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Isolation and characterization of potential probiotic bacteria from sahiwal cow gut

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Abstract

Farm animal productivity depends upon many factors including gut microbiome. It is well established that dysbiosis in the gut microbiome leads to compromised productivity and metabolic disorder. Microbial species with probiotic potential can be isolated from dung samples. From twelve cow dung samples total of nineteen bacterial strains were isolated on two different growth media (M17 and TSA). Only four gram-positive strains were isolated and conformed through 16S rDNA sequencing. Fully characterized strains QAULL04 (KP256013) and QAUEM01 (KP273582) were checked for their probiotic potential. QAULL04 and QAUEM01 has shown maximum mimic gut survival of 31.70% and 3.35% after two hours respectively while it reduces to 26.72% and 0% after 24 hours respectively. Cholesterol assimilation potential of QAULL04 was high (64.44%) in comparison with QAUEM01 (50%) while cell hydrophobicity of QAUEM01 was high (21.10%) than QAULL04 (4.712%). Cumulatively QAULL04 has shown better results for its probiotic application in animal feed.

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Introduction

Gut microbial diversity is one of the key factors of the ruminants which play important part in nutrient digestion. Healthy gut microbiota improves feed digestion and part significant impact on growth, health and many productivity patter (Fuller 1989). There is a diverse microbial population in the gut which can improve the nutrient digestibility which leads to increase productivity and the physiological parameters of animal (Guarner and Malagelada 2003).Healthy gut microbiota can also eliminate the pathogenic microbial load and provide the competition of resources for the pathogenic bacteria (Nurmi and Rantala 1973). Healthy gut microflora can stimulate the immune response and provide barrier to against the pathogenic bacteria colonization and reduces the infection rate. It also stops the unwanted and pathogenic bacteria from colonizing the gut (Cebra, H.-Q. *et al.* 1999).Healthy gut microbial diversity is very significant factor affecting many parameters of health so many strategies has been used to improve the gut microbial diversity in dairy animals to increase the milk yield and improvement of other parameters. Now a day, the use of probiotics to improve gastro-intestinal health has become a handy and economical method to enhance the overall health and productive performance of animals.

The "Probiotics supplements" are natural products which contain live microbiota that improves the health and production performance of the host by improving gut microbial flora (Klaenhammer, Kleerebezem *et al.* 2012). About a century ago, Metchnikoff was the first one who instigated the interests in probiotics(Metschnikoff 1907). Lactic acid bacteria are considered to be the primary source of probiotics. It includes *Lactobacillus* sp., *Bifidobacteriumsp., Enterococcus sp.* and *Pediococcussp., Bacillus sp.,Clostridium butyrium*., yeast "*Saccharomyces boulardii*(Klein, Pack *et al.* 1998, Elmer, Martin *et al.* 1999, Senesi, Celandroni *et al.* 2001, Takahashi, Taguchi *et al.* 2004). Bacterial probiotics yielded better results in young calves, chickens and pigs, whereas yeast/ fungal probiotics were more effective in adult ruminants (Musa, Wu *et al.* 2009). Consequently, probiotic strains should have potential such as survive in upper GIT, tolerate bile toxicity and highly acidic gastric environment andability of mucin-binding and adherence to intestinal-imitative epithelial linings (Dunne, O'Mahony *et al.* 2001, Del Piano, Morelli *et al.* 2006). Furthermore, probiotic strains must be assessed for their antibiotic susceptibility before applying them in food processing (Parvez, Malik *et al.* 2006).

Demand for safe and healthy food is increasing day by day worldwide and for this probiotic use is an alternative because it can enhance the productivity and inhibit the growth of pathogenic bacteria in the rumen. Probiotic are also in use to reduce the nitrogen-based pollutant and methane production (Strohlein 2003).

There are many potential sources for the isolation of probiotic including fermented food, healthy cow dung. Current research was planned to assess the *in vitro* probiotic potential of fully characterized isolates, isolated from Sahiwal cow dung.

Materials and 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, mimic gut survival, cell hydrophobicity, cholesterol assimilation.

Isolation of microbialstrains from cattle dung

A total of 12 cattle dung samples (10 gram) were collected in re-closable polythene bags aseptically from National Agriculture Research Center (NARC) experiment cattle farm and were transported to process in Laboratory for Microbial Isolation. All samples were inoculated on selected media including M-17 andTSA (tryptic soy agar). The inoculation was done by spread plate method and plates were

incubated at 32˚C for 24hours for bacteriaisolation.

