate and incubation was done for 10 days at 37˚C, aerobically. Positive results were interpreted by the formation of precipitation halo

around inoculation area, pervious to light. Esterase activity was calculated in millimetre (mm) by using a formula as described previously (Price *et al*., 1982). Results were interpreted according to criteria described in Table 6.4 (Koga-Ito *et al*., 2006; Galán-Ladero *et al*., 2010)

6.2.3.2.8.1.2- Phospholipase assay

Egg yolk media was used for determining phospholipase activity by *Candida* isolates. For media preparation, 65.0 g of SDA, NaCl 58.4 g and calcium chloride 5.5 g was added to 980 mL distilled water and autoclaved for 15 min at 121˚C and 15 psi. Sterile egg yolk was centrifuged for 30 min at 5000g, and 10% of this was added to media after cooling. Media was poured in glass plates under sterile conditions, inoculated with fungal suspensions (10 µL), and incubated for 04 days at 37˚C, aerobically. Activity was calculated in millimetre (mm), as a ratio of fungal colony diameter to diameter of fungal colony plus zone of precipitation formed around the colony. Results were interpreted according to the criteria as described previously (Sachin *et al*., 2012).

Table 6.4: Classification of esterase activity of *Candida* according to Ez index

Ez Index	**Esterase activity**
01	Negative
<0.81-0.99	Very low
0.61-0.80	Low
0.41-0.60	Moderate
<0.40	High

Table 6.5: Classification of phospholipase activity of *Candida* according to Pz index

Pz index	**Phospholipase activity**
01	Negative
<0.90- 0.99	Very Low
0.80-0.89	Low
0.70-0.79	Moderate
<0.70	Strong

6.2.3.2.8.2- Screening of biofilm formation by CRA

CRA media was prepared as described previously in chapter 5 (section 5.2.3.5.3.1). Freshly cultured colonies were streaked on CRA plates and incubation was done for 24-48 h at 37˚C, aerobically. After incubation, plates were checked for appearance of coloured colonies. Strong biofilm forming isolates produced dark red to black colonies, red by moderate biofilm formers, pink by weak biofilm formers and non-biofilm formers gave white to light pink colonies.

6.2.3.2.8.3- Quantification of biofilm formation by MTP

For preparation of yeast suspensions for MTP assay, freshly cultured *Candida* isolates were inoculated in test tube containing SDB supplemented with 10% glucose solution. Suspensions were incubated at 37°C for 24 h and later centrifuged at 5,000 rpm for 10 min at 4°C to get pellet. Sterile PBS (pH 7.4-7.6) was used for washing pellet, twice. Pellet was resuspended in 1.0 mL of SDB supplemented with 10% glucose. By using same media, three-fold dilutions were prepared from suspensions. Suspension with final cell density of 1.0×10^7 /mL was prepared by comparison with 0.5 McFarland standard and adjusting its OD to 0.38 at 520nm spectrophotometrically (Multiskan Go, Thermoscientific). Yeast suspension (200 µL) was added to wells of 96-well microtiter plate. Three wells containing sterile SDB without fungal cell suspension were used as negative controls. Plates were covered with lid, sealed with parafilm and incubated for 24 h at 37˚C, aerobically. Remaining procedure and criteria for biofilm results interpretation is discussed in chapter 5 (section 5.2.3.5.3.2).

6.2.3.2.9- Statistical analysis

Described previously in chapter 3 (section 3.2.3). Mann Whitneys U test was also done to determine significant associations for different variables, where $P<0.05$ was considered significant for analysis.

6.2.4- Part II: Study of oral fungi by culture-independent methods

6.2.4.1- Study population and sample collection

Described previously in chapter 5 (section 5.2.4.1).

6.2.5.2- DNA Extraction, sequencing, processing and analysis

Described previously in chapter 5 (section 5.2.4.2), however, *ITS* region from extracted DNA sample was amplified by using primers *ITS*1F (CTTGGTCATTTAGAGGAAGTAA) and *ITS*2R (GCTGCGTTCTTCATCGATGC) with forward primer having barcode in 30 cycle PCR instead of primers used for bacterial sequencing. Raw data sequences have been submitted in SRA database with accession number PRJNA530579.

6.3- RESULTS

6.3.1- Part I. Isolation of culturable fungi from saliva samples

Only colonies of *Candida* species were produced by cultured based method from saliva samples of all females, which were confirmed by biochemical and molecular analysis.

6.3.1.1- Isolation and identification of *Candida* species

6.3.1.1.1 Growth of *Candida* species on SDA

After 24-48 h of incubation at 37˚C under aerobic conditions, colonies were observed and purified on SDA. On SDA, *Candida* species produce smooth, creamy, pasty and convex colonies having characteristic yeast odour. In present study, out of 267 saliva samples from postpartum group, 147 (55.5%) were positive for *Candida* isolation. From saliva samples of 13 postpartum females, based on colony morphology, two types of *Candida* species were cultured. In total 160 isolates were purified. From the group of nonpregnant females, out of 54 saliva samples, 12 (22.22%) were *Candida* positive. From one sample two different *Candida* species were cultured, thus in total, 13 *Candida* isolates were purified.

6.3.1.1.2- Gram Staining

Suspected *Candida* isolates gave Gram positive reaction by Gram staining. They appeared as oval, budding cells of approximately 5.0 µm in diameter. In case of *C. krusei,* Gram-positive budding cells appeared ellipsoidal in shape.

6.3.1.1.3- Germ tube test

Germ tube is a tube-like long projection that appear without constriction at their origin from oval *Candida* cells. *C. albicans* is germ test tube positive as well as *C. dubliniensis*. Out of 160 isolates from postpartum females, 130 (81.25%) isolates, while among nonpregnant group 12 isolates were germ tube positive, which were further differentiated on basis of growth at high temperature (Figure 6.1).

6.3.1.1.4- Growth of germ tube positive *Candida* species at 45°C

To further differentiate between germ test tube positive *C. albicans* and *C. dubliniensis,* isolates were cultured at 45°C. Among 130 isolates from postpartum group plus 12 isolates from nonpregnant females, all showed growth at this temperature thus, confirming that none of the germ tube positive isolate was *C. dubliniensis.*

6.3.1.1.5- Growth of *Candida* species on Chromogenic agar

Based on change in colour of media after growth, among postpartum group, 81.25% (*n*=130) isolates were confirmed as *C. albicans*, 14.38% (*n*=23) as *C. glabrata* and 4. 38% (*n*=07) as *C. krusei*. Among isolates from nonpregnant group, 92.30% (*n*=12) were confirmed as *C. albicans* and one as *C. glabrata* (Figure 6.2).

6.3.1.1.6- Nitrate Test

All *Candida* isolates gave positive nitrate reduction test. In inoculated nitrate broth, after addition of sulfanilic acid and α-naphthylamine, red colour was not developed. Further, after addition of zinc in the broth also did not change colour, which confirmed positive nitrate reduction test for *Candida* species.

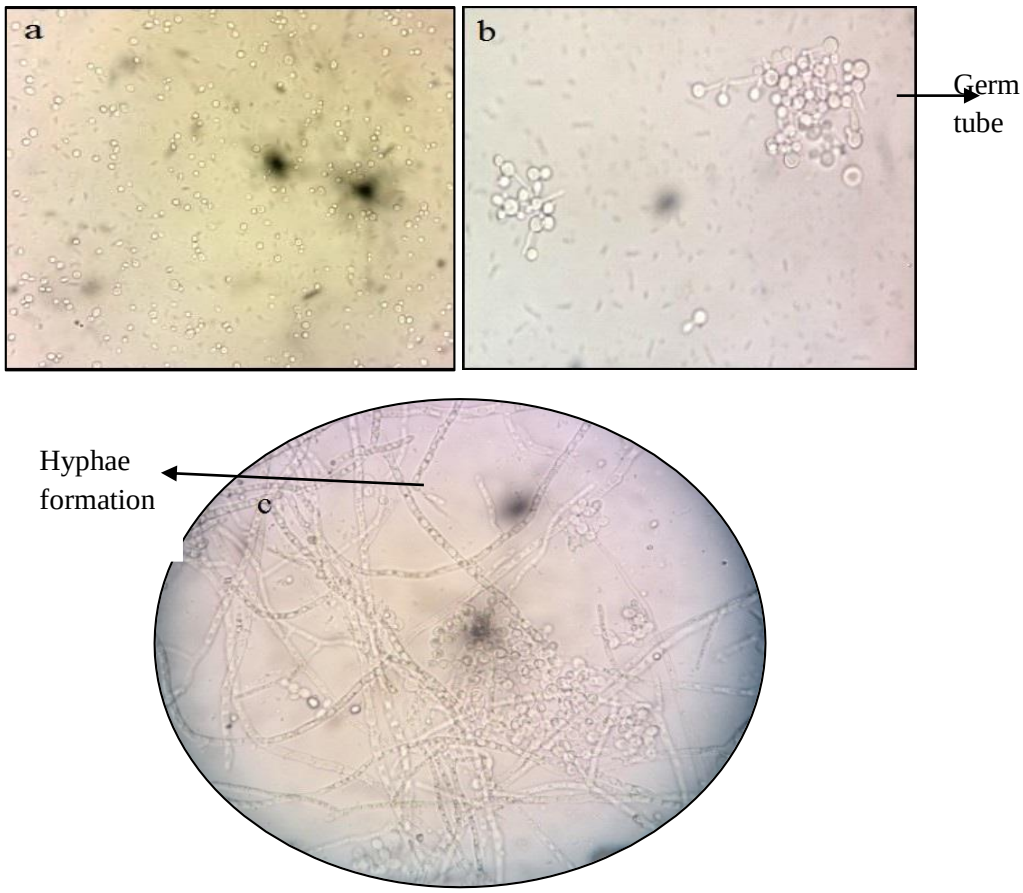

Figure 6.1: Germ tube test results for *Candida* isolate under 40X (A) sample BK27; Germ tube negative isolate (B) Sample BK6; Germ tube positive isolate (C) Sample BK39; Germ tube positive *Candida* with hyphae formation

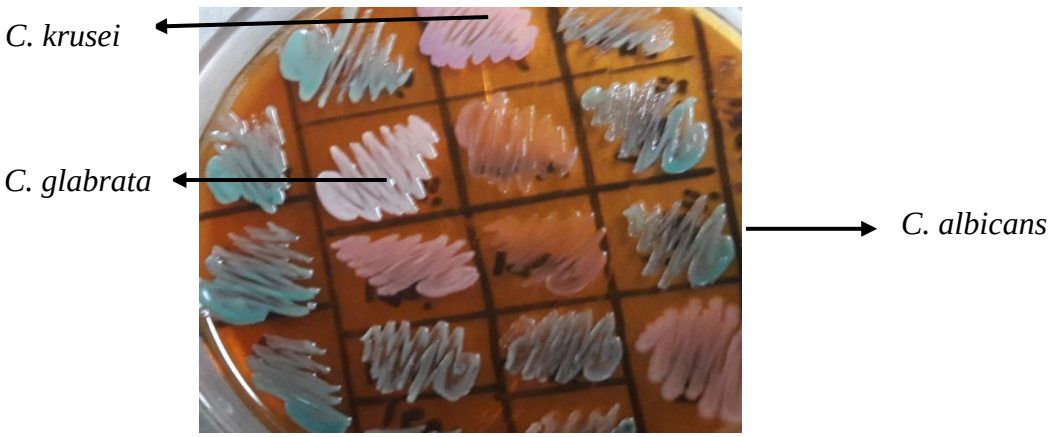

Figure 6.2: Growth of *Candida* isolates on Chrom agar. Green colour: *C. albicans*-BK6, pink dry colour: *C. krusei*-BK27, white colour: *C. glabrata*-BK45

6.3.1.1.7- Identification of *Candida* species by PCR-RFLP

To further confirm the specie level identification, amplification of *ITS* gene of *Candida* species followed by RFLP by 10U of using *Hpa*II enzyme was done. In Table 6.6 given size of fragments used for identification of various species.

Table 6.6: *Candida* species PCR-RFLP product sizes

***Candida* species**	**PCR amplification product size (bp) of *ITS* gene**	**RFLP product sizes (bp)**
C. albicans	535	338, 297
C. glabrata	871	557, 314
C. krusei	510	261, 249

6.3.1.1.8- Antifungal susceptibility testing

Three antifungal discs were used i.e. fluconazole, voriconazole and amphotericin B. Out of 160 *Candida* isolates from postpartum females, 136 (85.0%) were resistant to fluconazole and voriconazole, while 103 (64.38%) were resistant to amphotericin B. Among isolates from health control females, 6/12 isolates showed resistance to all three antifungals. Out of 130 isolates of *C. albicans* from postpartum females, 111 (85.38%) showed resistance to fluconazole, 112 (86.15%) to voriconazole and 79 (60.76%) to amphotericin B. Out of 23 *C. glabrata* isolates, 18 (78.58%) showed sensitivity to fluconazole, voriconazole and amphotericin B. Seven *C. krusei* showed resistance to fluconazole, while 06 (85.71%) were resistant to voriconazole and amphotericin B. Among control group, 6/12 *C. albicans* isolates (50.0%) and one *C. glabrata* isolate was sensitive to all three antifungals (Table 6.7).

Table 6.7: Antifungal susceptibility pattern of *Candida* species isolated from saliva samples of postpartum and nonpregnant females

Antibiotics		***C. albicans***		***C. glabrata***		***C. krusei***
		Postpartum females *n* (%)	**Non-pregnant females *n* (%)**	**Postpartum females *n* (%)**	**Non-pregnant females *n* (%)**	**Postpartum females *n* (%)**
Fluconazole sensitivity	R	111(85.38)	06(50.0)	18(78.58)	00(0.00)	07(100)
	S	19(14.62)	06(50.0)	05(21.42)	01(100)	00(0.00)
Voriconazole sensitivity	R	112(86.15)	06(50.0)	18(78.58)	00(0.00)	06(85.71)
	S	18(13.85)	06(50.0)	05(21.42)	01(100)	01(14.29)
Amphotericin B sensitivity	R	79(60.76)	06(50.0)	18(78.58)	00(0.00)	06(85.71)
	S	52(39.24)	06(50.0)	05(21.42)	01(100)	01(14.29)
Total		130(100)	12(100)	23(100)	01(100)	07(100)

R: Resistant; S: Sensitive

6.3.1.1.9- Screening of *Candida* species for expression of various virulence factors

6.3.1.1.9.1- Biofilm assay by CRA and MTP method

In CRA method, out of 160 isolates from postpartum group, 3 (1.88%) isolates showed strong biofilm forming ability, 53 (33.12%) moderate, 95 (59.38%) weak biofilm forming and 09 (5.62%) showed non-biofilm forming ability (Figure 6.6). By MTP method, 107 (66.87%) isolates showed biofilm forming ability. Strong activity was shown by three isolates, 10 (6.255) isolates were moderate biofilm formers, 94 (58.75%) were weak biofilm formers and 53 (33.13%) were non-biofilm formers. Among nonpregnant females, all isolate showed weak biofilm forming ability by both CRA and MTP method. Out of 130 *C. albicans* isolates, 66.87% were biofilm formers of which 02 (1.87%) showed strong biofilm forming ability and 07 (6.25 %) isolates showed moderate biofilm forming ability. Among *C. glabrata* isolates, 16 (69.57%) were biofilm formers with 01 (4.35%) isolate showing strong biofilm forming ability and 03 (13.04%) isolate showed moderate activity. All *C. krusei* isolate were weak biofilm formers (Table 6.8)

Table 6.8: Biofilm forming ability of isolated *Candida* species assessed by MTP assay

Category		***C. albicans***		***C. glabrata***		***C. krusei***
		Postpartum females *n* (%)	**Non-pregnant females *n* (%)**	**Postpartum female *n* (%)**	**Non-pregnant females *n* (%)**	**Postpartum females *n* (%)**
Biofilm forming activity	Yes	84(66.87)	12(100)	16(69.57)	01(100)	07(100)
	No	46(33.13)	00(0.00)	07(30.43)	00 (0.00)	00(0.00)
Strength of Biofilm forming activity	No	46(33.13)	00(0.00)	07(30.43)	00 (0.00)	00(0.00)
	Weak	75(58.75)	12(100)	12(52.18)	01(100)	07(100)
	Moderate	07(6.25)	00(0.00)	03(13.04)	00(0.00)	00(0.00)
	Strong	02(1.87)	00(0.00)	01(4.35)	00(0.00)	00(0.00)

6.3.1.1.9.2- Esterase assay

Esterase activity was indicated by the formation of zone of precipitation around *Candida* colony (Figure 6.3). In comparison to control group, esterase activity was high among *Candida* species isolated from group of postpartum females. In postpartum females, 132 (82.5%) isolates, while in control group 07 (53.85%) isolates were esterase producers. Most of the isolates from postpartum group exhibit moderate esterase activity (50%). Odds for esterase activity showed four-fold increased esterase activity among postpartum group compared to nonpregnant group. Chi-square (χ^2) analysis and Mann Whitney's test showed significantly raised esterase production by isolates cultured from postpartum group compared to nonpregnant group with P=0.01 and 0.004, respectively (Table 6.9 and Table 6.14). Both tests also showed significantly high enzyme activity among isolates of *C. albicans* from postpartum group (P=0.004 and P=0.001, respectively), with approximately seven-fold increased activity compared to isolates from control group. Out of 130 isolates, 115 (82.5%) *C. albicans* were esterase producers with 50% of the isolates were moderate esterase producers. Among nonpregnant group, 41.67% were not producing esterase (Table

6.10). Among *C. glabrata* isolates, 13 (56.52%) were esterase producers while among nonpregnant group none of the *C. glabrata* was positive for esterase activity. Like *C. albicans*, most of the *C. glabrata* (39.13%) were moderate esterase producers. Among *C. krusei* isolates, 04 (82.5%) were esterase producers and had strong esterase activity (Table 6.11).

6.3.1.1.9.3- Phospholipase Assay

Out of 160 *Candida* isolates, 109 (68.13%) produced precipitation zone around colony indicating phospholipase activity (Figure 6.4 and Table 6.12). Most of these isolates were strong phospholipase producers (34.36%). Phospholipase activity mean values were significantly high for *C. albicans* isolates (*P*=0.001) from postpartum females (Table 6.14). Among postpartum group, 101 (68%) isolates were phospholipase producers while among nonpregnant group 09 (75%) isolates showed this activity. Among *C. glabrata*, 08 (34.79%) were phospholipase producers of which majority expressed strong activity (17.40%). While the only isolate of *C. glabrata* from nonpregnant group had strong phospholipase activity. *C. krusei* isolates did not show any phospholipase activity (Table 6.13).

Table 6.9: Esterase production by *Candida* isolated from postpartum and nonpregnant females

Category		***Candida* species**		**P-value (χ^2)**	**OR (95% Cl)**
		Postpartum females *n* (%)	**Nonpregnant females *n* (%)**		
Esterase (Ez) activity	Present	132(82.5)	07(53.85)	0.01 (6.25)	4.04 (1.26-12.9)
	Absent	28(17.5)	06(46.15)		
Strength of Ez Activity	No	28(17.5)	06(46.15)	-	-
	Low	10(6.25)	02(15.38)		
	Medium	80(50.0)	00(0.00)		
	High	42(26.25)	05(38.47)		

Table 6.10: Esterase production by *C. albicans* isolated from postpartum and nonpregnant females

Category		***C. albicans***		**P-value (χ^2)**	**OR (95% Cl)**
		Postpartum females *n* (%)	**Nonpregnant females *n* (%)**		
Esterase (Ez) activity	Yes	115(82.5)	07(58.33)	0.004 (08.24)	6.84 (1.87-24.9)
	No	15(17.5)	05(41.67)		
Strength of Ez Activity	No	15 (17.5)	05(41.67)	-	-
	Low	10(6.25)	02(16.66)		
	Medium	71(50.00)	00(0.00)		
	High	34(26.25)	05(41.67)		

Table 6.11: Esterase production by *C. glabrata* and *C. krusei* isolated from postpartum and nonpregnant females

Category		***C. glabrata***		***C. krusei***
		Postpartum females *n* (%)	**Nonpregnant females *n* (%)**	**Postpartum females *n* (%)**
Esterase (Ez) activity	Yes	13(56.52)	00(0.00)	04 (82.5)
	No	10(43.48)	01(100)	03(17.5)
Strength of Ez Activity	No	10(43.48)	01(100)	03(17.5)
	Low	00(0.00)	00(0.00)	00(0.00)
	Medium	09(39.13)	00(0.00)	00(0.00)
	High	04(17.39)	00(0.00)	04(82.5)

Table 6.12: Expression of phospholipase activity by *Candida* isolated from postpartum and nonpregnant females

Category		***Candida***	
		Postpartum females *n* (%)	**Nonpregnant females *n* (%)**
Phospholipase (Pz) Activity	Present	109(68.13)	10(76.92)
	Absent	51(31.87)	03(23.08)
Strength of Pz activity	No	51(31.87)	03(23.08)
	Very low	05(3.13)	00(0.00)
	Low	14(8.76)	00(0.00)
	Medium	35(21.88)	00(0.00)
	Strong	55(34.36)	10(76.92)

Table 6.13: Expression of phospholipase activity by different species of *Candida* isolated from postpartum and nonpregnant females

Category		***C. albicans***		***C. glabrata***		***C. krusei***
		Postpartum females *n* (%)	**Non-pregnant female *n* (%)**	**Postpartum female *n* (%)**	**Non-pregnant female *n* (%)**	**Postpartum female *n* (%)**
Phospholipase (Pz) Activity	Yes	101(68.13)	09(75.0)	08(34.79)	01(100)	00(68.13)
	No	29(31.87)	03(25.0)	15(65.21)	00(0.00)	07(31.87)
Strength of Pz activity	No	29(31.87)	03(25.0)	15(65.21)	00(0.00)	07(31.87)
	Very low	05(3.13)	00(0.00)	00(0.00)	00(0.00)	00(3.13)
	Low	13(8.76)	00(0.00)	01(4.35)	00(0.00)	00(8.76)
	Medium	32(21.88)	00(0.00)	03(13.04)	00(0.00)	00(21.88)
	Strong	51(34.36)	10(75.0)	04(17.40)	01(100)	00(34.36)

Table 6.14: Statistical analysis of various virulence factors expressed by *Candida* isolates from saliva samples of postpartum and nonpregnant females

Category	***Candida* species**	***C. albicans***	***C. glabrata***	***C. krusei***
	Mean±SD (95% Cl)	**Mean±SD (95% Cl)**	**Mean±SD (95% Cl)**	**Mean±SD (95% Cl)**
Biofilm activity				
Postpartum females	0.363±0.194 (0.332-0.393)	0.352±0.182 (0.320-0.383)	0.414±0.273 (0.302-0.525)	0.397±0.039 (0.369-0.426)
Nonpregnant females	0.379±0.073 (0.340-0.418)	0.382±0.075 (0.340-0.424)	-	-
P-value*	0.258	0.141	-	-
Esterase activity				
Postpartum females	0.450±0.360 (0.393-0.505)	0.410±0.179 (0.379-0.440)	0.197±0.248 (0.096-0.299)	0.178±0.172 (0.050-0.304)
Nonpregnant females	0.198±0.200 (0.089-0.307)	0.215±0.200 (0.100-0.328)	-	-
P-value*	0.004	0.001	-	-
Phospholipase activity				
Postpartum females	0.369±0.207 (0.337-0.400)	0.496±0.300 (0.444-0.548)	0.218±0.313 (0.090-0.346)	-
Nonpregnant females	0.385±0.232 (0.259-0.511)	0.385±0.232 (0.259-0.511)	-	-
P-value*	0.7414	0.001	-	-

P-value* = For Mann Whitneys U test

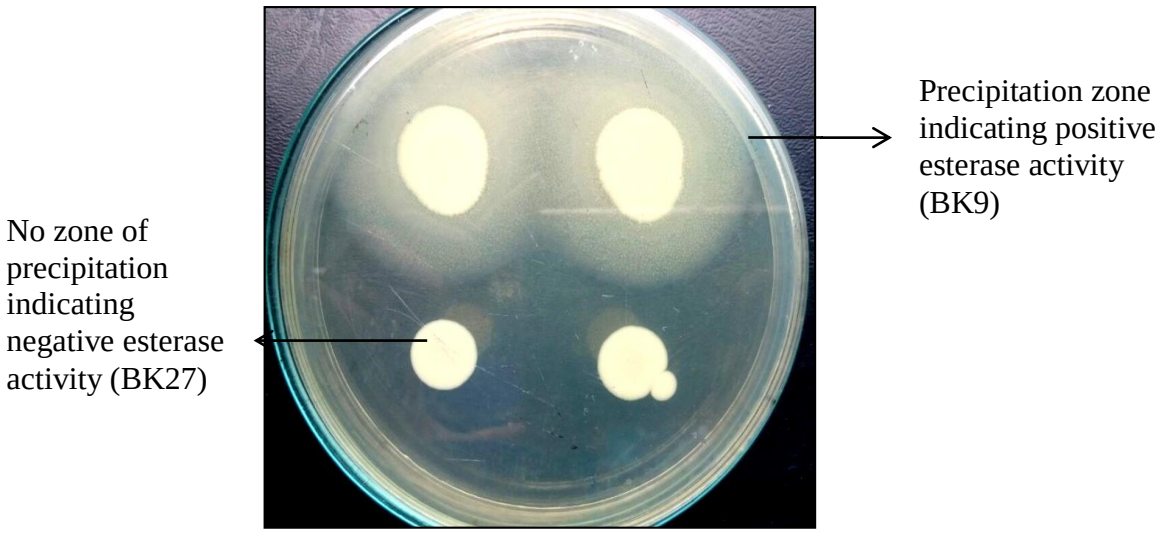

Figure 6.3: Esterase activities of *Candida* isolates BK9 and BK27 (in duplicates) on Tween-80 medium**.** BK9: showed precipitation zone around colony indicating positive esterase activity, BK27: showed no precipitation zone around colony indicating negative esterase activity

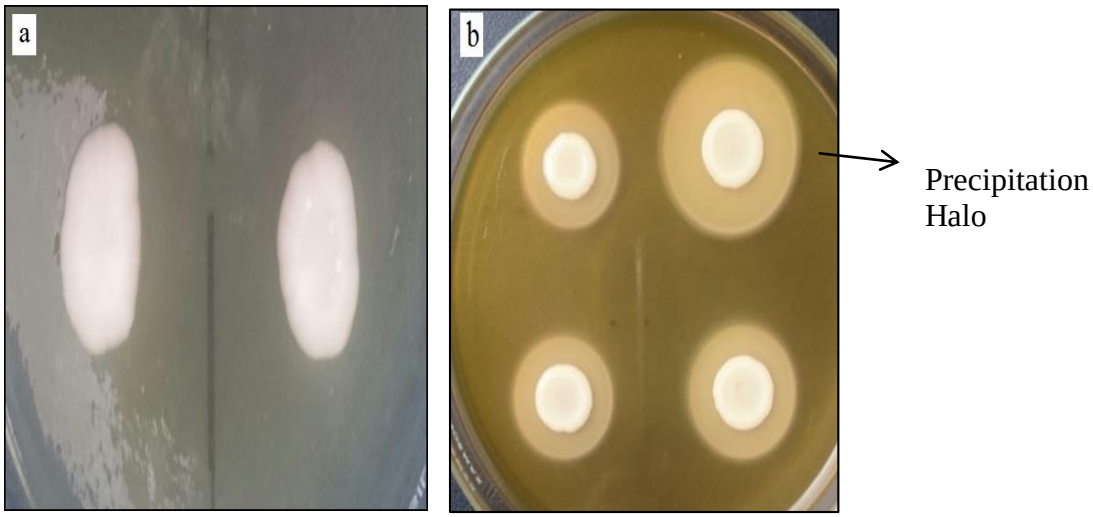

Figure 6.4: Phospholipase activities of different *Candida* isolates on Egg yolk medium (a) Negative phospholipase activity in isolate BK-221 (b) Positive phospholipase activity as shown by zone of precipitation formed around colony of *Candida* isolates BK-133 and BK-145

6.3.1.1.10- Comparison of *Candida* colonization in postpartum females vs nonpregnant group

Significantly high *Candida* colonization was found in postpartum group in comparison to nonpregnant females ($P<0.001$) and was seen to have four-fold increased risk for oral colonization (Table 6.15). In postpartum group, *C. albicans* colonization was significantly raised with approximately three-fold increased risk for colonization. *C. glabrata* also had five-fold increased risk for oral colonization in postpartum females (Table 6.16).

