Development of Microbial Based Probiotic Feed Supplement and Evaluation of its Impact on Growth, Production and Health of Dairy Cattle

by

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Islamabad

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

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In

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In the Name of That, Who Has Many Names, Who Can be Called, Remembered and Worshiped by Any Name.

My faith remains incomplete without the respect for Holy Muhammad (PBUH), the great Messenger, Who, conveyed the message of truth and showed us the right path.

Dedicated to my "Parents and Family"

For their prayers, endless support, love, encouragement and motivation throughout my PhD.

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List of Acronym/abbreviations

%	Percentage
µL	Microliter
Mm	Micrometer
%WG	Percentage weight gain
ATP	Adenosine triphosphate
ATR	Attenuated total reflectance
Ca	Calcium
CFU	Colony forming unit
Control	Cows feed without microbial feed additive
Cm	Centimeter
cm^{-1}	Per centimeter
CTAB	Cethyl trimethyl ammonium bromide
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acids
Eco	Ecology
EDTA	Ethylene diamide tetra acetic acid
Etc	Etcetera
°C	Degree celsius/Centigrade
Fig.	Figure
Fe	Iron
FTIR	Fourier transform infrared spectrophotometer
G	Gram
GCMS	Gas chromatography mass spectrometry
Hcl	Hydro chloric acid
Min	Minute
Hgb	Hemoglobin
Hrs	Hours
HPLC	High performance liquid chromatography
i.e.	That is
IR	Infrared

ITS	Internal transcribed spacer
Kg	Kilogram
L	Liter
LAB	Lactic acid bacteria
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration.
MCV	Mean corpuscular volume
Na	Sodium
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National center for biotechnology information
Mg	Milligram
Mg	Magnesium
MRS	De Man‘s Rogosa and Sharpe agar
ºC	Degree centigrade
OD	Optical density
OGA	Oxytetracycline glucose agar
OUT	Operational taxonomic unit
PCA	Principle component analysis
PCR	Polymerase chain reaction
RBCs	Red blood cells
PK	Proteinase K
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
Rpm	Revolutions per minute
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulfate
SGR	Specific growth rate
Spp	Species
TCA	Tri-Chloro acetic acid
TE	Tris- ethylenediaminetetraacetic acid
T-RFLP	Terminal restriction fragment length polymorphism

UV	Ultra violet
WBCs	White blood cells
Zn	Zinc
ATCC	American type culture collection
B-cells	Bone-marrow cells or bursa derived cells
BCATTLEST	Basic local alignment search tool
BSH	Bile salt hydrolase
CFU	Colony Forming Unit
CMC	Carboxy methyl cellulase
CO_2	Carbon dioxide
CTAB	Cetyl trimethyl ammonium bromide
CV	Crystal violet
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EPS	Exopolysaccharide
FAO	Food and agriculture organization
FOS	Fructo-oligosaccharide
GC	Guanine cytosine
GIT	Gastrointestinal tract
GOS	Galacto-oligosaccharide
GRAS	Generally recognized as safe
HMM	High molecular mass
IBD	Inflammatory bowel disease
Ig	Immune-globulin
IKB-α	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IL	Interleukin
CATTLAB	Cattle Latic acid bacteria
LB	Luria broth
LMM	Low molecular mass
M cells	Microfold cells
Ml	Milliliter

MR	Methyl red
MRS	de man Ragosa and Sharpe
MRVP	Methyl Red Vogues Proskauer
NaCl	Sodium chloride
NARC	National Agriculture Research Council
NCBI	National Center for Biotechnology Information
NF-β	Nuclear factor beta
Nm	Nano meter
OD	Optical density
OGA	Oxytetracycline glucose agar
Rpm	Revolution per minute
rRNA	Ribosomal Nucleic Acid
SDS	Sodium dodecyl sulphate
SIM	Sulfide indole motility
T-cells	Thymus cells
TGF	Transforming growth factor
THEIFER	Tryptic soy agar
TSI	Triple sugar iron
TSS	Trypticase sugar solution
UN	United nation
WHO	World health organization

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My PhD program from the very outset to the end has been a long strenuous and toilsome journey of incredible encounters and experiences. Whilst writing the last piece of this story, I am peering through every window into the past to recall all those lovely people who have been a part of this interesting story and made my journey nothing short of amazing. Now, I would like to say thanks to all those people without whom this journey would not have been accomplished. First and foremost, I wish to thank my supervisor, ***Dr. Muhammad Imran*** *Associate Professor, Department of Microbiology, Quaid-I-Azam University Islamabad, who I am deeply indebted for his support, encouragement, professional advice, patience and careful attention to detail during the whole program. I am grateful to* ***Faculty members*** *Department of Microbiology, Quaid-I-Azam University Islamabad, Pakistan for enabling me to build strong theoretical background, and* ***Pakistan Science Foundation*** *for providing me financial support. I also extend my special thanks to* ***Shafi Reso Chemical (SRC)*** *for providing experimental animals for this study and Centre of Animal Nutrition, Livestock Research and Development Station for providing the facility of testing the samples.*

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Abstract

Present research work was designed to evaluate the probiotic potential of indigenously isolated microorganisms. Later on selected microorganisms with best probiotic characteristics were applied in dairy cattle feed to check their impact on growth, productivity and health of dairy cattle. In phase I, previously isolated strains comprised of lactic acid bacteria, gram positive bacteria and yeasts (*S. cerevisiae*, *G. candidum*) in addition to *E. faecium* were characterized for probiotic potential. Characterization based on amylolytic, proteolytic and cellulolytic activity in addition to mimic gut survival, cell hydrophobicity, anti-pathogenic activity and cholesterol assimilation yield *G. candidum* (QAUGC01) as potential Probiotic to be used in dairy cattle. In total, ten lactic acid bacteria (including *Enterococcus, Lactococcus, Enterobacter* Sp.), three *Bacillus* species from cow dung, eighteen gram-positive cocci and bacilli from corn silage while five *Enterococcus faecium* and twelve *Geotrichum candidum* strains were previously isolated from Dahi. All previously isolated strains were checked for their probiotic potential. Strain with best cumulative probiotic properties was further used in cattle‘s feed as microbial supplement. It was found that among all the isolates only *Geotrichum candidum* strain has shown best cumulative abilities. Among all *G. candidum* strains sourced from Dahi, QAUGC02 has shown maximum survival rate of 53.9, 48.770% and 48.770% at 2, 4 and 24 hours respectively, followed by QAUGC10 (44.442%, 41.366% and 40.26%) and QAUGC01 (42.04%, 37.74% and 34.511%). *Geotrichum candidum* QAUGC12 has shown survival rate of 47.442% at 2 hours but later its survival decreased to 24.142% at 4 hours which is 20% less. *G. candidum* QAUGC07, QAUGC02, QAUGC06, QAUGC05, QAUGC01, QAUGC09 has shown maximum cholesterol assimilation respectively, QAUGC08 has maximum cell hydrophobicity of 86.10% followed by QAUGC10 (76.87%) and QAUGC03 (75.93%). *G. candidum* QAUGC01 has cell hydrophobicity of 46.6%. According to above results QAUGC02 has significant mimic gut survival and cholesterol assimilation while its cell hydrophobicity is very low. *G. candidum* QAUGC10 has low cholesterol assimilation. Only *G. candidum* QAUGC01 (KT280407) has significant cumulative results for all studied parameters. It is well established that dysbiosis in the gut microbiome leads to compromised productivity and metabolic disorder in dairy cows. The significant impact of gut microbiota on better feed utilization ability and resistance against diseases has been frequently reported. In phase II *G. candidum* QAUGC01 (KT280407) was used as microbial feed additive in basal diet of experimental cows and was compared with control cows to find the impact of QAUGC01 on dairy cow‘s health and

productivity. After ninety days of trail growth performance, nutrient digestibility, blood parameters, serum biochemistry and milk yield along with milk composition has been analyzed and compared between control and experimental groups. It was found that dry matter intake was high in control cows (14.16 kg/day) in comparison with experimental cows (13.46 kg/day), but average milk yield, and average feed efficiency (FE) was high in experimental cows (15.26 kg, 1.30 respectively) with 1.45 kg more milk as compared to control cows (14.77 kg, 1.12 respectively). The milk lactose was significantly high in milk of *G. candidum* supplemented dairy cows accompanied by increase in milk fat and protein content as compared to control. The digestibility of dry matter (DM), crude protein (CP), Neutral detergent fiber (NDF), acid detergent fiber (ADF), crude fiber (CF) and ether extract (EE) of probiotic supplemented feed in experimental cows was high as compared to control. Impact of feeding probiotic on hematological parameters was within normal range. RBC count has improved in experimental cows 7.7 $x10^6$-8.72 $x10^6$ cells/µl, while in the control cows it decreased from 7.6 $x10^6$- 6.9$x10^6$ cells/µl. WBC count decreased both in control and experimental cows, but this decrease was low in experimental cows (9922.2-9524.4 cells/µl) than control cows (9000-7393.3 cells/µl). Neutrophil count increased both in control and experimental groups, but this increase was high in control cows (53.3 to 55 %) than experimental cows (57.33 to 58.55 %). Blood serum profile depicted high glucose level in experimental cows while decreased in control group. The decrease in serum total cholesterol was significantly high while HDL level increased in experimental cows. The serum LDL was decreased in serum of experimental cows (126 mg/dl) after *G. candidum supplementation* than control serum LDL (176 mg/dl) at end of experiment. Serum butyrate of experimental cows increased than control cows. The milk cholesterol (mg/ 100gm fat) was reduced significantly in experimental cows during experimental period as compared to control milk cholesterol. After analysis of all parameters some cows were selected through principle component analysis (PCA) for metagenomics study in phase III. *G. candidum* was found in all three experimental cows with high abundance than control. TAC (Total aerobic count) and *Lactobacillus* count found high in experimental cows. Anaerobic count increased in experimental cows because of *G. candidum* QAUGC01 supplementation and resulted with high abundance of *Bifidobacterium choerinum, Bifidobacterium longum*, *Bacteroides spp.*, *Rikenella spp., Rikenella microfusus, Clostridial species*, *Ruminococcus albus, Ruminococcus sp., Eubacterium spp*. in gut of experimental cows. *Pseudomonas psychrophila*, *Pseudomonas sp.*, *Pseudomonas spp.*, *Pseudomonas trivialis*, *Pseudomonas*

veronii, *Pseudomonas syringae* also appeared in experimental cows which is a cellulolytic flora found on phylo-sphere of grasses. While *Klebsiella oxytoca*, *Achromobacter xyloxydanes Enterobacter sp*. *Salmonella enterica* and *Serratia quinivorans* showed negative co relation with *G. candidum* due to the antagonistic effect of yeast *G. candidum* QAUGC01. Shredding of pathogenic flora by experimental cows was less as compared to control cow. In current study *G. candidum* QAUGC01 appeared as a potential probiotic that could be categorized as pharmabiotic among dairy cattle. Since, it modulated rumen flora, improved blood physiology, serum and milk profile which indicate its direct effects on health and physiology. Molecular mechanisms of all these effects demands further investigation with more comprehensive approach.

Chapter 1

Introduction

1. Introduction

Probiotics either as nutribiotics and pharmabiotics (E.-S. Lee *et al.*, 2018) gaining expansion in the world market. Interactions between pathophysiological processes and microbiota helped probiotics to emerge as mediators in disease and health (Day *et al.*, 2019). While considering commensal microbes, host appeared as super organism with greater collective metabolic potential than commensal microbes individually. Manipulation of these commensals in a desirable way could be carried out by microbial, dietary and therapeutic interventions (C. Hill, 2010). Probiotics role as modulator of gut microbiota have gained a lot of attention while talking about prophylaxis and remedy of intestinal disorders (Celiberto *et al.*, 2018). Working Group of World Health Organization (WHO) and Food and Agriculture Organization (FAO) jointly come up with Probiotics definition as ―live micro-organisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). This definition of Probiotics accepted and used widely further International Scientific Association for Probiotics and Prebiotics adopted this definition of Probiotics (C. Hill *et al.*, 2014).

Further, heaved interest regarding probiotics has coincided with microbiome era; as we understood more about the gut microbiota and its relationship with health as well as disease, intervention option for gut microbiota modulation by the supplementation of probiotics has drawn-out a secure foundation. Fermented products such as cultured milk, yogurts are good source of probiotics and reported with strong health benefits (Sen, 2019). It is essential to investigate the unique probiotic strains that not only fulfill the demand of market but should also have the novel characteristics and functions as compared to already available probiotics in market (Kumar *et al.*, 2015). Production of Probiotics based food products found to be key research area focusing functional food market in future. Expected forecast regarding probiotics based dietary supplements have shown an increase ranging from 3.30 to 7 billion US$ during period from 2015 to 2025 (Terpou *et al.*, 2019). In selection of probiotic strain, number of aspects should be considered such as the general and technological features of the strains, and the functional characteristics that it exerts *in-vivo* (Shokryazdan *et al.*, 2017). To fulfill the requirements for selection of probiotics, probiotic strains have to survive during the food processing, in human gut and intestinal conditions; therefore probiotic strains need to be investigated under mimic conditions of gastrointestinal tract (Pham & Mohajeri, 2018). The

first harsh condition that a probiotic strain encounter is lysozyme present in the saliva (in mouth) then it goes to the stomach having pH ranging from 1.5 to 3; further it goes to small intestine, containing bile (Singhal *et al.*, 2019). The two fundamental properties that a probiotic strain must survive in gut, are the acidity tolerance and the bile tolerance during the passage through GI tract. Stress conditions of acidity and bile starts at the start of small intestine (Prasad *et al*., 1998, Park *et al*., 2002). Several static *in vitro* digestion models have been established to determine probiotic survival rate in intestine (Keller *et al.*, 2019; Shinde *et al.*, 2019). ‗Gastric-small intestine system TIM' is one of them (Blanquet *et al.*, 2005), it contains four compartments serially arranged that stimulate the stomach and small intestine consisting of three segments: duodenum, jejunum, and ileum. Another *in-vitro* digestion model is TIM-2 model; this model is more advanced than the previous one. In its flexible glass vessels are linked in a series way to stimulate the peristalsis (Joossens *et al.*, 2011).

Safety of Probiotic strains must be assessed carefully (Salvetti *et al.*, 2016), with the consideration to prevent the transferable resistance due to antibiotic (Bowler, 2018). In recent years, concern about the safety of probiotic strains has arisen for feed application. Recent literature has revealed the possibilities of their antibiotic-resistant gene-reservoirs. Much attention has been given to LAB group due to the widespread of transposons and conjugative plasmids (Morandi *et al.*, 2015). The exclusion of pathogenic micro-organisms from intestine is the primary benefit of the probiotic strains (M. Peng *et al.*, 2019). Antimicrobial activity (Ghori *et al.*, 2018) of the probiotic strains is another benefit that enhances and improves the fermented food quality. Adherence ability of probiotic strains to the mucosal surface and epithelial cells is considered as an important quality (FAO/WHO, 2001). Adherence to cell is complex process that involves the interaction between interacting surfaces and cell membrane of bacteria. Study of bacterial adhesion in the body is difficult, particularly in humans, for this purpose *in vitro* models have been developed to screen the potential microbes preliminary (Laparra & Sanz, 2009).

The exact number of microbial cells required for therapeutic benefits is not in the knowledge and their number varies according to function and desired health effects of the strain. Normally the minimum level for viable probiotic strain was 10^6/mL or gram was acceptable (Selim & Haider, 2014). The investigation on new probiotic microbes for their use in food items and technological relevance is important in industry and trade. The study of strains

showing more resistant to barriers in human GI tract, caring physiological characteristics that are compatible to probiotic properties, may lead eventually to the selection and identification of novel probiotic strains for functional food products (Day *et al.*, 2019; Shokryazdan *et al.*, 2017).

Thus, it can be elaborated as ~~"~~preparation of a product containing viable, defined microorganisms in sufficient numbers, which alters the micro-biota (by implantation or colonization) in a compartment of the host and that exert beneficial health effects in the host" (Frédéric Leroy & De Vuyst, 2004). There are various applications (Kerry *et al.*, 2018) of probiotics e.g. it can be used in pharmaceutical preparations (Bober *et al.*, 2018), infant formulas, fermented and non-fermented milk products. Probiotic exerts enormous beneficial effects upon its consumption i.e. balanced intestinal micro-biota, boost up the immune response, adjuvant vaccine effects, fecal enzyme reduction in cancer initiation, antibiotic therapy, diarrhea treatment, control of colitis because of *Clostridium difficile* and rotavirus, prevention of *Helicobacter pylori* induced ulcer. They also play their role in serum cholesterol reduction, antagonist to food-borne pathogens, the enhancement in mal-absorption lactose symptoms, candidiasis and oral diseases (Fontana *et al.*, 2015). Various properties and attributes are there for the microorganisms to enlist them in probiotic category (Kerry *et al.*, 2018). Some attributes of the probiotic strains are (a) adherence with the mucosal surface (Monteagudo-Mera *et al.*, 2019) (b) exclusion of already existing pathogenic microorganisms (c) multiplication and persistence in the GI tract (Perry & Doron, 2018) (d) production of H_2O_2 (K. Lee *et al.*, 2019), acids and bacteriocin (Chikindas *et al.*, 2018) thereby inhibit the growth of pathogenic micro-organisms (e) should be safe to use (non-pathogenic), do not disturb or damage the epithelial surface and non-carcinogenic and (f) co-interaction with other microbes to establish balanced flora (Ouwehand *et al.*, 2001; Rinkinen *et al.*, 2003).

There has been set forth the criteria of probiotics strains (Rivera-Espinoza & Gallardo-Navarro, 2010). A reported study manipulate the *in vitro* characteristics of lactic acid producing bacterial strains that include *L. sakei*, *L. curiatus* and *Staphylococcus carnosus* from meat, *L. paracasei* and *L. plantarum* from fruits exhibit the same metabolic and functional properties like human intestinal flora (Haller *et al.*, 2001). Additionally, *Lactobacillus* strains from brine of naturally fermented Aloren (green table olive) and *L.*

buchneri P2 from pickled juice have revealed some of the probiotic properties as, acid and bile tolerance, antimicrobial activity and cholesterol reduction (Zeng *et al.*, 2010).

The interest in microbial usage as a significant tool in probiotics has increased over the last few decades. The *Geotrichum* spp., yeast like filamentous fungus, is pervasive and is widespread *i.e*. found in soil, silage and plant tissues (Jacques & Casaregola, 2008). This genus also contributes to the existing micro-flora of human GI tract, mouth and skin. Fermented milk, cheese and different type of juices are also found as the source of various species belonging to this genus. *Geotrichum candidum* has been reported as technologically important species by the "International Dairy Federation and European Food and Feed Culture Association" that has previously been used in different dairy products (Cosentino *et al.*, 2001). *G. candidum* can grow in wide range of pH *i.e.* 5.5-7.0 and temperature *i.e*. 5-38^{o}C (optimal temperature 25^{o}C) (Boutrou & Guéguen, 2005). Majority of *G. candidum* strains can tolerate the salt range up to 2.5% (w/v) and cannot survive in 4% (w/v) salt concentration.

It is found as the important part of Camembert (soft cheese), St. Nectaire and artisanal's (semi hard cheese) and goat's raw milk cheeses (Hayaloglu & Kirbag, 2007; Vasdinyei & Deak, 2003). *G. candidum* not only take part in ripening of soft cheese but also in development of typical flavor due to lipolytic, peptidolytic and proteolytic activity of its strains. Besides that during production, *G. candidum* can contaminate pasteurized milk and dairy products (Boutrou & Guéguen, 2005; Torkar & Venguńt, 2008). *G. candidum* is able to survive in the presence of bile in the gut (Amir *et al.*, 2018). Cholesterol lowering ability (Syal & Vohra, 2014) in the serum by *G. candidum* increases its importance to be used as probiotic. Potential to colonize with the epithelial layer is important for a probiotic strain. *G. candidum* can colonize in the gut and compete with pathogens. Exclusion of pathogens by competitive inhibition improves the health and modulates the immune response (Ibrar *et al.*, 2017). Pathogenic bacteria may invade the epithelial layer of the intestine thus may cause diarrhea and bowel syndrome, *G. candidum* eliminate the pathogens and repair the layer and prevent the progression of pathogens by colonizing there. Thus, probiotic plays vital role as probiotics in context of betterment for the human health as there is elimination or removal of foodborne pathogens in the GI tact of human (Prado *et al.*, 2010).

Several species of the genus (*Lactobacillus*) make significant percentage of animal and human normal gut flora. Genus *Lactobacillus* being a heterogeneous and wide taxonomic unit

includes over 100 species. Being used in a variety of food and feed fermentations, different species of this genus deliberately introduced in food chain and supplemented as Probiotics in animals and humans (Hammes & Hertel, 2006). *Enterococci* used in food products like cheese as starter cultures, as probiotics among animals and humans, also as additives in silage (Perez-Moreno & Fuchs, 2006). Gastrointestinal tract is an effective and primary source of probiotics which inhabit almost more than 500 bacterial species. *L. gassei* and *L. reuteri* is the most commonly used probiotic specie that resides in the human gastro-intestinal tract (Ryan *et al.*, 2008). Similarly, *B.longum* (D. Srutkova *et al.*, 2011) and *L. acidophilus* (P. P. Lin *et al.*, 2009) strains were screened out respectively from healthy human adult and being marked as probiotics. Besides this, probiotic strains are also profound in animals‘ gastro intestinal tract, commonly in rats, pigs, and poultry sources (Petrof *et al.*, 2012). Bee gut induces beneficial response to honey bee colonies (Audisio & Benitez-Ahrendts, 2011). Moreover, GIT of marine and fresh water fish such as, carassius auratus gibelio (Chu *et al.*, 2011), rainbow trout (Perez-Sanchez *et al.*, 2011) and shrimp (J. E. Hill *et al.*, 2009) are also rich source of probiotics.

Furthermore, in a reported study, human breast milk also provides *Lactobacillus* strains that act as probiotic source. This helps in generation of T-cells and natural killer cells and also responsible for regulatory T cell expansion that simultaneously heighten natural and acquired immunity (Perez-Cano *et al.*, 2010).

The rumen and intestinal microbiota of cattle performs its vital role in the fermentation process. They help in methane emission by means of fermentation both from rumen and large intestine (Johnson & Johnson, 1995). The microbial diversity in the GIT of the dairy cattle has lot of impact on the productivity and well-being of the cattle (Dowd *et al.*, 2008; Eckburg *et al.*, 2005; Engelbrektson *et al.*, 2010; Guarner & Malagelada, 2003). Culture-dependent techniques have been widely applicable for proper characterization of microorganisms that resides in the bovine fecal material. These microorganisms can be easily cultured and manipulated on laboratory scale. The non-cultivated microorganisms are being revealed via ―culture-independent‖ techniques. 16S rDNA-based sequencing has been used to study this approach at molecular level. New trends and tools like ―pyrosequencing‖ have been developed for characterization of uncultured species of gastrointestinal tract. Pyrosequencing perform rapid characterization of large-sample data sets (Dowd *et al.*, 2008).

Milk in term of tonnage third and in term of value was the top agriculture commodity worldwide. Milk is also an important local commodity and in most of countries it ranked among top five agriculture commodities both in term of value and tonnage (FAO, 2016). 82.7% of whole fresh milk produced worldwide comes from cow followed by 13.3% from buffaloes, 2.3% from goats, 1.3% and 0.4% from sheep and camels respectively. Globally 14% of agricultural trade comprised of dairy products and milk (OECD *et al.*, 2016). Annually over 50 billion liters of milk produced by Pakistan makes it the third largest milk producer in the world. In livestock sector milk is the only product that exceeds the combined value of all the main cash crops in the country (Shaista Naz & Khan, 2018). Apart from ensuring food security and being source of income for over 8 million rural families, livestock also contribute 3.1% to export. Number of cows in Pakistan stands around 47.8 million and cow milk produced is 21691000 tones while cow milk comes under human consumption is 17353000 tones (PEC, 2019).

Ruminants hosted an intricate microenvironment in symbiotic terms in their unique digestive compartment known as rumen or fore-stomach which enabled ruminants to get benefit from dietary compounds considered as non-digestible (Bravo & Wall, 2016). Cattles have been equipped with a digestive system which is unique in terms of its structure, function and efficiency. Stomach in cattle comprising of rumen (fermentation vat), reticulum (honey comb lining), omasum and abomasum (true stomach). Ruminants (grazers, browsers and mix or intermediate feeders) named after rumen, cattle categorized as grazer (Robbins *et al.*, 1995). Naturally present microbial species in the GIT of the ruminants aid in fermentation and digestion of feed ingredients (Khan *et al.*, 2016). These symbiotic floras present in the rumen of cow delivered by new substrates when she ingested feed. In return microorganisms yield variety of valuable nutrients to the cow and for themselves via process of fermentation (B. A. Dehority, 2003; Zilber-Rosenberg & Rosenberg, 2008). Digestion is the unique feature of ruminants through which they got the ability of converting complex or indigestible plant fibrous materials into Volatile fatty acids (VFAs) having carbon number C2 to C6 through the process of fermentation. These VFAs (Acetic acid 50-60%, Butyric acid 18-20%, Propionic acid 12-18%) serves as major source of energy for host animals. Being a fermentation vat rumen house a huge number of microbial populations with vast diversity, bacteria being the dominants (McSweeney & Mackie, 2012). This natural fermentation vat provides a suitable environment to anaerobes, a pH ranging from 5.5 to 6.9 and a favorable

temperature of 100.4 to 105.8 Fahrenheit (B. A. Dehority, 2003). Micro-flora of the cattle rumen can be classified into three domains: eucarya (fungi & protozoa), archaea (methanogens) and predominantly present bacteria. Bacteria were dominating with viable count of 10^{10} to 10^{11} per gram. Other microbes housed by rumen are as protozoa 10^4 to 10^6, bacteriophages 10^7 to 10^9 (McSweeney & Mackie, 2012). Further include anaerobic fungi 10^2 to 10^4 (Ousama AlZahal *et al.*, 2017; Ishaq *et al.*, 2017) and archaea which are 5% of all bacteria and archaea populations (Frey *et al.*, 2010). Microbiome of the rumen possesses genes 100 times higher than the animal hosting all these microbes. So much magnitude of these genes armed the ruminants with enormous metabolic and genetic abilities to ferment and hydrolyze nutrients which are difficult or unable to digest (McSweeney & Mackie, 2012). Hence, cattle's performance greatly depends on the quality and extent of fermentation products at the end. Quality, quantity and range of these products further rely on the type of nutrition offered and diversity of rumen flora. As a result of all these phenomenon rumen microbiome plays a vital role in cattle and referred as cattle's ~~"s~~econd" genome (Bath *et al.*, 2013). If we want to conceptualize the herbivores digestive function or physiology, it appeared as dilemma to enhance diet quality, intake and digestibility (Clauss *et al.*, 2010). Poor quality hemicellulose, lignin and cellulose hydrolyzed in the rumen and transformed into good quality short chain fatty acids (SCFA) that are beneficial for the host and easily absorbed by the body of host. Meanwhile, this micro-flora of the cattle rumen helps in elimination of toxic substances produced by the host, during normal metabolic processes (Jouany, 2006). Until today, understanding about dynamics of rumen micro-flora is that microbiota of the rumen comprises of variable microbiota in addition to core microbiota (Creevey *et al.*, 2014; Henderson *et al.*, 2015). Core microbiota found over extended geographical range and comprises of diversity of taxa, their abundance decrease or increase as this very little deviation associated with type of diet fed (Henderson *et al.*, 2015). Several studies reported strong association between important production parameters of cattle like milk yield, quality or composition (Jami *et al.*, 2014; Lima *et al.*, 2015), feed efficiency (Carberry *et al.*, 2012; Guan *et al.*, 2008; Hernandez-Sanabria *et al.*, 2012; Jewell *et al.*, 2015b; F. Li, 2017; McCann, Wiley*, et al.*, 2014; P. R. Myer, Smith*, et al.*, 2015; Rius *et al.*, 2012; Shabat *et al.*, 2016; Zhou & Hernandez-Sanabria, 2009, 2010) and microbiota of rumen. Among diverse populations of microbes in rumen some are in more abundance than others like *Firmicutes* are the predominant phylum (Durso *et al.*, 2010; Khafipour *et al.*,

2009). Another dominant phylum of bacteria inhabiting the rumen of the dairy cattle includes *Bacteroidetes* (Fernando *et al.*, 2010; Kong *et al.*, 2010). Prevalence of another important bacterial population comprising of *Proteobacteria* is below 4% of overall ruminal community (Pitta *et al.*, 2014). Importance of this bacterial population can be assessed by the possibility that increased proportion of *Proteobacteria* in the ruminal community resulted in sub-acute ruminal acidosis (SARA) (Khafipour *et al.*, 2009). *Ruminococcus flavefaciens* found in the rumen produce cellulase as well as hemicellulase for the breakdown of plant fibers and considered as powerful bacteria amongst microbial community of rumen (Wood *et al.*, 1982). *Fibrobacter succinogenes* are powerful cellulolytic bacteria, normally inhabiting the GIT of ruminants and helping in digestion in a significant way (Kobayashi *et al.*, 2008). *Butyrivibrio fibrisolvens* belongs to fibrolytic bacteria based on their activity and play significant role in the production of butyrate by the utilization or breakdown of the sucrose and maltose (Fernando *et al.*, 2010).

Any disturbance in the balance of normal flora in the rumen of dairy cattle leads to different metabolic conditions like SARA (Khafipour *et al.*, 2009). Feeding diets with high grain concentration tends to enhance the milk yield, even diets with concentrates up to 75%. Ruminants have predominantly been adapted to metabolize and digest feeds like forages. Diets carrying high load of grains when consumed by the dairy cattle makes them critically expose to ruminal acidosis (Kennelly *et al.*, 1999). Though, ruminal acidosis mainly caused by heavy grain feeding among dairy cattle, it has been seen that cows only grazing on pasture are also susceptible to lower pH in the stomach (O'Grady *et al.*, 2008). Ruminal acidosis can be categorized into two main types as: SARA or sub-acute ruminal acidosis and acute ruminal acidosis (Owens *et al.*, 1998). Prevalence of SARA among dairy herds is common and can be estimated by spot samples, only practical method. While investigating the prevalence of the SARA among dairy herds using rumen liquor collected via process of rumeno centesis it has shown that percentage of cattle having pH < 5.5 was as follow US cows 20% (K. Krause & Oetzel, 2005), 14% and 20% among Danish and German cows respectively (Kleen *et al.*, 2013). Low pH of the rumen considered as de facto definition of sub-acute ruminal acidosis; nevertheless, clinical symptoms manifested by the SARA might not only associate with ruminal pH. Based on the type of diets offered to the animal, microbial flora might have produced metabolites harmful for the health status of cows

(González *et al.*, 2012). Decreased pH of rumen, ruminal inflammation, (Plaizier *et al.*, 2008; Zebeli & Metzler-Zebeli, 2012), elevated ruminal lactate, valerate, (Counotte & Prins, 1981; Enemark, 2008), lowered DMI (Desnoyers, Giger-Reverdin, Bertin*, et al.*, 2009), decreased efficiency of feed, changed behavior of the cows and selection of feed ingredients all these happened among cows suffering from SARA (Oetzel, 2017). Extensive fermentation of carbohydrates inside large intestine gave rise to organic acids accumulation and ultimately leads hindgut acidosis (Gressley *et al.*, 2011). Mucin casts presence in dung, frothy feces and diarrhea among the clinical manifestations of SARA (Nordlund *et al.*, 2004). The pathophysiology of sub-acute ruminal acidosis is complex, involving both local ruminal effects and systemic inflammation. It is difficult to diagnose sub-acute ruminal acidosis in dairy herds. There is no definitive herd test; instead, information about herd performance, clinical signs, and measured ruminal pH must be integrated. Prevention of sub-acute ruminal acidosis requires excellent feeding management and proper diet formulation. Feed additives may reduce (but not eliminate) the risk for sub-acute ruminal acidosis in dairy herds (Oetzel, 2017).

In addition to yeast, bacterial probiotics also have been supplemented in ruminant‘s nutrition to get beneficial effects on animal performance; they have shown to enhance feed efficiency (FE), weight gain (WG) and DMI (Elghandour *et al.*, 2015). Bacterial probiotics in ruminants might have inhibited the growth of pathogens, instigated immune response by bacteriocin‘s secretion and modulated the GIT microbial balance (Khan *et al.*, 2016). Bacterial supplementation in dairy cows enhanced milk fat, fat-corrected milk yield as well as overall milk yield (Elghandour *et al.*, 2015; Khan *et al.*, 2016).

In our study we will select best strain with probiotics potential in addition to enzymatic potential and its ability to release antimicrobials etc.

Aim

Characterization and application of indigenous probiotics in cattle feed to evaluate the impact on physiology, production and gut microbial modulation.

Objectives

The objectives of the project are given.

The research work is divided into three phases dedicated to:

1. The evaluation of the probiotic potential of indigenous bacterial and fungal isolates
2. The study of the impact of selected probiotic strains on physiology, productivity and health of dairy cattle
3. The determinations of dairy cattle gut microbial modulation after ingestion of *Geotrichum candidum*

Chapter 2

Review of Literature

2. Review of Literature

2.1 Dairy, Health and Microbiology Nexus

Foods are not only significant in consumers in terms of flavor and instant dietary needs, but also its capacity to deliver health benefits besides from their simple dietary value. Presently, the activity and the equilibrium of the intestinal microflora has been refined by the help of the major part of the practical food market (Saarela *et al.*, 2002). Ingestion of foods encompassing living bacteria is the oldest and still most extensively used way to upsurge the numbers of beneficial 17 different bacterial strains in the intestinal tract. The bacterial strains that are basically the innate inhabitant of the GIT are principally belonging to the genera of *Lactobacillus* and *Bifidobacterium* and such bacteria are called _Probiotics' (AFRC, 1989; Capela *et al.*, 2006). Milk a vastly nutritious liquid produced by the mammary glands in mammals to sustain newborns through their early months. Daily consumption of milk supplies a significant portion of nutrients required daily (Haug, 2007) in addition to other health benefits (Haug, 2007; A. Zulueta *et al.*, 2009). Several milk components play role in metabolism either by providing essential minerals, vitamins, amino acids and fatty acids, or by influencing nutrients absorption (Haug, 2007). Presence of about 400 different kinds of fatty acids in milk fat categorizes it as most complex fat in nature, lipids in cow milk found in globules in the form of oil in water emulsion. Almost half of short chain fatty acids is butyric acid (Lindmark Månsson, 2008) which is found to be good modulator of gene function and might have a role in cancer prevention (German, 1999). Dairy trans-fats also known as ruminant trans-fats found with positive health effects. Small amounts of trans fats found in milk like conjugated linoleic acid (CLA) and vaccenic acid (Lindmark Månsson, 2008). Milk fat also found associated with antioxidant quality of milk because correlation between antioxidant capacity and percentage of fat was significant (Zulueta *et al.*, 2009). Milk provides important proteins having health benefits such as casein forms the 80% of milk proteins having the ability to enhance minerals absorption like phosphorus and calcium (Holt *et al.*, 2013). It also has assessed that casein might promotes lower blood pressure (Ricci *et al.*, 2010). Whey proteins are 20% of milk proteins having branched chain amino acids (BCAAs). Whey proteins found associated with many beneficial health effects like mood improvement during stress periods and lower blood pressure (Markus *et al.*, 2000; Pal *et al.*, 2010). Casein appeared as main contributor to antioxidant capability of whole milk, while

albumen of whey protein. In deproteinized milk hydrophilic compounds uric acid and vitamin C are prime contributor to antioxidant capability (Zulueta *et al.*, 2009). Vitamin B12, an essential vitamin required by the body present in high quantity in milk. Diets of animal origin are rich in this essential vitamin (Pawlak *et al.*, 2014). Riboflavin or vitamin B2 mainly supply by dairy products in western diet (Powers, 2003). Milk is one of good sources of calcium an important mineral required by the body. Another advantage of milk is that calcium supply by milk absorbed easily. Higher bone density associated with cow‘s milk (Cashman, 2002), calcium and protein found in milk are the two major factors responsible for such effect (Hunt *et al.*, 2009). Cow milk is a source of insulin like growth factor 1 (IGF-1) only hormone from cow‘s milk absorbed by human. IGF-1 involved in regeneration and growth processes (Wiley, 2012). Among the risk factors leads to heart disease high blood pressure is the major one. Dairy products shown to have important role in reduced risk of high blood pressure (Engberink *et al.*, 2009; Wang *et al.*, 2015). This phenomenon of reduced high blood pressure thought to be resulted from a distinctive recipe of potassium, magnesium and calcium in milk (Griffith *et al.*, 1999; Massey, 2001), in addition to other factors such as peptides produced from casein‘s digestion (Ricci *et al.*, 2010). The main conventional ways are used for the fermentation of dairy product so that these ways can be used in a more better way and the numerous microorganisms, mainly species of *Geotrichum* and *Enterococcus*, have conventionally been exploited to encourage human health in addition to food functionality and taste and now a day several inquiries have done on these species to appraise their prospective as probiotics. Historical perspectives of probiotics colonize association *Escherichia* labeled as the microbiota of the newborn GIT and recommended advantages of their inhabiting in digestion. Doderlein hypothesized the lactic acid is made by the helpful relationship of 7 vaginal bacteria that are involves in preventing the development of pathogenic bacteria by making lactic acid at that place (Argyri *et al.*, 2013).

According to the studies of (Moro *et al.*, 2002), it has been evaluated that the valuable relationship of different bacterial strains with human host is helpful for preventing the different diseases (Moro *et al.*, 2002). It is clarified in the popular book –The Prolongation of Life” by Metschnikoff, 1907 described that the longevity and potential of Caucasians was related to the high consumption of fermented milk goods. The process of fermentation of starch yield the main metabolic end product of lactic acid by the use of bacterial genera

Enterococcus that fits to LAB 7 group of Gram positive, non- sporulating, non- respiring cocci or rods (Salminen *et al.*, 1998). Another group of bacteria that is involved in the lactic acid bacterial strain and is phylogenetically dissimilar, name, *Bifidiobacteria* that make bacteria frequently recognized as LAB. When we do a comparison with formula-fed infants, 9 different type of *Bifidiobacteria* were found that were basically linked with the faces of breast-fed toddlers and a small number of occurrences of intestinal upset was also observed for breast-fed infants (Meile *et al.*, 2008). In some applications, *Geotrichum candidum* has been used in fermented products particularly in villi manufacture (Timmerman *et al.*, 2005).

2.2. Milk Market Worldwide

In 2018 estimated global milk production was 843 million tones, 2.2 percent higher than 2017 (FAO, 2019). Globally 14% of agricultural trade comprised of dairy products and milk (OECD *et al.*, 2016). Over 0.15 billion farmers worldwide possess minimum one milking animal. Dairy cow particularly kept by 0.13 billion holdings worldwide (FAO, 2005). Cows being most common milk animal farmers kept them as herds comprised of 2 or 3 animals in developing countries (DEFRA, 2016; USDA, 2015). There are below 0.3% dairy farms worldwide having above 100 cows (IFCN, 2015). Livestock also contributes to rural women empowerment and support as it is among the well-known assets for the rural women. In 25% of the cattle possessing households dairy cows directly kept by women (Njuki *et al.*, 2013).

2.3. Dairy in Pakistan

In Pakistan contribution of agriculture to country‘s gross domestic product (GDP) is 18.5% and offers 38.5% employment to national labor force. During 2018-19 agriculture‘s performance in Pakistan remained subdue. Instead of this slow growth livestock which is 60.54% of agriculture and 11.22% of GDP maintained its growth at 4.0% against the targeted 3.8% (Laporte & Ahmed, 2019). This indicates the potential of livestock sector in Pakistan. Livestock plays major role in curbing and relieving rural poverty as livestock provided a large amount of disposable income helping marginal and disadvantages populations throughout developing countries worldwide. Due to major developments taking place over last two decades, increasing foreign investments dairy sector in Pakistan attaining the status of an industry. Inclination to branded, packaged dairy products enforcing dynamic product development. Further in Asia modern trade development propelling growth in dairy industry (Cargill, 2018). But in tropics as well as in developing countries the Cattle production suffers

a lot as compared to temperate and developed countries. In the tropics, ruminants are grazed or restricted to low-quality forages, crop residues and agro-industrial by-products, which adversely affect the cattle productivity. To overcome these problems during last two decades, the researchers have explored several methods to enhance the functions of rumen microbiota for efficient digestion and fermentation processes, as well as to enhance bioavailability and utilization of nutrients through supplementation of feed by probiotics for increasing milk production through good health (Arowolo & He, 2018).

2.4. Functional Aspects of Probiotics

Some clinical trials of probiotics were also implied on animal and human studies (Yan & Polk, 2011). Probiotic effects were analyzed and verified by number of trials. They show sudden responses in repressing diarrhea (Lye *et al.*, 2009), relieving lactose intolerance, anti-colorectal cancer (Liong, 2008; Rafter *et al.*, 2007) and antimicrobial activities, easiness in post-operative intricacies (Woodard *et al.*, 2009), reduction of irritable bowel symptoms (Moayyedi *et al.*, 2010), and hinder inflammatory bowel diseases (Golowczyc *et al.*, 2007).

Several studies have manipulated the beneficial effects of non-viable probiotics by means of fermentation (primarily by LAB), which include the procreation of secondary metabolites e.g. Vitamin B, Bioactive peptides, exopolysaccharides (EPS), bacteriocins and organic acids. These metabolites are soluble and can be spray-dried which are added in the form of dried powder in food milieu. Non-viable probiotics have certain benefits over viable ones including Pro-long shelf life, Easier handling, transportation and storage facilities, Lessen refrigerated storage conditions.

Probiotics have shown direct and indirect effect on functional (fermented) food stuffs. Direct effect indicates host-organism relationship while In-direct effect demonstrate the biogenic upshot (due to taking in of microbial metabolites as a result of fermentation)

This advances towards the efficient consequences of probiotics that seems to be applied in non-dairy food items as chocolate products, cereals, honey, biscuits, cakes, dressing, sweetness, tea, and chewing gum (Vinderola, 2008). In general, probiotic bacteria in the food industry provide somehow difficulty in their multiplication and survival rate because of the distress conditions of gastrointestinal tract. To ensure shelf-life of probiotics, novel probiotics are being designed through microencapsulation technology that opposes environmental

conditions. Various factors could contribute to the beneficial aspects of probiotics, but its proper mechanism of action is still vague.

2.5. Characteristics of Probiotics

Probiotics have the capability to do carbohydrate fermentation and make small fatty chain fatty acids, causes decrease in celiac pH. Small chains fatty acids ratify the expansion of intestinal cells and involved in cell differentiation, thus, encourage the acclimatization and preoccupation process. They also take part in toxin nullification. They hamper the development of several pathogens; act as a barrier via competitive removal (commensal species for the similar sources of nutrients as possible pathogens) (Chapman *et al.*, 2011). Intestinal bacteria promote the development of immune system together by physical and practical means, in host gut. They generate the competence in immunoglobulins to withstand the proficiency of the immune system (Todd R Klaenhammer *et al.*, 2012). The probiotics strains are resilient to the enzyme existing in oral cavity. These endure the gastric acid location, regardless of the contact with the bile and pancreatic juice (in upper small intestine). These should not be sensitive alongside antibiotics. Attachment to the intestinal cells and discharge of antimicrobial complexes are also significant properties of probiotics that act as a competitor beside entheogenic (pathogenic) which reduce their survival. Probiotics possessed definite features that justify the subsequent striking features, gastric acid and bile salt tolerance (gastro-intestinal conditions), adherence competence to the mucous coating in GIT tract, non-toxic, non-pathogenic and devoid of any harmful effects, in-vivo existence in GIT, and competitive removal of pathogens from the path maintenance of living cell product in adequate quantity (Garcia-Mazcorro & Minamoto, 2013).

2.6. Commercially Available Probiotics

The probiotics are mainly defined by Food and Agriculture organization (FAO) of United Nations in 2001 as live microorganisms that are when administered on the host in calculated amounts conferred a health benefit. According to U.S. Food and Drug Administration (FDA), there is no definite health claims regarding the probiotics. Moreover, there are also variations regarding health benefits of probiotics because it varies due to different strains to and different conditions.

The FDA reported about the regulations that are required for the dietary supplements that are produced to ensure the purity and quality manner. The supplements should be contamination

free and impurities are in lesser amount and are must be labeled accurately. The regulations of these characteristics will enhance the probiotic supplements quality. Different probiotic strains provide different health benefits.

Some of the commercially available probiotics are:

- ✓ *Bifidobacterium lactis* HN019, which helps in modulating the immune system present in elderly persons and it is available in markets as food supplement and dietary ingredient.
- ✓ *Lactobacillus reuteri* ATCC55730, provide gut health benefits and it is available in ―BioGaia".
- ✓ *Lactobacillus rhamnosus* GG (LGG) that is available as drinkable yogurt in Danimals and in culturelles capsules.
- ✓ *Lactobacillus casei* DN-114 001 available in Dan Active products.
- ✓ *Bifidobacterium lactis* Bb-12 is available in Yo-Plus yogurt and Live Active cheese. It is used as uncooked condition for the best result.

For antibiotic related diarrhea following probiotic strains are recommended:

- ✓ *S.cerevisiae* (*S. boulardii*) that is available in Lalflor capsulesand florastor powder.
- ✓ *Lactobacillus rhamnosus* GG (LGG) is available in culturelles capsules and as drinkable yogurt in Danimals.
- ✓ *Lactobacillus casei* DN-114 001 is present in Dan Active products.
- ✓ *Lactobacillus acidophilus* CL1285 plus and *Lactobicillus casei* Lbc80r are available as BioK + CL1285 soy milk, Biok + CL1285 fermented milk and as capsules.

For the selection of most appropriate probiotics available in market genus, specie and strain name is considered. Several commercially available probiotics only contain specie and genus name on the label e.g. *Bifidobacterium lactis* in Kraft's and Live Active in Cheddar Cheese. Label on the probiotic products will tell about the availability of strain in the product and the quantity of probiotic in each serving product, the health benefits of the selected probiotic product and the probiotic amount used in the research.

2.7. Routes for Probiotic Administration

The health benefits and beneficiary effects of probiotics on health have been approved from previous studies such as oral application of Lactic acid bacteria to prevent bacterial vaginosis in women (Anukam *et al.*, 2006). Mostly probiotics are taken as dietary and food supplements (Jack *et al.*, 2010). Traditionally, probiotic strains have been used as a part of fermented products and it is in use from ancient times with very little knowledge of their beneficiary effects on health. The valuable strains helped in maintain health and prevent them from encountering with disease-manifestations. Commercially, most probiotics are available in chewable tablets and lyophilized forms. The probiotics not only contain the live microbes, but it may also consist of protein or even DNA of the strain that can execute their health benefits. Commercially available probiotics are safer to use because of the adaptation of recommended standards. Moreover, in commercial probiotics, the dosage and number of microbes are also considered unlike the traditionally food item where there is no control over microbial amount. The viability and the number of microbial cells are of great concern with respect to the positive influence on host‘s health. Nowadays, with the advancement in research regarding probiotic, the best route for administering probiotic to attain friendly micro-biota is of great concern.

2.7.1 Oral vs Sub-Cutaneous Administration

To determine the best administrating route, *L. salivarius* was administered orally and sub-cutaneous. Anti-inflammatory response was observed when bacteria was administered systematically, revealing the fact that oral rout is not the eventual necessity for beneficial health effects. The two administering routs exhibited the same health benefits within the host and no major difference was observed. So, it can be suggested that advantageous effects of probiotic does not depend on the two mentioned routes. In addition, the ease of probiotic intake route varies from person to person and should be considered while selecting the probiotic (Sheil *et al.*, 2004).

2.7.2. Live or Dead Cells Administration

Live and dead cells are also administered through sub-cutaneous and oral routes to determine their effect on health. A group –Cesolari" conducted this research and found that gastrointestinal problems are prevented regardless of the form and route of the probiotic strain administration. Irrespective of the viable or non-viable, subcutaneous or oral,

noticeable response of anti-inflammation was observed. Recent studies described that *salmonella* infection can be cured by taking the probiotic orally. The oral intake route is most preferred one against several health-compromised conditions. In addition, some studies also revealed that under compromised conditions sub-cutaneous rout is quite beneficial. Strong immune response was observed when *Lactobacillus* strain was administered systematically. In another study, the overall general health effects that were previously achieved by using oral route can be attained using combinatorial approach by administrating *S. faecalis, L. casei, L. plantarum* and *B. brevis* systematically (Adams *et al.*, 2008).

2.7.3. Sub-Lingual Administration

The high rate of mortality and morbidity is mainly due to the influenza viruses which interferes with immune responses and make the host immune-compromised. The probiotics have been used extensively as the preventive measure against infections due to influenza virus. Recently, sub-lingual route for probiotic administering was used in individuals infected with influenza. When *L. rhamnosus* was administered under such conditions through this route, it enhanced the natural killer cell activity along with T-cells as well as increased the IgA production. Moreover, lung IL-2 level increased dramatically. With these evidences, sub-lingual route can be used definitely as promising alternative for probiotic administration to the existing routes against influenza administration (Kim *et al.*, 2013).

2.7.4. Nasal Administration

In recent research work in the internationally recognized journal, Antiviral Research scientists focused on the administration of lactobacilli through nasal cavity route and tried to figure out its effect in delivering the protection in human against common cold. The effective way of probiotic administration that exert beneficial effects by using various ways of probiotic uptake, amount of probiotic strains and the form of administered probiotic cells i.e. live or killed. They studied that when *Lactobacillus* strains were administered through intranasal route, efficient protection was attained against common cold when compared to the orally administrated probiotic, thus become the choice for quicker relief. Intranasal probiotic administration boost up the production of secretory IgA, furthermore, also enhance the production of cytokines when compared to other modes of administration. In addition, before the dose intake determination of dosage formulation are of necessity for their beneficial

effects. B-cell mediated immunity has been reinstating in patients having pneumococcal infection, when *L. rhamnosus* was given through intranasal route (Hori *et al.*, 2001).

There is still needed to find out the exact mechanism behind the quick protection against respiratory pathogens when probiotic is administrated through intranasal route. It can be stated that under these circumstances, it grasps high potential to fight against pathogens. Number of ways are known and well reported for the administration of the probiotic but there is till need to study the effective way of administrating the certain probiotic. Because each probiotic is unique and there are also variations in attributes that distributes differently from strain to strain. Therefore, choice must be made according to the potential of specific probiotic strain.

2.8. Mechanism of Action of Probiotics

There has been recommended numerous mechanisms of action of probiotics which demonstrate the upshots of probiotics in a positive way. Probiotics strain provide useful aspects with respect to metabolic activities and survival rate in the gut (F. Chaucheyras-Durand & Durand, 2010). The probiotics generate its mode of action based on the specifications of strains (Newbold *et al.*, 1995).

