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IDENTIFICATION OF LACTOBACILLUS FROM SET YOGHURT SAMPLES AND DETERMINATION OF THEIR ACID TOLERANCE ACTIVITY

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ABSTRACT

Lactobacillus is the most commonly used probiotic live bacteria in the dairy industry, that have health benefits to the host when consumed in sufficient concentrations. The aim of this study was to identify lactobacillus from set yoghurt samples and to determine their acid tolerance activity. Lactobacillus was isolated from five set yoghurt samples cultured on MRS agar and subjected to Grams staining and catalase test. The identified isolates were then sub-cultured in MRS broth. DNA was extracted by boil cell and kit-based methods followed by quantification, presumptive lactobacillus was further identified by PCR. Then the acid tolerance activity of the bacterial isolates was assessed. Creamy/white, shiny, mucoid colonies having an entire margin with flat, raised and convex elevations observed on MRS agar, purple rod-shaped bacteria observed under Grams staining and no oxygen bubble formation was observed in catalase test indicating catalase negative. The biochemical test results confirmed that all the samples contained lactobacillus. Higher DNA concentration was obtained via boil cell method compared to kit method. PCR products were obtained for all the samples which further confirmed the presence of lactobacillus. Acid tolerance activity assay indicated no significant difference in survival of lactobacillus between 0 hours and 3 hours incubation at pH 3.0 indicating lactobacillus a has good acid tolerance activity. In conclusion, lactobacillus bacteria isolated from all samples survived at pH3.0 confirming acid tolerance property. Incorporating acid tolerant lactobacillus into food products may enhance health benefits.

Keywords: Probiotics, yoghurt, Lactobacillus, Biochemical characterization, Acid tolerance activity.

INTRODUCTION

probiotics

Awareness towards human nutrition over the past few decades has led to the rapid growth of probiotic industry (Sahadeva et al., 2011). Elie Metchnikoff father of probiotics, discovered that the Bulgarian bacillus present in fermented milk product yoghurt enhanced health benefit in a certain group of Bulgarian people (Metchnikoff, 1907). Probiotic is defined as, live microorganisms when administered in adequate amounts confer a health benefit to the host (Oyeniran et al., 2020). The health benefits of the probiotic bacteria to the host includes improvement of intestinal health, enhancement of immune response, reduction of serum cholesterol, antiallergic and anticancer effect and also can be used as therapeutics (Kechangia et al., 2013). The most common fermented milk probiotic sources are yoghurt, curd and cheese which are rich in lactobacillus spp, bifidobacterium spp and enterococcus spp (Sornplang and Piyadeatsoontorn, 2016).

Lactobacillus as a probiotic

Lactobacillus bacterium belongs to the genus of Lactic Acid Bacteria (LAB) and is a normal flora of mouth, intestine and female genital tract (Karami et al.,2017). Lactobacillus is a rod shaped, Gram-positive, non-spore forming, catalase negative, facultative anaerobic/ microaerophilic and acid tolerant bacteria (Huang et al., 2018; Chowdhury et al., 2012).

Probiotic lactobacillus is used in industrial and medical applications as they are Generally Considered As Safe (GRAS) (Kumar and Kumar,2014). The most common properties of an ideal probiotic lactobacillus include, antimicrobial activity, adhesion to epithelial cells, bile salts hydrolase activity, antioxidant activity, acid tolerance activity, resisting antibiotics and production of exopolysaccharides (Fijan, 2014: Kechagia et al.,2013).

Acid tolerance activity of probiotic lactobacillus

An effective probiotic bacterium must survive in the gastrointestinal tract (GIT) in order to confer health benefit to the host. The survival and viability of the probiotic lactobacillus after consumption remains obscure as they are subjected to unfavourable physiological conditions of the GIT, such as acidic environment (low pH), bile salt concentrations, proteolytic enzymes and pancreatic juices (Shuhadha et al., 2017: Wang et al., 2017). Probiotic lactobacillus possesses the following acid resistance mechanism in order to have sufficient survival and viability in the GIT such as neutralization processes, biofilm and cell density, proton pumps, protection of macromolecules, pre adaptation and cross protection (Hassanzadazour et al.,2012).