6.3.1.1.11- Associations of *Candida* species isolation with obstetric factors, oral health problems, oral hygienic measures and drinking habits

Candida colonization did not show associations with APOs, oral health issues, oral hygienic measures and with sugary liquid intake among postpartum females. However, *C. albicans* colonization was approximately one-fold more in females with PTB, preeclampsia and oral disorders. Odds showed increased *C. albicans* colonization risk with decreasing brushing frequency (OR=2.78 to OR=1.37), increased intake of juices/drinks (OR=0.96 to OR=1.18) and tea and coffee intake (OR=1.56 to OR=1.80) (Table 6.17). *C. glabrata* colonization increased 1.65-fold risk for PTB, approximately one-fold for LWB, preeclampsia, gingivitis, and approximately two-fold for dental caries (Table 6.18). *C. krusei* increased 1.45-fold risk for PTB and approximately two-fold for dental caries. Increasing intake of juices/drinks and tea/coffee also increased risk for *C. krusei* colonization (Table 6.19).

Table 6.15: *Candida* colonization in postpartum (*n*=267) and nonpregnant females (*n*=54) saliva samples

Category	***Candida***		***P*-value (χ^2)**	**OR (95%Cl)**
	Case positive *n* (%)	**Case negative *n* (%)**		
Postpartum females	147(55.05)	120(44.95)	<0.001 (19.37)	4.28 (2.16-8.50)
Nonpregnant females	12(22.22)	42(77.78)		

Table 6.16: Colonization of different *Candida* species in postpartum (*n*=267) and nonpregnant female (*n*=54) saliva samples

Category		**Postpartum females *n* (%)**	**Nonpregnant females *n* (%)**	***P*-value (χ^2)**	**OR (95%Cl)**
C. albicans	Yes	130 (48.68)	12(44.95)	<0.001 (12.75)	3.32 (1.67-6.58)
	No	137(51.32)	42(77.78)		
C. glabrata	Yes	23(8.62)	01(1.86)	0.08 (2.96)	4.99 (0.66-37.81)
	No	244(91.38)	53(98.14)		
C. krusei	Yes	07(2.62)	00(0.00)	0.22 (1.44)	-
	No	260(97.37)	54(100)		

Table 6.17: Associations of *C. albicans* colonization in postpartum females (*n*=267) with different obstetric factors, oral health factors and drinking habits

Factors		***C. albicans n* (%)**		***P*-value (χ^2)**	**OR (95% Cl)**
		Case positive	**Case negative**		
Gestation Period	PTB	29(10.87)	29(10.87)	0.821 (0.05)	1.06 (0.59-1.91)
	FTB	101(37.82)	108(40.44)		
Baby weight (Kg)	<2.5	21(7.86)	39(14.60)	0.029 (7.05)	- 0.47 (0.26-0.86) -
	2.5-4	108(40.44)	95(35.60)		
	>4	01(0.38)	03(1.12)		
Preeclam-psia	Present	16(6.00)	15(5.62)	0.729 (0.12)	1.14 (0.54-02.41)
	Absent	114(42.69)	122(45.69)		
Dental problems	Yes	61(22.85)	65(24.34)	0.947 (0.007)	0.97 (0.60-1.58)
	No	69(25.85)	72(26.96)		
Gingivitis	Yes	38(14.24)	35(13.10)	0.499 (0.45)	1.20 (0.70-2.06)
	No	92(34.46)	102(38.20)		
Dental caries	Yes	40(15.00)	35(13.10)	0.342 (0.90)	1.29 (0.75-2.21)
	No	90(33.70)	102(38.12)		
Brushing frequency/ day	No	03(01.12)	1(00.38)	0.137 (05.51)	- 2.78(0.28-27.3) 1.37(0.83-2.28) -
	1 time	84(31.46)	78(29.22)		
	2 times	43(16.10)	55(20.60)		
	3 times	00(0.00)	03(1.12)		
Intake of juices/ drinks	No	29(10.86)	32(11.98)	0.815 (0.40)	- 0.96(0.50-1.85) 1.18(0.68-2.06)
	Not frequently	41(15.36)	47(17.60)		
	Frequently	60(22.48)	58(21.72)		
Tea and coffee intake	No	10(3.75)	17(6.36)	0.09 (6.45)	- 1.56 (0.68-3.59) 1.80(0.89-3.63) -
	1-2 times daily	95(35.58)	103(38.58)		
	3-4 times daily	25(9.36)	15(5.62)		
	>4 times daily	00(0.00)	02(0.75)		
Total		130(48.68)	137(51.32)	-	-

Table 6.18: Associations of *C. glabrata* colonization in postpartum females (*n*=267) with different obstetric factors, oral health factors and drinking habits

Factors		***C. glabrata n* (%)**		***P*-value (χ^2)**	**OR (95% Cl)**
		Case positive	**Case negative**		
Gestation Period	PTB	07(2.62)	51(19.10)	0.289 (1.12)	1.65 (0.64-4.24)
	FTB	16(6.00)	193(72.28)		
Baby weight (Kg)	<2.5	06(2.25)	54(20.22)	0.764 (0.53)	- 1.21(0.45-3.23) -
	2.5-4	17(6.36)	186(69.67)		
	>4	00(0.00)	04(1.50)		
Preeclam-psia	Present	04(1.50)	27(10.11)	0.365 (0.82)	1.19 (0.38-3.66)
	Absent	19(7.11)	217(81.28)		
Dental problems	Yes	12(4.50)	114(42.70)	0.616 (0.25)	1.24 (0.52-2.92)
	No	11(4.12)	130(48.68)		
Gingivitis	Yes	07(2.62)	66(24.72)	0.727 (0.12)	1.18 (0.46-2.99)
	No	16(6.00)	178(66.66)		
Dental caries	Yes	10(3.76)	65(24.34)	0.08 (02.95)	2.11 (0.88-05.06)
	No	13(4.86)	179(67.04)		
Brushing frequency/ day	No	00(0.00)	04(1.50)	0.790 (1.04)	- - 0.76 (0.32-1.82) -
	1 time	13(4.87)	149(55.80)		
	2 times	10(3.75)	88(32.96)		
	3 times	00(0.00)	03(1.12)		
Intake of juices/ drinks	No	03(1.12)	58(21.72)	0.556 (1.17)	- 2.20 (0.57-8.49) 0.90(0.35-2.28)
	Not frequently	09(3.38)	79(29.58)		
	Frequently	11(4.12)	107(40.08)		
Tea and coffee intake	No	02(0.75)	25(9.36)	0.948 (0.35)	- 1.25(0.27-5.71) 0.811(0.22-2.89)
	1-2 times daily	18(6.75)	180(67.42)		
	3-4 times daily	03(1.12)	37(13.85)		
	>4 times daily	00(0.00)	02(0.75)		
Total		23(8.61)	244(91.38)	-	-

Table 6.19: Associations of *C. krusei* colonization in postpartum females (*n*=267) with different obstetric factors, oral health factors and drinking habits

Factors		***C. krusei n* (%)**		***P*-value (χ^2)**	**OR (95% Cl)**
		Case positive	**Case negative**		
Gestation Period	PTB	02(0.75)	56(20.98)	0.656 (0.19)	1.45 (0.27-7.71)
	FTB	05(1.87)	204(76.40)		
Baby weight (Kg)	<2.5	01(0.38)	59(22.10)	0.814 (0.41)	- 0.55(0.06-4.71) -
	2.5-4	06(2.24)	197(73.78)		
	>4	00(0.00)	04(1.50)		
Preeclam-psia	Present	0(0.00)	31(11.62)	0.331 (0.94)	-
	Absent	07(2.62)	229(85.76)		
Dental problems	Yes	03(1.12)	123(46.06)	0.816 (0.05)	0.83 (0.18-3.80)
	No	04(1.50)	137(51.32)		
Gingivitis	Yes	01(0.38)	72(26.96)	0.432 (0.61)	0.43 (0.05-3.67)
	No	06(2.24)	188(70.42)		
Dental caries	Yes	03(1.12)	72(26.96)	0.378 (0.77)	1.95 (0.42-8.96)
	No	04(1.50)	188(70.42)		
Brushing frequency/ day	No	00(0.00)	04(1.50)	0.928 (0.45)	- - 1.52(0.29-08.03) -
	1 time	05(1.88)	157(58.80)		
	2 times	02(0.75)	96(35.95)		
	3 times	00(0.00)	03(1.12)		
Intake of juices/ drinks	No	03(01.12)	58(21.72)	0.427 (1.69)	- 0.45(0.07-2.77) 0.74(0.10-5.36)
	Not frequently	02(0.76)	86(32.20)		
	Frequently	02(0.76)	116(43.44)		
Tea and coffee intake	No	02(0.75)	25(09.36)	0.430 (2.75)	- 0.25(0.04-01.48) 1.24(0.13-11.42) -
	1-2 times daily	04(1.50)	194(72.65)		
	3-4 times daily	01(0.38)	39(14.60)		
	>4 times daily	00(0.00)	02(0.76)		
Total		07(2.62)	260(97.38)	-	-

6.3.2- Part II: Study of oral fungi by culture independent methods

6.3.2.2- Sequence characteristics

A total of 563,033 reads were obtained from four saliva samples. BK1 had total count of 1,690,93, BK2 had 1,269,15, BKC1 2,394,50 and BKC2 had 2,757,5. A total of 388 OTUs were detected in these samples. After data trimming, filtering and normalization, 219 OTUs were predominant with count ≥2.

6.3.2.3- Taxonomic analysis

6.3.2.3.1- Predominant fungal taxa in saliva samples

Ascomycota and Basidiomycota were the predominant phyla in all samples along with unclassified members of fungal groups. A total of 12 classes, 25 orders, 40 families, 55 genera and 92 species were observed in all samples. Subject BK1 had two main dominating phyla, these phyla were Ascomycota (77.54%) followed by Basidiomycota (5.52%) and unidentified fungal groups (16.92%). BK2 had 77.42% Ascomycota followed by 22.49% of Basidiomycota. In case of BKC1, again dominating phyla were Ascomycota (63.05%) followed by Basidiomycota (36.89%). Subject BKC2 had Ascomycota as dominated phyla (99.57%) followed by Basidiomycota (0.36%)

In these samples, abundance of major genera was recorded. In BK1 major genera were: *Phialosimplex* (28.73%) followed by *Olpidium* (16.92%), *Cochliobolus* (15.65%), *Candida* (7.07%), *Aspergillus* (5.76%), *Neurospora* (4.94%), *Debaryomyces* (4.76%), *Malassezia* (3.26%), *Penicillium* (2.08%), *Nakaseomyces* (2.02%), *Rhodotorula* (1.81%), *Fusarium* (1.48%), *Ramulispora* (1.27%), *Stachybotrys* (1.03%), *Eurotium* (0.85%), and *Cladosporium* (0.48%). BK2 had predominant genera including *Saccharomyces* (40.94%) followed by *Candida* (22.79%), *Hyphodontia* (10.17%), *Malassezia* (8.00%), *Cladosporium* (6.64%), *Fusarium* (4.86%), *Mrakia* (4.06%), *Eurotium* (1.35%), *Phialosimplex* (0.17%), *Aspergillus* (0.14%), *Termitomyces* (0.106%), *Penicillium* (0.09%), *Olpidium* (0.07%), *Cochilobolus* (0.036%), *Neurospora* (0.05%), *Cristinia* (0.04%), *Rhodotorula* (0.036%), and *Debaryomyces* (0.035%).

BKC1 had predominant genera including *Termitomyces* (16.17%) followed by *Penicillium* (14.53%), *Aspergillus* (11.121%), *Cristinia* (7.07%), *Neurospora* (6.53%), *Eutypella* (5.12%), *Psaththyrella* (4.54%), *Malassezia* (4.28%), *Candida* (3.95%), *Cladosporium* (3.08%), *Saccharata* (2.69%), *Trichoderma* (2.25%), *Schizophyllum* (2.14%), *Alternaria* (1.92%), *Aureobasidium* (1.82%), *Epicoccum* (1.66%), and *Fusarium* (1.55%). In BKC2, most abundant genera were: *Candida* (65.51%), followed by *Kluyveromyces* (16.91%), *Nakaseomyces* (8.35%), *Alternaria* (2.92%), *Aspergillus* (1.70%), *Phialosimplex* (1.02%), *Saccharomyces* (0.69%), *Hanseniaspora* (0.56%), *Sagenomella* (0.422%), *Simplicillium* (0.31%), *Sclerotinia* (0.20%), *Cladosporium* (0.16%), *Curvularia* (0.14%), *Penicillium* (0.11%), *Malassezia* (0.088%), *Cochilobolus* (0.08%), and *Termitomyces* (0.07%) (Figure 6.5).

Stachybotrys, Geotrichum, Talaromyces, Leucosporidium, Acremonium, Wallemia, Eupenicillium, Septoria, Zymoseptoria, Coniosporium, Phialophora, and *Mycosphaerella* genera were detected in postpartum group but were absent in nonpregnant female. Among postpartum group the genera, which were detected in postpartum females with FTB but absent from female having PLWB were *Zymoseptoria, Septoria, Wallemia,* and *Eupenicillium*. However, among postpartum female with different pregnancy outcomes but having oral health issues following genera were detected: *Sclerotinia, Curvularia, Wallemia, Eupenicillium, Septoria, Zymoseptoria. Coniosporium, Phialophora, Cercospora* and *Mycosphaerella*, only detected in postpartum female with healthy oral cavity and FTB. *Simplicillium* and *Myrothecium* were only found in females with healthy oral cavity, while *Pichia* was only detected in nonpregnant female (0.03%).

At species level, in BK1 predominant species were as follows: *Phialosimplex chlamydosporus* (28.73%) followed by *Olpidium brassicae* (16.92%), *Cochliobolus* spp. (15.65%), *Neurospora* spp. (4.94%), *Debaryomyces hansenii* (4.76%), *Aspergillus penicillioides* (4.38%), *C. albicans* (3.91%), *Malassezia restricta* (2.74%), *C. Tropicalis* (2.40%), *C. glabrata* (2.02%), *Rhodotorula* spp. (1.81%), *Penicicillium* (1.79%), *Ramulispora sorghi* (1.27%), *Stachybotrys* spp. (1.03%), *Eurotium* spp. (0.85%), *Fusarium* spp. (0.82%), *Aspergillus Tamarii* (0.78%), *Candida* spp. (0.75%), *Fusarium Oxysporum* (0.55%) and *Malassezia globosa* (0.52%). In BK2 predominant species were *Saccharomyces bayanus* (40.93%), *C.*

albicans (22.59%), *Hyphodontia sambuci* (10.17%), *Cladosporium cladosporioides* (6.62%), *Fusarium gibberella fujikuroi* (4.78%), *M. restricta* (4.60%), *Mrakia frigida* (4.06%), *M. globose* (3.06%), *Eurotium* spp. (1.35%), *Malassezia slooffiae* (0.33%), *P. chlamydosporus* (0.175%), *C. tropicalis* (0.17%), *Termitomyces* spp. (0.10%), *Aspergillus penicillioides* (0.08%), *O. brassicae* (0.07%), *Cochliobolus* spp. ((0.066%) and *Penicillium lanosum* (0.063%). In BKC1 predominant species were *Termitomyces* spp. (16.17%), *P. lanosum* (12.07%), *Cristinia* spp. (7.07%), *Neurospora* spp. (6.53%), *A. penicillioide* (6.50%), *Eutypella* spp. (5.12%), *Psathyrella candolleana* (4.54%), *C. albicans* (3.85%), *M. globosa* (3.09%), *Saccharate proteae* (2.69%), *Aspergillus oryzae* (2.62%), *Trichoderma aureoviride* (2.24%), *Penicillium* spp. (2.21%), *Schizophyllum commune* (2.14%), *Alternaria alternate* (1.91%) and *Aureobasidium pullulans* (1.82%).

In BKC2 major identified species were *C. albicans* (63.30%), *Kluyveriomyces marxianus* (16.91%), *C. glabrata* (8.35%), *A. alternata* (2.92%), *C. Tropicalis* (2.19%), *A. penicillioides* (1.633%), *P. chlamydosporus* (1.02*%), S. Bayanus* (0.68%), *Hanseniaspora thailandica* (0.56%), *Sagenomella* spp. (0.42%), *Simplicillium obclavatum* (0.31%), *Sclerotinia homoeocarpa* (0.20%), *C. cladosporioides* (0.15%), *Curvularia cochliobolus lunatus* (0.14%), *Cochliobolus* spp. (0.08%), *Termitomyces* spp. (0.07%), *Myrothecium* spp. (0.066%), and *O. brassicae* (0.062%).

6.3.2.3.2- Comparative fungal taxa abundance in postpartum females with oral health issues

Females with gingivitis and dental caries showed greater abundance of genus *Saccharomyces, Phialosimplex, Candida, Olpidium, Cochliobolus, Malaseezia, Hyphodontia, Debaryomyces, Mrakia,* and *Nakaseomyces*as compared to those with good oral health. However, level of *Termitomyces, Penicillium, Aspergillus and Cristinia*was was very high in females without gingivitis and dental caries. Females with PLWB had comparatively increased level of *Saccharomyces, Candida, Hyphodontia, Malassezia, Cladosporium, Fusarium,* and *Mrakia*, as to postpartum female with FTB and normal weight baby.

6.3.2.4- Community profiling

6.3.2.4.1- Alpha diversity

To measure alpha diversity Shannon and Simpson diversity index were calculated to determine the richness and evenness of the samples. Diversity analysis showed greater richness among postpartum group compared to nonpregnant female and among postpartum group even greater diversity was seen in postpartum female with healthy oral cavity and FTB compared to female with dental health issues. Among postpartum females, Shannon index for BK1 and BKC1 saliva samples showed relatively high richness and evenness compared to BK2 sample, which had oral health issues and were also PLWB (Table 6.20).

Table 6.20: Alpha diversity indices for salivary mycobiome of postpartum females (BK1, BK2, BKC1) and non-pregnant female (BKC2)

Sample ID	Shannon index	Simpson index
BK1	2.794	0.889
BK2	1.948	0.773
BKC1	3.331	0.937
BKC2	1.706	0.710

6.3.2.4.2- Beta diversity

Bray-Curtis dissimilarity showed greater dissimilarity between BK1 and BK2 sample (BC=0.919), also BK1 with BKC1 (BC=0.833) and BK1 with BKC2 (BC=0.888). Dissimilarity value between BK2 and BKC1 was also high (BC=0.895) compared to between BK2 and BKC2 (BC=0.872). High dissimilarity value was also obtained for BKC1 and BKC2 (BC=0.945). In PCOA ordination by using Bray-Curtis, all samples cluster separately and it was also statistically insignificant. Results of Statistical analysis had F-value: 0.70697; R-squared: 0.26117; p-value < 1, with Stress = 0 (Figure 6.7).

6.3.2.5- Core mycobiome analysis

At genera level, 36 out of 55 genera were shared between all four female saliva samples (Figure 6.6). At genera level *Candida, Phialosimplex, Malassezia, Fusarium, Eurotium, Cladosporium,* and *Aspergillus* were top genera present in all females.

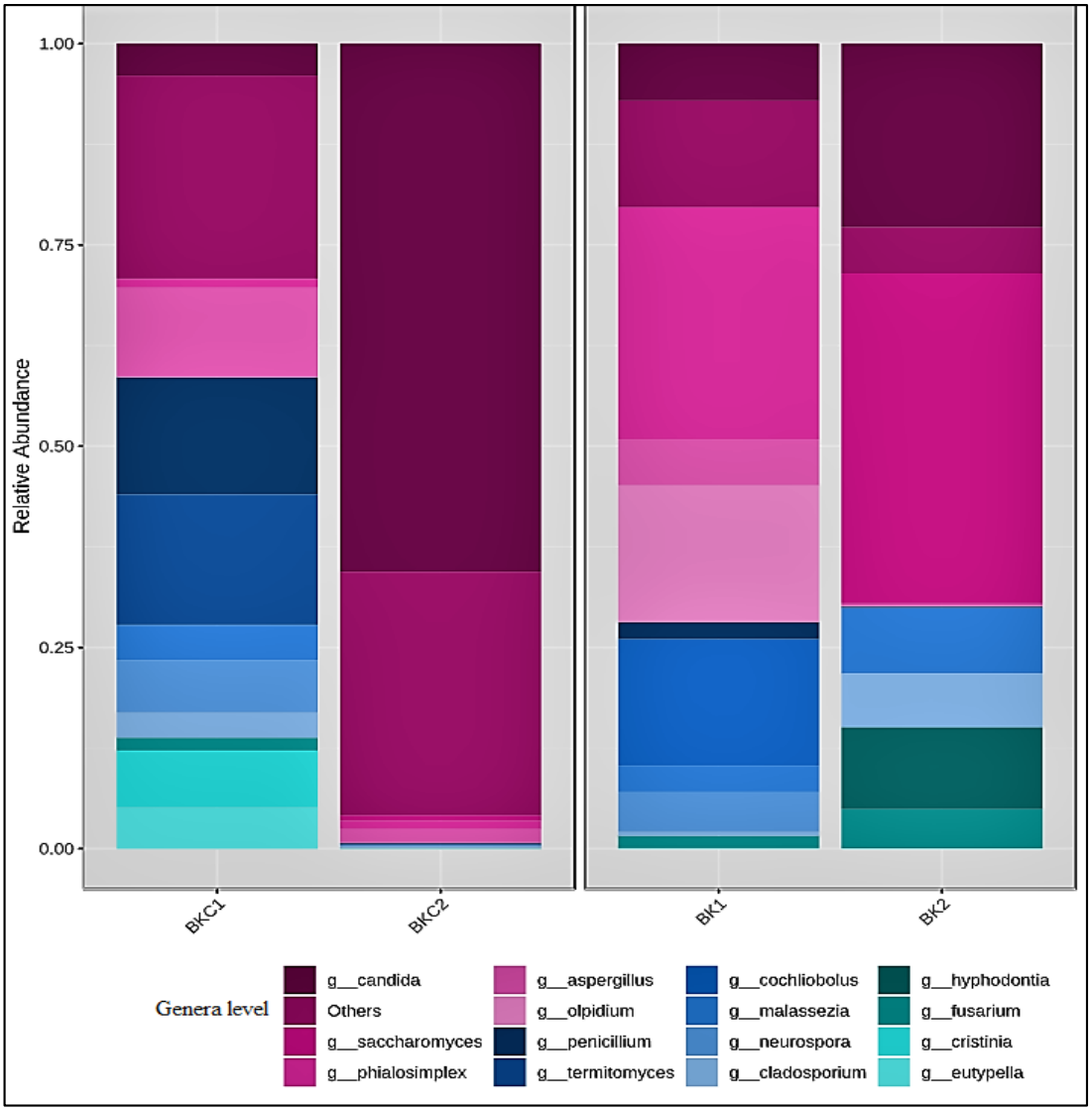

Figure 6.5: Relative abundance of predominant fungal taxa in saliva samples of postpartum and nonpregnant females at genera level; In BK1 major genera were: *Phialosimplex* followed by *Olpidium*, *Cochliobolus*, *Candida*, *Aspergillus*, *Neurospora*, *Debaryomyces* and *Malassezia*, In BK2: *Saccharomyces* followed by *Candida*, *Hyphodontia*, *Malassezia*, *Cladosporium*, *Fusarium*, *Mrakia* and *Eurotium*, In BKC1: *Termitomyces* followed by *Penicillium*, *Aspergillus*, *Cristinia*, *Neurospora*, *Eutypella*, *Psaththyrella*, *Malassezia* and *Candida* (3.95%), In BKC2: *Candida* followed by *Kluyveromyces*, *Nakaseomyces*, *Alternaria*, *Aspergillus*, *Phialosimplex*, *Saccharomyces* and *Hanseniaspora*

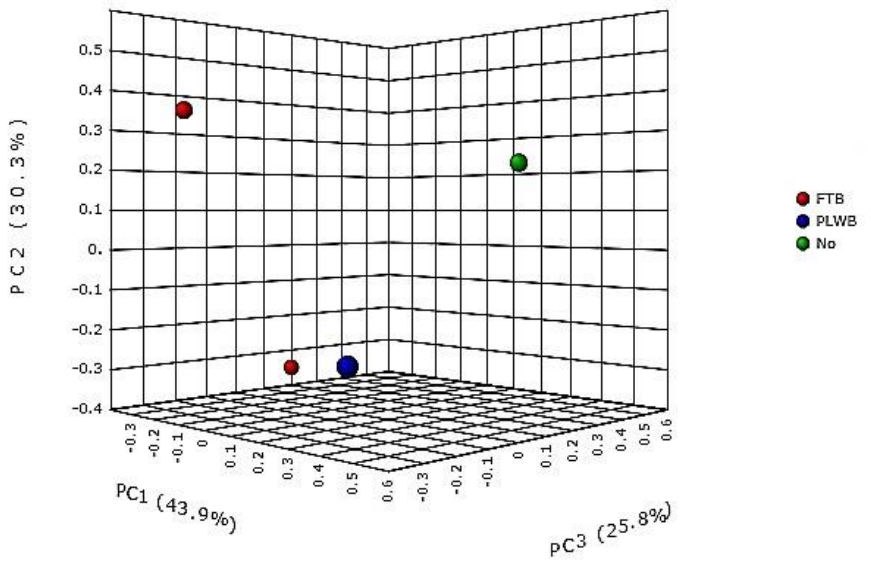

Figure 6.6: PCoA analysis by Bray-Curtis distance of OTU based clustering of microbial communities in postpartum and nonpregnant females. PCoA score for Axis 1, Axis 2 and Axis 3 were: BK1; -0.032626, 0.3561 and -0.2256, BK2; 0.37987, -0.22881 and -0.30326, BKC1; -0.38013, 0.34622 and 0.18551, BKC2; 0.32652, 0.21894 and 0.34336

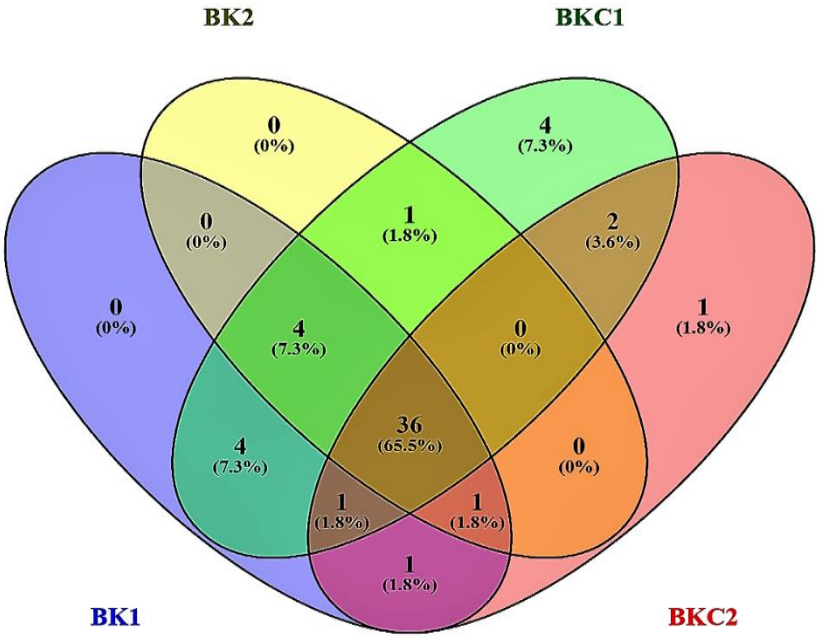

Figure 6.7: Core mycobiome analysis; Percentage abundance of core genera with all saliva samples sharing 65.5% of detected genera

6.4- DISCUSSION

During pregnancy female body undergo various reversible physiological and hormonal changes, which can modulate oral health status and microbial colonization. These modulations remain persistent in early postpartum period and return to normal conditions within 4-6 weeks of gestation. In present study, effect of pregnancy phase on oral fungal colonization in postpartum females post one day of delivery was investigated. As samples were collected after one day of delivery their flora was assumed to be more like that what exist in third trimester or in end of the pregnancy. Both culturable and unculturable fungal species were investigated. Species of *Candida* were the only fungus cultured from these samples. For analysing complete fungal diversity NGS was used. Although *Candida* is the most prevalent genus, and most of the fungal studies are based on identifying its role in disease developments, however, there is lack of data related to the isolation of oral *Candida* species from females in pregnancy and postpartum phase especially in case of oral disorders and its association with pregnancy complications (Nelson *et al*., 2013). For isolation of microbes and fungal diversity analysis in present work, saliva samples were collected, it is a good diagnostic sample as it contains microbes sheds from all oral surfaces (Chojnowska *et al*., 2018).