In case of monogastric, bacterial probiotics produce organic acids, lactic or acetic acid which helps in the reduction of gut pH and prevention of pathogens from colonization. Thus, it aids in setting of much approving ecological environment for the resident microbiota (Servin, 2004). The probiotic strains can release ―bacteriocins" which are antimicrobial peptides and help in growth inhibition of pathogenic bacteria. Probiotics have potential to produce enzymes which enables the hydrolyzation of bacterial toxins (Buts, 2004).

Several strains of probiotics show elimination of pathogenic bacteria (with respect to their elevated affinity for nutrients or adhesive sites) (La Ragione & Woodward, 2003). Various probiotics show generation of such growth factors and nutrients that stimulate the favorable microorganisms of gut microbiota. Probiotic also generate host interaction and produce components that influence the mucosal expansion and metabolism of host‘s intestinal cells (Johnson-Henry *et al.*, 2008). A few probiotics seem to have metabolic and detoxification phenomena of definite inhibitory compounds for instance, amines, nitrates, or hunting for oxygen (anaerobic system of gut). Thus, the mechanism of probiotics demonstrates beneficial, nutritious as well as healthful effects both for animal and human gut. Probiotic

bacteria incorporate varied and diverse effects on host. Though their exact mode of action is still unclear but they have applied its mode of action on the basis of their innate and adaptive immune responses (in case of epithelial cells, dendritic cells, monocytes or macrophages, B-cells, T-cells), intestinal luminal environment, epithelial barrier function, and the mucosal immunity (Neurath, 2007; Z. L. Zhang *et al.*, 2007).

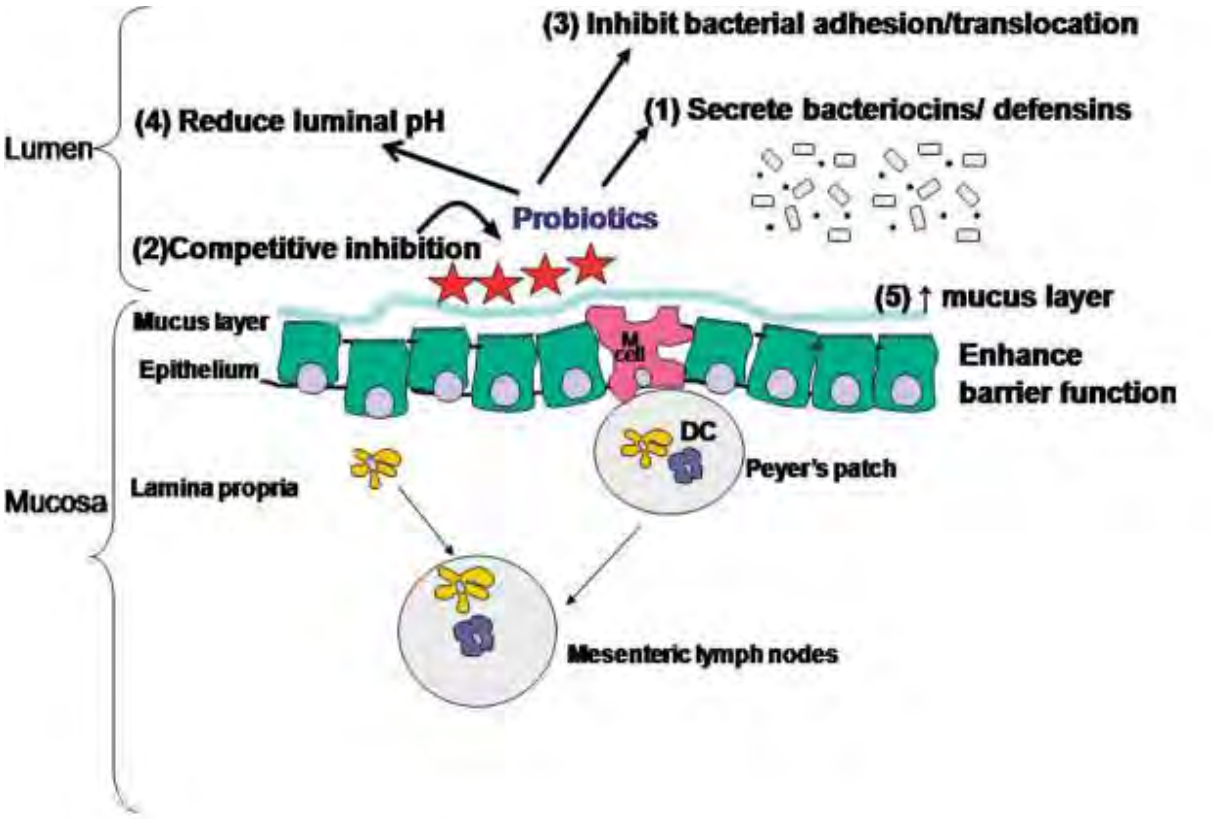


Figure 2. 1: Modes of action of probiotics (Zhang *et al.*, 2007)

Probiotic bacteria reduces the luminal pH, inhibits bacterial translocation and adherence properties, and produce antimicrobial substance or defensins. Thus, it aids in the alienation of pathogenic bacteria from gut. In the gut, the gut flora inhibits colonization of pathogenic bacteria by generating limitations in the physiologically restrictive environment such as pH, redox potential and hydrogen sulphide production.

2.8.1. Enhancement of Barrier Function

By means of enrichment of intestinal barrier properties in the course of cytoskeletal modulation and phosphorylation of tight junction proteins, probiotics promote mucosal (cell-cell) signaling and provides cellular stability. Numerous organized methods facilitate the maintenance of intestinal barrier purposes that primarily include chloride, mucus and water secretions and connection of epithelial cells to their apical junctions through tight junction proteins (Watts *et al.*, 2005). This system aids in health benefit to host and probiotic bacteria restrain it from number of diseases (Meddings, 2008).

2.8.2. Immunomodulation

2.8.2.1. Effect of Probiotic Bacteria on Epithelial Cells

The epithelial cells import significant variation at the point of signal transduction pathway and cytokine production which implicit the differentiation of probiotics and pathogenic bacteria. The signaling pathway permits different pathways to epithelial cells which enable the differentiation between probiotics and pathogenic organisms. In this mechanism, probiotics hinder the degradation of the counter regulatory factor (IKB), which in turn attenuates the pro inflammatory responses. On the contrary, pathogenic species stimulate the transcription factor (NF-B) that provokes pro-inflammatory reaction in the intestinal epithelial cells. Probiotic strains also facilitate in epithelial recovery or in case hinder apoptosis. In a study, a probiotic strain ―*Lactobacillus rhamnosus* GG", in the intestinal epithelial cells, aids in the prevention of apoptosis induced by cytokines (Lammers *et al.*, 2002; Otte & Podolsky, 2004).

2.8.2.2. Effects of Probiotic Bacteria on Dendritic Cells

Dendritic cells are antigen presenting cells used for the bacterial identification and determination of the successive T-cell response. Dendritic cells perform specific functions in the gut. They carry out oral tolerance induction via cytokines (IL-10 and TGF beta) that generate regulation of T cells and (Ig-A producing) B cells (Akbari *et al.*, 2001; Iwasaki & Kelsall, 1999; Williamson *et al.*, 2002). Intestinal DC's assist luminal bacteria directly and indirectly. By direct means, in the intestine lumen, they surpass their dendrites into the epithelial tight junctions and indirectly by passing through M cells.

Dendritic cells play its role in the junction of innate and adaptive immunity, which supports reorganization and reaction of bacterial components that cause initiation of primary immune

response and T and B cell responses. There has been studied a wide impact of probiotic bacteria on DC in various experimental structures (monocyte and bone marrow derived DC, whole blood DC, lamina propria DC) and in diverse species of human and mouse.

2.8.2.3. Effects of Probiotic Bacteria on Monocytes and Macrophages

Probiotic bacteria induce direct influence on lymphocytes, or they depict some nature of modifications in dendritic cells or on macrophages that alters the stimulation response of lymphocytes. These effects have been figured out in different sorts of lymphocytes (B Lymphocytes, Natural killer cells, T cells).

2.9. Classification of Probiotics

A diverse number of microbes considered and used as Probiotics. Based on this, classification of Probiotics can be done as below.

2.9.1. Non-bacterial vs. Bacterial Probiotics:

When talking about non-bacterial Probiotics such as fungal or yeast most commonly included *Saccharomyces cerevisiae* (SP Bai *et al.*, 2013), *Candida pintolopesii* (Daşkıran *et al.*, 2012), *Saccharomyces bourlardii* (Rahman *et al.*, 2013), *Geotrichum candidum* (Ghori *et al.*, 2018) and *Aspergillus oryzae* (Shim *et al.*, 2012). Apart from certain fungal and yeast Probiotics as describe before most of the microbes used as Probiotics are bacteria. Most commonly used bacterial Probiotics are belongs to many *Lactobacillus* species (Mookiah *et al.*, 2014), *Bacilus* (Abdelqader *et al.*, 2013), *Bifidobacterium* (Khaksar *et al.*, 2012; Pedroso *et al.*, 2013) and *Enterococcus* (Mountzouris *et al.*, 2010).

2.9.2. Non-spore Forming vs. Spore Forming:

Initially non-spore forming strains of Bifidobacterium and Lactobacillus predominated but now a days bacteria categorized as spore forming also used as Probiotics such as *Bacillus amyloliquefaciens* (Ahmed *et al.*, 2014) and *Bacillus subtilis* (Alexopoulos *et al.*, 2004; Cutting, 2011).

2.9.3. Single Specie or Strain Probiotics vs. Consortium (Multi-species or Strain):

Probiotics used come under different composition these could be consisted of a single species or strain or it could be a mixture or consortium of more than one species or strain. Most common and well-known Probiotics microbes used as single-species are *Saccharomyces servisia* and *E. faecium* (Abdel-Rahman *et al.*, 2013). Further, examples of Probiotics

consumed as mixture such as consortium of *L. salivarius, Lactobacillus reuteri, Enterococcus faecium* and *Pediococcus acidilactici* (Gaggìa *et al.*, 2010); consortium of *Bifidobacterium thermophilum, E. faecium* and *Lactobacillus spp.* (Pedroso *et al.*, 2013); and consortium of various species of *Streptococcus, Bifidobacterium, Lactobacillus, Bacillus* and *Saccharomyces* (Rahman *et al.*, 2013).

2.9.4. Autochthonous vs. Allochthonous Probiotics:

This classification of Probiotics based on the presence of the Probiotics microbe as normal inhabitant of host GIT or isolated somewhere else. Microbes which are normal inhabitant of the animals GIT and used as Probiotics are known as autochthonous Probiotics. While on the other hand microbes which not found normally in animals GIT and used as Probiotics referred as allochtchonous Probiotics (Bajagai *et al.*, 2016).

Probiotics used in the human and animals also comprised of spore forming bacteria, common example is *Bacillus subtilis* (Alexopoulos *et al.*, 2004). Advantage with the supplementation of the Probiotics comprising of bacterial spores is that they provide a safe passage through the stomach (Hoa *et al.*, 2000; Souza *et al.*, 2017). GIT of the ruminants contains a number of microbial species which help in fermentation and digestion of feedstuff (Stover *et al.*, 2016). Probiotics improve the feed digestibility, efficiency and ruminal fermentation by altering the gut microbiota of the animal (Seo *et al.*, 2010).

2.10. Common Genera Used as Probiotics

Enterococcus: These microbes are common commensals of animals and humans. *Enterococcus* genus like *Lactobacillus* are lactic acid bacteria (LAB) group. Human GIT most commonly harbors *E. faecalis* and *E. faecium,* while *E. faceium* in animals with high prevalence, further, food products found to be natural carrier of *Enterococci* (Fisher & Phillips, 2009).

Bacillus: Normally related with air, water and soil *Bacillus* species are spore forming Gram-positive bacteria. *Bacillus* species are mostly allochthonous, mostly they are not flora of intestinal tract but enter the GIT via contamination of feed. There are a lot of concerns regarding safety of *Bacillus* spores as Probiotics, seed coating or plant fortification products (Sanders *et al.*, 2003).

Saccharomyces: *Saccharomyces* are ubiquitous in nature found in soil, plants and fruits as part of intestinal flora. Due to its vital role in fermentation *S. cerevisiae* added in beverages, foods and health foods (van der Aa Kühle & Jespersen, 2003).

Bifidobacterium: They are considered as vital genera found in the GIT of the humans as well as animals. *Bifidobacterium* importance and potential role can be assessed by their presence in high being linked with good status of health. It is strongly believed that they help in maintaining balance of GIT microbiota and plummeting risk of infection with pathogens. A number of species belongs to this found to be host specific (Biavati & Mattarelli, 2006).

Probiotics With Undefined Microbes: Competitive exclusion (CE) or Nurmi concept come from the phenomenon when contents from the gut of a healthy chicken taken prepared and then suspension drenched to newly hatched chick as a result of which chick treated with suspension remain protected from the colonization of highly pathogenic *Salmonella* (Nurmi & Rantala, 1973). For successful achievement of the desire purpose CE bacteria should be introduced during early days of life, because CE bacteria to become antagonistic or competitive against opportunistic pathogenic microbes. Preparation of undefined suspensions from fecal or cecal contents could result in transmission of pathogenic microbes put regulatory concerns regarding such products. Though, products have CE potential carrying identified and distinct microbes being produced and applied in animals (Schneitz, 2005).

2.11. Selection Criteria for Potential Probiotics

2.11.1. Bile Tolerance Effect

Bile is a yellowish green aqueous solution mainly consists of cholesterol, phospholipids, biliverdin pigment and the bile acids (Carey & Duane, 1994; A. F Hofmann, 1994). Bile synthesis occurs in the pericentral liver cells, stored and accumulated in the gall bladder, and after ingestion, released into the duodenum. Bile plays its role in solubilization and emulsifying of lipid contents, supports fat assimilation. Thus, it can be act as a biological detergent which also represents strong antimicrobial activity by terminating bacterial membranes (Begley *et al.*, 2005).

Primarily, de novo synthesis of cholic, chenodeoxycholic and bile acids takes place in the liver (from cholesterol). There is an effective preservation of bile salts under usual conditions by means of –enterohepatic recirculation" process. By means of active transportation,

conjugated and unconjugated bile acids are assimilated in the terminal ileum while in the gut portion by passive diffusion (Batta *et al.*, 1990). Hepatocytes reabsorbed bile acids in the portal bloodstream, which is then re-conjugated and re-secreted in the form of bile. Native intestinal flora modifies the overall bile acid and around 5% of overall bile acid (0.3-0.6g/day) evades epithelial incorporation (Bortolini *et al.*, 1997). ―Deconjugation" is the fundamental step occurs before modifications. Bile salt hydrolase (BSH) enzyme catalyze the deconjugation process in which amide bond are hydrolyzed and glycine/taurine components are released from the steroid core. This results in liberation of deconjugated bile acids (Batta *et al.*, 1990).

2.11.2. Incidence of BSH Activity Among Bacteria

There has been reported BSH activity among number of bacterial species including *Bacteroide*, *Bifidobacterium*, *Clostridium*, *Enterococcus*, and *Lactobacillus*. Among them, *Bifidobacterium* and *Lactobacilli* are normally applied as a source of probiotics, whereas, *Enterococcus*, *Clostridium*, and *Bacteroides* are also remarked under commensal or probiotic category, and act as normal residents of gastrointestinal tract. Almost all gram-positive bacteria of intestinal tract (except for few Bacteroides) possess positive BSH activity, while gram negative bacteria lack this activity. (Ahn *et al.*, 2003; Elkins & Savage, 1998; Moser & Savage, 2001).

Listeria monocytogenes, a notorious pathogen of gastro-intestinal tract and is gram positive. Basically, it is not believed as a constituent of gastrointestinal flora, but it has BSH enzyme. On this basis, its position is recommended on the edges of commensal and pathogenic species. Besides this, *Enterococcus faecalis* act as an opportunistic pathogen also have a *bsh* homolog (EF0040; AAM75246) found near pathogenic boundaries, but it is not properly characterized up till now (Shankar *et al.*, 2002).

2.11.3. Cholesterol Assimilation

One of the properties revealed by probiotics is their lowering effect of cholesterol. The mechanism lies behind cholesterol assimilation is the deconjugation of bile slats through microbes that show rapid transit in the small bowel (Gilliland, 1990). Probiotic bacteria boost up or produced a number of factors by which cholesterol synthesis is restrained in the body (Mann, 1977). In addition to cholesterol lowering effect, probiotic bacteria also play its vital

functions by mounting phenol tolerance, neutralizing the latent carcinogens, provoking immune response and metabolic activities, and reducing constipations.

Cholesterol-lowering effect by probiotic bacteria has been suggested in vitro, based on recommended hypothesis which exhibit the enzymatic deconjugation of bile acids, binding of cholesterol to bacterial cell wall, lowering of cholesterol by bacteria, and the fermentation of short chain fatty acids (particularly propionate) and its end-products by physiological actions. These purposed mechanisms of cholesterol lowering effect have been applied on humans and animals studies but the exact mechanism of action on probiotic bacteria is still ambiguous (Gilliland *et al.*, 1985; Klaver & Vandermeer, 1993; M. Y. Lin & Chen, 2000; Noh *et al.*, 1997; Tahri *et al.*, 1996, 1997; Usman, 1999).

2.11.4. Anti-microbial Activity

The Lactic acid bacteria (as a potential probiotic) also contribute towards the advancement of anti-microbial compounds. Among these compounds, Bacteriocins are the most noticeable proteins or peptides, which are synthesized by ribosomes and destroy the pathogenic bacteria (Corr *et al.*, 2007). Hence, bacteriocins produced by LAB are being used as a putative agent for probiotics as well as biological control agents.

Unusual antimicrobial compounds by LAB are categorized under and high molecular mass (HMM) and low-molecular mass (LMM) compounds. HMM include bacteriocins like compounds, which can counter act the pathogenic and spoilage causing bacteria in the foods. LLM include uncharacterized compounds, CO_2, diacetyl (2,3-butanedione) and H_2O_2 (Jay, 1982; T. R. Klaenhammer, 1988; Piard & Desmazeaud, 1991, 1992).

Up till now, variety of bacteriocins have been ascertained such as: *Streptococcus salivarius* has produced a new type of bacteriocin, similarly, *Enterococcus avium* produced avicin A (class IIa) and another (class IIa) production from *Enterococcus faecalis* strains. Some sorts of unknown bacteriocins are also reported in which *Lactobacillus gasseri* generate two-peptide gassericin, *Lactobacillus fermentum* and *E. faecalis* encodes uncharacterized bacteriocins, two from *L. fermentum* and one from *E. faecalis* (Birri *et al.*, 2013).

A very large number of micro-organisms inhabit the gastrointestinal tract of animals. Approximately 1014 bacteria belonging to 200 species and 45-50 genera are present on the mucus membrane in gut of the animals (Savage, 1998). Gut micro-flora is assumed to be an

important and influential factor in maintaining and regulating health and pathological conditions. It plays an important role in development of immunity in the host and also results in many favorable effects on the host, therefore beneficial microbes can be administered to the animals as probiotics. The microorganisms which are mostly used as probiotic strains belong to *Lactobacillus, Bacteroides, Bifidobacterium, Eubacterium, Fusobacterium* and *Clostridium*. Some yeast strains are also appropriate for probiotic use (García-Hernández *et al.*, 2012). Some Lactic acid bacteria (LAB) such as strains of *Pediococcus* and *Enterococcus* species are members of gut micro-biota of many animals but are very often used as probiotic strains (Rahman *et al.*, 2013). Many probiotic strains can be isolated from fermented food items and are generally regarded as safe (GRAS). These microorganisms produce a diverse range of health beneficial compounds in host such as different aromatic compounds, antimicrobial peptides for pathogenic bacteria and organic acids without producing toxic effects (Zoumpopoulou *et al.*, 2018). The microbial ecosystem has effects on the metabolic pathways and productivity of host animals and to investigate the functional relationship between gut micro-flora and host metabolism is not very easy (Cani & Delzenne, 2009).

2.12. Sources of Probiotics

The probiotics are best available in the form of ~~"f~~ermented milk products", ~~"d~~airy products", ~~"y~~ogurt", and in some ~~"n~~on-dairy food items".

2.12.1. Food Items

For that matter, the food items including mayonnaise, soymilk, meat products, baby food, ice-cream, fruit drinks, and vegetables are being enriched with probiotic microorganisms. They are also delivered in the form of supplements as, capsules, tablets, and freeze-dried preparations (Homayouni *et al.*, 2008).

2.12.2. Fermented Food Items

Dairy and dairy-associated products provide excellent sources of probiotics (Liong, 2011). Variety of microorganisms, specifically Lactic acid bacteria, *Bifidobacterium* (from fermented milk) has been applied as primary source of probiotics since centuries.

The conventional milk fermentation process has intricated Lactic acid bacteria composition, thus act as a potent probiotic source. In a latest designed study, various LAB strains were isolated and identified from different dairy food stuffs such as from KURUT (fermented yak

milk), some yeast and lactobacillus strains from Koumiss (fermented milk drink), Kefir grains and Masai milk. Microorganisms screened out from these sources thus enhance their immunity level (Audisio & Benitez-Ahrendts, 2011; Patrignani *et al.*, 2006; Romanin *et al.*, 2010; Ya *et al.*, 2008). Naturally, Lactobacillus specie is evaluated as source of probiotics that is primarily accessible from traditional and natural fermented products (S. M. Lim & Im, 2009; Won *et al.*, 2011) which includes "*Weisella*" specie isolated from Nigerian fermented food and is behaved as a potential probiotic (Ayeni *et al.*, 2011).

2.12.3. Non-dairy Fermented Products

They also set forth the criteria of probiotics strains (Rivera-Espinoza & Gallardo-Navarro, 2010). A reported study manipulate the *in vitro* characteristics of bacterial strains that include *L. sakei*, *L. curiatus* and *Staphylococcus carnosus* from meat, *L. paracasei* and *L. plantarum* from fruits exhibit the same metabolic and functional properties like human intestinal flora (Haller *et al.*, 2001). Additionally, *Lactobacillus* strains from brine of naturally fermented Aloren (green table olive) and *L. buchneri* P2 from pickled juice have revealed some of the probiotic properties as, acid and bile tolerance, antimicrobial activity and cholesterol reduction (Zeng *et al.*, 2010).

2.12.4. Other Sources

Gastrointestinal tract is an effective and primary source of probiotics which inhabit almost more than 500 bacterial species. *L. gassei* and *L. reuteri* is the most commonly used probiotic specie that resides in the human gastro-intestinal tract (Ryan *et al.*, 2008). Similarly, *B.longum* (D. Srutkova *et al.*, 2011) and *L. acidophilus* (P. P. Lin *et al.*, 2009) strains were screened out respectively from healthy human adult and being marked as probiotics. Besides this, probiotic strains are also profound in animals' gastro intestine, commonly in rats, pigs, and poultry sources (Petrof *et al.*, 2012). Bee gut induces beneficial response to honey bee colonies (Audisio & Benitez-Ahrendts, 2011). Moreover, GIT of marine and fresh water fish such as, carassius auratus gibelio (Chu *et al.*, 2011), rainbow trout (Perez-Sanchez *et al.*, 2011) and shrimp (J. E. Hill *et al.*, 2009) are also rich source of probiotics.

Furthermore, in a reported study, human breast milk also provides *Lactobacillus* strains that act as probiotic source. This helps in generation of T-cells and natural killer cells and also responsible for regulatory T cell expansion that simultaneously heighten natural and acquired immunity (Perez-Cano *et al.*, 2010).

2.13. Probiotics in Animal Nutrition

2.13.1. Poultry

As the demand for the animal‘s origin food products on the rise worldwide, poultry playing its significant role by contributing as the cheap source of animal origin protein (Furtula *et al.*, 2013). Poultry meat consumption climbs to second position after pork meat globally, in addition trade and consumption of poultry foods rapidly increasing as the population of the globe. Probiotics supplementation in the feed of broiler resulted in increased growth rate (Afsharmanesh & Sadaghi, 2014; Lei *et al.*, 2015; Mookiah *et al.*, 2014; H. Zhang *et al.*, 2014), feed efficiency and good health status. Elevated antibody titer in the blood observed following vaccination against Newcastle disease in broilers supplemented with components of yeast cell wall and hydrolyzed yeast in the feed (Muthusamy *et al.*, 2011). Probiotics maintain health status in the broiler by preventing it against several important diseases such as salmonellosis (Biloni *et al.*, 2013; Haghighi *et al.*, 2008; Tellez *et al.*, 2012), coccidiosis (Dalloul *et al.*, 2003) and necrotic enteritis (Jayaraman *et al.*, 2013). But outcomes are not consistent in every study supplementing Probiotics. Several types of Probiotics including spore forming to non-spore formers as well yeasts have been investigated for their role in improving growth rates of commercial poultry birds (Afsharmanesh & Sadaghi, 2014; SP Bai *et al.*, 2013). As the feed cost in poultry production systems is the largest portion of cost, feed cost can be controlled by optimizing feed efficiency (Shim *et al.*, 2012). Growth rate improvement in response to Probiotics treatment among broiler birds found related with enhanced feed intake. In addition to enhanced efficiency of feed. According to a previous study (Afsharmanesh & Sadaghi, 2014) there wasn‘t any substantial increase in feed conversion ratio (FCR) in response to improved feed intake. Contrary to this there wasn‘t any improvement in feed intake while FCR was increased in another study (H. Zhang *et al.*, 2014). But also significant improvement in FCR and feed intake has been reported (Landy & Kavyani, 2013). There are few studies related to carcass quality and yield. Effects of Probiotics supplementation on quality and yield of the carcass are still ambiguous. Increase in carcass yield or ready to cook weight of carcass found in response to commercially available Probiotics strains both in the form of consortia and single strain (Abdel-Raheem *et al.*, 2012). Proper functioning of the intestine strongly associated with the mucosa of the intestine and ultimately to the growth performance of poultry bird as nutrients absorption takes place in intestine. As ratio of the crypt and villus height increases absorption capacity of the nutrients

also increases due to the availability of large surface area (Afsharmanesh & Sadaghi, 2014). In intestine ration between villus and crypt found increased during histological studies of intestinal mucosa in response to supplementation of Probiotics carrying lactic acid bacteria *P. parvulus, L. salivarius* (Biloni *et al.*, 2013), *B. subtilis* (Afsharmanesh & Sadaghi, 2014; Jayaraman *et al.*, 2013), *E. faecium* (Abdel-Raheem *et al.*, 2012) and *B. coagulans* (Hung *et al.*, 2012). Probiotics also influence the egg production and quality, but the results are inconsistent (Bajagai *et al.*, 2016). Studies exist showing both enhanced egg production (Gallazzi *et al.*, 2008; Kurtoglu* *et al.*, 2004; Xu *et al.*, 2017; Yörük *et al.*, 2004) and no effect regarding egg production (Asli *et al.*, 2007; Capcarova *et al.*, 2010; Dizaji & Pirmohammadi, 2009; Mikulski *et al.*, 2012) in response to supplementation of feed with Probiotics. In addition, while dealing with mounting problem of antibiotic resistance, substitutions are the most favorable options under consideration these days. Probiotics are one of the best alternatives in poultry production systems while dealing with antibiotics resistance. Probiotics found helpful in lowering the pathogens load in intestine of poultry by inhibiting the colonization and dispersion of both zoonotic as well as other pathogens of intestine (Bajagai *et al.*, 2016).

2.13.2. Pigs

Pork meat is the mostly consumed meat globally. Use of antibiotics as growth and performance promoters in swine industry is still rather common. So, antibiotics alternatives for performance improvement among pig production systems considered necessary. Substitution of antibiotics with Probiotics among monogestrics mostly studied in poultry but its use in swine industry less studied. Distinctions in dose, microbes used, husbandry practices and duration of use among pigs make it difficult while we make generalization with other livestock (Kenny *et al.*, 2011). There are less consistent results regarding growth rate among pigs as compare to poultry in response to Probiotics introduction in feed (Bajagai *et al.*, 2016). Bettered *Bifidobacteria* to *E. coli* ratio as well as positive effects on the growth performance among piglets achieved as a result of *B. animalis subsp. lactis* introduction in the feed (Modesto *et al.*, 2009). In different production systems farm animals frequently challenged with stresses (environmental, diet, management protocols etc.), which leads to imbalance in the ecosystem of the intestine and consequently rise of risk factors regarding pathogens infection (Gaggìa *et al.*, 2010). In most instances Probiotics imparts their effects on health rather than other parameters in pig production systems. Probiotics have benefited

the health parameters of pigs by reducing the incidence of post weaning diarrhea major cause of death during early lives and also led to reduced morbidity and mortality incidence among adult pigs (Bajagai *et al.*, 2016). Improvement in average daily weight gain, lowered incidence of diarrhea and enhanced performance observed among piglets supplemented with *E. faecium* starting from birth to start of weaning (Zeyner & Boldt, 2006). Post weaning piglets with diarrhea induced by *E. coli* K88 were supplemented with *L. rhamnosus* GG and as a result of this supplementation diarrhea was ameliorated possibly by enhanced antibody defenses of intestine, intestinal microenvironment modulation and regulation of inflammatory cytokines (L. Zhang *et al.*, 2010). Effects of Probiotics supplementation on *E. coli* colonization, shedding and load in the GIT of the pig reported by many studies, like *P. acidilactici* and *S. cerevisiae* introduction in the feed of pigs at the rate of 2×10^9 cfu/kg for consecutive four weeks reduce the load of *E. coli* and other coliforms for a temporary period (Le Bon *et al.*, 2010). Addition of Probiotics in pig feeding practices enhanced the numbers of bacteria producing lactic acid and lowered the numbers of *E. coli, Clostridium* and *Enterobacterium spp.* in the microenvironment of pigs GIT (Bajagai *et al.*, 2016). *Lactobacillus rhamnosus* and *Bifidobacterium lactis* individually lowered the mucosal adherence of *Salmonella, Clostridum spp.* and *E. coli* in the intestine of swine. While using these two in the form of consortia deliver more effective outcomes in addition to reduction of each other‘s adherence (Collado *et al.*, 2007). Further an interdisciplinary study demonstrate the Probiotics mode of action in swine, showed lowered pathogenic bacteria load among healthy sows and piglets in response to *E. faecium* NCIMB 10415 supplementation (Lodemann, 2010).

2.13.3. Probiotics in Dairy Cattle

GIT flora of the animals as well as humans can be categorized an organ with metabolic activity due to its immense diversity both in term of high number of cells as well as species, cells number might reach to 10^{14} (Bäckhed *et al.*, 2005). These microbes are crucial in maintaining the physiology of the host, because without a healthy microbiota of the rumen, proper functioning of the ruminants is impossible (Jami *et al.*, 2014). The GI tract of a calf at birth is sterile and immediately colonization of intestine begins after birth. After that as animal grows complex microbial ecosystem establishes in large intestine (Stewart *et al.*, 1988). Molecular level monitoring of bacterial communities in intestine shows that it experiences dynamic changes in first twelve weeks (Y Uyeno *et al.*, 2010). *Clostridium*

coccoides, Eubacterium rectale group and *Bacteroides, Prevotella* group make a major fraction of about 50-70% of microbiota during 12 weeks after birth while the number of *Faecalibacterium* and some probiotic bacterial groups e.g. *Lactobacillus* & *Bifidobacterium* decrease in number with the age of animal. Major microbial groups in rumen of cattle are *Archea, Bcteriods, Fermicutes, Fibrobacter* and Protozoan species while in the large intestine of pre-weaned calves are *Bifidobacterium, Bcteriods, Fermicutes* and *Atopobium* are dominating. However, in the large intestine of adult cattle *Fibrobacter, Bacteroides* and *Firmicutes* (including un-culturable groups) dominate. Changes in gut microbiota occur according to physiological as well as metabolic development of gastrointestinal tract (Dehority, 2003a). In rumen animals have a complex microbial ecosystem having diverse range of anaerobic microorganisms which play important role in digesting fibrous plant polymers and their fermentation (Dehority, 2003a). Many factors can-strongly affect metabolic activities and structure of microbial communities which can impair the health status and performance of livestock animals (Dehority, 2003b). For example excess fat deposition in dairy cattle is a great risk factor-for digestive, metabolic, reproductive and infectious disorders (Schröder & Staufenbiel, 2006).

Commensal bacteria considered one of the essential assets regarding health under normal circumstances, because they have a protective effect on intestinal homeostasis, integrity and structure in addition to nutritional function (O'Hara & Shanahan, 2007). Association regarding role of the rumen microbiome with the overall performance of the dairy cows like production and metabolic traits well-established and therefore provided valid reason to investigate as well as correlate interactions between rumen microbiome and host. Strong and vital correlations between different production traits like milk production, feed efficiency etc. and rumen microbiome have been confirmed in addition to association of the microbial diversity with rate and quality of fermentation in the cattle rumen (Schären *et al.*, 2018). Mucosal immunity instructed and primed by intestinal flora acting via actively exchanging regulatory developmental signals which consequently protects host against infections. Microbiota of the intestine is an intricate environment comprising of diversity of microbes and beneficial role of most bacteria on the host still not clear, many bacterial species have shown protection from enteric infections like species of bacteria belonging to genera *Bifidobacterium* and *Lactobacillus*. By increasing the beneficial components of microbial

flora of the gut makes it possible to deal with several GIT disorders and sustain well-being of host (O'Hara & Shanahan, 2007).

Administration of Probiotics in the feed of dairy cows yield significant benefits, by maintaining the balance of gut flora as well as increase the overall bacterial diversity in the rumen (Pinloche *et al.*, 2013; Stein *et al.*, 2006). Live yeasts are among the most efficient and common Probiotics utilized in the nutrition of ruminants due to its ability in stabilizing the environment of rumen which would favor the proper functioning of rumen flora, more importantly fibrolytic bacteria. Ability of yeast cells to maintain their viability throughout the GIT makes it good Probiotics. Yeast *S. cerevisiae* provided vitamins and organic acids to encourage the LAB growth when introduced in the feed of ruminants (Khan *et al.*, 2016) which in return bettered metabolism of the rumen by enhancing the cellulolytic bacterial populations, stabilizing the pH of rumen, enhancing anaerobiosis via oxygen scavenging in the rumen and competing for substrate against lactate producing bacteria (Chiquette, 2009; Marden *et al.*, 2008; Vibhute *et al.*, 2011). Inclusion of yeast culture (YC) in the ration of the dairy cows becomes a common feeding practice due its positive effects on milk composition and milk yield (Poppy *et al.*, 2012). Enhanced utilization of feed or FE found associated with YC supplementation (Schingoethe *et al.*, 2004), apparently due to the improved digestion of organic matter (OM) digestion by *S. cerevisiae* (Desnoyers, Giger-Reverdin, Duvaux-Ponter*, et al.*, 2009) and more rumen pH stability (Bach, 2012). Supplementation of YC to dairy cows create more stable environment of the rumen which enhance the digestion rate of fiber by reducing physical effectiveness fiber and it justify the enhanced DMI that resulted in improved milk composition in addition to yield (Oelberg & Stone, 2014). A major challenged faced while formulating rations for dairy animals is the inherited variability regarding nutrients present in ration either due to the altered ingredient's composition or due to mixing errors while making total mix ration that leads to high proportion of starch in diets already high in starch ultimately resulted in SARA (O AlZahal *et al.*, 2014). SARA is an important metabolic disorder among dairy herds affecting mostly cows while they are in their early or mid-lactation period or periods of high production. It affects the fermentation of the rumen, productivity, animal welfare and overall profitability of the farm (Colman *et al.*, 2013). Diets high in concentrates during high production period of lactation leads to lower pH of the rumen resulted from VFA's accumulation in the rumen and ultimately to SARA (Plaizier *et*

al., 2008). Impact of SARA on dairy production can be assessed by the fact that culling rate could be 0.45 among dairy herds having problem of SARA, further, this rate of culling 0.20 high than recommended culling rate. Inclusion of yeast products, either in the form of YC or just live yeast cells, shown to significantly attenuate the shifts in ruminal pH, bettered fiber digestion milk fat. That inclusion of yeast to feed of dairy cows might have influence the rumen flora composition and number and leads to improved carbohydrate digestion. Incidence of SARA significantly reduced among dairy cows provided with yeast supplementation, especially when cows were on high starch diets. These improvements in fiber digestion, high microbial nitrogen yield and composition of ruminal flora might explain the improvements in milk composition as well as milk yield (Dias *et al.*, 2018). Compositional changes in the ruminal flora upon Probiotics inclusion in the feed leads to balanced microbiota in the rumen and good health status which potentially improved fermentation and curb the disease risk in lactating cows (Nocek & Kautz, 2006). There is need to increase the efficiency of fiber breakdown and utilization because, regardless the fibrolytic microorganisms‘ presence, fiber is not digested properly as fiber present in the dung can be further fermented (D. O. Krause *et al.*, 2003). Structural carbohydrates like hemicellulose and cellulose availability as energy source in addition to increased digestion could be achieved by Probiotics inclusion in feed of ruminants. Enhanced activities of the bacteria associated with cellulolytic functions and stimulation of the fungus responsible for lignin tissues solubilization observed while SC I-1077 live yeast supplemented in ruminant‘s nutrition (Frédérique Chaucheyras-Durand *et al.*, 2012). Cows provided with live yeast having Probiotics potential enhanced the population of fibrolytic bacteria *Ruminococcus flaveafaciens* and *Fibrobacter succinogens* increased by 85% and 45% respectively in the rumen of the cows (Mosoni *et al.*, 2007). Digestion of silages with poor fiber degradation enhanced by 24% in response to yeast supplementation and it has been found that supplementation of specific yeasts might enhance availability of metabolizable energy from silages comprising of low-quality maize in addition to glucogenic potential of diet, both of these ultimately would enhance the performance of dairy cows (Guedes *et al.*, 2008).

In addition to yeast, bacterial Probiotics also have been supplemented in ruminant‘s nutrition to get beneficial effects on animal performance; they have shown to enhance feed efficiency (FE), weight gain (WG) and DMI (Elghandour *et al.*, 2015). Bacterial Probiotics in ruminants

might have inhibited the growth of pathogens, instigated immune response by bacteriocin's secretion and modulated the GIT microbial balance (Khan *et al.*, 2016). Bacterial supplementation in dairy cows enhanced milk fat, fat-corrected milk yield as well as overall milk yield (Elghandour *et al.*, 2015; Khan *et al.*, 2016). According to an experimental study supplementation of Probiotics among animals did not imparted significant effects on fecal microbial diversity and load but significantly affected the populations of fermentative bacteria in rumen (*Coprococcus, Bacteroides, Clostridium, Ruminococcus, Dorea and Roseburia*) and increase the beneficial bacterial (*Faecalibacterium prausnitzii*) load. Further, it suppressed the load of pathogens opportunistic in nature like *Cronobacter sakazakii*, *Bacillus cereus* and *Alkaliphilus oremlandii* (Xu *et al.*, 2017). Inclusion of *S. bovis* in the diet of ruminants prevented ruminal acidosis, (Niu *et al.*, 2018) while during acidosis facultative anaerobe *S. bovis* was among predominated flora (Owens *et al.*, 1998), indicated its potential as Probiotics. Information regarding mechanism as well as mode of action needed further investigation.

Chapter 3

Isolation and Characterization of Potential Probiotics Strains from Different Sources

3. Isolation and Characterization of Potential Probiotics Strains from Different Sources

3.1. Introduction

Nurturing of microflora beneficial to host (Sánchez *et al.*, 2017), toning up effects on the host health (Kerry *et al.*, 2018) and curbing the threats to global health like antibiotic resistance by probiotics (Relman & Lipsitch, 2018) making it trendy to hunt for novel Probiotics. Link between brain and gut quite evidenced now a days (Santos *et al.*, 2019) and in addition contribution of gut dysbiosis in chronic diseases fairly evidenced (M.-F. Sun & Shen, 2018). There is vast array of niches (conventional and unconventional) (Sornplang & Piyadeatsoontorn, 2016) that could be source of strains with Probiotics potential, like, human feces (Gharbi *et al.*, 2019; Gheziel *et al.*, 2019) and breast milk (Rajoka *et al.*, 2018; Zacarías *et al.*, 2019) could be good source of Probiotics to be used in humans. Foods of animal origin like dairy fermented products (Jeong *et al.*, 2019), raw milk (Kalhoro *et al.*, 2019), yogurt (Islam *et al.*, 2018) and foods of plant origin including fermented plant products (Yerlikaya, 2019) another good source of strains having potential Probiotics effects. Many probiotics were isolated from aquaculture like *Kocuria* SM1, *Rhodococcus* SM2, (Sharifuzzaman *et al.*, 2018) *Lactococcus lactis* WFLU12 (Nguyen *et al.*, 2017) Probiotics were isolated from the gut of fish, isolation of Probiotics from donkey milk have been reported (Rashmi *et al.*, 2018). Screening of microbes with probiotics potential also carried out from sources like soil (Mohkam *et al.*, 2016), grains (Gut *et al.*, 2019; Mantzourani *et al.*, 2018), juices (Naeem *et al.*, 2012) and honeycomb (Pajor *et al.*, 2018; Tajabadi *et al.*, 2013). Like *Lactobacillus plantarum* has been found and isolated from variety of juices that includes both citrus as well as solid fruits. Contrary to this *Leuconostoc mesenteroides* having Probiotics potential found associated with tomatoes but not in previously mentioned fruits (Naeem *et al.*, 2012). In addition, Probiotics sourced from tomato juices and pineapple wastes (Amorim *et al.*, 2018; Patel *et al.*, 2014) can be grown on the medium used to grow LAB. In addition to honeycomb probiotics strains have also been isolated from honeybee (Mathialagan *et al.*, 2018) and other insects (Borah *et al.*, 2019). Belonging to genera *Enterococcus* and *Lactobacillus*, lactic acid bacteria (LAB), among most commonly used bacterial probiotics posing positive effects on host health in addition to fight against pathogens (Imperial & Ibana, 2016). *Enterococci* used in food products like cheese as starter cultures, as probiotics among animals and humans, also as additives in silage (Moreno *et al.*, 2006). *Saccharomyces* are ubiquitous in nature found in soil, plants, fruits and as part of intestinal flora. Due to its vital role in fermentation *S.*

cerevisiae added in beverages, foods and health foods (Brysch-Herzberg & Seidel, 2017). *Bifidobcaterium* are considered as vital genera found in the GIT of the humans as well as animals. *Bifidobcaterium* importance and potential role can be assessed by their presence in high being linked with good status of health (Gotoh *et al.*, 2018; Dagmar Srutkova *et al.*, 2015). Following isolation characterization for the Probiotics potential has been based on the selection criteria that includes amylolytic, proteolytic and cellulolytic activity in addition to mimic gut survival, cell hydrophobicity, anti-pathogenic activity and cholesterol assimilation (Thakur *et al.*, 2016). Further, safety assessment regarding isolated potential probiotics should be made. Susceptibility to antimicrobials used in veterinary and human medicine, identification of virulence related factors, cytotoxic effects on epithelium of intestine, transmission of resistant genes and adherence capacity must be factors of prime focus regarding safety assessment (Ayala *et al.*, 2019).

The present study was designed to isolate the probiotic bacterial and fungal strains from local fermented products i.e. Dahi and corn silage as well as from cows‘ dung with promising probiotic characteristics in dairy cows of tropical regions.

3.2. Materials and Methods

In first phase, pre isolated strains from Dahi and silage isolates were re-inoculated and characterized by using routine microscopic and biochemical methods. Cow dung samples were selected for isolation of microbial strains with probiotic potential. The isolates were purified and characterized by using routine microscopic and biochemical methods. Selected isolates were further assessed for their enzymatic potential by performing different enzymatic activities like; amylolytic activity, cellulolytic activity, proteolytic activity, bile salt tolerance, cholesterol assimilation. Antimicrobial capacity of selected isolates was also tested.

3.2.1. Isolation of Microbial Strains

A total of 12 cattle dung samples (10 gram) were collected in re-closable polythene bags aseptically from NARC experiment cattle farm and were transported to process in laboratory for microbial isolation. Isolation from silage has been done by taking10g of twelve silage samples which were grind in a sterilized way. Homogenized samples were dissolved in 90ml of trypticase salt solution and mixed through vortex for 15 minutes. In the similar way Dahi samples were taken from different Dahi shops and were processed for isolation of microbial

strains. All samples were inoculated on selected media including TSA (tryptic soy broth) and M-17 for bacterial isolation and Oxy Tetracycline Glucose Agar (OGA) for yeast isolation. The inoculation was done by spread plate method and plates were incubated at 32˚C for 24hours for bacteria and at 25˚C 48 hours for fungus isolation. All the samples were treated with same methodology.

3.2.2. Identification of Microbial Strains

Bacterial and yeast strains isolated by using different media were preliminary identified phenotypically and were further confirmed through 16S rRNA gene sequencing while the yeast strains were identified by Internal Transcribed Spacer (ITS).

3.2.3 Phenotypic Characterization

All isolates were examined according to Berge‘s Manual of Systematic Bacteriology. On each plate, colony morphology was observed for the selection of microbial isolate for further study. Cell morphology of pure colonies was examined microscopically after gram staining and phenol cotton blue staining, for bacterial and yeast isolates. Further these isolates where phenotypically characterized through different biochemical testing.

3.2.4. Biochemical Characterization

Routine biochemical tests included catalase test, oxidase test, citrate utilization test, methyl red (MR) test, sulfide indole motility (SIM) test and triple sugar iron test (TSI) were performed for the identification of bacterial and yeast isolates.

For catalase test, a smear of 24-hour fresh growth isolates was prepared. Bubble formation after putting 2-3 drops of H_2O_2 counts for positive results. In oxidase test, sterilized filter paper was soaked in oxidase reagent. A single isolated colony was picked with red hot sterile loop and rubbed against it. Change in color indicate positive while no change support negative results. The citrate utilization test provides the mean to study either the isolated strains utilize sodium citrate as a sole source of nitrogen (inorganic) and carbon (organic) source or not. To analyze this, loop full culture of freshly prepared isolated strains was streaked out on Simon‘s citrate agar slants. These slants were then placed in incubator (37˚C±24 hours). If slant‘s color changes from green to blue, its indication of positive result, if not means negative result.

In Methyl Red test this test, autoclaved MRVP broth was inoculated with freshly pure isolates and incubated at 37˚C for 24 hours. After incubation, few drops of methyl red used as an indicator was added in the inoculated tubes and results were examined. If color changes from pink to red, its indication of positive result.

In SIM test is used to test motility of organisms, its Indole production characteristic and H2S production ability. SIM test configured three types of things i.e. motility, Indole production and H2S production. Basically, media used for this is semi-solid media. For motility purpose, point inoculation of desired strains was performed. After 24-hour incubation, haziness pattern from the stab line indicate positive result for motility.

In case of Indole test, few drops of kovac‘s reagent was added, appearance of cherry-red ring points out its positive result and no color shows negative result. Formation of black residues represents H2S production. In Triple sugar iron (TSI) agar media was autoclaved and slants were prepared. Pure isolates were then streaked out on these slants tubes and then allowed to incubate at 37˚C±24 hours. The fermenting conditions were noted down, Slant red / Butt yellow indicated Glucose fermentation; Butt yellow / Slant red indicated Lactose and sucrose fermentation Red only indicated no fermentation.

3.2.5. Identification by Partial Sequencing of 16S rDNA

Isolates were confirmed by determination of 16S rRNA gene sequences of bacterial isolates and 23S rRNA gene sequences for representative fungal isolates. Initially, DNA of bacterial and yeast isolates was extracted as following.

3.2.5.1. DNA Extraction

Kate Wilson method was used for bacterial DNA extraction (Wilson, 2001). The bacterial strains were revived in 10ml liquid culture media. The fresh bacterial strains were centrifuged at 14,000 rpm for 10 minutes. This step was repeated twice until the formation of complete and dense pellet. Supernatant was discarded, and pallet was resuspended in 567µl TE buffer by repeated pipetting. After that 30µl of 10% SDS, 3µl of 20mg/ml of proteinase K was added and mixed gently. It was then incubated for 1 hour at 37°C. The solution thus formed must be viscous. After its incubation period, 100µl of 5M NaCl and 80µl of CTAB/NaCl solution were added and mixed thoroughly. It was then incubated at 65°C for 10 minutes. Then 800µl of chloroform-isoamyl alcohol was appended in these Eppendorf vials by

repeated pipetting. It was again centrifuged at 10,000 rpm for 8-10 minutes. After centrifugation, upper aqueous phase was removed and shifted to another Eppendorf. Added equal concentration of phenol- chloroform-isoamyl alcohol same by repeated pipetting and allowed to centrifuge at 8,000rpm for 8-10 minutes. Again, supernatant was removed and transferred gently to next Eppendorf vial. Then 600µl of iso-propanol was added and mixed gently to precipitate the nucleic acid. This was set on spinning for about 5 minutes. Supernatant was removed and washed with 400µl of 70% ethanol. It was again centrifuged at 10,000 rpm for 2 minutes. Supernatant was again removed, and pallet was re-suspended in 100µl TE buffer and 4µlRNAase. The extracted DNA was preserved overnight and stored at -4°C.

3.2.5.2. Phylogenetic Analysis

After DNA extraction, DNA from all isolates were sequenced for 16s rRNA gene. Sequencing of samples was performed by Macrogen, Commercial Seoul, South Korea. 16S rRNA gene sequences of the isolated strains and the most similar sequences from Gen Bank were identified through BLAST from NCBI. The alignments were thoroughly analyzed and corrected manually. The ambiguous aligned regions were removed from the sequence analysis. Finally, Phylogenetic trees were constructed for all isolates using neighbor joining method with Bootstrap values to identify the most probable similarity with reference strains.

3.2.6. Enzymatic Analysis of Lactic Acid Bacteria (LAB) and Fungi

The isolates screened out were analyzed for their ability to produce extracellular enzyme. This qualitative assay includes cellulase, protease, and amylase activity. This assay procedure has been described below.

3.2.6.1. Detection of Amylolytic Activity

To determine the amylolytic activity nutrient agar supplemented with 1-gram of starch was used (amylase media plates). These plates were inoculated by isolates by means of point inoculation and then allowed to incubate for 48 hours. After incubation period, iodine crystals were sprinkled over the amylase plates and then let them for few minutes. Formation of luminous zones around the inoculation point indicates positive result and no zone is indication for negative result.

3.2.6.2. Detection of Cellulolytic Activity

To determine cellulolytic activity of isolates nutrient agar supplemented with 1-gram CMC was prepared. Point inoculation was done, and plates were incubated. After incubation period, plates were stained firstly with Congo red dye for about 15 minutes and then stained with NaCl for 15 minutes. The presence of clear zone around the inoculated colony is indication of positive result and absence of this shows negativity effect.