Identification of microbial strains

Bacterial strains isolated by using different media were preliminary identified phenotypically and were further confirmed through16S rDNA sequencing.All isolates were examined according to Bergey'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. 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. Isolates were confirmed by determination of 16S rDNA gene sequencing for representative isolates. Initially, DNA of bacterial isolates was extracted through Kate Wilson method*(Wilson 2001)*.

Phylogenetic analysis

Phylogenetic analysis was performed on 16S rDNA gene sequence basis. Macrogen, Commercial Seoul, South Korea was used for sequencing and same sequences from Gen Bank were further recognizedby using BLAST from NCBI. Alignments were carefully analyzed and manually corrected and ambiguate aligned regions were removed. Finally, Phylogenetic trees were constructed using neighbor joining method with bootstrap values.

Determination enzymatic activity

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 described below.

Detection of amylolytic activity

To determine the amylolytic activity nutrient agar supplemented with one 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(Cotta 1988).

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(Johnsen and Krause 2014).

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.

Determination of bacterial isolates' survival under mimic cattle gutconditions

100 µL of bacterial suspension at their log phase were inoculated in 10 ml of Tryptic soy broth (TSB).1g/l0ml of bile salts stock solutions and 0.01g/10ml of lysozyme 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. TSB with neutral pH having 100 µL of bacterial samples, without the addition of bile salt and lysozyme, were set out as a control media. After the interval of 2, 4 and 24 hours, the comparative survival of isolates were measured by using

spectrophotometer at 600nm (Holzapfel, Haberer *et al.* 1998). Experiment was done in triplicate.

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

Determination of cholesterol assimilation

Assimilation of cholesterol was determined according to Pereira and Gibson(Pereira and Gibson 2002). 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). The 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 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 × 100 × 0.2 / OD of known × 0.05.

Cell surface hydrophobicity

The ability of the microbes to adhere with the intestinal cell layer can be evaluated by cell surface hydrophobicity test. Bacterial cultures were grown in the TSB 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:

Hydrophobicity Percentage (%) = $[(A_o - A_1) / A_o] \times 100$.

Where,

A_o =Optical density before mixing the xylene

A_1 = Optical density of the aqueous layer

Results

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.

Table 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	+vecocci	-	-	-	-	-	+	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	-vecocci	+	-	-	-	-	-	M, No H_2S
13	-vecooci	+	-	-	+	-	+	M, No H_2S

Thirteen bacteria strains were isolated on MRS and further examined for gram stain 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 1, Fig. 1).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 2).

Table 2. Biochemical characterization of isolated strains from cow dung onM-17 Media.

S. No.	Gram Staining	Catalase	Oxidase	Indole	Simmon's Citrate	Methyl Red	Triple sugar iron	Sulfide Indole Motility test
1	-ve, rods	+	-	-	-	-	-	NM,NoH$_2$S
2	-ve rods	+	-	-	+	-	-	M,No H$_2$S
3	-ve, cocci	+	-	-	-	-	+	NM, No H$_2$S
4	-ve rods	+	-	-	+	+	+	NM, No H$_2$S
5	-ve, cocci	+	-	-	-	-	-	NM, H$_2$S
6	-ve rods	+	-	-	-	-	-	M, No H$_2$S

Molecular identification

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 asthese 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).

Table 3. Molecular Identification 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

Phylogenetic analyses of bacterial isolates

The blast search revealed that The *Lactococcus* QAULL04 (KP256013) had the highest sequence similarity with the *Lactococcus lactisssp.tructae* L105^T (EU770697) and The Bacterium QAULG02 (KP256011) had the highest sequence similarity with the *Lactococcus garvieae* ATCC 49156^T (AP009332) (Fig. 2). 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).

Probiotic characterization

After 16SrDNA sequencing, fully characterized strains were preceded for probiotic characterization. *Enterococcus mundtii* QAUEM01 and *Lactococcus lactis* QAULL04 were checked for their enzymatic potential, cholesterol assimilation and mimic gut survival at different time intervals.