Candida colonization was significantly raised among group of postpartum females (55.05%). *C. albicans* was the most prevalent as 48.68% of the females has this fungus and it was followed by *C. glabrata* and *C. krusei*. Postpartum females also showed risk of four-fold for *Candida* colonization compared to control group. Previously, most of the studies were based on isolation of *Candida* species from either plaque or oral rinse samples of patients suffering from either diabetes or HIV. Only study is found in literature, which was conducted on oral *Candida* for its isolation was from Chinese healthy pregnant females. *Candida* colonization was studied in different pregnancy phases. However, in that study subgingival plaque samples were used for analysis of *Candida* and was found that there was increased colonization in these females from 40% prevalence detected in middle pregnancy phase to 45.9% seen in late phase of pregnancy. This rate was high compared to nonpregnant group (27.5%) (Fujiwara *et al*., 2017). These results are in agreement with the present study as lower colonization of *Candida* was found in nonpregnant females compared to postpartum

group. There was slight difference in prevalence rates, which might be due to change in source and method of sample collection in Chinese and present study. Similar findings of high *Candida* colonization were also reported from a gestational diabetic pregnant females, where colonization of *Candida* was 57% in mouthwash samples from the females (Nowakowska *et al*.,2004). High *Candida* carriage (56.6%) in oral rinse samples, was also reported from Thai population infected with HIV (Thanyasrisung *et al*., 2014). These studies indicated that colonization of *Candida* species is affected by disease and host immune status. Its colonization increased with decreasing host immunity. Pregnancy, gestational diabetes and HIV, all these conditions compromised the host immune status and predisposes them to increased *Candida* colonization.

Compared to present work findings, relatively low prevalence of *Candida* (32%), was seen in buccal swab samples from Indian diabetic patients compared to healthy control group (8.5 %). Like present study, various *Candida* species were isolated where high level of *C. albicans* (61%) was seen among these Indian patients followed by *C. tropicallis, C glabrata* and *C. krusei*. Most effective drug against *Candida* species was fluconazole (95.6%) in Indian population (Rajakumari and Kumari, 2016). However, in present study *Candida* isolates from postpartum females were sensitive to amphotericin B and 85% isolates were resistant to fluconazole and voriconazole, suggesting increased antibiotic resistance pattern in postpartum females.

Candida species colonizes oral cavity as commensal, but they possess various virulence factors that under favourable conditions cause their transition to opportunistic pathogen. Most important virulence factors are their ability to show phenotypic switching, production of enzymes and biofilm formation. Depending upon the type and source of isolation, *Candida* species showed variable degree of enzymatic activity (İncİ *et al*., 2012). Significantly raised esterase activity was exhibited by the isolates from the postpartum females, with highest activity expressed by *C. albicans* isolates (82.5%). These results are in accordance with the previous reports of high esterase activity expressed by *C. albicans* (92.3%) isolated from immunocompromised individuals (Kumar *et al*., 2006).

Mostly isolates had strong phospholipase activity from both postpartum and nonpregnant group, strong phospholipase activity was seen in isolates from previous

report from diabetic Turkish population (Sahin *et al*., 2005). Literature showed, variable phospholipase production activity by oral *C. albicans* ranging from 30-100% (Barros *et al*., 2008; Hannula *et al*., 2000). Most of the isolates from present study were also biofilm formers. Results of both CRA and MTP methods were not corroborating. On CRA method, more isolates were found to be biofilm formers compared to MTP. This difference could be due to difference in the protocol sensitivity as CRA is a qualitative method based on colour interpretation that could be misleading in case of false positive or negative interpretation. Frequency of biofilm formers were high for *C. albicans* and *C. glabrata*, in contrast to previous report which showed that isolates of *C. albicans*, isolated from blood cultures are less biofilm formers detected by MTP method (Gültekin *et al*., 2011).

The sample source also effect the detection method as a study conducted in children infected with HIV, all isolated *Candida* species from saliva expressing biofilm forming ability in *in vitro* assay showed varies results (Portela *et al*., 2017). Biofilm forming ability was not significantly changed in postpartum group and nonpregnant females. However, compared to postpartum females all isolates from nonpregnant group were weak biofilm formers, while isolates from postpartum group were moderate to strong biofilm former. Overall, *Candida* species from postpartum group were expressing high esterase, phospholipase and biofilm forming activity compared to healthy controls, highlighting that there might of role of immunosuppression enabling the *Candida* to have virulence enhancement.

Candida species colonization in postpartum females did not showed associations with APOs, however, risk was seen in postpartum females with *Candida* carriage for APOs and oral disorders. No data is available in literature about the role of oral *Candida* in APOs. Studies which are available are based on finding the association between vaginal carriage of *Candida* with APOs (Roberts *et al*., 2011). Although, *Candida* was not significantly associated with oral disorders, however, it was increasing the risk for gingivitis and dental caries. This can be explained by the fact that in etiology of periodontal disease and dental caries, along with bacterial species it is often found to be involved, however, role of *Candida* in these condition cannot be neglected, because they help the periodontopathogens and acid producing bacteria in disease progression (Rio *et al*., 2017).

Candida colonization among postpartum females was associated with higher sugary liquid intake. Like aciduric bacteria, *Candida* can also produce acid by carbohydrate fermentation, promoting its growth in oral cavity which favours development of dental plaque. It is a common thought that for preventing plaque development and oral disorders, maintaining oral hygiene is important. Like present work, a study from Jordan showed that oral *Candida* carriage was not significant (*P*=0.94) among this population and was not significantly associated with oral plaque status and moderate level of gingivitis (Darwazeh *et al*., 2010).

In second part of the study, fungal diversity was studied by using sequencing technique to explore mycobiome associated with health and disease. Fungi are emerging as potential threat, as more than 600 fungal species are now known for causing infections in humans. To understand pathogenic potential of unculturable fungi, it is important to fully characterize all fungi both as commensal and pathogen in human health and diseases (Dupuy *et al*., 2014). Although greater efforts have been made in identifying oral bacteriome, knowledge of oral mycobiome is still incomplete and lacking in comparison (Kragelund & Keller, 2019). Majority of the research on oral mycobiome is mainly focused on *Candida*, but with introduction of NGS technologies in the past few decades has greatly increased our knowledge about the oral microbial communities. By using these culture independent technologies, it become possible to know that oral mycobiome is much more diverse as not expected previously (Diaz *et al*., 2017; Välimaa *et al*., 2018)

In present study by using MiSeq illumina platform, salivary mycobiome of postpartum females with different pregnancy outcomes after first day of delivery and of healthy nonpregnant female was identified. It is the 1st study that is conducted to determine the mycobiome associated with different oral health conditions and its association with pregnancy outcomes in postpartum females. In present study, detected predominant genera were *Candida* (12.2%) followed by *Saccharomyces* (9.27%), *Phialosimplex* (9.19%), *Termitomyces* (6.96%), *Penicillium* (6.85%), *Aspergillus* (6.56%), *Olpidium* (5.15%), *Cochliobolus* (4.78%), *Malassezia* (4.61%), *Neurospora* (4.3%), *Cristinia* (3.04%), *Cladosporadium* (2.95%) and *Hyphodontia* (2.3%) in all samples. This trend was quite different from the findings of first landmark study on oral mycobiome by using multitag pyrosequencing and *ITS*

primers, conducted by Ghannoum *et al*., in 2010, characterizing the basal oral mycobiome in oral rinse of 20 healthy participants. Main finding of the study was 74 culturable and 11 nonculturable fungal genera, and 101 species were identified. Percentages of different genera in their mycobiome were as follow: *Candida* (22.2%), *Cladosporium* (19.4%), *Saccharomycetales* (13.9%), *Aspergillus* (11.1%), *Glomus* (5.6%), *Fusarium* (5.6%), *Alternaria* (4.2%), *Penicillium* (4.2%), *Ophiosoma* (2.8%), *Cryptococcus* (2.8%), *Phoma* (2.8%), *Zygosaccharomyces* (2.8%) and *Schizosaccharomyces* (2.8%). *Candida* was the most frequent detected genera in 75% of participants, followed by *Cladosprium* (65%), *Saccharomycetales* (50%), *Aureobasidium* (50%), *Aspergillus* (35%) and *Fusarium* (30%).

Although in present study *Candida* was the predominant genera but abundance and presence of other characterized genera was quite different. The reason might be that in Ghannoum *et al*., study, samples were collected from healthy individuals while in present study saliva sample were collected from postpartum females, an immunocompromised group having both physiological and hormonal imbalance which affect microbiome. Normally microbiome returns to its normal states within 4-6 weeks of postpartum period, and in present study samples were collected post one day of delivery, so it is presumably different from the microbiome seen in normal nonpregnant conditions. Although mycobiome of nonpregnant female was also different from Ghannoum *et al*., findings, however, in their study the mycobiome profile was detected for both healthy male and females by using different sample and extraction techniques as well as sequencing method.

Following Ghannoum et al study, Dupuy *et al*., (2014) carried out study on oral mycobiome of oral rinse from six healthy subjects. A massively parallel, high throughput sequencing method was used by targeting *ITS*1 region from saliva. In their study, they have found high prevalence and abundance of the genus *Malassezia*, the one not detected in Ghannoum *et al*., study. It was the first study that reported presence of *Malassezia* in oral cavity, a commonly known skin pathogen and also detected in nostrils, and in back of head and ear. Similar to Dupuy *et al*., findings, in present study *Malassezia* was detected in all samples, however, its abundance was high in postpartum females compared to nonpregnant female. Within postpartum group, even its abundance was comparatively very high (8.0%) in female with oral

health issues and PLWB, compared to postpartum females with FTB and normal weight baby. In Dupuy's work, major detected core genera include *Alternaria/Lewia*, *Aspergillus/Emericella/Eurotium*, *Candida/Pichia*, *Cladosporium/Davidiella*, *Cryptococcus/Filobasidiella*, and *Fusarium/Gibberella*. While in present study top core genera were *Candida, Phialosimplex, Malassezia, Fusarium, Eurotium, Cladosporium,* and *Aspergillus.*

In 2014, Mukherjee *et al*., explore oral mycobiome in 12 HIV patients and 12 healthy individuals. Among HIV infected patients, most common detected genera were; *Candida, Epicoccum,* and *Alternaria* (affecting 92%, 33%, and 25% patients, respectively), while in healthy individuals, the most abundant fungi were *Candida, Pichia,* and *Fusarium*. In present study, in nonpregnant subject, level of *Candida* species (65.51%) was comparable to findings of Mukherjee. *Pichia* was only detected in nonpregnant female, and its prevalence was very low (0.03%). Vesty *et al*., (2017) conducted a study on pooled saliva samples from 12 healthy volunteers and their DNA was extracted by different extraction techniques to check their efficiency, followed by MiSeq illumina sequencing. Similar to the present study, in that study by using phenol chloroform extraction method, the major detected genera were *Candida* and *Penicillium*. *Saccharomyces* and *Malassezia* were found at <10% and <1%, respectively. However, in contrast to previous studies and also to the present work, in the work of Vesty *et al*., *Cladosporium, Fusarium*, *Aureobasidium* and *Epicoccum* were absent in the saliva samples.

The only study available in the literature based on oral mycobiome in periodontal disease patients was carried out by Peters *et al*., (2017). They characterize fungal diversity in oral wash samples of 15 periodontal disease patients and 15 healthy individuals by using sequencing of *ITS* gene. In their study, they have found 81 genera and 154 fungal species in all samples. In their study frequently, detected genera included *Candida* and *Aspergillus*, followed by *Penicillium, Schizophyllum*, *Rhodotorula*, and *Gibberella*. They have also detected genus *Malassezia*. Similarly, studies on oral mycobiome of dental caries are also very rare, only one study is available in literature on plaque samples of children (*n*=17) with and without caries, which employed the same technique for sequencing as used in present work. Ascomycota, Basidiomycota and Zygomycota were the most abundant phyla in their

study, in which a total of 23 genera and 46 fungal species were isolated, among these *C. albicans* was the most abundant in both children with or without caries. Overall fungal diversity was also same in both groups, however, presence of caries influenced the abundance of specific fungi. Core microbiome was composed of *C. albicans,* unclassified *Saccharomyces, N. diffluens, R. mucilaginosa* and *M. globose* (Fechney *et al*., 2018). Herein, females with different health status and pregnancy outcomes had different taxon with change in their relative abundance.

Among the studied postpartum female in this work having oral health issues (gingivitis and dental caries), there were some genera that were absent in postpartum female with healthy oral cavity including: *Sclerotinia, Curvularia, Wallemia, Eupenicillium, Septoria* and *Zymoseptoria.* Females with gingivitis and dental caries showed greater abundance of genus *Saccharomyces, Phialosimplex, Candida, Olpidium, Cochliobolus, Malaseezia, Hyphodontia, Debaryomyces, Mrakia, and Nakaseomyces*. The level of *Termitomyces, Penicillium, Aspergillus* and *Cristinia* was very high in females without gingivitis and dental caries. Females with PLWB had pronounced increase in the genera like *Saccharomyces, Candida, Hyphodontia, Malassezia, Cladosporium, Fusarium,* and *Mrakia,* compared to postpartum female with FTB and normal weight baby. Beta diversity analysis of these female showed greater dissimilarity values between females having dental issues but different pregnancy outcomes, and also decrease in alpha diversity in female having both oral health issues and PLWB. Postpartum female with oral health issues and FTB showed diversity closer to the healthy postpartum female with FTB. These findings imply that the presence of oral health issues like gingivitis and dental caries can be associated with diversity change, and there might be association between APOs like PTB and LWB with oral fungal diversity, which needs further validation.

The findings from present work provided for the first time the snapshot of oral health conditions of postpartum females with regards to both culturable and unculturable fungal isolates. *Candida* colonization was significantly raised in group of postpartum females and had possibly risk for development of APOs and oral diseases. Isolates of *Candida* were expressing enhanced virulence characteristics compared to isolates from nonpregnant females. Oral mycobiome of nonpregnant female was quite different from postpartum group. Comparatively greater diversity was seen in

postpartum group. Among postpartum group female with PLWB, reduced richness and evenness but elevated levels of *Saccharomyces* and *Candida* compared to the female having FTB was recorded, suggesting that there might be possible role of changing diversity as risk factor in affecting pregnancy outcomes beside other factors. Although the small size of the study population for mycobiome may have limited power of detecting significant associations, the findings from the present study will provide a baseline information on individual variabilities with different health conditions in postpartum group as well as will support in adding information to make comprehensive oral fungal databases.

CHAPTER 7: FUNGAL-BACTERIAL INTERACTION IN DUAL-SPECIES BIOFILM FOR PATHOGENESIS

7.1- INTRODUCTION

Microbial cells can exist as free living planktonic forms or associated with each other and get attached on surfaces are called biofilms (Schrøder *et al*., 2018), in which microorganisms are embedded themselves in a self-produced hydrated polysaccharide and protein matrix (Chandra *et al*., 2018; Jamal *et al*., 2018). Due to biofilm formation several benefits are conferred to the microorganisms, ranging from shared metabolic pathways to protection from host defence mechanisms, environmental factors and toxic substances such as antimicrobials (Ranieri *et al*., 2018; Felipe *et al*., 2019). These biofilm structures are of great interest and importance in clinical scenarios as these biofilm play role in development of various infectious disorders and increased resistance to significantly higher concentration of antimicrobial agents (Chakraborty *et al*., 2018; Klauck *et al*., 2018). Biofilm can form on both abiotic (medical devices) and biotic surfaces (mucosal surfaces). National Institute of Health, USA, estimated that approximately 80% of the human infections arise due to pathogenic biofilms (Goikoetxea *et al*., 2018).

In biofilm development commensal flora are mainly involved, including viral, bacterial and fungal species. In more than 70% of the biofilm related infections, Gram-positive especially *Staphylococcus* species are involved. Among fungal species commonly studied biofilm forming specie is *C. albicans*. Other biofilm forming *Candida* species include *C. parapsilosis*, *C. krusei*. *C. tropicalis* and *C. glabrata*. Fungal biofilm related infection commonly effects oral cavity, lungs, burn wounds, reproductive tract, gastrointestinal tract, intravascular catheters, skin and urinary catheters (Íñigo and Pozo, 2018).

Among other, oral biofilms are known to be polymicrobial in nature and are spontaneously formed on teeth and its surrounding periodontal surfaces. In this condition, these communities interact with each other via specific signalling mechanisms, which lead to development of either symbiotic or antagonistic relationship between various microbes. Oral microbes specifically colonize different ecological niche, and still now it is not clear whether non-commensal and transient

bacteria can also efficiently grow in these oral biofilms (Thurnheer and Belibasakis, 2015; Berger *et al*., 2018; Kriebel *et al*., 2018)

C. albicans is common human oral commensal specie, which has been a best studied fungal model for biofilm development. This fungus can become pathogen under unhygienic and immunocompromised conditions. It also is commonly isolated from biofilms of root caries and infected gingival crevices. *C. albicans* has several cariogenic characteristics like high acid tolerance, ability to bind collagen and dentin collagen degradation by secreted aspartyle proteinase. Despite its known role in caries, its significance is often ignored because of its small number in oral microbiota (Cannon and Villas-bôas, 2011; Yang *et al*., 2012; Sanchez-vargas *et al*., 2013)

There is need to study these polymicrobial associations in development of biofilms. Mostly *C. albicans* biofilm based studies are *in vitro* as mono-specie biofilms developed on abiotic surfaces (Harriott and Noverr, 2011). Recent studies on biotic surfaces, such as oral and vaginal mucosal tissues show that these surfaces are excellent for biofilm development especially for such multiple microbial biofilms (Gulati and Nobile, 2016). *C. albicans* is involved in mixed-species infections like in periodontitis (Montelongo-Jauregui and Lopez-Ribot, 2018), in which it interacts with several Gram-negative bacteria, which exacerbates periodontitis. It also enhances proinflammatory cytokines secretions in response to LPS secreted by Gram negative bacteria (Tamai *et al*., 2011). *C. albicans* also act synergistically with *S. mutans* in inducing more aggressive form of dental caries by increased acid production, EPS production and by providing abundance of binding sites for exoenzyme *Gfts* (Fernandes *et al*., 2018; Rocha *et al*., 2018). *C. albicans* and *S. aureus* are also common bloodstream pathogens and are associated with high rates of morbidity and mortality in hospitalized patients and are difficult to treat. These both microbes are frequently co-cultured from vaginal and oral mucosal biofilms (Peters *et al*., 2010; Zago *et al*., 2015; Kong *et al*., 2016).

Currently, dual-species biofilm formation between *C. albicans* and *S. aureus* is commonly studied for their pathogenic potential (Schlecht *et al*., 2018). However, limited data is available in literature related to oral commensal *Candida* species interaction with each other and with CoNS as well as with Gram-negative rods in dual-species biofilm. There is a need to explore these interactions in more

comprehensive way to assess the possible intra and inter-species effects of these oral microbial communities in biofilms development leading to oral infections. In present study, interaction between different *Candida* species and their interaction with other oral Gram-positive and negative bacteria was dissected as oral dual species-biofilm model by qualitative and quantitative analysis (CV and XTT assay). In addition, estimation of total protein content and aspartyl proteinase activity in single and dual-species biofilm were also evaluated to explore their role in survival and development of pathogenesis.

7.2- MATERIAL AND METHODS

7.2.1- Isolates used for mono and dual-species biofilm assays

Single and dual-species biofilm and its analysis by detecting different virulence factors contributing in biofilm development were assessed by using 96-well microtiter plates and spectrophotometric technique. For these assays multiple drug resistant weak biofilms former *Candida* species and bacterial isolates from saliva samples of postpartum females were studied in mono and different combinations of dual-species biofilm (Table 7.1).

Candida isolates from postpartum females were inoculated from 48 h old stock cultures in test tubes containing SDB supplemented with 10% glucose while bacterial isolates were inoculated in NB supplemented with 10% glucose and incubated for 24 h at 37°C for CV and XTT reduction assay. After incubation, centrifugation was done for 10 min at 5,000 rpm (4°C) to get pellet. Supernatant was removed and washing was done twice in sterile PBS (pH 7.4 -7.6). The cells were resuspended in 1.0 mL of RPMI 1640 media. Inoculum was prepared with final cell density of 1.0×10^7 cells/mL, compared with 0.5 McFarland and adjusted to OD of 0.38 at wavelength 520 nm spectrophotometrically (Multiskan Go, thermo scientific).

Table 7.1: Fungal and bacterial combinations used for mono and dual-species biofilm assays

Mono-specie biofilm	**Dual-species biofilm**
ATCC *C. albicans* (90092)	ATCC *C. albicans* (90092) + ATCC *S. aureus* (25923)
ATCC *S. aureus* (25923)	ATCC *C. albicans* (90092) + ATCC *K. pneumoniae* (700603)
ATCC *K. pneumoniae* (700603)	ATCC *C. albicans* (90092) + ATCC *E. coli* (25922)
ATCC *E. coli* (25922)	*C. albicans* BK239+ *C. glabrata* BK214
C. albicans BK239	*C. albicans* BK239+ *C. krusei* BK229
C. glabrata BK214	*C. glabrata* BK214+ *C. krusei* BK229
C. krusei BK229	*C. albicans* BK239+ *S. aureus* BK79
S. aureus BK79	*C. albicans* BK239+ *S. epidermidis* BK155
S. epidermidis BK155	*C. albicans* BK239+ *S. saprophyticus* BK134
S. saprophyticus BK134	*C. albicans* BK239+ *K. pneumoniae* BK166
K. pneumoniae BK166	*C. albicans* BK239+ *E. coli* 203
E. coli 203	*C. glabrata* BK214+ *S. aureus* BK79
	C. glabrata BK214+ *S. epidermidis* BK155
	C. glabrata BK214+ *S. saprophyticus* BK134
	C. glabrata BK214+ *K. pneumoniae* BK166
	C. glabrata BK214+ *E. coli* 203
	C. krusei BK229+ *S. aureus* BK79
	C. krusei BK229+ *S. epidermidis* BK155
	C. krusei BK229+ *S. saprophyticus* BK134
	C. krusei BK229+ *K. pneumoniae* BK166
	C. krusei BK229+ *E. coli* 203

7.2.1.1- CV assay

CV assay was done by using 96-well microtiter plates, which contained 75 µL of cell suspension and 75 µL of RPMI 1640 for each strain. In dual-species assay, 75 µL of microorganism's suspension was used in the above mention combinations. Three sterile RPMI 1640 containing wells without fungal and bacterial cultures were used as negative control. Plates were incubated for 24 h and 48 h at 37°C, separately. Media was removed carefully from wells after incubation period. Washing of wells were done three times with 200 µL PBS (pH 7.2). For fixing adherent cells, 95% ethanol was added to each well and incubated (30 min) at room temperature. The adherent cells were stained with 1% w/v CV (200µL) and again incubated at room temperature (15 min). The wells of microtiter plate were washed to remove excess stain with deionized water. After washing, wells were air dried and to solubilize the CV stain, treated with 33% acetic acid followed by incubation for 15 min at room temperature. For each well, OD was determined at wavelength 492 nm (Multiskan Go, Thermoscientific). Isolates were inoculated in triplicates for biofilm formation and average of three reading was taken for result analysis by comparing ODs of isolates (Dhanasekaran *et al*., 2014; Zago *et al*., 2015).

7.2.1.2- XTT reduction assay

For this assay, 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazoliumhydroxide (XTT) was used to assess metabolic activity of adhere biofilm cells. XTT (Sigma) reagent solution was prepared (1.0 mg/mL) in ultrapure water, filtered and stored at -70°C until use. Solution of phenazine methosulfate (PMS) stock was prepared freshly (0.32 mg/mL in deionized water) and stored in a dark. XTT assay was done by using 96-well microtiter plates. Biofilm were developed after 24 h and 48 h of incubation by following the procedure used for CV assay. Media was removed from each well after incubation and washed thrice with 150 µL of PBS (0.1 M, pH 7). Later, 90 µL of XTT solution and 10 µL of freshly prepared PMS were added. Plates were incubated for one h at 37°C in dark and absorbance was measured at 492 nm (Multiskan Go, Thermoscientific). All isolates were tested for XTT reduction assay in triplicates.

7.2.2- Tyrosine aspartyl proteinase activity

Candida species and bacterial strains were firstly cultured in 5.0 mL BHI broth (CM 1135) and incubation was done for 24 h at 37 °C. After incubation, washing with PBS was done twice by centrifugation for 5 min at 5000 rpm. The cells suspension density of 10^7 cells/mL was inoculated in 5.0 mL of BHI broth and incubated for 24 h and 48 h at 37°C. After incubation, 0.1 mL broth was transferred to other eppendroff and 0.4 mL of 0.1 M sodium citrate buffer (pH 3.2) containing 1% w/v bovine serum albumin (BSA) was added and incubation was done for 15 min at 37°C. After incubation, 5% w/v trichloroacetic acid (0.5 mL) was added to stop the reaction and centrifugation was done for 10 min at 3000 rpm, and OD was measured at 280 nm against distilled water.