3.2.6.3. Detection of Proteolytic Activity

1% casein agar media is used for Proteolytic activity (Vermelho *et al*., 1996). Point inoculation was performed on these plates and set on incubation for 48 hours. After incubation, the plates were immersed in 1% glacial acetic acid. Bright zone formation brings out positive result and no zone for negative result.

3.2.7. Percentage Survival in Cattle Gut Conditions

In this assay, the 100 µL of bacterial strains at their log phase were inoculated in 10 ml of sterilized Tryptic soy broth (TSB) present in test tubes while the 100 µL of yeast strains at their log phase were inoculated in 10ml of sterilized oxy-tetracycline glucose broth (OGB) present in the test tubes. Stock solutions of bile salts (1g/l0ml) and lysozyme (0.01g/10ml) were prepared. 150µl from the stock solution of bile salt and 1ml from the stock solution of lysozyme were added in all the test tubes to have their final concentration as (1.5g/l) and (100µg/ml) respectively. pH was adjusted at 3. Bacterial samples were incubated at 37˚C, 150 rpm while yeast samples were incubated at 30˚C, 150 rpm. TSB and OGB medias of a neutral pH having 100 µL of bacterial and yeast samples respectively, without the addition of bile salt and lysozyme, were set out as a control media. After 2 hours, 4 hours and 24 hours interval, samples were successively taken out and the comparative survival of the strains was measured by using spectrophotometer at 600nm. Experiment was done in triplicate.

% Survival = [OD of bile media / OD of control media] x 100

3.2.8. Cholesterol Assimilation

The ability of the microbial strains to assimilate the cholesterol was determined by Zak‘s method (Shankara, 2008). Selected strains were inoculated in tryptic soy broth (TSB) in Erlenmeyer flasks and set on incubation at their appropriate conditions. After their incubation period, about 0.1ml of each sample was taken from flasks, transferred in 10ml $FeCl_3$-acetic

acid in the falcon tubes, and then allowed to vortex for 5-10 minutes. Samples were then left for about 15 minutes until its complete protein precipitation. For its comparison, standard was prepared by appending physiological saline (0.1ml) and cholesterol standard solution (10ml). 5ml of FeCl3-acetic acid was taken as a blank and then 3ml of H2SO4 was added in these and mixed well. These were then left for 30 minutes and OD was taken at 560 nm. Percentage of cholesterol assimilation assay was estimated with the help of following formula

Cholesterol (mg/100ml) = OD of unknown x 100 x 0.2 / OD of known x 0.05

3.2.9. Cell Surface Hydrophobicity

The ability of the microbes to adhere with the intestinal cell layer can be evaluated by cell surface hydrophobicity test. Bacterial and yeast cultures were grown in the TSB and OGB media for 15hrs and 24hrs respectively. Two milliliters of the cultures were taken in the 2mL graduated Eppendorf‟s tubes. These tubes were then subjected to centrifuge at 6000rpm for 5min. After performing centrifugation, the supernatant was discarded, and pellets were taken. To remove the media contents pellets were washed twice with normal saline. After washing, the pellet was suspended in 3mL of Nano water in separate test tubes. Optical densities of these samples were taken at 600nm. Then 0.6mL of xylene was added into these tubes and vortex gently at 20rpm to avoid foaming. These tubes were then incubated for 20-30 min. Two layers were formed, the aqueous layer was taken from it and OD of aqueous layer of each sample was taken at 600nm. The percentage hydrophobicity of the samples was calculated by using the given formula:

$$\textbf{Hydrophobicity Percentage (\%)} = [(A_0 - A_1) / A_0] \times 100$$

Where,

A_0 =Optical density before mixing the xylene

A_1 = Optical density of the aqueous layer

3.2.10. Determination of Antimicrobial Activity

The indicator ATCC strains, *Listeria monocytogenes* (ATCC13932), *E. coli* (ATCC8739), *Staphylococcus aureus* (ATCC6538) and *Pseudomonas aeruginosa* (ATCC9027), were used against testing strains (Lactic acid bacteria). Soft agar suspension was poured into freshly prepared TSA plates and allowed it for solidification. Plates were then placed in incubator at 37°C for about 2-3 hours. Sterile disks were set on the lawn of indicator strains carefully.

After that, 10µl of cell or supernatant was taken from overnight culture of testing strains (lactic acid bacteria) and carefully poured on filter paper disks. All plates were placed in incubator at 37°C for 24-48 hours. Yeast strains were treated likely, irrespective of its variation in incubation conditions i.e. 30°C for 48 hours. Results of antimicrobial activity were observed in terms of its zone diameter (mm). A clear zone formation around the disks, determine the antimicrobial behavior.

3.2.11. Statistical Analysis

The statistical analysis was carried out by using XLSTAT 2014.5.03. Principal component analysis was applied by using Pearson correction analysis (n) method. The results are shown in distance biplot.

3.3. Results

From cow dung samples Thirtteen strains (ten lactic acid bacteria, three *Bacillus* species were isolated, while eighteen-gram positive bacteria (16-Gram positive rod and 2-Gram positive cocci) were isolated from corn silage and from Dahi five strains of *Enterococcus faecium* and twelve yeast strains (*Geotrichum candidum*) were isolated. All these isolates were identified through biochemical tests. Identified strains were checked for their extracellular enzymatic activity all those isolates which were positive for protease, amylase and cellulase activity were checked for mimic gut survival rate, and anti-pathogenic activity.

3.3.1. Microbial Isolates from Cow Dung

Cow dung samples were inoculated on two different growth media (TSA and M-17), all the isolates were biochemically characterized and confirmed by Bergey‘s manual of bacteriology. Thirteen bacteria strains were isolated on TSA and further examined for gram staining and biochemical tests including Simmon‘s citrate test**,** Triple sugar iron (TSI) test**,** Methyl Red (MR) test**,** Sulfideindole motility (SIM) test**,** Indole test. Among thirteen isolates only four were gram positive while nine were gram negative (Table 3.1, Fig.3.1 & 3.2). Six bacterial strains were isolated on M-17 media and were further characterized through gram staining and biochemical testing. All the isolates were gram negative.

Table 3. 1: Biochemical characterization of isolated strains from cow dung on TSA

S. No	Gram's staining	Catalase	Oxidase	Indole	Simmon's Citrate	Methyl Red	Triple sugar iron	Sulfide, Indole Motility test
1	-ve cocci	+	-	-	-	+	-	NM, NO H_2S
2	-ve cocci	-	-	-	-	+	+	NM, NO H_2S
3	+ve cocci	-	-	-	-	+	+	NM, NO H_2S
4	-ve cocci	+	-	-	-	-	+	NM, NO H_2S
5	+ve cocci	-	-	+	-	-	+	NM, NO H_2S
6	-ve cocci	-	-	-	-	+	+	NM, No H_2S
7	+ve cocci	-	-	-	-	-	+	NM, No H_2S
8	-ve cocci	-	-	-	-	-	+	NM, No H_2S
9	-ve cocci	+	-	-	+	+	+	NM, No H_2S
10	-ve cocci	+	-	+	+	-	-	M, No H_2S
11	+ve cocci	+	-	-	-	-	-	M, No H_2S
12	-ve cocci	+	-	-	-	-	-	M, No H_2S
13	-ve cooci	+	-	-	+	-	+	M, No H_2S

Table 3. 2: Biochemical characterization of isolated strains from cow dung on MRS Media

S. No.	Gram Staining	Catalase	Oxidase	Indole	Simmon's Citrate	Methyl Red	Triple sugar iron	Sulfide Indole Motility test
1	+ve cocci	-	-	-	-	-	+	NM, No H_2S
2	+ve cocci	-	-	-	-	+	+	NM, No H_2S
3	+ve cocci	-	-	-	-	+	+	NM, No H_2S
4	+ve cocci	-	-	-	-	+	+	NM, No H_2S
5	+ve cocci	-	-	-	-	+	+	NM, No H_2S
6	+ve cocci	-	-	-	-	+	+	NM, No H_2S
7	+ve cocci	-	-	-	-	+	+	NM, No H_2S
8	+ve cocci	-	-	+	-	+	+	NM, No H_2S
9	+ve cocci	-	-	-	-	+	+	NM, No H_2S

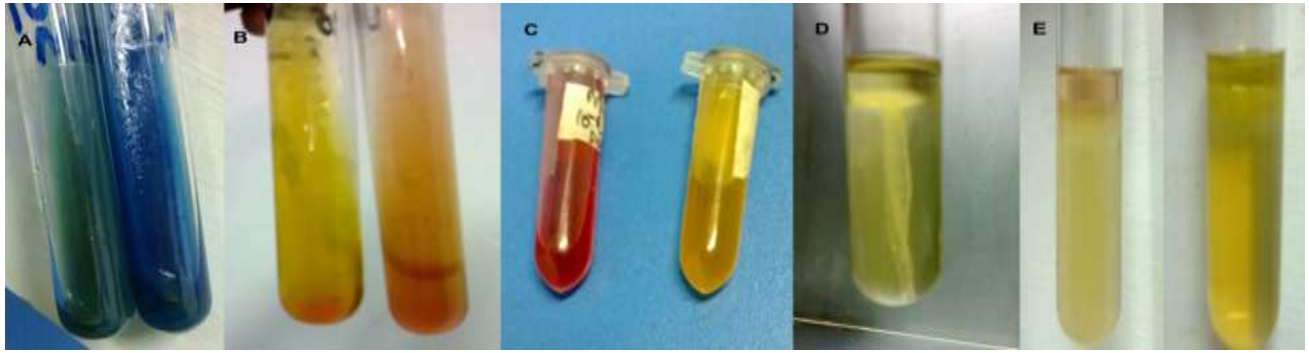


Figure 3. 1: Biochemical analysis; (A) Simmon's citrate test (B) Triple sugar iron (TSI) test (C) Methyl Red (MR) test (D) Sulfideindole motility (SIM) test (E) Indole test.

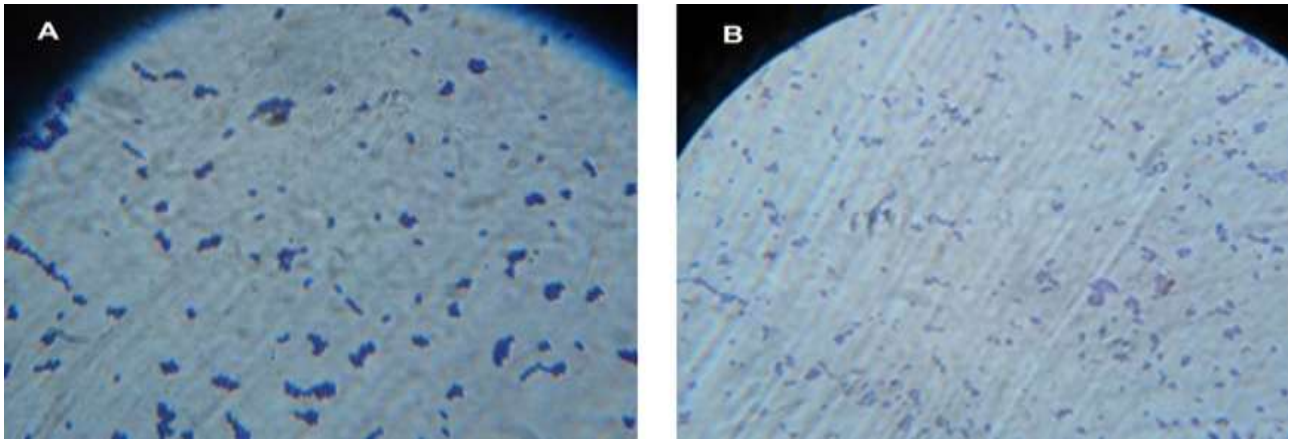


Figure 3. 2: Gram staining of *Lactococcus and Enterococcus* strains on MRS; (A) QAULG03, gram positive cocci (B) QAULG10 gram positive cocci.

3.3.2. Molecular Identification of Isolates

All the selected gram-positive isolates (4) and one-gram negative isolate were further assed through 16S rDNA gene sequencing. Phylogenetic tree shows the evolutionary origin as these bacterial isolates mainly belong to *Enterococcus*, within the *Enterococcus*; experimental strains were observed to be distantly related to *Enterococcus faecium* specie as they were lying at a separate branch of the tree. However, the experimental strains were clustering with a clade that contains two members of *Enterococcus mundtii*. This pattern demonstrates that our isolated bacterial strains most probably belongs to *E. mundtii* species as their highest sequence similarity within Enterococcus genus was observed with this specie (Table 3.3).

Table 3. 3: Accession number of selected strains isolated from cow dung

S. No.	NCBI Accession Numbers	Name of Isolate	Strain Codes
1	KP256018	*Enterococcus sp.*	QAUSK01
2	KP273582	*Enterococcus mundtii*	QAU EM01
3	KP256013	*Lactococcus lactis subsp. lactis*	QAULL04
4	KP256011	*Bacterium*	QAULG02

3.3.3. Phylogenetic Analyses of Bacterial Isolates

The blast search revealed that The *Lactococcus* QAULL04 (KP256013) had the highest sequence similarity with the *Lactococcus lactis ssp. tructae* L105^{T} (EU770697) and The Bacterium QAULG02 (KP256011) had the highest sequence similarity with the *Lactococcus garvieae* ATCC 49156^{T} (AP009332) (Fig. 3.3). The blast search revealed that *Enterococcus* QAUSKO1 (KP256018) had the highest sequence similarity with the *Enterococcus faecium* ATCC CGMCC 1.2136^{T} (AJKH01000109) (Fig. 3.4).

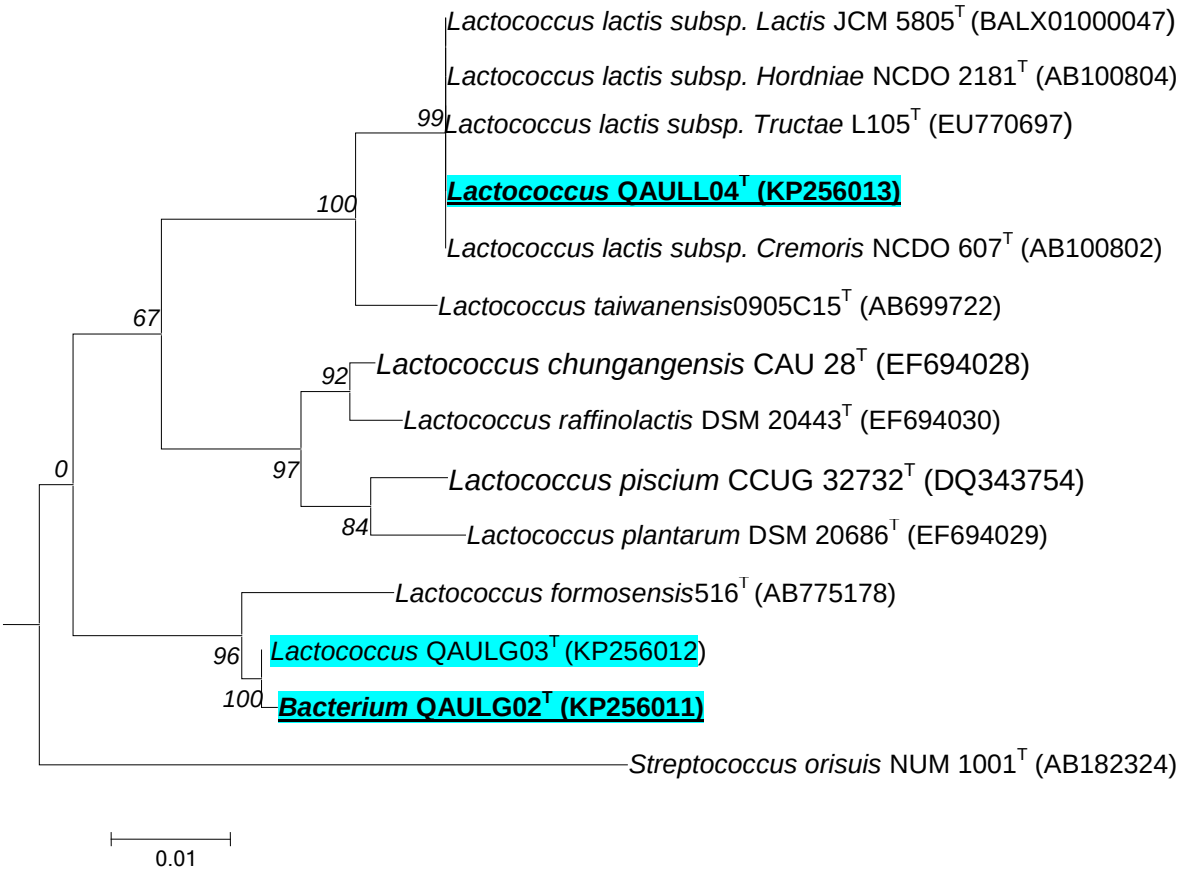


Figure 3. 3: Phylogenetic tree of the *Lactococcus* QAULL04, QAULG03, and QAULG02 species based on 16S rRNA gene sequence.

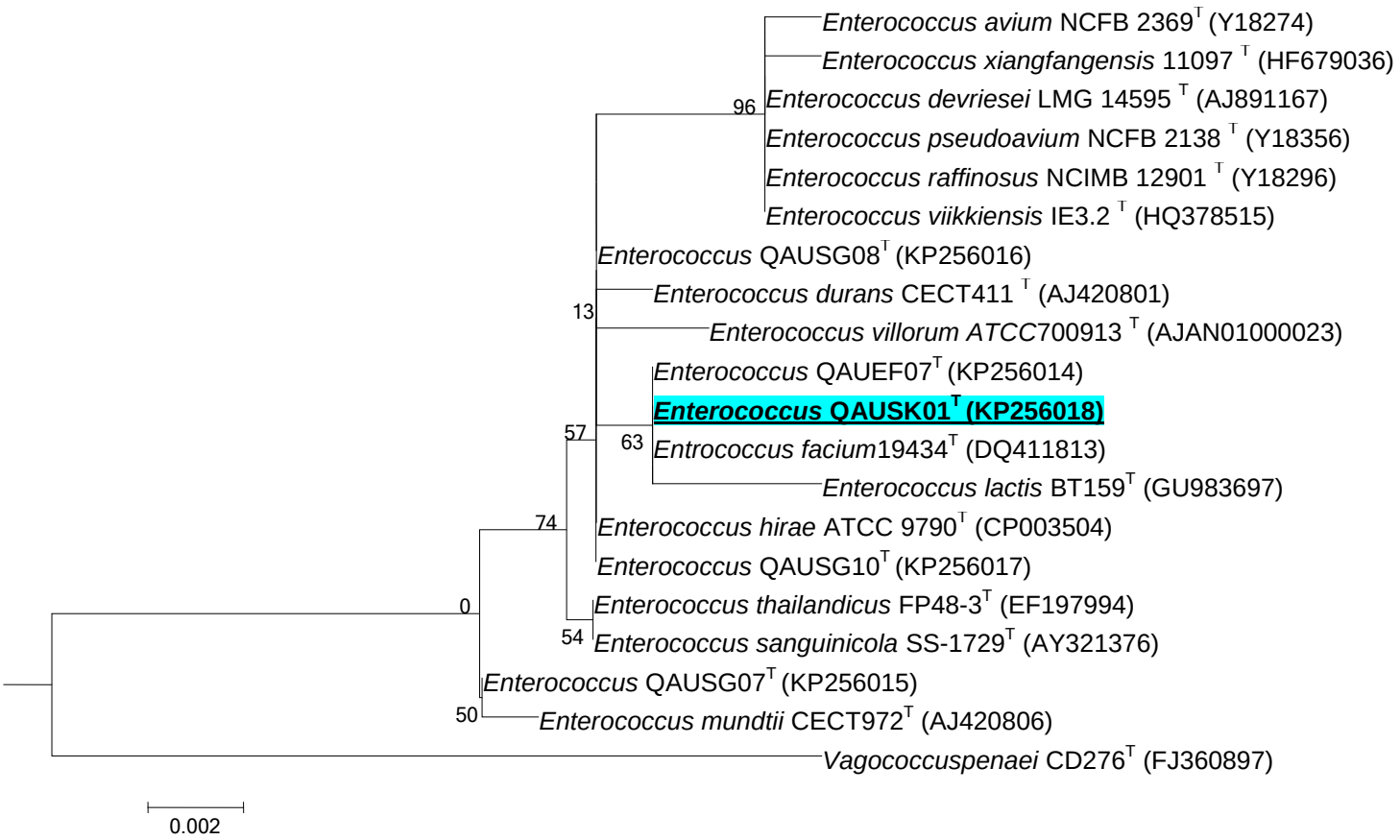


Figure 3. 4: Phylogenetic tree of the *Enterococcus* (KP256016, KP256017, KP256014, KP256015, KP256018) species based on 16S rRNA gene sequence.

3.3.4 Microbial Isolation from Corn Silage

Recovered isolates (isolated from corn silage) were phenotypically identified and were assessed for their bioactive properties and mimic gut survival. All the silage samples were homogenized and were inoculated on growth media. Gram staining and biochemical tests were performed for identification of isolated bacteria. Eighteen bacterial strains were recovered from corn silage from which sixteen were gram-positive rods and two were Gram positive cocci. Figure 3.5 showed few of isolated gram-positive bacteria while figure 3.6 showed gram staining. After gram staining Gram positive isolates were confirmed through biochemical tests including catalase, oxidase, simmon's citrate, motility and methyl red tests (Table 3.4).

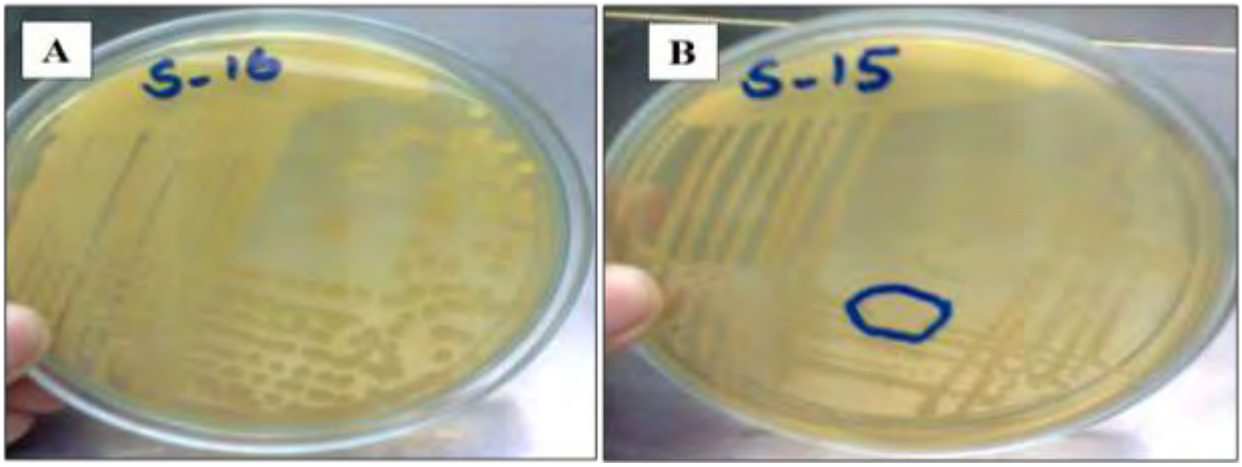


Figure 3. 5: Pure culture of lactic acid bacteria isolated from corn silage; (A) S-16 isolate from corn silage (B) S-15 isolate from corn silage

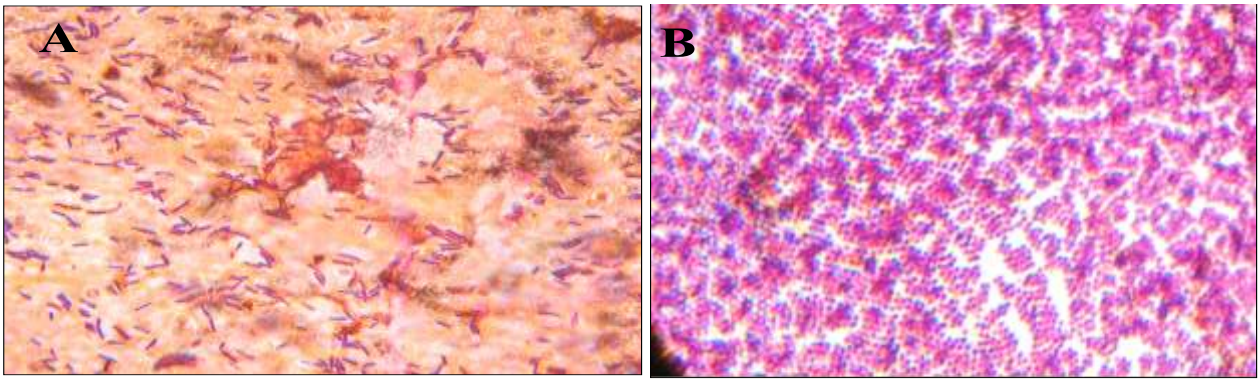


Figure 3. 6: Gram staining of bacterial strains isolated from corn silage; (A) Gram Positive Rods (B) Gram Positive cocci

Table 3. 4: Gram staining and biochemical tests of strains isolated from corn silage

Sample	Gram Staining	Catalase	Oxidase	Simmon's citrate	Motility	Methyl Red
S-10 (LAB-100)	Gram +ive rods	-ive	+ive	+ive	-ive	-ive
S-11 (SGQAU01)	Gram +ive rods	-ive	+ive	+ive	-ive	-ive
S-12 (LAB-11)	Gram +ive rods	-ive	+ive	+ive	-ive	-ive
S-13 (LAB-23)	Gram +ive rods	-ive	+ive	+ive	-ive	-ive
S-14 (LAB-01)	Gram +ive rods	-ive	+ive	+ive	-ive	-ive
S-15 (SGQAU2)	Gram +ive cocci	+ive	+ive	+ive	-ive	-ive
S-16 (LAB-10)	Gram +ive rods	-ive	+ive	+ive	-ive	-ive
S-17 (LAB-13)	Gram +ive rods	-ive	+ive	+ive	-ive	-ive
S-18 (LAB-16)	Gram +ive rods	-ive	+ive	+ive	-ive	-ive
S-19 (Lis20)	Gram +ive rods	-ive	+ive	+ive	-ive	-ive
S-20 (LAB-17)	Gram +ive rods	-ive	+ive	+ive	-ive	-ive
S-21 (LAB-18)	Gram +ive rods	-ive	+ive	+ive	-ive	-ive
S-22 (QAUBS01)	Gram +ive rods	-ive	+ive	+ive	-ive	-ive
S-23 (LAB-20)	Gram +ive rods	-ive	+ive	+ive	-ive	+ive
S-24 (LAB-22)	Gram +ive rods	-ive	+ive	+ive	-ive	-ive
S-25 (LAB-24)	Gram +ive rods	-ive	+ive	+ive	-ive	-ive
S-1 (Lis-17)	Gram +ive rods	+ive	+iv	+ive	-ive	+ive
S-2 (Lis-18)	Gram +ive cocci	-ive	+ive	+ive	-ive	-ive

Morphologically distinct colonies were selected for identification by Fourier transform infrared spectroscopy (FTIR) (Bruker, Karlsruhe, Germany). For data processing, OPUS software v.6 (Bruker) was used (Wenning *et al.*, 2010). Dendrogram was calculated according to the average linkage algorithm. Agglomerative hierarchical clustering (AHC) was used to group the FTIR peaks and the distance between different groups was calculated with Pearson correlation coefficient. Representative isolates from each cluster were selected

for species level identification. The representative silage isolates selected based on FTIR Spectroscopy were identified by 16S rDNA sequencing. Three strains were identified as *Bacillus subtilis* (KT033701) *Bacillus licheniformis* (KT033702), and *Bacillus amyloliquefaciens* (KP826775) (Figure 3.7)

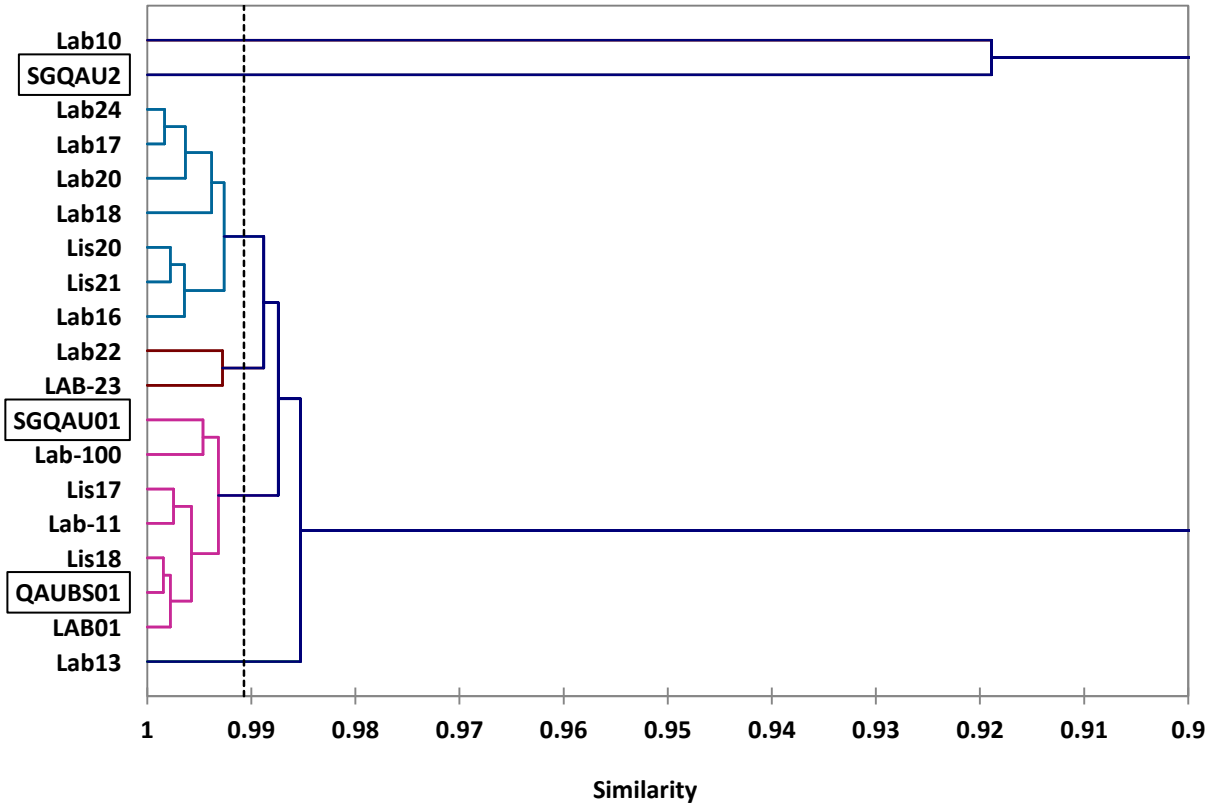


Figure 3. 7: FTIR based agglomerative hierarchical clustering (AHC) of silage isolated Lactic acid bacteria

3.3.5. Microbial Isolates from Fermented Dairy Product (Dahi)

Dahi samples were inoculated on different media for isolation of bacterial and yeast strains. Five strains of *Enterococcus faecium* were isolated from local Dahi, while twelve strains of *G. candidum* were isolated. Total five isolates (QAUEF01, QAUEF03, QAUEF04, QAUEF5 and QAUEF06 were selected from M17 media based on creamy/white, round, centrally raised colonies of bacteria. The isolates after Gram positively stained, while all were negative for catalase and simmon-citrate tests, while QAUEF05 strain was catalase and simmon-citrate positive. All the strains were lactose positive. Based on growth pattern and biochemical characteristics, the strains QAUEF01, QAUEF03, QAUEF04, QAUEF5 and QAUEF06 were

initially identified as they belong to Genus *Enterococcus* (Table 6). The isolated colonies QAUEF01, QAUEF03, QAUEF04, QAUEF5 and QAUEF06 strains were identified to the species level by using the API 20E kit (Bio-Merieux, Germany). The results were recorded after 4 and 24 hours, when incubated at 37°C and 8-digit numeric codes were generated from these results. The digital codes were read by the API web software, which showed that five of the strains; QAUEF01QAUEF03, QAUEF04 QAUEF05 and QAUEF06 were *E. faecium*, while strain QAUEF5 was 48.7% similarity with *E. faecium.*

Table 3. 5: *Enterococcus faecium* strains isolated from Dahi

Strain ID	**NCBI Accession No.**	**Identity**
QAUEF01	KP256006	*Enterococcus faecium*
QAUEF03	KP256007	*Enterococcus faecium*
QAUEF04	KP256008	*Enterococcus faecium*
QAUEF05	KP256009	*Enterococcus faecium*
QAUEF06	KT021871	*Enterococcus faecium*

The twelve already isolated strains of *Geotrichum candidum* have been analyzed on the morphological basis. All the three media used in the present study for optimization i.e. tryptone soya agar (TSA), oxytetracycline glucose agar (OGA) and potato dextrose agar (PDA) have adequately supported the growth of *G. candidum*. Typical white velvety colonies were observed for all the *G. candidum* strains. However, microscopy was also performed for further confirmation. With cotton blue staining, cylindrical sub-globose chains of colonies were observed (Figure 11).

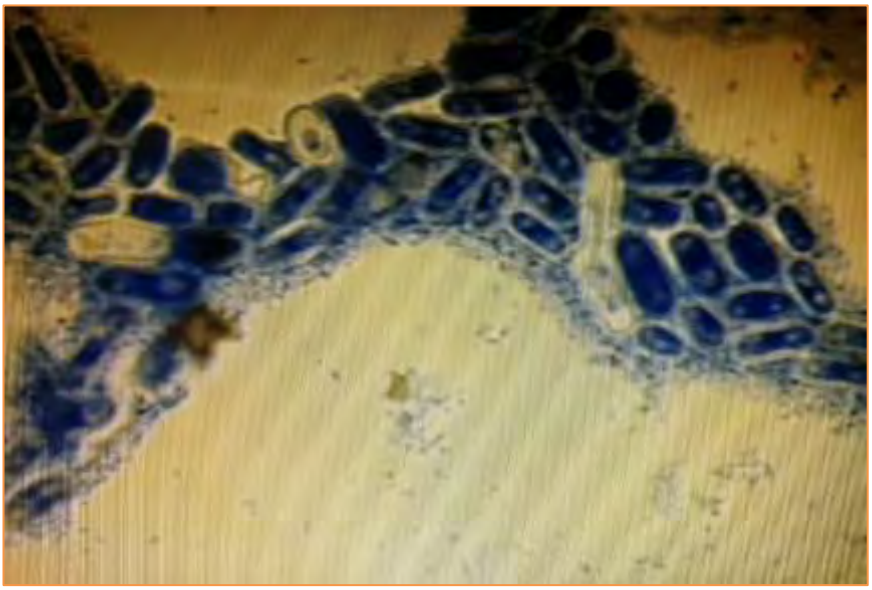


Figure 3. 8: Microscopic appearance of *Geotrichum candidum* strain QAUGC01after cotton blue staining

All locally isolated *G. candidum* strains have been identified on morphological basis. All of them have typical yeast like white velvety colonies. However, strain *G. candidum* QAUGC01 has been sequenced using ITS region and Accession No. KT280407 (Figure 12).

Three strains of *G. candidum* (UCMA 91, UCMA 103 and UCMA 322), acquired from France, and two commercially available probiotic products; Enflor (*S. cerevisiae*) and Enterogermina (*Bacillus subtilis* spores) were purchased from market and were used as reference strains. *Enterococcus faecium* strains were labelled from (*E. faecium*) E1 to E5, similarly, Geotrichum sp. strains were categorized as (*G. candidum*) GC1-GC3. The remaining three GC strains were named as GC 91, GC 103 and GC 322.

3.3.6. Extracellular Enzymatic Activity of Microbial Isolates

All biochemically confirmed isolates were checked for their extracellular enzymatic activity including cellulolytic, amylolytic and proteolytic activities. All strains isolated from cow dung have shown different amylolytic, cellulolytic and proteolytic activities. Bacterial isolates recovered from corn silage had shown different extracellular enzymatic activity as they have shown positive cellulolytic and amylolytic activities while their proteolytic activity was zero so, they were not further proceeded for mimic gut survival. Strains isolated from Dahi were positive for amylolytic, cellulolytic and proteolytic activities.

3.3.6.1. Cellulolytic Activity

CMC carboxymethyl cellulose was used as a substrate for cellulolytic test of lactic acid bacteria, clear zone formation around the inoculation point will represent the positive results (Figure 3.9). All cow dung isolates were positive for cellulolytic activity. From corn silage, sixteen bacterial isolates were positive for cellulolytic activity (Table 3.6). Cellulolytic activity was checked against bacterial and yeast strains isolated from Dahi and it was found that all isolates have cellulolytic activity (Table 3.7)

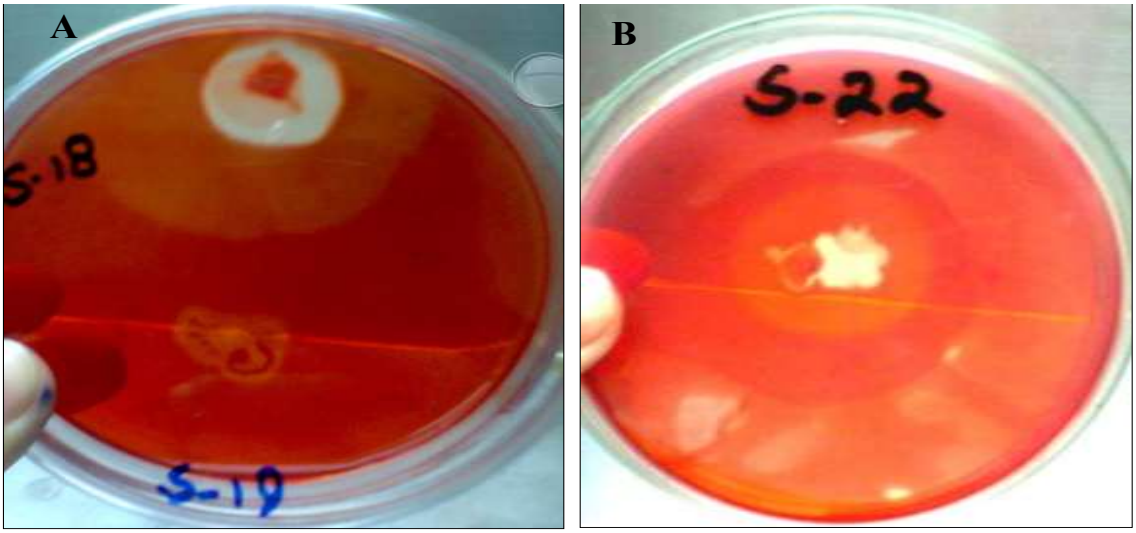


Figure 3. 9: Cellulolytic activity of lactic acid bacteria isolated from corn silage; (A) Clear zone around the bacterial colony S-18 (B) Clear zone around the bacterial colony S-22

3.3.6.2 Amylolytic Activity

Amylolytic activity was determined on starch agar media. Sprinkling of iodine crystals on culture plate of starch agar resulted in the formation of clear zones with in few seconds around the lactic acid bacteria colonies (Figure 3.10). All isolated bacterial strains from cow dung had shown positive results for amylolytic activity in our conditions. From silage isolates fourteen isolates showed positive amylolytic activity (Table 3.6). All bacterial and yeast isolates isolated from Dahi show positive amylolytic activity (table 3.7).

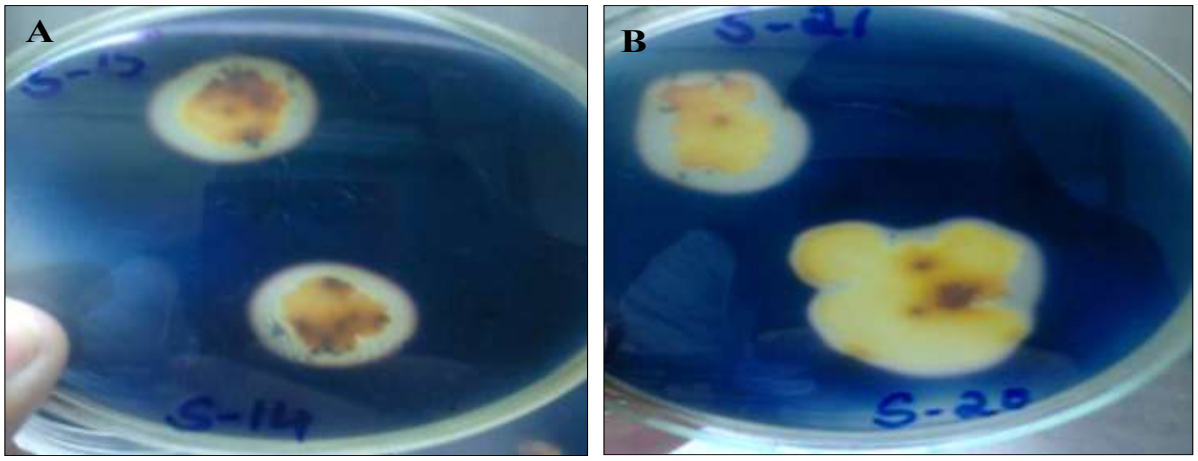


Figure 3. 10: Amylolytic activity of lactic acid bacteria isolated from corn silage; (A) clear zone around colonies S-14 and S- 15 (B) Clear zone around colonies S-21 and S-20

3.3.6.3. Proteolytic

Casein agar media was used as a substrate for detection of proteolytic activity by lactic acid bacteria. It was found that all cow dung and Dahi isolates were positive for proteolytic activity while all silage isolated bacteria showed negative proteolytic activity (Table 3.6).

Table 3. 6: Enzymatic activity of lactic acid bacteria isolates from corn silage

Isolates	Proteolytic	Cellulolytic	Amylolytic
LAB-100	-	+	+
SGQAU01	-	+	+
LAB-11	-	+	+
LAB-23	-	+	+
LAB 01	-	+	+
SGQAU2	-	+	+
LAB 10	-	+	+
LAB 13	-	+	+
LAB 16	-	+	+
Lis20	-	+	+
LAB 17	-	+	+
LAB 18	-	+	+
QAUBS01	-	-	+
LAB 20	-	+	+
LAB 22	-	+	+
LAB 24	-	-	+
Lis17	-	-	+
Lis18	-	+	+
Lis21	-	+	+

Table 3. 7: Enzymatic activity of lactic acid bacteria isolates from Dahi

Isolates	Proteolytic	Cellulolytic	Amylolytic
QAUEF01	+	+	+
QAUEF03	+	+	+
QAUEF04	+	+	+
QAUEF05	+	+	+
QAUEF06	+	+	+
QAUGC01	+	+	+
QAUGC02	+	+	+
QAUGC03	+	+	+
QAUGC04	+	+	+
QAUGC05	+	+	+
QAUGC06	+	+	+
QAUGC07	+	+	+
QAUGC08	+	+	+
QAUGC09	+	+	+
QAUGC10	+	+	+
QAUGC11	+	+	+
QAUGC12	+	+	+

Table 3.8: lactic acid bacterial and yeast isolates from Cow Dung and Dahi.

S. No.	Name of Isolate	Strain Codes	NCBI Accession Numbers	Source
1	*Enterococcus sp.*	QAUSK01	KP256018	Cow Dung
2	*Enterococcus mundtii*	QAU EM01	KP273582	
3	*Lactococcus lactis subsp. lactis*	QAULL04	KP256013	
4	*Bacterium*	QAULG02	KP256011	
5	*Enterococcus faecium*	QAUEF01	KP256006	Dahi
6	*Enterococcus faecium*	QAUEF03	KP256007	
7	*Enterococcus faecium*	QAUEF04	KP256008	
8	*Enterococcus faecium*	QAUEF05	KP256009	
9	*Enterococcus faecium*	QAUEF06	KT021871	
10	*Geotrichum candidum*	QAUGC01-QAUGC12	KT280407- 418	
11	*Geotrichum candidum*	UCMA91	ATCC 204307	Milk (Normandy)
12	*Geotrichum candidum*	UCMA322		Cheese (French)
	Geotrichum candidum	UCMA 103		

3.3.7. Mimic Gut Survival

Survival of isolated strains was determined in the cattle gut mimic conditions that was low pH of stomach of cattle that is 3, bile concentration 1.5g/L and lysozyme concentration 100µg/ml. Percentage survival was determined after different time intervals that are after 2hrs, 4hrs and 24hrs by comparing the growth rates of bacterial and yeast strains in an experimental and in control media possessing neutral pH that is 7 with no lysozyme enzyme and bile salts.

After 2 hours QAULL04 showed the highest survival rate of 31.708% while the least percentage survival was shown by *Enterococcus mundtii* strains QAUEM01 of 3.359%. After four- and 24-hours incubation in mimic gut conditions survival rate of QAULL04 decreases to 29.82% and 26.72% respectively. The survival rate of QAUEM01 reduces to zero percent after 4 hours.

After two hours three strains showed % survival more than 50%. Two of them were of *Enterococcus faecium* species that were QAUEF04 having 58.551 and QAUEF06 showing

52.614 % survival. % survival by the other 3 strains of *Enterococcus faecium* was QAUEF01 (45.854%), QAUEF03 (47.908%) and QAUEF05 (43.580%). Among the twelve yeast strains only one strain showed % survival more than 50% that is QAUGC02 showing survival 53.925%. Seven strains showed the survival of moderate level ranging from 30-50%. Those strains were QAUGC12 (47.442%), QAUGC10 (44.432%), QAUGC01 (42.041%), QAUGC08 (41.142%), QAUGC07 (36.718%), QAUGC05 (33.754%) and UCMA322 (31.821%). Other four strains of yeast showed a very low % survival ranging from 10 -30 %. Those strains were QAUGC06 (26.559%), QAUGC03 (25.829%), QAUGC11 (24.455%) and UCMA91 showed minimum survival that is 11.857% (Figure 11).

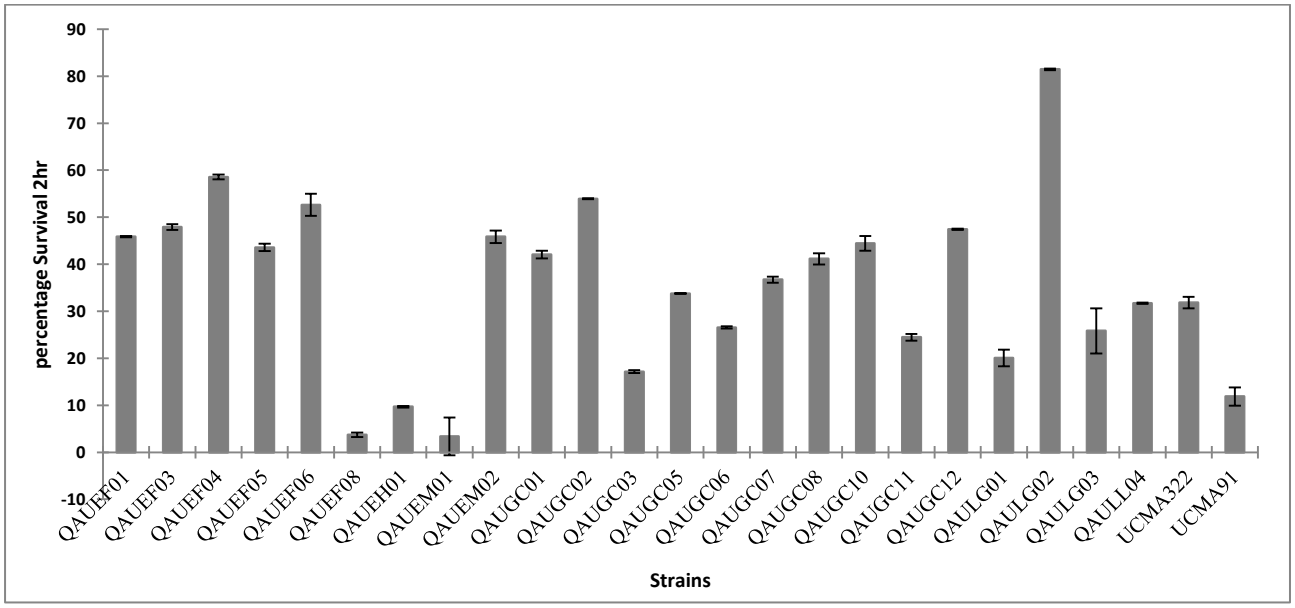


Figure 3. 11: Mimic Gut Survival of Dahi isolate at 2hrs

The bacterial strains that survived well after 2 hours also tended to survive in gut conditions after 4 hours with the slight decrease in their survival percentage. Two *Enterococcus faecium* species that showed more than 50% survival after 2 hours their survival had reduced to QAUEF04 (50.153%) and QAUEF06 (45.470%). QAUEM02 showed almost no survival after two hours by showing the decrease in % survival of 32.54%. Yeast strains also showed the same behavior as bacterial strains. The maximum survival value showed by QAUGC02 was 53.925% that had reduced to 48.770% after 4 hours. Maximum decrease in survival their percentage was 8.548% while the minimum decrease was 1.465% after 4 hours. One strain

(QAUGC12) among them showed the exceptional behavior showing 20.03% decrease in percentage survival (Figure 3.12).

