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DETERMINATION OF ANTIBACTERIAL ACTIVITY OF LACTOBACILLUS IN DRINKING YOGURT SAMPLES

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ABSTRACT

Probiotics are live bacteria that provide health benefits to the host when consumed in required amounts. Lactobacillus is the commonest genus of probiotic organism found in most of the fermented foods and dairy products. This project was intended to isolate Lactobacillus from the commercially available yogurt drink samples and to estimate the antibacterial activity. The process was initiated by culturing five different yogurt drink samples on MRS agar and the presence of pure Lactobacillus colonies was identified by biochemical tests. Afterwards the antibacterial activity against Escherichia coli and Staphylococcus aureus was detected using well-diffusion method for Lactobacillus cell-intact and cell-free samples. According to the results, a proper bacterial growth was observed in all five samples on MRS agar but the biochemical tests confirmed the presence of Lactobacillus only in four samples. According to the results, antibacterial activity against Escherichia coli and Staphylococcus aureus were confirmed as statistically significant (p-value < 0.05). Findings of this study are useful in developing treatments for infectious diseases.

Keywords: Probiotics, Lactobacillus, Yogurt, Antibacterial activity

INTRODUCTION

Probiotics are viable non-pathogenic microorganisms that are used in various food products to improve nutrition and to prevent various disease conditions. This concept was initially published by Elie Metchnikoff in 1907 that explained the ingestion of living organisms which favorably alters the gastrointestinal microflora (Gogineni et al., 2013). In 2001, The Food and Agriculture Organization and World Health Organization defined probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Hill et al., 2014).

Lactic acid bacteria are commonly known probiotic organisms that produce lactic acid during fermentation process that contain various species such as Lactobacillus (Karami et al., 2017). These bacteria can be introduced to the human gut by the consumption of dairy products and other fermented foods (Negi et al., 2018). Lactobacillus is the largest genus of lactic acid bacteria (LAB) that consists of 196 published species. These species are commonly found in fermented foods and also gastrointestinal and vaginal tracts of both humans and animals (Huang et al., 2018). Lactobacillus is long, straight, and slender rod-shaped bacteria with the size of 1.0 – 10.0 micrometers in length. They are vegetative cells which mean that they do not sporulate and form spores (Arasu et al., 2015).

The colonies of these bacteria on agar are usually 2 -5 micrometers with convex, entire, opaque, and without pigments (Knox and Holmwood, 1967). Lactobacillus is non-motile but rarely shows motility by peritrichous flagella. They are facultative anaerobes or microaerophilic organotrophs because

they can grow either in an aerobic or anaerobic condition (Fijan, 2014). S-layer has been found in some species of Lactobacillus which are outermost proteinaceous envelope structure. Also they have a long anionic polymers containing thick peptidoglycan cell wall that consists of teichoic acids (Silhavy, Kahne and Walker, 2010).

Lactobacillus is Gram's positive bacteria due to its thick cell wall. They are catalase-negative. Therefore, they will not produce oxygen radical from hydrogen peroxide. Also they are not producing oxidase and urease enzymes. Lactobacillus do not reduce nitrates as well as they are H2S-negative, indole-negative, and gelatin hydrolysis negative (Aryal, 2019). Lactobacillus is a homofermentative bacterium because, it converts sugar completely into lactic acid in an anaerobic condition (Karami et al., 2017). Lactobacillus containing foods are being used in the therapeutic field due to their antimutagenic, anticarcinogenic, hypocholesterolemic, anti-hypertensive, anti-osteoporosis, and immune-modulatory effects (Hawaz, 2014). US Food and Drug Administration and European Food Safety Authority approved that the LAB species are generally regarded as safe to be consumed (Guo et al., 2017).Yogurt drink is a dairy product rich in Lactobacillus species. Nowadays, nutritional and therapeutic properties of yogurt drinks are very impressive (Islam et al., 2016). Regular intake reduces the excessive fat and reduces the probability of having atherosclerosis, heart diseases, and hypertension also they increase the digestive capability (Mercenier et al., 2003).

Probiotic properties of Lactobacillus are the most important characteristics to enhance the health benefits such antibacterial activity, antioxidant activity, cholesterol reducing ability, acid and bile tolerance (Ramila et al., 2016). Lactobacillus species produces bactericidal bioactive agents that control the growth of pathogenic and spoilage microorganisms (Maragkoudakis et al., 2006). The most important antibacterial mechanisms are increased adhesion to intestinal mucosa, enrichment of the epithelial barrier, and simultaneous inhibition of pathogen adhesion. It also produces anti microorganism substances and stimulates the immune system (Brito et al., 2012).

Lactobacillus produces organic compounds and antimicrobial substances such as bacteriocins, hydrogen peroxide, diacetyl, bactericidal proteins and reutericyclin. Organic acids such lactic acid, acetate, formic acid, and caproic acid have a strong inhibitory effect against other pathogens especially Gram-negative bacteria (Akpinar, Yerlikaya and Kilic, 2011).Bacteriocins are biologically active, low molecular-weight proteins including lactacin B and nasin inhibit the growth of a variety of pathogenic bacteria (Brito et al., 2012). These bacteria have an ability to interact with dendritic cells (DCs), macrophages and lymphocytes thereby they enhance the immune response of the host to kill harmful pathogens such as Escherichia coli, Staphylococcus aureus, Clostridium difficile and Helicobacter pylori (Petti et al., 2008).

