EFFECT OF INDIGENOUSLY ISOLATED *SACCHAROMYCES CEREVISIAE* PROBIOTICS ON MILK PRODUCTION, NUTRIENT DIGESTIBILITY, BLOOD CHEMISTRY AND FECAL MICROBIOTA IN LACTATING DAIRY COWS

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ABSTRACT

Inadequate and unbalanced diet is considered a major constraint for livestock promotion in developing countries which leads to disturbance in ruminal gut microbial flora. Ruminal gut microbes play a vital role in overall health performance of dairy animals, and conversely, imbalance of the microbiota may lead to metabolic diseases. Probiotics have the capability to manipulate the balance/function of the gut microbes resultantly gives a beneficial effect to the host animal. The study aimed to evaluate the effect of commercial versus indigenously isolated strains of *Saccharomyces cerevisiae* as probiotics on nutrient digestibility, blood chemistry, fecal microbiota, and milk production and its milk composition of lactating dairy cattle. Two strains of *S. cerevisiae* (SCQAU03 and SCQAU05) were isolated from cattle dung and fermented food respectively. In the preliminary screening QAU03 shown comparatively higher enzymatic and probiotic potential under mimic rumen gastric conditions. Therefore, was selected for further supplementation in cattle feed. Nine lactating dairy cows were randomly divided into three equal groups. In Group I (served as control group; CON), lactating cows were fed 3 kg concentrate feed, 8 kg silage and 20 kg fodder as control diet. In group II, lactating cows were fed control diet plus commercially (COM) available yeast, while in group III, lactating cows were fed control diet plus laboratory (LAB) produced yeast, for 60 days. Results revealed that neutral detergent fiber and acid detergent fiber digestibility were significantly ($p<0.05$) better in LAB fed group (group III) as compared to other groups. LAB fed cows produced significantly ($P<0.05$) more milk with higher fat contents. The outcomes of the ruminal gut microflora showed that the average, *Lactococcus* species (CFU/g) counts were increased ($p<0.05$) while *Enterococcus* and coliform ssp (CFU/g) counts were ($p<0.05$) decreased in LAB yeast fed groups than other groups which leads to improved GIT microbial balance. It can be concluded that indigenous isolated strain has ability to modulate the GI microbial balance resultantly give an enhancing effect on milk production and milk fat contents in lactating cows.

Keywords: dairy cattle, digestibility, probiotics, milk composition; fecal flora.

INTRODUCTION

Probiotics supplements are natural products containing live microbiota that beneficially affect the health and production performance of the host by improving gut microbial flora (Klaenhammer *et al.,* 2012). Therefore, presently livestock industry is showing great interest in the use of probiotics for improving growth and production performance of the dairy animal. This ultimately helps in combating the food security challenge (Puniya *et al.,* 2015). In Ruminants, a major portion of the energy recovery from dietary carbohydrates that cannot be digested by the host has been attributed to the function of the rumen microbial flora; Moreover, this digestion process also depends on the structure and numbers of the GIT microbiota. Probiotics have a beneficial effect on the GIT health by stimulating the development of a healthy microbiota, and preventing enteric pathogens from colonizing the intestine, increasing digestive performance (Uyeno *et al.,* 2015).Yeast culture as probiotic may improve growth rate and health status, increases feed efficiency of poor quality forages and high grain diets (Arambel and Kent1990) by increasing the desirable bacterial population in the rumen and stabilizes the rumen pH and improve GIT health (Shehu *et al.,* 2014; Uyeno *et al.,* 2015). Yeast produce many important fermentation metabolites like protein, carbohydrates, high potency vitamins, different types of important minerals and enzymes (Szucs *et al.,* 2013) that make it useful and highly nutritive feed supplement for ruminants (Yalcın *et al.,* 2011). These fermentation metabolites may have a number of positive effects on the rumen including stimulation of the function of desirable bacterial species, increased pH, increase the number of cellulolytic bacteria and alter the volatile fatty acid concentration (Dolezal *et al.,* 2012). It promotes fermentation by secreting various digesting enzymes within the ruminant stomach and

stabilizes the flora in the stomach and intestines by supplying the nutrients which are necessary for their growth. Earlier scientists reported that yeast culture has significant effects on daily feed intake, feed conversion ratio and growth and nutrient digestibility (Lascano *et al.,* 2012; Ayad *et al.,* 2013; Agazzi *et al.,* 2014). Production and growth efficiency of the dairy animals have been improved by probiotic yeast supplementation (Sabbia *et al.,* 2012; Shakira *et al.,* 2015). The addition of yeast culture has many positive effects in the absorption of some minerals that the metabolic health of animals (Cole *et al.,* 1992; Doleza *et al.,* 2012). The response of animals on the yeast supplementation is highly dependent upon yeast strain, stage of lactation, age, diet, breed and the geographical location of the animal. Many imported (Western or European origin) yeast cultures based products are available in the local market, but their use as dietary supplement in local breeds, particularly under tropical environment may not be so effective and economical, therefore, there is a strong need for studies to explore the new ingenious based probiotic strains for local use. This study planned to isolate and compare ingenious probiotic candidate with commercial probiotics on fecal microbiota, blood chemistry, nutrients digestibility, milk production and its composition in lactating dairy cattle.

MATERIAL AND METHODS:

Selection and probiotic characteristics of *S. cerevisiae*: Two strains of *S. cerevisiae* previously isolated from fermented food and dung, and were identified morphologically and biochemically (Barnett *et al.,*1985).

Enzymatic potential of *S. cerevisiae*: For determination of amylolytic activity, bone-gram nutrient broth (NB), 2g agar, 1g starch was used to prepare the amylase media plates. These plates were inoculated by isolates and incubated for 48 hrs. After incubation, iodine crystals were sprinkled over the plates. Formation of luminous zones around the inoculation point indicated positive result and no zone appeared for negative results. The media prepared for cellulolytic activity by dissolving 0.9g NB, 2g agar, 1g carboxymethyl cellulose (CMC) in 100ml distilled water. The plates were treated same by means of point inoculation. After the incubation period, plates were stained firstly with Congo red dye and stained with NaCl. The presence of clear zone around the inoculated colony is an indication of the positive result and absence of this shows negativity effect. 1% casein agar media is used for proteolytic activity. Point inoculation was performed on these plates and set on incubation for 48 hrs. After incubation, the plates were immersed in 1% glacial acetic acid. Bright zone formation brings out positive result and no zone for the negative result.

Survival of the *S. cerevisiae* in mimic gut condition: Strains were inoculated in sterilized tryptic soy broth (TSB) and then kept in a shaker incubator at 37˚C, 150 rpm for 48 hrs. Stock solutions (SS) of bile salts (1.5g/l) and lysozyme (100µg/ml) were prepared. After the incubation period, 750µl from the SS of bile salt and 500µl from the SS of lysozyme were added in the 50ml cultured TSB flask. The pH of the solution was adjusted at 3. TSB media without the addition of bile salt and lysozyme was set out as a control medium. After 30, 60 and 90-minute interval, samples were taken out and spread out on tryptic soy agar (TSA) plates. These plates were incubated at 37˚C for 48 hrs. Colony formation unit (CFU) was calculated by the formula:

CFU/ml = no. of colonies x dilution factor

Tolerance rate = CFU/ml of bile media÷ CFU/ml of control media

Cholesterol lowering ability of the *S. cerevisiae:* Strains were inoculated in TSB and set on incubation at their appropriate conditions. After incubation, about 0.1ml of each sample 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 $FeCl_3$-acetic acid was taken as a blank and then 3ml of H_2SO_4 was added in these and mixed well. These were then left for 30 minutes and optical density (OD) was taken at 560 nm. Percentage of the cholesterol assimilation assay was estimated by following formula:

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

Antimicrobial activity of the *S. cerevisiae*: The indicator strains *Listeria monocytogenes* (ATCC13932), *E.coli* (ATCC8739), *Staphylococcus aureus* (ATCC6538) and *Pseudomonas aeruginosa* (ATCC9027) (against testing strains were revived, and 100µl of each indicator strain was suspended in 2.5ml of (0.75% TSA) soft agar. In order to prepare the lawn of indicator strains, soft agar suspension was poured into freshly prepared TSA plates and allowed it for solidification and incubated at 37°C for about 2-3 hrs. Sterile disks were set on the lawn of indicator strains carefully. After that, 10µl of the cell or supernatant was taken from an overnight culture of testing strains and carefully poured on filter paper disks. Then plates were placed in incubator at 37°C for 48 hrs. Yeast strains were treated likely, irrespective of its variation in incubation conditions i.e. 30°C for 48 hrs. Results of antimicrobial activity were observed in terms of its zone diameter (nm). A clear zone formation around the disks, determine the antimicrobial behavior.