The acid tolerance assay helps to determine the viability and survival rate of probiotic bacteria at varying levels of acidic conditions (Fouchi et al., 2015). According to a study by Qian et al., 2017, showed that lactobacillus plantarum has high acid tolerance activity (68.05%) than lactobacillus bulgaricus (30.35%) at pH3.0 which were probiotic bacteria of yak yoghurt samples, this indicates that the probiotic lactobacillus can survive at low pH levels and confers health benefits to the host. The study carried out by Jatmiko, Horwath and Barton (2017), subjected 20 strains of lactobacillus plantarum present in fermented milk to two set of pH levels which were slightly acidic media (pH 5-6) and acidic media (pH2-4), the results obtained by the study showed that 35% out of 20 strains tolerated slightly acidic media but whereas 5% higher tolerance was observed in acidic media of pH 2-4. This indicates that the probiotic bacteria can survive the GI transit while resisting low pH levels of about pH 3.0 and enhance the health benefits to the host.

Set yoghurt as a source of Probiotic lactobacillus.

Yoghurt is a widely marketed milk fermented dairy product and is known for its taste, ease of production and the health benefits. Yoghurt is a good source of several micronutrients and consumption of it has shown to promote health status due to the presence of live probiotic bacteria such as streptococcus thermophiles and lactobacillus delbrueckii subsp as starter cultures (Oyeniran et al., 2020).

Metchnikoff (1907), discovered that the Bulgarian bacillus present in the fermented milk product yoghurt enhanced health in certain group Bulgarians, which is now characterised as lactobacillus bulgaricus. A study carried out by Ranasinghe and perera (2016) discovered, isolates cultured on MRS gar were all bacilli with long and rounded ends, indicating presence of lactobacillus bulgaricus in eight different yoghurt brands from different markets in Sri Lanka.

Significance of the study

Survival and viability of a probiotic bacteria is an important consideration because they must survive in food

supplements during the shelf life of the probiotic fermented milk product and in the unfavourable environment during the GIT transit by resisting low pH in order to actively elicit the probiotic functions of the bacteria and improve health status of the host. (Wang et al., 2017: Ranasinghe and perera ,2016).

This study aims to determine the acid tolerance activity of the probiotic bacteria in five different yoghurt samples and to identify which yoghurt samples have the potential probiotic lactobacillus bringing about health benefits to the host by maintaining their survival and activity at acidic environment of the GIT. The purpose of this study identify lactobacillus from set yoghurt samples and determine their acid tolerance activity. Therefore, the findings of this study will provide useful information to the dairy industry to develop new technologies to ensure the supply of high-quality milk products to the consumers

METHODOLOGY

Sample preparation and isolation of lactobacillus bacteria

Five set yoghurt samples were purchased from the local market. Two grams (2g) of each sample was homogenized into beakers labelled as A to E. A loop full of each sample was streaked on MRS agar using quadrant streaking method. The processed plates were then incubated for 48 hours at 37℃.

Biochemical characterization of presumptive lactobacillus

A presumptive isolated lactobacillus colony was selected and a thin smear was prepared and gram staining procedure was followed. Purple rod-shaped bacteria were observed.

In catalase test the presumptive colony was treated with 3% H2O2 and observed for evolution of bubbles.

Subculture

The isolated presumptive lactobacillus confirmed via catalase and grams staining were then sub cultured in 35ml of MRS broth. These were incubated at 37℃ for 48 hours

DNA extraction

The DNA extraction procedure was carried out for all samples along with the positive control via Qiagen D Neasy Blood and Tissue Kit and boil cell method (Abdulamir et al., 2010). The DNA extracts were then stored at 4℃ until further use.

DNA quantification

Absorbance of DNA was measured using UV visible spectrophotometer at 260nm. The following equation was used to calculate DNA concentration (Abdulamir et al., 2010).

DNA concentration (µg ml^{-1}):

$$\frac{(A260\ in\ OD\ units \times 50 \mu g\ ml^{-1} \times DF)}{1000}$$

Identification of lactobacillus using genus specific PCR assay.