7.2.3- Total protein estimation (Lowery method)

For total protein estimation, isolates of *Candida* species and bacterial strains were cultured in 5.0 mL BHI medium for 24 h and 48 h at 37 °C. After incubation, culture medium was centrifuged for 5 min at 5000 rpm. Culture supernatant (100 µL) and biuret reagent (200 µL) was added to each well of MTP, mixed thoroughly by pipetting and incubated for 10-15 min at room temperature. Biuret reagent was prepared by adding 0.5 mL cupric sulfate (1.0%) and 0.5 mL of sodium potassium tartrate (2.0%) i.e. solution B, with 50 mL of 2.0% sodium carbonate in 0.1 N NaOH solution i.e. solution A and stored in dark. After incubation, to each well, 20 µL of 1N Folin and Ciocalteu's reagent was added followed by gently pipetting for mixing. This step was performed in dark as Folins phenol is light sensitive. Change in colour was detected after incubation at room temperature (30 min). ODs were recorded at wavelength of 650 nm. Concentration of protein was measured in µg/ml by using equation Y=0.5034x+0.1246, derived from BSA curve. Here X=concentration of unknown sample and Y=Absorbance of unknown sample-absorbance of control sample (Appendix D).

7.2.4 Statistical analysis

Means, SD and mean standard error was calculated for descriptive data and categorical data by using IBM SPSS (version 21). ANOVA analysis was used to

determine associations between different combinations, where $P<0.05$ was used as the level of significance.

7.3- RESULTS

7.3.1- *Candida-Candida* interactions in dual-species biofilm

7.3.1.1- CV biofilm biomass and XTT reduction assays for *Candida-Candida* biofilms

CV assay results showed increased in biofilm biomass after 48 h of incubation in mono and dual-species biofilm. For *C. albicans* BK239 and *C. glabrata* BK214 decrease in biofilm biomass (OD=0.172±0.010 after 24 h and OD=0.270±0.016 after 48 h) was recorded, when co-cultured. Alone *C. krusei* BK229 showed maximum biofilm biomass formation among *Candida* isolates (OD=0.534±0.02 after 24 h and OD=0.971±0.18 after 48 h), but its biofilm biomass was markedly decreased in dual-species biofilm with *C. albicans* BK239 (OD=0.333±0.05 after 24 h and OD=0.847±0.097 after 48 h) and *C. glabrata* BK214 (OD=0.187±0.02 after 24 h and OD=0.688±0.320 after 48 h) (Table 7.2, Figure 7.1). One-way ANOVA analysis showed significant change in mono and dual-species biofilm biomass production after 24 h and 48 h in CV assay (Table7.4).

XTT assay showed overall decreasing trend in metabolic rate in biofilms after 48 h of incubation compared to 24 h. Metabolic activity of *C. albicans* (BK239)-*C. glabrata* (BK214) dual-species biofilm increased after 24 h and 48 h of incubation compared to mono-specie assay. *C. krusei* (BK229) biofilms showed highest metabolic rate in mono-species biofilm, which increased further after 24 h of incubation in dual-species biofilms. However, after 48 h of incubation their biofilms showed slight reduction in metabolic rate in dual-species biofilm with both *C. albicans* 239BK (from OD=0.338±0.035 to OD=0.300±0.032) and *C. glabrata* (OD=0.338±0.035 to OD=0.272±0.004) (Table 7.2, Figure 7.2). ANOVA analysis showed significant change in metabolic rate between single and dual-species biofilm after 24 h and 48 h (Table 7.4).

7.3.1.2- Total protein estimation and aspartyl proteinase activity of *Candida* in mono and dual-species biofilm

Increase in protein content in developed biofilms was seen after 48 h of incubation compared to 24 h except for *C. albicans* BK239-*C. krusei* BK229 and *C. glabrata* BK214-*C. krusei* BK229 dual-species biofilm, which showed slight decrease. Protein concentration of *C. albicans* BK239-*C. glabrata* BK214 dual-species biofilm was less affected compared to mono-specie after 24 h and 48 h of incubation. However, protein concentration was greatly reduced in *C. albicans* BK239-*C. glabrata* BK214 (3.380±0.15) dual-specie biofilms after 48 h of incubation. In dual-species biofilm of *C. krusei* BK229, protein concentration increased after 24 h of incubation. ANOVA analysis indicated significant change in protein concentration in mono and dual-species cultures (Table 7.3 and 7.4, Figure 7.3).

Aspartyl proteinase activity was not much affected after 48 h of incubation both in mono and dual-species biofilm. Highest aspartyl proteinase activity was expressed by *C. albicans* BK239 (0.147±0.010) after 24 h of incubation. *C. albicans* BK239-*C. glabrata* BK214 and *C. albicans* BK239-*C. glabrata* BK229 dual-species biofilm showed decrease in activity after 24 h and 48 h of incubation. Decrease activity was also seen in *C. glabrata* BK214-*C. krusei* BK229 dual-species biofilm after 24 h and 48 h of incubation. ANOVA analysis showed significant change in enzymatic activity after 24 h and 48 h of incubation in mono and dual-species combinations (Table 7.3 and 7.4, Figure 7.4).

Table 7.2: Detection of biofilm formation **(**CV assay) and metabolic activity (XTT reduction assay) expressed by *Candida* mono and dual-species biofilm. All experiments were performed in triplicates, mean ODs and standard deviation from the mean ODs was calculated after 24 h and 48 h of incubation

***Candida* mono and dual-species combination**		**CV assay ODs after 24 h**	**CV assay ODs after 48 h**	**XTT assay ODs after 24 h**	**XTT assay ODs after 48 h**
C. albicans BK239	Mean±SD Std. error	0.239±0.037 0.021	0.320±0.023 0.0133	0.142±0.006 0.003	0.119±0.018 0.010
C. glabrata BK214	Mean±SD Std. error	0.165±0.016 0.009	0.390±0.092 0.053	0.105±0.002 0.0011	0.112±0.011 0.006
C. krusei BK229	Mean±SD Std. error	0.534±0.026 0.015	0.971±0.188 0.108	0.403±0.054 0.031	0.338±0.035 0.020
C. albicans BK239+*C. glabrata* BK214	Mean±SD Std. error	0.172±0.010 0.006	0.270±0.016 0.009	0.306±0.041 0.024	0.300±0.032 0.018
C. albicans BK239+*C. krusei* BK229	Mean±SD Std. error	0.333±0.05 0.030	0.847±0.097 0.056	0.511±0.041 0.023	0.303±0.050 0.028
C. glabrata BK214+*C. krusei* BK229	Mean±SD Std. error	0.187±0.02 0.013	0.688±0.320 0.185	0.423±0.017 0.009	0.272±0.004 0.002
Blank	Mean±SD Std. error	0.083±0.005 0.003	0.168±0.001 0.0005	0.063±0.003 0.001	0.059±0.002 0.001

Table 7.3: Estimation of total protein concentration and aspartyl proteinase activity displayed by *Candida* mono and dual-species biofilm combinations after 24 h and 48 h of incubation (in triplicates). Mean concentration and SD from mean concentration was calculated for total protein estimation by Lowery method and for aspartyl proteinase activity, mean ODs and SD from the mean ODs was calculated

***Candida* mono and dual-species combinations**		**Protein concentration after 24 h (µg/mL)**	**Protein concentration after 48 h (µg/mL)**	**ODs of aspartyl proteinase activity after 24 h**	**ODs of aspartyl proteinase activity after 48 h**
C. albicans BK239	Mean±SD Std. error	3.048±0.58 0.33	3.97±1.30 0.75	0.147±0.010 0.006	0.125±0.008 0.005
C. glabrata BK214	Mean±SD Std. error	2.925±0.29 0.17	4.31±0.36 0.20	0.111±0.005 0.003	0.131±0.01 0.005
C. krusei BK229	Mean±SD Std. error	2.85±0.19 0.10	4.39±0.89 0.51	0.097±0.005 0.003	0.113±0.008 0.005
C. albicans BK239+ *C. glabrata* BK214	Mean±SD Std. error	3.105±0.49 0.28	3.380±0.15 0.089	0.116±0.01 0.006	0.116±0.008 0.004
C. albicans BK239+ *C. Krusei* BK229	Mean±SD Std. error	4.052±0.96 0.55	3.143±0.465 0.268	0.109±0.006 0.003	0.119±0.007 0.004
C. glabrata BK214+ *C. krusei* BK229	Mean±SD Std. error	4.143±0.58 0.33	2.897±0.44 0.25	0.095±0.002 0.001	0.098±0.006 0.003

Table 7.4: Estimation of statistically significant change between and within groups (combinations) in biofilm formation, metabolic activity, total protein concentration and aspartyl proteinase activity by *Candida* mono and dual-species combinations after 24 h and 48 h of incubation (in triplicates) using ANOVA

Assays		**DF***	**F**	***P* value**
CV assay ODs after 24 h (492nm)	Between groups Within groups	07 16	69.908	<0.001†
CV assay ODs after 48 h (492nm)	Between groups Within groups	07 16	13.666	<0.001†
XTT reduction assay ODs after 24 h (492nm)	Between groups Within groups	07 16	96.708	<0.001†
XTT reduction assay ODs after 48 h (492nm)	Between groups Within groups	07 16	50.667	<0.001†
Total protein concentration after 24 h (µg/mL)	Between groups Within groups	07 16	10.634	<0.001†
Total protein concentration after 48 h (µg/mL)	Between groups Within groups	07 16	14.885	<0.001†
Aspartyl proteinase activity ODs after 24 h (280nm)	Between groups Within groups	06 14	140.384	<0.001†
Aspartyl proteinase activity ODs after 48 h (280nm)	Between groups Within groups	06 14	10.572	<0.001†

DF*****=Degree of freedom

†Significant difference at value <0.05

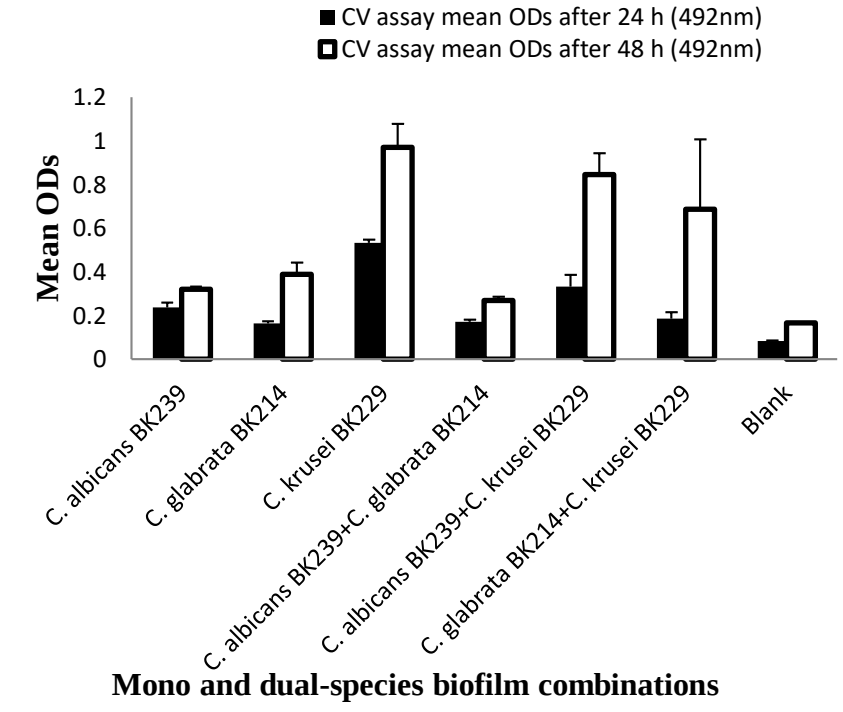

Figure 7.1: Comparison of biofilm biomass production by CV assay for *Candida* mono and dual-species combinations after 24 h and 48 h of incubation

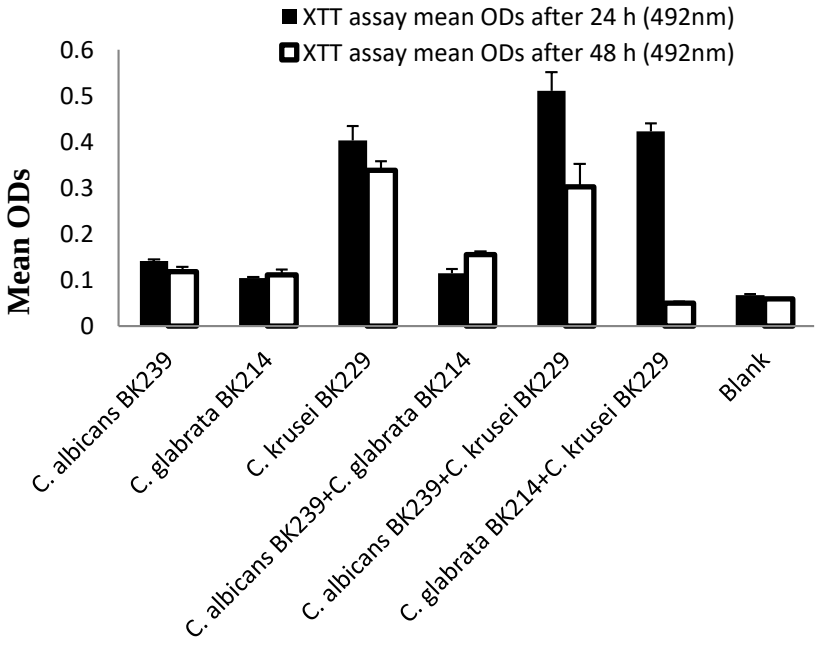

Figure 7.2: Comparison of metabolic activity by XTT assay expressed by *Candida* mono and dual-species combinations in developed biofilms after 24 h and 48 h of incubation

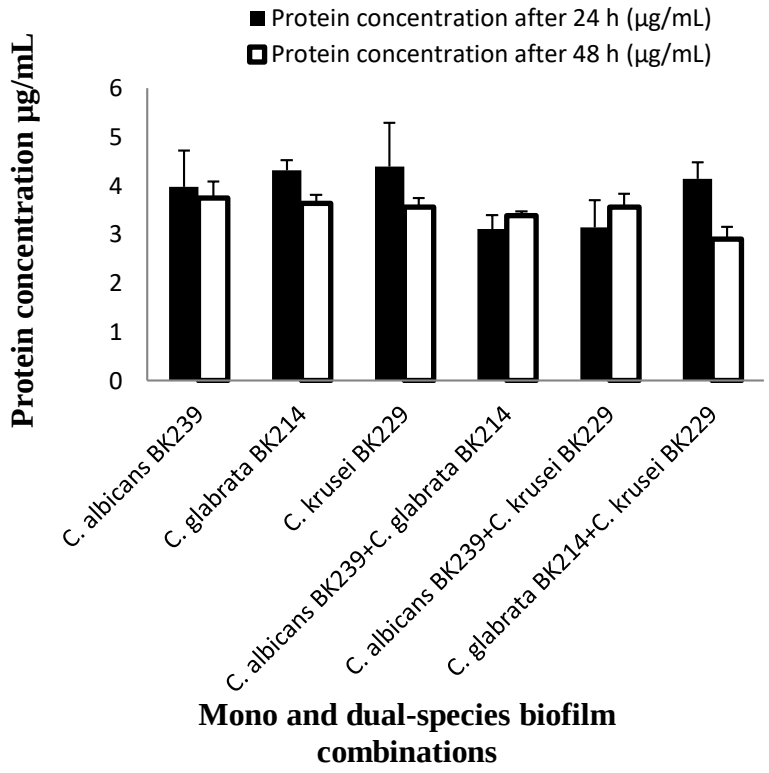

Figure 7.3: Total protein estimation by Lowery method for *Candida* mono and dual-species combinations in developed biofilms after 24 h and 48 h of incubation

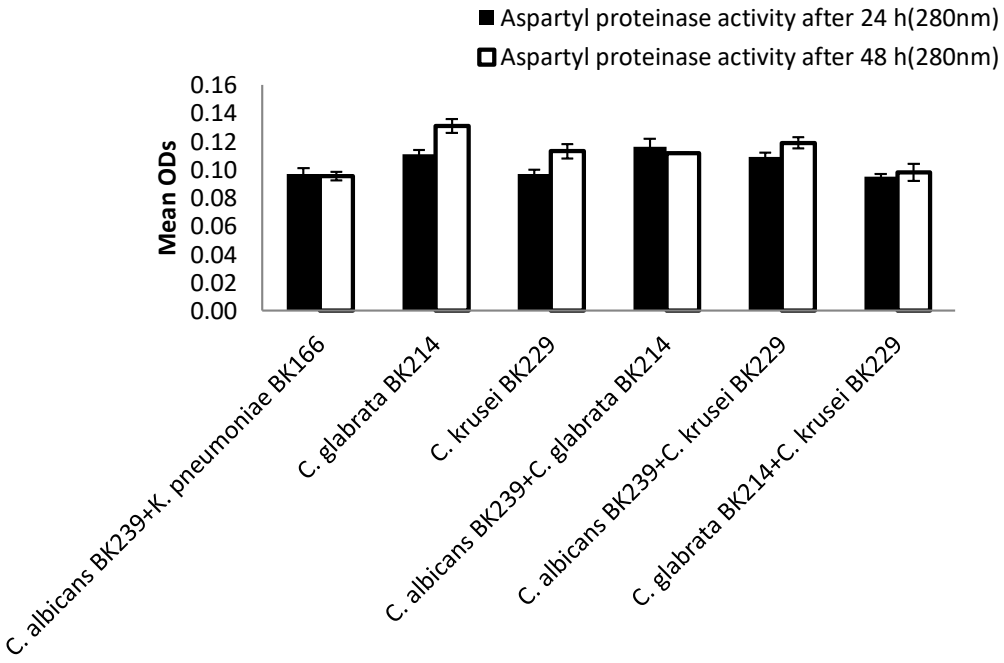

Figure 7.4: Comparison of aspartyl proteinase activity expressed by *Candida* in mono and dual-species combinations in developed biofilms after 24 h and 48 h of incubation

7.3.2 *Candida-Staphylococcus* interactions in dual-species biofilm

7.3.2.1 CV biofilm biomass and XTT reduction assays for *Candida-Staphylococcus* biofilms

Biofilm biomass production was enhanced after 48 h of incubation for both mono and dual-species biofilm. CV assay results showed that in mono-specie biofilm of ATCC *C. albicans* (90029), absorbance of 0.282±0.0261 after 24 h and 0.8773±0.0743 after 48 h were recorded, which was greatly increased when co-cultured with ATCC *S. aureus* (25923) (OD=0.425±0.042 after 24 h and OD=1.100±0.208 after 48 h). *C. albicans* (BK239) showed increased in biofilm biomass in dual-species biofilm with *Staphylococcus* species except for *S. aureus* BK79, in which it showed reduction in biofilm biomass after 24 h and 48 h of incubation (OD=0.239±0.037 after 24 h and OD=0.320±0.023 after 48 h to OD=0.190±0.027 after 24 h and 0.283±0.136 after 48 h) compared to mono-specie biofilm (Table 7.5a, Figure 7.5). Among other *Candida* species, *C. glabrata* (BK214) showed increased in biofilm biomass in dual-species biofilms with *Staphylococcus* species except for *S. aureus* BK79, in which it showed reduction in biofilm biomass after 48 h of incubation (OD=0.390±0.092 to OD=0.283±0.136) compared to mono-specie biofilms (Table 7.5b, Figure 7.6). *C. krusei* BK229 showed reduction in biofilm biomass in dual-species biofilm with all *Staphylococcus* species (Table 7.5b, Figure 7.7). One-way ANOVA analysis showed significant change in biofilm biomass in mono and dual-species biofilm after 24 h and 48 h CV assay (Table7.7).

Metabolic activity was reduced overall after 48 h of incubation compared to 24 h, except for *Staphylococcus* mono-species biofilms and *C. glabrata* BK214-*S. aureus* BK79 dual-species biofilm. Metabolic rate of ATCC *C. albicans* (90029) was enhanced after 24 h and 48 h of incubation in dual-species biofilm with ATCC *S. aureus* 25922 (from OD=0.368±0.011 to OD=0.511±0.007 after 24 h and from OD=0.245±0.003 to OD=0.430±0.059 after 48 h). Metabolic activity of *C. albicans* (CA239) with all *Staphylococcus* species was enhanced after 24 h and 48 h of incubation compared to mono-specie, however, *S. saprophyticus* showed decrease in metabolic rate when co-cultured with *C. albicans* BK239 (Table 7.5a, Figure 7.8). Metabolic activity of *C. glabrata* (BK214) was not much affected in dual-species biofilm except with *S. saprophyticus*, with which it showed enhanced activity (from

OD=0.105±0.002 to 0.292±0.04 after 24 h and OD=0.112±0.011 to OD=0.238±0.103 after 48 h) (Table 7.5b, Figure 7.9). *C. krusei* BK229 showed increase in metabolic rate in dual-species biofilm with *Staphylococcus* species except for *S. saprophyticus* BK134, with which it showed reduction after 24 h incubation. *S. saprophyticus* showed decreased in metabolic activity in dual-species biofilm with all *Candida* species (Table 7.5b, Figure 7.10). ANOVA analysis showed significant change in metabolic activity in mono and dual-species biofilm after 24 h and 48 h XTT reduction assay (Table 7.7).

Table 7.5a: Detection of biofilm formation **(**CV assay) and metabolic activity (XTT reduction assay) expressed by *C. albicans-Staphylococcus* mono and dual-species biofilm combinations. All experiments were performed in triplicates, mean ODs and standard deviation from the mean ODs was calculated after 24 h and 48 h of incubation

***C. albicans-Staphylococcus* species combinations**		**CV assay ODs after 24h**	**CV assay ODs after 48h**	**XTT assay ODs after 24h**	**XTT assay ODs after 48h**
ATCC C. *albicans* (90029)	Mean±SD Std. error	0.282±0.261 0.015	0.877±0.128 0.074	0.368±0.011 0.006	0.245±0.003 0.002
ATCC *S. aureus* (25922)	Mean±SD Std. error	0.137±0.022 0.01	0.257±0.052 0.03	0.298±0.068 0.039	0.090±0.003 0.001
ATCC *C. albicans* (90029) +ATCC *S. aureus* (25922)	Mean±SD Std. error	0.425±0.042 0.02	1.100±0.208 0.12	0.511±0.007 0.004	0.430±0.059 0.034
C. albicans BK239	Mean±SD Std. error	0.239±0.037 0.0217	0.320±0.023 0.0133	0.142±0.006 0.003	0.119±0.018 0.010
S. aureus BK79	Mean±SD Std. error	0.159±0.016 0.009	0.365±0.036 0.02	0.110±0.013 0.007	0.112±0.003 0.001
S. epidermidis BK155	Mean±SD Std. error	0.116±0.016 0.009	0.329±0.063 0.03	0.159±0.082 0.047	0.070±0.014 0.008
S. saprophyticus BK134	Mean±SD Std. error	0.151±0.014 0.008	0.189±0.017 0.01	0.852±0.030 0.01	1.101±0.061 0.035
C.albicans BK239+*S. aureus* BK79	Mean±SD Std. error	0.190±0.027 0.015	0.283±0.136 0.078	0.754±0.066 0.038	0.762±0.029 0.01
C. albicans BK239+ *S. epidermidis* BK155	Mean±SD Std. error	0.299±0.017 0.009	0.506±0.110 0.064	0.293±0.019 0.01	0.178±0.001 0.0005
C. albicans BK239+ *S. saprophytics* BK134	Mean±SD Std. error	0.243±0.014 0.008	0.369±0.041 0.02	0.315±0.006 0.003	0.203±0.098 0.05
Blank	Mean±SD Std. error	0.083±0.005 0.003	0.168±0.001 0.0005	0.063±0.003 0.001	0.059±0.002 0.001

Table 7.5b: Detection of biofilm formation **(**CV assay) and metabolic activity (XTT reduction assay) expressed by non-albicans *Candida-Staphylococcus* mono and dual-species biofilm combinations. All experiments were performed in triplicates, mean ODs and standard deviation from the mean ODs was calculated after 24 h and 48 h of incubation

***C. albicans-Staphylococcus* species combinations**		**CV assay ODs after 24 h**	**CV assay ODs after 48 h**	**XTT assay ODs after 24 h**	**XTT assay ODs after 48 h**
C. glabrata BK214	Mean±SD Std. error	0.165±0.016 0.009	0.390±0.092 0.053	0.105±0.002 0.0011	0.112±0.011 0.006
C. krusei BK229	Mean±SD Std. error	0.534±0.026 0.015	0.971±0.188 0.108	0.403±0.054 0.031	0.338±0.035 0.020
C. glabrata BK214+ *S. aureus* BK79	Mean±SD Std. error	0.190±0.027 0.015	0.283±0.136 0.078	0.107±0.009 0.005	0.125±0.005 0.003
C. glabrata BK214+ *S. epidermidis* BK155	Mean±SD Std. error	0.299±0.017 0.009	0.506±0.110 0.064	0.116±0.016 0.009	0.115±0.017 0.009
C. glabrata BK214+ *S. saprophytics* BK134	Mean±SD Std. error	0.243±0.014 0.008	0.369±0.041 0.02	0.292±0.04 0.02	0.238±0.103 0.059
C. krusei BK229+ *S. aureus* BK79	Mean±SD Std. error	0.190±0.027 0.015	0.283±0.136 0.078	0.459±0.077 0.04	0.357±0.023 0.013
C. krusei BK229+ *S. epidermidis* BK155	Mean±SD Std. error	0.299±0.017 0.009	0.506±0.110 0.064	0.443±0.001 0.0008	0.366±0.045 0.02
C. krusei BK229+ *S. saprophytics* BK134	Mean±SD Std. error	0.243±0.014 0.008	0.369±0.041 0.02	0.370±0.004 0.002	0.353±0.045 0.02
Blank	Mean±SD Std. error	0.083±0.005 0.003	0.168±0.001 0.0005	0.063±0.003 0.001	0.059±0.002 0.001

7.3.2.2 Total protein estimation and aspartyl proteinase activity of *Candida-Staphylococcus* biofilms

In comparison to 24 h, protein content was reduced in 48 h ATCC isolates in both mono and dual-species biofilm. Comparatively, an increase in protein content was observed in dual-species biofilm of ATCC *C. albicans* and ATCC *S. aureus* isolates than mono-species biofilms (4.908±0.207 after 24 h and 4.793±0.109 after 48 h). *C. albicans* BK239-*Staphylococcus* species biofilms showed increased protein content after 24 h, however, reduction was seen in *C. albicans* BK239-*S. epidermidis* and *S. saprophyticus* dual-species biofilm combinations after 48 h of incubation (Table 7.6a, Figure 7.11). All other dual-species biofilm of *Candida* with *Staphylococcus* species showed increase in protein content after 24 h of incubation. Both *C. glabrata* BK214 and *C. krusei* dual-species biofilm with *S. epidermidis* BK155 and *S. saprophyticus* BK134 showed reduction in protein content (Table 7.6b, Figure 7.12 and 7.13). ANOVA analysis indicates significant change in mono and dual-species cultures for protein content after 24 h of incubation (Table 7.7).