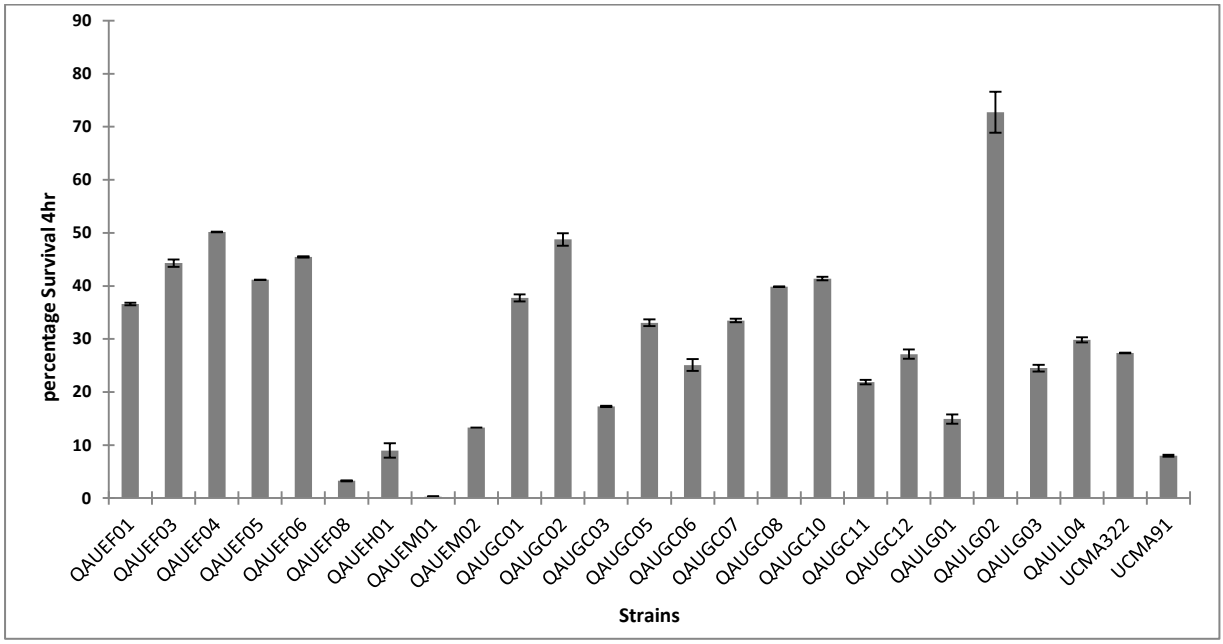


Figure 3. 12: Mimic Gut Survival of Dahi isolate at 4hrs

Bacterial strains showed again a slight decrease in their percentage survival after 24 hours. Two *Enterococcus faecium* species that showed maximum survival after 4 hours were QAUEF04 (50.153%) and QAUEF06 (45.470%) their survival had reduced to. QAUEF04 (49.459%) and QAUEF06 36.216 (%). The only one strain showed no decrease that is QAUEM06. Its decrease in survival value was in negative that is -0.061%. Yeast strains also showed the same behavior as bacterial strains again after 24 hours. The maximum survival value showed by QAUGC02 was 48.770% % that had reduced to 46.001% after 24 hours. A slight decrease in the survival of yeast strains was ranging from 0.322 %-10.676% (Figure 3.13).

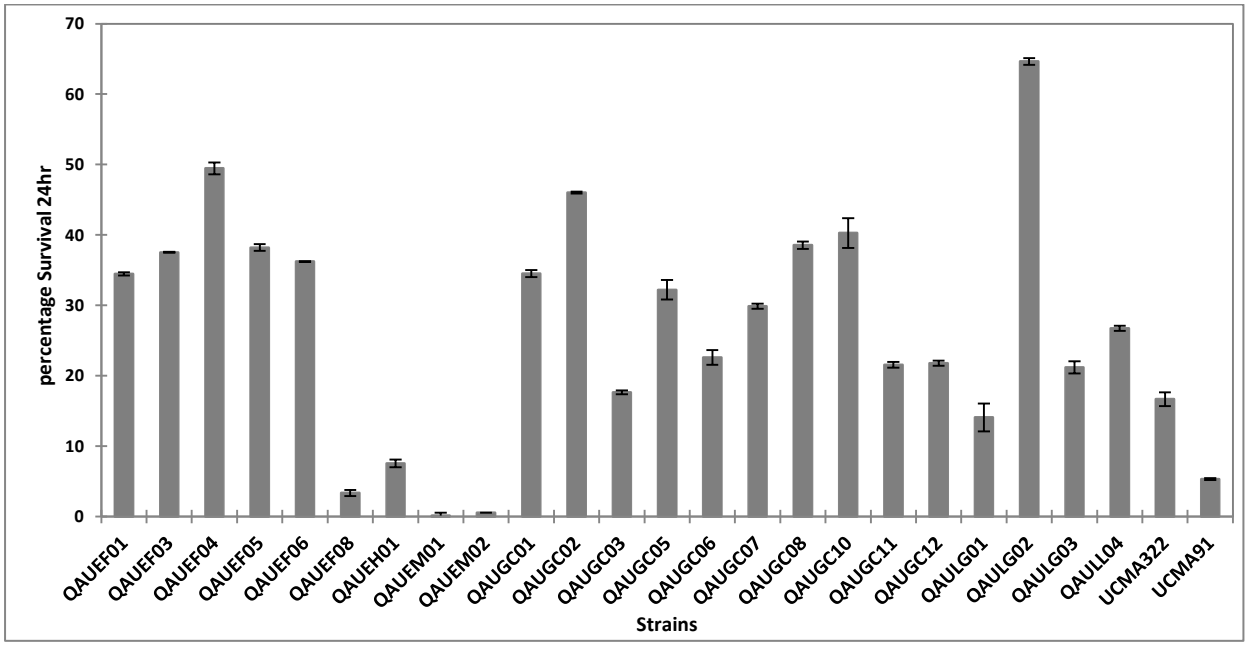


Figure 3. 13: Mimic Gut Survival of Dahi isolate at 24hrs

3.3.8. Cholesterol Assimilation

Both bacterial and yeast strains isolated from cow dung were checked for their ability to reduce the cholesterol level in the media. Among all bacterial strains two strains showed cholesterol reduction more than 50%. QAULL04 cholesterol reduction values was 64.442% while QAUEM01 showed 50% cholesterol reduction. QAULG03 showed 36.444% cholesterol reduction. Cholesterol reduction of remaining strains were very low ranging from 13.615% - 19.871%.

Enterococcal and *G. candidum* strains isolated from Dahi were check for their ability to reduce the cholesterol level in the media. These results are displayed in figure 3.14 and figure 15. Maximum cholesterol reduction was observed by the strain E1 which lowered down the cholesterol level from 400mg/100mL to 135.14mg/100mL. Isolates E2 and E3 reduced the cholesterol level to 141.78 and 141.81 mg/100mL respectively. Although there was no significant difference between the cholesterol lowering ability of E2 and E3, both strains assimilate cholesterol to the almost same level. Among the tested *Enterococcal* strains, lowest assimilation power was exhibited by E4 and E5, which assimilated the cholesterol to 150.72 and 150.48 mg/100mL. Hence, most effective cholesterol was assimilated by *E. faecium* 01 strain. The assimilated cholesterol quantity by *Geotrichum* and commercial strains (*S. cerevisie* and *B. subtilis)* during 24 h of incubation revealed wide range of

variations among isolates (Figure 3.15). The final concentration of cholesterol in the control media was 400mg/100mL. All the *G. candidum* strains exposed the characteristic of lowering the cholesterol level below 137mg/100mL. Highest assimilation power was shown by QAUGC12 with the cholesterol lowering ability to 74.66mg/100mL. 41.17% of the tested strains displayed significant characteristic of decreasing the cholesterol level below the 100mg/100mL. Although GC 07 assimilated the lowest cholesterol i.e. 136.88mg/100mL among the tested strains but it was significant result when compared to the control. Commercially available strains Enflor (*S. cerevisiae*) and *B. subtilis* assimilated the cholesterol to the almost same level i.e. 108.44mg/100mL and 110.22mg/100mL disclosing the fact that some of our isolated strains are more efficient in cholesterol lowering ability.

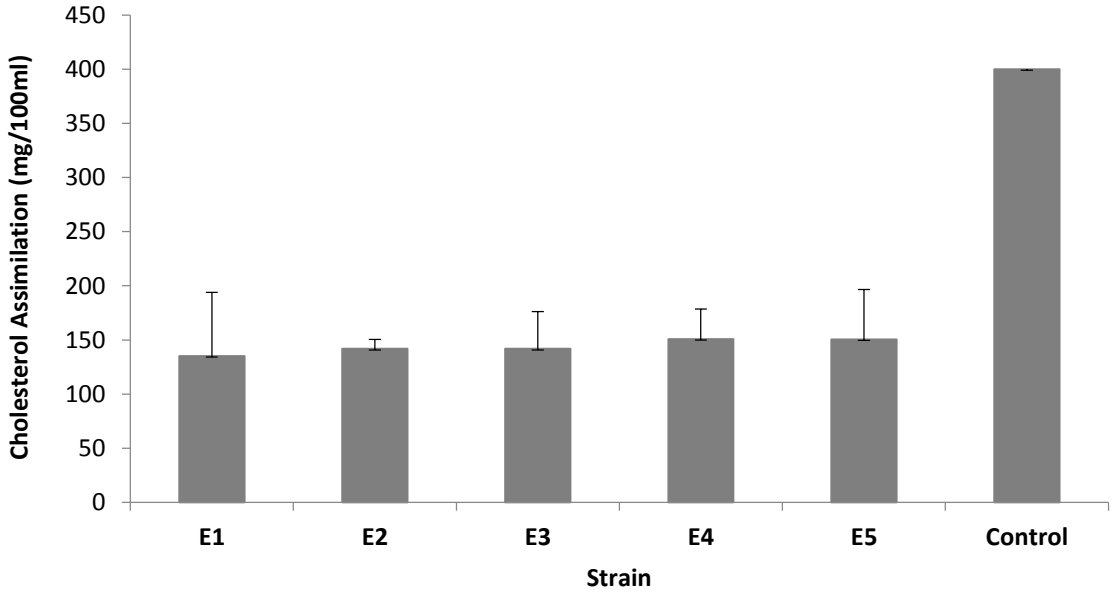


Figure 3. 14: Cholesterol assimilation by *E. faecium*

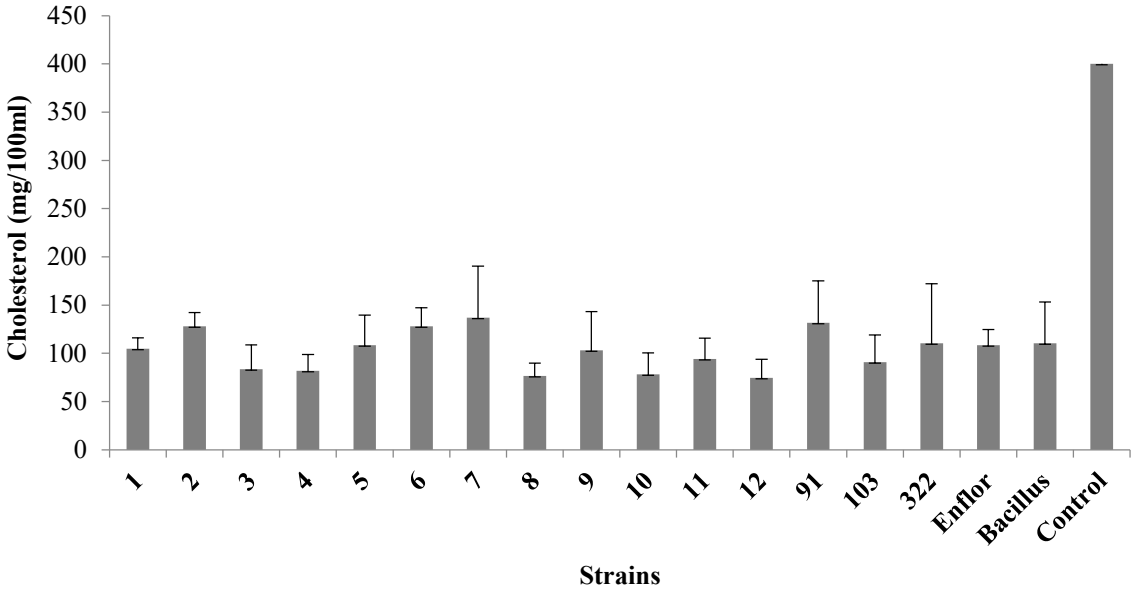


Figure 3. 15: Cholesterol Assimilation by *G. candidum* and Commercial Strains

3.3.9. Cell Surface Hydrophobicity

Hydrophobicity activities among the bacterial and yeast strains were measured after incubating them for 24hrs and 48hrs accordingly to reduce the number of dead cells. The adhering ability varied from strain to strain. This capability was determined by measuring the number of cells present in xylene layer. Cell hydrophobicity among cow dung isolates was measured as; among all bacterial isolates QAULG03 was maximum 30.845% followed by *E. mundtii* QAUEM01 showed 21.104% while QAULL04 has minimum value of 4.712% for cell hydrophobicity. Hydrophobic activities among *Enterococcal* and *G. candidum* strains isolated from Dahi varied from strain to strain (figure 3.16 and 3.17). All the *Enterococcal* strains had adherence ability >50%. Highest adhering ability was found in *E. faecium* strain E1 which exhibited 79.13% of hydrophobicity revealing that strains has highest ability to colonize to epithelial layer among the tested *Enterococcal* strain. The strain E4 showed significant result by revealing the 77.94% of hydrophobicity activity. The result displayed by E3 strain was also remarkable when compared to other strains i.e. 70.67%. E3 and E5 exhibited the same activity of hydrophobicity as they showed 70.67% and 70.16% adhering capability (Fig.3.16). Among *G. candidum* strains QAUGC12 has shown maximum cell hydrophobicity (86.50%) followed by QAUGC08 (86.10%), QAUGC06 (85.48%). QAUGC01 has shown cell hydrophobicity of 46.64% (Fig.3.17).

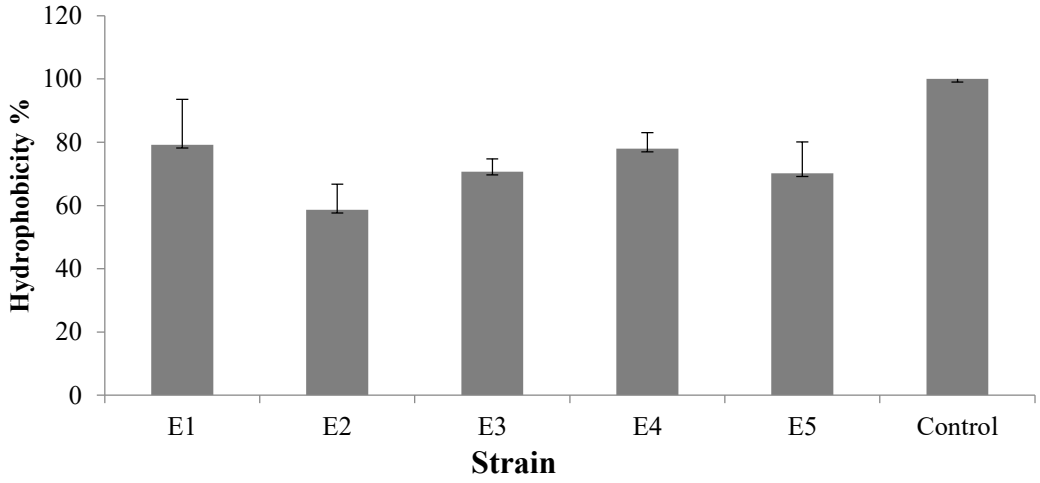


Figure 3. 16: Hydrophobicity of *E. faecium* strains.

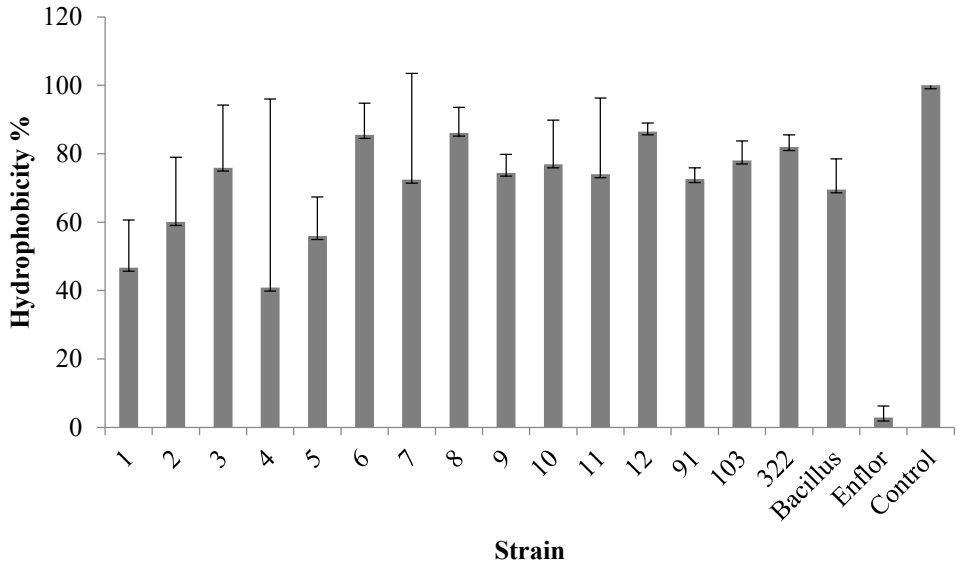


Figure 3. 17: Hydrophobicity of *G. candidum* and Commercial Strains

3.3.10. Anti-pathogenic Activity

The antipathogenic activity of selected isolates were done against four ATCC culture strains of *L. monocytogenes* (ATCC13932), *E. coli* (ATCC8739), *S. aureus* (ATCC6538) and *p. aeruginosa* (ATCC9027). Results have shown that all *G. candidum* strains isolated from Dahi have showed anti-pathogenic activity against *E. coli* (ATCC8739), *P. aeruginosa* (ATCC9027), *S. aureus* (ATCC6538) but not against *L. monocytogenes* (ATCC13932).

While *G. candidum* QAUGC01 has shown maximum antipathogenic activity among all isolated *G. candidum* (Table 3.9).

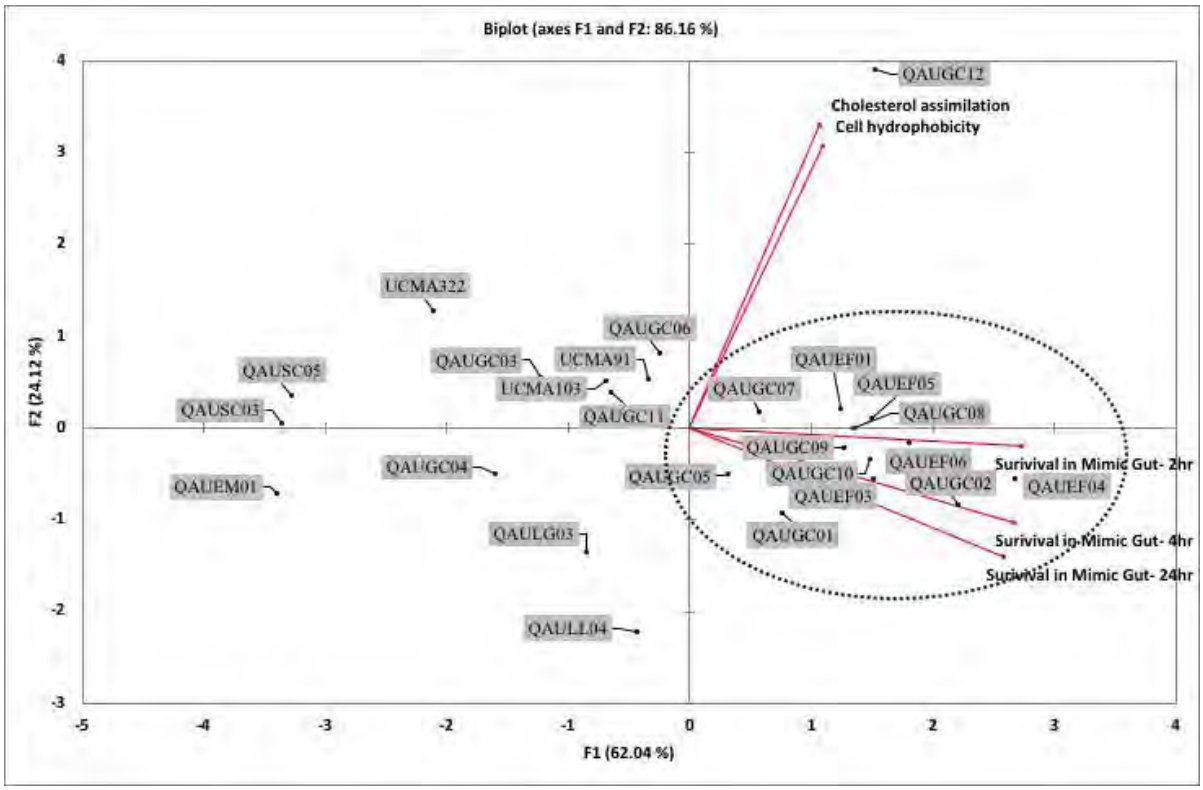
Table 3.9: Inhibitory activity of *G. candidum;* Inhibition zone was ranked as: no inhibition (-), visible inhibition (+), inhibition zone ≤ 5mm (++), zone of 4-8 mm (+++), zone of 8-12 mm or above (++++)

***G. candidum* QAU GC01 Accession No. KT280407**	***E. coli* ATCC8739**	***P. aeruginosa* ATCC9027**	***L. monocytogenes* ATCC13932**	***S. aureus* ATCC6538**
Supernatant	++	+++	-	++
Live Cell	++	+++	-	++

3.4 Statistical Comparison

Tolerance in mimic gut of all microbial strains after initial screening was compared statisticayl. The PCA helped in understanding the trend of the tested strains in presence of tested parameter of mimic gut (Figure 3.18.A). The Agglomerative Hierarchical Clustering helped in grouping the strains into cluster based on similarities in tested parameters (Figure 3.18.B).

A.



B.

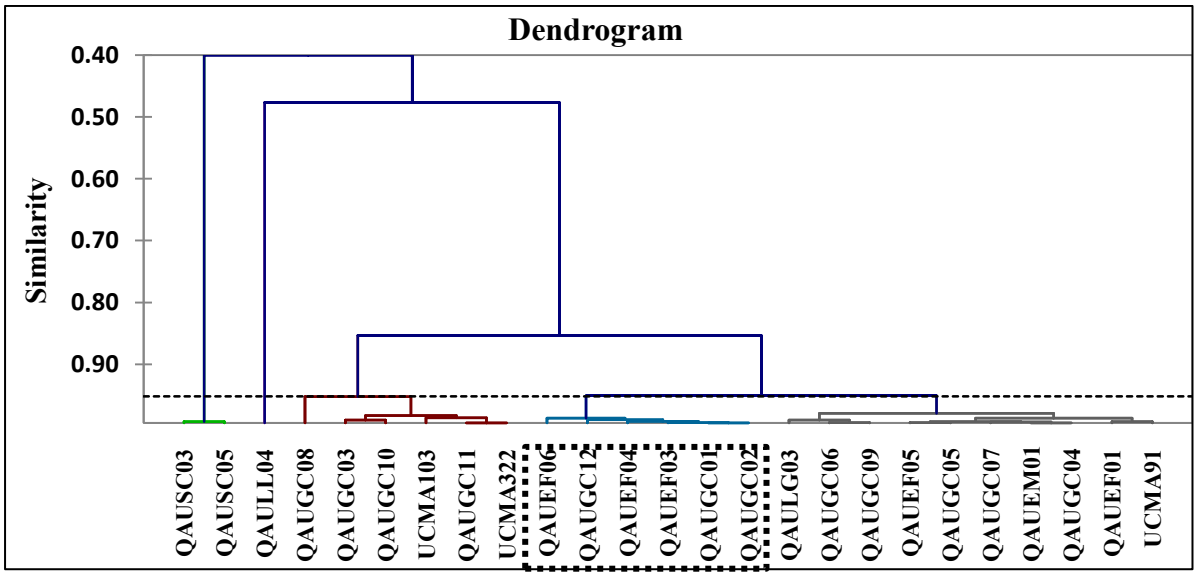


Figure 3.18: A. Principle Components Analysis (PCA) B. Agglomerative Hierarchical Clustering (AHC) indicating tolerance of tested strains in mimic gut condition, cell hydrophobicity and cholesterol assimilation potential.

3.5. Discussion

Imbalanced feeding rendered dairy animals with less productivity. Yeast based Probiotics can help in mitigating adversative effects of imbalanced feed. Many of commercial yeast based probiotics are available in the market, but unsuitable for our local breeds regarding cost and impact on physiology as well as productivity. Experiment was planned to analyze the impact of indigenously isolated microbial strains as probiotic on health, physiology and productivity of dairy cattle.

The microorganisms used in this project were isolated from cow dung, silage and Dahi samples. After their phenotypic and molecular based identification, they were evaluated for extracellular enzymatic activity, mimic gut survival and bioactive properties subsequently. The strain with best enzymatic abilities, mimic gut survival and bioactive properties, were further used in cattle‘s feed as microbial supplement.

In total thirteen lactic acid bacteria were isolated from cow dung samples and were subsequently checked for their probiotic potential. Biochemically all isolates were gram positive cocci and negative for catalase, oxidase, indole and simmon‘s citrate. They were non-motile as well as they all lack the ability of gas production. They were positive only for triple sugar iron test and methyl red test. All these properties indicate that these strains probably belong to lactic acid bacterial family and in agrement with the studied data reported by (Cullimore, 2008; Roos *et al.*, 2005). Based on phylogenetic analysis of experimental bacterial strains, they were characterized to be the member of Enterococcus genera. From corn silage only bacteria were recovered while no yeast isolate was found on OGA, this might be due to the favourable growth condition for lactic acid bacteria. It was reported that in well fermenative silage, Lactic acid should be 65% to 70% of the total silage acids which is because of high concentration of lactic acid bacteria in silage (Shaver & Garrett, 1997). All the bacterial isolates were identified biochemicaly through catalase, oxidase, simmon‘s citrate, motility and methyl red test. Isolated lactic acid bacteria from corn silage, after identification were assesed for their exteracellular enzyme activity. Physiochemistry of all bacterial isolates were determined by using FTIR spectroscopy followed by clustering into groups. The three representative bacteria of all isolates were identified as *Bacillus subtilis* (KT033701) *Bacillus licheniformis* (KT033702), and *Bacillus amyloliquefaciens* (KP826775). From Dahi five *E. faecium* and twelve *G. candidum* were recovered and were

checked for their enzymatic activities and survival in mimic gut conditions. After identification, all the isolated strains were firstly assessed for extracellular enzymatic activates including amylolytic, cellulolytic and proteolytic activities. Those isolates which were positive for amylolytic, cellulolytic and proteolytic activities were further checked for mimic gut survival and bioactive properties.

It was found that cow dung isolates has good enzymatic activities. All isolates from corn silage have shown amylolytic enzyme activity as it was also reported that amylolytic lactic acid bacteria in corn silage (Agati *et al.*, 1998; Sanni *et al.*, 2002). Production of amylases increases the digestibility of fiber in corn silage (Weinberg *et al.*, 2007). All lactic acid bacteria isolates showed amylolytic activity. Moreover, the production of cellulase during ensilage process helps in degrading the cellulosic mass of plants. Sixteen out of eighteen lactic acid bacteria showed cellulase activity. Cellulase activity of lactic acid bacteria from many substrates has been reported in many studies (Saraswati Bai *et al.*, 2012; Mohamed *et al.*, 2010). None of the silage origin isolates showed proteolytic activity in our conditions. It is also reported earlier that lactic acid bacteria isolated from fermented crops showed no proteolytic activity (Chahrour *et al.*, 2013). While Matthews *et al.* (2004) reported protease activity of lactic acid bacteria during vilification. Due to vital role of proteases in digestion, these microbial strains were not processed further for survival in mimic gut conditions. The *E. faecium* isolated from Dahi has minimum extra cellular enzymatic activity in our conditions while all twelve *G. candidum* strains has shown cellulolytic, amylolytic and proteolytic activities.

Only cow dung and Dahi isolates were further proceeded for mimic gut survival, probiotic strains must be resistant to bile salts and survive at low pH as stomach maintains the pH from 2.5-3.3 (Holzapfel *et al.*, 1998). Cow dung isolated bacterial strains QAULL04 has shown maximum survival rate (31.70%) followed by QAUEM01 (3.359%). These results fully supports the bile tolerance activity and showed that these tested strains have the capability of hydrolyzing bile salts by the activity of bile salt hydrolase (BSH) enzyme (A. F. Hofmann & Mysels, 1992). Among Dahi isolates, QAUEF04 has shown maximum survival in mimic gut conditions 58.551%, 50.153% and 49.459% at 2, 4 and 24 hours respectively followed by QAUEF06 (52.614%, 45.47% and 36.21%), while remaining isolates has shown maximum decrease in survival at 4 and 24 hours. Among *G. candidum* strains, Dahi isolates, QAUGC02

has shown maximum survival rate of 53.925%, 48.770% and 48.770% at 2, 4 and 24 hours respectively, followed by QAUGC10 (44.442%, 41.366% and 40.26%) and QAUGC01 (42.04%, 37.74% and 34.511%). QAUGC12 has shown survival rate of 47.442% at 2 hours but later its survival decreases to 24.142% at 4 hours which is 20% decrease.

F0F1ATPase system helps the bacterial cells to survive in acidic stress by using ATP and translocation of protons from the cells through the membranous channels thus raising the intracellular pH (Kullen & Klaenhammer, 1999) Increased expression of general stress protein such as GroESL operon were also detected in low pH (Lorca *et al.*, 2002).Proton pumps, elevated expression of regulators, repairing proteins, regulatory proteins and alterations composition of membranes are few survival strategies adopted by cell during acid shocks (Cotter & Hill, 2003).

Cholesterol assimilation was also observed among all bacterial isolates of cow dung and Dahi samples. All the strains significantly reduced cholesterol level when compared with the standard value. All the cow dung strains showed better cholesterol assimilation. *Lactococcus lactis subsp. lactis* QAULL04 has shown maximum cholesterol assimilation (64.44%) followed by *Enterococcus mundtii* QAUEM01 (50%) while *Lactococcus sp.* QAULG03 showed minimum values of 36.4%. This reduction in cholesterol level is probably assumed due to the deconjugation of bile acids in the liver. (Liong & Shah, 2005) reported that using probiotics strains is one of the most effective ways to control cholesterol level. Formerly, it was suggested that *S. boulardii, P. kudriavzevii* and *S. cerevisiae* have been estimated as potential probiotics for reduction of cholesterol over the past few years (Razin *et al.*, 1980). It is reported that the cholesterol reduction is a consequence of deconjugation of bile salts (Fukushima & Nakano, 1996). This results in the increased excretion of bile acids. Cholesterol is used as a precursor for the synthesis of new bile acids due to which serum cholesterol reduces (Driessen & de Boer, 1989; Tamai *et al.*, 1996). An *invitro* study demonstrated the cholesterol lowering effect by *L. fermentum* probiotics strain (Pereira *et al.*, 2003). (Klaver & Vandermeer, 1993) in their study also illustrated that some *Lactobacillus* sp., undergo cholesterol assimilation by in-vitro deconjugation of bile salts. Cell hydrophobicity of cow dung isolates were measured as QAULG03 has maximum value of 30.845% followed by QAUEM01 (21.104%) and QAULL04 (4.712%). Among Dahi isolates *E. faecium* QAUEF4 has maximum cholesterol assimilation (150.75%) followed by

QAUEF05 (150.48%) while among *G. candidum* QAUGC07, QAUGC02, QAUGC06, QAUGC05, QAUGC01, QAUGC09 has shown maximum cholesterol assimilation respectively. Cell hydrophobicity of *E. faecium* strains (Dahi isolates) were measured as, QAUEF01 has maximum value of 79.13% followed by QAUEF04 (77.94%) and QAUEF03 (70.67%) While QAUGC08 has maximum cell hydrophobicity of 86.10% followed by QAUGC10 (76.87%) and QAUGC03 (75.93%). QAUGC01 has cell hydrophobicity of 46.6%.

Antimicrobial activity of all isolates was checked against *E. coli* (ATCC8739), *P. aeruginosa* (ATCC9027), *S. aureus* (ATCC6538) and *L. monocytogenes* (ATCC13932) which was probably due to the production of some antimicrobial compounds (bacteriocin-like or enteroccin-like). Rest of ATCC strains get control over the Enterococcus might be due to the inactivation of proteins (bacteriocins-like compounds) under the growth conditions. Same results were also reported in a study by several authors (Callewaert *et al.*, 2000; Cintas *et al.*, 1997). (F. Leroy *et al.*, 2003). They demonstrated the antimicrobial activity of *Enterococcus faecium* against *L. monocytogenes* and *S. aureus* pathogenic species which consists of the hypothesis that bacteriocin-like cells would unable to contact with the cells of indicator organisms for inhibitory actions. It was also suggested the use of *E. faecium* in food fermentation as a co-culture (Callewaert *et al.*, 2000). In a reported study by (Cintas *et al.*, 1995), *E. faecium* was responsible for production of some antimicrobial compounds. The compounds in turn prevent the growth of pathogenic bacteria *L. monocytogenes, S. aureus,* which somehow support our data. The *G. candidum* QAUGC01 has maximum antipathogenic activity among all the isolates strains from Dahi. There this strain could be more suitable for application in dairy cattle.

The Principle components Analysis (PCA) indicated that the most tolerant tested strains in mimic gut condition were belong to *E. faecium* and *G. candidum*. The similar trend was also confirmed by Agglomerative Hierarchical Clustering (AHC) shown grouping of these strain in similar cluster.

3.6. Conclusion

At the end of phase one of this project ten lactic acid bacteria and two *S. cerevisiae* from cow dung, eighteen gram-positive bacteria from corn silage while five *E. faecium* and twelve *G.*

candidum strains from Dahi were isolated. All these isolates were checked for their probiotic potential after identification. *Lactococcus lactis subsp. lactis* QAULL04 has maximum gut survival and cholesterol assimilation while its cell hydrophobicity was very low in comparison with *Lactococcus sp.* QAULG03, which has maximum cell hydrophobicity, but its other parameters were low. However, yeast isolates from cow dung have shown notable antipathogenic activity while its amylolytic activity was mild. All the isolated gram-positive bacteria from silage has no proteolytic activity. According to current results we didn‘t get any suitable isolate from cow dung and silage having probiotic potential while isolates from Dahi has shown mixed probiotic properties. From five *E. faecium* E1 has maximum pH tolerance, bile salt tolerance and cell hydrophobicity while E4 has high cholesterol assimilation ability as compare to E1. Among *G. candidum* strains isolated from Dahi, QAUGC03 has maximum pH, bile tolerance and hydrophobicity but its cholesterol assimilation, proteolytic and lipolytic activity was not significant. The *G. candidum* QAUGC12 has tolerance to pH, bile salt, cell hydrophobicity and lipolytic activity but its cholesterol assimilation and proteolytic activity was not reported. Among all isolates only *G. candidum* strain QAUGC01 has all probiotic properties as it showed comparatively significant pH tolerance, bile salt tolerance, cholesterol assimilation, cell hydrophobicity, proteolytic and lipolytic activity.

Chapter 4

Impact of G. candidum QAUGC01 on the Health Status, Physiology and Productivity in Sahiwal Cross Dairy Cow

4. Impact of feeding *G. candidum* QAUGC01 on the Health Status, Physiology and Productivity in Sahiwal cross dairy cow

4.1. Introduction

Health as well as performance improvement of ruminants is among the prime objectives in the livestock production (Puniya *et al.*, 2015). Distinct range of microbial groups found present in the GIT of cattle. (Garcia-Mazcorro & Minamoto, 2013). These microbes are vital in sustaining the physiology of the host, as without a healthy microbiota of the rumen, appropriate functioning of the ruminants seems impossible (Jami *et al.*, 2014). Nutrient digestibility in ruminants found coupled with performance; improvement in performance required bettered nutrient digestibility. Probiotics strains such as strains of *E. faecium* thought to increase digestion and dry matter intake (DMI) by supporting the flora of rumen resulted from the production of lactic acid (Nocek & Kautz, 2006). Supplementation of Probiotics in the feed of dairy cows yield significant benefits, by maintaining the balance of gut flora as well as increase the overall bacterial diversity in the rumen (Pinloche *et al.*, 2013; Stein *et al.*, 2006). Live yeasts are among the most efficient and common Probiotics utilized in the nutrition of ruminants due to their ability in stabilizing the environment of rumen which would favor the proper functioning of rumen flora, more importantly fibrolytic bacteria. Ability of yeast cells to maintain their viability throughout the GIT makes it good Probiotics. Yeast *S. cerevisiae* provided vitamins and organic acids to encourage the LAB growth when introduced in the feed of ruminants (Khan *et al.*, 2016). Inclusion of yeast culture (YC) in the ration of the dairy cows becomes a common feeding practice due its positive effects on milk composition and milk yield (Poppy *et al.*, 2012). Supplementation of YC to dairy cows create more stable environment of the rumen which enhance the digestion rate of fiber by reducing physical effectiveness of fiber and it justify the enhanced DMI that resulted in improved milk composition in addition to yield (Oelberg & Stone, 2014). In dairy animals Probiotics are used to produce more milk, consortia of 2×10^9 cells of *S. cervisiae* and *E. faecium* at 5×10^9 cfu per day enhanced the milk yield at the rate of 2.3 liter per head per day (Nocek & Kautz, 2006). Another consortium comprising of *P. freudenreichii* NP24 and *L. acidophilus* NP51 at the rate of 4×10^9 cfu/head/day positively affect the average milk production with an increase of 7.6% daily (Boyd *et al.*, 2011). Weight gain in dairy calves is very important because it leads to early maturity and more productive lifespan, Probiotics found effective in daily weight gain improvement as supplementation of *B. amyloliquefaciens* H57

resulted in 39% increase in growth rate and 14% increase in feed efficiency (Le *et al.*, 2017). Similarly, 25% and 50% increase in growth rate during pre-weaning and weaning period respectively achieved in response supplementation of *P. jensenii* 702 a novel strain to Holstein calves (Adams *et al.*, 2008). Costs at farms needed to be minimize, these low costs can be achieved by increasing the efficiency of feed as feed costs goes to 70% of total farm costs. To improve the feed efficiency and overall performance of the animals feed additives are the suitable options. In addition, efficiency of feed offers reflection of health, management, nutrition quality and reproductive performance of animals (Bach, 2012). *Geotrichum candidum* (GC), yeast like fungus richly found in dairy products and ubiquitous in nature (Boutrou & Guéguen, 2005). While assessing the Probiotics effects of *G. candidum* QAUGC01 (strain isolated from yogurt) in combination with *B. cereus*, it has shown to improve survival, growth, resistance against diseases and muscle composition of aquaculture (*Labeo rohita*) (Ghori *et al.*, 2018). Increased colonization of the GC in the gut has associated with enhanced digestibility in addition to increased production of antimicrobial substances and improved non-specific immune responses. 22% increase in live weight in response to GC inclusion the feed attributed to enzymatic potential carried by the GC which help in enhanced efficacy (Ibrar *et al.*, 2017).

Imbalanced feeding rendered dairy animals with less productivity. Yeast based Probiotics can help in mitigating adversative effects of imbalanced feed. Many of commercial yeast based probiotics are available in the market, but unsuitable for our local breeds regarding cost and impact on physiology as well as productivity. Experiment was planned to analyze the impact of indigenously isolated *G. candidum* QAUGC01strain as probiotic on health, physiology and productivity of dairy cattle. The selection of experimental and control dairy cows was unbiased and were selected on the basis of similar parity, same lactation phase (Mid Lactating) and Body condition score (BCS).

4.2. Materials and Methods

All the strains isolated in phase I were identified and characterized for their probiotic potential, and among all isolates *Geotrichum candidum* QAUGC01 was selected for cattle feed as microbial base feed supplement. In current study QAUGC01 was given to experimental cows as a feed additive to check its impact on cattle health status, feed efficiency, milk yield and alteration in milk composition in comparison with control group of cows.

4.2.1. Selection of Animals

In this study twelve, three to four months lactating *Sahiwal-Friesian* cross bred cows receiving similar basal diet were randomly selected based on Body Condition Score (BCS) (2.5 to 3.0), body weight (400 to 475 kg) and milk yield. Body condition score **(**BCS) was recorded before start of experiment and at end day of experiment, using a 5-point scale (1=thin and 5=fat), as described by Edmondson (Edmonson *et al.*, 1989). The cows were grouped into Experimental cows (n=9) and Control cows (n=3).

4.2.2. Animals, Diets and Experimental Design

Twelve cows of *Sahiwal*- Friesian cross bred in mid lactation phase were used in the experiment. These cows were selected from Shafi Reso Chemicals (SRC) (Pvt) Ltd. dairy farm, Lahore. Cows were housed in individual pens and fed the basal diet at 09:00 and 16:00 hours while Concentrate feeding was done twice a day (0500 and 1800 hrs) before each milking by keeping experimental animals in separate groups. Fresh and *ad libidum* supply of water was available for 18hrs of a day except for 6hrs during milking, controlled concentrate feeding, and other practices done to ensure control feeding and proper data collection. The diet was balanced according to the recommendations of National Research Council NRC (2001) to fulfill the production and maintenance needs of all lactating cows. For the proper feed intake and digestibility monitoring up to 10% feed refusal was ensured. All the practices (housing, nutrition, experimental procedures etc.) were according to ―Guide for the Care and Use of Agricultural Animals in Research and Teaching" and approved by the ethical committee of Quaid I Azam University, Islamabad. The experiment lasted for 90 days.

Table 4. 1: Selection and grouping of animals

Treatments	Tag No	Milk Yield (Kg/day)	BCS (Body Condition Scoring)	Weight (Kg)	Remarks
Control Cows (without Yeast supplement) **N = 3**	603	19	3	465	Healthy
	605	18.49	3	475	Healthy
	606	17	3	460	Healthy
Experimental Cows (GC01yeast supplemented feed) **N = 9**	602	19	3	469	Healthy
	604	20.92	3	472	Healthy
	613	12.4	2.5	457	Healthy
	617	20	2.5	449	Healthy
	620	15.06	2.5	400	Healthy
	621	17	2.5	406	Healthy
	624	17.8	3	415	Healthy
	632	19	3	414	Healthy
	640	16.6	3	428	Healthy

4.2.3. Analysis of Feed Ingredients and Feed Formulation

Feed ingredients used in basal diet were analyzed for nutritional profile prior to feed formulation. Feedstuffs i.e. concentrate 17% (Master‘s Feed ®) and roughages (Berseem 43%, Corn Silage 35%, Wheat straw 5%) were included and analyzed for proximate composition. Feed samples were analyzed at Centre of Animal Nutrition (CAN), Directorate of Livestock Research and Development, Peshawar. Proximate analysis including Dry matter (DM), crude protein (CP), ether extract (EE), Ash and Crude Fiber (CF) for feed samples was performed by methods

(AOAC, 2000). The levels of ADF (acid detergent fiber) and NDF (neutral detergent fiber) were analyzed by the method of Goering and Van Soest, 1991 and P. Van Soest & Mason, 1991).

4.2.4. Preparation of Yeast Inoculum for Probiotic Feed

The glycerol preserved culture of QAUGC01 was used as microbial based probiotic feed additive for dairy cows. This strain of *G. candidum* QAUGC01 was locally isolated and has been previously screened for probiotic attributes and characterized in Phase I. The fresh culture of QAUGC01 was prepared in Oxy-tetracycline Glucose Broth (OGB) by inoculating it with glycerol preserved yeast culture. After incubation for 48 hours at 30c, the culture was washed with Normal saline to purify the inoculum seed. This probiotic culture was produced on daily basis for 90 days according to the dose requirement.

4.2.5. Preparation of *G. candidum* QAUGC01 Supplemented Feed

For experimental cows probiotic feed was prepared by mixing the freshly prepared yeast inoculum with the concentrate feed as a microbial feed additive. Feed was supplemented with *G. candidum* QAUGC01, 10^8 CFU/ml, at the rate of 100 ml/day/cow for three months. Experimental cows were fed with the feed supplemented with *G. candidum* QAU GC01 at the rate of 10^8CFU/ml and 100 ml/ cow/ day for three months i.e. 90 days. While the control cows were fed with normal feed i.e. without probiotic supplementation.

4.2.6. Growth Performance and Body Weight of Control and Experimental Cows

Individual feed intake was recorded daily. This was done by subtracting the amount of feed refused from the feed offered before the morning feeding. Animals were weighed at 0-Day, 30-Day, 60-Day and 90-Day after restriction of feed and water intake for 16 hours throughout the experimental period by using an electronic scale (Avery Berker L122, USA).

4.2.7. Nutrients Digestibility

Apparent digestibility of experimental and control cows was determined at the end of experiment. Three animals of similar body weight from each group were placed in individual digestibility pens equipped with the facility to collect faces. Animals were fed at two intervals (09:00 and 16.00 hours) at the rate of 90% of voluntary intake for five day‘s collection period. Feed samples were collected daily. Samples were composited by days, dried at 60˚C for 48 hours, ground through a 2mm screen Wiley mill (standard model 4) and analyzed for Dry Matter

(DM), Crude Protein (CP) and Crude Fiber (CF) according to (AOAC, 2000). Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) were determined according to Van Soest et al. (1991) (P. v. Van Soest *et al.*, 1991).

For five days, total faeces voided by the selected cows were collected, weighed and mixed daily, and a representative sample (2%) was taken, stored at -20˚C, and subsequently thawed, dried at 60˚C for 48 hours, ground through a 1mm screen (Wiley mill) for chemical analysis by method of (AOAC, 2000). Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) were determined according to Van Soest et al. (1991) (P. v. Van Soest *et al.*, 1991).

$$\textbf{Dry matter digestibility (\%)} = \frac{\text{Dry matte intake} - \text{Dry matter out go}}{\text{Dry matter intake}} \times 100$$

4.2.8. Blood Collection and Analysis

4.2.8.1. Hematological Parameters

Blood samples were drawn before the start of experiment (0 day) and at the end of experiment (90^{th}-day) from jugular vein of animals between 09.00 and 10.00 hour after morning feeding and immediately put into 5 ml heparinized Venoject® EDTA (K_3) tube and mixed thoroughly for hematological studies. Various hematological parameters i.e., red blood cell (RBC), white blood cell (WBC), hemoglobin (Hb), Packed cell volume (PCV), monocytes (Mono) and eosinophils were determined by method of Benjamin (1985) by using Beckman Coulter® AcT Diff™ Hematology Analyzer at University Diagnostic Lab, University of Veterinary and Animal Sciences Lahore**.**

4.2.8.2 Serum Biochemical Parameters

The blood samples were collected and allowed to clot at 4ºC, and sera were separated by centrifugation at 3000 rpm for 10 minutes and were stored at -20ºC for analysis of serum biochemistry. The serum samples were analyzed at University Diagnostic Lab, University of Veterinary and Animal Sciences Lahore**.**

4.2.8.2.1. Serum Glucose

Serum glucose concentration (mg/dl) was determined with an enzymatic procedure with a commercial kit (kit BD2901-E V1.2-CE, AMP Medizintechnik GmbH, Austria). The absorbance was measured at a wavelength of 500 nm on UVD-2960 spectrophotometer.

4.2.8.2.2. Serum Cholesterol

Serum cholesterol concentration (mg/dl) was measured with an enzymatic procedure with a commercial kit (kit BD2601-E V1.4-CE, AMP Medizintechnik GmbH, Austria). The reaction was employed, and absorbance was read on UVD-2960 spectrophotometer at a wavelength of 500 nm.

4.2.8.2.3 Serum High Density Lipid (HDL)

Serum HDL concentration (mg/dl) was measured with an enzymatic procedure with a commercial kit (kit BD2601-E V1.4-CE, AMP Medizintechnik GmbH, Austria). After reaction the absorbance was read on UVD-2960 spectrophotometer at a wavelength of 500 nm.

4.2.8.2.4. Serum Triglyceride (TG)

Serum triglyceride concentration (mg/dl) was also measured with an enzymatic procedure with a commercial kit (kit BD2601-E V1.4-CE, AMP Medizintechnik GmbH, Austria). The absorbance was read on UVD-2960 spectrophotometer at a wavelength of 500 nm.

4.2.8.2.5. Serum Low Density Lipids (LDL)

Serum LDL concentration (mg/dl) was measured with the Friedewald formula (Warnick *et al.*, 1990)

LDL = Total Cholesterol – HDL – TG/5

4.2.8.2.6 Serum Alanine Aminotransferase (ALT)

Hepatic enzyme Alanine aminotransferase (ALT) and minerals (Ca and P) were determined by using commercial quantification kits (Centronic GmbH, Germany) and the absorbance was determined at a wavelength of 500 nm and was read on UVD-2960 spectrophotometer.

4.2.8.2.7. Short Chain Fatty Acids Analysis in Serum samples of Experimental and Control Cows at Zero-Day and End-Day

4.2.8.2.7.1. Standard Curve

The commercial standard of Butyric acid (TCI no. B0745) was purchased to generate standard curve. Six dilutions 5, 10, 15, 20, 25, 30µg/ 100 µl were prepared by dissolving standard into water. A calibration curve was generated through GC-FID.

4.2.8.2.7.2. Extraction of Short Chain Fatty Acids (SCFAs)

Short chain fatty acids were extracted from cattle serum both at zero and end day of experimental trial by using protocol given by (Skoglund, 2016). According to the protocol, 200 µl of serum, 100 µl of 150 µM Acrylic acid (Sigma-Aldrich no. 147230), and 100 µl of 1500 µM m-Phosphoric acid (Merck no. 100456) were added in a microliter tube followed by vertexing on high speed for 5 minutes and centrifugation at 14,000 rpms (HERMLE Z236K). 100 µl supernatant was separated in a new microliter tube after half hour refrigeration at -20 ^{o}C and 100 µl of pure Propyl format (Sigma-Aldrich no. W294306-1KG-K), was added followed by the second cycle of centrifugation at 14,000 rpms. The supernatant was collected and directly analyzed by GC-FID (Agilent Technologies. 6890N).

4.2.8.2.7.3. Quantitative Analysis of SCFAs by GCFID

To determine SCFAs in the samples Gas Chromatography-Flame Ionization Detector was used. The protocol used was adopted from study reported by (Baltierra-Trejo *et al.*, 2015). 1 µl of the sample was injected in the inlet valve fitted with the capillary column. Nitrogen (N_2) gas was used as carrier gas at a constant flow rate of 15.0 ml min^{-1}. An oven temperature of GC was maintained at 200^{o}C while that of FID was maintained at 250^{o}C. For the injector, the temperature started from 120^{o}C with a ramp of 10^{o}C until reached 200^{o}C was used (Baltierra-Trejo *et al.*, 2015).

In the preparation of samples, propyl format was used which causes loss of SCFAs from the sample. That‘s why method‘s accuracy was evaluated by adding the calculated amount of 20µg/ml of acrylic acid (Ac) into the sample before the propyl format according to the protocol (Skoglund, 2016). After the estimation of SCFAs, the amount of acrylic acid was added in the amount of SCFAs detected by GC-FID as no peak for acrylic acid was detected.

Actual Concentration = Observed concentration + Amount of Acrylic acid added

4.2.9. Milk Production and Composition

Animals were milked twice daily at 0500 and 1800 hrs and milk production was recorded daily. Milk samples (200 ml) were collected from morning and afternoon milking, pooled and frozen for subsequent analysis at fortnightly intervals. Milk samples were used for quality analysis i.e Fat%, Protein %, Lactose %, Total Solids, Solid Non-Fat % and milk pH by using Milk analyzer

(Lactoscan, Bulgaria) at University Diagnostic Lab, University of Veterinary and Animal Sciences Lahore**.** And the collected milk samples were analyzed for determination of milk cholesterol.