Selection and propagation of best performing microbial inoculant: QAUSC03 yeast strain was

selected for further study because of its better enzymatic activity as well as probiotic characteristics. A stock culture of yeast stain was inoculated into 5mL trypticase soya broth (Merck) TSB and incubated at and aerobically incubated at 25ºC for 24 hrs. Samples were taken for viable cell count and analysis of each strain. Pour plate counts in TSA agar were used to the numeration of each culture. 1mL of cultures were transferred into 1 liter TSB and allowed to grow for 48hrs at 25 ºC. The culture was moved into a freeze dried for 72 hrs. The producers of freezer drier cultures were mixed and added to whey as a carrier. Samples were taken to determine the total viable count of the finished product. The stability of the product was determined by the counting of viable and total cell counts. Surviving yeast was numerated by pour plate counts in TSA agar after incubation at 25ºC and counts were expressed as mean log CFU/g. The product was prepared every week and was kept at 4ºC.

Effect of indigenous isolated probiotic on milk production of cows

Experimental layout: The study was carried out at National Agricultural Research Centre, Islamabad (NARC), Pakistan. Nine lactating dairy cattle of mix breed (*Sahiwal* and *Sahiwal×Jersey*) at first and second lactation and producing 4-5 liters/day were used. The nutrient requirements of the animals were fulfilled by offering 3 kg concentrates feed, 8 kg maize silage and 20 kg oat fodder (Control feed). The concentrate feed was prepared at NARC by using following formula; 18 % maize grain, 12 % rape seed cake, 18 % rice police, 24 % what bran, 10 % maize gluten (30%), 10 % sugar cane molasses, 5 % sun flower meal, 1.5 % Dicalcium phosphate and 0.5 % each; sodium chloride, mineral premix and limestone power. The nutrient composition of concentrate feed, maize silage and oat fodder was given in the Table 3. Animals were randomly divided into three equal groups, following completely randomized design. The group I cows were fed control diet (CON) without yeast addition, group II cows were fed control diet plus commercially (COM) available yeast (Yac-Sac^{1026}, 10g/day/animal; corresponding to 2.5×10^{07}CFU/g *S. cerevisiae* while group III cows were fed control diet plus laboratory (LAB) produced yeast (8g/day/animal, corresponding to 3.13×10^{07}CFU/g *S. cerevisiae)* for 60 days.

Milk production and feed digestion study: Milk yield was recorded daily. Milk samples (200 ml) collected at 0, 15, 30, 45 and 60 days for milk composition study using milk analyzer (Lactoscan). During last week for five consecutive days, feces were weighed and mixed daily, and a representative fecal sample was taken for proximate analysis (AOAC, 1990) and neutral detergent fiber and acid detergent fiber determination (Van Soest *et al.,* 1991).

Blood study: Blood samples were taken after 0, 30 and 60 days of the experiment. Samples were refrigerated for 1 h and then centrifuged at 3000 rpm for 15 min. The serum was separated and stored at −20°C until analyzed. Serum mineral for Calcium (Ca) and Phosphorus (P) were analyzed by commercial kits (Kit AMP Medizintechnik GmbH, Austria, BD7202-E and BD3702, 650 and 340 nm). Sodium (Na) and potassium (K) in the serum was determined using flame photometer (AFP 100, Biotech Engineering\Management Co. Ltd. U.K).

Microbial profiling of fecal samples: Fecal samples were taken after 0, 30 and 60 days of the experiment. Microbial profiling was done in term of counts by using standard microbiological techniques in the fecal samples (Benson, 2002). Total bacterial count (TBC) was performed on TSA medium by using spread plate technique. M-17 growth medium (Oxoid, Basingstoke, UK) was used specifically for *Lactococcus* and *Enterococcus* species. Macconkey agar was used for coliform isolation according to the procedure given by Shakira *et al.* (2015).

16S rRNA gene sequencing and phylogenetic analysis: The bacterial isolates (QAUBL11, QAULG02, QAULL04, QAUSG07, QAUSG08 and QAUSK01) were distinguished according to Bergey‘s manual of systematic bacteriology. These isolates were sequenced for 16S rRNA gene to confirm their future identification. Sequencing was done by using commercial service of Macrogen Inc. Korea. Bio-Edit software (Hall, 1999) was used to assemble the fragment sequences of 16S rRNA gene sequencing. The gene sequences were submitted to DDBJ (www.ddbj.nig.ac.jp). Using 16S rRNA gene sequences, the strains were identified by BLAST search on EZ Taxon Server. The sequences of closely related type strains were retrieved for constructing phylogenetic trees. Phylogenetic and molecular evolutionary analyzes were conducted using MEGA version 5 (Tamura *et al.*, 2011). A phylogenetic tree was built from unambiguously aligned nucleotides using the neighbor-joining algorithm (Saitou and Nei, 1987). The stability of the relationship was assessed by boots trap analysis by performing 1000 re-samplings for the tree topology of neighbor-joining data. The sequenced data was submitted to NCBI gene data base and accession numbers were obtained.

Statistical analysis: Data collected on different parameters (daily feed intake, daily milk yield, nutrient digestibility) was subjected to statistical analysis by using analysis of variance technique under completely randomized design. Means of different parameters were tested by using least significant difference (Steel *et al.,* 1997). Principle factor analysis was applied by using Pearson correlation (*n*) method. The results are shown in distance biplot.

RESULTS

Identification of isolated yeast strains: Both yeasts belonged to *S. cerevisiae* according to their morphological (Figure.1), biochemical characteristics (Table1) resemblance with *S. cerevisiae.*

Probiotic and enzymatic potential of *S. cerevisiae*: Yeast strains (QAUSC03 and QAUSC05) displayed significant cellulolytic activity. Formation of clear zone indicated the cellulolytic activity in yeast strains. Yeast strains (QAUSC03 and QAUSC05) indicated the proteolytic activity with the formation of clear zones around the colonies displayed no amylolytic activity (Table 1).Yeast strains also showed a high resistance pattern to bile i.e., the mean values of bile tolerance rate of strain no. 3 at 30, 60, and 90 minutes were 0.848, 1.042, and 1.146, respectively. Similarly, the mean values of strain no.5 at the same time duration were 0.966, 1.07, and 1.176 respectively. This data thus analyzed the increasing pattern of bile tolerance effect in yeast strains and the strains were resistant to bile (Figure 2). The cholesterol lowering effect of yeast strains was determined and results showed that the calculated values of yeast strains *S. cerevisiae* QAU03 was lower than that of *S.cerevisiae* QAU05. The cholesterol level of yeast strains (S. cerevisiae QAU03 and *S. cerevisiae* QAU05) was mentioned in (Figure 3). The antimicrobial activity of yeast strains (*S. cerevisiae* QAU03 and *S. cerevisiae* QAU05) were identified against four ATCC culture strains of *L. monocytogenes* (ATCC13932), *E. coli* (ATCC8739), *S. aureus* (ATCC6538) and *P. aeruginosa* (ATCC9027). Both yeast strains exhibit inhibitory activity against all pathogens except *L. monocytogenes* (Figure 4). Diameters of inhibition zones are mentioned in Table 2.