Extracellular enzymatic activity of microbial isolates

QAUEM01 and QAULL04 were positive for cellulolytic, proteolytic and amylolytic activity (Table 4), and were confirmed through formation of clear zone around the point inoculation on respective media.

Cholesterol assimilation

Both strains showed cholesterol reduction more than 50% as QAULL04 cholesterol reduction values was 64.442% while QAUEM01 showed 50% cholesterol reduction (Table 4).

Mimic gut survival

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 (Table 4).

Cellsurfacehydrophobicity

Hydrophobicity activities of bacterial strains were measured after incubating them for 24hrs. The adhering ability varied from strain to strain. This capability was determined by measuring the number of cells present in xylene layer. *E. mundtii* QAUEM01 showed maximum hydrophobicity of 21.104% while QAULL04 has minimum value of 4.712% for cell hydrophobicity (Table 4).

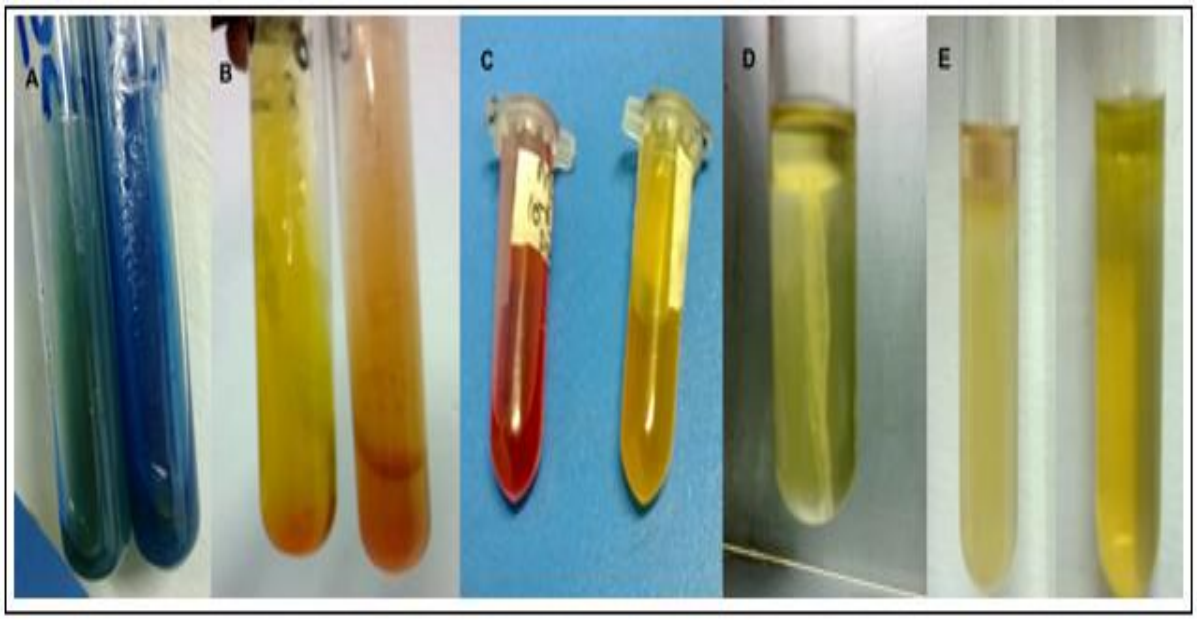

Fig. 1. Biochemical analysis; (A) Simmon's citrate test (B) Triple sugar iron (TSI) test (C) Methyl Red (MR) test (D) Sulfideindole motility (SIM) test (E) Indole test.

Discussion

Probiotic strain isolation from cow dung samples

The microorganisms isolated from cow dung, after their phenotypic and molecular based identification, they were evaluated for extracellular enzymatic activity, mimic gut survival and bioactive properties subsequently. In total nineteen bacteria were isolated from cow dung samples and were subsequently checked for their probiotic potential. Biochemically all isolates were 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 belong to lactic acid bacteria family and agreed with the studied data reported by (Roos, Engstrand *et al.* 2005, Cullimore 2008). On microscopic examination after gram staining only four strains were found to be gram positive while remaining were gram negative.

Based on phylogenetic analysis of these four gram-positive bacterial strains, they were characterized to be the member of *Enterococcus*and *Lactococcus*genera. While among four isolates two were fully confirmed isolates QAULL04 and QAUEM01 and were checked for probiotic potential.