4.2.9.1. Milk Cholesterol

Frozen milk samples were thawed in a water bath and then homogenized for 5 min. The milk cholesterol was determined by using methods described by (Kessler *et al.*, 2014), according to method, 9 mL of ethanol and 1 mL of KOH (8.9 M KOH solution) were added to 2 mL of raw milk. The mixture was vortexed for 20 seconds. The test tube was then placed at 60°C in a water bath, saponification was performed, and stirred at 200 rpm for 1 hour. Then the samples were cooled at room temperature. For extraction 5 mL of deionized water and 10 mL of hexane were added and vortexed for approximately 2 min. The sample was centrifuged at 2,500 × *g* for 3 min at 4°C. The upper hexane layer was collected into a new sterilized vial. The extraction process was repeated, and the upper hexane layer was also transferred into the test tube. The tubes are placed in water bath for drying to evaporate the hexane extract to dryness at 70°C and the sample was re-dissolved in 2 mL of isopropanol. The extracted samples were then analyzed to measure total cholesterol in milk by using Semi-Automatic Biochemistry analyzer (MicroLab300, Merck Germany) by using the kits of Centronic GmbH/ Germany.

4.2.10. Statistical Analysis

The statistical analysis of data was done by using XLSTAT 2014.5.03. All the Data was expressed as Mean ± SD. T test was used for the comparison of control and experimental samples while ANOVA followed by tukey‘s test ($P<0.05$) was used for the comparison between different samples. Pearson Correlation was applied to determine the correlation between different parameters.

4.3. Results

A total of twelve mid lactating dairy cows were selected for the current study. They were divided into control (n= 3) and experimental cows (n= 9). Basal diet meeting the nutrient allowances of NARC (2001) was used in the study. Control feed consisted of normal feed while probiotic feed consisted of normal feed supplemented with probiotic strain of *G. candidum* QAUGC01. Experimental cows were fed with probiotic feed for 90 days while control cows were fed without probiotic yeast. Blood and Serum samples were collected at zero day (before feeding *G. candidum* QAUGC01 supplemented feed) and at the end day (90^{th}-Day), which were then subjected to blood profiling, serum biochemistry. The dung samples were collected before *G. candidum* QAUGC01 supplementation and Five days after end day (90^{th}) of experiment in sterilized condition, and immediately analyzed for microbiological study in phase III. Milk was collected for milk yield, milk composition, milk cholesterol and for determination of feed efficiency of control and experimental group of dairy cows.

4.3.1. Basal Diet Composition

Ingredients and Chemical Composition of Basal diet and water availability for experimental and control cows has been mentioned in Table 4.2. The basal diet of control and experimental cows was composed of berseem 19.73%, Corn silage 35.33%, Wheat straw 10.31% and Concentrate Feed 34.32% on dry matter basis.

Table 4. 2: Ingredients and Chemical Composition of Basal diet and water availability for experimental and control cows

Ingredients of Diet	Portion (%)	Chemical Composition						
		DM (%)	CP (%)	NDF (%)	ADF (%)	CF (%)	E.E (%)	Ash (%)
Corn (Silage)	35	44	8.5	44.5	27.5	28	3.2	4
Wheat Straw	5	92.5	4.8	73	49.4	36	1.6	7.6
Commercial feed (Master's Feed®)	17	88	18	29.33	14.17	7.8	4.91	9.06
Berseem (*Trifolium alexandrinum*)	43	20	19	46	34	25.1	3	9
Mineral Mixture (Energizer ®)	100gm/cow/day							
Water	Fresh water available for 24hrs with free excess							

4.3.2. Preparation of Probiotic Feed

Probiotic feed is a part of basal diet i.e. concentrates feed supplemented with fresh culture of locally isolated and characterized probiotic yeast *G. candidum* QAUGC01 daily for ninety days of experiment.

4.3.3. Nutrient Digestibility

The Apparent Digestibility of Dry matter and nutrients of Experimental and Control cows is given in Table 4.3.

Table 4.3: Apparent Digestibility of control and experimental cows at Day 90

Components	Cows		
	Experimental	Control	*P*-Value
No. of cows	9	3	
Apparent Digestibility (%)			
DM	70.1	66.2	0.0164
CP	72.3	67.1	0.0001
NDF	70.2	68.3	0.1423
ADF	71.2	67.8	0.0019
CF	67.9	61.2	0.0000
E.E	66.5	61.4	0.0000

4.3.4. Dry Matter Intake and Feed Efficiency of Control and Experimental Cows

Feed efficiency was determined as kg of milk produced per kg of dry matter consumed. Among control cows maximum feed efficiency recorded was 1.203 with the dry matter intake of 14 kg. Among experimental cows maximum feed efficiency recorded was 1.50 with dry matter intake of 14 kg followed by 1.47 and 1.46 with the dry matter intake of 14, 13.125 kg respectively. The Feed efficiency of all cows has been mentioned in following Table 4.4.

Table 4. 4: Dry matter intake and feed efficiency among control and experimental cows

Cow no	DM Intake Kg	Feed Efficiency
603C	14	1.119132071
605C	14.5	1.035871183
606C	14	1.203458132
602E	14	1.124417054
604E	14	1.506062688
613E	13.125	0.808923673
617E	14	1.344672489
620E	12.6875	1.255776955
621E	13.125	1.470796823
624E	13.125	1.343616432
632E	14	1.467509583
640E	13.125	1.412793188

4.3.5. Impact of Feeding *G. candidum* QAUGC01 on Dry Matter Intake, Milk Yield and Feed Efficiency

Table 4.5 shows that the experimental cows produced more milk with significant (p=0.042) difference as compared to the average milk production of control cows. The control cows consumed more average dry mater (DM) than experimental cows but the average feed efficiency of experimental cows was significantly (p=0.032) high than control cows.

Table 4. 5: Comparison of average Dry matter intake, Milk yield and feed efficiency among control and experimental cows

Parameter	Control (SD)	Experimental (SD)	Significance (p-value)
Dry Matter (Kg/day)	14.16(0.37)	13.46(0.59)	0.045
Milk Production (Kg /day)	14.77 (0.30)	15.26 (2.96)	0.042
Feed Efficiency (%)	1.12 (0.089)	1.30 (0.22)	0.032

4.3.6. Impact of *G. candidum* QAUGC01 Supplementation on Body Weight of Experimental and Control Cows

The body weight of experimental and control dairy cows increased slightly from start to end of experiment and no significant difference was found between body weight of experimental and control cows.

Table 4.6: Body weight of control and experimental cows on zero day till end day

Cow Group	Cow Weight (Kg)				
	(0-Day)	**(30-Day)**	**(60-Day)**	**(90-Day)**	**P= 0.05**
Control	466.7	470.7	474.3	478.7	0.12
Experimental	434.4	439.0	444.2	447.2	0.34

4.3.7. Impact of Feeding *G. candidum* QAUGC01 on Hematological Parameters

Blood samples from all the experimental and control cows were tested before the start of experiment i.e. 0-Day and at the end of the experiment i.e. 90^{th} day to monitor the health status of dairy cows.

Blood sample was collected in sterile conditions from both control and experimental cows and was analyzed to check the impact of *G. candidum* QAUGC01 on blood profile. All the blood parameters were found with in normal ranges, both in control and experimental cows.

Fluctuation in RBC, WBC and hemoglobin has shown the good health status of experimental cows. Neutrophils increase above normal range represents the infectious state of animal but in this study, neutrophils were within normal range both in control and experimental cows and showed noninfectious condition of cows

Table 4. 7: Hematological Parameters at the Zero-day and End-day of Experiment in Control and Experimental animals

Parameter (mg/dl)	Time Period	Control Cows		Treated cows		Normal Range mg/dl
		Mean	P-value	Mean	P-value	
Red Blood Cells	0-Day	7623333	0.7183	7707778	0.0091	5---10 Millions/ Micron L
	End-Day	6976667		8772222		
White Blood Cells	0-Day	9000.0	0.2485	9922.2	0.4941	4000---12000/ Micro L
	End-Day	7393.3		9524.4		
Hemoglobin	0-Day	7.73	0.7231	8.53	0.0032	8---15 g/dL
	End-Day	8.30		10.25		
Neutrophils	0-Day	53.33	0.6500	57.33	0.4255	26---70 %
	End-Day	55		58.55		
Monocytes	0-Day	4.33	0.9275	4.11	0.6634	1---10 %
	End-Day	4.66		4.77		
Eosinophils	0-Day	7.00	0.9275	7.22	0.8277	1----15 %
	End-Day	7.33		6.88		
MCV	0-Day	48.33	0.5263	46.36	0.7006	34---58 fL
	End-Day	46		45.77		
PCV (HCT) %	0-Day	34.7100	0.9164	34.1389	0.0117	32---48 %
	End-Day	33.7667		39.4944		

Reference range according to Merck Veterinary Manual

4.3.7.1. Red Blood Cells (RBCs)

The normal range of red blood cells in cattle blood is 5-10 million per micro liter (10^6//µL). Blood samples from experimental as well as control animals were tested for RBC count before and at the end of the experiment. At the start of experiment i.e. at 0-Day RBC count in experimental animals was very slightly greater than control animals while at the end of experiment i.e. at 90^{th} day RBC count increased more in experimental animals. In control animal's RBC count decreased from 7.69 x 10^6 /µL to 6.9 x 10^6/µL for 90 days experimental period while in the experimental animals, fed with probiotic yeast, it increased from 7.7 x 10^6//µL to 8.72 x 10^6//µL.

4.3.7.2. White Blood Cells (WBCs)

The normal range of white blood cells in cattle blood is 4000-12000 WBCs per micro liter. Blood samples from experimental as well as control animals were tested for WBC count before and at the end of the experiment. At the start of experiment i.e. at 0-Day WBC count in experimental animals was slightly greater than control animals while at the end of experiment i.e. at 90^{th} day WBC count increased more in experimental animals. In control animals WBC count decreased from 9000 to 7393.3 for 90 days' experimental period while in the experimental animals it also decreased from 9922.2 to 9524.4, But this decrease in experimental animals was almost negligible and very low as compared to decrease in control cows.

4.3.7.3. Percent Neutrophils Count

The percent neutrophils was increased both in experimental and control blood samples within normal range (26% to 70%). It increased (P= 0.42) more than percent Neutrophils count of control blood (P= 0.65).

4.3.7.4. Hemoglobin & Packed Cell Volume (PCV)

The normal range of hemoglobin in cattle blood is 8-15 g/dL while that of PCV is 24-46%. Similar to RBCs and WBCs they were also tested in animals' blood samples. The hemoglobin in experimental cows increased from 8.53 to 10.25 g/dL while in control cows it also increased from 7.73 to 8.30 g/dL. The PCV increased in experimental cows while decreased in control cows from the start to the end of experiment. The PCV before the start of experiment was 34.71% & 34.13% and at the end of experiment it was 33.766% & 39.49% in control and experimental cows respectively.

4.3.8. Serum Biochemical Parameters

All serum parameters were also analyzed by using semi-automatic Bio Analyzer. Table 4.8 summarizes the impact of feeding *G. candidum* QAUGC01 probiotic strain on serum biochemical parameters.

Table 4.8: Serum biochemistry at the zero-day and end-day of experiment in control and experimental animals

Parameter	Time Period	Control Cows		Treated cows		Normal Range
		Mean	P-value	Mean	P-value	
Glucose mg/dl	0-Day	**59.0**	**0.6963**	52.67	0.001	42-75 mg/dl
	End-Day	57.33		58.00		
Cholesterol mg/dl	0-Day	235.33	0.8267	192.22	0.0024	62-193 mg/dl
	End-Day	230.67		166.55		
Triglycerides mg/dl	0-Day	41.33	0.0891	37.22	0.001	0-200 mg/dl
	End-Day	57.33		53.00		
HDL mg/dl	0-Day	43.33	0.624	38.89	0.3987	40-60 mg/dl
	End-Day	42.33		39.89		
LDL mg/dl	0-Day	183.73	0.7336	156.00	0.1225	<130 mg/dl
	End-Day	176.87		126.95		
Phosphorus Mg/dl	0-Day	6.00	0.6433	5.22	1	4.3-7.8 mg/dl
	End-Day	5.33		5.22		
Calcium Mg/dl	0-Day	4.67	0.7142	3.77	0.3664	8.4-11 mg/dl
	End-Day	4.57		4.29		
ALT	0-Day	26	0.7335	28.66	0.1476	7-35 Micron/L
	End-Day	28		32.11		

4.3.8.1. Serum Glucose Level

Biochemical analysis of serum glucose level was measured by using commercial kit. The normal glucose range in cattle is 42-75 mg/dl. In experimental cow‘s glucose level significantly increased ($P<0.05$) from 52.6 to 58.1 mg/dl from start to the end of experiment. In control cows it decreased a little ($P>0.05$) i.e. from 59 to 57.33 mg/dl. The increase of glucose level in blood was considerably greater in experimental cows as compared to control cows.

4.3.8.2. Serum Hepatic Enzyme ALT

Hepatic enzyme Alanine aminotransferase (ALT) was also measured using quantification kit. The normal range of ALT in serum is 7-35 micron per liter. At end of experiment ALT level in serum increased in both experimental as well as control cows. The increase was greater ($P<0.05$)

in experimental cows. Serum ALT level increased from 28.67 to 32.11 micron/L in experimental cows and increased from 26 to 28 micron/L in control cows.

4.3.8.3. Serum Lipid Profile

Serum lipid profile such as triglyceride (TG), high density lipoprotein (HDL) and cholesterol were measured using kits (Spinreact, Spain). The LDL was determined by Friedewald equation. Serum lipid profile shows that cholesterol level decreased in experimental as well as the control cows during experimental period. Normal serum cholesterol value ranges from 62 to 193mg/dl. This decrease was very little ($P>0.05$) in control cows (235.3 to 230.6 mg/dl) while the total cholesterol reduced significantly ($P<0.05$) in experimental cows (202.3 to 177.4 mg/dl). Triglycerides increased in both experimental and control cows with in normal range (0 to 200 g/dl). This increase was significant ($P<0.05$) in experimental (37.22 to 53.0 g/dl) as compared to control ($P>0.05$) (41.33 to 57.33 g/dl). Serum HDL decreased in control cows (43.33 to 42.33 mg/dl) but increased in experimental cows from 38.89 to 39.89mg/dl. Significant effect ($P<0.05$) to decrease bad cholesterol (LDL) was reported in experimental cows while very less decrease ($P>0.05$) in control cows.

4.3.8.4. Minerals Calcium and Phosphorus in Serum Samples of Experimental and Control Cows at Zero-Day and End-Day of Experiment

Calcium and phosphorus are two of the main minerals in the body required for important functions. Minerals i.e. Calcium (Ca) and Phosphorus (P) were measured by using commercial kit (Centronic GmbH, Germany). Calcium increased in experimental as well as control cows, but the increase was greater in experimental cows. It increased from 3.77 to 4.29 mg/dl in experimental cows while from 4.27 to 4.57 mg/dl in control cows. Phosphorus remains same in experimental cows 5.22 mg/dl while decreased in control cows at the end of the experiment (6 to 5.33 mg/dl).

4.3.8.5. Butyric Acid Concentration in Serum Samples of Experimental and Control Cows at Zero-day and End-day of Experiment

Serum samples were processed by using GC-FID to determine the quantity of butyrate in cow‘s serum. Retention time for sample was 10 min and peak at 3.71 min retention time was observed for butyrate as per butyrate standard retention time. Observed concentration and peak area of butyrate in control and experimental cows at zero and end day of experiment is given in below

table. In experimental cows serum butyrate concentration was higher except 613 at the end of experiment as compare to zero day. While opposite trend was observed in control cow‘s butyrate concentration was low in their serum at the end of experiment (Table 4.9 & Fig.4.1).

Table 4.9: Peak area (A) and concentration mM/ml of Serum butyrate in Experimental and Control cows at Zero-Day and End-day.

Serum Samples	Group	Butyrate Peak Area (A)		Butyrate Concentration mM/ml	
		Zero Day	End Day	Zero Day	End Day
602E	**Experimental Cows**	4.62	21.03	0.279	0.466
604E		0	4.27	0.227	0.275
613E		15.98	0	0.408	0.227
617E		0	12.61	0.227	0.370
620E		15.16	22.90	0.399	0.487
621E		11.11	71.97	0.353	1.044
624E		0	10.36	0.227	0.345
632E		10.77	9.979	0.349	0.340
640E		11.32	25.97	0.356	0.522
603C	**Control Cows**	10.66	11.73	0.348	0.360
605C		11.89	11.08	0.362	0.353
606C		32.51	14.19	0.596	0.388

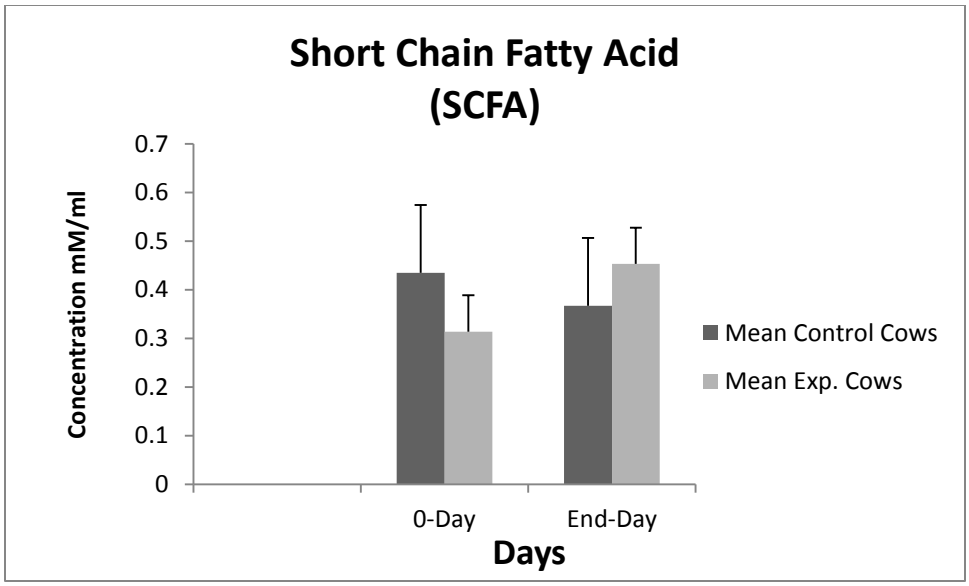


Figure 4. 1: Mean Butyrate concentration mM/ml in serum of experimental and control cows at zero-day and end-day of experiment

4.3.8.6. Statistical Comparison of Butyrate Concentration mM/ml in Serum of Experimental and Control cows

The average serum butyrate concentration increased in experimental cows from zero-day to end day of experiment under the influence of G. candidum QAUGC01probiotic yeast while in case of control cows the average butyrate level decreased highly from zero day to end day of experiment by applying t-Test (Fig.4.1).

Table 4. 10: Statistical comparison of butyrate concentration mM/ml in serum of experimental and control cows at zero-day and end-day of experiment

Parameter	**Butyrate Concentration mM/ml**		**Significance**
Cows	Zero-Day	End- Day	**(p-value)**
Control (SD)	0.435	0.367	0.447
Experimental (SD)	0.314	0.453	0.119

4.3.9. Milk Yield and Composition

Milk production and composition varies throughout the experimental trails both in control and experimental groups. In milk composition, percentage of milk protein, lactose, fats, solid nonfat (SNF), milk solids, milk density and milk pH and cholesterol were measured on different time interval. The trend of milk yield throughout the experimental period varies between control and experimental group. Milk yield fluctuate during experimental period is due to seasonal variation. As it was previously reported that in hot summer milk yield decreases along with variation in milk composition, due to hormonal changes. This effect can be overcome by using probiotic supplements in cattle‘s feed.

4.3.9.1. Impact of feeding *G. candidum* QAUGC01 on Milk Yield During High Temperature Humidity Index from Day-0 to 90th day of Experiment

After supplementation of *G. candidum* QAUGC01 in experimental cow‘s feed, it was found that milk yield in control cows decreased from 18.31 to 17.54 L/day/cow in starting fifteen days while it increases once to 18.97 L/day/cow on thirtieth day and later on it decreases up to 10.45 L/cow at the end of experiment while in experimental cows‘ trend of milk yield is a bit different. Among experimental cow‘s milk yield first increased from 17.53 to 18.17 L/day/cow while further milk yield decreased till end day by 11.90 L/day/cow. As this experiment was conducted in hot summer days and due to heat stress milk yield decreased gradually, while it was clearly found that *G. candidum* QAUGC01 has reduced the heat stress among experimental cows and decrease in milk was low in experimental cows. In comparison, throughout experimental period average milk yield was high among experimental cows then control cows. The drop-in milk yield in control cows was 42.92% as compared to experimental cows (32.11%) that showed more than 10% less drop in average milk yield in experimental cows than control. therefore, the experimental cows maintained good feed efficiency than control during 3 months of hot season with high temperature humidity index (THI). So, the experimental dairy group produced 1.45 kg/day/cow more milk than control group under high temperature humidity index (THI) (Figure 4.2).

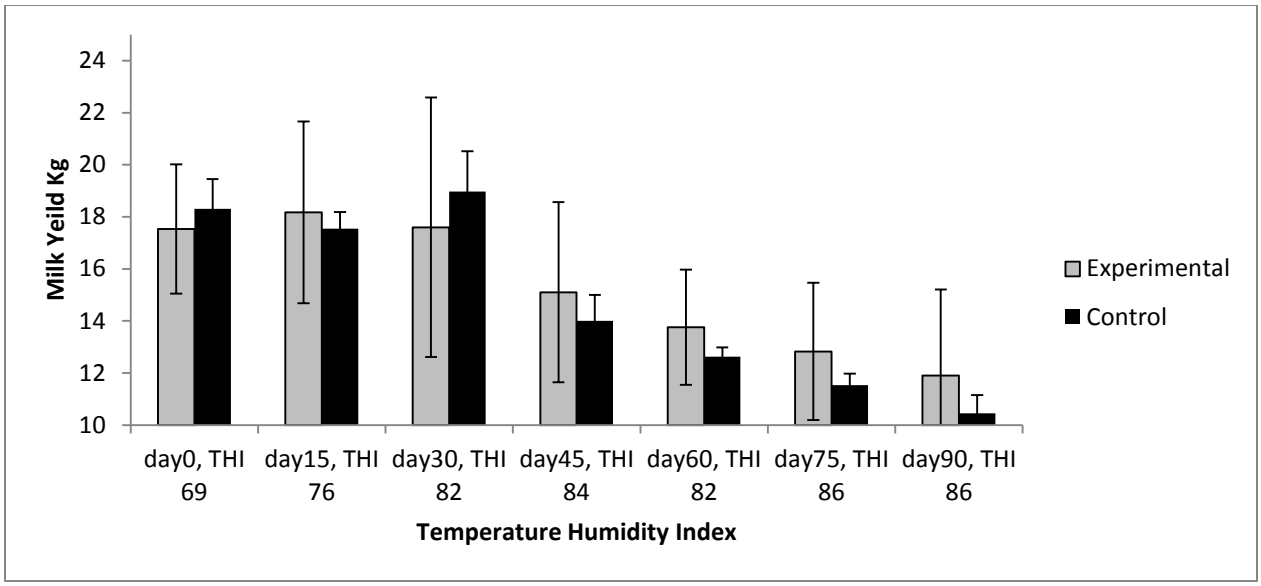


Figure 4. 2: Impact of *G. candidum* QAUGC01 supplementation on milk yield during high temperature index from day-0 to 90th day of experiment

The current trial showed that experimental cows produced more milk with significant (p=0.042) difference as compared to the average milk production of control cows, while the control cows consumed more average dry mater (DM) than experimental cows but the average milk yield (P=0.042) and feed efficiency of experimental cows was significantly (P=0.032) high than control cows (Table 4.11).

Table 4. 11: Average Milk yield of each experimental and control cows during 90 days of experiment

Cow no	Average milk, kgs	DM Intake Kg
603C	15.05714	14
605C	14.44714	14.5
606C	14.82714	14
602E	13.24286	14
604E	17.35286	14
613E	9.272857	13.125
617E	17.04714	14
620E	14.01429	12.6875
621E	15.37571	13.125
624E	16.05286	13.125
632E	19.83571	14
640E	15.16286	13.125

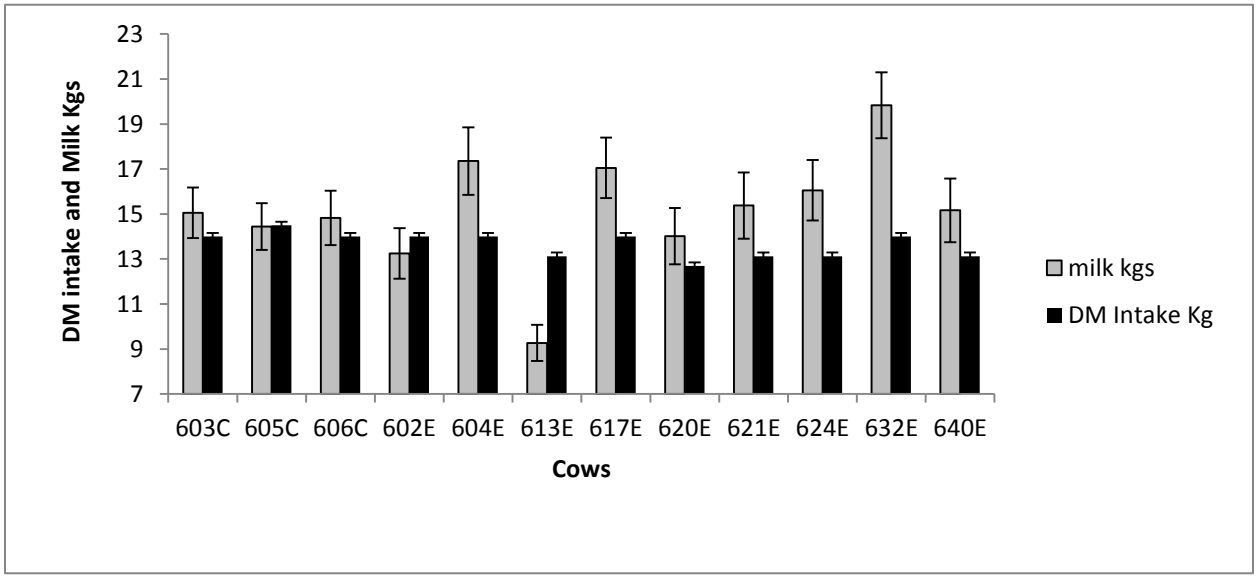


Figure 4. 3: Milk yield among control and experimental cows with dry matter intake

E= Experimental cow C= Control cow

4.3.9.2. Milk Composition

In milk composition different parameter has been analyzed including milk protein, lactose, fats, solid nonfat (SNF), milk solids, density, cholesterol, HDL, TG and pH (Kanwal *et al.*, 2004).

4.3.9.2.1. Milk Protein

Throughout the experimental trail, it was found that milk protein in experimental cows were high as compare to control after fifteenth day of experiment. Variation in milk protein percentage is different between control and experimental cows. In the start of experiment milk protein were high in control cow's (3.4) while low in experimental cows (3.28%) (Appendix1). After feeding experimental cows on *G. candidum* QAUGC01 supplemented feed throughout experiment till end milk protein were high in experimental cows as compared to control cows. In control cow's milk protein decreased from 3.4 % to 2.9 % from start of experiment to forty-fifth day. Afterwards it remained constant as 2.9%. In experimental cow's milk protein increased from 3.3% to 3.6% in starting fifteen days, later on it has decreased up to 3.1 on seventy-fifth day while small increase was noted on last day (3.2%).

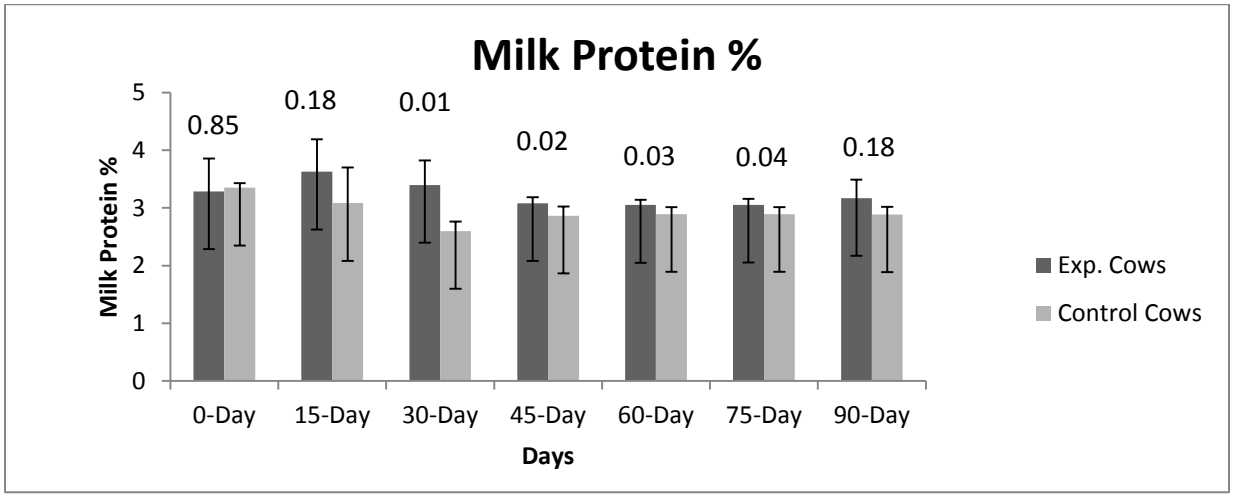


Figure 4. 4: Milk protein alteration among control and experimental cows

4.3.9.2.2. Milk Fat

Milk fat percentage was measured among control and experimental cows to check the impact of *G. candidum* QAUGC01 in alteration of total fat level in milk. It was observed after experiment that *Geotrichum candidum* QAUGC01 has changed the fat percentage in experimental cows as it

increased from 4.2% to 4.7%, while in control group milk fat percentage decreased from 4.2% to 4.1%. The figure 4.5 described that the milk fat % increased significantly (p=0.036) in experimental cow's milk than control at 45th day and remained high non significantly at 65th day (p=0.08) and at 75th day (p=0.07) of experiment. At the end day there was no significant difference in the milk fat content of experimental cows increased to 4.7% as compared to control 4.09% (Appendix 2).

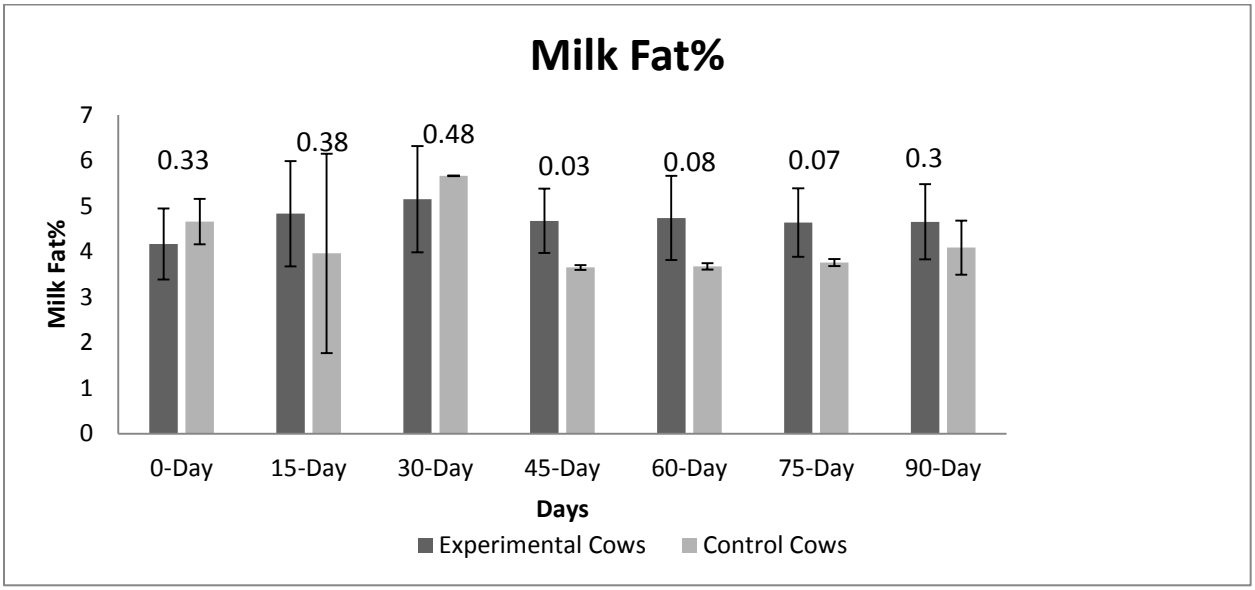


Figure 4. 5: Milk Fat content in control and experimental cows

4.3.9.2.3. Milk Lactose

Milk lactose is one of the important components; percentage of milk lactose was measured both in control and experimental cows to check the impact of *Geotrichum candidum* QAUGC01 in altering milk composition. Milk lactose in control cows increased from zero day to 90th day (4.21 to 4.32%) but significantly (p=0.008) increased in milk of experimental cows (4.12 to 4.61%). (Appendix 3)

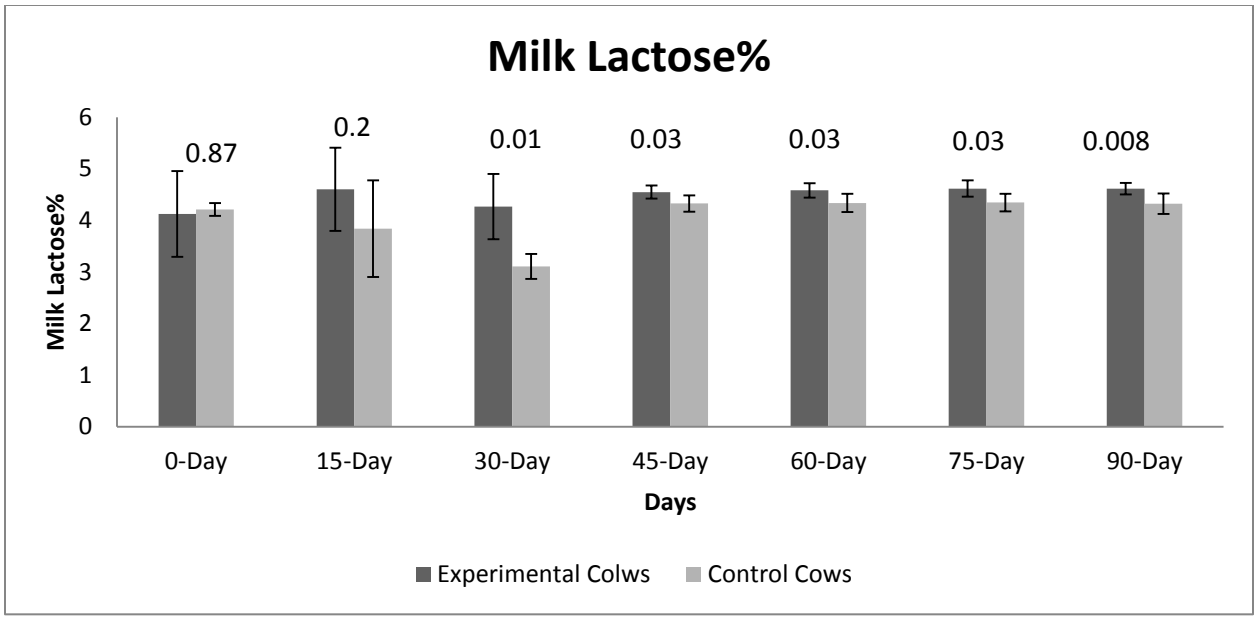


Figure 4. 6: Milk lactose variation in control and experimental cows

4.3.9.2.4. Milk Solid Non Fat (SNF)

Milk solid Non Fat was calculated of both control and experimental cows. It was found that percentage of SNF decreased both in control and experimental cows, but this decrease was in normal range (8-14%) among experimental cows. Milk SNF percentage decreased from 8.80 to 8.36% in experimental cows while in control cows this decrease was from 8.97 to 7.87%. There was significant (p=0.01) increase in SNF in experimental cow‘s milk (Appendix 4).

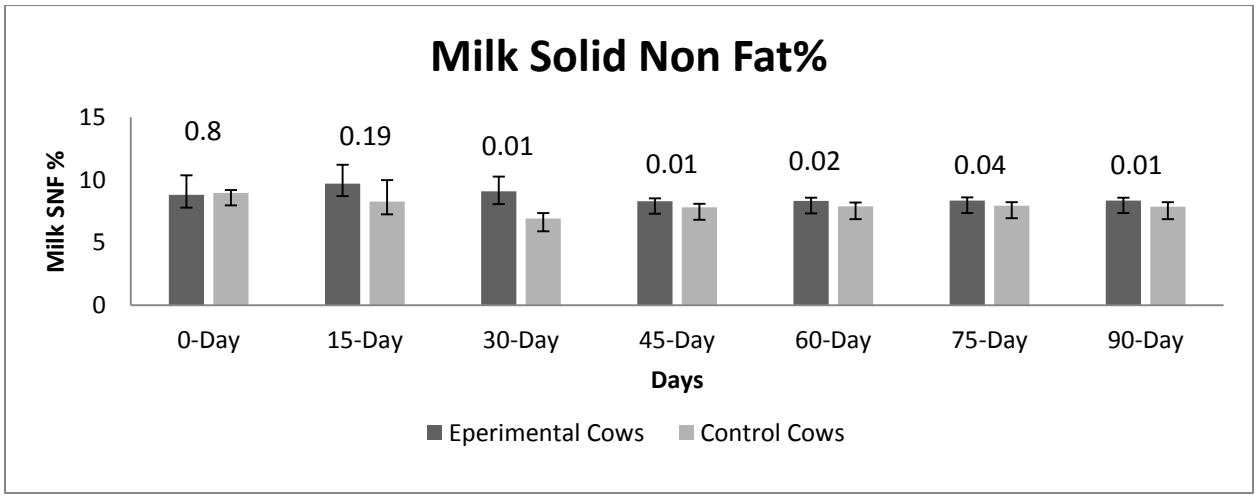


Figure 4. 7: Milk SNF % in control and experimental cows

4.3.9.2.5. Milk Density

Milk Density of milk was determined in control and experimental cows to check the impact of supplementation of *Geotrichum candidum* QAUGC01 in altering milk composition. Milk density has increased both in control (27.1 to 27.5%) and experimental cows (26.7 to 28.8%). The alterations has been shown by figure 4.8.

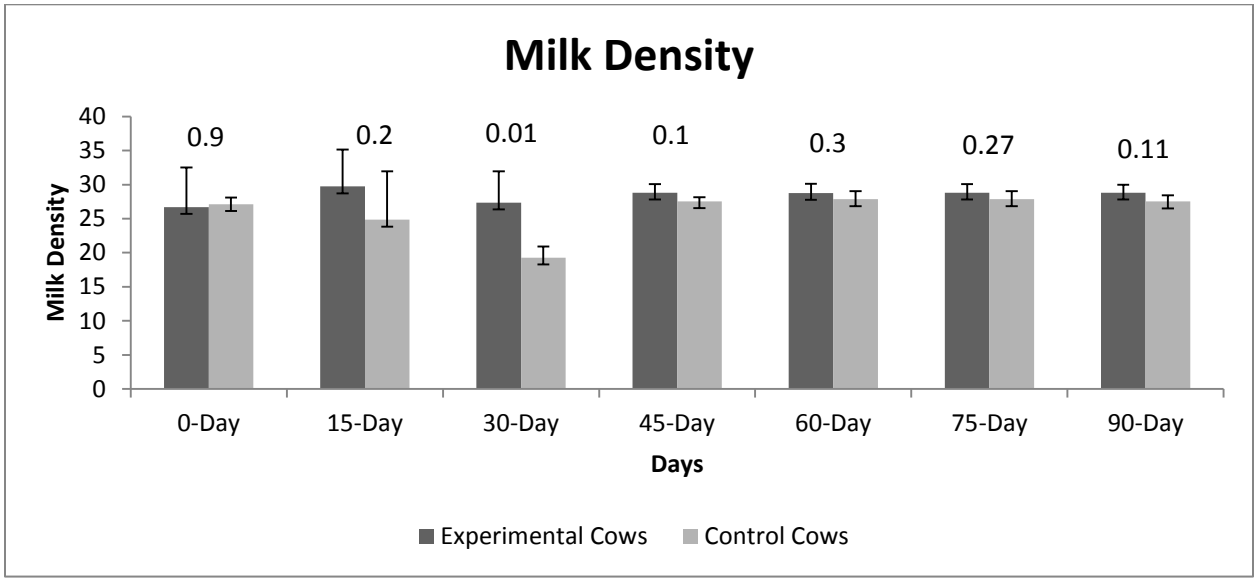


Figure 4. 8: Milk density in control and experimental cows

4.3.9.2.6. Milk Solids

Total milk solid was measured in the milk samples of control and experimental cows. Total milk solid was in normal range (13.43 to 14.34%) among experimental cows (12.97 to 13.02%) throughout the experiment but in control cows total milk solid decreased from 13.63 to 11.96%.

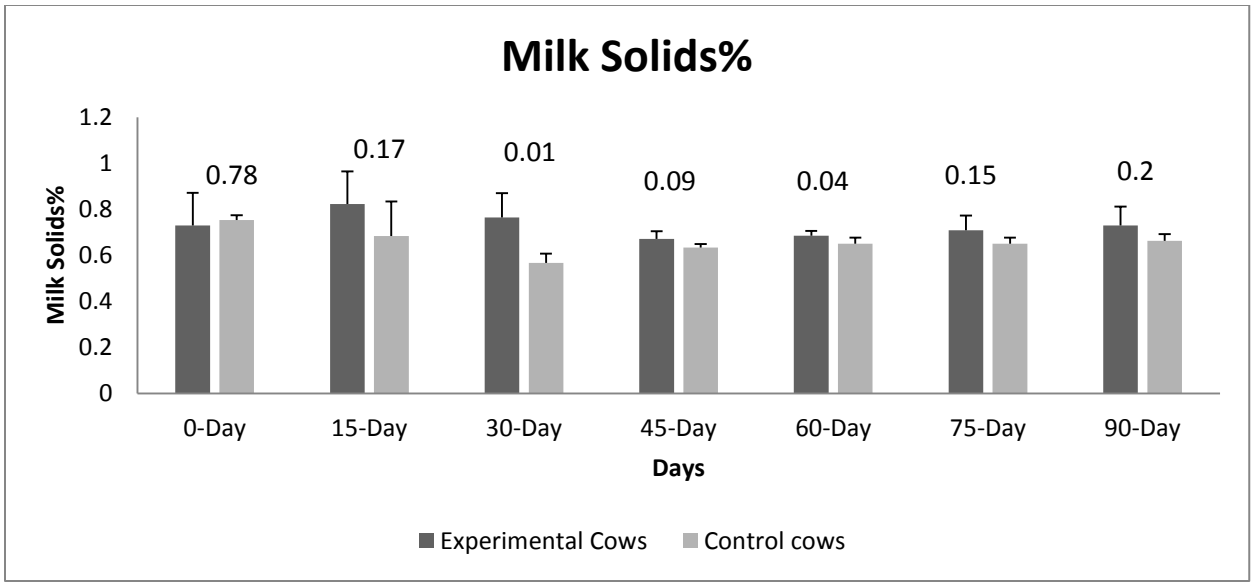


Figure 4. 9: Milk Total solid in control and experimental cows

4.3.9.2.7. Milk pH

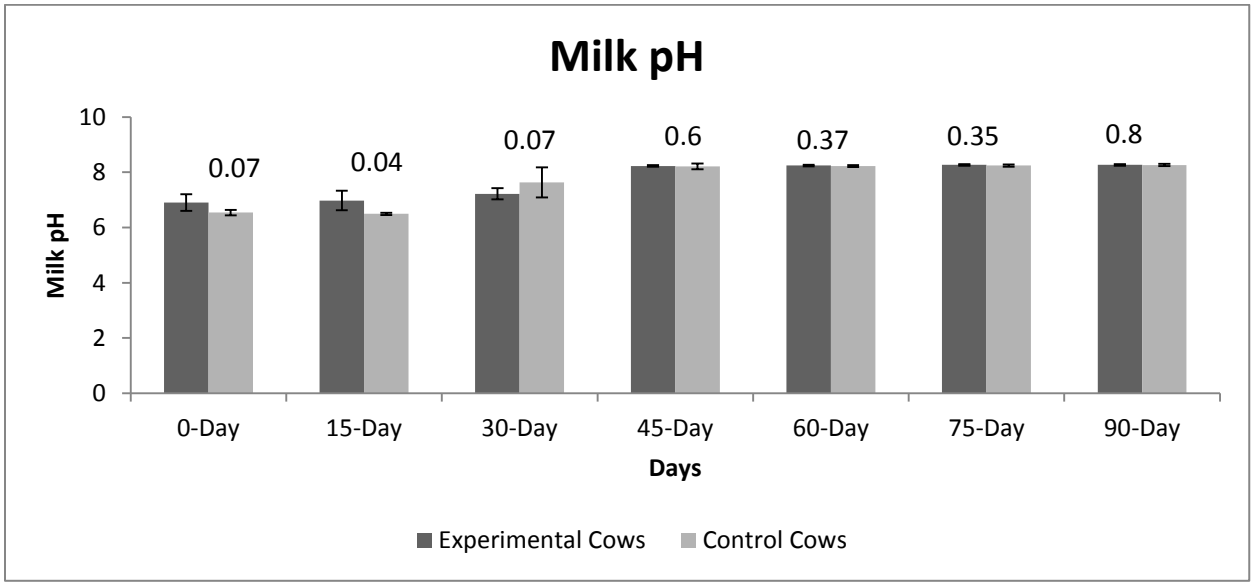


Figure 4. 10: Milk pH in control and experimental cows

4.3.9.2.8. Milk Cholesterol

Total cholesterol content in milk was determined after every fifteenth day. Trend of cholesterol level variation was different in milk of control and experimental cows. Among control cows cholesterol level increased from zero day (161.7mg/100gm fat) to seventy fifth day (188.7mg/100gm fat) of experiment while in last fifteen days it decreases from 188.7mg/100gm fat to 183.7mg/100 gm fat

but this value was still high from zero-day reading. Experimental cow's cholesterol level decreased from zero day (230.7mg/100gm fat) to seventy fifth day (124.3mg/100gm fat). At the end day slight increase was recorded (135.3mg/100gm fat) but still this level was low than measured at zero day. Cholesterol level in both control and experimental groups has shown that *Geotrichum candidum* QAUGC01 supplementation has significantly decreased cholesterol content in milk of experimental cows. The statistical comparison of milk cholesterol at zero day showed that milk cholesterol of experimental cows was near to significantly increased (P= 0.05) than control milk cholesterol at day zero. The same trend was recorded at 15th day (P= 0.018) showed that still the milk cholesterol content was high in experimental cows than control. While on 30th day of experiment the milk cholesterol in experimental cows decreased to a level of 188.7 mg/100gm while steady increase measured in control milk samples (P= 0.16). But on 60th day the milk cholesterol significantly decreased (P= 0.00) in experimental milk samples (128.22 mg/100gm fat) as compared to control milk cholesterol (182 mg/100gm fat). Similarly, on 75th and 90th day of *G. candidum* QAUGC01supplementation the significant decrease (P= 0.002) and (P= 0.04) respectively in milk cholesterol of experimental cows' milk samples was recorded as compared to control milk cholesterol (Table 4.12).

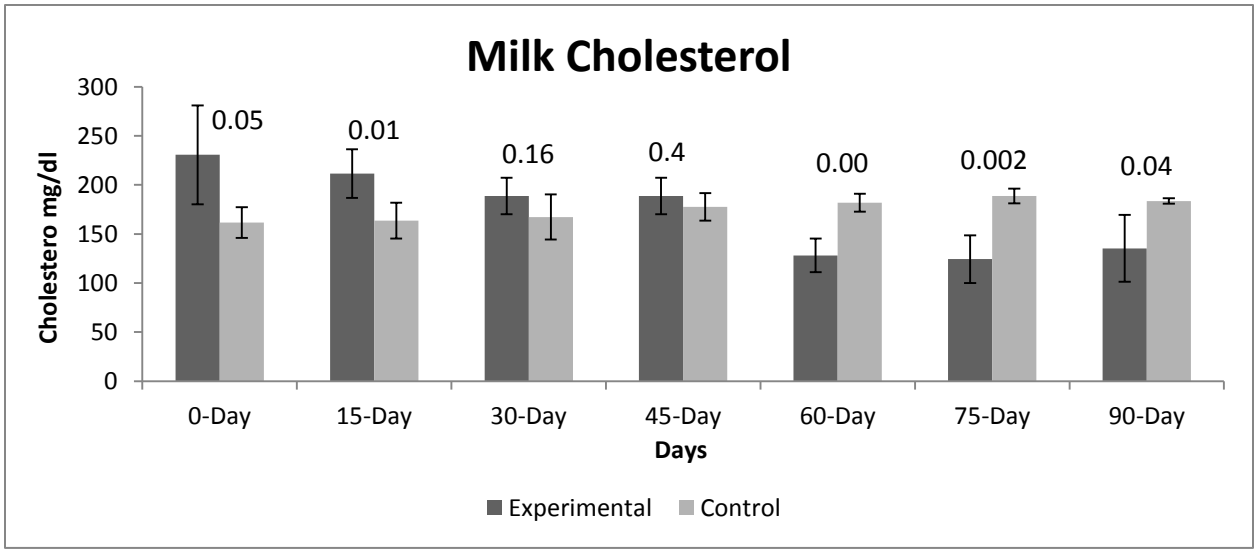


Figure 4. 11: Milk cholesterol content in milk samples of control and experimental cows

Table 4. 12. Statistical comparison of alteration in Milk cholesterol (mg/dl) of Experimental and Control Cows during experimental period of 90 days

Time Period	Cows	Mean Milk Cholesterol mg/ 100gm fat	P- value (<0.05)
0-Day	**Control**	161.667	0.058
	Experimental	230.667	
15th Day	**Control**	163.667	0.018
	Experimental	211.555	
30th Day	**Control**	167.333	0.168
	Experimental	188.778	
45th Day	**Control**	177.667	0.40
	Experimental	188.778	
60th Day	**Control**	182.000	0.000
	Experimental	128.222	
75th Day	**Control**	188.667	0.002
	Experimental	124.333	
90th Day	**Control**	183.667	0.04
	Experimental	135.333	

4.4. Discussion

Livestock plays an important role in economy by providing essential food items and dairy sector is its important component. Dairy cattle serving human by renovating low grade materials such as plant fibers, proteins into high grade food materials such as meat, milk in addition to leather and wool. While talking exclusively about milk a large portion of milk consumed globally come from dairy cattle (Britt *et al.*, 2018). Dairy in Pakistan prove to be back bone of agriculture sector contributing 58.3% in agriculture sector‘s GDP (gross domestic production). Milk alone exceeds the combined value of all major cash crops in the country. Pakistan being third largest milk producing country yields over 50 billion liters of milk annually. Apart from contributing one-fifth to countries GDP dairy sector employs >40% of rural workforce (Tahir *et al.*, 2019). The health and performance improvement of ruminants is one of the primary objectives in the livestock production (Puniya *et al.*, 2015).