Higher aspartyl proteinase activity was shown by ATCC *C. albicans* 90029 (OD=0.247±0.012) and local isolate of *C. albicans* CA239 (OD=0.147±0.010) after 24 h of incubation, which was reduced after 48 h of incubation. All other isolates showed increased in enzyme activity after 48 h of incubation. In dual-species biofilm, both ATCC and clinical isolate of *Candida* showed markedly decrease activity with all *Staphylococcus* isolates compared to when it was cultured alone. ANOVA analysis showed significant change in aspartyl proteinase activity after 24 h incubation for mono and dual-species biofilm (Table 7.6a and 7.6b, Figure 7.14).

Table 7.6a: Estimation of total protein concentration and aspartyl proteinase activity displayed by *C. albicans-Staphylococcus* mono and dual-species biofilm combinations after 24 h and 48 h of incubation (in triplicates). Mean concentration and SD from mean concentration was calculated for total protein estimation by Lowery method and for aspartyl proteinase activity, mean ODs and SD from the mean ODs was calculated

***C. albicans-Staphylococcus* species combinations**		**Protein concentration after 24 h (µg/mL)**	**Protein concentration after 48 h (µg/mL)**	**ODs of aspartyl proteinase activity after 24 h**	**ODs of aspartyl proteinase activity after 48 h**
ATCC C. *albicans* (90029)	Mean±SD Std. error	3.461±0.429 0.24	2.28±0.33 0.19	0.247±0.012 0.007	0.120±0.003 0.001
ATCC *S. aureus* (25922)	Mean±SD Std. error	4.890±0.283 0.16	4.604±0.387 0.22	-	-
ATCC *C. albicans* (90029) +ATCC *S. aureus* (25922)	Mean±SD Std. error	4.908±0.207 0.119	4.793±0.109 0.063	0.062±0.023 0.013	0.072±0.017 0.009
C. albicans BK239	Mean±SD Std. error	3.048±0.585 0.338	3.97±1.30 0.75	0.147±0.010 0.006	0.125±0.008 0.005
S. aureus BK79	Mean±SD Std. error	4.252±0.423 0.244	4.297±0.329 0.190	-	-
S. epidermidis BK155	Mean±SD Std. error	5.024±0.039 0.02	4.957±0.00 0.00	-	-
S. saprophyticus BK134	Mean±SD Std. error	4.890±0.236 0.136	4.738±0.096 0.05	-	-
C. albicans BK239+ *S. aureus* BK79	Mean±SD Std. error	4.715±0.059 0.03	4.502±0.787 0.454	0.070±0.019 0.01	0.093±0.003 0.002
C. albicans BK239+ *S. epidermidis* BK155	Mean±SD Std. error	4.465±0.462 0.267	2.814±0.881 0.50	0.057±0.018 0.01	0.084±0.004 0.002
C. albicans BK239+*S. saprophyticus* BK134	Mean±SD Std. error	4.543±0.321 0.18	2.171±0.607 0.350	0.063±0.011 0.006	0.091±0.018 0.01

Table 7.6b: Estimation of total protein concentration and aspartyl proteinase activity displayed by non albicans *Candida-Staphylococcus* mono and dual-species biofilm combinations after 24 h and 48 h of incubation (in triplicates). Mean concentration and SD from mean concentration was calculated for total protein estimation by Lowery method and for aspartyl proteinase activity, mean ODs and SD from the mean ODs was calculated

***C. albicans*-*Staphylococcus* species combinations**		**Protein concentration after 24 h (µg/mL)**	**Protein concentration after 48 h (µg/mL)**	**ODs of aspartyl proteinase activity after 24 h**	**ODs of aspartyl proteinase activity after 48 h**
C. glabrata BK214	Mean±SD Std. error	2.925±0.29 0.172	4.31±0.362 0.2	0.1111±0.005 0.003	0.131±0.01 0.005
C. krusei BK229	Mean±SD Std. error	2.85±0.19 0.10	4.39±0.89 0.51	0.097±0.005 0.003	0.113±0.008 0.005
C. glabrata BK214+*S. aureus* BK79	Mean±SD Std. error	4.470±0.793 0.45	4.643±0.449 0.25	0.019±0.008 0.004	0.129±0.037 0.02
C. glabrata BK214+*S. epidermidis* BK155	Mean±SD Std. error	4.494±0.391 0.22	4.119±0.264 0.15	0.054±0.007 0.004	0.090±0.008 0.004
C. glabrata BK214+*S. saprophyticus* BK134	Mean±SD Std. error	3.756±1.374 0.79	3.609±0.126 0.07	0.072±0.022 0.01	0.080±0.005 0.002
C. krusei BK229+*S. aureus* BK79	Mean±SD Std. error	3.953±1.522 0.87	4.657±0.255 0.14	0.053±0.014 0.008	0.068±0.006 0.003
C. krusei BK229+*S. epidermidis* BK155	Mean±SD Std. error	4.873±0.068 0.03	2.615±0.801 0.46	0.069±0.013 0.007	0.096±0.010 0.005
C. krusei BK229+*S. saprophytics* BK134	Mean±SD Std. error	4.565±0.618 0.35	3.412±0.471 0.27	0.072±0.028 0.016	0.109±0.021 0.012

Table 7.7: Estimation of statistically significant change between and within groups (combinations) in biofilm formation, metabolic activity, total protein concentration and aspartyl proteinase activity by *Candida-Staphylococcus* mono and dual-species combinations after 24 h and 48 h of incubation (in triplicates) using ANOVA

Assays		**DF***	**F**	***P* value**
CV assay ODs after 24 h (492nm)	Between groups Within groups	18 38	93.355	<0.001^{+}
CV assay ODs after 48 h (492nm)	Between groups Within groups	18 38	26.381	<0.001^{+}
XTT reduction assay ODs after 24 h (492nm)	Between groups Within groups	18 38	94.654	<0.001^{+}
XTT reduction assay ODs after 48 h (492nm)	Between groups Within groups	18 38	110.400	<0.001^{+}
Total protein concentration after 24 h (µg/mL)	Between groups Within groups	18 38	8.152	0.016^{+}
Total protein concentration after 48 h (µg/mL)	Between groups Within groups	18 38	14.744	0.348
Aspartyl proteinase activity ODs after 24 h (280nm)	Between groups Within groups	17 36	25.548	<0.001^{+}
Aspartyl proteinase activity ODs after 48 h (280nm)	Between groups Within groups	17 36	0.879	0.601

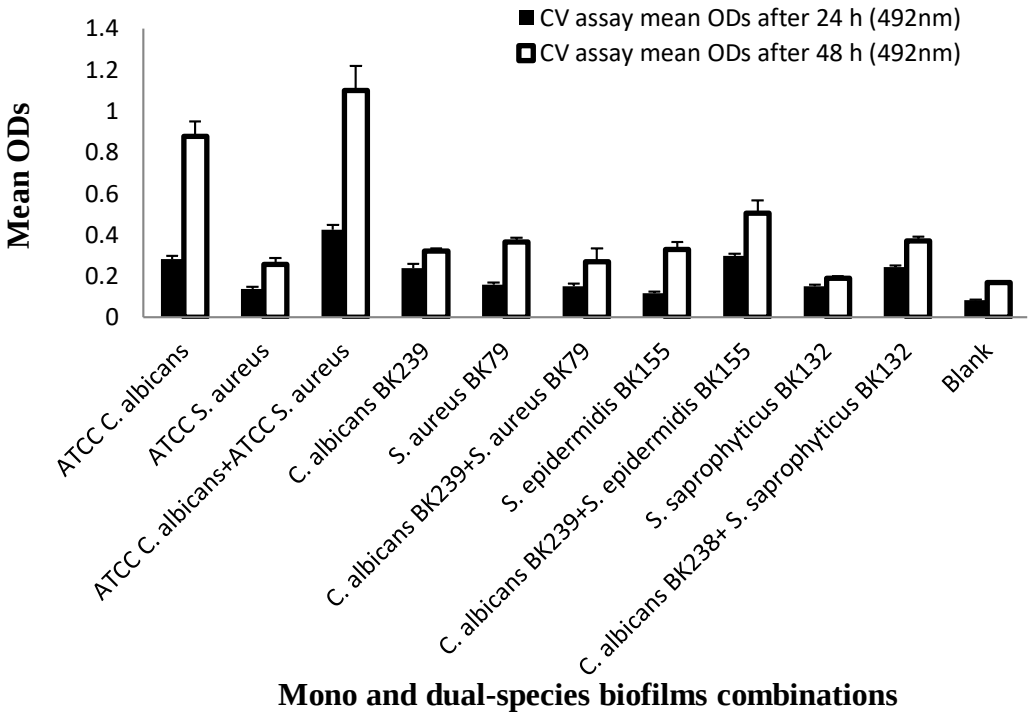

Figure 7.5: Comparison of biofilm biomass production by CV assay for *C. albicans-Staphylococcus* mono and dual-species combinations after 24 h and 48 h of incubation

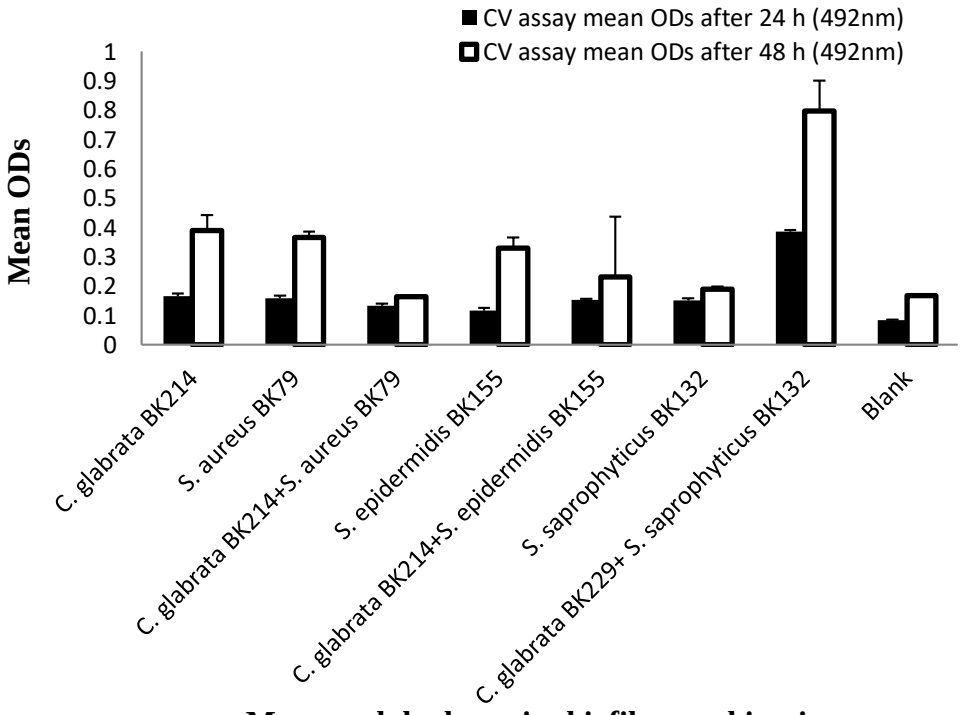

Figure 7.6: Comparison of biofilm biomass production by CV assay for *C. glabrata-Staphylococcus* mono and dual-species combinations after 24 h and 48 h of incubation

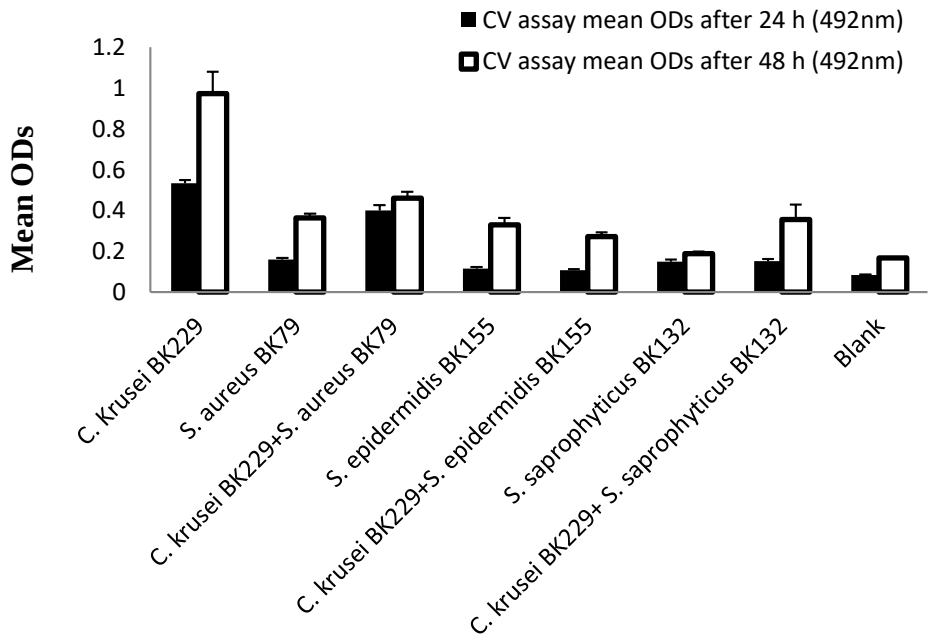

Figure 7.7: Comparison of biofilm biomass production by CV assay for *C. krusei-Staphylococcus* mono and dual-species combinations after 24 h and 48 h of incubation

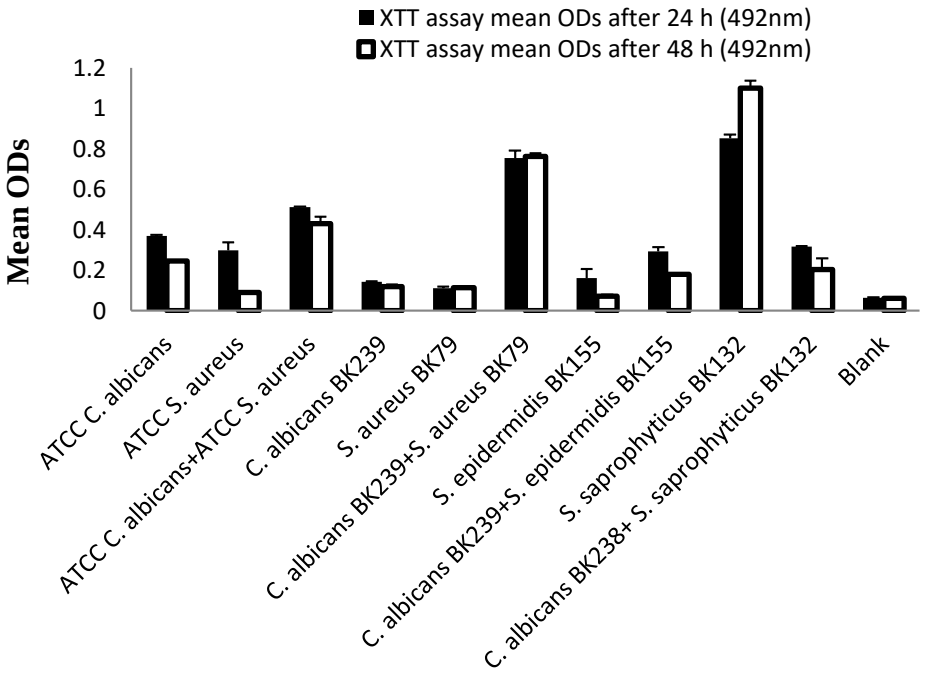

Figure 7.8: Comparison of metabolic activity by XTT assay expressed by *C. albicans-Staphylococcus* mono and dual-species combinations in developed biofilms after 24 h and 48 h of incubation

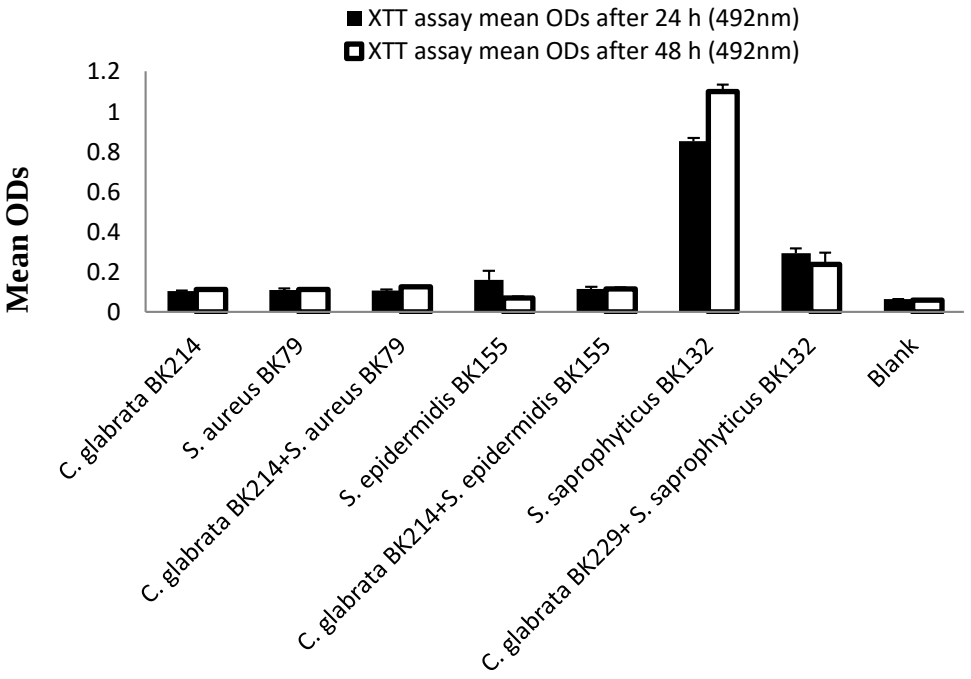

Figure 7.9: Comparison of metabolic activity by XTT assay expressed by *C. glabrata-Staphylococcus* mono and dual-species combinations in developed biofilms after 24 h and 48 h of incubation

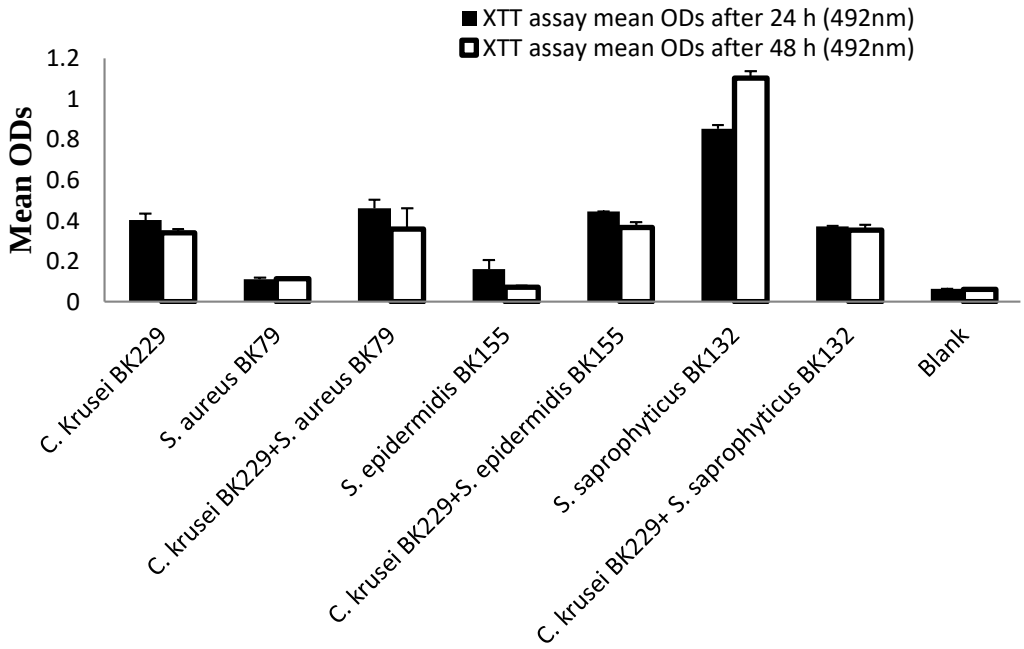

Figure 7.10: Comparison of metabolic activity by XTT assay expressed by *C. krusei-Staphylococcus* mono and dual-species combinations in developed biofilms after 24 h and 48 h of incubation

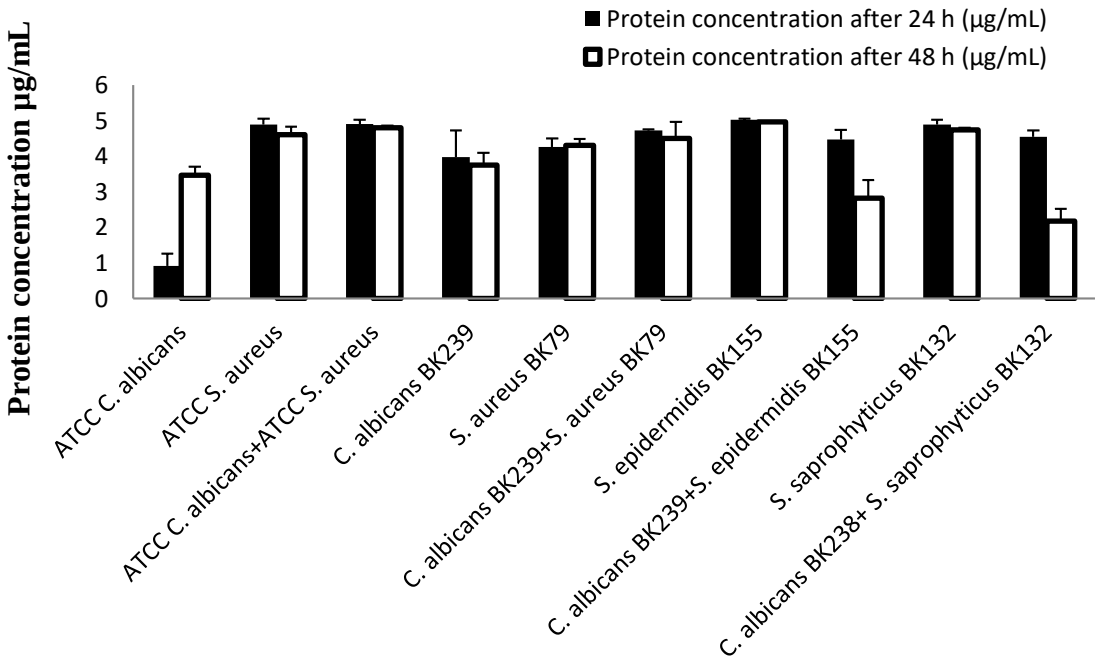

Figure 7.11: Total protein estimation by Lowery method for *C. albicans-Staphylococcus* mono and dual-species combinations in developed biofilms after 24 h and 48 h of incubation

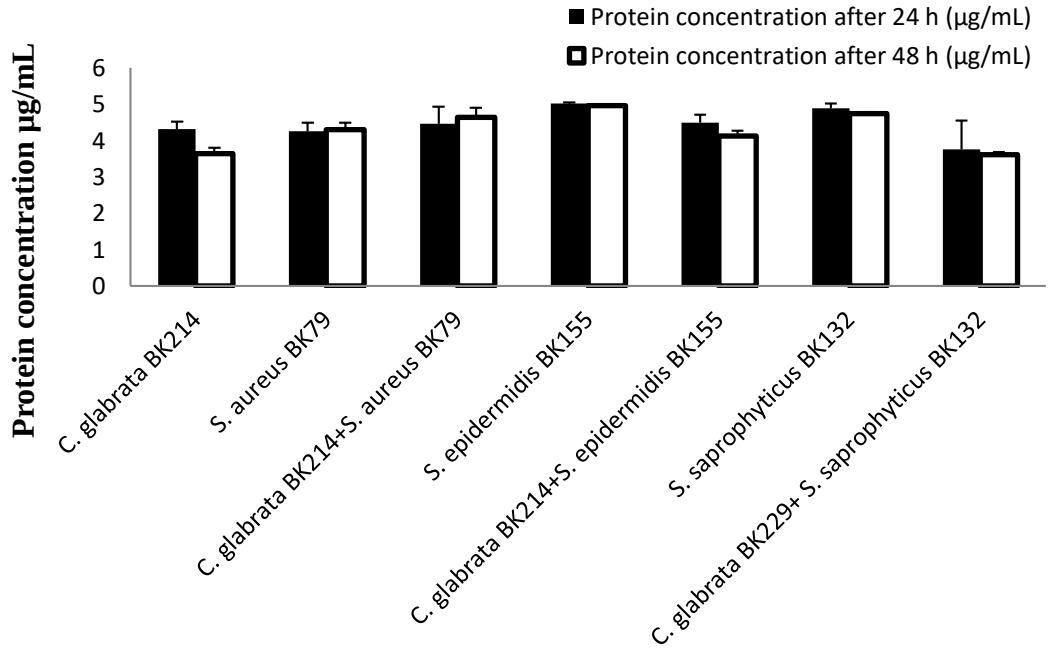

Figure 7.12: Total protein estimation by Lowery method for *C. glabrata-Staphylococcus* mono and dual-species combinations in developed biofilms after 24 h and 48 h of incubation

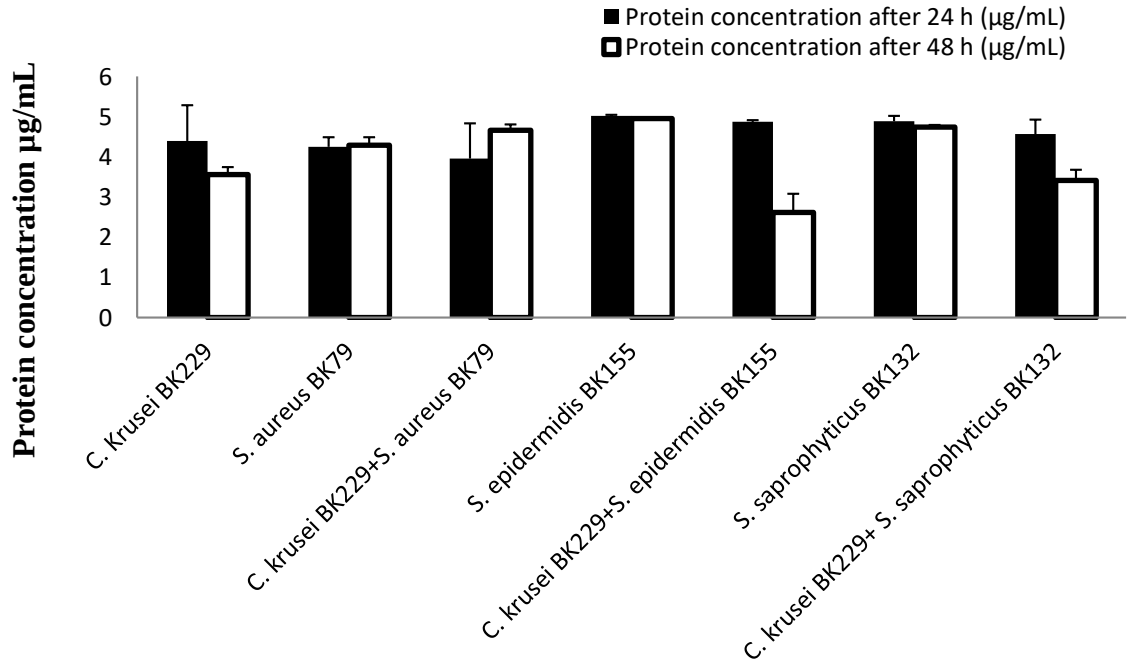

Figure 7.13: Total protein estimation by Lowery method for *C. krusei-Staphylococcus* mono and dual-species combinations in developed biofilms after 24 h and 48 h of incubation

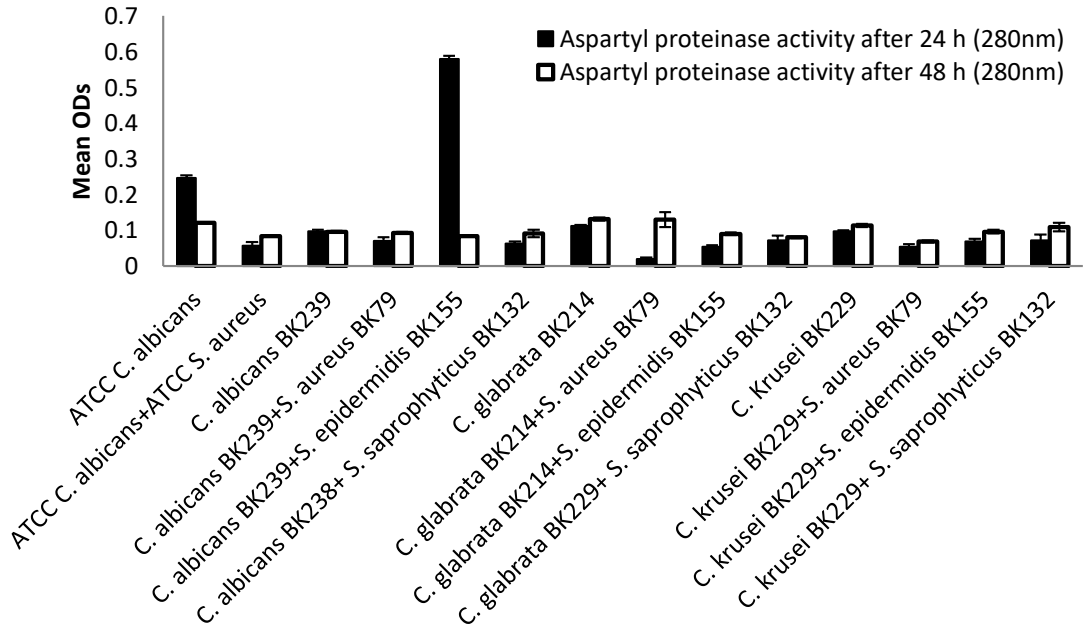

Figure 7.14: Comparison of aspartyl proteinase activity expressed by *Candida* in mono and *Candida-Staphylococcus* dual-species combinations in developed biofilms after 24 h and 48 h of incubation

7.3.3- *Candida-K. pneumoniae* interactions in dual-species biofilm

7.3.3.1- CV biofilm biomass and XTT reduction assays for *Candida-K. pneumoniae* biofilms

CV assay results showed that ATCC *C. albican*s (90029) showed reduction in biofilm biomass when it was co-cultured with ATCC *K. pneumonia* 700603 (OD=0.191±0.028 after 24 h and OD=0.465±0.027 after 48 h). Next dual-species assay of *Candida* species [*C. albicans* (BK239), *C. glabrata* (BK214) and *C. krusei* (BK229)] with *K. pneumoniae* (BK166) isolated from saliva samples were performed. *C. albicans* BK239 showed reduction in biofilm biomass in dual-species biofilm after 24 h of incubation, however, both *C. albicans* BK239 and *K. pneumoniae* BK166 showed slight increase in biomass after 48 h of incubation (Table 7.8, Figure 7.15). *C. glabrata* BK214 was not much affected in dual-species biofilm after 24 h of incubation, however, biomass was greatly reduced for both isolates after 48 h of incubation. *C. krusei* BK229 was also not much affected in dual-species biofilm after 24 h of incubation, however, biomass was greatly enhanced after 48 h of incubation (OD=0.971±0.188 to 1.088±0.260) (Table 7.8, Figure 7.16). One-way ANOVA analysis showed significant change in mono and dual-species biofilm after 24 h and 48 h CV assay (Table 7.10).