Milk production and nutrients digestion: Highest value of average DMI was observed in lactating dairy cattle fed on LAB probiotic feed whereas lowest and almost equal values were observed in lactating dairy cows fed on control feed and dairy cows cattle fed on COM probiotic feed. The difference in the DMI was non-significant ($p>0.05$) among all the treatments (Table 4). Highest average milk yield was observed in lactating dairy cattle fed on LAB probiotic feed. There was a significant difference ($p<0.05$) in milk production of lactating dairy cattle fed on LAB probiotic feed than fed on control and COM probiotic feed. Results of cow milk analysis showed that, there was no significant ($p>0.05$) difference in MP, TS, SNF and lactose % among the treatments. On the other hand, a significant ($p<0.05$) value of milk fat has been recorded in dairy cattle fed on LAB probiotic feed as compared to other treatments (Table 4). NDF and ADF digestibility significantly ($p<0.05$) improved in animal fed on LAB probiotic feed due to indigenously isolated yeast strains.

Blood biomarker of health: Probiotic yeast exerted no effect on the Ca, Na, K and P levels of blood. All cattle have shown healthier values which is a sign of good health (Table 5).

Microbial profiling of fecal sample: The microbial profile of the fecal samples showed that, probiotic yeast significantly ($p<0.05$) decreases the coliform and *Enterococcus* (CFU/g) species counts and significantly ($p<0.05$) increases the *Lactococcus* (CFU/g) species counts. The aerobic plate count in the control animals at day 0 was 7.98±1.23 CFU/g and at day 60 were 8.12±0.54 CFU/g. On the other hand in the COM fed animals at day 0 aerobic counts were 6.98 ±1.98 CFU/g and at day 60 were 7.71±0.38 CFU/g. In LAB fed at day 0 aerobic counts were CFU/g and at day 0 were 6.12±1.11 CFU/g and at day 60 were 7.97±0.81 CFU/g. (Figure 5).

Identification of microbial isolates and phylogenetic analysis: On the basis of data obtained from physical and biochemical test results we concluded that bacterial isolates on the MRS agar were identified as *Lactococcus* or *Enterococcus* species. Biochemical test of the bacterial isolates on macconkey agar showed that these are catalase, oxidase and MR positive while citrate, MR, indole and citrate negative. These strains were motile and also there were no gas (H_2S) production. According to the experimental data, the isolated bacteria were identified as coliform (Figure 6,7,8). The blastn search revealed that *Lactococcus* QAUBLL04 (KP25613) had the highest sequence similarity with *Lactococcus lactis* subsp. Cremoris NCDO 607^T (AB100802). The blastn search revealed that *Bacterium* QAULG02 (KP25611) had the highest sequence similarity *Lactococcus garvieae* ATCC 49156^T (AP009332). The blastn search revealed that *Enterococcus* QAUSG07 (KP25615) had the highest sequence similarity with *Enterococcus mundtii* $CECT972^T$ (AJ420806). The blastn search revealed that *Enterococcus* QAULG08 (KP25016) had the highest sequence similarity *Enterococcus hirae* ATCC 9790^T (CP003504). The blastn search revealed that *Enterococcus* QAULSK01 (KP25018) had the highest sequence similarity *Enterococcus faecium* ATCC CGMCC 1.2136^T (AJKH0100010). The blastn search revealed that *Bacillus* QAUBL11 (KP25619) had the highest sequence similarity with the *Bacillus Licheniformis* ATCC 14580^T (AE017333) (Figures 9, 10) (Table 6).

Correlation analysis of milk yield, nutrient digestibility, blood and fecal biomarkers: Results of correlation analysis between milk yield, nutrient digestibility, blood chemistry and fecal flora are showed in the figure 11. There was a positive correlation to nutrient digestibility and the prevalence of the gut

microbiota. The most fermentation metabolites were positively correlated with the nutrient digestibility.

Table 1. Morphological and biochemical characteristics of isolated strains.

Parameters	Yeast strains	
	QAUSC03	QAUSC05
Morphological characteristics		
Cell shape	Ellipsoid to elongate	Ellipsoid to elongate
Colony morphology	Circular	Circular
Colony surface	Smooth/Slimy	Smooth
Colony colour	Off-white	Pinkish
Colony elevation	Pulvinate	Umbonate
Colony margin	Entire	Filamentous
Biochemical characteristics		
Alcohol production	+	+
Glucose fermentation	+	+
Catalase	+	+
Fructose	+	+
Urease	-	-
Enzymatic activities		
Amylolytic activity	-	-
Cellulolytic activity	+++	++
Proteolytic activity	++	+

Table 2. The antipathogenic activity of isolated yeast strains against ATCC strains and their inhibitory zones diameter (mm).

Strains	*E. coli* (ATCC8739)	*Pseudomonas aeruginosa* (ATCC9027)	*Staphylococcus aureus* (ATCC6538)	*Listeria monocytogenes* (ATCC13932)
QAUSC03	10	18	14	Nil
QAUSC05	10	14	16	Nil

Table 3. Chemical composition of concentration feed, fodder and silage (%DM basis).

Nutrient	Concentrate feed	Fodder (Oat)	Silage (Maize)
Dry Matter	92.12	14.28	34.35
Crude Protein	15.23	12.29	8.04
Neutral detergent fibre	27.88	54.23	50.6
Acid detergent fibre	18.04	37.39	31.3

Table 4. Effect of yeast supplementation on dry matter intake, milk yield its composition and nutrient digestibility (Means±SEM) in lactating dairy cattle.

Parameters	Groups		
	Control	LAB*	COM**
Dry matter intake (Kg/day)	10.01± 0.18	10.08 ± 0.21	10.04± 0.19
Milk yield (Lit/day)	05.40^{b} ± 0.65	06.12^{a} ±0.70	05.80^{b} ± 0.83
Feed conversion ratio	1.87±0.12	1.64±0.11	1.73±0.04
Milk composition %			
Protein	3.39 ± 0.11	3.71 ± 0.05	3.50 ± 0.12
Fat	4.60 ± 0.46^{b}	5.46 ± 0.36^{a}	4.78 ± 0.27^{b}
Total solids	14.40 ± 0.61	14.51 ± 0.59	15.22 ± 0.23
Solid not Fat	9.84 ± 0.39	9.05 ± 0.74	10.44 ± 0.50
Lactose	5.09 ± 0.15	5.26 ± 0.07	5.23 ± 0.08
Nutrient digestibility%			

Dry matter	56.34±1.13 [b]	61.11±1.94 [a]	62.81±0.94 [a]
Crude protein	57.50±1.19 [b]	61.43±1.04 [a]	63.56±1.12 [a]
Neutral detergent fibre	58.33±0.68 [b]	60.89±0.55[a]	61.55±0.58[b]
Acid detergent fibre	51.47±0.34 [b]	54.27±0.56[a]	55.27±0.77[b]

[a, b] Values on the same row with different superscripts differ significantly ($p<0.05$); [1]n=3 per treatment; [2]Control feed without yeast; [3]LAB compose of control feed supplemented with 3.13×10^{07} cfu/g laboratory produces probiotic yeast (QAUSC03) at the rate of 8g /day/animal [4] COM compose of control feed supplemented with 2.5×10^{07} cfu/g commercially probiotic yeast (Yac-Sac[1026]) at the rate of 10g /day/animal; [5]Before treatment (day 0);[6]after treatment (day 120); [*]±SEM = standard error of the mean

Table 5. Effect of dietary yeast supplementation on blood serum metabolites (Means ± SEM) in lactating dairy cattle.

Parameters	**Feeding regime**		
	Control[2]	**LAB-Probiotic[3]**	**COM-Probiotic [4]**
Calcium (mg/dl)[1]			
Before treatment[5]	8.68±[*]0.05	9.38±0.03	8.71±0.08
After treatment[6]	8.65±0.03	9.28±0.03	8.86±0.09
Phosphorus (mg/dl)			
Before treatment	7.58± 0.04	7.89 ± 0.02	7.85± 0.04
After treatment	7.81 ± 0.04	8.00 ± 0.02	7.87 ± 0.02
Potassium (meq/l)			
Before treatment	5.60±0.132	5.58±0.27	5.64±0.15
After treatment	5.65± 0.107	5.25 ± 0.10	5.32 ± 0.05
Sodium (meq/l)			
Before treatment	123.86 ± 0.90	126.53 ± 2.40	125.86 ± 1.75
After treatment	127.10 ± 1.03	130.64 ± 1.59	127.17 ± 1.49

[a, b] Values on the same row with different superscripts differ significantly ($p<0.05$); [1]n=3 per treatment; [2]Control feed without yeast; [3]LAB compose of control feed supplemented with 3.13×10^{07} cfu/g laboratory produces probiotic yeast (QAUSC03) at the rate of 8g /day/animal [4] COM compose of control feed supplemented with 2.5×10^{07} cfu/g commercially probiotic yeast (Yac-Sac[1026]) at the rate of 10g /day/animal; [5]Before treatment (day 0);[6]after treatment (day 120); [*]±SEM = standard error of the mean

Table 6. Identification of isolated strains based on 16SrRNA gene sequence and their accession numbers published in DNA database.