Rumen microbiota consists of a diverse range of bacteria, fungi, viruses and protozoa. About 95% of whole microbiota is estimated to be bacterial communities (Pitta *et al.*, 2014). These microorganisms present in the rumen contribute greatly to the digestion of feed stuffs and its conversion into other substances (Puniya *et al.*, 2015). Thus, the productivity and well-being of animals can be improved by changing the metabolic activities and composition of gut microbiota. From many years the studies on supplementation of diet with microbial additives for improving health and progress of animals are in progress and have shown that useful microorganisms such as probiotics in the diet can improve feed efficiency, feed intake and also weight gain (Adams *et al.*, 2008; Timmerman *et al.*, 2005). For ruminants such as cattle, yeasts have been used successfully to improve the growth rate and also production efficiency (Puniya *et al.*, 2015). Numerous studies have reported positive impacts of *Aspergillus oryzae* and *Saccharomyces cerevisiae* on milk production and feed intake of the lactating cows (Cakiroglu *et al.*, 2010; de Ondarza *et al.*, 2010; Schingoethe *et al.*, 2004).

Imbalanced feeding rendered dairy animals with less productivity. Yeast based Probiotics can help in mitigating adversative effects of imbalanced feed. Many of commercial yeast-based probiotics are available in the market, but unsuitable for our local breeds regarding cost and impact on physiology as well as productivity. In the present study trial the selection of cows is unbiased and all the experimental and control dairy cows were randomly selected on the basis of

same BCS, parity, body weight, same lactation phase (mid lactating). All the cows were cross breed Sahiwal. Present study was designed to check the impact of *G. candidum* QAUGC01 with potential probiotics attributes to improve Feed Efficiency (FE), milk yield, milk composition and health of mid-lactating dairy cows of Sahiwal cross fed a high forage diet. *G. candidum* QAUGC01 is supposed to be a probiotic yeast strain which has been screened through different probiotic attributes in Phase I. The health status of animals used in this experiment was monitored by analyzing serum biochemistry and other important hematological parameters.

The health status and productivity of cattle is greatly associated with the role of GIT microbial community. Healthy gut microbiota can have impact on different health parameters and functions such as feed stuff digestion, conversion and metabolic activities etc. But underlaying mechanisms, includes feed stuff digestion, conversion, immunomodulation and improved metabolism, need to be determined (Yu *et al.*, 2017). The underlying dynamics of cause and effect still need to be determined (Song *et al.*, 2017; Weimer, 2015).

In current study experimental cows have shown impact of *G. candidum* QAUGC01supplementation in improving FE and milk yield. Table 4.5 shows that the experimental cows produced more milk with significant (p=0.042) difference as compared to the average milk production of control cows. The control cows consumed more average dry mater (DM) than experimental cows but the average feed efficiency of experimental cows was significantly (p=0.032) high than control cows. This leads to significant increase of milk lactose content as well as desirable increase of milk fat%, milk protein%, milk solids and solid non fats in experimental cows than control. Thus the *G. candidum* QAUGC01 probiotic yeast supplementation improved the composition and quality of experimental cow‘s milk as the milk cholesterol content also significantly decreased.

Analysis of blood samples showed that all the animals remained healthy during the experimental period of three months. Values of all the analyzed blood parameters were in the normal physiological range which is a sign of good health. In the experimental cows RBC count significantly (p= 0.0091) increased while in control animals it decreased (p= 0.7183). White blood cells (WBCs) contribute to the immunity of animals. In control cows WBC count decreased from 9.00 x 10^3 to 7.393 x 10^3/µl as well as in experimental cows 9.922 x 10^3 to 9.524 x 10^3/µl. while the decrease in experimental cows is negligible and very low. The percent Neutrophils increased in experimental cows as compared to control. Adjei-Fremah, Ekwemalor,

Asiamah et al. measured percent neutrophil count that decreased in probiotic-treated animals and also identified 87 bovine pathways impacted by probiotic treatment such as on genes involved in immunity and homeostasis (Adjei-Fremah *et al.*, 2018). The PCV value increased in experimental group than control but this increase was within normal range. while one recent study reported no effect on PCV of cattle (Adjei-Fremah *et al.*, 2018). The good health status of dairy cows (experimental) is also indicated by good hemoglobin normal range was also significantly increased within normal range and shows the excellent proteolytic activity performed by added probiotic yeast *G. candidum* QAUGC01 in current study. Dietary yeast supplementation may benefit for microbial protein synthesis in the rumen (Zhu *et al.*, 2017). Earlier study showed the significant increase in packed cell volume, hemoglobin concentration and white blood cell count in sheep fed a legume diet supplemented with *S. cerevisiae* (Osita *et al.*, 2019). Similarly One study clearly showed the good effect of Yeast-supplementation by increasing erythrocytes and leukocytes count as well as hemoglobin level significantly in dairy heifers (S Ghazanfar *et al.*, 2015). The current results are in accordance with the study of Agazzi and Heinrichs (Agazzi *et al.*, 2014; Heinrichs *et al.*, 2003) who reported that by the probiotic addition hematological parameters were being affected.

The serum glucose significantly increased (P=0.001) in treated group (52.6667mg/dl to 58.0000 mg/dl) that resulted not only in the maintenance of body condition score (BCS) and body weight of experimental group and also prevented the herd from the condition of negative energy balance (Ebtehag *et al.*, 2011) and subsequent ketosis during the mid-lactation phase. The optimum level of glucose in treated cows during mid-lactation maintained the body weight and BCS as progression to late lactation phase during 3 months of trial. Glucose from dietary source is also needed for milk lactose synthesis because in the present trial the milk lactose also significantly increased (P= 0.001) in yeast supplemented cows. While in control cows there was no significant variation noted in serum glucose while decreased at end of three months of experiment (P > 0.05). Milk lactose in control cows increased from zero day to 90th day (4.21 to 4.32%) but significantly (P=0.008) increased in milk of experimental cows (4.12 to 4.61%). (Appendix 3). It has also found earlier with significant increase in serum glucose and decrease in cholesterol of probiotics-fed group of 10 cows in comparison with control group of 6 cows (Bakr *et al.*, 2015).

Serum glucose is the main precursor for lactose synthesis that increases the demand of lactating dairy cows for large quantity of glucose. Milk yield greatly depends on mammary lactose synthesis due to its osmoregulatory property for mammary uptake of water. Thus, glucose availability to the mammary gland could be a potential regulator of milk production. Glucose supply in sufficient quantity to Bovine Mammary epithelial Cells (BMEC) in vitro may promote glucose metabolism, and affect the synthesis of milk composition (Liu *et al.*, 2013). The addition of yeast culture significantly ($P < 0.01$) increased concentration of serum glucose (Petr Doležal *et al.*, 2011) and increased glucose level within normal range may be attributed to the improved efficiency as energy requiring for physiological processes for example respiration.

In the present study the total serum cholesterol level was decreased significantly (P= 0.0024) in experimental dairy cows than control cows non-significantly (P=0.8267). This decrease was very little in control cows (235.33 to 230.66 mg/dl) while the reduction was significantly greater in experimental cows (202.3 to 177.4 mg/dl) could be attributed to hypocholesterimic effect of *G. candidum*. The serum LDL was also decreased in experimental cows than control cows. Cholesterol lowering ability (Syal & Vohra, 2014) in the serum by *G. candidum* increases its importance to be used as probiotic. The strains of Geotrichum and Galactomyces species have been previously reported to show highest adhesive ability and assimilate cholesterol from YPD-CHOL broth containing bile salt and cholesterol after 72 h growth at 37 °C (Chen *et al.*, 2010). In earlier study *saccharomyces cerevesiae* reduced the serum cholesterol significantly in 10 experimental cows in comparison with six control dairy cows and Serum triglycerides, high density lipoproteins, and low density lipoproteins concentrations were lower in treated group (Bakr *et al.*, 2015), but in current study under the influence of *G. candidum* QAUGCO1supplementation the serum triglycerides significantly increased in experimental (P= 0.001) but non-significant increase in control cows (P= 0.0891) within normal range (0 to 200mg/dl). Serum samples were processed by using GC-FID to determine the quantity of butyrate in experimental and control cows. In experimental cows serum butyrate concentration was higher at the end of experiment as compared to concentration at zero day. While opposite trend was observed in control cows the butyrate concentration was decreased in their serum at the end of experiment. The current study reported the increased serum butyrate level in mid lactating cows strongly supported by the results of Zhu *et al.* (2017) fed mid lactating dairy cows with high dose of *S. cerevisiae* yeast supplementation in high forage diet increased milk yield

and significantly increased concentrations of ruminal total volatile fatty acids, acetate, propionate, and butyrate with increased population of fungi and certain cellulolytic bacteria but decreased lactate-producing bacteria (*Streptococcus bovis*) indicated the desirable effects of yeast supplementation that enhanced the synthesis of microbial protein in the rumen (Zhu *et al.*, 2017). importantly, the host requires ruminal fermentation products for body maintenance and growth (Qumar *et al.*, 2016; Van Houtert, 1993) and milk production (Bauman *et al.*, 2011). The efficient growth of gut microbiome results in increased production of short chain fatty acids in rumen provides energy (Malmuthuge, 2017)

The significant decrease of serum cholesterol in experimental cows within normal range while they maintained the BCS. This could be due to lipolytic ability of *G. candidum* QAUGC01in gut that has been already determined in earlier studies (Boutrou *et al.*, 2006; Helmy *et al.*, 2019; Litthauer *et al.*, 1996; Muhammad, Syed Ali Imran*, et al.*, 2019; Piegza *et al.*, 2014). This may also be due to increase serum butyrate concentration and percent digestibility of NDF, ADF, CF, crude protein and total dry matter in experimental dairy cows after supplementation of *G. candidum* QAUGC01 in feed in our study. The yeast supplementation improved digestibility may benefit for microbial protein synthesis by increasing the fungal and cellulolytic bacterial population in rumen of mid lactating dairy cows (Zhu *et al.*, 2017). In Another study Yeast supplementation showed benefits in early lactation by improving digestibility and protein synthesis preventing the loss of BCS of dairy lactating cows (Mumbach *et al.*, 2017). In current study the supplementation of G. candidum QAUGC01yeast significantly improved average milk yield in experimental group than control group fed diet without probiotic addition. Recently described the effects of Direct fed microbials (DFM) of *Propionibacterium, Saccharomyces cerevisiae*, *Lactocbacillus acidophilis*, the mixture of yeast products and *Enterococcu*s and combination of *L. ecidophilus, L. casei* and *Enterococcus faecium* to dairy cows, have significantly improved milk yield and well as the milk composition (Tesfaye & Hailu, 2019). The results coincide with findings of (Bakr *et al.*, 2015) reported higher milk production and milk fat percentage, whereas milk protein percentage and somatic cell count were decreased in *Saccharomyces cereviceae* supplemented ten cows during early lactation throughout the study. The increased milk yield along with increased serum glucose and milk lactose has been reported earlier (Szucs *et al.*, 2013). The increase of serum glucose is because of increase production of propionic acid that is the precursor of Glucose to supply energy to lactating cows. It may show

the stimulation effect of GC01 yeast in rumen that stimulated the fibrolytic flora /cellulolytic flora indicating the stable pH of cow gut during the current study. This might be due lactate degrading effect of *G. candidum* (Choisy *et al.*, 1997; Lenoir, 1984).. yeast supplementation stabilize the rumen pH favorable for growth of cellulolytic bacteria and prevented the dairy cow from negative energy balance and subsequent metabolic disorders i.e. ketosis (P Doležal *et al.*, 2005) . In current study trial the milk cholesterol content decreased in milk of experimental cows while very little decrease in milk cholesterol reported in control cows. That might be due to significant decrease in serum cholesterol in experimental cows in our study. Serum HDL decreased in control cows (P= 0.624) by 1 mg/dl while increase in experimental cows (P= 0.3987) by 1 mg/dl within normal range. Minerals i.e. Calcium (Ca) and Phosphorus (P) were measured and found increase of ca in experimental as well as control cows but the increase was greater in experimental cows. Phosphorus remain same in experimental cows while decreased in control cows at the end of the experiment. One study recently described the Influence of feed additives on minerals (calcium, phosphorus) concentration in the cattle blood was positive and evaluated in the normal physiological limits of concentrations (Dailidavičienė *et al.*, 2018).

Total cholesterol content in milk was determined after every fifteenth day of experiment. Trend of cholesterol level variation was different in milk of control and experimental cows. Among control cows cholesterol level increased from zero day (161.7mg/100gm fat) to seventy fifth day (188.7mg/100gm fat) of experiment while in last fifteen days it decreases from 188.7mg/100gm fat to 183.7mg/100 gm fat but this value was still high from zero-day reading. Experimental cow‘s cholesterol level decreased from zero day (230.7mg/100gm fat) to seventy fifth day (124.3mg/100gm fat). At the end day slight increase was recorded (135.3mg/100gm fat) but still this level was low than measured at zero day. Cholesterol level in both control and experimental groups has shown that *Geotrichum candidum* QAUGC01 supplementation has significantly decreased cholesterol content in milk of experimental cows. The statistical comparison of milk cholesterol at zero day showed that milk cholesterol of experimental cows was near to significantly increased (P= 0.05) than control milk cholesterol at day zero. The same trend was recorded at 15^{th} day (P= 0.018) showed that still the milk cholesterol content was high in experimental cows than control. While on 30^{th} day of experiment the milk cholesterol in experimental cows decreased to a level of 188.7 mg/100gm while steady increase measured in control milk samples (P= 0.16). But on 60^{th} day the milk cholesterol significantly decreased (P=

0.00) in experimental milk samples (128.22 mg/100gm fat) as compared to control milk cholesterol (182 mg/100gm fat). Similarly, on 75th and 90th day of *G. candidum* QAUGC01supplementation the significant decrease (P= 0.002) and (P= 0.04) respectively in milk cholesterol of experimental cows' milk samples was recorded as compared to control milk cholesterol (Table 4.13). The content of cholesterol in milk fat varied from 232 to 373 mg/100 g fat in cow milk (Reklewska *et al.*, 2002). the cholesterol concentration in cow milk decreased with the advancement of the lactation (Strzałkowska *et al.*, 2010).The previous study regarding milk and serum cholesterol homeostasis detected higher amount of cholesterol in milk fat fraction during first week of lactation compared with week 4 of lactation, can be explained by an elevated fat content in week 4 in parallel to low cholesterol levels. Interestingly, the milk cholesterol concentration reached its highest levels in week 1 lactation whereas it was concomitantly decreased during this period in blood (Kessler *et al.*, 2014) suggesting that cholesterol content in milk decreased from 284.33mg/ 100gm of fat in first week of lactation to 171.55 mg/100gm of fat during 4th week of lactation but with the progression of lactation to 14th week (mid-lactation) the milk cholesterol level increased i.e 272.35 mg/100gm of fat. The mammary tissue synthesize only a small part of milk cholesterol by de novo synthesis, whereas cholesterol uptake from the blood is far more pronounced during this period (Long *et al.*, 1980). It is likely that the high cholesterol mass in milk in first week of lactation is partly responsible for the low cholesterol concentration in the blood. In earlier study no significant correlation was found between cholesterol in milk and serum in lactating dairy cows. At reverse a positive significant correlation was observed between cholesterol and fat in milk ($r = 0.636$; $P < 0.001$) and between fat in milk and cholesterol in serum ($r = 0.344$; $P < 0.01$) (Faye *et al.*, 2015).

According to our findings *G. candidum* has significantly modulated the gut microbiota with its positive impact on other physiological parameters resulted in significant increase in FE ($p<0.05$) and average milk yield ($p<0.05$). Moreover all the serum biochemical values were in normal physiological range with significant increase in blood hemoglobin and erythrocytes which showed good health status of experimental cows yielded milk with good qualitative enhancement in milk composition.

In current study milk production decreased both in control and experimental cows due to stress of hot summer but this stress was reduced in experimental cows fed yeast supplemented feed resulted in less decrease in milk yield in experimental cows than control as evidenced by 1.45 kg

more milk produced by experimental cows as compared to control cows at the end day of experiment and these results were in agreement to the studies reporting 1 to 2 kg/d increase in milk production by yeast *Saccharomyces cerevisiae* (Bruno *et al.*, 2009; Dailidavičienė *et al.*, 2018; Robinson & Garrett, 1999; Yalcin *et al.*, 2011). Previous studies have highlighted the positive effects of probiotic supplementation to neutralize thermal impact in dairy cows and numerically increased (1.26 kg, P = 0.11) milk yield in cows fed Calsporin during thermo neutral but was reduced under heat stress (-2.67 kg, P < 0.01) accompanied by decrease (P = 0.05) in milk protein content (Amaral-Phillips, 2019; Hall, 2014). In mid lactating dairy cows the high dose of *S. cerevisiae* supplementation in high forage diet increased milk yield and significantly increased concentrations of ruminal total volatile fatty acids, acetate, propionate, and butyrate with increased population of fungi and certain cellulolytic bacteria but decreased lactate-producing bacteria (*Streptococcus bovis*) indicated the desirable effects of yeast supplementation that enhanced the synthesis of microbial protein in the rumen (Zhu *et al.*, 2017). The increase in milk protein, fat, lactose and solid non fats content resulted in significant increase in milk yield ($p<0.05$) and Feed Efficiency of experimental cows than control of present study.

Milk yield affected by many factors including heat stress because of high humidity in tropical summer season could lead to decrease in milk production. The thermoneutral zone (TNZ) of dairy animals ranges from 16°C to 25°C, and they maintain a body temperature of 38.4-39.1°C (Leonard, 1986). However, air temperatures above 25-37°C in a tropical climate, increase the heat gain beyond that lost from the body and induces heat stress (Vale, 2007). Dairy cows undergo heat stress (HS) whenever an imbalance between metabolic heat production inside the animal body and its dissipation to the surroundings under high air temperature and humid climates. The HS increases the respiration rate, rectal temperature and heart rate. It directly affect feed intake thereby, reduces growth rate, milk yield, reproductive performance, and even death in extreme cases (Das *et al.*, 2016). In contrast, some studies found no increase in milk production in response to yeast *(Saccharomyces cerevisiae)* supplementation (Bagheri *et al.*, 2009; Dann *et al.*, 2000; Schingoethe *et al.*, 2004; Soder & Holden, 1999). Supplementation of DFM (direct fed microbials) such as combination of *Lactobacillus plantarum, Enterococcus faecium,* and *Saccharomyces cerevisiae* and *Bacillus licheniformis* may enhance milk yield (Nocek & Kautz, 2006; Nocek *et al.*, 2002; Qiao *et al.*, 2010). Some studies highlighted improvement in milk yield by DFM commercial product (Probios® Chr. Hansen) contains

combination of *Enterococcus faecium* and yeast (Nocek & Kautz, 2006; Nocek *et al.*, 2003). According to one study utilizing *Pediococcus acidilactici* and *Bacillus subtilis* as probiotics found no effect on milk yield(Thomas, 2017). But, in same study supplementation with *P. acidilactici* has positive effect on milk yield as a result of increase DMI during first five weeks. Another study reported no effect on milk yield with *Bacillus subtilis* and increase in milk production with *Bacillus licheniformis* (Qiao *et al.*, 2010). Supplementation of indigenously isolated *Saccharomyces cerevisiae* (SCQAU03) resulted in significant increase in milk production (Shakira *et al.*, 2018). Supplementation with combination of probiotics in a study resulted in both increase in milk yield and suppression of somatic cell count (SCC) in milk (Xu *et al.*, 2017). Therefore, the impact of probiotic on milk yield is highly dependent on condition, herd and feeding pattern.

The probiotic could also have impact on milk composition. Previously, also, effect of probiotics on milk composition has been reported (Bagheri *et al.*, 2009; Desnoyers, Giger-Reverdin, Bertin, *et al.*, 2009; Shakira *et al.*, 2018; Soder & Holden, 1999; Yalcin *et al.*, 2011). Many researchers (Erasmus *et al.*, 1992; Giger-Reverdin *et al.*, 1996; Günther, 1990; Lehloenya *et al.*, 2008; Moallem *et al.*, 2009; Piva *et al.*, 1993; Putnam *et al.*, 1997; Shakira *et al.*, 2018; P. Sun *et al.*, 2011; Wohlt *et al.*, 1998; Yalcin *et al.*, 2011) have reported beneficial effects of probiotics on milk composition. The significant increase in milk lactose ($p<0.05$) with desirable increase in milk fat and milk protein was measured in experimental dairy cows during ninety days of current study supported by the findings of study with significant increase in milk yield along with milk fat and protein (Dailidavičienė *et al.*, 2018). In present study the serum concentration of butyrate significantly influenced the milk fat contents of experimental cows. Recently described the Direct fed microbials (DFM) of *Propionibacterium, Saccharomyces cerevisiae, Lactocbacillus acidophilis*, the mixture of yeast products and *Enterococcus* and combination of *L. ecidophilus, L. casei* and *Enterococcus faecium* fed to dairy cows, have significantly improved milk yield and well as the milk composition (Tesfaye & Hailu, 2019). On the other hand different experimental studies (Arambel & Kent, 1990; Bagheri *et al.*, 2009; H. Peng *et al.*, 2012; Soder & Holden, 1999; Swartz *et al.*, 1994) and meta-analysis (Desnoyers, Giger-Reverdin, Bertin, *et al.*, 2009) reported no effect of probiotics on milk composition. Similarly Shakira et al. (2018) reported that supplementation of *S. cerevisiae* SCQAU03 imparted no significant effect on milk protein (Shakira Ghazanfar *et al.*, 2017), while in current study milk protein has increased in the cows

fed on QAUGC01 supplemented feed. This could be due to proteolytic ability of *G. candidum* QAUGC01 as the *G. candidum* known for proteolytic activity in chesses (Boutrou *et al.*, 2006; Helmy *et al.*, 2019; Litthauer *et al.*, 1996; Muhammad, Syed Ali Imran*, et al.*, 2019; Piegza *et al.*, 2014).

In our study we found beneficial effects of *G. candidum* QAUGC01 as potential probiotic on milk composition, reduced the serum and milk cholesterol in experimental cows and increased total volatile fatty acids concentration by increasing the serum butyrate concentration in mid lactating dairy cows fed high forage diet. Earlier study on mid lactating dairy cows supplemented with high dose of *S. cerevisiae* supplementation in high forage diet increased milk yeild and significantly increased concentrations of ruminal total volatile fatty acids, acetate, propionate, and butyrate with increased population of fungi and certain cellulolytic bacteria but dcreased lactate-producing bacteria (*Streptococcus bovis*) indicating that yeast supplementation may benefit for microbial protein synthesis in the rumen (Zhu *et al.*, 2017)

4.5. Conclusion

In current study selected probiotic strain of *G. candidum*, QAUGC01, was supposed as potential probiotic after in vitro characterization. Selected *G. candidum* strain QAUGC01 was used in dairy cow‘s feed as microbial based probiotic feed supplement to check its impact on modulating blood parameters, feed digestibility, growth parameters, milk yield and milk composition. Cows were grouped into Control cows and experimental cows, Experimental cows were fed on QAUGC01 supplemented feed while control cows were given normal feed for three months. Samples were collected after equal interval of time and were analyzed to check the impact of our locally isolated strain as potential probiotic. On the basis of all the analyzed parameters it was found that our locally isolated *G. candidum* strain QAUGC01 has good probiotic potential in improving blood and serum parameters, nutrient digestibility, milk yield and milk composition in comparison with control cows. RBC‘s, Hemoglobin and PCV level raised in experimental cows while WBC count decreased both in experimental and control cows. Decrease in WBC was more in control than experimental cows. Neutrophil count increased in both groups while this increase was high in control group. All the identified values of blood parameters were in normal range which depict healthy role of *G. candidum* QAUGC01 on blood parameters. Serum biochemistry has also shown increase of glucose and HDL in experimental cows and decrease of cholesterol, LDL and TG in blood serum. Serum samples were processed by using GC-FID to determine the

quantity of butyrate in cow‘s serum. In experimental cows serum butyrate concentration was higher at the end of experiment as compared to zero day. While opposite trend was observed in control cows butyrate concentration was low in their serum at the end of experiment. Feed Efficiency, dry matter intake and milk yield was measured, and it was found that FE was significantly high in experimental cows, produced more milk with significant (p=0.042) difference as compared to the average milk production of control cows. The control cows consumed more average dry mater (DM) than experimental cows but the average feed efficiency of experimental cows was significantly (p=0.032) high than control cows. This may be due to significant increase of serum glucose, milk lactose content as well as desirable increase of milk fat%, milk protein%, milk solids and solid non fats in experimental cows than control cows. These all effects attributes to supplementation of locally isolated yeast *G. candidum* QAUGC01 that has been already reported for its best enzymatic ability resulted in increased apparent digestibility of dry matter (DM), CP, CF, NDF and ADF in experimental cows. Thus, QAUGC01 has significantly improved the milk yield and milk composition in experimental cows as compared to control cows, fed on normal feed.

Chapter 5

Impact of G. candidum QAUGC01 in Gut Modulation of Sahiwal-Friesian Cross Dairy Cow

5. Impact of *G. candidum* QAUGC01 in Gut Modulation of Sahiwal Cross Dairy Cows

5.1. Introduction

Number and diversity of microbes is of significant importance in GIT (Bao *et al.*, 2010). When probiotics are administered, they interact with other micro-biota in the gastrointestinal tract and use different diverse mechanisms which affect the gut micro-biota (Bron *et al.*, 2012; J. Dias *et al.*, 2018; Neish, 2009; Yutaka Uyeno *et al.*, 2015). Pathogens in the intestine produce different substances and toxins such as adhesins, invasions and mucinases, these substances affect the epithelial metabolic processes (Gaggìa *et al.*, 2010). Thus, probiotics can have potential effects on pathogenic bacteria in gut, immune system, modulate the gut microbiome for desirable metabolism and digestion impart a great impact on the health improvement and productivity of animals (Adjei-Fremah *et al.*, 2018). Many proposed modes of action of probiotics are available. All differ and vary from each other as several mechanisms associate probiotics to good GIT health by preventing enteric pathogens, while others associated with improved and enhanced animal performance. As GIT harbors a large number of microbes, microbial composition of this considered one of the prime factors regarding its good health. One of the phenomenon exhibited by probiotics is the establishment of more desirable flora by changing the dynamics of the GIT microflora which results in stability of beneficial microbes against harmful ones (An *et al.*, 2008; Mountzouris *et al.*, 2009; Mountzouris *et al.*, 2007). Reduction in the load of pathogenic flora can be attributed to the substances like bacteriocins having antimicrobial activity produced by probiotic microbes in the GIT (Shim *et al.*, 2012) in addition to probiotic microbe‘s adhesion to the epithelium of the intestine which results in the exclusion of the pathogenic flora either directly competitively or indirectly by induction of immune response (An *et al.*, 2008; Mountzouris *et al.*, 2009; Mountzouris *et al.*, 2007). This phenomenon of alteration or modification of GIT resident microflora shown by most commonly used bacteria with probiotic potential like spore forming *Bacillus spp.* (Abdelqader *et al.*, 2013; Shim *et al.*, 2012), lactic acid bacteria (LAB) (Cao *et al.*, 2013; Mookiah *et al.*, 2014; Mountzouris *et al.*, 2010), clostridial bacteria *C. butyricum* (Yang *et al.*, 2012; B. Zhang *et al.*, 2011) and by both Gram positive as well as Gram negative different bacterial strains (Hashemzadeh *et al.*, 2013). Further, probiotics might enhance the population of microbes like *Lactobacilli* and *Bifidobactria* which help in curbing load of harmful flora by producing inhibitory substances (organic acids or/and bacteriocins) and leads to competitive exclusion (Bajagai *et al.*, 2016). Probiotics also affect

pathogens and their pathogenicity by influencing the phenomenon of quorum sensing among pathogenic bacteria. Quorum sensing of an important serotype O157:H7 of *E. coli,* causative agent of entero-hemorrhagic in humans successfully disrupted by *L. acidophilus* La-5. This disruption resulted from the inhibition of autoinducer-2, a chemical signal released as an extracellular secretion, by the fermentation products of *L. acidophilus* La-5. Further, it suppressed the expression of gene LEE (locus of enterocyte effacement) associated with virulence. Disruption of quorum sensing halted colonization of GIT by serotype O157:H7 of *E. coli* (Medellin-Peña *et al.*, 2007). Further, adhesion of the probiotic microorganisms to the epithelium of the intestine done concealment of receptor binding sites and discourages the adherence of important pathogens such as *Salmonella*, *E. coli* O157:H7 consequently prevent colonization of host GIT by pathogenic flora (Bernet *et al.*, 1994; Hudault *et al.*, 1997; Johnson-Henry *et al.*, 2007).

In current study the modulation of gut microbiota was investigated by culture dependent analysis of dung samples before supplementation of yeast culture as well as at end of experiment. The gut microbial diversity was evaluated through metagenomic study of dung samples of experimental and control cows. All the dairy cows from control and experimental groups were same in their production parameters and the selection was unbiased because all dairy cows were randomly selected from Sahiwal cross in mid lactation phase with similar BCS, parity, body weight and age.

5.2. Materials and Methods

5.2.1. Experimental Design

A total of twelve dairy cows were selected for the current study. They were divided into control (n=3) and experimental (n=9) cows. Experimental cows were fed with basal feed, supplemented with probiotic strain of *G. candidum* QAUGC01 for 90 days, while control fed basal diet. Dung samples were collected before and after feeding of *G. candidum* QAUGC01, but the end dung samples were collected 5 days after 90^{th} day (End-Day) of experiment. The probiotic supplementation was stopped at 90^{th} day and the dung samples were collected at 95^{th} day. The dung samples were then subjected to culture dependent as well as culture independent microbiological analysis respectively.

5.2.2. Microbiological Analysis of Dung Samples

Fresh dung samples of each cow were subjected to analyze the microbial load. For culture-based analysis the dung samples were collected in sterile plastic bags at zero day i.e. before yeast supplementation and at 95th day (End-Day) after supplementation of probiotic yeast and stored at -20ºC for subsequent analysis. Both culture dependent and culture independent techniques were used (Jewell *et al.*, 2015a; Pinloche *et al.*, 2013).

5.2.2.1 Culture Dependent Analysis of Dung Samples of Experimental and Control Cows

Dung samples were subjected to microbiological analysis to study the rumen micro-biota and impact of *G. candidum* QAUGC01 on it. Twelve dung samples i.e. 9 experimental and 3 control samples were cultured on five different media for analysis of microbial load and diversity. Colony Forming Units (CFU) analysis of dung samples, collected before and after administration of *G. candidum* QAUGC01 was done.

Five different culture media were used for CFU analysis. Those were as follows: Tryptic Soy agar (TSA), it was used as general purpose media, supporting the growth of all types of microbes, de Man Rogosa and Sharpe agar (MRSA) is a selective medium for the enumeration of Lactic acid bacteria and *Lactobacillus sp.* M17 is also a selective medium for enumeration of *Enterococcus sp., Streptococcus sp.* and *Lactococcus sp.*, Oxy-tetracycline glucose agar (OGA) was used for enumeration of yeast and mold species and MacConkey (MacCk) agar was used for enumeration of *Enterobacteriaceae* members (David *et al.*, 2014; M. M. Lim *et al.*, 2004). These agar media were prepared according to the manufacturer‘s recommendations and were then autoclaved for 35-45 minutes. After autoclaving 1% Nilstatin was added to MacConkey agar and MRSA to avoid fungal contamination and 1% Oxy-Tetracycline Dehydrate (OTD, antibacterial solution) to OGA to avoid bacterial contamination.

Serial dilution was done by preparing ten-fold dilutions of the samples. For each sample three plates of each media were prepared. From each sample 0.1 ml of 10^{-4}, 10^{-5} and 10^{-6} dilution were poured and inoculated. TSA, MRS and MC plates were incubated at 37°C for 24 hours while OGA plates were incubated at 30°C for 48 hours in inverted position. After incubation plates were observed under a colony counter and CFUs were counted with number below 30 designated

as Too Few to Count (TFC) and over 300 were designated Too Numerous to Count (TNC) and was calculated by using the following formula:

CFU/ml = No. of colonies x Dilution factor / volume plated

5.2.2.2 Metagenomic Analysis of Dung Samples of Experimental and Control Cows

The impact of *G. candidum* QAUGC01 supplementation on the microbial diversity of cow gut was also assessed by metagenomic analysis of end day dung samples. Based on results of physiological and production parameters as well as the culture based microbial profile of experimental and control cows the Principal component analysis (PCA) was done to select the dung samples of cows for metagenomic studies to check that how *G. candidum* QAUGC01 has changed the gut microbial diversity. According to PCA one control (Cow 605) and three experimental Cows i.e Cow 604, 620 and 640 were selected for gut microbial diversity analysis (Fig.5.2).

5.2.2.2.1 DNA Extraction from Dung Samples

DNA was extracted from dung samples using FavorPrep^{TM} Stool DNA Isolation Mini kit by performing following steps: 200 mg of glass beads were added into a 2 ml bead tube and transferred 100 mg of dung sample into bead tube then placed on ice. Then added 300 µl of SDE1 buffer and 20 µl of proteinase K (10 mg/ml) in the sample and vortexed for 5 minutes. Samples were incubated at 70°C for 10 minutes and vortexed twice during incubation. After that cooled down the samples and added 100 µl of SDE2 buffer to the sample, mixed well by vertexing. Incubated the samples on ice for 5 minutes and centrifuged at 10,000 rpm for 5 minutes. The supernatant was then transferred to a 1.5 ml microtube and the pellet was discarded. Added 200 µl of SDE3 buffer to the sample and mixed well by vertexing. Samples were incubated at room temperature for 2 minutes, after that centrifuged at 10,000 rpm for 2 minutes. 250 µl of supernatant was transferred to a 1.5 ml microtube and the pellet was discarded. 250 µl of SDE4 buffer and 250 µl of ethanol were added to the sample, mixed thoroughly by pulse-vertexing. After this a SDE column was placed into a collection tube and all of the sample mixture was transferred to the SDE column. The column was then centrifuged at 10,000 rpm for 1 minute and the flow-through was discarded. After this the SDE column was placed into the new collection tube. 750 µl of wash buffer was added to the SDE column and centrifuged at 10,000 rpm for 1 minute. The flow-through was again discarded and repeated the

above step one more time. Then the column was centrifuged at 10,000 rpm for 3 minutes in order to dry the SDE column. The SDE column was placed into an elution tube and 60 µl of elution buffer was added to the center of SDE column. SDE column was allowed to stand for 2 minutes at room temperature and then centrifuged for 1 minute at 10,000 rpm to elute DNA. After elution DNA was stored at – 20°C.

5.2.2.2.2 Gel Electrophoresis

To confirm the presence of DNA after extraction, agarose gel electrophoresis was conducted. Gel casting tray was rinsed thoroughly, the edges were taped properly to avoid leakage and was placed at leveled surface. The comb was adjusted in its place, leaving a difference of few millimeters between the teeth of comb and base of tray that aids in fine well formation. About 1% agarose gel was prepared by mixing 1 g of agarose in 10 ml of 10X TBE buffer and 90 ml distilled water. The mixture was heated in microwave and then allowed to cool for 5 minutes. The agarose gel was then poured into the casting tray having well comb in it. The gel was allowed to cool at 4°C for 10-15 minutes so that it completely solidifies.

The chamber was filled with 1X TBE buffer and casting tray containing solidified gel was submerged in electrophoresis chamber. About 2µl of sample was mixed with 2 µl of loading dye i.e. bromophenol blue, by gentle pipetting and loaded carefully in the wells. The electrophoresis was run at 120 volts, for 30 minutes and 400 mA. Gel was observed under UV illuminator when bromophenol reached quarter of gel, ethidium bromide stained DNA produced flourescence in UV light.

5.2.2.2.3. DNA Quantification

DNA samples were quantified by using Nano-Drop 1000 spectrophotometer (Thermo Scientific) at 260nm wavelength. 1µl of illusion buffer was used as blank. After running blank 1 µl of sample was loaded with the help of micropipette and results were recorded.

5.2.2.2.4. PCR Amplification, *Illumina MiSeq* Sequencing and Data Processing

A total of four end day (5 days after end day of experiment) dung samples, one control sample (cow 605) and three experimental samples were selected for meta-genomic study by PCA on the basis of best results in Phase I (Fig.5.2). The DNA extracted from these samples was subjected to

meta-genomic analysis. The genome sequencing was performed at Molecular Research (MR DNA), Shallowater, Texas.

Meta-genome or genome sequencing steps include isolation and purification of genomic DNA, fragmentation, ligation to sequencing adapters and then purification. Variable V4 region of 16S rRNA for bacteria and ITS region for fungal diversity was amplified in a 30 cycle PCR using primers 515/806 (GTGCCAGCMGCCGCGGTAA/GGACTACHVGGGTWTCTAAT) and ITS primers respectively and HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. PCR amplification was confirmed, and the relative intensity of bands was observed in 2% agarose gel. Amplicons were pooled together in equivalent ratios on the basis of DNA concentration and molecular weight and purified by using Ampure XP beads. DNA libraries from purified PCR amplicons were prepared by using Nextera DNA Sample Preparation Kit (Illumina) and following Illumina TruSeq DNA library preparation protocol. Library insert size was determined by Experion Automated Electrophoresis Station (Bio-Rad) with an average size of 500 bp. DNA libraries were pooled and loaded to a 600 Cycles v3 Reagent cartridge (Illumina) and the sequencing was performed on MiSeq (illumina).

Sequenced data was processed according to MR DNA analysis pipeline. Evidence based annotation approach was used to annotate the sequences. Sequences were BlastX against protein databases at an E value cutoff $1x10^{-5}$. Predicted genes were classified into functional categories from lower to higher orders. Relative abundance for each gene was calculated by dividing the similarity hits for an individual gene by total hits against any of the database. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence i.e. 97% similarity. OTUs were then taxonomically classified by using BLASTn against a curated database derived from GreenGenes, RDPII and NCBI (DeSantis et al. 2006). Shannon and Simpson indexes were used to estimate dominance, richness and evenness of bacterial and fungal community biodiversity.

5.2.3. Statistical Analysis

The statistical analysis of data was done by using XLSTAT 2014.5.03. All the Data was expressed as Mean ± SD. T test was used for the comparison of pre-treatment (0-day) and post-treatment (End-day) samples while ANOVA followed by Tukey‘s test was used for the variance

between different samples. Probability value of < 0.05 was considered significant. Pearson Correlation was applied to determine the correlation between different parameters.

5.3. Results

5.3.1. Microbiological Analysis of Dung Samples of Control and Experimental Cows.

5.3.1.1. Impact of *G. candidum* QAUGC01 Supplementation on Abundance of Culturable dung Microbiota of Experimental Cows in Comparison with Control

Enumeration of microbial load in fecal samples was done on five culture growth media i.e. Tryptic Soy Agar (TSA, Oxoid), deMan Rogosa Sharpe Agar (MRSA, Oxoid), M17 (Oxoid), Oxy-tetracycline Glucose Agar (OGA, Oxoid) and MacConkey (MC, Oxoid) Agar by serial dilution method. TSA, MRSA, M17 and MCA plates were incubated at 37°C for 24 h while OGA plates at 30°C for 24-48 h. Colony Forming Units (CFU) were counted by using colony counter with number below 30 designated as Too Few to Count (TFC) and over 300 were designated Too Numerous to Count (TNC).

For this purpose, a total of 12 dairy cows dung samples were analyzed to determine total microbiological load in the samples including yeasts & molds and also specific bacterial groups i.e. members of *Enterobacteriaceae, Lactobacillus sp., Enterococcus sp., Streptococcus sp.* and *Lactococcus sp.* at zero day i.e. before feeding *G. candidum* QAUGC01 and at 90^{th} day after feeding GC01 was done by CFU analysis. After analysis, the results of control and experimental group of cows were compared to check changes in the microbiology of gut and the impact of *G. candidum* QAUGC01supplementation on the microbial composition and diversity in the cattle gut. To the control animals *G. candidum* QAUGC01 was not fed and samples were collected at zero day and 90^{th} day like the experimental cows.

5.3.1.1.1. Impact of *G. candidum* QAUGC01 Supplementation on Total Aerobic Count of Experimental Cows in Comparison with Control

Total aerobic count increased in experimental cows ($3.13 \times 10^7 – 6.2654 \times 10^7$) ($P$= 0.299) while decreased in control cows (9.89×10^7 -- 9.00×10^7) (P=0.89) at the end day of experiment as depicted by table 5.1and Fig.5.1.

5.3.1.1.2. Impact of *G. candidum* QAUGC01 Supplementation on Lactic Acid Bacteria (LAB) and *Lactobacillus sp.* of Experimental Cows in Comparison with Control

Table 5.1 showed that LAB and *Lactobacillus sp.* count increased both in experimental (2.2624×10^7- 5.0548×10^7) (P=0.32) and in control cow's gut (5.32×10^6-5.96×10^6) (P=0.11).

5.3.1.1.3. Impact of *G. candidum* QAUGC01 supplementation on *Enterococcus sp., Streptococcus sp.* and *Lactococcus sp.* Of dung microbiota of experimental cows in comparison with control

CFU analysis of samples on M17 showed the impact of *G. candidum* QAUGC01 on *Enterococcus sp., Streptococcus sp.* and *Lactococcus sp.* in cattle gut as this media is specific for the growth of these species. CFU/g of experimental and control dung samples on M17 at zero day i.e. before supplementation and at end day i.e. 90th day after supplementation of the probiotic yeast is compared (Table 5.1, Fig.5.1).

The following table represents that in all the experimental cows, members of *Enterococcus sp., Streptococcus sp.* and *Lactococcus sp.* increased in number than control. *Enterococcus sp., Streptococcus sp.* and *Lactococcus sp.* Count in cattle gut increased in experimental cows (9.1982×10^7 - 1.4948×10^8) (P= 0.25) and remained almost same in control cows (1.86×10^8 - 1.85×10^8) (P= 0.89).

5.3.1.1.4. Impact of feeding *G. candidum* QAUGC01 on *Enterobacteriaceae* Members in Gut of Experimental Cows in Comparison with Control by Culture Dependent Analysis

To analyze the effect of *G. candidum* QAUGC01 on *Enterobacteriaceae* members in the cow gut MacConkey (MC) agar was used. *Enterobacteriaceae* also contains many pathogenic microorganisms so by CFU analysis on MacConkey agar it can be assessed that what is the impact of this probiotic feeding on the pathogenic microbial community in the experimental cow gut. The microbial load of *Enterobacteriaceae* before and after feeding probiotic yeast was compared between experimental and control samples and found that *Enterobactericeae* count decreased in experimental cows (2.128×10^7- 3.874×10^6) non significantly (P= 0.16) and increased in control cows (1.19×10^7- 2.23×10^7) (P=0.39).

The decrease in number of *Enterobacteriaceae* members in samples of experimental cows indicated a decrease in pathogenic load in the experimental cattle gut, as depicted by the

following table 5.1. This comparison before supplementation and after supplementation of *G. candidum* QAUGC01 showed that its use as microbial based feed additive also helps in decreasing the pathogenic load in the cattle gut.

5.3.1.1.5. Impact of Feeding *G. candidum* QAUGC01 on Yeast and Fungal Microbiota in Cattle Gut of Experimental in Comparison with Control Cows by Culture Dependent Analysis

To check the impact of feeding *G. candidum* QAUGC01 on yeast and fungal microbiota in cattle gut the dung samples were analyzed and compared on OGA. CFU/g of experimental and control samples on OGA at zero day i.e. before feeding and end day i.e. 90^{th} day after feeding probiotic yeast was compared and shown in table 5.1and Fig.5.1. Yeast and fungal count increased in both experimental (2.396×10^7- 3.5556×10^7) (*P*= 0.57) and in control cows (1.22×10^7- 1.28×10^7) (*P*= 0.88). In all of the 90^{th} day samples *G. candidum* QAUGC01 was found to be present which was confirmed by microscopy. The presence of this strain shows that after feeding it established and survived successfully in the gut of experimental cows.

In control cow's gut there was a little decrease in overall microbial count at 90^{th} day while the population load of lactic acid bacteria, yeasts & molds remained almost the same. The number of members belonging to *Enterobacteriaceae* increased a little while that of *Enterococcus sp., Streptococcus sp.* and *Lactococcus sp.* increased very slightly (Fig.5.1).

Table. 5.1: Comparative culture based microbiological analysis of control and experimental dung samples at 0-Day and 90^{th} Day

Parameter	Time Period	Control Cows			Experimental cows		
		Mean	*P-value*	S. D	Mean	*P-value*	S. D
TAB count	0-Day	9.89×10^7	0.89	1.19E+08	3.13×10^7	0.29	2.88E+07
	90^{th} Day	9.00×10^7		1.05E+08	6.26×10^7		5.45E+07
LAB count	0-Day	5.32×10^6	0.11	5.80E+05	2.26×10^7	0.32	2.43E+07
	90^{th} Day	5.96×10^6		6.36E+04	5.05×10^7		5.28E+07
Lacto/ Enterococcus count	0-Day	1.86×10^8	0.89	7.78E+06	9.19×10^7	0.25	7.09E+07
	90^{th} Day	1.85×10^8		1.41E+06	1.49×10^8		7.80E+07
Enterobactericeae count	0-Day	1.19×10^7	0.39	7.13E+06	2.12×10^7	0.16	2.30E+07
	90^{th} Day	2.23×10^7		2.37E+07	3.87×10^6		1.61E+06
Yeast/ Fungal count	0-Day	1.22×10^7	0.88	6.21E+06	2.39×10^7	0.57	2.59E+07
	90^{th} Day	1.28×10^7		7.74E+06	3.55×10^7		3.55E+07

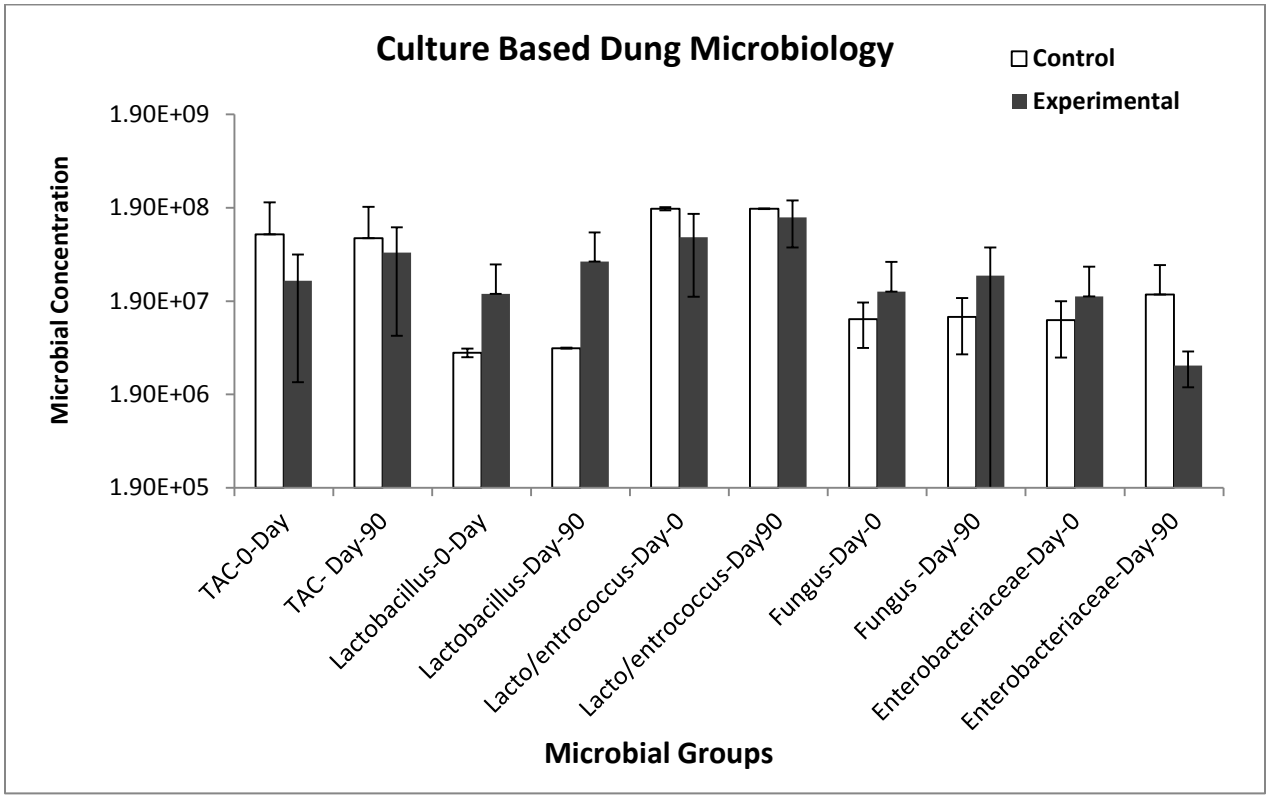


Figure 5.1: Culture based Dung Microbial analysis of experimental and control cows at 0-Day and 90th Day.

5.3.1.2. Culture Independent Analysis by 16SrDNA and ITS Sequencing.

5.3.1.2.1. Gel Electrophoresis

Gel electrophoresis was conducted to confirm the extraction and presence of DNA. In all the samples clear bands were observed on gel under UV illuminator which confirmed the presence of DNA, shown in Figure 5.2.

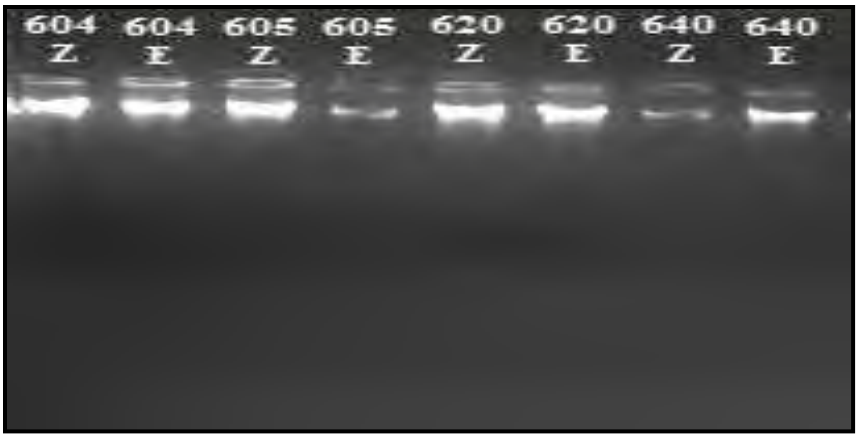


Figure 5. 2: DNA bands on Agarose Gel extracted from control & experimental dung samples. Z = Zero day E = End day

5.3.1.2.2. DNA Quantification

DNA samples were quantified by using Nano-Drop spectrophotometer. Concentration of DNA (ng/µl) and its purity values are given in the following Table 5.2.