XTT reduction assay showed overall decreasing trend for metabolic activity after 48 h of incubation compared to 24 h. Metabolic activity of ATCC *C. albicans* was unaffected after 24 h and 48 h of incubation in dual-species biofilm compared to mono-specie. ATCC *K. pneumoniae* showed reduction in metabolic activity after 24 h incubation in dual-species biofilm (OD=0.407±0.040 to OD=0.369±0.034). Metabolic activity of *C. albicans* (CA239) was increased after 24 h and 48 h of incubation in dual-species biofilm compared to mono-specie (Table 7.8, Figure 7.17). Metabolic activity of *C. glabrata* BK214 was not much affected in dual-species biofilm, however, activity of *K. pneumoniae* BK166 was reduced in dual-species biofilm. *C. krusei* BK229 showed increased metabolic rate after 24 h of incubation (OD=0.403±0.054 to OD=0.445±0.010), however, it showed decrease in activity in dual-species biofilm with *K. pneumoniae* BK166 (Table 7.8, Figure 7.18). ANOVA analysis showed significant changed in metabolic activity in mono and dual-species biofilm combinations for 24 h and 48 h XTT reduction assay (Table 7.10).

7.3.3.2 Total protein estimation and aspartyl proteinase activity of *Candida-K. pneumoniae* biofilms

In dual-species biofilm of ATCC strains, a slight increase in total protein estimation was seen compared to mono-specie biofilms after 24 h and 48 h of incubation (4.613±0.323 after 24 h and 3.239±0.207 after 48 h). *C. albicans* BK239-*K. pneumoniae* BK166 biofilms showed increased protein content after 24 h incubation (3.463±0.335), while slight increase was seen after 48 h of incubation. In *C. glabrata* BK214-*K. pneumoniae* BK166 biofilms protein content was reduced in dual-species consortia (2.940±0.670). For *C. krusei* BK229-*K. pneumoniae* BK166 protein content was increased in dual-species biofilm after 24 h of incubation, however, content was reduced after 48 h of incubation (3.317±0.523) (Table 7.9, Figure 7.19). ANOVA analysis indicate significant change in mono and dual-species cultures protein content after 24 h of incubation (Table 7.10).

Aspartyl proteinase activity by ATCC *C. albicans* 90029 was reduced in dual-species biofilm with ATCC *K. pneumoniae* 700603 (OD=0.247±0.012 to 0.147±0.010) after 24 h of incubation, while it was not affected after 48 h of incubation in dual-species biofilm. *C. albicans* BK239 and *C. krusei* BK214 showed increased in enzyme activity after 24 h of incubation when co-cultured with *K. pneumoniae* BK166, while decrease was seen after 48 h of incubation. With *C. glabrata* BK214 enzymatic activity was decreased in dual-species biofilm after 24 h and 48 h of incubation (Table 7.9, Figure 7.20). ANOVA analysis showed significant change in aspartyl proteinase activity after 24 h of incubation (Table 7.10).

Table 7.8: Detection of biofilm formation **(**CV assay) and metabolic activity (XTT reduction assay) expressed by *Candida-K. pneumoniae* mono and dual-species biofilm combinations. All experiments were performed in triplicates, mean ODs and standard deviation from the mean ODs was calculated after 24 h and 48 h of incubation

***Candida- K. pneumoniae* mono and dual-species combinations**		**CV assay ODs after 24 h**	**CV assay ODs after 48 h**	**XTT assay ODs after 24 h**	**XTT assay ODs after 48 h**
ATCC *K. pneumoniae* (700603)	Mean±SD Std. error	0.131±0.012 0.006	0.491±0.082 0.047	0.407±0.040 0.023	0.238±0.010 0.006
ATCC *C. albicans* (90029) +ATCC *K. pneumoniae* (700603)	Mean±SD Std. error	0.191±0.028 0.01	0.465±0.027 0.01	0.369±0.034 0.01	0.253±0.008 0.005
K. pneumoniae BK166	Mean±SD Std. error	0.130±0.026 0.01	0.355±0.031 0.01	0.322±0.021 0.01	0.342±0.021 0.01
C. albicans BK239+ *K. pneumoniae* BK166	Mean±SD Std. error	0.147±0.019 0.01	0.359±0.092 0.05	0.327±0.005 0.003	0.301±0.009 0.005
C. glabrata BK214+ *K. pneumoniae* BK166	Mean±SD Std. error	0.165±0.021 0.01	0.273±0.027 0.01	0.115±0.007 0.009	0.155±0.014 0.008
C. krusei BK214+ *K. pneumoniae* BK166	Mean±SD Std. error	0.521±0.039 0.02	1.088±0.260 0.150	0.445±0.010 0.005	0.246±0.005 0.002
Blank	Mean±SD Std. error	0.083±0.005 0.003	0.168±0.001 0.0005	0.063±0.003 0.001	0.0590±0.002 0.001

Table 7.9: Estimation of total protein concentration and aspartyl proteinase activity displayed by *Candida-K. pneumoniae* mono and dual-species biofilm combinations after 24 h and 48 h of incubation (in triplicates). Mean concentration and SD from mean concentration was calculated for total protein estimation by Lowery method and for aspartyl proteinase activity, mean ODs and SD from the mean ODs was calculated

***Candida-K. pneumoniae* mono and dual-species combinations**		**Protein concentration after 24 h (µg/mL)**	**Protein concentration after 48 h (µg/mL)**	**ODs of aspartyl proteinase activity after 24 h**	**ODs of aspartyl proteinase activity after 48 h**
ATCC *K. pneumoniae* (700603)	Mean±SD Std. error	2.014±1.729 0.998	3.407±0.059 0.03	-	-
ATCC *C. albicans* (90029) +ATCC *K. pneumoniae* (700603)	Mean±SD Std. error	4.613±0.323 0.18	3.239±0.207 0.119	0.147±0.010 0.006	0.125±0.008 0.005
K. pneumoniae BK166	Mean±SD Std. error	3.356±0.346 0.20	2.338±1.266 0.731	-	-
C. albicans BK239+ *K. pneumoniae* BK166	Mean±SD Std. error	3.463±0.335 0.19	3.710±0.559 0.32	0.380±0.484 0.27	0.088±0.008 0.004
C. glabrata BK214+ *K. pneumoniae* BK166	Mean±SD Std. error	2.940±0.670 0.38	3.318±0.165 0.09	0.095±0.010 0.006	0.102±0.009 0.005
C. krusei BK214+ *K. pneumoniae* BK166	Mean±SD Std. error	3.674±0.709 0.40	3.317±0.523 0.302	0.119±0.004 0.002	0.108±0.011 0.006

Table 7.10: Estimation of statistically significant change between and within groups (combinations) in biofilm formation, metabolic activity, total protein concentration and aspartyl proteinase activity by *Candida-K. pneumoniae* mono and dual-species combinations after 24 h and 48 h of incubation (in triplicates) using ANOVA

Assays		**DF***	**F**	***P* value**
CV assay ODs after 24 h (492nm)	Between groups Within groups	10 22	110.627	<0.001^{+}
CV assay ODs after 48 h (492nm)	Between groups Within groups	10 22	21.407	<0.001^{+}
XTT reduction assay ODs after 24 h (492nm)	Between groups Within groups	10 22	98.878	<0.001^{+}
XTT reduction assay ODs after 48 h (492nm)	Between groups Within groups	10 22	110.679	<0.001^{+}
Total protein concentration after 24 h (µg/mL)	Between groups Within groups	10 22	7.336	<0.001^{+}
Total protein concentration after 48 h (µg/mL)	Between groups Within groups	10 22	6.478	<0.001^{+}
Aspartyl proteinase activity ODs after 24 h (280nm)	Between groups Within groups	09 20	1.078	0.419
Aspartyl proteinase activity ODs after 48 h (280nm)	Between groups Within groups	09 20	8.972	<0.001^{+}

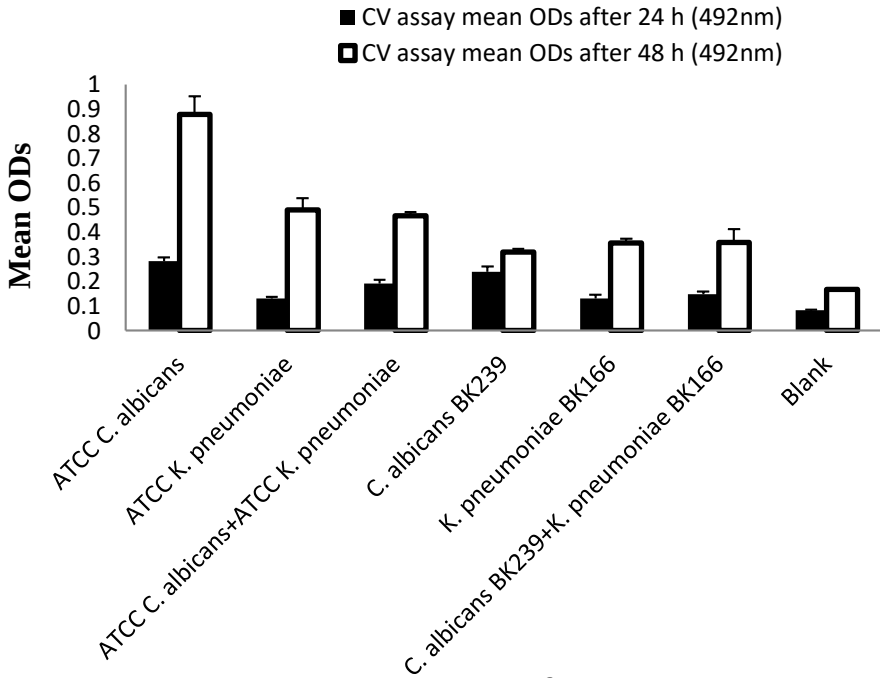

Figure 7.15: Comparison of Biofilm biomass production by CV assay for *C. albicans-K. pneumoniae* mono and dual-species combinations after 24 h and 48 h of incubation

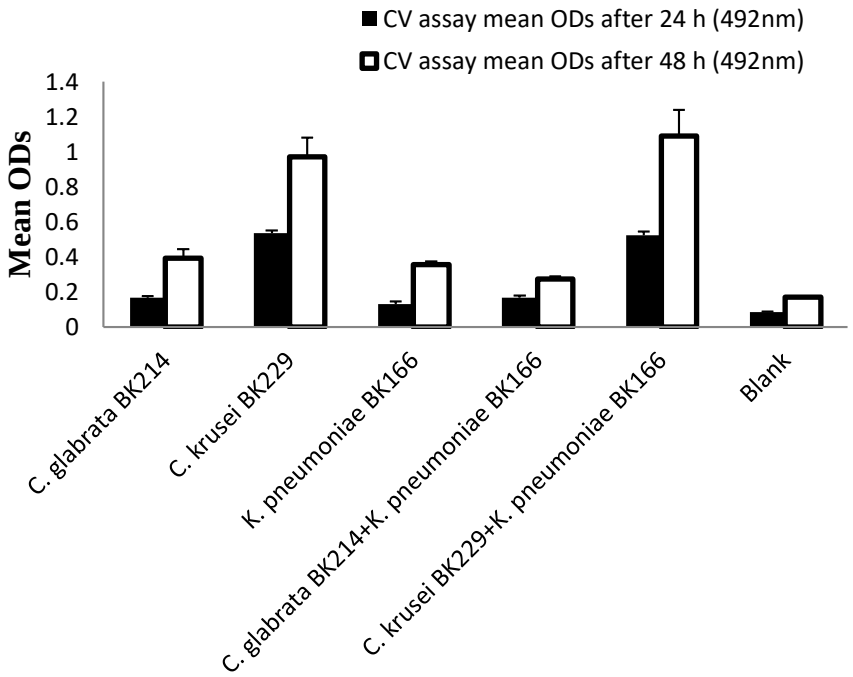

Figure 7.16: Comparison of Biofilm biomass production by CV assay for non-albicans *Candida-K. pneumoniae* mono and dual-species combinations after 24 h and 48 h of incubation

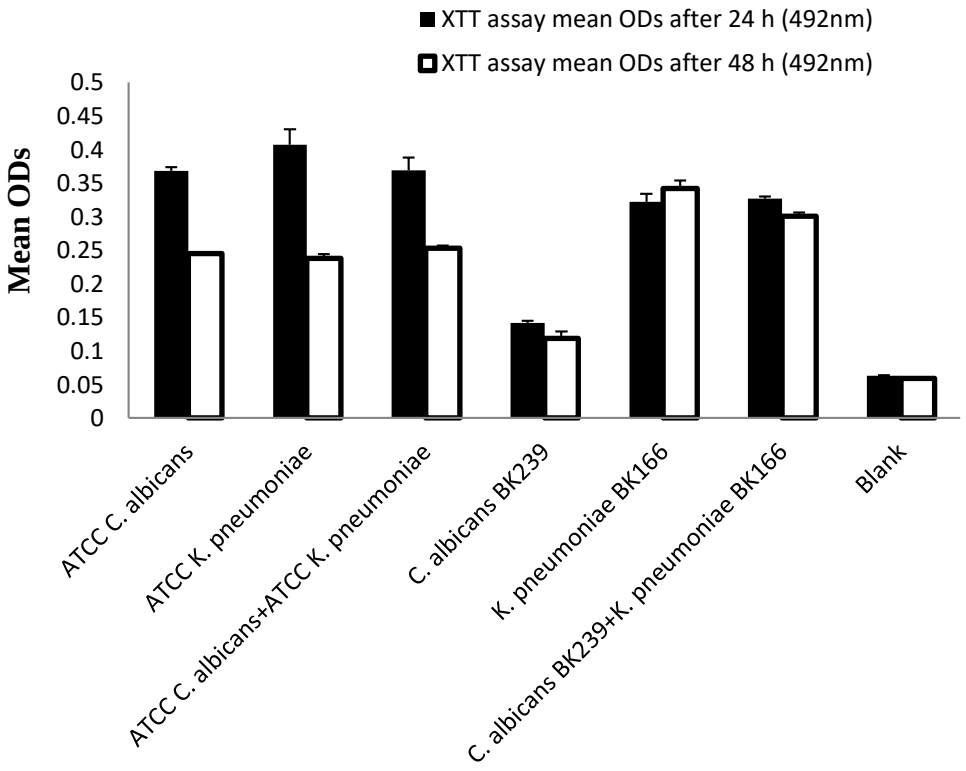

Figure 7.17: Comparison of metabolic activity by XTT assay expressed by *C. albicans-K. pneumoniae* mono and dual-species combinations in developed biofilms after 24 h and 48 h of incubation

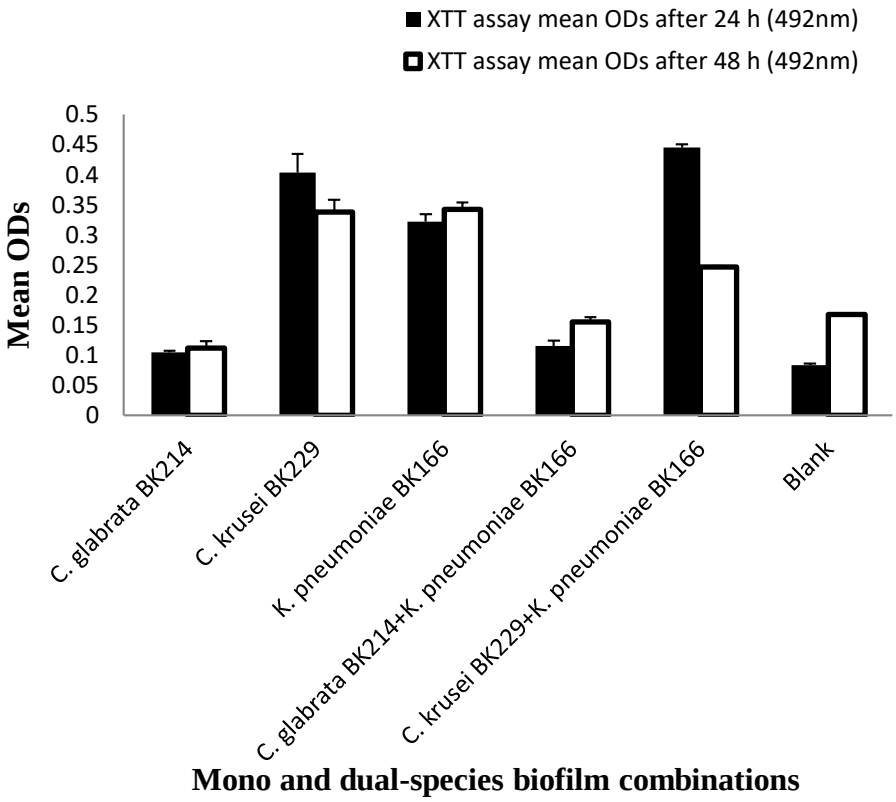

Figure 7.18: Comparison of metabolic activity by XTT assay expressed by non-albicans *Candida-K. pneumoniae* mono and dual-species combinations in developed biofilms after 24 h and 48 h of incubation

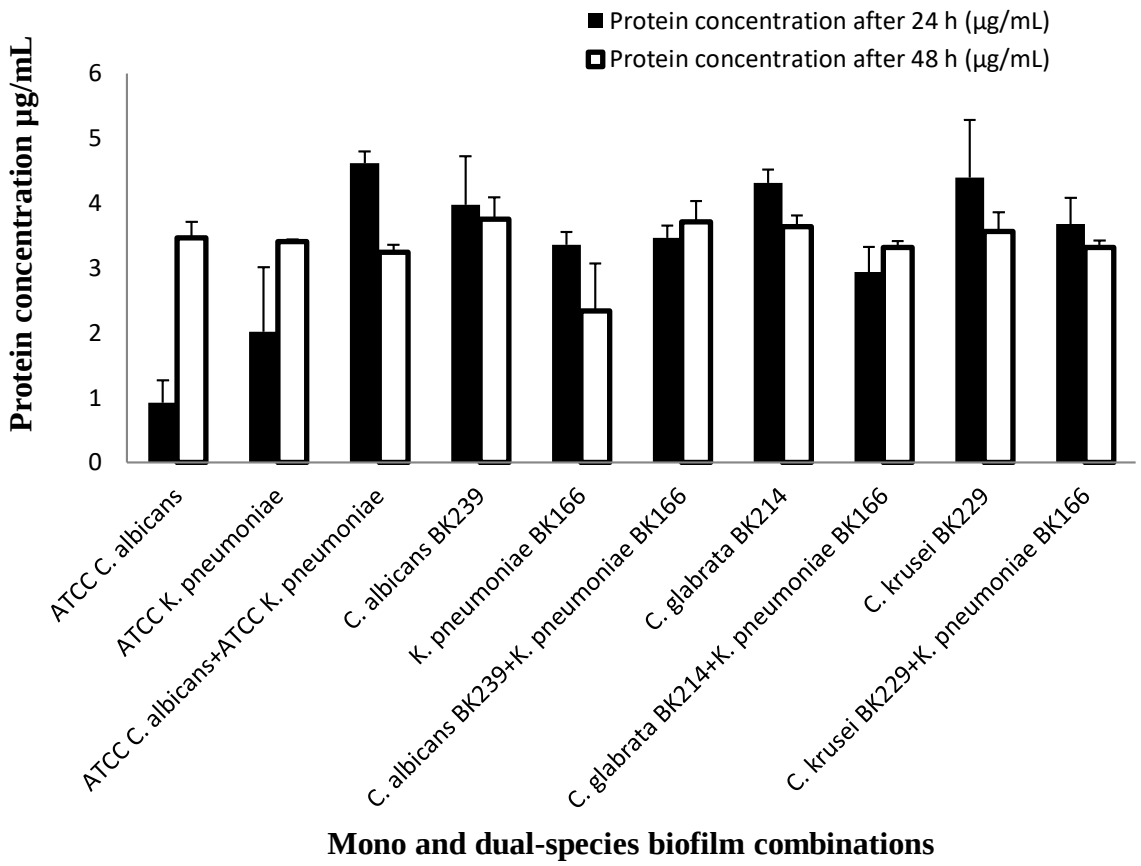

Figure 7.19: Total protein estimation by Lowery method for *Candida-K. pneumoniae* mono and dual-species combinations in developed biofilms after 24 h and 48 h of incubation

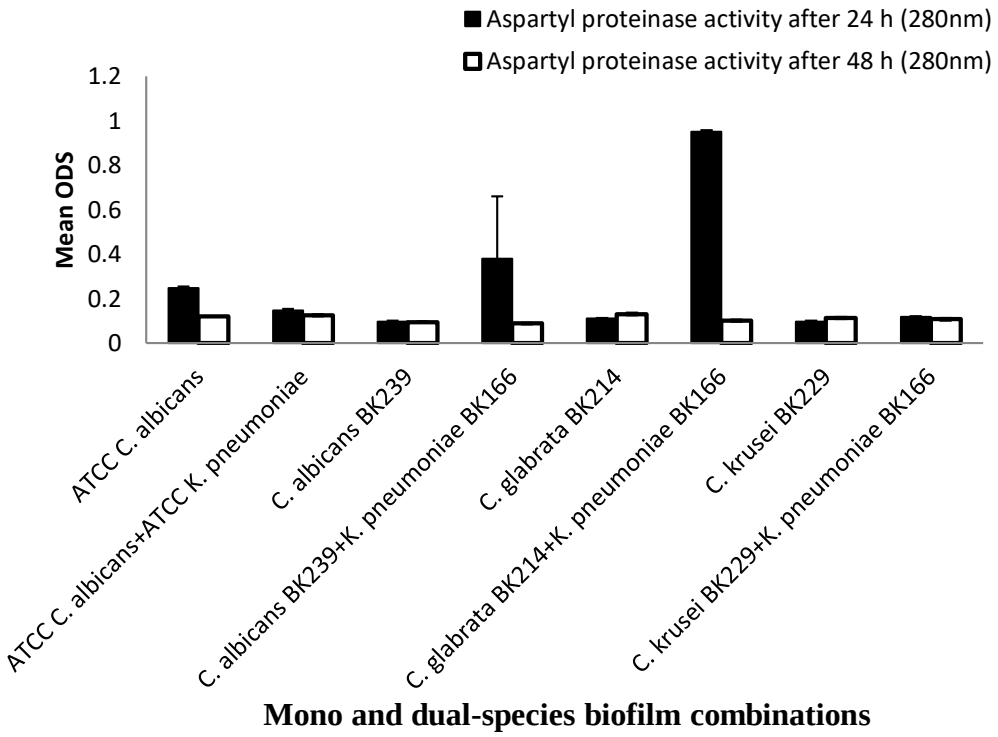

Figure 7.20: Comparison of aspartyl proteinase activity expressed by *Candida* in mono and *Candida- K. pneumoniae* dual-species combinations in developed biofilms after 24 h and 48 h of incubation

7.3.4- *Candida-E. coli* interactions in dual-species biofilm

7.3.4.1- CV biofilm biomass and XTT reduction assays for *Candida-E. coli* biofilms

CV assay results showed that biofilm biomass of ATCC *C. albicans* (90029) after 24 h and 48 h incubation was reduced when it was co-cultured with ATCC *E. coli* 25922 (OD=0.176±0.036 after 24 h and OD=0.451±0.0743 after 48 h). In dual-species biofilm of isolates from postpartum females, all *Candida* species showed reduction in total biofilm biomass as indicated by ODs values, when co-cultured with *E. coli* BK203. Isolate of *C. albicans* BK239 (OD=0.239±0.037 after 24 h and OD=0.320±0.0231 after 48 h) showed slight decrease in its biofilm biomass (OD=0.190±0.027 after 24 h and OD=0.283±0.136 after 48 h) when co-cultured with *E. coli* BK203 (Table 7.11, Figure 7.21). ODs for *C. glabrata* (BK214) were also decreased (from OD=0.165±0.016 after 24 h and OD=0.390±0.092 after 48 h to OD=0.152±0.015 after 24 h and OD=0.241±0.066 after 48 h) in dual-species biofilm. However, for *C. krusei* BK229 marked decrease was seen in ODs in dual-species biofilm (OD=0.231±0.002 after 24 h and OD=0.388±0.006 after 48 h) compared to mono-specie biofilm (OD=0.534±0.026 after 24 h and OD=0.971±0.188 after 48 h) (Table 7.11, Figure 7.22). One-way ANOVA analysis showed significant change in mono and dual-species biofilm biomass production in 24 h and 48 h CV assay (Table 7.13).