Strain ID	**Strain name/ genus**	**Length of 16S r RNA (ntds)**	**Accession number of 16S rRNA gene**	**Closely related Validly published species**	**Similarity % of 16S r RNA gene sequencing**
QAUBL11	*Bacillus*	885	KP256019	*Bacillus licheniformis* ATCC 14580[T] (AE017333)	99.66
QAULG02	*Bacterium*	871	KP256011	*Lactococcus garvieae* ATCC 49156[T] (AP009332)	99.89
QAULL04	*Lactococcus*	913	KP256013	*Lactococcus lactis* subsp. ATCC *Cremoris* NCDO 607[T] (AB100802)	100
QAUSG07	*Enterococcus*	963	KP256015	*Enterococcus mundtii* CECT972[T] (AJ420806)	99.9
QAUSG08	*Enterococcus*	925	KP256016	*Enterococcus hirae* ATCC 9790 [T] (CP003504)	100
QAUSK01	*Enterococcus*	903	KP256018	*Enterococcus faecium* ATCC CGMCC 1.2136[T] (AJKH01000109)	100

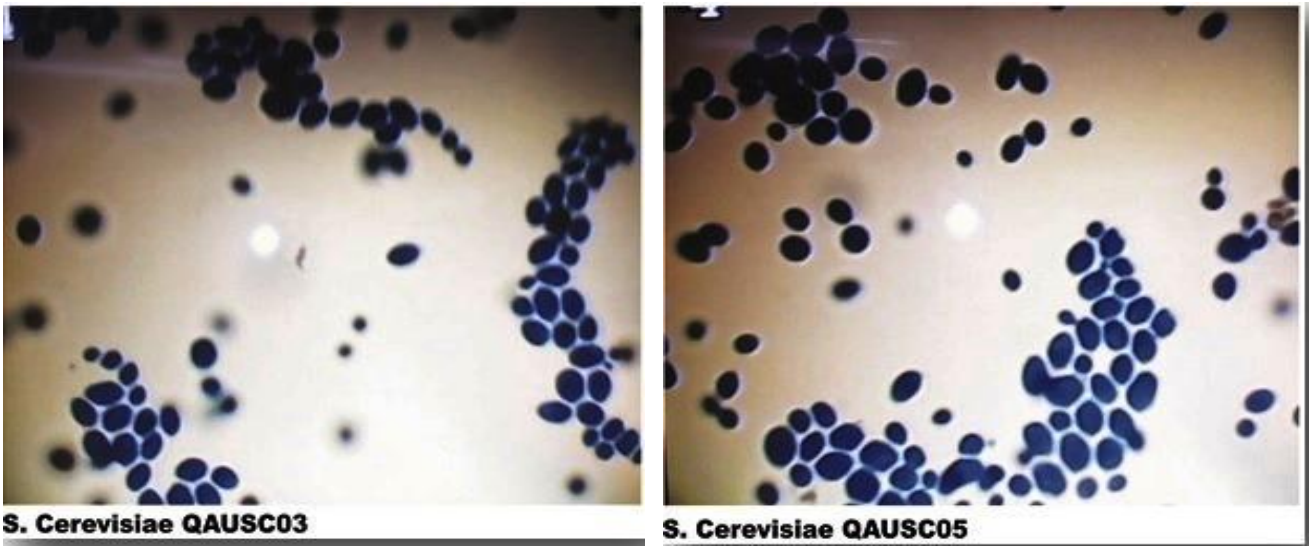

Figure 1. Simple staining of yeast strains (L) QAUSC05 and (R) QAUSC03

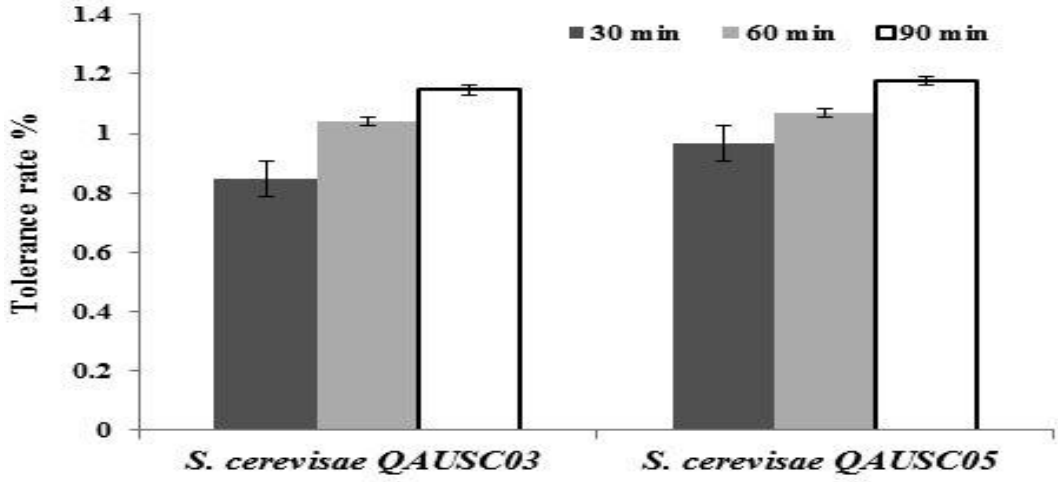

Figure 2/ Tolerance rate of isolated yeasts strains in bile salt (% + SEM)

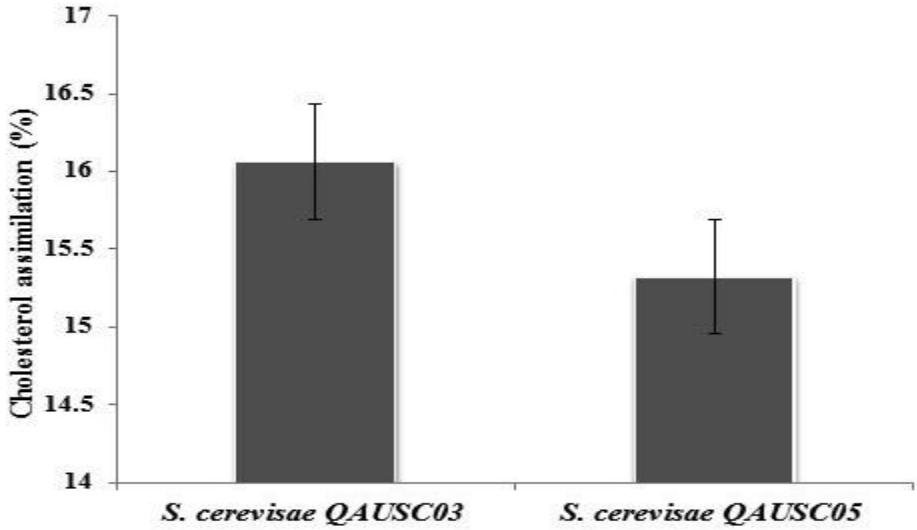

Figure 3: Cholesterol assimilation of isolated yeast strains (%+SEM)