Enzymatic potential of cow dung isolates

It was found that cow dung isolates have good enzymatic activities. Both isolates showed amylolytic activity. Moreover, the production of cellulase during ensilage process helps in degrading the cellulosic mass of plants. Both bacteria showed cellulase activity. Cellulase activity of lactic acid bacteria from many substrates has been reported in many studies (Mohamed, Shabeb *et al.* 2010, Bai, kumar *et al.* 2012).

Mimic gut survival

Cow dung 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, Haberer *et al.* 1998).

Cow dung isolated bacterial strains QAULL04 has shown maximum survival rate (31.70%) than 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 (Hofmann and Mysels 1992).

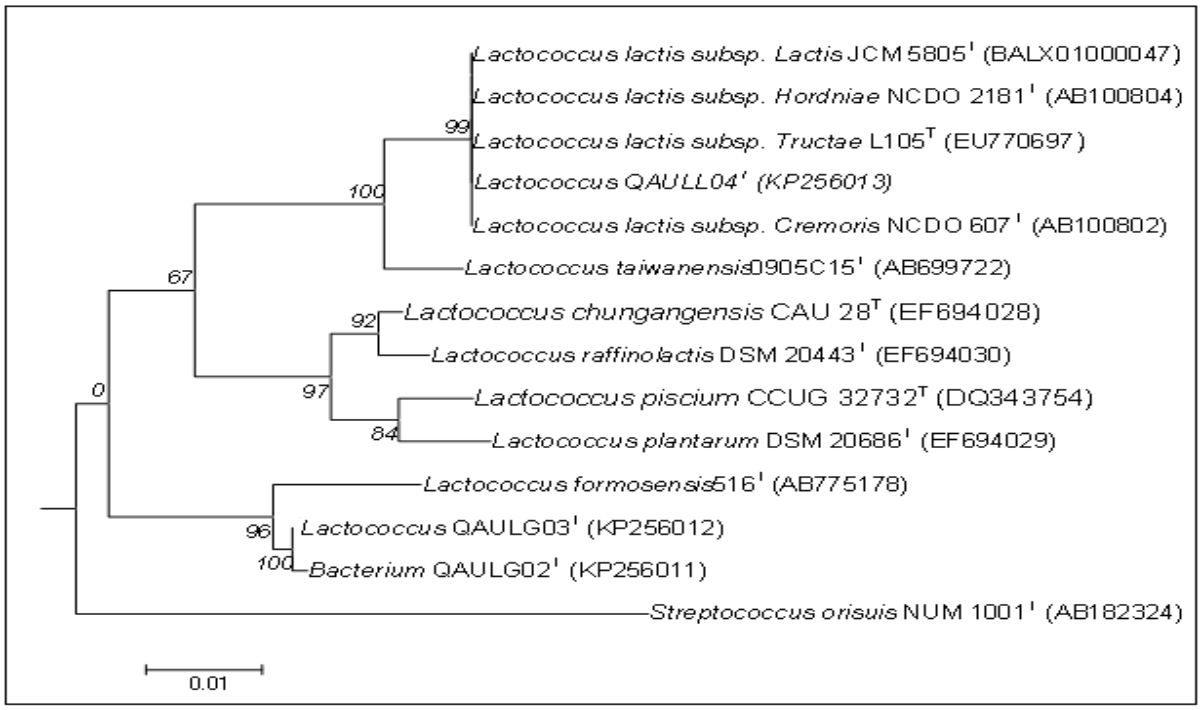

Fig. 2. Phylogenetic tree of the *Lactococcus* QAULL04 and QAULG02 species based on 16S rDNA gene sequence.

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 and Klaenhammer 1999).

Increased expression of general stress protein such as GroESL operon were also detected in low pH (Lorca, de Valdez *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 and Hill 2003).

Cholesterol assimilation

Cholesterol assimilation was also observed among all bacterial isolates of cow dung. All the strains significantly reduced cholesterol level when compared with the standard value. All the cow dung strainsshowed better cholesterol assimilation.*Lactococcus lactis ssp. lactis*QAULL04 has shown maximum cholesterol assimilation (64.44%) than*Enterococcus mundtii* QAUEM01 (50%). This reduction in cholesterol level is probably assumed due to the deconjugation of bile acids in the liver. (Liong and 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, Kutner *et al.* 1980).