XTT assay showed overall decreasing metabolic activity after 48 h of incubation compared to 24 h. Metabolic activity of ATCC *C. albicans* (90029) was unaffected after 24 h of incubation in dual-species biofilm compared to mono-specie, however, increased was seen after 48 h of incubation in dual-species biofilm (OD=0.245±0.003 to OD=0.3703±0.005 after 48 h). ATCC *E. coli* (25922) showed decreased metabolic activity in dual-species biofilms after both 24 h and 48 h of incubation. Metabolic activity of *C. albicans* (BK239) was unaffected after 24 h and 48 h of incubation in dual-species biofilm compared to mono-specie, while like ATCC strain, local isolate of *E. coli* (BK203) also showed marked decrease in metabolic rate in dual-species

biofilm compared to mono-specie biofilms with *C. albicans* (BK239) and slight decrease with *C. krusei* (BK229) (Figure 7.23 and 7.24). With *C. glabrata* BK214, *E. coli* BK203 showed decreased metabolic activity after 48 h of incubation (Figure 7.24). Overall, decreasing metabolic activity trend was seen for *E. coli* BK203 (Table 7.11). ANOVA analysis showed significant change in mono and dual-species biofilm for 24 h and 48 h XTT reduction assay (Table 7.13).

7.3.4.2- Total protein estimation and aspartyl proteinase activity of *Candida-E. coli* biofilms

Comparatively increased protein content was seen in developed biofilms after 48 h of incubation compared to 24 h except for ATCC strains, which showed decrease after 48 h of incubation. In dual-species biofilm of ATCC strain slight increase in total protein estimation was seen compared to mono-specie biofilms after 24 h and 48 h of incubation. However, for all other isolate's protein concentration decreased in dual-species biofilm compared to mono-species biofilm (Table 7.12, Figure 7.25). ANOVA analysis indicate significant change in mono and dual-species cultures protein content after 24 h of incubation (Table 7.13).

In dual-species biofilm, both ATCC and local isolate of *C. albicans* BK239 showed decrease aspartyl proteinase activity (ODs=0.131±0.006 at 24 h to 0.124±0.006 at 48 h and ODs=0.1094±0.020 at 24 h to 0.1095±0.009 at 48 h, respectively) compared to when they were cultured alone. Among other isolates activity in *C. glabrata* BK214 and *C. krusei* BK229 were changed slightly in dual-species biofilm compared to single-specie biofilms after 24 h and 48 h of incubation. After 48 h incubation both ATCC and local isolate of *C. albicans* BK239 showed decreased activity compared to 24 h. Both *C. glabrata* BK214 and *C. krusei* BK229 in a mono and dual-species biofilm showed slight increased enzyme activity after 48 h of incubation (Table 7.12, Figure 7.26). ANOVA analysis showed significant change after 24 h and 48 h of incubation for mono and dual-species biofilm aspartyl proteinase activity (Table 7.13).

Table 7.11: Detection of biofilm formation **(**CV assay) and metabolic activity (XTT reduction assay) expressed by *Candida-E. coli* mono and dual-species biofilm combinations. All experiments were performed in triplicates, mean ODs and standard deviation from the mean ODs was calculated after 24 h and 48 h of incubation

***Candida-E. coli* mono and dual-species combinations**		**CV assay ODs after 24 h**	**CV assay ODs after 48 h**	**XTT assay ODs after 24 h**	**XTT assay ODs after 48 h**
ATCC *E. coli* (25922)	Mean±SD Std. error	0.154±0.007 0.004	0.723±0.067 0.039	0.409±0.014 0.008	0.394±0.14 0.008
ATCC *C. albicans* (90029) +ATCC *E. coli* (25922)	Mean±SD Std. error	0.176±0.036 0.021	0.451±0.074 0.042	0.382±0.01 0.006	0.370±0.005 0.003
E. coli BK203	Mean±SD Std. error	0.112±0.018 0.010	0.212±0.047 0.027	0.502±0.001 0.0005	0.605±0.018 0.01
C. albicans BK239+*E. coli* BK203	Mean±SD Std. error	0.190±0.027 0.015	0.283±0.136 0.078	0.167±0.022 0.012	0.113±0.021 0.012
C. glabrata BK214+*E. coli* BK203	Mean±SD Std. error	0.152±0.026 0.015	0.241±0.066 0.038	0.584±0.047 0.027	0.530±0.018 0.01
C. krusei BK214+*E. coli* BC203	Mean±SD Std. error	0.231±0.002 0.001	0.388±0.006 0.003	0.430±0.005 0.002	0.357±0.009 0.005
Blank	Mean±SD Std. error	0.083±0.005 0.003	0.168±0.001 0.0005	0.063±0.003 0.001	0.0590±0.002 0.001

Table 7.12: Estimation of total protein concentration and aspartyl proteinase activity displayed by *Candida-E. coli* mono and dual-species biofilm combinations after 24 h and 48 h of incubation (in triplicates). Mean concentration and SD from mean concentration was calculated for total protein estimation by Lowery method and for aspartyl proteinase activity, mean ODs and SD from the mean ODs was calculated

***Candida-E. coli* mono and dual-species combinations**		**Protein concentration after 24 h (µg/mL)**	**Protein concentration after 48 h (µg/mL)**	**ODs of aspartyl proteinase activity after 24 h**	**ODs of aspartyl proteinase activity after 48 h**
ATCC *E. coli* (25922)	Mean±SD Std. error	3.461±0.429 0.248	2.28±0.33 0.19	-	-
ATCC *C. albicans* (90029) +ATCC *E. coli* (25922)	Mean±SD Std. error	3.139±0.445 0.257	2.820±1.61 0.929	0.1316±0.006 0.003	0.124±0.006 0.003
E. coli BK203	Mean±SD Std. error	3.760±0.90 0.521	3.357±1.25 0.725	-	-
C. albicans BK239+ *E. coli* BK203	Mean±SD Std. error	2.599±0.52 0.3	3.72±1.0 0.61	0.1094±0.006 0.003	0.1095±0.009 0.004
C. glabrata BK214+ *E. coli* BK203	Mean±SD Std. error	2.86±0.2 0.16	3.31±1.4 0.82	0.100±0.047 0.027	0.118±0.007 0.01
C. krusei BK214+ *E. coli*	Mean±SD Std. error	1.94±0.38 0.22	3.48±1.4 0.8	0.113±0.009 0.005	0.102±0.005 0.003

BC203					

Table 7.13: Estimation of statistically significant change between and within groups (combinations) in biofilm formation, metabolic activity, total protein concentration and aspartyl proteinase activity by *Candida-E. coli* mono and dual-species combinations after 24 h and 48 h of incubation (in triplicates) using ANOVA

Assays		**DF***	**F**	***P* value**
CV assay ODs after 24 h (492nm)	Between groups Within groups	10 22	77.247	<0.001^{+}
CV assay ODs after 48 h (492nm)	Between groups Within groups	10 22	25.955	<0.001^{+}
XTT reduction assay ODs after 24 h (492nm)	Between groups Within groups	10 22	161.385	<0.001^{+}
XTT reduction assay ODs after 48 h (492nm)	Between groups Within groups	10 22	342.831	<0.001^{+}
Total protein concentration after 24 h (µg/mL)	Between groups Within groups	9 20	2.688	0.031^{+}
Total protein concentration after 48 h (µg/mL)	Between groups Within groups	9 20	3.119	0.016^{+}
Aspartyl proteinase activity ODs after 24 h (280nm)	Between groups Within groups	7 16	62.090	<0.001^{+}
Aspartyl proteinase activity ODs after 48 h (280nm)	Between groups Within groups	7 16	4.411	<0.001^{+}

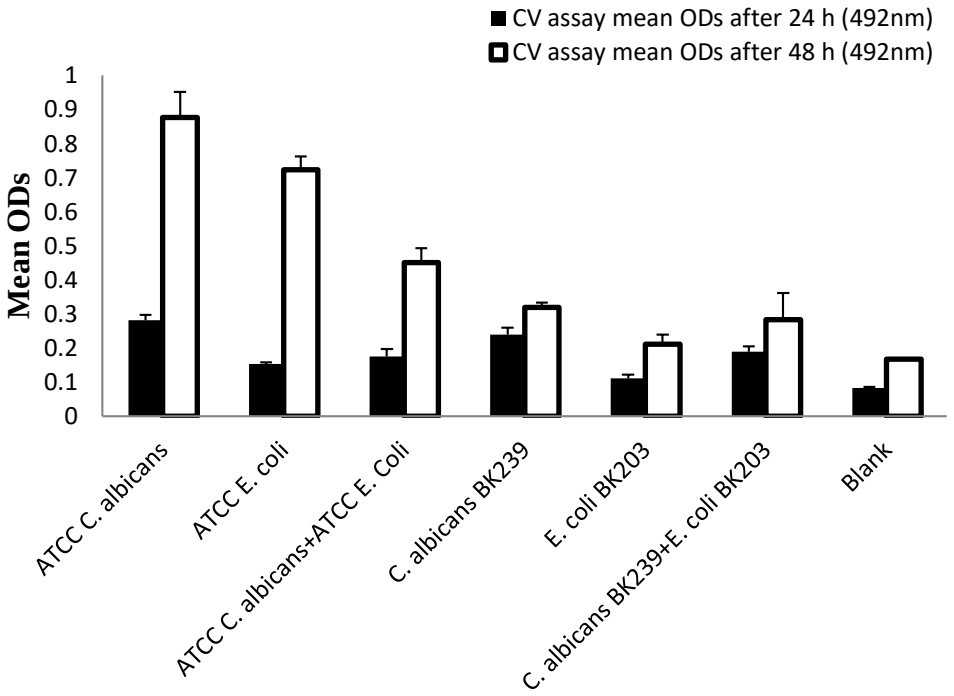

Figure 7.21: Comparison of biofilm biomass production by CV assay for *C. albicans*-*E. coli* mono and dual-species combinations after 24 h and 48 h of incubation

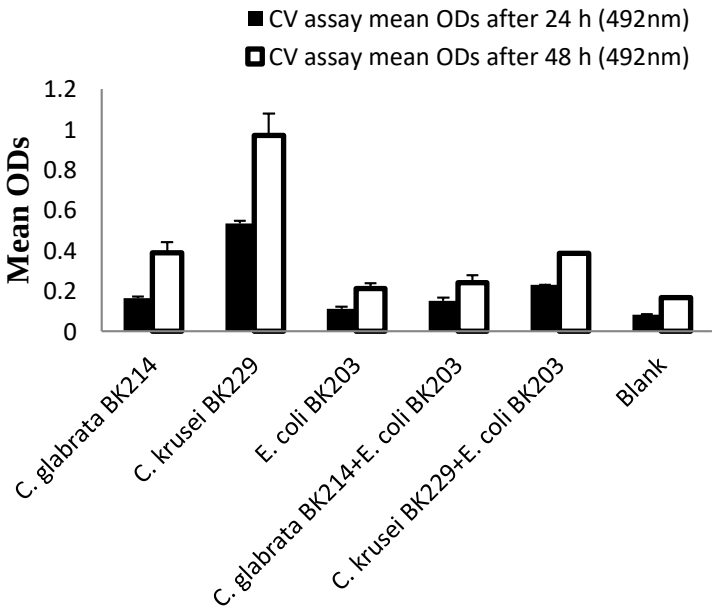

Figure 7.22: Comparison of biofilm biomass production by CV assay for non-albicans *Candida*-*E. coli* mono and dual-species combinations after 24 h and 48 h of incubation

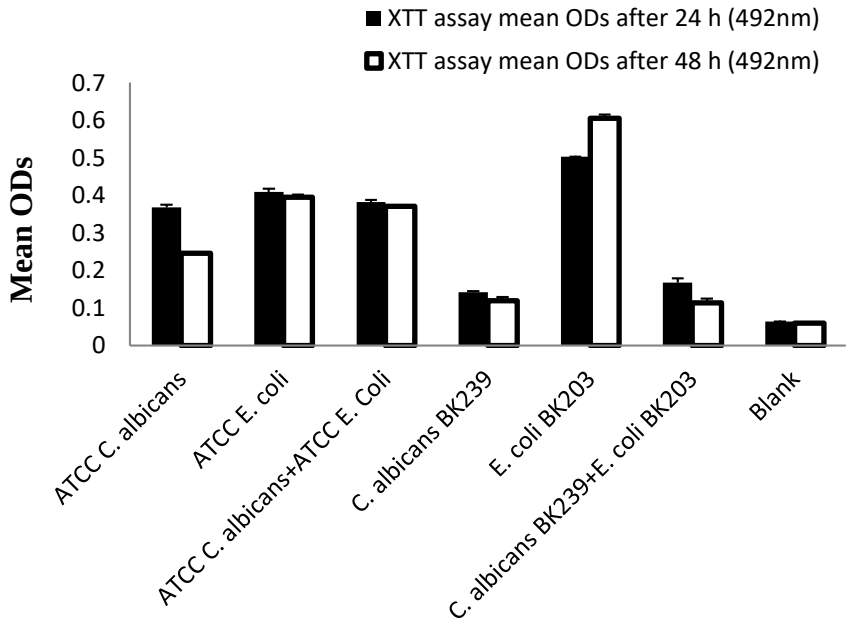

Figure 7.23: Comparison of metabolic activity by XTT assay expressed by *C. albicans-E. coli* mono and dual-species combinations in developed biofilms after 24 h and 48 h of incubation

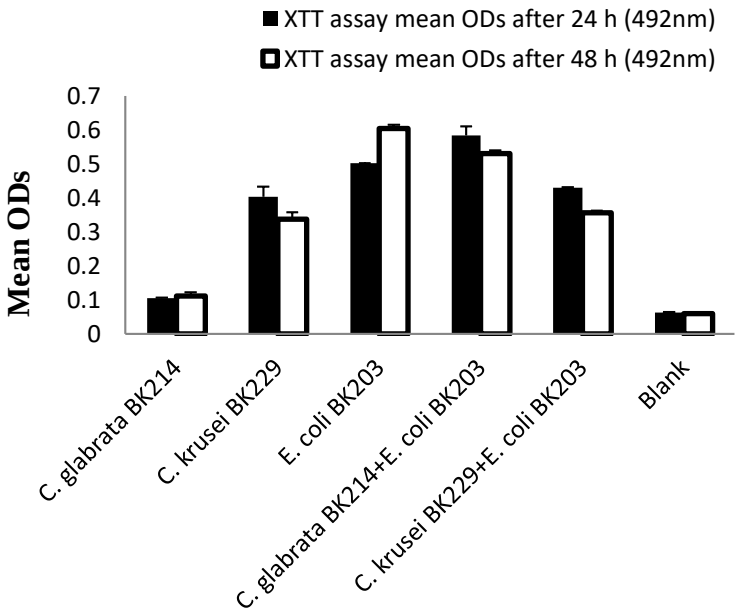

Figure 7.24: Comparison of metabolic activity by XTT assay expressed by non-albicans *Candida-E. coli* mono and dual-species combinations in developed biofilms after 24 h and 48 h of incubation

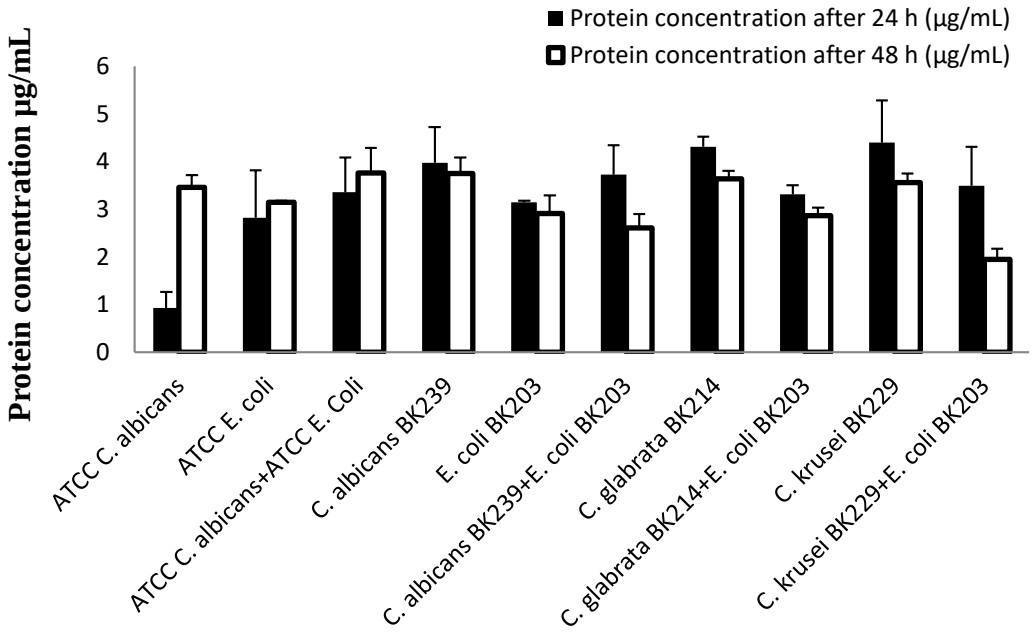

Figure 7.25: Total protein estimation by Lowery method for *Candida-E. coli* mono and dual-species combinations in developed biofilms after 24 h and 48 h of incubation

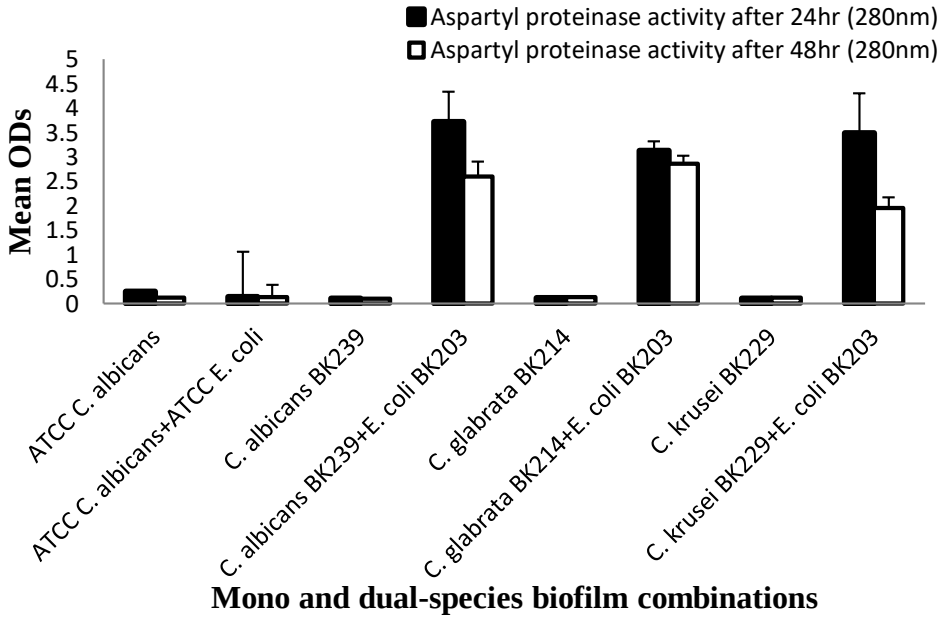

Figure 7.26: Comparison of aspartyl proteinase activity expressed by *Candida* in mono and *Candida- E. coli* dual-species combinations in developed biofilms after 24 h and 48 h of incubation

7.4- DISCUSSION

Biofilm on biotic surfaces are mostly polymicrobial in nature, in which eukaryotic and prokaryotic members of normal flora and nosocomial pathogens can be also involved, thus making it difficult to diagnose and treat. These microbial communities are understudied and are clinically challenging health problems (Harriott and Noverr, 2011). Biofilm forming microorganisms can easily access oral cavity and form biofilm throughout the soft mucosal and hard tissues. Regular shedding of epithelial cells from soft tissues, is an effective control of oral biofilms. Inside biofilm, microbes are protected from host innate immune response and microbes still thrive even when good oral hygienic practices are maintained. These microbial communities if not regularly removed, can lead to oral disorders (Jakubovics, 2015).

Most of the studies related to *Candida* biofilm development are *in vitro* are based on mono-microbial biofilm models on abiotic surfaces. As ability of *C. albicans* to grow on various biotic surfaces as well as now on the indwelling catheters (abiotic surface) is becoming a significant issue, which can lead to fatal systemic infections, therefore, their role in oral surfaces is also needed to be deciphered. Species heterogeneity within polymicrobial biofilm makes it difficult to understand individual species contribution in disease pathogenesis. However, new advance molecular techniques make it possible to understand interaction in these communities. Effect of bacterial species on *Candida* biofilm formation are now becoming available in literature, which is giving insight into the both agonistic and antagonistic relationships among these communities (Harriott and Noverr, 2011; Gulati and Nobile, 2016) .

Besides *C. albicans*, other non-albicans species like *C. glabrata*, *C. Krusei* and *C. parapsilosis* are also frequently cultured from mouth and are involved in biofilm development on soft and hard tissues. In addition to fungal species, oral cavity also harbours many other microbial species. These oral residents can perform several functions within biofilm community, they either interact synergistically with other resident flora or antagonistically to inhibit growth of other resident (Ovchinnikova *et al*., 2013).

In present study, potential of multidrug resistant weak biofilm former *Candida* species and other commensal aerobic bacteria isolated from saliva samples of postpartum

females to grow and survive in dual-species biofilm was analysed by *in vitro* biofilm model using CV biofilm biomass and XTT reduction assay. Dual-species biofilm between *Candida* species and oral bacteria were developed on polystyrene surface and was investigated along with ATCC strains.

Biofilm forming ability of *C. albicans* is a significant issue especially in consortia (Gulati and Nobile, 2016). Mostly polymicrobial biofilm studies focus on relationship between bacterial and Candidal species, however, data is rare related to co-existence of more than two *Candida* species within a biofilm. In present work, dual-species biofilm formation between different *Candida* species was studied. Analysis showed that both *C. albicans* and *C. glabrata* exhibit reduction in biofilm biomass and protein content, however, their metabolic activity was raised in dual-species biofilm. Another indicator of metabolism that is aspartyl proteinase activity, which was reduced in dual-species biofilm of *C. albicans* and *C. glabrata* compared to their individual biofilms. Among *Candida* species maximum biofilm biomass was produced by *C. krusei*, which was greatly reduced in dual-species biofilm assay with both *Candida* species. Also, their metabolic activity was decreased in dual-species biofilm after 48 h of incubation. *Candida* species might have antagonistic relation in developed polymicrobial biofilms.

Like present work, reduction in *C. albicans* biofilm forming ability was also reported by Rossoni *et al*., (2015), when *C. glabrata* (77% reduction) and *C. krusei* (89% reduction) were co-cultured. Santos *et al*., (2016), also showed similar trend of reduction in biofilm forming ability and metabolic activity in dual-species assays (*C. albicans* with *C. glabrata* and *C. krusei)*. Another study reported that *Candida* isolates from dental prostheses show reduction in biofilm forming ability and express antagonistic relationship in dual-species biofilm and their metabolic activity also significantly differ in mono and dual-species biofilm. In their study on *Candida-Candida* dual-species biofilm, hyphal production was reduced which is required for more invasive form of yeast (Martins *et al*., 2016). Possible reason for low biofilm biomass and metabolic activity expressed by *Candida* isolates in the present study could be due to microbial competitive behaviour for limited supply of nutrients in microtiter plates and for colonization sites available for *Candida* to grow in mixed-species biofilms. Furthermore, decreased survival due to oxygen stress in multi-

layered biofilms might be contributing factor for antagonistic behaviour. Expression of high *C. krusei* biofilm biomass compared to other *Candida* species could be due to high initial colonization rate, high cell surface hydrophobicity and adherence to acrylic surfaces compared to other *Candida* species.

Contrary to present study, results were reported by a study based on *in vivo* mice model injected sublingually with *C. albicans* or *C. glabrata* alone and in combinations to check their ability to grow and develop into oropharyngeal candidiasis. These experiments showed that *C. glabrata* alone was unable to develop aggressive tongue infection; however, with pre-established *C. albicans* infection was greatly enhanced. There was also reduction in *C. albicans* along with decreased *C. glabrata* colonization (Tati *et al*., 2016). In present work, biofilms were developed *in vitro* on polystyrene surfaces. Effect of substratum on microbial biofilms development is already reported, like tongue as substratum provides nutritionally rich and balanced environment for greater support, growth and hyphal production of *Candida* species compared to *in vitro* polystyrene surfaces.

In next part, *Candidal-Staphylococcal* dual-species biofilm were analysed. Both ATCC isolates of *Candida* and *Staphylococcus* showed increased biofilm biomass production, metabolic activity and protein content in dual-species biofilm. Isolate of *C. albicans* and *C. glabrata* expressed increased biofilm biomass production and metabolic activity in dual-species biofilm with *Staphylococcus* species (except with *S. aureus*). Their protein content was also seen to increase after 24 h of incubation. Although *C. krusei* showed decrease in biofilm biomass, however, its metabolic activity was raised with *Staphylococcus* species except with *S. saprophyticus*. Enzyme activity was reduced for all *Candida* isolates in dual-species biofilm.

Mostly literature, which is available is based on involvement of *C. albicans-S. aureus* dual-species biofilm in different pathologies such as periodontitis, keratitis, denture stomatitis, cystic fibrosis, UTIs, ventilator associated pneumonia and burn wound infections. A study by Zago *et al*., (2015), reported synergistic growth effect in dual-species biofilm between *C. albicans*-MRSA and MSSA strains. A high biofilm biomass and metabolic activity was found in *C. albicans-S. aureus* dual-species biofilm. Another study demonstrated similar agonistic relationship in an *in vitro* biofilm assay of *C. albicans-S. aureus* in murine oral cavity. It was shown that *S.*

aureus adhered more to *Candida* hyphal due to adhesion molecules called Als3p. Moreover, due to this adhesion transport of *S. aureus* across mucosal barrier and disseminate to other body site to cause systematic infections is enhanced (Schlecht *et al*., 2015). ATCC strains employed in present work exhibited the similar agonistic relationship in dual-species biofilm, however, local isolates used produced antagonistic relationship. The reason might be the differences in biofilm forming conditions (*in vitro* vs *in vivo*), short time duration required for *Candida* to showed phenotypic switching in a nutrient deficient culture media and to produces excessive EPS. Also strains used in both studies were from different sources as isolates from saliva samples were used in current work in comparison to ATCC cultures.