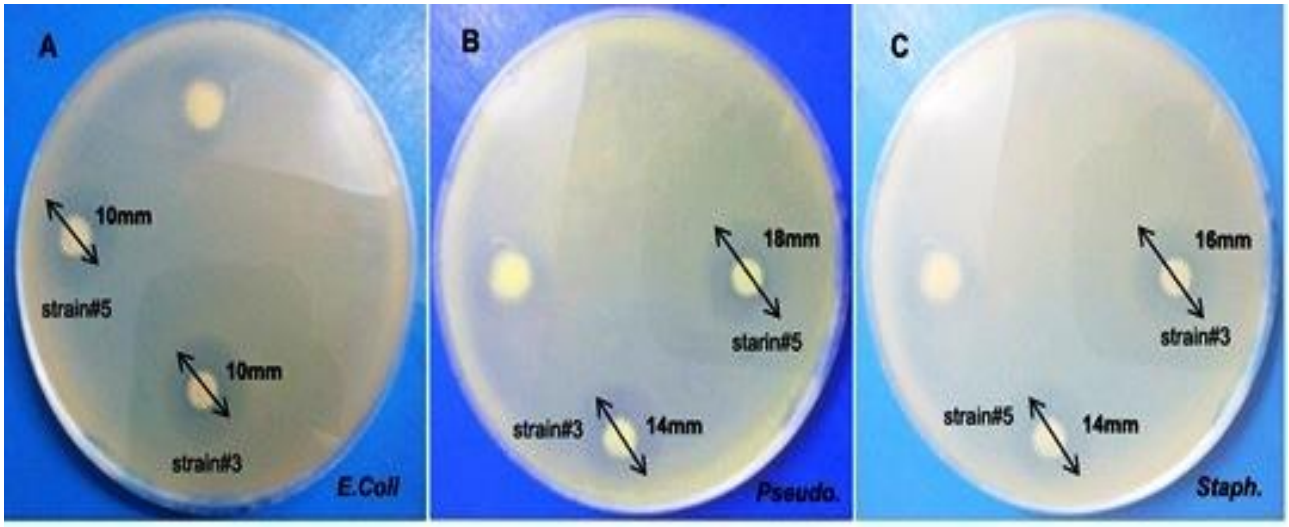

Figure 4. Anti-pathogenic activity of isolated yeast strains QAUSC03 (Strain #3) and QAUSC05 (Strain #5) against ATCC strains with their zones of inhibition

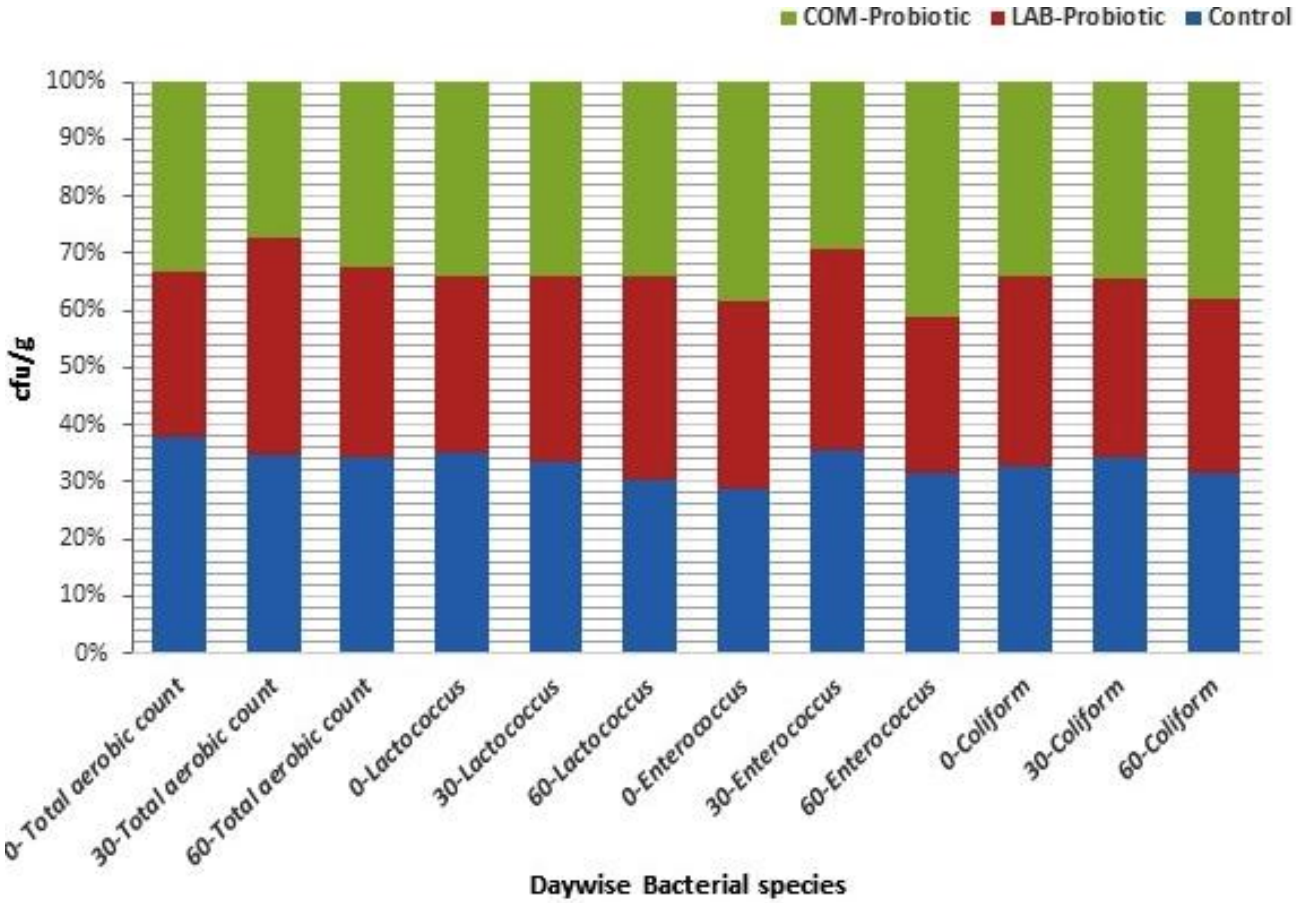

Figure 5. The graphical representation of CFU counts (cfu/g) of ruminal gut microbial biomarkers of health of dairy cows fed on probiotic yeast at different time interval.