The DNA were amplified using genus specific primers. The components of a PCR mix contained 5 µl of PCR buffer, 1.5 µl of MgCl2, 0.5 µl of dNTPs, 2.5 µl of forward ad reverse primers 0.25 µl of taq polymerase , 10.75 µl of distilled water and 1 µl of DNA sample. The total volume of a PCR mix was 24 µl. The PCR tubes were then subjected to the following thermal cyclic conditions in the PCR reaction initial denaturation at 94℃ for 3 minutes, denaturation at 94℃ for 1 minute, annealing at 62℃ for 1 minute, extension at 72℃ for 2 minutes, and final extension at 72℃ for 12 minutes. The steps of denaturation, annealing and extension was repeated for 35 cycles.

Visualization of PCR fragments by agarose gel electrophoresis

The PCR amplicons were analysed by electrophoresis on a 1% agarose gel and was visualized using the gel documentation system.

Acid tolerance assay

3-5 ml of overnight bacterial culture was centrifuged at 3000rpm for 5 minutes. Then the cell pellet was resuspended in 2ml of peptone water, the suspension was mixed well and 1ml of suspension was transferred into two tubes labelled as of pH 3.0 and 7.2. The pH was adjusted to 3.0 by addition of HCL. pH 7.2 was used a control. Suspensions of both pH was incubated for 0hours and 3hours. After incubation, all samples of both pH were subjected to 1:10 dilution. Absorbance was measured at 600nm in duplicates and average absorbance was calculated (Azat *et al*., 2010).

Statistical analysis

The results of DNA quantification and acid tolerance assay was analysed by oneway ANOVA in SPSS software. P value less than 0.05 was considered as statistically significant.

RESULTS

First streak on MRS agar

Morphological observation of bacterial colonies on MRS agar after 48-hour incubation.

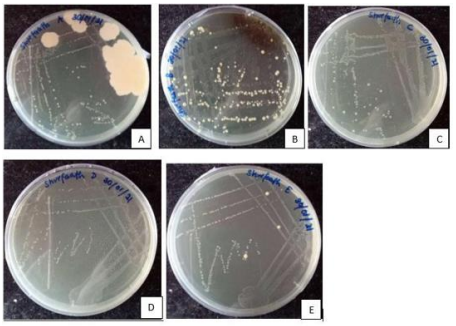

Figure 1: Bacterial growth on MRS agar

Creamy/white, shiny, mucoid colonies having an entire margin with flat, raised and convex elevations were observed in all five (5) MRS agar plates

Gram's staining

Biochemical identification of presumptive lactobacillus isolates

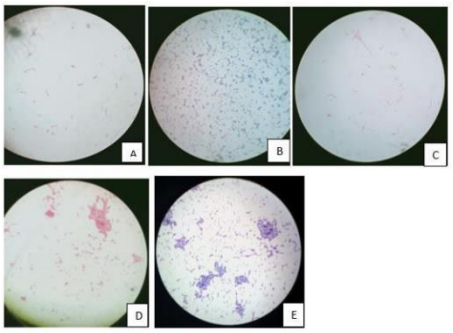

Figure 2: Gram's staining images of presumptive lactobacillus bacteria (100X).

Purple rod-shaped gram-positive bacteria were observed in all the samples.

Catalase test

Biochemical identification of presumptive lactobacillus isolates

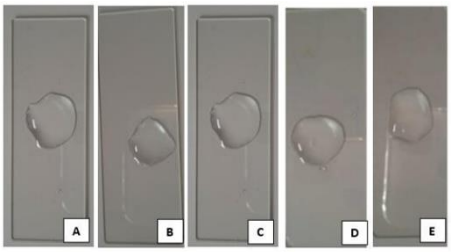

Figure 3: Catalase test results of presumptive lactobacillus isolates

No O2 bubbles were formed in all the samples, indicated the bacterial isolates were catalase negative.

According to Gram's staining and catalase test results all the samples may contain lactobacillus.

DNA concentration

Comparison of DNA concentrations of boil cell and kit based extraction methods.