Table 5. 2: Quantification of DNA samples of three experimental and one control cow

Sr. No	Sample	Conc. of DNA (ng/µl)	Absorbance (A260)	Absorbance (A280)	Purity (260/280)
1	604 Z	9.14	0.183	0.143	1.28
2	604 E	16.39	0.328	0.282	1.16
3	605 Z	20.49	0.410	0.424	0.97
4	605 E	11.56	0.231	0.241	0.96
5	620 Z	7.75	0.155	0.114	1.36
6	620 E	9.08	0.182	0.140	1.29
7	640 Z	7.92	0.158	0.161	0.98
8	640 E	8.90	0.178	0.123	1.45

5.3.1.2.3. Metagenomic Analysis

Principal component analysis (PCA) was done to select the dung samples of cows for metagenomic studies to check that how *G. candidum* QAUGC01 has changed the gut microbial diversity. According to PCA one control (Cow 605) and three experimental cows‘ i.e cow 604,

620 and 640 were selected for rumen microbial diversity analysis through 16SrDNA sequencing and ITS (Fig.5.3).

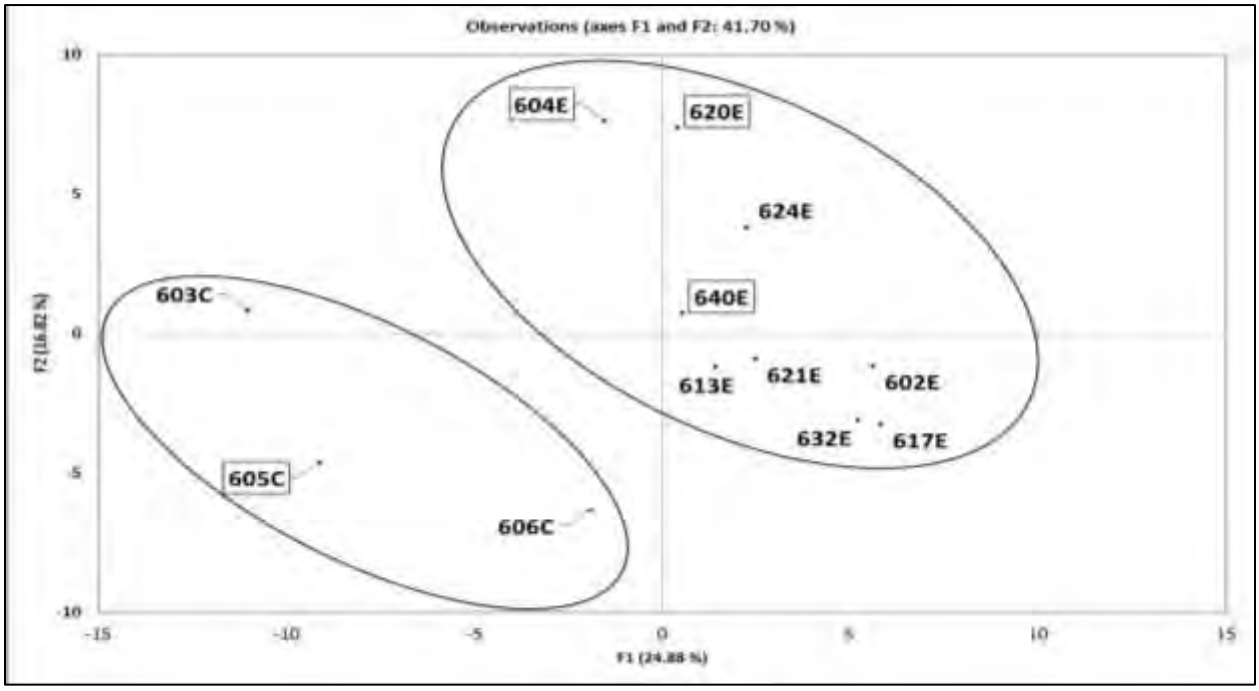


Figure 5.3: Principal component analysis of experimental (E) and control cows ©.

5.3.1.2.4. Gut Microbial Biodiversity by Using 16S rDNA Based Analysis

5.3.1.2.4.1. Bacterial Phyla Diversity of Experimental and Control Cows

Among the taxonomic level (phylum) a total of 11 bacterial phyla were detected and 5 were shared by GIT of both control and experimental cows. The four dominating phyla were *Proteobacteria, Firmicutes, Actinobacteria & Bacteroidetes* (Fig.5.4).

Proteobacteria population was low in control cow gut (5.99%) while high in the experimental cows GIT (59.68, 69.82, 99.15%) at the end day of experiment. The second abundant phylum was *Firmicutes*, its population was high in control cow GIT (93.4%) while low in experimental cow (35.01, 11.88, 0.63%). *Bacteroidetes* percentage was low in GIT of control (0.07%) than that of experimental cows (3.77, 1.07,0.08%). *Actinobacteri*a and *Cyanobacteria* were found to be higher in two experimental cows' GIT (604 and 640) (0.42,16.93% and 0.38, 0.05% respectively) than control cow (0.28, 0.01% respectively), while their percentage was low in the

experimental cow 620l (0.07, 0.005%) than control. Phylum *Spirochaetes* was identified with abundance of 0.5% and 0.15% in dung samples of experimental cow 604 and 640.

The rest of identified phyla i.e. *Planctomycetes, Candidatus, Saccharibacteria, Tenericutes, Verrucomicrobia* and *Elusimicrobia* were not present in control cow's GIT while found to be present in all three experimental cows each of which represented <0.1 % of total bacterial population/ abundance.

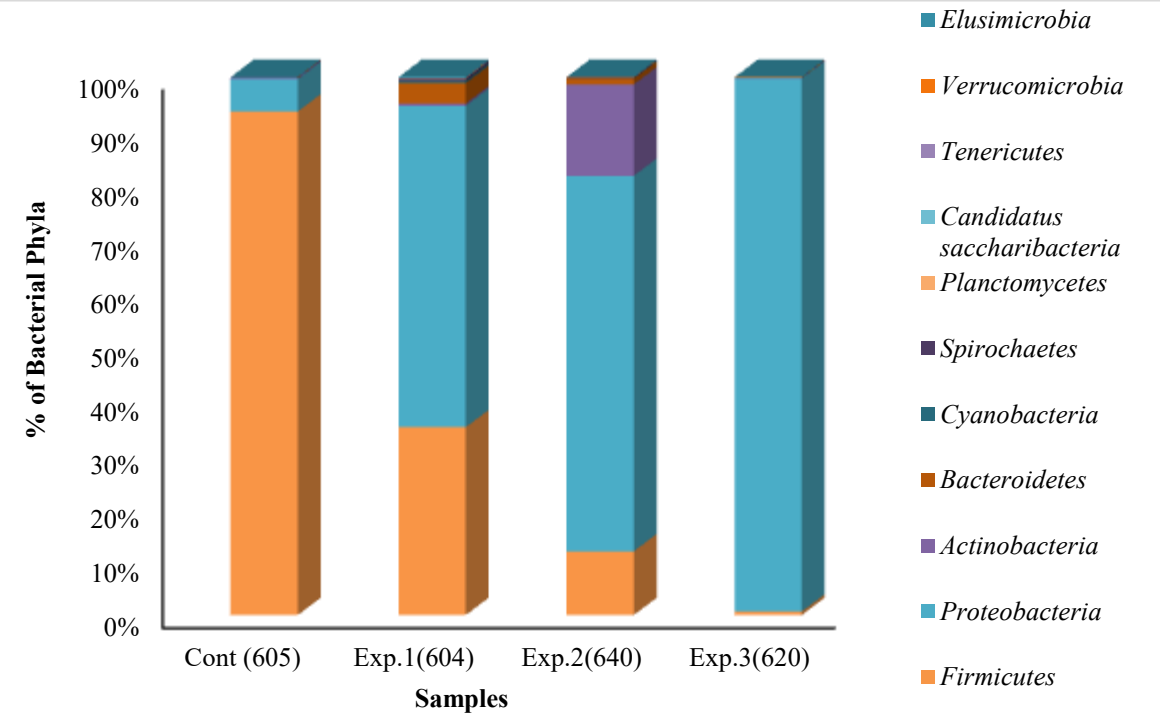


Figure 5. 4: Percentage relative abundance of Bacterial Phyla in control and experimental samples.

5.3.1.2.4.2. Bacterial Species Level Diversity of Experimental and Control Cow

At species level a total of 220 bacterial species were detected in all four dung samples through metagenomics study at the end of experiment. The total bacterial diversity in all four dung samples was represented by 62 species (Fig.5.5). Bacterial species diversity of control (605) sample was represented by 64 bacterial operational taxonomic units (OTUs) with only 7 (10.9%) bacterial OTUs found with relative abundance ≥ 0.1%. While the total observed bacteria OTUs in dung samples of experimental cows 604, 640 and 620 were 176, 148 and 105 OTUs respectively in which 43(24.43%), 28(18.9%) and 17(16.1%) respectively were present with

relative abundance ≥ 0.1% of the total bacterial abundance at the end of experiment (Table 5.3). The Anaerobic bacterial OTUs comprised high abundance in experimental cows gut than control. The anaerobic microbiota constituted 65.11 % and 64.28% abundance in experimental cows 604 and 640. In control sample the anaerobe abundance was 42.85%. The very less concentration of anaerobic OTUs was 35.29% in dung sample of experimental cow 620. The Aerobic OTUs concentration was 57% in control, 64.70% in experimental cow 620 while very less abundance of aerobic OTUs identified in experimental cow 604 and 640 with relative abundance of 34.88% and 35.71% (Table 5.3).

Table 5. 3: Percent abundance and total numbers of anaerobic and aerobic bacterial OTUs in experimental and control cows

Cow's Dung Sample (Metagenome)	Total Observed Bacterial OTUs	Bacterial OTUs with ≥ 0.1% abundance	Total Anaerobic OTUs	Total Aerobic OTUs	%age of Anaerobic OTUs	%age of Aerobic OTUs
Control (605)	64	7	3	4	42.85	57.14
Exp. Cow (604)	176	43	28	15	65.11	34.88
Exp. Cow (640)	148	28	18	10	64.28	35.71
Exp. Cow (620)	105	17	6	11	35.29	64.70

Among Gamma *Proteobacteria*, genus *Pseudomonas* was low in control (0.35%) while high 57.45%, 68.44% and 2.32% in experimental cows' GIT. The OTUs like *Pseudomonas veronii* (Exp.604:0.96%, Exp.640: 37.65%, Exp.620: 0.89%) and *Pseudomonas trivialis* (Exp.640: 50.23%, Exp.640: 27.24%, Exp.620: 1.05%) significantly contributed to high *Pseudomonas* spp. percentage (Fig.5.4). *Enterobacter* spp. was higher only in GIT of gut of Exp. 620 (0.43%) than control (0.005%) at the end of experiment. *Klebsiella oxytoca* was significantly higher in GIT of experimental cow 620 (25.99%) as compared to experimental 604 (0.11%) and 640 (0.15%) also present in GIT of control cow (0.13%). Similar pattern was observed for *Serratia quinivorans* (Control: 0.1%, Exp. 604, 640, 620: 0.07, 0.06, 13.52%). *Raoultella ornithinolytica* was higher (4.44%) in Exp.620 than control cow 605 (0.01%) (Fig.5.5). In *Betaproteobacteria* class the genus *Achromobacter* was significantly higher (51.22%) in Exp.620 than in control cow GIT (1.89%).

In class *Betaproteobacteria* the genera *Achromobacter* contributed high concentration 51.22% in dung metagenome of Exp. cow 620 and 1.89% in control while 0.28% and 0.21% in Exp. cows 604 and 640 respectively. And in class *Gammaproteobacteria* the genera *Klebsiella, Serratia* and *Enterobacter* were detected as 26%,13.56% and 0.43% respectively in intestinal microbiota of Exp.620 and in control cow 605 (0.8%, 0.62% and 0.03%) respectively while very less abundance of genus *Klebsiella* i.e 0.11%, 0.15% detected in dung metagenome of Exp. cows 604 and 640. The representative species was *Achromobacter xylosoxydans* and *Klebsiella oxytoca* excreted in concentration of 48.49% and 25.98% respectively in faeces of exp. cow 620 followed by *Serratia quinivorans* 13.52% and *Enterobacter* species 0.4% associated with *Salmonella enterica* 0.15% and very less concentration of these pathogenic flora appeared in dung samples of experimental dairy cows no. 604 and 640 (Fig.5.5).

On the other hand the phylum *Firmicutes* accommodated with highest abundance the fecal microbiota of control dairy cow with the members of the dominant genus *Paenibacillus* excreted with very high concentration of more than 89% in dung of control cow, followed by genus *Bacillus* (2.8%) with dominant species *Bacillus szutsauensis* appeared with high abundance of 84.96% in GIT of control cow while observed with lower percentage in experimental cows‘ GIT (Exp.604,640,620: 0.18%, 1.31%, 0.12%) respectively (Fig.5.5). In phylum *Firmicutes* the *Clostridium* spp. and *Eubacteria* spp. were both low in control cow GIT (0.25% and 0.03% respectively) and high in Exp. cows 604, 640, 620 GIT (17.71, 4.88, 0.24% and 0.21, 0.3, 0.009% respectively). The resident (core) rumen *Firmicutes* genera including *Ruminococcus* found with abundance of 0.16%, 0.16% and 0.001% in treated dairy cows while 0% in control dung samples associated with species of genus *Ruminiclostridium* 0.2% in dung samples of experimental dairy cow 604 and genus lactobacillus (0.16%) with only one type species *Lactobacillus coryniformis* in dung sample of exp. cow 640. *Psychrobacillus, peptoclostridium*, are important genera of Firmicutes appeared with abundance of 0.1%, 0.1% in experimental cow 640 and *Lachnoclostridium* 0.35% in cow 604 (Fig.5.5).

In Phylum *Bacteriodetes*, the *Bacteroides* spp. were low in control cow‘s GIT (0.001%) than in Exp. 604, 640, 620 GIT (0.54, 0.78, 0.02%) associated with species of genus *Rikenella* identified with abundance of 1.4%, 0.02% and 0.009% in experimental cows respectively with members like *Rikenella spp.* 1.34% and *Rikenella microfusus* 0.14% identified only in dung samples of

exp. Cow 604. The genus *Paludibacter* appeared with abundance of 0.6%, 0.05% and 0.01% in experimental cows with type species. The metagenomic study of dung metagenome of control dairy cow didn‘t show members of *Bacteroidetes*. The species of genus *Paludibacter* also observed in dung metagenome showed their presence in gut of dairy cows. Species of genus *Mucilaginibacter* (0.27%) such as *Mucilaginibacter oryzae* (0.11%) and *Mucilaginibacter herbaticus* (0.15%) accompanied by genus *Nubsella* (0.18%) with type species *Nubsella sp.* also detected with percentage abundance of 0.18% (Fig.5.5).

In the phylum *Actinobacteria, Bifidobacterium choerinum* and *Bifidobacterium longum* both were low in GIT of control cow 605 (0.03 and 0.003% respectively) than that in case of experimental cows (Exp. 604, 640, 620: 0.27%, 15.22%, 0.05% and 0.01%, 0.74%, 0.003% respectively). The genus *Arthrobacter* with species *Arthrobacter luteolus* 0.18% identified only in dung metagenome of exp. cow 640 (Fig.5.5). The percentage of the remaining species was considerably low.

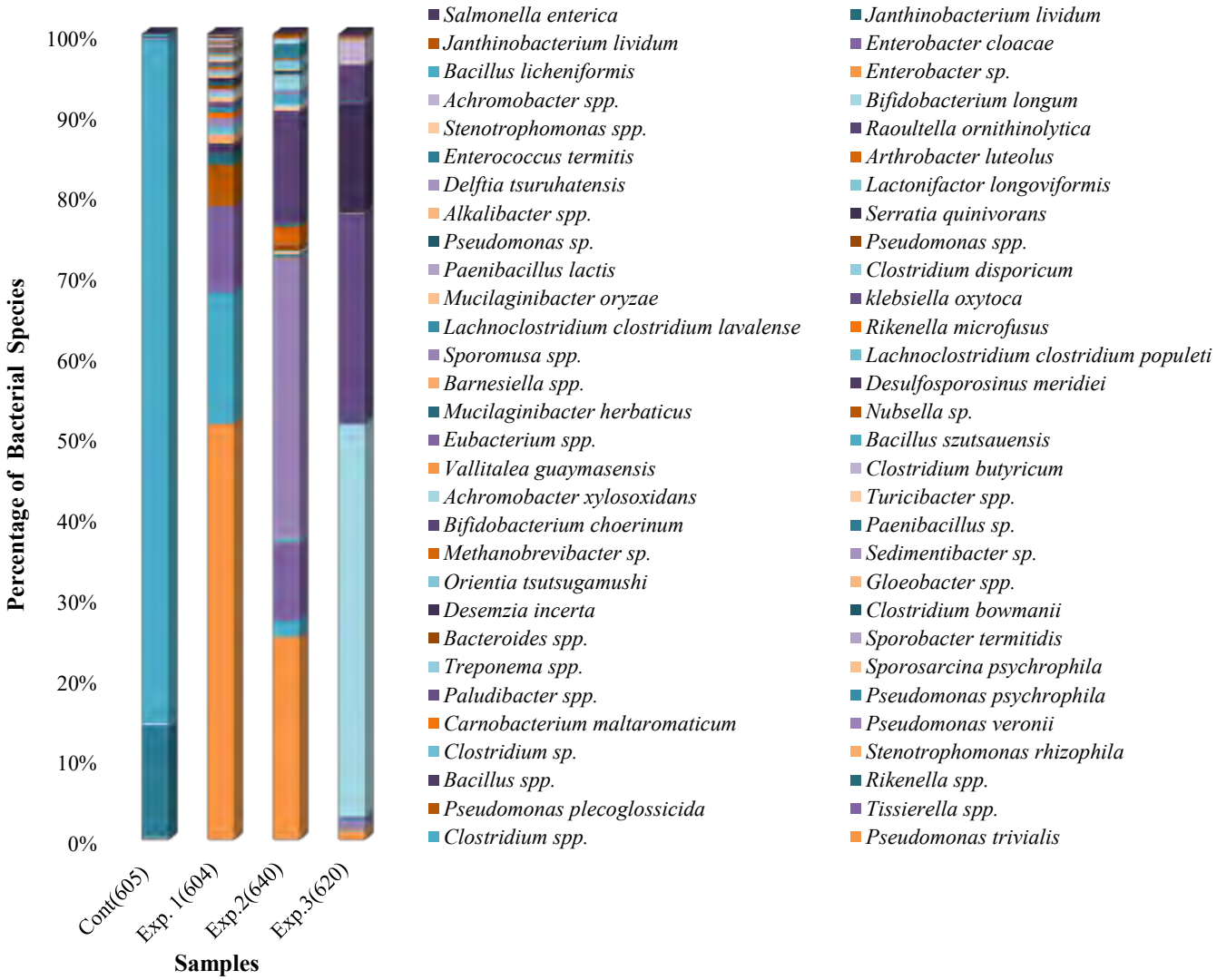


Figure 5.5: Bacterial communities in GIT of control and experimental cows at the end of experiment. Data represents bacterial species with reads accounting for >0.1 % of total reads in at least one sample.

5.3.1.2.4.3. Bacterial Genera Level Diversity of Experimental and Control Cows

The genera level diversity of experimental and control samples showed overall 45 bacterial genera with abundance > 0.1%. Similarly, *G. candidum* QAUGC01 supplementation also increased the genus level diversity in experimental cows with 32, 22 genera in experimental cows 604 and 640 respectively than control cow (13) and one experimental cow 620 (13) with abundance ≥ 0.1% (Table 5.4). The anaerobic genera increased in experimental cow 604 and 640 while decreased in control and experimental cow 620

Table 5. 4: Percent abundance of bacterial genera of experimental and control dung samples

No of Bacterial Genera	Bacterial Genera	Bacterial Genera Abundance %			
		604.EE	640.EE	620.EE	605.EC
1	*Pseudomonas*	57.459	68.443	2.319	2.320
2	*Clostridium*	17.807	5.077	0.243	0.258
3	*Tissierella*	10.466	0.369		0.135
4	*Rikenella*	1.494			
5	*Bacillus*	1.269	0.134		2.885
6	*Stenotrophomonas*	1.083		0.375	
7	*Carnobacterium*	0.680			0.123
8	*Paludibacter*	0.619			
9	*Sporosarcina*	0.568	0.304		0.135
10	*Bacteroides*	0.533	0.813		
11	*Treponema*	0.509	0.158		
12	*Paenibacillus*	0.492	0.474	0.119	89.429
13	*Sporobacter*	0.466			
14	*Desemzia*	0.403			
15	*Gloeobacter*	0.379			
16	*Orientia*	0.379			
17	*Lachnoclostridium*	0.359			
18	*Sedimentibacter*	0.336			
19	*Bifidobacterium*	0.288	16.591		0.209
20	*Achromobacter*	0.283	0.217	51.299	1.890
21	*Mucilaginibacter*	0.275			
22	*Turicibacter*	0.273	0.608		0.110
23	*Alistipes*	0.227			
24	*Ruminiclostridium*	0.219			
25	*Eubacterium*	0.206	0.304		
26	*Vallitalea*	0.194			
27	*Nubsella*	0.186			
28	*Ruminococcus*	0.168	0.169		
29	*Desulfosporosinus*	0.150			
30	*Barnesiella*	0.147			
31	*Sporomusa*	0.145			
32	*Klebsiella*	0.117	0.155	26.035	0.847
33	*Serratia*			13.564	0.626
34	*Enterococcus*		1.978	0.176	0.233
35	*Lactonifactor*		1.290		
36	*Janthinobacterium*		0.649	0.160	
37	*Arthrobacter*		0.199		
38	*Alkalibacter*		0.194		
39	*Lactobacillus*		0.169		

40	*Psychrobacillus*		0.107		
41	*Peptoclostridium*		0.104		
42	*Raoultella*			4.442	
43	*Enterobacter*			0.439	
44	*Salmonella*			0.159	
45	*Delftia*			0.108	

EE = End day dung sample of experimental cow

EC = End day dung sample of control cow

5.3.1.2.5. Fungal Phyla Diversity of Control and Experimental Cows at End-day of Experiment

Total of three phyla were identified in dung samples of experimental and control cows. Phylum *Ascomycota* was most dominating in fungal biodiversity, contributing >95% of fungal population in all samples followed by *Basidiomycota* and highly anaerobic *Neocallimastigomycota*. The population of *Ascomycota* was low in GIT of Exp. cow 604 (96.38%) while high in Exp.640 (98.61%) and Exp.620 (99.36%) cow's GIT than control (97.73%) at end of experiment. Members of *Basidiomycota* were low in all the three experimental cow's GIT (Exp. 604, 640, 620:1.85, 1.38, 0.62%) as compared to control (2.26%). Conversely *Neocallimastigomycota* population was high in the entire three experimental cow's GIT (Exp. 604, 640, 620:1.76, 0.018, 0.019%) than that in control (0.003%) (Fig.5.6).

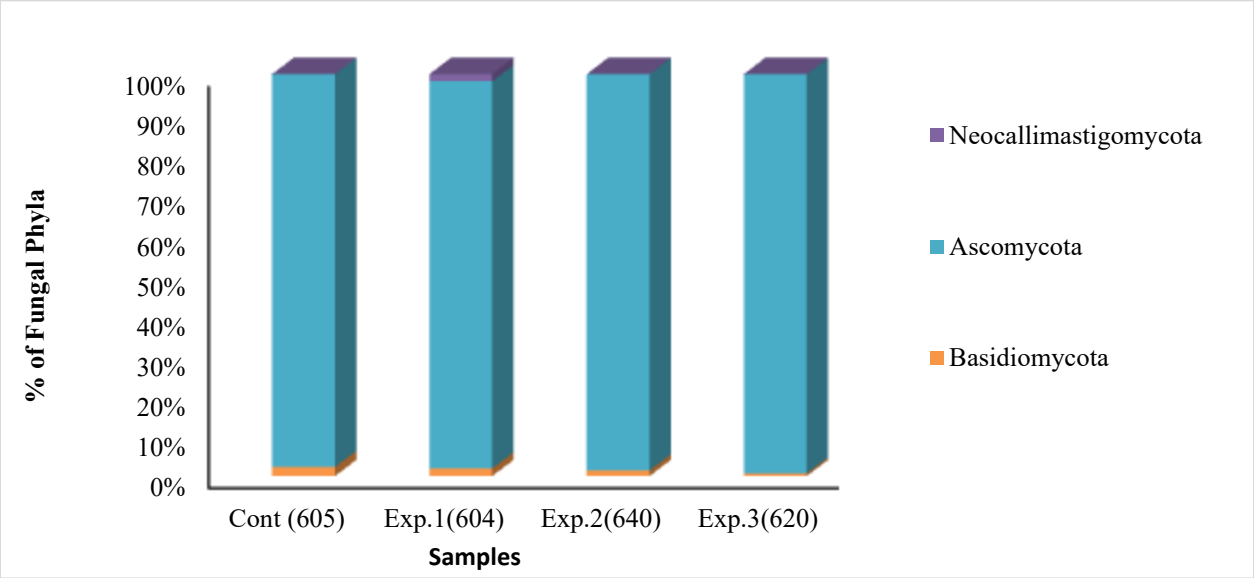


Figure 5.6: Percentage relative abundance of fungal phyla in control (Cont.) and experimental (Exp.) samples.

5.3.1.2.5.1 Fungal and Yeast Species Level Diversity of Experimental and Control Dung Metagenome

Fungal and yeast species (OTUs) diversity was represented by the relative abundance of total 64 species (Fig.5.6). Metagenomic study revealed 44 fungal species in control cow 605 while 61, 52 and 42 species detected in Exp. Cows.604, 640 and 620 at the end day of experiment. *Geotrichum* species were detected both in control and experimental cows' GIT but their percent abundances were remarkably high in experimental cows; *Galactomyces geotrichum* abundance was 59.30%, 66.32%, 27.81% in Exp. cows 604, 640 and 620 showing too high abundance even the dung samples of experimental cows were collected at 95th day while the G. candidum supplementation was stopped at 90th day. Very less abundance of *Galactomyces geotrichum* in Control cow 605 3.73%, The *G. candidum* was present in high abundance 37.06% in cow 620 than experimental cows 604 and 640 i.e 4.50% and 6.02% while very less abundance of *Geotrichum candidum* was in control cow 0.83%. In the phylum Ascomycota the most prominent and promising affect was on the growth of *Debaryomyces hansenii* as its percentage was remarkably high in the GIT of control cow (82.215%) while it showed controlled and reduced growth in Exp.640, 640 and 620 cows (2.85%, 3.90%, 2.81% respectively). *Thelebolus globosus, Dipodascus australiensis, Pseudeurotium bakeri, Candida* spp. and *Aspergillus* spp. were low in the control (0.04%, 0.02%, 0.004%, 0.03%, 0.01%) and their percentages were found to be higher in experimental cows' GIT (Exp. 604, 640, 620: 16.34, 0.40, 0.08; 0.11, 1.59, 0.05; 0.33, 1.05, 0.02; 0.13, 0.18, 0.09; 0.31, 0.92, 0.03% respectively) *Meyerozyma guilliermondii* growth was lower in Exp. 604 (0.005%) while higher in Exp. 640 and 620 (0.44, 1.64%) than in control (0.02%). Percentage of *Fusarium gibberella fujikuroi* was same i.e. 0.03% in the GIT of Exp. 604 and control cow and higher in Exp. 640 and 620 cow (0.04, 3.61%).

Preussia sp. identified and found in GIT of experimental cow 604 was 0.29% and 0.0089% in GIT of cow 640 while not detected in control sample.

Among the members of Basidiomycota, *wallemia* spp. were higher in GIT of Exp. 604 and 640 (0.22, 0.11%) while lower in Exp. 620 GIT (0.09%) than in control (0.10%). *Rhodotorula* spp.

were higher and *Cryptococcus* spp. were lower in Exp. 604, 640 and 620 (0.61, 0.45, 0.07%; 0.28, 0.47, 0.27% respectively) in comparison with that in control (0.003% and 1.59%) (Fig.5.6). The members of Phylum *Neocallimastigomycota* were also observed and detected with high % abundance in dung metagenome of Exp. Cows 604 and 640 while not identified in control and Exp. Cow 620 such as *Cellamyces spp*., In the present study the 3 genera of rumen anaerobic fungi i.e *Neocallimastix frontalis, Orpinomyces sp.* and *Cyllamyces sp.* found/observed with relative high abundance of 0.711765 %, 0.364706 and 0.673529 in the GIT of Exp. cow 604 while 0% in control cow no 605 and other two experimental cows.

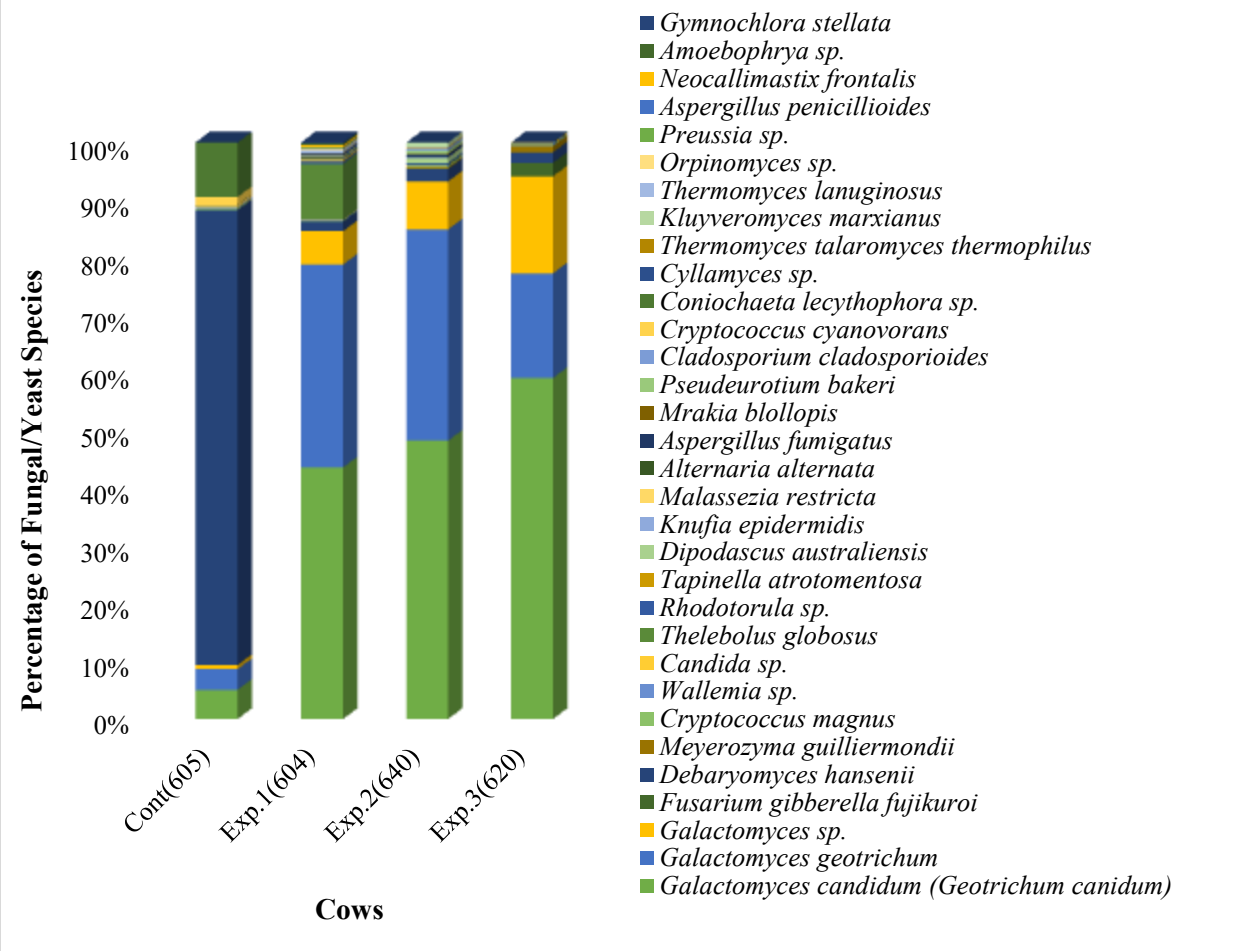


Figure 5.7: The yeast communities in the GIT of control and experimental cows at End day of experiment. Data represents yeast species with reads accounting for >0.1 % of total reads in at least one sample

5.3.1.2.6. Shared and Unique Bacterial Diversity of control and experimental cows at End day of experiment.

Heatmap analysis of bacterial diversity at genus level revealed that 604EE and 640EE clustered most closely to each other and then they clustered with 605EC which is control cow fed on basal diet. This clustering pattern statistically approved that 604EE and 640EE have highly similar diversity as compared to 605EC and 620EE. 620EE is also experimental cow but its diversity profile was distant from the other experimental cows, however its profile is somewhat similar to 605EC control cow (Fig.5.8).

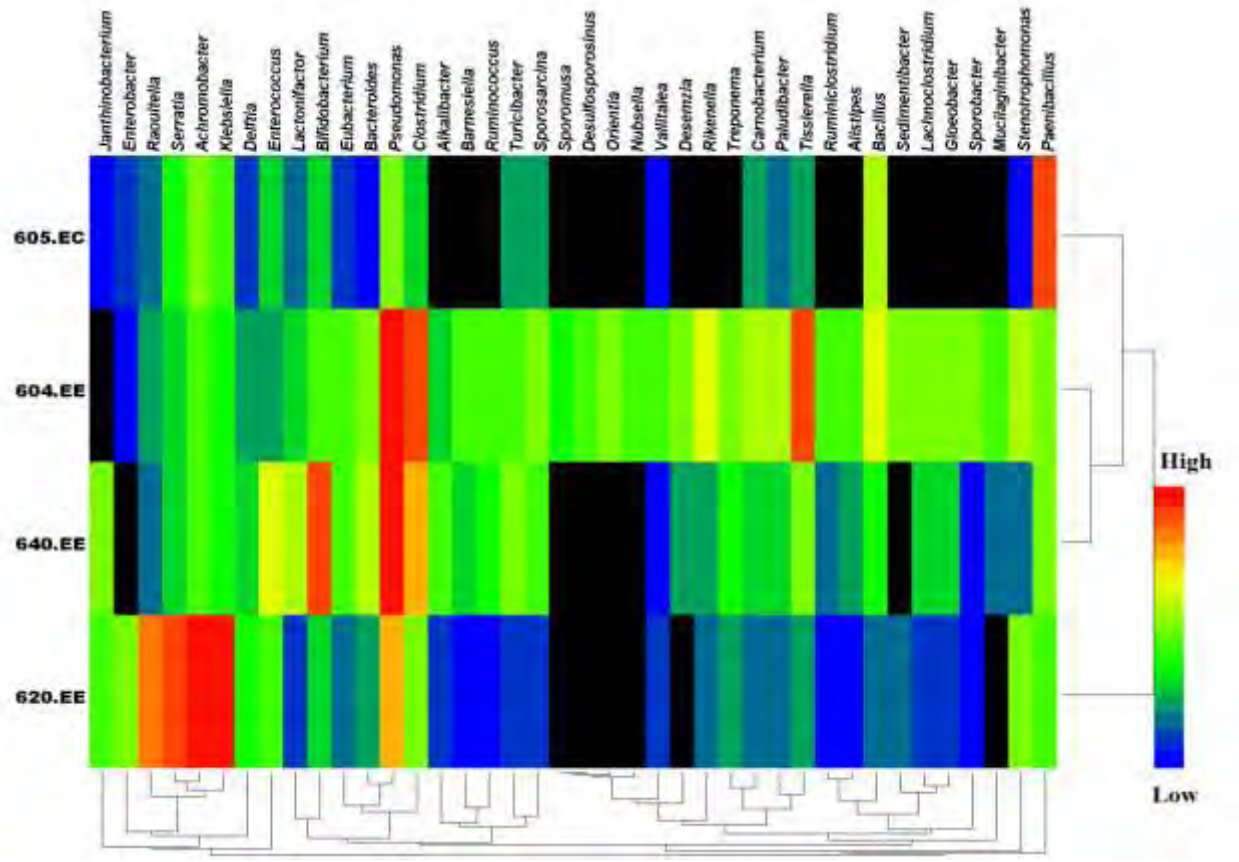


Figure 5. 8: Heat Map analysis of bacterial genera OTUs shared between experimental cows 604,640, 620 and control 605.

Fungal diversity was analyzed by heat map analysis at genus level showed different pattern as compared to bacterial diversity. In this analysis 620EE and 640EE cow metagenome clustered closely, then they clustered with 604EE and 605EC. It showed that experimental cows fed on *G. candidum* supplemented diet showed higher similarity as compare to control (Fig.5.9).

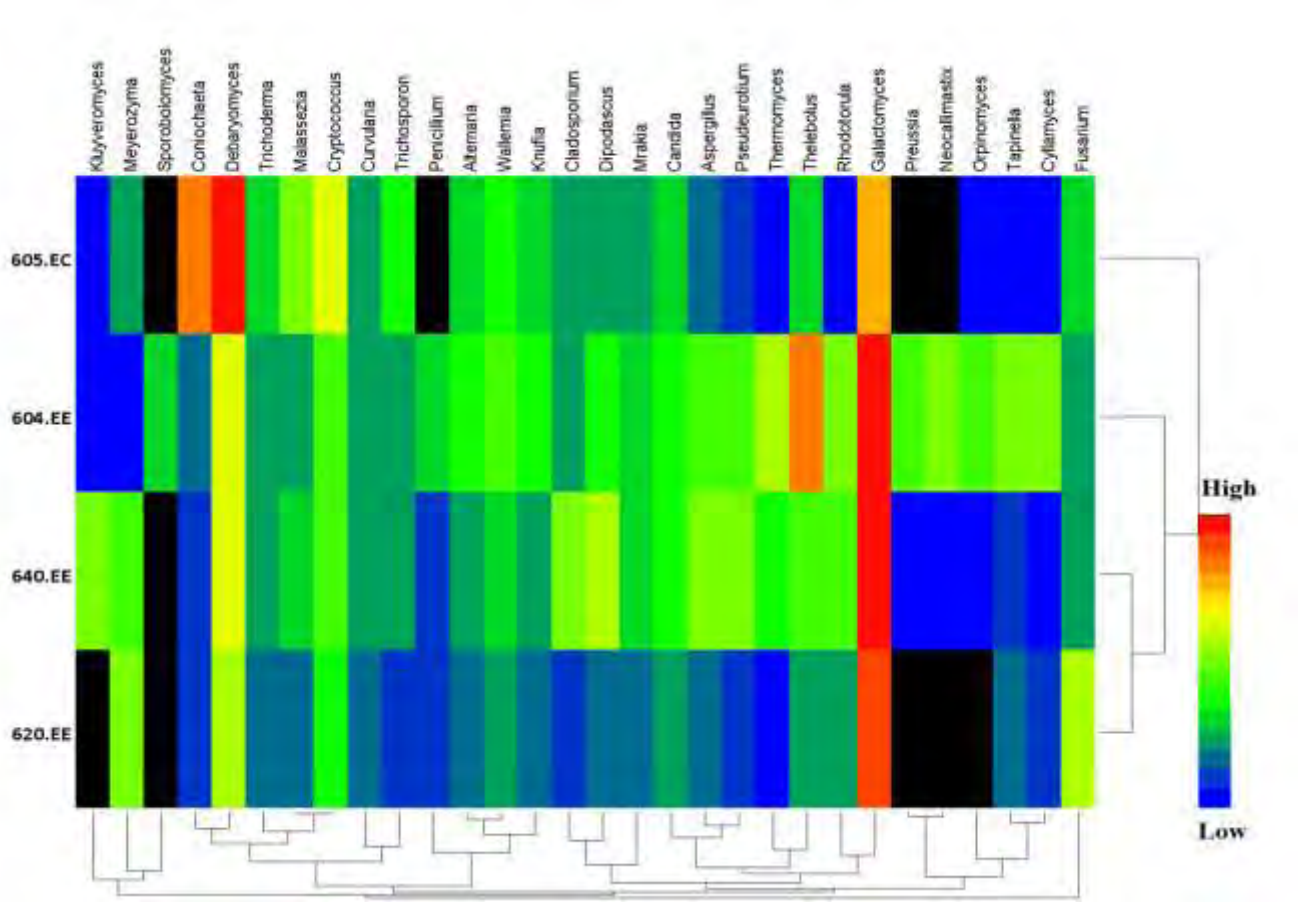


Figure 5. 9: Heat Map analysis of fungal genera OTUs shared between experimental cows 604,640, 620 and control 605

5.3.1.2.7. Common and Unique Microbiome Diversity Among Experimental and Control Cows

Common and unique microbiome diversity among experimental and control cows was evaluated by plotting Venn diagrams. Out of total observed bacterial species 27 species were common in all experimental and control cows that comprised of 21.3%. The 27 bacterial species comprised 21.3% were found to be common in all 604EE, 640EE and 620EE experimental cows. The 22 bacterial species were found to be shared in 604EE and 640EE experimental cows. The 25 (19.7%), 5 (3.9%), 1 (0.8%) and 0 (0%) species were uniquely identified in 604EE, 640EE, 620EE and 605EC cow respectively (Fig.5.10).

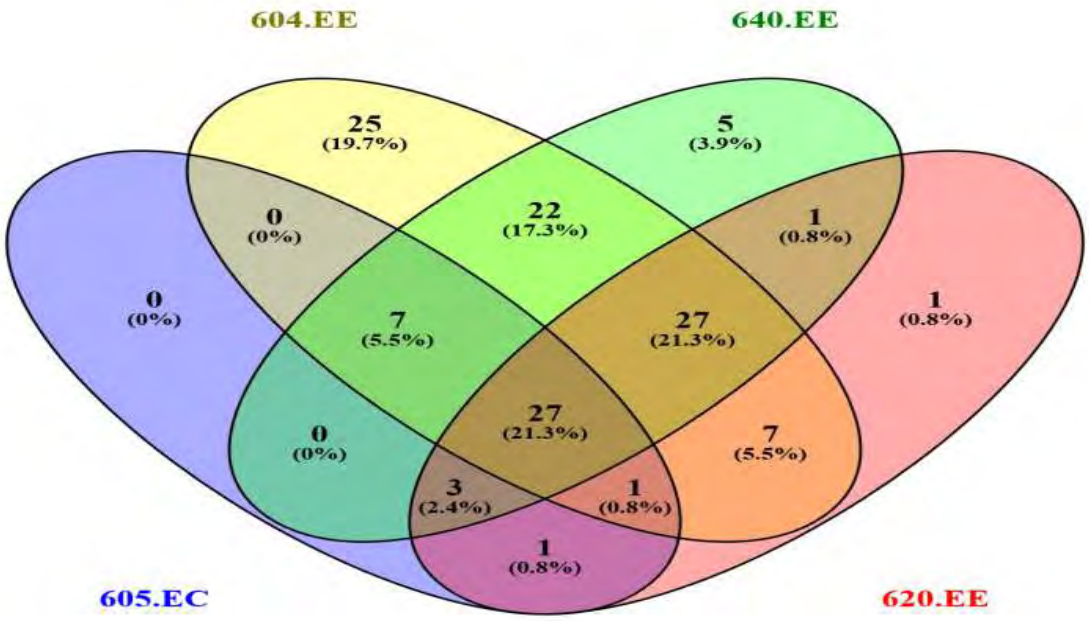


Figure 5.10: Venn diagram analysis of bacterial species shared between experimental cows 604,640, 620 and control 605

Common and unique fungal microbiome diversity among experimental and control cows was evaluated by plotting Venn diagrams. Out of total observed fungal species 27 species were common in all experimental and control cows that comprised of 6.8% of total percent microbiome. The 2 fungal species comprising 4.7% were found to be common in all 604EE, 640EE and 620EE experimental cows. 4 species were found to be shared in 604EE and 640EE experimental cows. 4 (9.3%), 0 (0%), 0 (0%) and 0 (0%) species were uniquely identified in 604EE, 640EE, 620EE and 605EC cow respectively (Fig.5.11).

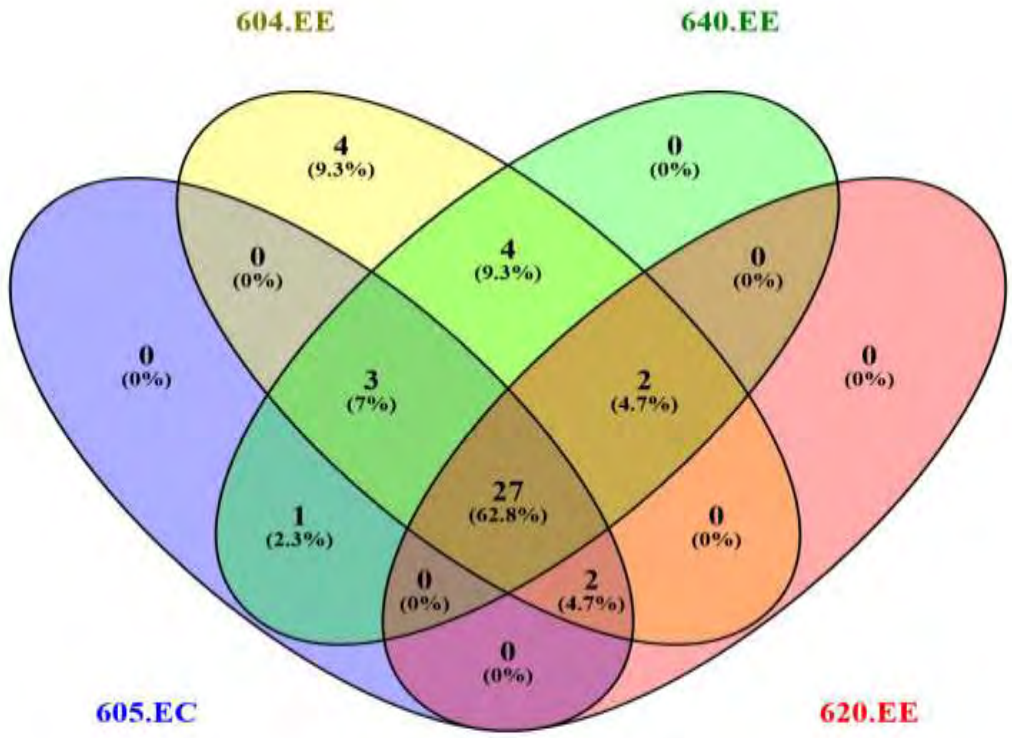


Figure 5.11: Venn diagram analysis of fungal species shared between experimental cows 604, 640, 620 and control 605:

5.3.1.2.8. Microbial Diversity Index of Experimental and Control Cows

Richness, alpha diversity and dominance of the bacterial and yeast communities were calculated by using number of reads, operational taxonomy units (OTUs), Shannon and Simpson index. Total number of bacterial OUTs were higher in experimental cows than in control animal's GIT, which showed that overall richness of bacterial OTUs increased in *G. candidum* QAUGC01supplemented experimental cows at end of experiment. The richness of fungal OTUs also increased in experimental cow 604 because the number of fungal OTUs was higher in Exp. cow 604 than in control 605 and experimental cow 640 and 620. The Exp. cow 640 and control 605 both of which showed to have same numbers of fungal OTUs. i.e.109. Alpha diversity of bacteria and fungi according to Shannon Index was high in experimental cows than control at the end of experiment. Similarly, the Simpson index was also calculated to see the dominance of bacteria and fungi in experimental and control cows. According to Simpson index the bacterial as well as the yeast dominance was high in experimental cows than control. Alpha diversity of both bacteria and fungi in terms of richness and evenness was higher in all the three experimental cows than in control according to Shannon and Simpson index (Table 5.5).

Table 5. 5: Number of analyzed sequences, diversity richness (OTU's), diversity index (Shannon and Simpson) for 16SrDNA and ITS (Internal Transcribed spacer) region sequencing libraries in GIT of Control (Cont.) and experimental (Exp.) cows.

Sample ID	Numbers of reads		Number of OTU's		Shannon Index		Simpson Index		Observed Species	
	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
Control (605)	52623	60432	105	109	0.69	0.73	0.22	0.31	105	109
Exp. (604)	39436	34000	356	141	2.05	1.45	0.71	0.60	356	141
Exp. (640)	34886	22452	242	109	1.87	1.29	0.74	0.53	242	109
Exp. (620)	51050	15373	220	91	1.49	1.45	0.67	0.72	220	91

5.4. Discussion Phase III

The health status and productivity of cattle is greatly associated with the role of GIT microbial community. The efficient growth of gut microbiome results in increased production of short chain fatty acids in rumen that provides energy (Malmuthuge, 2017; P. Van Soest *et al.*, 1984). Beneficial modulation of gut microbiome has prominent impact on host physiology, productivity and health. The link between production parameters such as feed efficiency and milk yield and composition, and the rumen microbiota has been established in recent studies (Carberry *et al.*, 2012; Hernandez-Sanabria *et al.*, 2012; Jami *et al.*, 2014; Jewell *et al.*, 2015b; Lima *et al.*, 2015; McCann, Wickersham*, et al.*, 2014; Shabat *et al.*, 2016; Song *et al.*, 2017; Weimer, 2015). Probiotic supplementation is one of the healthy and promising way to modulate GIT microbiota beneficially. In cattle, yeast supplementations have improved growth rate and production efficiency successfully (Puniya *et al.*, 2015). Improved milk production and feed intake in lactating cows have been reported by *A. oryzae* and *S. cerevisiae* supplementation (Cakiroglu *et al.*, 2010; de Ondarza *et al.*, 2010; Schingoethe *et al.*, 2004). But underlying mechanisms, includes feed stuff digestion, conversion, immunomodulation and improved metabolism, need to be determined (Yu *et al.*, 2017). The molecular dynamics of cause and effect of gut microbiome still need to be determined (Song *et al.*, 2017; Weimer, 2015).

This study used an *in vitro* characterized *G. candidum* QAUGC01yeast as probiotic feed additive for *in vivo* application through supplementation in dairy cow‘s ration. The investigation of its survival in gastrointestinal tract (GIT) and interaction with gut microbiome was also investigated. The possible impact was investigated by analyzing the production parameters such as milk yield, milk composition, blood and serum biochemistry and gut microbial structure through analysis of dung microbial profile using culture dependent techniques.