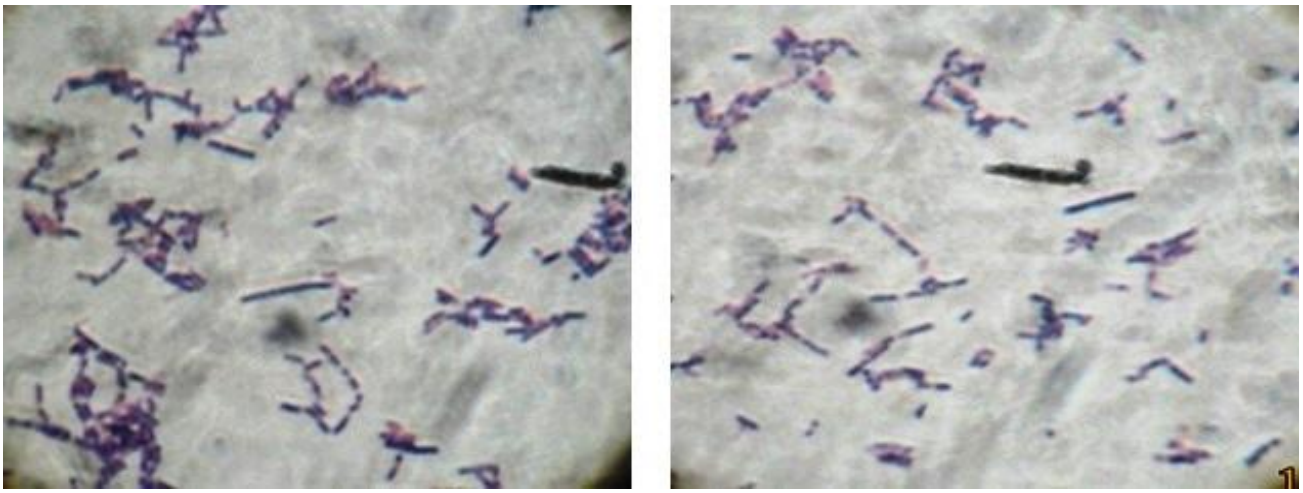

Figure 6. Gram staining of *Lactobacillus* strains on MRS; gram positive rod

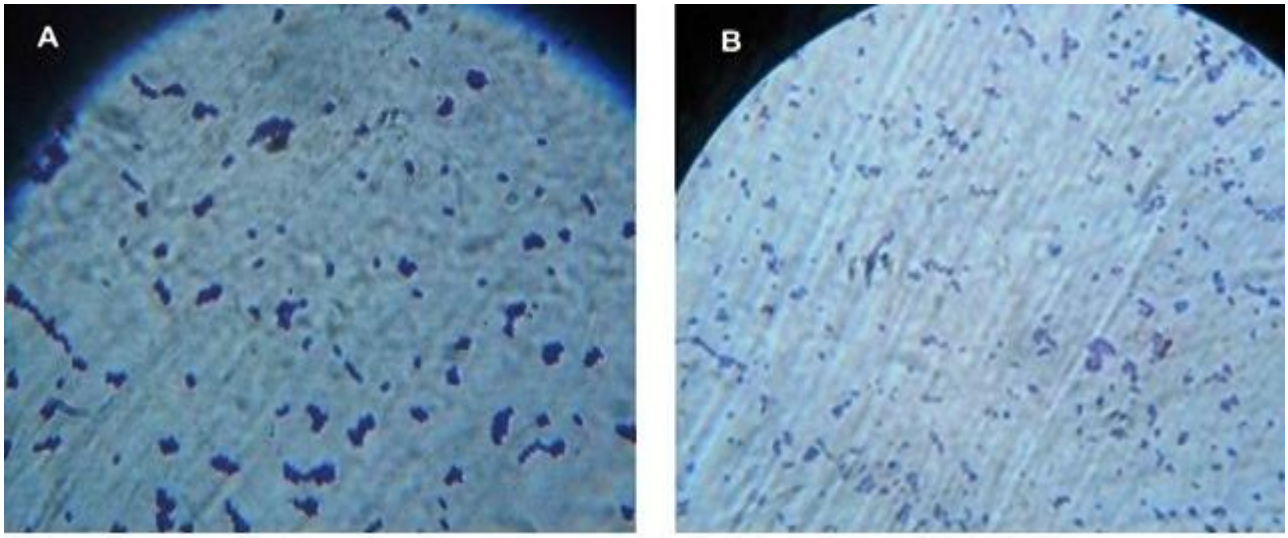

Figure 7. Gram staining of *Lactococcus* and *Enterococcus* strains on MRS; (A) Strain QAULG03 and (B) Strain QAULG10. Both are gram + cocci

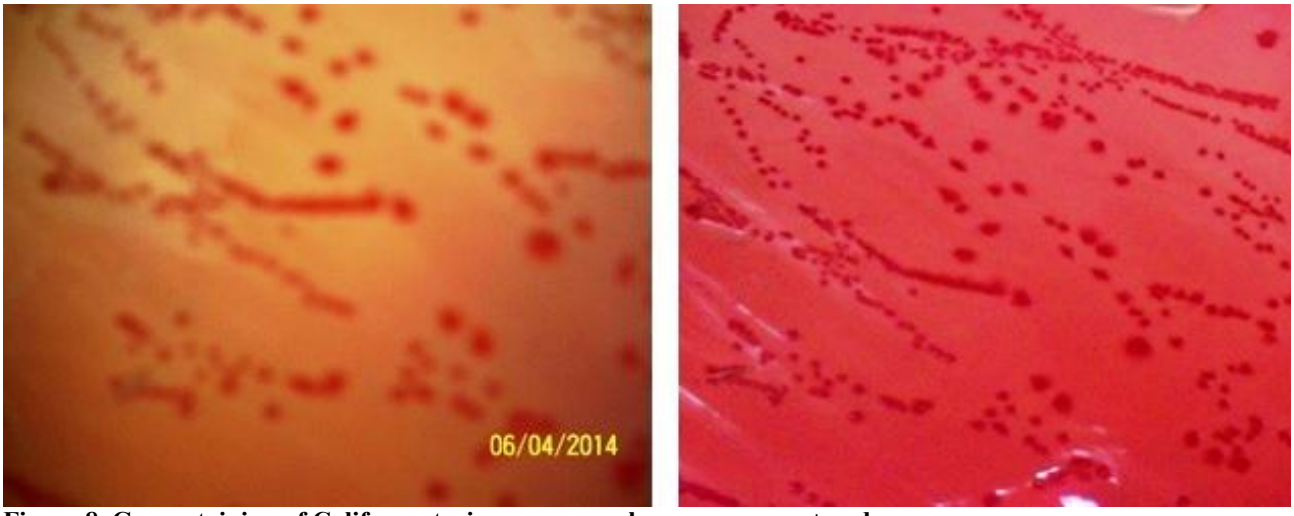

Figure 8. Gram staining of Coliform strains on macconkey agar; gram + rod.