It is reported that the cholesterol reduction is a consequence of deconjugation of bile salts (Fukushima and 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 and de Boer 1989, Tamai, Yoshimitsu *et al.* 1996). An *invitro* study demonstrated the cholesterol lowering effect by *L.fermentum* probiotics strain (Pereira, McCartney *et al.* 2003).

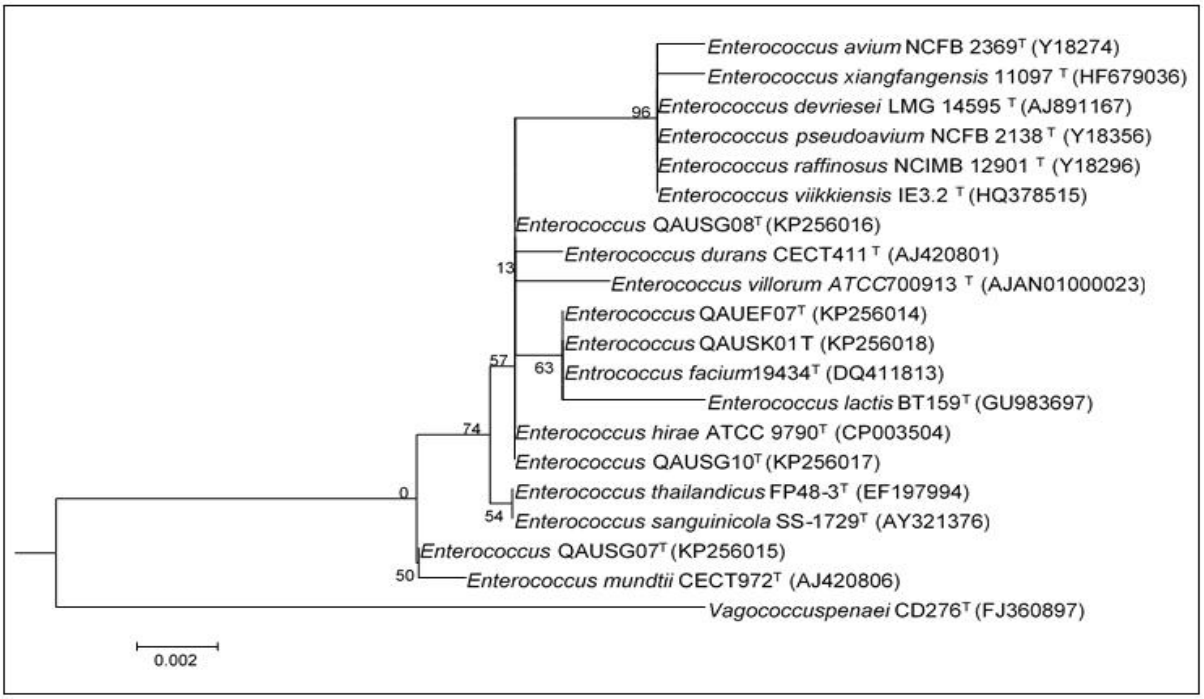

Fig. 3. Phylogenetic tree of the *Enterococcus* (KP256018) species based on 16S rDNA gene sequence.

One of a study also illustrated that some *Lactobacillus ssp.*, undergo cholesterol assimilation by *in-vitro* deconjugation of bile salts (Klaver and Vandermeer 1993).

Cell hydrophobicity

Cell hydrophobicity of cow dung isolates were measured as QAUEM01 has maximum value of 21.104% than QAULL04 (4.712%).

The protein molecules on the surface of microbes enhance the hydrophobicity activity whereas Lipopolysaccharide presence results in hydrophilic activity.

The charge and hydrophobicity have been recognized as important parameters for adhesion (Krepsky, Ferreira *et al.* 2003). However, the study described that hydrophobicity and charge are not required for microbe-surface interaction (Vacheethasanee, Temenoff *et al.* 1998).

Conclusion

Among nineteen bacterial strains, isolated from twelve Sahiwal cow dung samples, only *Lactococcus lactis*QAULL04 (KP256013) depicted better cumulative results for potential probiotic application in animal feed. *L. lactis*QAULL04 also exhibited strong proteolytic, amylolytic and lipolytic potential that can improve the digestibility in dairy cattle results in the feed efficiency (FE) and feed conversion ratio (FCR).*L. lactis*QAULL04 has also potential to assimilate 64.44% cholesterol which significantly contribute in health status and productivity of dairy

cattles.