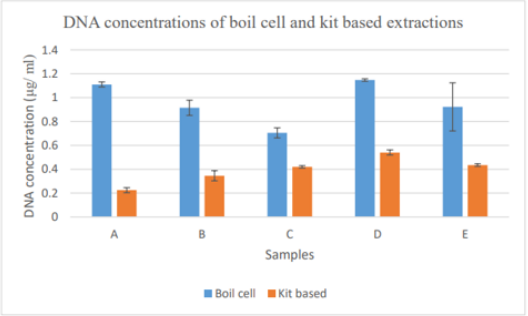

Figure 4 : The graph showing bacterial DNA concentration of boil cell and kit based extraction methods .

According to above figure 4 the DNA concentration obtained via boil cell extraction method is higher than kit-based extraction.

Table 1: The oneway ANOVA output in the comparison of DNA concentration.

ANOVA

Concentration

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	804289.600	1	804289.600	35.570	.000
Within Groups	180892.800	8	22611.600		
Total	985182.400	9			

P value is less than 0.05 therefore there is a significant difference between DNA concentrations of the boil cell method and kit based.

PCR gel images

Visualization of PCR products under UV using the gel documentation system after gel electrophoresis.

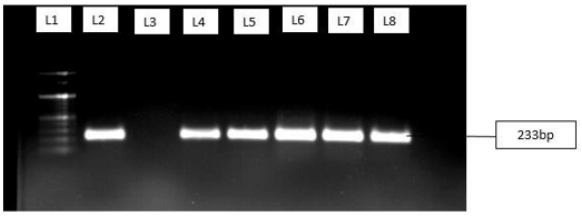

Figure 5: Gel images of PCR amplicons of boiled cell method (loading order: L1- 100bp DNA ladder, L2- positive control, L3- negative control, L4-8- samples A-E).

According to figure 5 all samples (L4-8) along with the positive control (L2) yielded a PCR product of 233bp long but no band was observed for negative control.

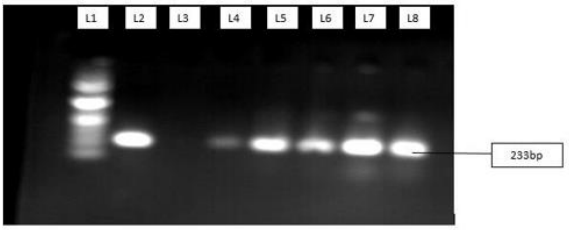

Figure 6: Gel images of PCR amplicons of kit based method (loading order: L1- 100bp DNA ladder, L2- positive control, L3- negative control, L4-8- samples A-E).

According to figure 6 all samples (L4-8) along with the positive control (L2) yielded a PCR product of 233bp long but no band was observed for negative control

Acid tolerance

Acid tolerance activity was assessed for all samples as PCR confirmed the presence of lactobacillus

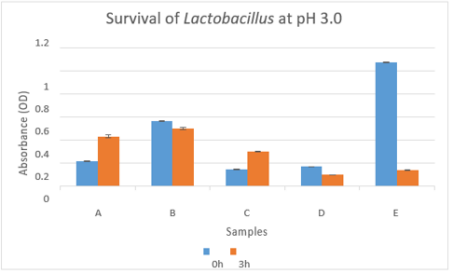

Figure 7: The graph showing the survival of lactobacillus at pH3.0 in two-time interval

According to figure 7 samples A and C shows an increase in survival of the bacteria after 3 hours but whereas samples B, D and E shows a decrease in survival.

Table 2: The oneway ANOVA output of the survival of lactobacillus at pH 3.0 at two time intervals

ANOVA

pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.098	1	.098	1.175	.293
Within Groups	1.503	18	.084		
Total	1.601	19			

P value is higher than 0.05 therefore there is no significant difference in survival of lactobacillus between 0 hours and 3 hours at pH 3.0

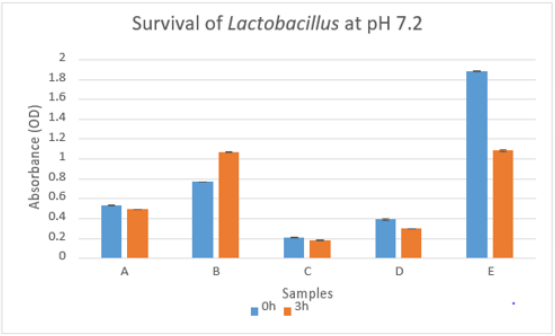

Figure 8 : The graph shows survival of lactobacillus at pH 7.2(control).