S. aureus attach best to hyphal cell but not with yeast cell, also hyphal associated cells showed increased resistance to antimicrobials (Scheres and Krom, 2016; Weidt *et al*., 2016). Similar trend was also exhibited by *S. aureus* in *Candida-S. aureus* dual-species biofilm developed by Kong *et al*., (2016), where *S. aureus* grown in EPS produced by *C. albicans* showed increase antimicrobial tolerance. Kong et al., (2017), also found that fernesol produced by *C. albicans* enhanced the antimicrobial activity of *S. aureus* in mixed-species biofilm. In another study, PGE2 produced by *C. albicans* was found to enhance the growth of *S. aureus* in *C. albicans-S. aureus* biofilms (Krause *et al*., 2015). In present study the source of isolates were early postpartum females, a condition during which females have faced several challenges including enhanced hormone mediated inflammatory response especially in oral cavity. PGE2 is normally raised in pregnancy and returns to normal in postpartum phase. It can create an environment to enhance the growth and survival of *C. albicans* and *S. aureus* to establish in a mixed-species oral biofilm and to be involved in oral pathologies. However, as in present work *in* vitro conditions were used which were different from the environment provided by oral cavity, there might be a possibility that its effects on the biofilm forming activity of isolates was varied.

A study by Holt *et al*., (2017), demonstrated a role of dual-species biofilm of *C. albicans-S. epidermidis* in nematode model. After mono and dual-species inoculation, survival of nematode after infection was assessed. In comparison to single-specie inoculation, dual-species inoculation significantly enhanced the virulence and reduced nematode survival rate. Hyphal production by *C. albicans* and EPS overproduction by

S. epidermidis together enhanced the virulence in dual-species biofilm (Harriott and Noverr, 2011). An enhanced biofilm forming activity of dual-species *C. albicans-S. epidermidis* was also reported by El-Azizi *et al*., in 2004. *C. albicans* from present study also showed enhanced biofilm biomass, protein content and metabolic activity in dual-species biofilm with *S. epidermidis*, indicating synergistic relationship between these two microorganisms in dual-species biofilm.

In last portion of the present work, dual-species biofilm between *Candida-K. pneumoniae* and *C. albicans-E. coli* were investigated. ATCC *C. albicans*- ATCC *K. pneumoniae* and ATCC *C. albicans*-ATCC *E. coli* isolates showed reduction in biofilm biomass in dual-species biofilm. In all *Candida* species dual specie assay with *K. pneumoniae* biofilms initially showed decrease in biofilm biomass, however, later on increase was seen after 48 h of incubation. In dual-species biofilm of all *Candida* species- *E. coli*, reduction in biofilm biomass was seen. Their protein was also reduced in dual-species biofilm and aspartyl proteinase activity was overall reduced in dual-species biofilm also.

Candida-K. pneumoniae and *Candida-E. coli* biofilms are mostly studied for their role in catheters associated biofilms development, but in current work it was studied for oral infections. It is estimated that about 80% of the catheter associated UTIs are caused by the polymicrobial biofilms developed by the combinations of uro-pathogenic *E. coli*, *Enterococcus* specie, *K. pneumoniae, Proteus mirabilis, P. aeruginosa, and C. albicans* (Galván *et al*., 2016). *E.coli* (21.4%) and *C. albicans* (21.0%) are considered as the most common pathogens associated with polymicrobial biofilms on catheters (Koves *et al*., 2017).

E.coli and *K. pneumoniae* are ubiquitous Gram negative bacteria that are involved in biofilm production (Piperaki *et al*., 2017; Oliveira *et al*., 2017). *E. coli* can develop biofilm with the help of flagella, type 1 fimbriae, by production of colonic acid and coordinated expression of several transcriptional regulators like OmpR, FlhD/FlhC and RcsB (Sule *et al*., 2009). *K. pneumoniae* is the 2nd most common cause of nosocomial infection next to *E. coli.* (Abdulhasan, 2015), and its biofilm forming ability is considered as a the key factor for its pathogenesis in nosocomial infections (Lin *et al*., 2017; Piperaki *et al*., 2017). *K. pneumoniae* expressed two types of

fimbrial adhesions (type 1 and 3) for surface attachment that help in biofilm formation (Abdulhasan, 2015; Schumacher and Zeng, 2016; Bandeira *et al*., 2017).

Like present work, Park *et al*., (2014), also demonstrated a decrease in biofilm formation in dual-species biofilm between isolates of *Candida* and *E. coli*. A dual-species biofilm was established between *C. albicans* isolated from oral and *E. coli* cultured from urine. It was found that co-culturing of *C. albicans* with this bacterium leads to suppression of several genes associated with hyphal transition. Another study conducted in 2006, also found a significant negative correlation in biofilm development ($P<0.05$) between the co-cultured *C. albicans* and *E. coli* isolated from oral cavity (Thein *et al*., 2006). Like present work, a study by El-Azizi *et al*., (2004), also reported decrease in *C. albicans* biofilm forming ability when co-cultured with *K. pneumoniae*.

Bandara *et al*., (2009), conducted a study on interaction of ATCC *E. coli* 25922 strain (same as used in present work) with ATCC strain of *C. albicans* 90028, *C. glabrata* 90030 and *C. krusei* 6258 in dual-species biofilm developed on polystyrene surfaces. A significant reduction in biofilm activity after 24 h of incubation in *C. krusei* with *E. coli* dual-species biofilm was reported, while activity of *C. albicans* and *C. glabrata* was found to be unaffected, however, after 48 h of incubation the activity was unaffected for all isolates. ATCC *E. coli* in present study, showed decrease in metabolic activity in dual-species biofilm after both 24 h and 48 h of incubation. Contrary to the present work, a study by Piva *et al*., (2011), demonstrated in XTT assay of dual-species biofilm interactions between ATCC *E. coli* (25922) and *C. albicans* (18804), showed increase in metabolic rate after 24 h of incubation in dual-species biofilm compared to mono-specie biofilm. This difference in interaction might be due to the use of different ATCC *C. albicans* strain expressing different biofilm forming behaviour in *in vitro* biofilms.

Cabral *et al*., (2018), in their study showed reduction in dual-species biofilm between non-oral *C. albicans* and *E. coli* strains. *E. coli* reduced *C. albicans* in a time dependent manner. The only study found in the literature based on oral commensal *C. albicans* interaction with non-oral *E. coli* isolate was conducted by Thurnheer and Belibasakis (2015), by using *in vitro* supragingival biofilm model. This study showed that non-commensal *E. coli* was able to survive in dual-species biofilm and caused

slight increase in *C. albicans*. These results are contrary to the present work, this might be due to difference in the experimental conditions, which needs to be further investigated for possible mechanisms.

As biofilm formation play important role in development of dental caries and gingivitis, this was an attempt to develop a model to evaluate role of different microorganisms in the biofilm's development of these attached communities. Limited data is available in literature related to association of oral *Candida* species with commensal or pathogenic oral bacteria and ultimately their role in biofilm related oral disorders. In present study, overall, decrease in biofilm biomass was seen in dual-species biofilm of *Candida-Candida* and *Candida-E. coli* dual-species biofilm. In *Candida-K. pneumoniae* dual-species biofilm, slight increase in biofilm biomass was seen except in *C. glabrata-K. pneumoniae* dual-species biofilms. However, with *Staphylococcus* species increase in biofilm biomass was seen in all combinations except for clinical isolates of *C. albicans-S. aureus, C. glabrata-S. aureus* and *C. glabrata-S. epidermidis* dual-species biofilm. These findings suggest that in dual-species consortia microorganisms can behave synergistically or antagonistically for their survival and disease pathogenesis. The present study was carried out to explore the possible interaction of the *Candida* and oral bacteria in mixed-species biofilm formation as a virulence factor for pathogenesis of dental disorder by using *in vitro* model. Oral cavity provides the ecological niche which is totally different from *in vitro* models. To understand the interaction between microbes in the multi-species consortia, there is a need to develop appropriate *in vivo* animal models which should be coupled with advance metagenomic, metaproteomic and metametabolomic techniques.

CONCLUSION

Postpartum females from the present work were prevalently suffering from various oral disorders. Gingivitis was present in 27.3% females, while 28.1% were presented with dental caries. Incidence of APOs was also high (LWB=22.5%, PTB=21.7% and preeclampsia=11.6%). Postpartum females showed high *S. mutans* (p<0.0001) and *S. sobrinus* colonization, especially in females with oral disorders, having poor oral hygiene and females with APOs. Although *S. mutans* and *S. sobrinus* are the predominantly known cariogenic bacteria, however, by culture independent method low abundance of *S. mutans* in all samples (BK1=0.004%, BK2 and BKC2=0.001%, and BKC1=0.004%) were detected. Instead females suffering from dental caries and gingivitis showed predominance of *Streptococcus, Yersinia, Haemophilus, Prevotella, Neisseria, Fusobacterium, Gemella, Prevotella, Aggregatibacter, Rothia, Veillonella, Granulicatella* and *Actinomyces,* indicating the possible cariogenic role of these isolates.

Frequently detected bacterial genera by cultured based method in both healthy and postpartum females belonged to *Staphylococcus*, *Streptococcus,* Enterobacteriaceae, *Neisseria* and *Lactobacilli* group. Out of 267 postpartum females, 65.16% were culture positive for *Staphylococcus* species. *S. epidermidis* colonization was significantly raised in postpartum group (P=0.005). Out of 210 *Staphylococcus* isolates from postpartum females, most of the phenotypically biofilm positive isolates were also expressing biofilms forming genes and had high antibiotic resistance against majority of the tested antibiotics. Prevalence of *Streptococcus* species were also significantly high in postpartum compared to nonpregnant females. Prevalence of other detected bacterial species in this group was as follow: *Lactobacilli* 12.73%, *N. meningitides*10.48%, *K. pneumoniae* 6.36%, *Enterobacter* species 5.61% and 2.62% for *E. coli*. On MTP method 58.82% *K. pneumoniae*, 100% *E. coli* and 80% isolates of *Enterobacter* species were confirmed as biofilm formers. *K. pneumoniae* and *E. coli* showed high resistance against most of the commonly used antibiotics.

Only detected fungal specie by culture-based method in studied females belonged to *Candida*. In postpartum females, prevalence of *Candida* (55.05%) was significantly raised. *C. albicans* isolated from saliva samples of postpartum females showed high esterase and phospholipase activity compared to control nonpregnant group. Majority

of the isolates from postpartum group showed biofilm forming ability (66.87%) and increased antifungal activity in comparison to control group. Postpartum females were at high risk for oral Candidal carriage, which expressed enhanced virulence.

Culture independent method showed that core predominant bacterial phyla were Firmicutes followed by Proteobacteria, Bacteroidetes, Actinobacteria and Fusobacteria, which were shared by all females. At genera level, top genera in all females included; *Streptococcus* followed by *Prevotella, Gemella, Rothia, Neisseria, Haemophilus, Veillonella, Granulicatella, Fusobacterium* and *Porphyromonas*. Alpha diversity was decreased in salivary microbiome of postpartum female having gingivitis and dental caries with PLWB. Beta diversity difference was highest between female with dental issues and different pregnancy outcomes. Female with oral health problem and which gave FTB showed more alpha diversity compared to female with PLWB and oral disorders, and was dominated by phyla Proteobacteria followed by Firmicutes, Bacteroidetes, Fusobacteria, Actinobacteria and Spirochaetes. At genera level, predominance of *Streptococcus* followed by *Yersinia, Haemophilus, Neisseria, Fusobacterium, Gemella* and *Prevotella* was seen. Female with PLWB and oral health issues showed predominant phyla of Firmicutes followed by Proteobacteria, Bacteroidetes, Actinobacteria, Fusobacteria and Spirochaetes, while, common identified genera were *Streptococcus,* followed by *Gemella, Prevotella, Rothia, Veillonella, Haemophilus, Neisseria, Granulicatella, Actinomyces* and *Fusobacterium*. This pattern of microbial diversity is quite different from the one seen in healthy population or in patients suffering from gingival and dental disorders. In dental caries commonly reported genera are *Streptococcus, Actinomycetes, Lactobacillus, Veillonella*, *Propionibacterium, Atopobium, Pseudoramibacter, Olsenella, Selenomonas* and *Propionibacterium*. While in case of gingivitis, microbial flora shifted from *Streptococcus* to *Actinomycetes, Capnocytophaga, Eikenella, Campylobacter, Prevotella,* and *Fusobacterium.*

Predominant fungal genera were *Candida* followed by *Saccharomyces, Phialosimplex, Termitomyces, Penicillium, Aspergillus, Olpidium, Cochliobolus* and *Malassezia* in all samples. *Stachybotrys, Geotrichum, Talaromyces, Leucosporidium, Acremonium, Wallemia, Eupenicillium, Septoria, Zymoseptoria, Coniosporium, Phialophora,* and *Mycosphaerella* were genera detected only in postpartum group. Postpartum female with gingivitis and dental caries showed abundance of genus

*Saccharomyces, Phialosimplex, Candida, Olpidium, Cochliobolus, Malaseezia, Hyphodontia, Debaryomyces, Mrakia, and Nakaseomyces*as compared to those postpartum females with good oral health. Among postpartum group female with oral health issues as well as who had PLWB, showed reduced richness, evenness with elevated levels of *Saccharomyces, Candida, Hyphodontia* and *Malassezia* compared to the female having FTB.

Both culture dependent and independent methods produce quite different results, as some of the isolates which were isolated by cultured based method were not isolated by culture independent method e.g. *S. aureus, S. saprophyticus, K. pneumoniae, E. coli, N. meningitides* and *C. krusei*. This difference of identifying different diversity by culture dependent and independent methods rises several questions, which challenges that there is need to improve sensitivity of DNA extraction method from samples, costume design better conditions for culturing which mimics the oral cavity and ultimately reliable sequencing method to detect these isolates, as different protocols still yield variable results.

Further, an *in vitro* model of mono and dual-species were assessed for biofilm formation. In present study, overall, decrease in biofilm biomass was seen in dual-species biofilms of *Candida-Candida, C. glabrata-K. pneumoniae* and *Candida-E. coli* dual-species biofilms. However, with *Staphylococcus* species increased in biofilm biomass was seen in all combinations except for clinical isolates of *C. albicans-S. aureus, C. glabrata-S. aureus* and *C. glabrata-S. epidermidis* dual-species biofilms. These findings suggest that in dual-species consortia microorganisms can behave synergistically or antagonistically for their mutual survival and oral disease pathogenesis.

Findings from the present work showed that microbial diversity changes during pregnancy, with predominance of microbes having more pathogenic potential. Both cultured bacterial and fungal species expressed enhanced virulence characteristics and antimicrobial resistance. In addition to, known pathogens (*C. albicans* and *S. aureus*), *C. glabrata* and *S. epidermidis* also emerged as pathogenic species. Unculturable microbial diversity also showed microbial shift with oral disorders, suggesting that there might be an association of this changing microbial diversity with oral disorders leading to APOs.

FUTURE PROSPECTS

The present work was conducted to explore pathogenic potential of oral culturable and unculturable bacterial and fungal species, colonizing the postpartum mothers with oral diseases to determine their possible association with pregnancy outcomes. However, a detailed and comprehensive evaluation of oral health status of Pakistani pregnant females in both health and disease states regarding culturable and unculturable microbial species and their possible effects on pregnancy outcomes is the need of time to avoid pregnancy related complications. As culture based methods and metagenomic based methods yield quite different results, so there is a need to explore a more reliable method of DNA extraction and sequencing for even strain level identification of microbes. In addition to explore diversity there is also a need to study their functional potential to assess their role on microbial consortia. As in present study only explored site was a saliva, there is also a need to explore other oral sites as well for more comprehensive study of oral ecology. Study on the prevalence of MS in infants along with their mothers should also be studied. MS virulence factors and their interaction with immune system should be studied for a better understanding of the association of *S. mutans* colonization with oral disorders and APOs. Follow up monitoring of the postpartum women should be carried out to determine the vertical transmission of *S. sobrinus* and *S. mutans* from mother to babies. Dental caries is usually not given importance during pregnancy, the results of this study shed light on this issue, that oral health should be regularly monitored to keep mother and child safe. Further detection of virulence factors of bacterial and *Candida* species using expression and molecular based studies are needed to dissect their role in oral disorders and APOs. Also work should be done on the antibacterial and antifungal resistance mechanisms in these species to avoid emergence of multidrug resistance. There is further need of molecular and animal-model based studies on *C. albicans* and other oral bacterial mixed-species biofilms to explore their interaction in consortium and there is a need for interventional studies to evaluate the role of different species in enhancing or inhibiting the biofilm development for therapeutic purposes.

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APPENDIX A

PATIENT CONSENT FORM

شرکت کنندہ:

تاریخ پیدائش:

ایم آر نمبر:

ریسرچ میں رضامندی کا فارم:

سٹڈی کا عنوان :

Microbial diversity in oral microbiome of postpartum females

تحقیق کنندگان :**Bibi Khadija**

ریسرچ کا مقصد:

ہم امیدکرتےہیں کہ یہ سٹڈی ہمیں ان بیکٹیریازکی تلاش میں مددکرےگی جوحاملہ خواتین کی صحت پراثرانداز ہوتے اور قبل از وقت زچگی کا باعث بنتےہیں۔اس ریسرچ سے قبل از وقت پیدائش جیسی پیچیدگیوں تشخیص اور علاج میں مدد ملے گی اور اس بات کی نشاندہی کرے گی کہ بیکٹیریا کس طرح سے زچہ و بچہ کی صحت کو متاثر کر سکتے ہیں ۔ یہ تفصیلات نئے طریقہ علاج متعارف کروانے میں مدد گار ثابت ہو سکتی ہیں جس سے دوران پیدائش ہونے والی شرح اموات کو کم کیا جا سکتا ہے۔

ریسرچ کی تفصیل:

آپ کے ماضی میں ہونے والےعلاج معالجے کی معلومات کی ضرورت پڑ سکتی ہےاور ان کا مطالعہ کیا جاسکتاہےتاکہ اس بات کا پتہ چلایا جا سکے کہ بیکٹیریا کس طرح سےزچہ و بچہ کی صحت کو متاثر کر آپ کے تھوک کا نمونہ لیا جائے گ سکتے ہیں۔

ممکنہ تکالیف:

سٹڈی کے دوران اگر آپ کے تھوک کا نمونہ لیا جائے گا جس سے کسی قسم کی تکلیف کا سامنا نہ ہو گا۔ ہمیں آپ کے نمونے میں کوئی خاص بات نظر آئی تو تحقیق کرنے والے عملے کا کوئی فرد آپ سے رابطہ کرے گا۔

ممکنہ فوائد:

ہو سکتا ہے اس سٹڈی سے آپ کو فائدہ نہ ہو لیکن اس کے نتائج مستقبل میں دوسری حاملہ خواتین کے لئے مفید ثابت ہو سکتے ہیں اور معلومات میں اضافے کا باعث بن سکتے ہیں ۔

رازداری:

آپ کی شناخت صیغہء راز رکھی جائے گی اور کسی کو بھی اس بارے نہیں بتایا جائے گا۔ یہ اجازت نامے کا فارم آپ کی فائل میں لگایا جائے گا۔ آپ کی جانچ پڑتال کے نتائج صرف آپ کے علاج کے لئے کی کئے جائیں گے۔ کوئی بھی بات آپ کے علاج اور اس میں شامل عمل کو جانے والی ریسرچ میں استعمال بتانے کے لئے آپ سے اجازت لی جائے گی۔آپ کے خون اور تھوک کے نمونوں پر آپ کے نام کی بجائے کوڈ نمبر لگایا جائے گااور ان کوڈ نمبروں کی فائل کومحفوظ رکھا جائے گا جس تک صرف تحقیق کرنے والے افراد کی رسائی ہوگی۔کوئی بھی معلومات جس سے آپ کی شناخت ظاہر ہو آپ کی اجازت کے بغیر کسی کو نہیں بتائی جائے گی۔آپ کے نمونوں سے حاصل شدہ ڈی این اے اور خلیوں کو کوڈ نمبر لگا کر غیر معینہ مدت کے لئے محفوظ کر لیا جائے گا تاکہ اس سے نئی معلومات حاصل کی جا سکے۔

رضا کارانہ شرکت:

اس ریسرچ میں شرکت رضاکارانہ ہے اور اگر آپ کسی بھی وقت اپنے خون یا تھوک کا نمونہ اس سٹڈی سے نکلوانا چاہیں تو آپ کے خون یا تھوک کا نمونہ ضائع کر دیا جائے گااور کسی کو بھی اس سٹڈی سے انفرادی مالی فائدہ نہیں ہوگا۔

APPENDIX B

QUESTIONNAIRE

Title: Microbial diversity in oral microbiome of postpartum females

Patient # Name Age			
Hospital			
Income			
Residential area	□ Rural		□ Urban
Social economic status	□ Low	□ Middle	□ High
History of prior pregnancies	□ if yes how many _____	□ No	
Mode of delivery	□ C-section	□ Vaginal	
Gestational period of current pregnancy	□ FTB (≥ 37 weeks)	□ PTB (< 37 weeks)	
Baby weight at the time of delivery (Kg)	□ Low (<2.5)	□ Normal (2.5-4)	□ High (>4)
Presence of preeclampsia	□ Yes	□ No	
BP at the time of delivery	□ Low	□ Normal	□ High
Antibiotic use in last three months	□ Yes Describe: _____		□ No
Presence of other medical conditions	□ Asthma □ Diabetes □ Stress □ Hypertension	□ Allergy □ Depression □ Anxiety □ Others_____	

Brushing frequency/day	□ Yes If yes how many times? □ once □ twice □ thrice			□ No
Use of mouth wash	□ Yes			□ No
Oral health data	□ Tooth decay □ Gum pain □ Bleeding gums □ Dental caries □ Lose teeth		□ Missing teeth □ Pocket depth____ □ Mouth sores □ Tumor lesions □ Others	
Orthodontic treatments during pregnancy	□ No □ Root canal		□ dental filling □ Tooth replacement	
Intake of juices/drinks	□ No	□ Frequently	□ Not frequently	
Intake of tea and coffee/day	□ No	□ 1-2 times	□ 3-4 times	□ >4 times
Do you ever used?	□ Cigarettes smoking □ Hookah		□ Naswar (Snuff) □ Betalnut (Chalian)	

APPENDICES C

Appendix C1: Compositions of stock solutions used for DNA extraction and gel electrophoresis

S #	Solutions	Chemicals	Weight (mg)	Water (mL)	Final volume (mL)
1	Tris HCl	Tris HCl (1M/1000 mM)	14.532	120	150
		Tris HCl (0.2M/200 mM)	2.9064	120	150
		Tris HCl (0.01M/10 mM)	0.14532		
2	EDTA	NaOH	Few drops to adjust PH	450	500
		EDTA (0.5M/500 mM)	73		
		EDTA (0.2M/200 mM)	29.2		
		EDTA (0.01M/10 mM)	1.46		
3	TBE Buffer 10 X	Tris-base	27.0	220	250
		EDTA	2.325		
		Boric acid	14.5		
4	Ethidium bromide staining solution	Ethidium bromide	0.05	40	50

Appendix C2: Composition of working solutions used for DNA extraction and gel electrophoresis

S#	Solution	Chemicals	Concentration	Final volume (mL)
1	Protienase K	Protienase K	100 mg	10
		Tris HCl	10 mM	
		Glycerol	50%	
		$CaCl_2$	20 mM	
2	Sodium dodecyl sulphate (20%)	SDS	20 g	100
		H_2O	50 mL	
3	Ethanol (70%)	Absolute ethanol	70 mL	100
		H_2O	30 mL	
4	Sodium acetate	CH_3COONa	9.84 g	40
		H_2O	25 ml	
5	T.E Buffer	EDTA (0.2 M)	10 mL	200
		Tris HCl (1M)	20 mL	
6	Loading Dye	Bromophenol blue	0.05 g	30
		Sucrose	8.0 g	
		H_2O	20 mL	

Appendix C3: Composition of PBS

NaCl	0.8 g
Na2HPO4	1.44 g
KH2PO4	0.24 g
Distilled Water	1000 mL

Adjust pH to 7.2-7.4 and autoclaved for optimal results

Appendix C4: Composition of CV (1% w/v)

CV	0.1g
Distilled Water	100mL

Dissolve and filter through 0.44 µm filter

Appendix C5: Preparation of 33% acetic acid

For 50 mL

Acetic acid	16.5 mL
Distilled water	33.5 mL

APPENDIX D

Appendix D1: Protocol for preparation of BSA standard curve

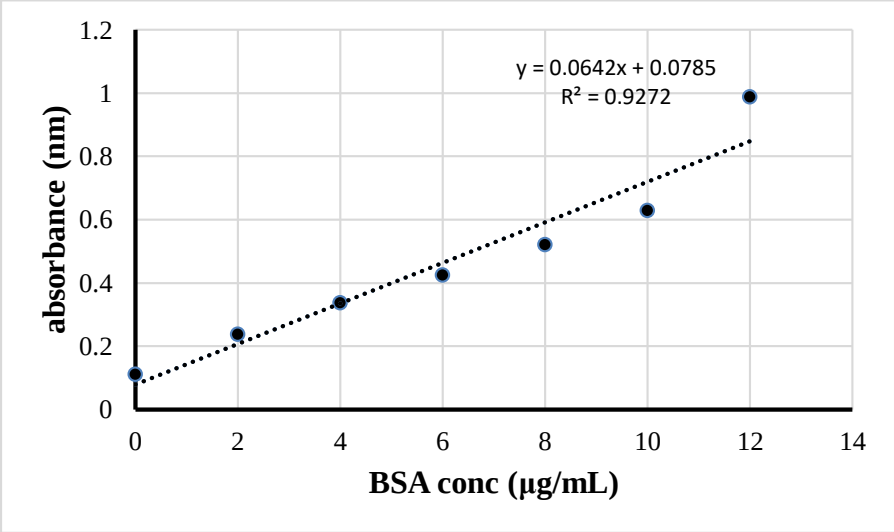
For BSA curve, BSA stock solution was prepared by dissolving 0.1 g of BSA in 80 mL distilled water and final volume was adjusted to 100 mL having final concentration of 1 mg/mL. Dilutions were prepared from this stock solution (Appendix D2). Solution C (Appendix D3) was then added in prepared dilutions and incubated for 10 min. After incubation, solution D (Appendix D4) was added (100 µL) and incubated for 30 min. Absorbance was measured at 650 nm using spectrophotometer after incubation and values were compared with blank. Standard curve was plotted by BSA concentration given in Appendix D5 and absorbance values and slope was calculated (Appendix D6) to determine protein concentration.

Appendix D2: Concentration of BSA µg/mL used for protein estimation by lowery method

BSA (mL)	**Water (mL)**	**BSA conc. (µg/mL)**	**BSA volume (mL)**	**Alkaline CuSO4 (mL)**	**Lowery reagent (mL)**
0.2	0.8	0.2	0.2	2.0	0.2
0.4	0.6	0.4	0.2	2.0	0.2
0.6	0.4	0.6	0.2	2.0	0.2
0.8	0.2	0.8	0.2	2.0	0.2
1.0	0.0	1.0	0.2	2.0	0.2

Appendix D3: Absorbance of BSA at different concentration

Concentration of BSA (µg/mL)	**Absorbance (650nm)**
0	0.1109
2	0.2371
4	0.3369
6	0.424
8	0.5211
10	0.6279
12	0.987



Appendix D4: BSA standard curve

Appendix D5: Solution C preparation

Solution B	1 mL
Solution A	50 mL

Appendix D6: Solution D preparation

Folin phenol	1 mL
Distilled water	1 mL