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References

Bai S. 2012. Cellulase Production by Bacillus subtilis isolated from Cow Dung. Archives of Applied Science Research scholars research library **4(1),** 269-279.

Cebra JJ. 1999. e role of mucosal microbiota in the development and maintenance of the mucosal immune system. New York, Academic Press.

Cotta M. 1988. Amylolytic activity of selected species of ruminal bacteria. Applied and Environmental Microbiology **54(3),** 772-776.

Cotter PD, Hill C. 2003. Surviving the acid test: responses of gram-positive bacteria to low pH. Microbiology and Molecular Biology Reviews **67(3),** 429-453.
https://doi.org/10.1128/mmbr.67.3.429-453.2003

Cullimore R. 2008. Practical atlas for bacterial identification.
https://doi.org/10.1201/97814200879.87

Del Piano M. 2006. Probiotics: from research to consumer. Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver **38 Suppl 2,** S248-255.
https://doi.org/10.1016/s1590-8658(07)60004-8

Driessen FM, de Boer R. 1989. Fermented milks with selected intestinal bacteria: a healthy trend in new products.Netherlands Milk Dairy Journal **43,** 367–382.

Dunne C. 2001. In vitro selection criteria for probiotic bacteria of human origin: correlation with in vivo findings.The American Journal of Clinical Nutritin **73(2 Suppl),** 386S-392S.
https://doi.org/10.1093/ajcn/73.2.38.6s

Elmer GW. 1999. Survival of Saccharomyces. boulardii in the rat gastrointestinal tract and effects of dietary fiber.Microbial Ecology in Health and Disease **11,** 29–34.
https://doi.org/10.1080/089106099435.899

Fukushima M, Nakano M. 1996. Effects of a mixture of organisms, Lactobacillus acidophilus or Streptococcus faecalis on cholesterol metabolism in rats fed on a fat- and cholesterol-enriched diet. British Journal of Nutrition **76(6),** 857-867.
https://doi.org/10.1079/bjn19960.092

Fuller R. 1989. Probiotics in man and animals. The Journal of applied bacteriology **66(5),** 365-378.
https://doi.org/10.1111/j.1365-2672.1989.tb051.05.x

Guarner F, Malagelada JR. 2003. Gut flora in health and disease.The Lancet **361(9356),** 512-519.
https://doi.org/10.1016/s0140-6736(03)124.89-0

Hofmann AF, Mysels KJ. 1992. Bile-Acid Solubility and Precipitation Invitro and Invivo - the Role of Conjugation, Ph, and Ca2+ Ions. Journal of Lipid Research **33(5),** 617-626.

Holzapfel WH. 1998. Overview of gut flora and probiotics.International Journal of Food Microbiology **41(2),** 85-101.
https://doi.org/10.1016/s0168-1605(98)00.044-0

Johnsen H, Krause K. 2014. Cellulase activity screening using pure carboxymethylcellulose: application to soluble cellulolytic samples and to plant tissue prints. International journal of molecular sciences **15(1),** 830-838.
https://doi.org/10.3390/ijms15010830

Klaenhammer TR. 2012. The impact of probiotics and prebiotics on the immune system. Nature Reviews Immunology **12(10),** 728-734.
https://doi.org/10.1038/nri33.12

Klaver FAM, Vandermeer R. 1993. The Assumed Assimilation of Cholesterol by Lactobacilli and Bifidobacterium-Bifidum Is Due to Their Bile Salt-Deconjugating Activity. Applied and Environmental Microbiology **59(4),** 1120-1124.