According to figure 8 the survival of lactobacillus at pH 7.2 is similar between the two-time intervals.

DISCUSSION

In this study lactobacillus bacteria were isolated from set yoghurt samples and was investigated for their acid tolerance activity. In the initial step of isolation, a highly selective culture media was used which is MRS agar. The colony morphology observed was of creamy/white, shiny, mucoid colonies having an entire margin with flat, raised and convex elevations as shown in figure 1. These observations were in concurrence with the observation of a previous study by Chowdury et al.,2012 indicating the identification of this morphology as presumptive lactobacillus.

The presumptive lactobacillus was then subjected for biochemical characterization by Gram's staining and catalase test. The results observed for the presumptive isolates in the current study was purple rod shaped bacteria and no oxygen bubble formation in Gam's staining and catalase test respectively as shown in figures 2 and 3. Gram's staining was performed in order to distinguish between gram positive and gram negative bacteria and catalase test was carried out to observe whether oxygen bubbles are formed when reacted with hydrogen peroxide (Kumar and Kumar,2014). A study carried out by Mannan et al., 2017 had proven that lactobacillus is a purple rod shaped gram positive bacteria. Observations of the study by Goyal et al., 2012 had proven that there was no oxygen bubble production from lactobacillus during catalase test. No oxygen bubble formed during catalase test is due to lack of catalase enzyme that converts hydrogen peroxide into water and oxygen (Ismail, Yulvizar and Mazhitor,2019).

The statistical analysis in comparison of DNA concentration between the two extraction methods indicates, the p value (0.00) less than 0.05 therefore there is a significant difference in DNA concentrations of the DNA extracts between boil cell and kit based method. In figure 4 shows that DNA concentration of all samples are higher in boil cell method extracts than in kit based method, this confirms the DNA concentration of boil cell method is significantly higher than the kit based concentration which is indicated by the statistical analysis shown in table 1. A similar observation was seen in a previous study conducted by Yahya, Firmansyah and Arlisyan ,2017 states that the DNA obtained through conventional method gave very high yield and was of good quality in comparison to DNA obtained via kit based extraction. The study carried out by Dimitrakopoulou et al.,2020, assessed comparison of DNA yield and purity between DNA extraction methods, the data obtained by the study

proved that boil cell method was the most efficient and produced DNA of high concentration in comparison to other kit based extraction methods.

Polymerase chain reaction was carried out to further confirm presence of lactobacillus. According to the PCR gel images shown in figures 5 and 6 the positive control and all five samples yielded a 233bp long PCR product, confirming the presence of probiotic lactobacillus in all yoghurt samples. The absence of a band in the lane-3 of negative control indicates no contamination. The genus specific primers LactoF and LactoR are highly specific for all lactobacillus producing an amplicon size of 233bp (Byun et al., 2004) The confirmation of the isolates as lactobacillus by the genus specific PCR led to the next step in determining the acid tolerance activity of the bacterial isolates. pH is an important factor which can dramatically affect bacterial growth and survival.

The statistical analysis of acid tolerance activity in table 2 shows that p value (0.293) is higher than 0.05 therefore there is no significant difference in survival of lactobacillus bacteria between 0hours and 3hours at pH3.0. According to figure 7 the absorbance of lactobacillus in samples A and C is higher in 3 hours than in 0hours, but whereas the absorbance of bacteria is lower in samples B, D and E after 3-hour incubation period, this difference in survival is not statistically significant. A higher absorbance in samples A and C may be due to presence of high number lactobacillus after 3hours indicating growth and survival compared to 0hours. In the survival of lactobacillus at pH 7.2 the control a similar absorbance value was obtained in samples A, C and D with a slight decrease after 3hours, but whereas in sample B a higher absorbance and in sample E a lower absorbance value was obtained.