Based on culturable microbiology, physiological, hematological and production parameters, statistical clustering was done to make the unbiased allocation of dairy cows. Four cows‘ (one control and three experimental) gut microbiota was analyzed by using 16SrDNA and ITS sequencing. The health status of animals for probiotic safety was monitored periodically by hematological and serum biochemical analysis (Phase II). The serum and blood parameters were reported within normal ranges and all experimental dairy cows were found healthy throughout

the experimental period of ninety days. Moreover, all cows were clinically examined by certified veterinary physician regularly.

All cows gut was monitored by culturing while based on variation in culture based microbiological profile the selective cows gut microbiota was further monitored by metagenomic study. Results of both culture dependent as well as culture independent analysis showed a variation in the GIT microbiota of all four cows at the end of current experiment and it has been already speculated that the variances in feed efficiency and nutrient utilization in dairy ruminants is partially due to variations in gut microbiota and the modulation of the microbiota composition can promote livestock development and sustainability (Delgado *et al.*, 2019). Culture dependent analysis confirmed the increase in total aerobic plate count in experimental cows which indicated that the probiotic supplementation increased total microbial population in GIT of experimental animals (Kawakami *et al.*, 2010). This increase may be attributed to the yeast cells, which in this case may provide different growth factors e.g. oligosaccharides, vitamins B, amino acids and organic acids to stimulate growth of rumen microbes (Retta, 2016). The yeast supplementation effects are also visible by an increase of *lactobacillus* count and *lactococcus* count in experimental cows than control and these results are in agreement with many studies, reported that with supplementation of probiotic increased lactobacilli population in fecal samples (Agazzi *et al.*, 2014; Ayad *et al.*, 2013; Bayatkouhsar *et al.*, 2013; S Ghazanfar *et al.*, 2015; Stella *et al.*, 2007) and *Lactococcus* count (Shakira Ghazanfar *et al.*, 2017). Coliform count was decreased in experimental (*P*= 0.16) while found increased in control cows (P=0.39) at the end of experiment, this could be attributed to competitive exclusion and cell binding as reported earlier (F Chaucheyras-Durand *et al.*, 2008; Shakira Ghazanfar *et al.*, 2017; Stella *et al.*, 2007) . The results of present study indicated that *G. candidum* QAUGC01 culture may have a role in colonization resistance and inhibit growth of pathogens as speculated by previous studies (Dieuleveux *et al.*, 1998; Muhammad Imran *et al.*, 2013; M Imran *et al.*, 2010; Mefteh *et al.*, 2017; Saima Naz *et al.*, 2013; Samuel *et al.*, 2011). The Yeast and fungal count increased in gut of experimental cows non-significantly (P= 0.57) and in control cows it didn‘t fluctuate (P= 0.89) which clearly support the findings of Stella *et al*. (Stella *et al.*, 2007). In all the 90th day samples *G. candidum* QAUGC01 was found to be present which was confirmed by microscopy and 23SrDNA sequencing with high abundance. The presence of this strain (*G. candidum* QAUGC01) with high abundance shows that after feeding it established and survived

successfully in the gut of experimental cows. Based on culture based results, it was inferred that control and experimental cows may have different gut microbiology. So, on culturing based statistical analysis, representative four cows were selected for metagenomic analysis.

The metagenomics analysis of dung samples of one control and three experimental cows‘ samples showed that total number of observed bacterial OUTs were higher in experimental group than in control animal‘s GIT. Richness, alpha diversity and dominance of the bacterial and yeast communities were calculated by using number of reads, OTUs, Shannon and Simpson index. Total numbers of bacterial OUTs were higher in experimental cows than in control animal‘s GIT, which showed that overall richness of bacterial OTUs increased in *G. candidum* QAUGC01supplemented experimental cows at end of experiment. The richness of fungal OTUs was increased in experimental cow 604 because the number of fungal OTUs was higher in cow 604 than in control 605 and experimental cow 640 and 620. The experimental cow 640 and control 605 both showed almost same numbers of fungal OTUs. i.e. 109. Alpha diversity of bacteria and fungi according to Shannon Index was high in experimental cows than control at the end of experiment. Similarly, the Simpson index was also calculated to see the dominance of bacteria and fungi in experimental and control cows. According to Simpson index the bacterial as well as the yeast dominance was high in experimental cows than control. Alpha diversity of both bacteria and fungi in terms of richness and evenness was higher in all the three experimental cows than in control according to Shannon and Simpson index. The current study results are in agreement to early study depicted the dominancy of bacterial OTUs during prepartum and postpartum period of dairy cows (Lima *et al.*, 2015). In another study the members of bacterial family *Lachnospiraceae* were found predominant in Holstein cows compared to Jersey cows and OTUs belonging to *Prevotellaceae* were differentially abundant in the two breeds (Paz *et al.*, 2016).

According to results of present metagenomic study, the number of bacterial OTUs with relative abundance ≥ 0.1% were increased. There were 43, 28 and 17 bacterial OTUs (≥ 0.1%) identified in experimental cows 604, 640 and 620 respectively out of total observed species (OTUs) 57, 52 and 48 respectively, while only 7 bacterial OTUs detected in control sample with abundance ≥ 0.1% out of 40 observed OTUs that demonstrate the increase in abundance and number of bacterial OTUs in experimental samples as compared to control at the end of experiment. The anaerobic flora was dominated in experimental cows 604 and 640 with abundance of more than 65% and 64% after supplementation of *G. candidum* QAUGC01 as compared to control and

third experimental cow 620 harbored 42.85% and 35.29% anaerobic flora at the end of experiment. Probiotics are recommended in all cattle life-stages to increase rumen microflora, improve anaerobiosis, and production efficiency.

Similarly, *G. candidum* QAUGC01 supplementation also increased the genus level diversity in experimental cows with 32, 22 genera in cows 604 and 640 respectively than control cow (13 genera) and experimental cow 620 (13 genera) with abundance ≥ 0.1%. It has also been speculated by earlier studies that development of strict anaerobic environment by yeast additives enhanced the population of anaerobic fungi and anaerobic cellulolytic bacteria in the rumen provides not only energy sources in the form of short chain fatty acids (SCFA) but also microbial protein that can be assimilated by the ruminant further down the GI tract during mid-lactation (Zhu *et al.*, 2017). While in early lactation period the yeast supplemented animals maintained body condition score (BCS) and it could be attributed to the yeast in enhancing ruminal microorganisms‘ cellulolytic capacity, increasing fiber digestibility and starch utilization (Mumbach *et al.*, 2017).

In current study the phylum level diversity showed that a total of 11 bacterial phyla were detected while only 5 were shared by both control and experimental cows. The four dominating phyla were Proteobacteria, Firmicutes, Actinobacteria and Bacteriodetes. Same findings were reported by other studies where Firmicutes, Proteobacteria and Bacteroidetes were dominant phyla. However, the abundance of the two main phyla—Bacteroidetes and Firmicutes showed large variation between the different animals (Girija *et al.*, 2013a; Jami & Mizrahi, 2012; X.-X. Li *et al.*, 2009; Mao *et al.*, 2015; P. R. Myer *et al.*, 2016). Previous study identified 28 phyla in at least 20 samples with 13 phyla composed the core microbiome accompanied by two predominant phyla Firmicutes and bacteriodetes identified in rumen fluids during prepartum and postpartum periods with conclusion that structure of rumen microbiome also shifts between the prepartum and postpartum of dairy cows (Lima *et al.*, 2015). Members belonging to these Phyla contribute efficiently to the -degradation of organic matter such as cellulose and lignin, and play role in improving the physiological parameters including milk yield and composition etc (Girija *et al.*, 2013a).

After supplementation of *G. candidum* QAUGC01 to the experimental cows, metagenomic results of two treated cows were similar regarding aerobic, anaerobic gut bacterial and

fungal/yeast flora. It has been reported earlier that each cow in a herd has distinct microbial communities and this variation is even more evident among different breeds (Paz *et al.*, 2016). The dominating species in two experimental cows were of genus *pseudomonas* (proteobacteria phylum) followed by anaerobic *clostridium*, *Ruminococcus*, *Bacteroides* and *Bifidobacterial, Rekinella* species. Third experimental cow 620 was dominated by pathogenic members of Phylum Proteobacteria. Our results are supported by earlier study analyzed the dung microbiota with dominant phyla i.e Bacteriodetes including genera *Bacteroides, Paludibacter*, Firmicutes with *Clostridium*, *Ruminococcus*, *Bacillus* and Alpha and Gamma Proteobacteria phylum with genera *Pseudomonas* and *stenotrophomonas* (Girija *et al.*, 2013b). Pathogenic bacteria *Achromobater xylosoxydanes, Klebseila oxytoca, Salmonella enterica* and enterobacter species were detected in high abundance in dung microbiota of control cow and experimental cow 620 and this finding is in agreement with results of Dowd et al. (2008) also reported the foodborne pathogenic bacteria such as *Salmonella enterica* and *Campylobacter lanienae* with ubiquitous bacteria from the cattle feces included *Clostridium*, *Bacteroides, Porpyhyromonas, Ruminococcus, Alistipes, Lachnospiraceae, Prevotella, Lachnospira, Enterococcus, Oscillospira, Cytophage, Anaerotruncus*, and *Acidaminococcus* spp (S. E. Dowd *et al.*, 2008). According to an experimental study supplementation of Probiotics among animals did not imparted significant effects on fecal microbial diversity and load but significantly affected the populations of fermentative bacteria in rumen (*Coprococcus, Bacteroides, Clostridium, Ruminococcus, Dorea and Roseburia*) and increase the beneficial bacterial (*Faecalibacterium prausnitzii*) load. Further, it suppressed the load of pathogens opportunistic in nature like *Cronobacter sakazakii*, *Bacillus cereus* and *Alkaliphilus oremlandii* (Xu *et al.*, 2017)(H. Xu *et al.*, 2017). Various studies have confirmed the usefulness of probiotics, prebiotics and competitive exclusion products to establish stable gastrointestinal flora in animals that improved animal performance and prevented colonization with zoonotic pathogens (Dunkley *et al.*, 2007; Ricke & Pillai, 1999; Vanbelle *et al.*, 1990) that is reflected by microbial profile of experimental cows 604 and 640 in current study that harbored more than 65% and 64% anaerobic OTUs respectively than control and experimental cow 620 which were dominated by aerobic gram negative pathogenic flora (Table 5.3). The yeast can easily survive and remain metabolically active in the gut, exert probiotic effects by interacting with the autochthonous microbial species responsible for feed digestion (F Chaucheyras-Durand *et al.*, 2008). The most prominent effect

of *G. candidum* supplementation as microbial feed additive was increase in anaerobic flora in experimental cows in our study.

The present study results also showed that the control cow was dominated by phylum *Firmicutes* with abundance 93.64 % with dominancy of the genus bacillus represented by *Bacillus szeutansis* and at genus level *by Paenibacillus* Low abundance of anaerobic *Closteridial* species and none of the member of phylum *Bacteriodetes* detected in control cow. But the phylum *Firmicutes* showed good abundances of 35%, 11.88% and 0.63% in experimental cows 604, 640 and 620 respectively. In experimental cows' very high diversity of anaerobic bacterial species belonging to genera *Clostridium, Ruminococcus, Ruminoclostridium*, *Bacteroides, Rekinella Lachnospiraceae* i.*e Lachnoclostriium* were identified. The *Clostridium* (*Cl*) species *Cl. disporicum, Cl. bowmanii, Cl. butyricum* and *Bacteroides sp. Janthinobacterium lividum* found with high abundance as compared to control cow microbiota. *Cl. butyricum* has been reported as a probiotic to stimulate increase resistance of the gut to pathogenic microflora by inducing the secretion of anti-inflammatory cytokines(Gao *et al.*, 2012; Qadis *et al.*, 2014) The *Bacillus* species were found with very low abundance in experimental cows supplemented by *G. candidum* that highlighted the anti-bacillus activity of *Geotrichum candidum* (Mefteh *et al.*, 2017). Phylum *Proteobacteria* appeared dominant in experimental dairy cows with dominant species *Pseudomonas trivalis* and *P. vironii* with significant increase in digestibility, milk yield. Milk lactose, fat%, Protein% and serum glucose and haemoglobin as well as serum butyrate that is supported by earlier findings of study where combination of corn silage and alfalfa silage feeding increased the abundance of *Proteobacteria* that improved the rumen digestion of feedstuffs and milk yields (Indugu *et al.*, 2017).

The Phylum *Actinobacteria* also detected with good abundance sharing two OTUs of *Bifidobacterium longum*, and *Arthrobacter luteolus* in experimental sample (640) while *Bifidobacterium choerinum* in two experimental cows 604 and 640 but not found in dung microbiota of experimental cow 620 and control 605. *B. choerinum* has also been isolated from young ruminant faeces and sewage (Buneňová *et al.*, 2012; Scardovi *et al.*, 1979; Vlková *et al.*, 2010). *Arthrobacter* species is widely present in the soil environments and were found to utilize several lignin derivatives for its energy (Niewerth *et al.*, 2012).

The members of phylum *Bacteriodetes* detected with very promising abundance of 3.77% and 1.07% in GIT of experimental cows 604 and 640 respectively, while negligible concentration 0.07% control and 0.08% in one experimental cow 620. Although in the present trial the *Bacteroidetes* was only present in dung samples of two experimental cows, while control cow exhibited a higher percentage of *Firmicutes* compensating for a lower abundance of *Bacteroidetes*. The species reported are already found in the cow gut however *G. candidum* fed cows were significantly different in the diversity. In this context the modulation in gut microbiome under the influence of yeast *G. candidum* GC01culture supplementation reflected by significant and no significant increase in production parameters including milk yield, milk composition, feed efficiency and physiological parameters (serum biochemistry and blood) have been determined in Phase II of the current study. Earlier one of the species of genus *Geotrichum* i.e *Geotrichum klebahnii* has been reported for probiotic attributes (Syal & Vohra, 2014) and the indigenously isolated *G. candidum* QAUGC01 strain has already been reported to enhance the *Enterococcus faecium* impact on modulating gut microbiome of *Labeo rohita* in mimic aquaculture conditions (Ghori *et al.*, 2018). Due to increased total bacterial count account the increase flow of microbial protein to intestine significantly increased milk protein content at different intervals during 90 days of experiment. Despite the small number of cows studied, the data suggested a key role of gut bacterial community as a determinant of production Efficiency. In the current study the average feed efficiency has been found significantly increased in experimental cows than control while all the cows received the same ration based on dry matter intake according to percentage body weight additionally the experimental diet was supplementation with *G. candidum* yeast culture. One previous study demonstrated the rumen bacterial community composition in dairy cows correlates with feed efficiency (Jewell *et al.*, 2015b). In case of control cows that received high dry matter than experimental cows yielded less milk while the average milk yield of experimental cows was significantly high than control.

In current study a total of three fungal phyla were identified via metagenomic approach including *Ascomycota, Basidiomycota and Neocallimastigomycota.* Phylum *Ascomycota* was most dominating in fungal biodiversity, contributing to >95% of fungal population in all samples followed by *Basidiomycota* and *Neocallimastigomycota*. The population of *Ascomycota* was low in GIT of Exp. cow 604 (96.38%) while high in Exp.640 (98.61%) and Exp.620 (99.36%) cow‘s GIT than control (97.73%) at end of experiment. Members of *Basidiomycota* were low in all the

three experimental cow‘s GIT (Exp. 604, 640, 620:1.85, 1.38, 0.62%) as compared to control (2.26%). Conversely *Neocallimastigomycota* population was high in the entire three experimental cow‘s GIT (Exp. 604, 640, 620:1.76, 0.018, 0.019%) than that in control (0.003%).

The fungal and yeast species (OTUs) diversity was represented by the relative abundance of total 64 species. Metagenomic study revealed 44 fungal species in control cow 605 while 61, 52 and 42 species detected in 604, 640 and 620 respectively at the end day of experiment. *Geotrichum* species were detected both in control and experimental cows‘ GIT but their percent abundances were remarkably high in experimental cows; *Galactomyces geotrichum* abundance was 59.30%, 66.32%, 27.81% in Exp. cows 604, 640 and 620 showing too high abundance even the dung samples of experimental cows were collected at 95th day while the *G. candidum* supplementation was stopped at 90th day. Very less abundance of *Galactomyces geotrichum* in control cow 3.73%, The *G. candidum* was present in high abundance 37.06% in cow 620 than experimental cows 604 and 640 i.e 4.50% and 6.02% while very less abundance of *Geotrichum candidum* was in control cow 0.83%. In the phylum Ascomycota the most prominent and promising affect was on the growth of *Debaryomyces hansenii* as its percentage was remarkably high in the GIT of control cow (82.215%) while it showed controlled and reduced growth in Exp.640, 640 and 620 cows (2.85%, 3.90%, 2.81% respectively). *Debaromyces hansenii* produce proteinaceous toxins & may be dominated in control cow rumen due to competition with other community. The reduce growth of *Debaryomyces hansenii* in experimental cows gut may be due to supplementation of *G. candidum QAUGC01* that compete well there.

Thelebolus globosus, Dipodascus australiensis, Pseudeurotium bakeri, Candida spp. and *Aspergillus spp.* were low in the control (0.04%, 0.02%, 0.004%, 0.03%, 0.01%) and their percentages were found to be higher in experimental cows‘ GIT (Exp. 604, 640, 620: 16.34, 0.40, 0.08; 0.11, 1.59, 0.05; 0.33, 1.05, 0.02; 0.13, 0.18, 0.09; 0.31, 0.92, 0.03% respectively) *Meyerozyma guilliermondii* growth was lower in Exp. 604 (0.005%) while higher in Exp. 640 and 620 (0.44, 1.64%) than in control (0.02%). Percentage of *Fusarium gibberella fujikuroi* was same i.e. 0.03% in the GIT of Exp. 604 and control cow and higher in Exp. 640 and 620 cow (0.04, 3.61%). *Preussia sp.* identified and found in GIT of Exp cow 604 was 0.29% and 0.0089% in GIT of Exp. cow 640.

Among the members of Basidiomycota, *wallemia* spp. were higher in GIT of Exp. 604 and 640 (0.22, 0.11%) while lower in Exp. 620 GIT (0.09%) than in control (0.10%). *Rhodotorula* spp. were higher and *Cryptococcus* spp. were lower in Exp. 604, 640 and 620 (0.61, 0.45, 0.07%; 0.28, 0.47, 0.27% respectively) in comparison with that in control (0.003% and 1.59%).

The members of Phylum *Neocallimastigomycota* were also observed and detected with high % abundance in dung metagenome of Exp. Cows 604 and 640 while not identified in control and Exp. Cow 620 such as *Cellamyces spp*., In the present study the 3 genera of rumen anaerobic fungi i.e *Neocallimastix frontalis, Orpinomyces sp.* and *Cyllamyces sp.* found/observed with relative high abundance of 0.711765 %, 0.364706 and 0.673529 in the GIT of Exp. cow 604 while 0% in control cow no 605 and other two experimental cows.

Fungal diversity was analyzed by heat map analysis at genus level showed different pattern as compared to bacterial diversity. In this analysis the experimental cows 620 and 640 cow metagenome clustered closely, then they clustered with experimental 604 and control 605. It showed that experimental cows fed on *G. candidum* supplemented diet showed higher similarity as compare to control. Common and unique microbiome diversity among experimental and control cows was evaluated by plotting Venn diagrams. Out of total observed fungal species 27 species were common in all experimental and control cows that comprised of 62.8% of total percent microbiome. The 2 fungal species comprising 4.7% were found to be common in all experimental cows 604EE, 640EE and 620EE. 4 species were found to be shared in 604EE and 640EE experimental cows. The unique fungal species 4 (9.3%) were identified as core microbiome of experimental cow 604 while 0 (0%), 0 (0%) and 0 (0%) fungal species were uniquely identified in other two experimental cows 640EE, 620EE and control 605EC cow respectively. Various studies have concluded unknown effects of yeast supplementation on ruminal protozoal and fungal diversity, despite their roles in fiber degradation (A. Belanche *et al.*, 2012; D. O. Krause *et al.*, 2003; S. Lee *et al.*, 2000; Y. Sun *et al.*, 2006; Williams & Withers, 1993). High-fiber diets favor rumen fungal diversity (D. Belanche *et al.*, 2012) supports the current study results as high forage diet was fed to experimental cows that increased the richness of fungal OTUs in experimental cow 604 because the number of fungal OTUs was higher in cow 604 than in control 605 and experimental cow 640 and 620. The Exp. cow 640 and control 605 both of which showed to have same numbers of fungal OTUs i.e.109. Alpha diversity of bacteria and fungi according to Shannon Index was high in experimental cows than control at the end of

experiment. Similarly, the Simpson index was also calculated to see the dominance of bacteria and fungi in experimental and control cows. Active dried yeast treatment increased fungal richness (OTUs) but not overall diversity while significantly affected the abundance of numerous fungal genera as seen in the high-fiber diet: *Lewia, Neocallimastix*, and *Phoma* were increased, while *Alternaria, Candida Orpinomyces*, and *Piromyces* spp. were decreased (Ishaq *et al.*, 2017). According to Simpson index the bacterial as well as the yeast dominance was high in experimental cows than control. Alpha diversity of both bacteria and fungi in terms of richness and evenness was higher in all the three experimental cows than in control according to Shannon and Simpson index. Yeast, a natural feed additive, has the potential to enhance microbial growth and subsequent fiber degradation and increased the volatile fatty acids level during fermentation processes leading to yield a greater amount of microbial protein to the duodenum. Single-celled fungi yeast has been considered to efficiently enhance fiber digestibility and lower fecal output with improved digestion of organic matter, which improves animal production efficiency (Elghandour *et al.*, 2019).

5.5 Conclusion

By feeding *Geotrichum candidum* QAUGC01 survived and established well in the dairy cattle gut. During the experimental period all the animals remained healthy which showed that it didn‘t affect animal‘s health. Enhanced the anaerobic gut flora in experimental cows with good diversity of anaerobic bacterial species belonging to genera *Clostridium, Ruminococcus, Ruminiclostridium*, *Bacteroides, Rikenella, Lachnospiraceae* i.*e. Lachnoclostridium and Clostridium* (*Cl*) species i.e. *Cl. Disporicum, Cl. Bowmanii, Cl. Butyricum* that promoted the digestibility and absorption of nutrients by increasing the level of serum butyrate. Reduced pathogenic load in gut microbiota of experimental cows also showed probiotic attributes to a healthy level. Dietary supplementation of *G. candidum* QAUGC01 to dairy cows improved their gut microbiology which can further boost up their metabolic activities and thus their milk production and feed efficiency. It improved the milk quality by improving the milk fat, protein, lactose and solid non-fat. It also increased milk yield and prevented the loss of body weight of cows by improving the physiology by increased RBCs, Hemoglobin, and blood glucose level. *G. candidum* showed prominent effect on serum lipid profile viz significantly reduced total cholesterol and LDL as well as reduce the milk cholesterol contents thus improved the quality of milk. The quality enhancement high light the probiotic attributes of locally isolated *G. candidum*

QAUGC01. In present study results displayed the presence of environment friendly bacteria such as *Paludibacter spp., Stenotrophomonas rhizophila*, *Mucilaginibacter oryzae, Mucilaginibacter herbaticus, Tissierella spp., Carnobacterium maltaromaticum, Turicibacter spp., Lactonifactor longoviformis, Janthinobacterium lividum* and *lactobacillus coryniformis* identified in good abundance in dung samples of experimental cows. Then it could be inferred from current study that *G. candidum* QAUGC01would be a potential microbial supplement for enhanced growth and health of dairy cattle.

Chapter 6

General Discussion

(Phase I, II and III)

6.1. General Discussion (Phase I, II and III)

Cross breeding of indigenous cattle with exotic such as Holstein is underway in Pakistan for the last four decades. The crossbred dairy cows in Pakistan have been generally produced as a result of crossing exotic *Bos taurus* breeds with local less productive *Bos indicus* breeds for purpose of more milk production in Pakistan. In Pakistan the local breed like Sahiwal, Red Sindhi, Tharparker and Hariana were crossed with exotic semen of Holstein and Jersey because of best performance of Holstein Friesian and Jersey crossbreds (Hassan & Khan, 2013). In tropics as well as in developing countries the Cattle production suffers a lot as compared to temperate and developed countries. In the tropics, ruminants are grazed or restricted to low-quality forages, crop residues and agro-industrial by-products, which adversely affect the cattle productivity. To overcome these problems during last two decades, the researchers have explored several methods to enhance the functions of rumen microbiota for efficient digestion and fermentation processes, as well as to enhance bioavailability and utilization of nutrients through supplementation of feed by probiotics (Arowolo & He, 2018). Imbalanced feeding rendered dairy animals with less productivity. Yeast based probiotics can help in mitigating adversative effects of imbalanced feed. Many of commercial yeast-based probiotics are available in the market, but unsuitable for our local breeds regarding cost and impact on physiology as well as productivity. Present study was designed to check the impact of *G. candidum* QAUGC01 with potential probiotics attributes to improve feed efficiency (FE), milk yield, milk composition and health of mid-lactating dairy cows of Sahiwal-Friesian cross fed a high forage diet. *G. candidum* QAUGC01 was screened through different probiotic attributes in Phase I. The health status of animals used in this experiment was monitored by analyzing serum biochemistry and other important hematological parameters. Based on physiological, hematological and production parameters, clustering was done and selective four cows (one control and three experimental) gut microbiota was analyzed by using 16SrDNA and ITS (Internal Transcribed spacer) region sequencing.

Strains isolated indigenously sourced from Dahi, silage and cow dung, niches overflown with microbes. In phase I, previously isolated strains comprised of lactic acid bacteria, gram positive bacteria and yeasts (*S. cerevisiae*, *G. candidum*) in addition to *E. faecium* were characterized for probiotic potential. Characterization was based on amylolytic, proteolytic and cellulolytic activity in addition to mimic gut survival, cell hydrophobicity, anti-pathogenic activity and cholesterol assimilation yielded *G. candidum* QAUGC01 as potential feed additive to be used in

dairy cattle. It was used in feed of experimental cows to investigate its survival in gastrointestinal tract (GIT) for 90 days. Based on high hydrophobicity % age of *G. candidum* QAUGC01 *in vitro* favored its adhesion with epithelial cells of cow gut. The metagenomic study of current experiment revealed its high abundance and concentration in dung samples of experimental cows. The qualitative effect was the desirable *in vivo* reduction of serum and milk cholesterol content revealed its cholesterol assimilation ability as determined *in vitro* in Phase I. The *G. candidum* strains have been known for assimilating cholesterol *in vitro* could be attributed to hypo cholesterimic effect of *G. candidum*. The serum LDL was also decreased in experimental cows than control cows. Cholesterol lowering ability in the serum by *G. candidum* increases its importance to be used as probiotic (Syal & Vohra, 2014). The strains of *Geotrichum* and *Galactomyces* species have been reported earlier with high adhesive ability and assimilate cholesterol from YPD-CHOL broth containing bile salt and cholesterol after 72 h growth at 37 °C (Chen *et al.*, 2010). *Bifidobacterium choerinum* and *Bifidobacterium longum* identified with good abundance in experimental cow gut, showed negative correlation (-0.770) and (-0.766) respectively with serum total cholesterol. The serum and milk cholesterol of experimental dairy cows reduced for 90 days highlight the cholesterol assimilation effect of *G. candidum* (QAUGC01) as well as *Bifidobacterial* species with increased abundance in response to *G. candidum* (QAUGC01) supplementation. Earlier study described the reduction of serum total cholesterol and LDL by *Bifidobacterium longum* while to some extent increasing serum HDL (Parvez *et al.*, 2006). The *Galactomyces geotrichum* created positive correlation with abundance of *Bifidobacterium choerinum* (0.632) and *Bifidobacterium longum* (0.629) as earlier reported with good cholesterol assimilation property (Grill *et al.*, 2000). *Saccharomyces cerevisiae* administration to rats reduced serum total cholesterol, low-density lipoprotein cholesterol, and triglyceride (Saikia *et al.*, 2018). Earlier study reported *Saccharomyces cerevesiae* reduced the serum cholesterol significantly in 10 experimental cows in comparison with six control dairy cows (Bakr *et al.*, 2015)**.** This property of G. candidum could be attributed to lipolytic potential as reported earlier (Muhammad, Bokhari*, et al.*, 2019) however detail mechanism is still unknown. The milk yield in experimental cows increased with desirable increase in protein, fat and lactose content. In current study milk production decreased both in control and experimental cows due to stress of hot summer but this stress was reduced in experimental cows fed yeast supplemented feed resulted in less decrease in milk yield in experimental cows than control as

evidenced by 1.45 kg more milk produced by experimental cows as compared to control cows at the end day of experiment. These results were in agreement to the studies reporting 1 to 2 kg/d increase in milk production by yeast *S. cerevisiae* (Bruno *et al.*, 2009; Dailidavičienė *et al.*, 2018; Robinson & Garrett, 1999; Yalcin *et al.*, 2011). Previous studies have highlighted the positive effects of probiotic supplementation to neutralize thermal impact in dairy cows and numerically increased (1.26 kg, $P = 0.11$) milk yield in cows fed Calsporin during thermo neutral but was reduced under heat stress (-2.67 kg, $P < 0.01$) accompanied by decrease ($P = 0.05$) in milk protein content (Amaral-Phillips, 2019; Hall, 2014). In mid lactating dairy cows the high dose of *S. cerevisiae* supplementation in high forage diet increased milk yield and significantly increased concentrations of ruminal total volatile fatty acids, acetate, propionate, and butyrate also enhanced the synthesis of microbial protein in the rumen (Zhu *et al.*, 2017). The increase in milk protein, fat, lactose and solid non fats content resulted in significant increase in milk yield ($p<0.05$) and Feed Efficiency of experimental cows than control of present study.

Milk yield affected by many factors including heat stress because of high humidity in tropical summer season could lead to decrease in milk production. The thermoneutral zone (TNZ) of dairy animals ranges from 16°C to 25°C, and they maintain a body temperature of 38.4-39.1°C (Leonard, 1986). However, air temperatures above 25-37°C in a tropical climate, increase the heat gain beyond that lost from the body and induces heat stress (Vale, 2007). The HS increases the respiration rate, rectal temperature and heart rate. It directly affect feed intake thereby, reduces growth rate, milk yield, reproductive performance, and even death in extreme cases (Das *et al.*, 2016). In contrast, some studies found no increase in milk production in response to yeast *(Saccharomyces cerevisiae)* supplementation (Bagheri *et al.*, 2009; Dann *et al.*, 2000; Schingoethe *et al.*, 2004; Soder & Holden, 1999). Supplementation of DFM (direct fed microbials) such as combination of *Lactobacillus plantarum, Enterococcus faecium,* and *S. cerevisiae* and *Bacillus licheniformis* may enhance milk yield (Nocek & Kautz, 2006; Nocek *et al.*, 2002; Qiao *et al.*, 2010). The active dry yeast supplemented dairy ration significantly improved production parameters and immunity of growth-retarded yaks by stimulating the *Prevotella, Butyrivibrio and Fibrobacter* groups in rumen beneficial bacterial populations (Hu *et al.*, 2019).

The antagonism potential of the probiotics inhibit and minimize the virulence of enteric pathogens mostly involved in foodborne infections. The same antagonistic effect of *G. candidum* QAUGC01 was observed *in vitro* as well as *in vivo* in current study. The decrease in number of *Enterobacteriaceae* in experimental cows indicated a decrease in pathogenic load in the experimental cattle gut, as depicted by the following ta. This comparison before supplementation and after supplementation of *G. candidum* QAUGC01 showed that its use as microbial based feed additive also helps in decreasing the pathogenic load in the cattle gut. The results of present study indicated that *G. candidum* QAUGC01yeast culture may have a role in colonization resistance and inhibit growth of pathogens as speculated by previous studies (Dieuleveux *et al.*, 1998; Muhammad Imran *et al.*, 2013; M Imran *et al.*, 2010; Mefteh *et al.*, 2017; Saima Naz *et al.*, 2013; Samuel *et al.*, 2011). . Yeast and fungal count increased in both experimental (P>0.57) and in control cows (P > 0.88). In all the 90th day samples *G. candidum* QAUGC01 was found to be present which was confirmed by microscopy. The metagenomic study also revealed the high abundance of *Galactomyces candidum*, *Galactomyces geotrichum* in experimental cows and very less concentration identified in control samples. The presence of this strain shows that after feeding it established and survived successfully in the gut of experimental cows supported the increase of total bacterial count in gut of experimental cows. The strains of *Geotrichum* and *Galactomyces* species have been previously reported showing highest adhesive ability in broth containing bile salt and cholesterol for 72 h growth at 37°C (Chen *et al.*, 2010). The *Galactomyces candidum* and *Galactomyces geotrichum* sequences derived from dung metagenome in high abundance in association with *Galactomyces* species in experimental cows. While control samples were dominated by sequences of *Debaryomyces hansenii*. The increase in microbial concentration in cattle gut may be attributed to the yeast cells by providing different growth factors e.g. oligosaccharides, vitamins B, amino acids and organic acids to stimulate growth of rumen microbes (Retta, 2016). The *G. candidum* was characterized for probiotic attributes showed the good survival capacity in harsh conditions of high acidity and high bile concentrations *in vivo*.

In addition to these basic probiotic characteristics, *G. candidum* QAUGC01 is also able to produce a variety of extracellular enzymes including protease, lipase, cellulase (Muhammad *et al.*, 2017; Muhammad, Bokhari*, et al.*, 2019). *G. candidum* QAUGC01 has shown positive lipase, protease and cellulase activity *in* vitro in phase I of current study. These enzymes have

been used in animal nutrition to assist in the digestion of feed and improve nutrient absorption. In current study following the *G. candidum* QAUGC01 supplementation in experimental cows' diet, the digestibility of dry matter including neutral detergent fiber, acid detergent fiber, crude fiber and crude protein significantly increased than control cows.

G. candidum can utilize available lactate in-vitro and thus might exerted the same effect of degrading lactic acid in cattle gut environment to prevent its accumulation in gut and thus increase the gut pH and made it stable for good growth of cellulolytic flora in experimental cows gut in present study. Previously reported the presence of lactate oxidase enzymes in *G. candidum* which degrades the lactate in pyruvate and hydrogen peroxide in presence of oxygen has been reported (Sztajer *et al.*, 1996). NAD dependent lactate dehydrogenase was also reported in *G. candidum* (Hang & Woodams, 1992). *G. candidum* and other yeasts degrade lactate and liberate ammonia; these two phenomena contribute to pH increase, which promotes the implantation of acid sensitive microorganisms (Choisy *et al.*, 1997; Lenoir, 1984). The stable pH favored the growth of cellulolytic flora of cattle gut with increase production of organic acids such as Acetic acid, propionate and butyrate as evidenced by significant increase in serum butyrate, serum glucose, milk lactose and milk fat content in current study because the propionate is the only precursor of glucose availability in serum of ruminants, and it is the principal gluconeogenic substrate (Beitz, 2004; P. Van Soest, 1994). Due to increased total bacterial count account for increase flow of microbial protein to intestine significantly increased milk protein content at different intervals during 90 days in milk of experimental cows. Despite the small number of cows studied, the data suggested a key role of gut bacterial community as a determinant of production Efficiency. In the current study the average feed efficiency has been found significantly increased in experimental cows than control while all the cows received the same ration based on dry matter intake according to percentage body weight while the diet of experimental cows supplemented with *G. candidum* yeast culture. One previous study demonstrated the rumen bacterial community composition in dairy cows correlates with feed efficiency (Jewell *et al.*, 2015b). In case of control cows that received high dry matter than experimental cows yielded less milk while the average milk yield of experimental cows was significantly high than control resulted in significant increase in feed efficiency of experimental cows under the influence of hot summer.

The rumen microbial community affect Feed Efficiency in beef production systems (Carberry *et al.*, 2012; Hernandez-Sanabria *et al.*, 2012; P. R. Myer *et al.*, 2016; P. R. Myer, Wells*, et al.*, 2015; Zhou & Hernandez-Sanabria, 2010). Myer et al., (2017) identified the relationship between the microbiomes within segments of the cattle gastrointestinal tract and feed efficiency (P. Myer *et al.*, 2017). Similar but very limited studies have determined the relationship between microbial community composition and FE in dairy cattle. Jami et al. (2014) revealed differences in Bacterial Community Composition (BCC) among dairy cows with different feed efficiency (Jami *et al.*, 2014) while other demonstrated differences in BCC in both ruminal solids and liquids in cows that differed in dry matter intake at equivalent levels of energy corrected milk (ECM) production within 3 discrete ranges of days in milk DIM (68–72, 151–157, and 251–257 d) over 2 lactations (Jewell *et al.*, 2015b). More recently, Shabat et al. (2016) explored differences in composition of bacterial and archaeal community between high and low efficient cows during mid-lactation (50–150 DIM). Recently analyzed the milk production efficiency (MPE) within the 2 efficiency groups revealed a transient increase in MPE for the Low Efficient cows upon exchange with ruminal contents from highly efficient donor cow and demonstrated that in terms of microbial community composition, all the previous studies were limited to determining correlations between FE and the relative abundance of specific taxa. Moreover, specific OTU whose abundance is related to feed efficiency across multiple studies have not been identified (Weimer *et al.*, 2017).

The ratio of *Firmicutes* to *Bacteroidetes* has effect on energy harvesting and body fat in humans and mice (Ley *et al.*, 2006; Turnbaugh *et al.*, 2006). Previous study demonstrated the correlation between cattle physiological parameters and a change in this ratio. This ratio was found to be strongly correlated with daily milk-fat yield and cleared, where a decreased amount of *Bacteroidetes* in the microbiota was correlated with increased fat in the blood and tissue (Turnbaugh *et al.*, 2006). The connection was found by strong correlation between the ratio of the phyla *Firmicutes* to *Bacteroidetes* and cow‘s milk-fat yield and the same correlation remained evident at the genus level. This suggests that the Gut bacterial community has a role in shaping host physiological parameters (Jami *et al.*, 2014). Similarly the current study results of production parameters and abundance of different groups of bacteria of dung metagenome were correlated by applying Pearson correlation and clearly depicted that the *clostridium sp.* showed significant positive correlation with cow‘s Milk production (0.952), Feed efficiency (0.624),

milk fat% (0.855), milk triglycerides (1.000), (0.959) and also showed positive correlation with *Galactomyces geotrichum* (0.776), total fungus count (0.977, 0.998) in dung samples but showed negative correlation (-0.476), (-0.359) with serum cholesterol level before and after supplementation respectively. The clostridium species also showed the significant positive correlation with rumen anaerobic fungi *Cyllamyces* sp. *Orpinomyces* sp. and *Neocallimastix frontalis* of phylum *Neocallimastigomycota* (≥ 0.1% abundance) identified only in gut of experimental cows, which are efficient fiber degrading fungi inside rumen of dairy cows (Sehgal *et al.*, 2008; Thareja *et al.*, 2006). Previously described that a complex set of interactions between fibrolytic microbes and the other actors of feed digestion does impact fiber degradation with significant homology between the xylanases of rumen anaerobic fungi and bacterial xylanases demonstrated by Sequence analysis, which implied the horizontal transfer of genes between bacteria and anaerobic fungi in the rumen (Xue *et al.*, 1992).

The *Bacteroides spp.* of current experiment have been found to be positively correlated with milk production (0.953) and with abundance of *Galactomyces geotrichum* (0.935) that found dominant in dung metagenome of experimental dairy cows while negatively correlated with *Debaryomyces hansenii* (-0.565) identified dominant in control. The *Bacteroides spp.* were found to be significantly positively correlated with Total Aerobic count (**0.955)** and *Lactobacillus count* (**0.988**) of dung samples of experimental cows which were found increased after yeast supplementation while the *bacteroides spp.* were not detected in control‘s dung metagenome.

Cow dung is a cheap and easily available bioresource on earth. Cow dung has been used as fuel, mosquito repellent and as cleansing agent are already known in India. It harbors a diverse group of microorganisms that may be beneficial to humans due to their ability to produce a range of metabolites. Along with the production of novel chemicals, many cow dung microorganisms have shown natural ability to increase soil fertility through phosphate solubilization (Gupta *et al.*, 2016). In present study results the dung metagenome displayed the presence of environment friendly bacteria such as *Paludibacter spp., Stenotrophomonas rhizophila*, *Mucilaginibacter oryzae, Mucilaginibacter herbaticus, Tissierella spp., Carnobacterium maltaromaticum, Turicibacter spp., Lactonifactor longoviformis, Janthinobacterium lividum* and *lactobacillus coryniformis* identified in good abundance in dung samples of experimental cows. *S. rhizophila*

has ability to both promote plant growth and protect roots against biotic and a-biotic stresses. It produce glucosylglycerol (GG), phyto hormones and osmoprotectants, play a key role in stress protection in high saline land. But Cattle manure harbors microbial constituents that make it a potential source of pollution in the environment and infections in humans. Physical, chemical and biological methods to reduce pathogen population in manure do exist. In addition the safe solution should be found out to further reduce bacterial pathogens excretion in dung to a significant level to prevent microbial contamination of the environment, animals and humans (Manyi-Loh *et al.*, 2016). Spiehs and Goyal recommended that to reduce the pathogens in the animals is the best management practices to reduce pathogens in livestock wastes (Spiehs & Goyal, 2007). Therefore, it will lower the risk of pathogen transfer from manure during land application (Hutchison *et al.*, 2005).

Various studies have concluded unknown effects of yeast supplementation on ruminal protozoal and fungal diversity, despite their roles in fiber degradation (A. Belanche *et al.*, 2012; D. O. Krause *et al.*, 2003; S. Lee *et al.*, 2000; Y. Sun *et al.*, 2006; Williams & Withers, 1993). High-fiber diets favors rumen fungal diversity (D. Belanche *et al.*, 2012) supports the current study results as high forage diet was fed to experimental cows that increased the richness of fungal OTUs in experimental cow 604 because the number of fungal OTUs was higher in cow 604 than in control 605 and experimental cow 640 and 620. The Exp. cow 640 and control 605 both have same numbers of fungal OTUs i.e.109. Alpha diversity of bacteria and fungi according to Shannon Index was high in experimental cows than control at the end of experiment. Similarly, the Simpson index was also calculated to see the dominance of bacteria and fungi in experimental and control cows. Active dried yeast treatment increased fungal richness (OTUs) but not overall diversity while significantly affected the abundance of numerous fungal genera as seen in the high-fiber diet including *Lewia, Neocallimastix*, and *Phoma* were increased, while *Alternaria, Candida Orpinomyces*, and *Piromyces* spp. were decreased (Ishaq *et al.*, 2017). According to Simpson index the bacterial as well as the yeast dominance was high in experimental cows than control. Alpha diversity of both bacteria and fungi in terms of richness and evenness was higher in all the three experimental cows than in control according to Shannon and Simpson index. Yeast, a natural feed additive, has the potential to enhance microbial growth and subsequent fiber degradation and increased the volatile fatty acids level during fermentation processes leading to yield a greater amount of microbial protein to the duodenum. Single-celled

fungi and yeast has been considered to efficiently enhance fiber digestibility and lower fecal output with improved digestion of organic matter, which improves animal production efficiency (Elghandour *et al.*, 2019). Ruminants and gut microbes have a symbiotic relationship results in microbial interactions for systemic digestion of plant fibers and subsequently ferment the monomers into end products, used as energy sources by the host ruminants (Qumar *et al.*, 2016; Russell & Rychlik, 2001).

Based on all the analyzed parameters it was found that our locally isolated *G. candidum* strain QAUGC01 has good probiotic potential in improving blood and serum parameters, nutrient digestibility, milk yield and milk composition in comparison with control cows. RBC‘s, Hemoglobin and PCV level increased in experimental cows while WBC count decreased both in experimental and control cows. Decrease in WBC was more in control than experimental cows. Neutrophil count increased in both groups while this increase was high in control group. All the identified values of blood parameters were in normal range which depicted healthy role of *G. candidum* QAUGC01 on blood parameters. Serum biochemistry has also shown increase of glucose and HDL in experimental cows and decrease of cholesterol, LDL and TG in blood serum. The milk cholesterol was also decreased in experimental cows milk than control cows. The Serum samples were processed by using GC-FID to determine the quantity of butyrate in cow‘s serum. In experimental cows serum butyrate concentration was higher at the end of experiment as compared to zero day. While opposite trend was observed in control cows where butyrate concentration was low in their serum at the end of experiment. Feed Efficiency, dry matter intake and milk yield was measured, and it was found that FE was significantly high in experimental cows, produced more milk with significant (p=0.042) difference as compared to the average milk production of control cows. The control cows consumed more average dry mater (DM) than experimental cows but the average feed efficiency of experimental cows was significantly (p=0.032) high than control cows. This may be due to significant increase of serum glucose, milk lactose content as well as desirable increase of milk fat%, milk protein%, milk solids and solid non fats in experimental cows than control cows. These all effects attributes to supplementation of locally isolated yeast *G. candidum* QAUGC01 that has been already reported for its best enzymatic ability resulted in increased apparent digestibility of dry matter (DM), CP, CF, NDF and ADF in experimental cows. Thus, QAUGC01 has significantly improved the milk

yield and milk composition in experimental cows as compared to control cows, fed on normal feed.

Conclusion

After feeding *Geotrichum candidum* QAUGC01 supplemented feed the yeast survived and established well in the dairy cattle gut. During the experimental period all the animals remained healthy which showed that it didn‘t affect animal‘s health. Enhanced the anaerobic gut flora in experimental cows with good diversity of anaerobic bacterial species belonging to genera *Clostridium, Ruminococcus, Ruminiclostridium*, *Bacteroides, Rikenella, Lachnospiraceae* i.*e. Lachnoclostridium and Clostridium* (*Cl*) species i.e. *Cl. Disporicum, Cl. Bowmanii, Cl. Butyricum* that promoted the digestibility and absorption of nutrients by increasing the level of serum butyrate. Reduced pathogenic load in gut microbiota of experimental cows also showed probiotic attributes to a healthy level. Dietary supplementation of *G. candidum* QAUGC01 to dairy cows improved their gut microbiology which can further boost up their metabolic activities and thus their milk production and feed efficiency. It improved the milk quality by improving the milk fat, protein, lactose and solid non-fat. It also increased milk yield and prevented the loss of body weight of cows by improving the physiology by increased RBCs, Hemoglobin, and blood glucose level. *G. candidum* showed prominent effect on serum lipid profile viz significantly reduced total cholesterol and LDL as well as reduce the milk cholesterol contents thus improved the quality of milk. The quality enhancement high light the probiotic attributes of locally isolated *G. candidum* QAUGC01. In present study results displayed the presence of environment friendly bacteria such as *Paludibacter spp., Stenotrophomonas rhizophila*, *Mucilaginibacter oryzae, Mucilaginibacter herbaticus, Tissierella spp., Carnobacterium maltaromaticum, Turicibacter spp., Lactonifactor longoviformis, Janthinobacterium lividum* and *lactobacillus coryniformis* identified in good abundance in dung samples of experimental cows. Then it could be inferred from current study that *G. candidum* QAUGC01would be a potential microbial supplement for enhanced growth and health of dairy cattle.

Future Prospects

In current study probiotic strain has been isolated to apply as microbial feed supplement in dairy cattle. *G. candidum* has been selected and applied in feed of dairy cattle. *G. candidum* has improved the cattle health and significantly alter the gut microbiota. Further studies can be conducted to study the detailed mechanisms by which this probiotic yeast strain *G. candidum (*QAUGC01) affect other microbial groups in the gut are required to be explored. Studies can be conducted to study the application and efficacy of this probiotic strain in other animals. Studies are required to explore that how the changes in gut microbiology affect different metabolic processes. Application of (QAUGC01) probiotic strain to treat rumen acidosis in high grain diet as it can degrade Lactate or to study its impact on lactate utilizing bacteria such as *Megasphaera elsdenii and Selenomonas ruminantium.* Whole metagenomic studies can give details on active metabolic pathway in composition with metabolomics studies. Further studies are needed to optimize the growth parameters of *G. candidum (*QAUGC01) for commercialization.

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Appendixes

Appendix. 1. Statistical comparison of alteration in milk protein (%) of experimental and control cows during experimental period of 90 days

Time Period	Cows	Mean Milk Protein %	P- value
0-Day	Control	3.3500	0.8540
	Experimental	3.2856	
15th Day	Control	3.0833	0.1866
	Experimental	3.6267	
30th Day	Control	2.6000	0.0111
	Experimental	3.3989	
45th Day	Control	2.8633	0.0227
	Experimental	3.0800	
60th Day	Control	2.8933	0.0365
	Experimental	3.0500	
75th Day	Control	2.8933	0.0491
	Experimental	3.0544	
90th Day	Control	2.8867	0.1802
	Experimental	3.1678	

Appendix. 2. Statistical comparison of alteration in milk Fat (%) of experimental and control cows during experimental period of 90 days

Time Period	Cows	Mean Milk Fat (%)	P- value (<0.05)
0-Day	Control	4.6600	0.3373647
	Experimental	4.1689	
15th Day	Control	3.9633	0.3807292
	Experimental	4.8344	
30th Day	Control	5.6633	0.4811342
	Experimental	5.1533	
45th Day	Control	3.6533	0.036131
	Experimental	4.6778	
60th Day	Control	3.6767	0.0832324
	Experimental	4.7411	
75th Day	Control	3.7600	0.0785751
	Experimental	4.6389	
90th Day	Control	4.0900	0.3042153
	Experimental	4.6567	

Appendix. 3. Statistical comparison of alteration in milk Lactose (%) of experimental and control cows during experimental period of 90 days

Time Period	Cows	Mean Milk Lactose %	P- value (<0.05)
0-Day	Control	4.2100	0.8670
	Experimental	4.1244	
15th Day	Control	3.8400	0.2012
	Experimental	4.6011	
30th Day	Control	3.1067	0.0126
	Experimental	4.2678	
45th Day	Control	4.3267	0.0329
	Experimental	4.5478	
60th Day	Control	4.3367	0.0321
	Experimental	4.5822	
75th Day	Control	4.3467	0.0311
	Experimental	4.6167	
90th Day	Control	4.3233	0.0080
	Experimental	4.6144	

Appendix. 4. Statistical comparison of alteration in milk solid Nonfat (%) of experimental and control cows during experimental period of 90 days

Time Period	Cows	Mean Milk SNF %	P- value (<0.05)
0-Day	**Control**	8.9700	0.8626
	Experimental	8.8044	
15th Day	**Control**	8.2700	0.1971
	Experimental	9.7067	
30th Day	**Control**	6.9167	0.0122
	Experimental	9.0878	
45th Day	**Control**	7.8267	0.0121
	Experimental	8.3111	
60th Day	**Control**	7.8867	0.0297
	Experimental	8.3378	
75th Day	**Control**	7.9533	0.0444
	Experimental	8.3567	
90th Day	**Control**	7.8667	0.0162
	Experimental	8.3633	