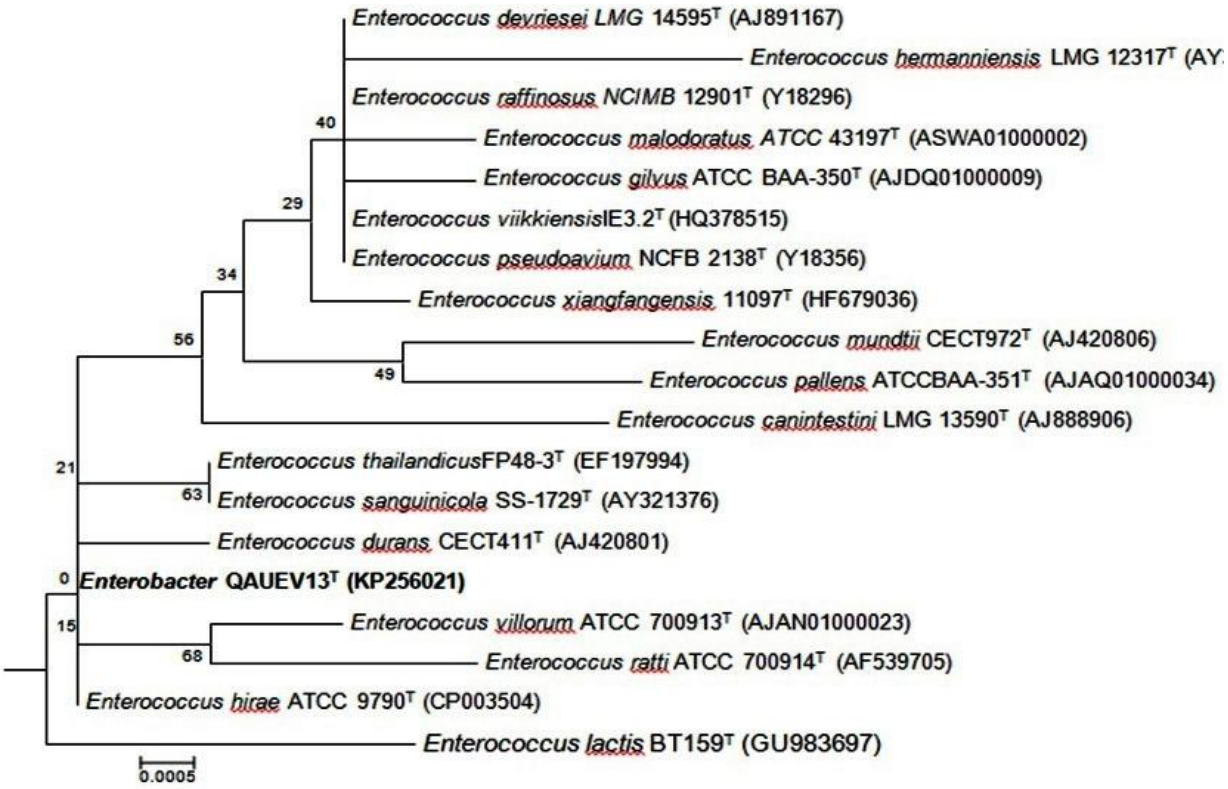

Figure: 9. Phylogenetic tree of the *Lactococcus* QAULL04, QAULG03, QAULG02

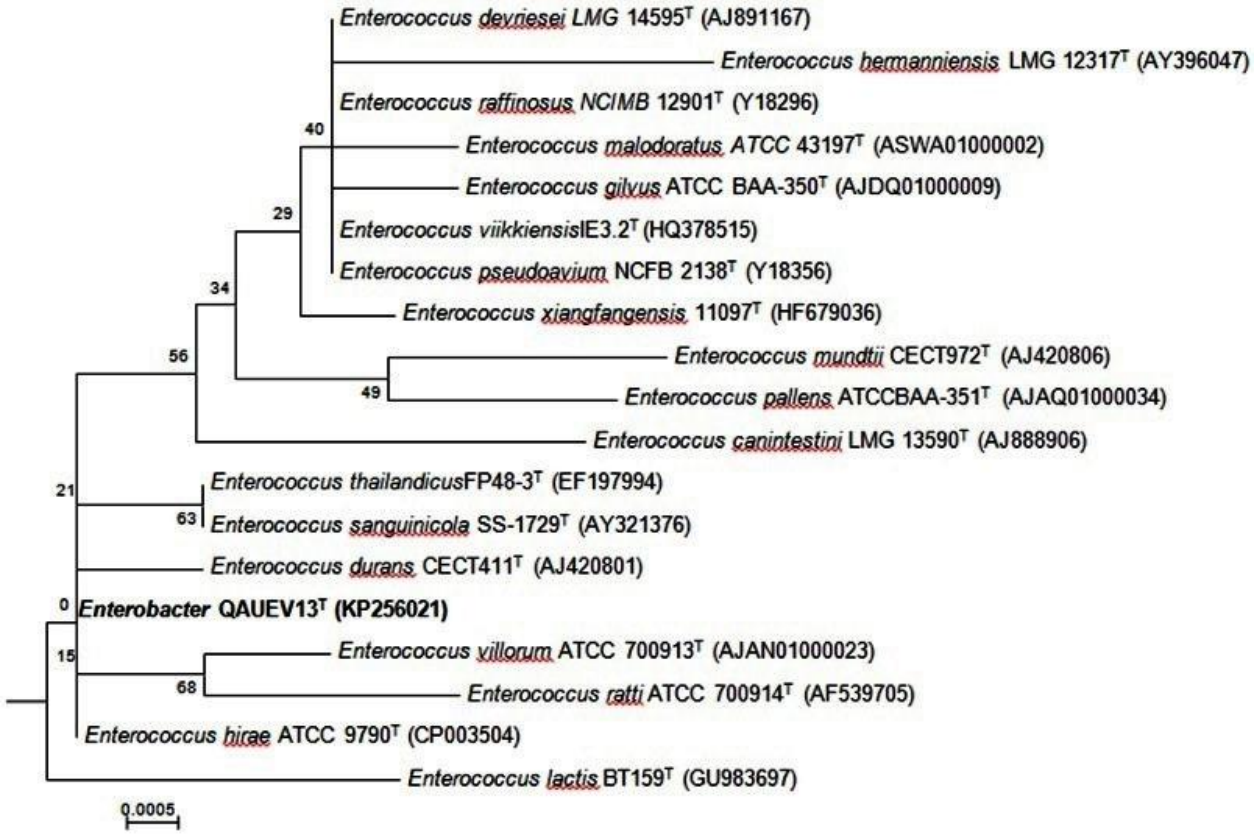

Figure: 10. Phylogenetic tree of the *Enterobacter* QAUEV13 (KP25621)

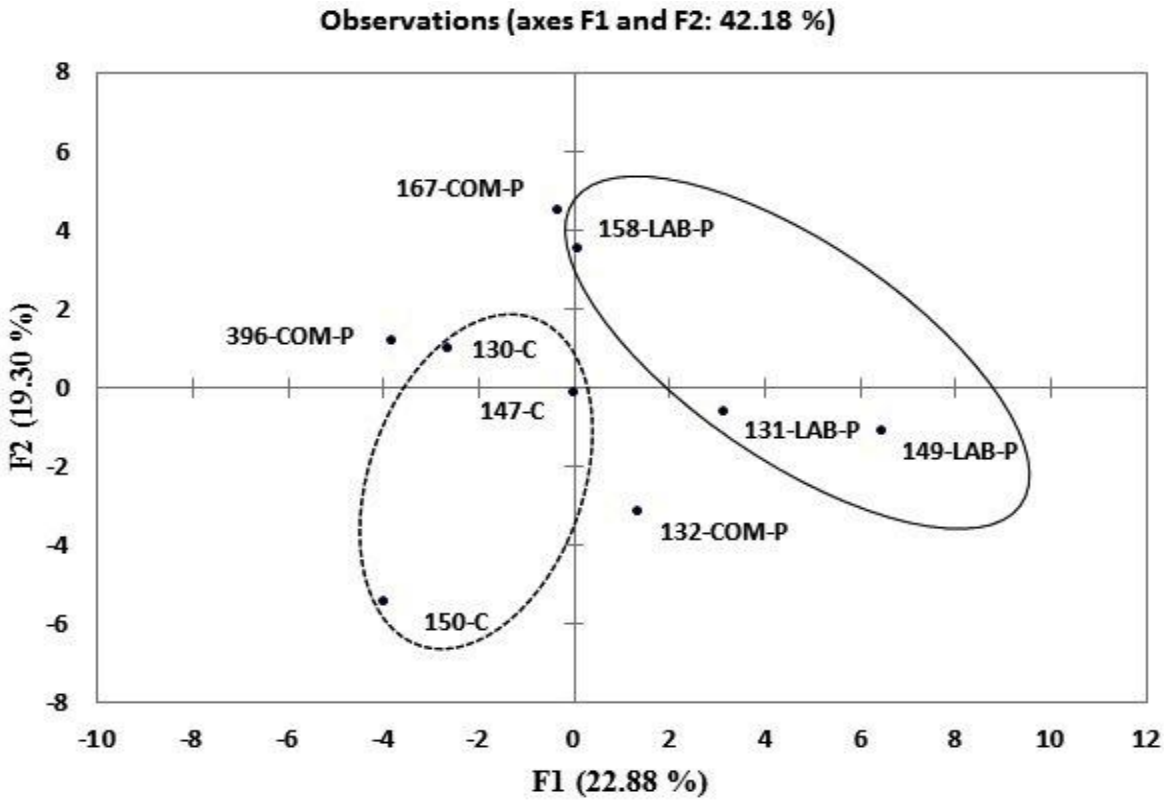

Figure 11. Multifactorial analysis of haematological parameter, milk yield and its quality of lactating dairy cows fed on probiotic yeast. C: Control feed without yeast. LAB-P: Control feed supplemented with 3.13×10^{07} cfu/g laboratory produces probiotic yeast (QAUSC03) at the rate of 8g /day/animal. COM-P: Control feed supplemented with 2.5×10^{07} cfu/g commercially probiotic yeast (Yac-Sac^{1026}) at the rate of 10g /day/animal.

DISCUSSION

Probiotic microbiota based feed supplements are the natural, save and sustainable strategy to combat recent challenges with productivity of livestock sector. It is noted that each probiotic strain may have its own specific ability affecting the host performance. That ability is mainly depending on the strains isolation source and its target. Many probiotic yeast strains available in market, but that may not be suitable for any local use due to their site and target specific nature. We see each strain is unique and different therefore there should be a clear knowledge on the complex microbial flora as well as a clear definition of the selection criteria of the probiotic strain leads to isolated and characterized new site specific probiotic strains. Bacterial probiotics produced better results in young calves, chickens and pigs, whereas yeast/ fungal probiotics were effective in adult ruminants (Musa *et al.*, 2009). One of the major benefits of the probiotic yeast is that yeast has no antibiotic resistance gene (Czerucka *et al.*, 2007). It has also ability to tolerate to bile salt and gastric acid and to neutralized enterotoxin and colonizes in the GIT resultantly improve health status and production efficiency of the dairy animals.