However, the absorbance value obtained at pH 3.0 and pH 7.2 indicated that the lactobacillus bacteria were viable in all samples after 3-hour incubation. To be used as probiotic, organisms have to tolerate low pH of human gut. The isolated Lactobacillus spp. can tolerate a wide range of pH (1-9) and grow well at acidic pH (1-5) (Chowdury et al.,2012). The study conducted by Hassanzadazar et al., 2012 examined survival of probiotic lactobacillus at pH 3.0 between 1 and 3-hour incubation period, the results of the study showed similar absorbance values between 1 hour and 3 hours, indicating similar pH tolerance between two incubation periods and no significant growth of bacteria was observed, but it confirmed that lactobacillus could survive at pH 3.0 after 3-hour incubation.

According to a study by Shuhadha et al.,2017 states that results obtained showed the isolates had an ability to grow at pH 3.0, but however viability of isolates decrease with time and most of the isolates showed no significant growth at pH 3.0 after 5 hours of incubation. The results obtained by the current study has a similar pattern to the data of pre published study mentioned above, showing that isolates of all 5 samples showed viability at pH 3.0 after 3-hour incubation but a decreased viability and no significant growth was observed. The study by Hashemi et al.,2014 determined the exposure of lactobacillus to acidic pH levels 2.0 and 3.0 for a 2-hour incubation period, it was observed that lactobacillus cells incubated at pH 3.0 showed higher resistance to acid giving a high colony count than in pH 2 acidic condition. The probiotic bacterial cell count decreases on exposure to PH 3.0 after 3hour incubation but whereas exposure to pH 7.2 (control) has a fairly constant count (Sahadeva et al., 2011). The above mentioned pattern was also observed in the current study as shown in figure 8 which shows a high and fairly similar absorbance value at pH 7.2 between the two time intervals in comparison to figure 7 which shows a

lower absorbance value at pH3.0 after 3 hour incubation but this difference at pH 3.0 is not statistically significant.

The acid tolerance activity results obtained of the current study showed that the lactobacillus present in all samples survived the low acidic pH level 3.0 after 3-hour incubation indicating viability of the bacterial cells but no significant growth was observed it could be due to decrease in bacterial count. The decrease in cell count is due to failure of acid resistance mechanism of lactobacillus in maintaining internal pH and the internal acidification leads to decreased enzyme activity and protein, DNA damage leading to cell death (Ng et al.,2015) Consumption of probiotic bacteria along with food or dairy products increases the stomach pH to 3.0 or higher, this increase in pH levels of the stomach environment enhances viability of probiotic lactobacillus and increase survival and colonization in the GI tract confirming health benefit to the host (Hashemi et al.,2014). The survival and viability of probiotic bacteria in acidic environment of the stomach is achieved due to acid resistance mechanisms as mentioned earlier . However, acid tolerance activity of probiotic bacteria in vitro cannot determine the performance of the bacteria in situ due to other physiological factors that might affect the survival and viability (Sahadeva et al., 2011). Survival of a lactobacillus at pH 3.0 for 2hours is one of the requirements for bacteria to be considered as a potential probiotic (Ng et al.,2015).

CONCLUSION

This study aimed to isolate probiotic lactobacillus from set yoghurt samples. The biochemical characterization tests confirmed the presence of lactobacillus which was further proved by the PCR amplicons yielding a 233bp long product. The boil cell method was proved to be a better extraction procedure since it produced a greater yield compare to kit-based method. The lactobacillus isolates of all samples confirmed the acid tolerance activity by surviving at a low pH of 3.0. Determination of acid tolerance activity is the most common and preliminary selection criteria for a probiotic bacterium.

REFERENCE

Abdulamir, A.S., Yoke, T.S., Nordin, N. and Abu-Bakar, F. (2010) 'Detection and quantification of probiotic bacteria using optimized DNA extraction, traditional and real-time PCR methods in complex microbial communities', African Journal of Biotechnology, 9(10), pp. 1481-1492 [Online] Available

at http://www.academicjournals.org/AJB (Accessed: 12 November 2020)

Azat, R., Liu, Y., Li, W., Kayir, A., Lin, D., Zhou, W. W., and Zheng, X.D. (2016) 'Probiotic properties of lactic acid bacteria isolated from traditionally fermented Xinjiang cheese', Journal of Zheijiang University,17(8), pp. 597-609 [Online] DOI: 10.1631%2Fjzus.B1500250 (Accessed: 12 November 2020)