Klein G. 1998. Taxonomy and physiology of probiotic lactic acid bacteria.International Journal of Food Microbiology**41(2),** 103-125. https://doi.org/10.1016/s0168-1605(98)00.049-x

Krepsky N. 2003. Cell surface hydrophobicity and slime production of Staphylococcus epidermidis Brazilian isolates. Current microbiology **46(4),** 0280-0286. https://doi.org/10.1007/s00284-002-386.8-5

Kullen MJ, Klaenhammer TR. 1999. Identification of the pH-inducible, proton-translocating F1Fo-ATPase (atpBEFHAGDC) operon of Lactobacillus acidophilus by differential display: gene structure, cloning and characterization. Molecular microbiology **33(6),** 1152-1161. https://doi.org/10.1046/j.1365-2958.1999.0155.7.x

Liong MT, Shah NP. 2005. Acid and bile tolerance and cholesterol removal ability of lactobacilli strains. Journal of Dairy Science **88(1),** 55-66. https://doi.org/10.3168/jds.s0022-0302(05)72.662-x

Lorca GL. 2002. Characterization of the protein-synthesis dependent adaptive acid tolerance response in *Lactobacillus acidophilus*. Journal of molecular microbiology and biotechnology **4(6),** 525-532.

Metchnikoff E. 1907. The prolongation of life. Optimisticstudies. London, UK, William Heinemann.

Mohamed SA. 2010. Production of cellulase in Low Cost Medium by Bacillus Subtilis KO strain. World Applied Sciences Journal **8(1),** 35-42.

Musa H. 2009. The potential benefits of probiotics in animal production and health. Journal of Animaland Veterinary Advances **8(2),** 313-321.

Nurmi E, Rantala M. 1973. New aspects of Salmonella infection in broiler production. Nature **241(5386),** 210-211. https://doi.org/10.1038/241210.a0

Parvez S. 2006. Probiotics and their fermented food products are beneficial for health. Journal of Applied Microbiology **100(6),** 1171-1185. https://doi.org/10.1111/j.1365-2672.2006.02963.x

Pereira DI, Gibson GR. 2002. Cholesterol assimilation by lactic acid bacteria and bifidobacteria isolated from the human gut.Applied and Environmental Microbiology **68(9),** 4689-4693. https://doi.org/10.1128/aem.68.9.4689-4693.20.02

Pereira DIA. 2003. An in vitro study of the probiotic potential of a bile-salt-hydrolyzing Lactobacillus fermentum strain, and determination of its cholesterol-lowering properties.Applied and Environmental Microbiology **69(8),** 4743-4752. https://doi.org/10.1128/aem.69.8.4743-4752.2.003

Razin S. 1980. Phospholipid and Cholesterol Uptake by Mycoplasma Cells and Membranes. Biochimica Et Biophysica Acta(BBA) – Biomembranes **598(3),** 628-640. https://doi.org/10.1016/0005-2736(80)900.42-5

Roos S. 2005. Lactobacillus gastricus sp nov., Lactobacillus antri sp nov., Lactobacillus kalixensis sp nov and Lactobacillus ultunensis sp nov., isolated from human stomach mucosa. International Journal of Systematic and Evolutionary Microbiology **55,** 77-82. https://doi.org/10.1099/ijs.0.6308.3-0

Senesi S. 2001. Molecular characterization and identification of Bacillus clausii Strains marketed for use in oral bacteriotherapy. Applied and Environmental Microbiology **67(2),** 834-839. https://doi.org/10.1128/aem.67.2.834-839.2001

Strohlein. 2003. Back to nature.Live yeasts in feed for dairy cows. DMZ,Lebensm. Ind.Milcheirtsh **124,** 68-71.

Takahashi M. 2004. The effect of probiotic treatment with Clostridium butyricum on enterohemorrhagic Escherichia coli O157:H7 infection in mice. Federation of European Microbiological Societies Immunology and Medical Microbiology**41(3),** 219-226. https://doi.org/10.1016/j.femsim.2004.03.010

Tamai Y. 1996. Effects of milk fermented by culturing with various lactic acid bacteria and a yeast on serum cholesterol level in rats. Journal of Fermentation and Bioengineering **81(2),** 181-182. https://doi.org/10.1016/0922-338x(96)87.601-x

Vacheethasanee K. 1998. Bacterial surface properties of clinically isolated Staphylococcus epidermidis strains determine adhesion on polyethylene. Journal of Biomedical Materials Research: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and the Australian Society for Biomaterials **42(3),** 425-432. https://doi.org/10.1002/(sici)1097-46.36(19981205)42:3<425::aid-jbm12>3.3.co;2-j

Wilson K. 2001. Preparation of genomic DNA from bacteria. Current protocols in molecular biology **56(1),** 2.4. 1-2.4. 5. https://doi.org/10.1002/0471142727.mb0204.s56