The main criterion for selecting a probiotic strain is the assessment of its pH and bile tolerance since the first biological barriers to be overcome after ingestion to reach its place of action are the acid in the stomach and bile salts in the intestine (Fuller, 1989). The possible use of yeasts as feed supplements is encouraged by the observation of the ability of yeast to survival passage through the GIT. Yeast strains showed a higher resistance to bile in present experiment, the growth pattern of QAUSC03 strain was not affected in the presence of bile salt. Similarly, the growth of QAUSC05 strain was also remained undisturbed in the presence of bile salt. The number of surviving strains implied that both strains had a relatively high tolerance to bile salts. Ability of *S. cerevisiae* strains to survive the passage through the intestinal tract is prerequisite to be use as probiotic for ruminates (Guillot, 1998). Animal digestibility, therefore characteristics for this parameter which indicated the enhanced feed adsorption in the yeast fed groups. The enzymatic activity of probiotic strains increases feed utilization efficiency. In this study *Saccharomyces cerevisiae* (QAUSC03 and QAUSC05) displayed cellulolytic and proteolytic activity but no amylolytic activity.

Results indicated that the QAUSC03 strain having comparatively better bile tolerance. The cholesterol lowering capacity of *S. cerevisiae* strains were determined as 16.1 and 15.5 percent for QAUSC03 and QAUSC05 respectively. The degradation of cholesterol in rumen leads to lower blood cholesterol in cattle; this ultimately decreases in cholesterol secretion in milk**.** The antimicrobial activity of yeast strains was estimated against four reference pathogens strains commonly associated with rumen GIT disorders. It was observed that both strains showed anti-pathogenic activity, against *Escherichia coli* (ATCC8739), *Staphylococcus aureus* (ATCC6538) and *Pseudomonas aeruginosa* (ATCC9027) but it was absent in case of *Listeria monocytogenes* ATCC1393. Although, both *S.cerevisiae* strains shown potentially acceptable bioactive properties. However, *S. cerevisiae* QAUSC03 showed significantly higher potential ability in antimicrobial, tolerance in acid until pH 2-2.5 and bile salt up to 1.5% (w/v). Therefore *S. cerevisiae* QAUSC03 was selected for further application as a probiotic in dairy cattle feed. The higher ($P<0.05$) milk production in LAB probiotic fed group might be due to cellulolytic activity of the *S. cerevisiae* QAUSC03. This activity enhanced the cellulose digestion rate and help in the milk synthesis (Dolezal *et al.,* 2012). Yeast culture significantly ($P<0.05$) increased the fiber digestibility, resulting in increased supply of absorbed nutrients for milk synthesis in our experiment. Yalcin *et al.* (2011) found 6.3 % more milk production in lactating dairy Holstein cattle fed with yeast culture than the control group. More recently, Hossain *et al,* (2014) found 1.5 kg (4.1%) more milk production in lactating dairy cattle due to yeast cultures addition. Milk composition results showed a significant ($P<0.05$) higher milk fat in LAB fed group, followed by COM yeast and control group. The increased milk fat in the LAB yeast fed group might be due to the positive effect of the yeast culture on the milk yield and fiber fermentation**.** Yeast culture enhances the cellulolytic bacteria population (especially, *Ruminococcous albus* and *Ruminococccus falivifavis*) that ultimately degrades the cellulose and enhanced the acetic acid production inside rumen. In agreement with this some researcher, Chiquette (1995) and Vibhute *et al.* (2011) found significant effects of supplementing yeast in milk fat. Yeast supplementation has no significant effect on the protein, total solid, milk lactose and solid not fat content in our study. Similar results were reported by Yalcın *et al.* (2011). Reproductive performance of the dairy animals is directly and indirectly related to Ca concentration (Bansal, 1978) and involved in steroid biosynthesise in ovaries (Vibhute *et al.*, 2011; Schingoethe *et al.,* 2004; Shemesh *et al.,* 1984). Dietary YC did not affect serum Ca, P, Na and K in our study. Similar results were reported by Piva *et al.* (1993) and Nursoy and Baytok (2003), On the other hand, Dolezal *et al.* (2011) reported that YC significantly ($P<0.01$) increased the Ca concentration in dairy animal. He reported that significant difference in the blood parameters might be due to the effect of diet and individuality of cow.

Increased digestibility of nutrients in diet may be due to increased enzyme activity in the intestine due to probiotics in present study. The improved nutrients digestibility in diet may be due to stable rumen pH and removal of oxygen from the rumen resultantly anaerobic rumen conditions might have increased total microbial population. On the other hand, increased fiber digestibility in diet may be due to the increased cellulolytic activity of the indigenous probiotic yeast which increased cellulose degradation. Moreover, yeast also increases the cellulose degrading bacterial population that appears to be the main mechanism by which yeast improves fiber digestion (Bitencourt *et al.,* 2011). The GIT inhabits multifarious microbial diversity that helps in generating impassive response regarding nutrition, health, physiology, productivity and well beings of animals (Guarner and Malagelada, 2003; Dowd *et al.,* 2008). The physiological, anatomical, and immunological status of the host is strongly dependent upon micro-biota of GIT which facilitate essential functions to host. It also restrains the injurious and pathogenic bacteria in gut colonization (Cebra *et al.,* 1999). Diet is the major factor which can change the microbiota of the GIT and rumen (Bilal, 2004; Russell and Hino, 1985).With respect to days considerably there was significance ($p<0.05$) differentiation in the total aerobic count in cattle fed on probiotic-yeast supplemented feed. This indicates that in dairy animals, yeast puts positive influence on the total aerobic count. On the other hand, the pathogenic *Enterococcus* and coliform counts (*Escherichia, Enterobacter,* and *Citrobacter*) were significantly ($p<0.05$) lower in yeast fed animals. It indicated that the probiotics yeast has an ability to lower the pathogenic bacteria in GIT. Some researchers also reported that feeding probiotics significantly decreased the pathogenic bacteria (Agarwal *et al.*, 2002). Gram positive and catalase negative cocci *Lactococcus* species were significantly ($p<0.05$) affected by the yeast supplementation at 60 days of the experiment in present study. That showed that probiotic yeast has ability to increase the beneficial bacteria which ultimately reduce the infection in the dairy animals. A similar finding has been reported by (Jatkauskas and Vrotniakiene, 2010) who reported that probiotic has an ability to reduce the *Enterococcus* species in GIT. Likewise, Rada *et al.* (2006) reported that probiotic fed group decreased the population of *Enterococcus* species. Results from this study indicate that probiotic yeast may have an ability to reduce the pathogenic bacteria (i.e. *E.coli*) and increase the beneficial bacteria (*Lactococcus* ssp). It may see that yeast may bind to pathogenic cell and interactions with gut constituents (Elmer and

McFarland, 1987), which may have given the suitable microbial dynamic inside GIT as the fecal microbial population in dairy animals are future quantified, scientists might be in the position to correlate microbial populations of kingdoms or nutrient-utilization guidelines or both with production parameters like feed intake, growth performance, milk production and well beings of the animals. Future research is needed for identification of nutrients that shape the microbes and their interaction with the host animals for getting better performance.

Conclusion: Ruminal-gut microbes play a crucial role in overall health of dairy animals, and conversely, imbalance of the microbiota may lead to metabolic diseases. Probiotic alters the relative proportions of the bacterial species in the ruminal-gut. Modification of the ruminal gut microbial population may have profound digestive consequences which alternatively enhanced production performance. Ruminants live in different parts of the world with different feeding and managemental condition therefore different probiotic strains may exhibit markedly different effects upon the ruminal fermentation. It is suggested that the animal origin probiotic strains act more precisely to ruminal gut microorganisms as compares to probiotic strains from any unknown origin. Therefore we should identify indigenous probiotic strains by using modern molecular techniques for getting best results on the rumen fermentation in their own living condition. Indigenous isolated probiotic strains might be used to promote the explosion of beneficial microbiota to maximize sustainable alter in the ruminal-gut microbiota of local breeds.

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