Chowdhurry, A., Hossain, M.N., Fakruddin, M. and Mostazir, N.J (2012) ' Screening of lactobacillus spp from Buffalo Yoghurt for probiotic and antibacterial activity ', bacteriology and parasitology, 3(8), pp.1-6 RESERACHGATE [Online] DOI: 10.4172/2155-9597.1000156 (Accessed: 12 November 2020)

Dimitrakopolou, M.E., Stavrou, V., Kotsalou, C. and Vantarakis, A. (2020)' Boiling exctraction method VS commercial kits of bacterial DNA isolation from food samples', Journal of food science and nutrition research, 3 (4) ,pp.311-319 [Online] DOI: 10.26502/jfsnr.2642-11000057 (Accessed:1 May 2021).

Fijan, S. (2014) 'Microorganisms with Claimed Probiotic Properties: An Overview of Recent Literature', International Journal of Environmental Research and Public Health, 11(5), pp.4745–4767 [Online] DOI: 10.3390/ijerpH110504745 (Accessed: 12 November 2020)

Fouchi, V., Pertronio, G.P., Lissandrello, E. and Furneri, P.M. (2015) ' Evaluation of resistence to low pH and bile salts of Human Lactobacillus spp. Isolates', International Journal of immunopathology and pharmacology,

28(3), pp.426-433. [Online] DOI: 10.1177/039463201550948 (Accessed: 22 November 2020)

Goyal, R., Dhingra, H., Bajpai, P. and Joshi, N. (2012) 'Characterization of the Lactobacillus isolated from different curd samples', African Journal of Biotechnology, 11(79), pp.14448- 14452 [Online] DOI: 10.5897/AJB11.310 (Accessed: 23 April 2021)

Hashemi, S.M.B., Shahidi, F., Mortazavi, S.A., Milani, E. and Eshagi, Z. (2014) 'Potentially Probiotic Lactobacillus Strains from Traditional Kurdish Cheese', probiotics and antimicrobial properties, 6, pp.22-31.[Online] DOI: 10.1007/s12602-014-9155-5 (Accessed:1 May 2021)

Hassanzadazar, H., Ehsani, A., Mardani, K. and Hesari. J (2012) 'Investigation of antibacterial, acid and bile tolerance properties of lactobacilli isolated from Koozeh cheese', veterinary research forum, 3(3), pp.181-185 PUBMED [Online]. DOI: 10.5713/ajas.15.0849 (Accessed: 18 November 2020)

Huang, C.H., Li, S.W., Huang, L. and Watanabe, K. (2018) 'Identificationand classification for the lactobacillus casei group', front microbiology, 9, pp1-13. [Online] DOI: 10.389/fmicb.2018.01974 (Accessed: 22 November 2020)

Ismail, Y.S., Yulvizar, C. and Mazhitov, B. (2019) ' Characterization of lactic acid bacteria from local cows milk kefir', earth and environmental science, 130, pp.1-9 [Online] DOI: 10.1088/1755-1315/130/1/012019 (Accessed:1 May 2021).

Jatmiko, Y.D., Howarth, G.S. and Barton, M.D. (2017) ' assessement of probiotic properties of lactic acid bacteria isolated from Indonesian naturally fermented milk', AIP, pp.1-15 [Online] DOI: 10.106/1.5012732 (Accessed:1 May 2021).

Karami, S., Roayaei, M., Hamzavi, H., Bahmani, M., Hassanzad-Azar, H., Leila, M., Kopaei, M.R (2017) ' Isolation and Identification of probiotic Lactobacillus from local diary and evaluating their antagonistic effect on pathogens', International Journal of PHarmaceutical Investigation, 137(41), pp.1-5 WOLSTER KLUWER [Online] DOI : 10.4103/jpHi.JPHI_8_17 (Accessed: 12 November 2020)

Kechagia, M., Basoulis, D., Konstantopoulou, S.,Dimitradi, D., Gyftopoulou, K., Skarmoutsou, N. and Fakiri, E.M (2013) ' Health Benefits of Probiotics ', ISRN nutrition, pp.1-8 [Online] DOI:10.5402/2013/481651 (Accessed: 12 November 2020) Kumar, A. and Kumar, D. (2014) 'Isolation and characterization of bacteria from dairy samples of solan in Himachai Pradesh for identification of Lactobacillus spp', International journal of pHarmaceutical sciences, 25(2), pp.110-114. [Online] Available at : www.globalresearchonline.net (Accessed: 12 November 2020)

Mannan, S.J., Rezwan, R., Rahman, S. and Begum, K. (2017) 'Isolation and Biochemical Characterization of Lactobacillus species from Yogurt and Cheese samples in Dhaka Metropolitan Area', Bangladesh Pharmaceutical Journal, 20(1), pp.27-33 [Online] Available at: www.banglajol.info (Accessed: 23 April 2021) Metchnikoff, E. (1907) 'The prolongation of Life' [Online] Available at: https://openlibrary.org/books/OL14036242M/The_prolongation_of_life (Accessed: 22 November 2020)

Ng, S.Y., Koon, S.S., Padam, S.S. and Chye, F.Y. (2015) ' Evaluation of probiotic potential of lactic acid bacteria isolated from traditional fermented Bambangan (Magifer pajang)', Journal of food , 13(4), pp.563-572 [Online] DOI: 10.1080/19476337.2015.1020342 (Accessed:1 May 2021).

Oyeniran, A., Zimmerman, T., Aljaloud, S., Krastanov, A. and Ibrahim, S. (2020) ' Probiotic Characteristics and Health benefits of yoghurt Bacterium Lactobacillus delbrueckii sp. Bulgaricus', Journal of Dairy Research, 87(1), pp.123-126. INTECHOPEN [Online] DOI: 10.5772/intechopen.86939 (Accessed: 12 November 2020).

Qian, Y., Long, X., Pan, Y., Li, G. and Zhao, X. (2018)' Identofication and isolation of Lactic acid bacteria (Lactobacillus Plantarum YS2) from Yak yogurt and its probiotic properties' Biomedical Research, 29(4), pp.815-820. [Online] DOI: 10.4066/biomedicalresearch.29-17- 3418 (Accessed: 22 November 2020) Ranasinghe, J.G.S. and Perera, W.T.R. (2016) 'Prevalence of lactobacillus bulgaricus and streptococcus thermopHiles stability in commercialy available yogurts in Sri Lanka' Asian Journals of Medical Sciences, 7(5), pp.97-101. [Online] DOI:10.3126/ajms.v7i5.14326 (Accessed: 22 November 2020

Sahadeva, R.P.K., Leong, S.F., Chua, K.H., Tan, C.H., Tong, E.V., Wong, S.Y.W. and Chan, H.K.(20110 ' Survival of commercial probiotic strains to PH and bile salts', International Food research Journal, 18(4), pp.1515-1522 [Online] Available at: www.ifrj.upm.edu.my/18%20(04)%2020 11/(44)IFRJ-2011-285.pdf (Accessed: 22 November 2020)

Shuhadha, M.F.F., Panagoda, G.J., Madhujith, T. and Jayawardena, N.W.I.A. (2017) 'Evaluation of probiotic attributes of lactobacillus sp. Isolated from cow and buffalo curd samples collescted from kandy', Ceylon medical journals, 62, pp.159-166. [Online] DOI:10.4038/cmj.v62i3.8519 (Accessed: 22 November 2020)

Sornplang, P. and Piyadeatsoontorn, S. (2016) ' probiotic isolates from unconventional sources', journal of anmal science and technology, 58, pp.1-26. PUBMED. [Online] DOI: 10.1186/s40781-016-0108-2 (Accessed: 22 November 2020)

Wang, C., Cui, Y. and Qu, X. (2017) 'Mechanism and improvement of acid resistance in lactic acid bacteria', Archives Microbiology, 200(2), pp.1-7 SPRINGER. [Online] DOI:10.1007/s00203-1446-2 (Accessed: 22 November 2020)

Yahya, A., Firmansyah, M. and Arlisyah, A. (2017)' 'Comparison of DNA Extraction Methods Between Conventional, Kit, Alkali and Buffer-Only for PCR Amplification on Raw and Boiled Bovine and Porcine Meat', journal of life science, 7(2), pp.1-6 [Online] DOI: 10.21776/ub.jels.2017.007.02.09 (Accessed:1 May 2021)