This study aimed to identify Lactobacillus and to analyze its antibacterial activity against harmful pathogens. The findings of this study may benefit the society by providing the information regarding the antibacterial activity of Lactobacillus species in commercially available yogurt drinks. Therefore, this study can be helpful in the future to discover effective medicines or treatments instead of using antibiotics to treat life-threatening disease.

METHODOLOGY

Sample Collection and Preparation

Five different commercially available yogurt drink samples were purchased from local market and labeled from A to E. 13 mL of yogurt drink sample was used for culturing.

The aseptic conditions were maintained for all the following techniques.

Isolation of Lactobacillus

A loop full of yogurt drink sample was streaked on the prepared De Man, Rogosa and Sharpe (MRS) agar plates using quadrant method. The streaked plates were incubated at 37℃ for 48 hours.

Biochemical tests

After 48 hours of incubation, colony morphologies were investigated and marked to perform biochemical tests on each colony.

Gram's staining: After 48 hours incubation, one drop of distilled water was mixed with a loop of bacterial inoculum on the glass slide to make a thin smear and the smear was heat fixed. Dried smear was stained with Crystal violet for 60 seconds, Gram's iodine for 90 seconds, Gram's decolourizer for 5 seconds and finally with Safranin for 20 seconds in order. After each steps of staining, the smear was gently washed with running water. The smear was air dried and observed under microscope at 100X.

Catalase test: A small amount of bacteria was mixed with a drop of 100% Hydrogen peroxide on a glass slide and observed for bubble formation.

Acid-fast staining: A thin bacterial smear was prepared and heat fixed. Dried smear was initially covered with Carbol fuchsin stain and heated until the vapour begins. The heated stain was allowed to stay for 5 minutes and washed with water. The smear was covered with 20% sulfuric acid for 5 minutes followed by Methylene blue for 2 minutes and the stain was washed with running water. The smear was allowed to air dry and observed under 100X.

Endospore staining: A thin smear was prepared and heat fixed aseptically. Filter paper was placed on the dried smear and Malachite green was added. The stain was heated and allowed to stay for 5 minutes. The paper was carefully removed. The smear was allowed to cool down for 2 minutes and washed with water. The smear was stained with Safranin for 2 minutes and washed with running water. The smear was air dried and observed under 100X.

Subculturing to the MRS broth

Isolated pure Gram-positive and catalase-negative colonies from four samples were subcultured in 10 mL MRS broth and incubated at 37°C for 24 hours and stored at 4°C.

Determination of the Antibacterial activity (Ramila et al., 2016)

Escherichia coli and Staphylococcus aureus were subcultured in Nutrient broth. Lactobacillus cell-intact and cell-free samples were prepared. The Escherichia coli and Staphylococcus aureus test suspensions were swabbed equally on the separate nutrient agar plates and four wells were made. 50µL of Gentamicin solution (positive control), 100 µL of cell-intact, 100 µL of cell-free suspension, and 100 µL of autoclaved distilled water (negative control) was added to the wells respectively and incubated for 24 hours at 37℃. The inhibition zones were measured.

DATA ANALYSIS

Antibacterial activity of cell-intact and cell-free suspensions of Lactobacillus against Escherichia coli and Staphylococcus aureus were statistically analyzed using one-way ANOVA in SPSS software.

RESULTS

Isolation of Lactobacillus on MRS agar

The colony formation of bacteria on MRS agar after 24 hours of incubation is shown below (Figure 1).

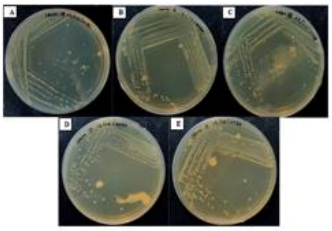

Figure 1: Colony formation of yogurt drink samples (A-E) on MRS agar plates after 48 hours of incubation.

Creamy coloured, round, entire in margin and raised colonies were observed in all five samples. A contamination was observed only in sample D.

Gram's staining

The Gram's staining results are shown below (Figure 2).

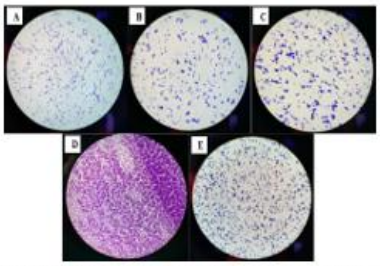

Figure 2: Gram's staining results of isolated colonies from cultured yogurt drink samples (A-E) (x100).

Purple coloured (Gram-positive) rod-shaped bacteria were observed in all samples except sample D. Purple coloured cocci (Gram-positive) were observed in sample D.

Catalase test

The catalase test results are shown below (Figure 3).

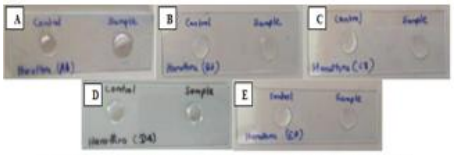

Figure 3: Catalase test results for selected colonies from cultured yogurt drink samples (A-E).

Bubble formation was not (catalase-negative) observed in sample A, B, C, and E but bubbles were observed in sample D (catalase-positive).

Endospore staining

The Endospore staining results are shown below (Figure 4).

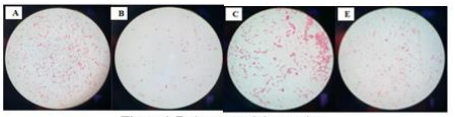

Figure 4: Endospore staining results.

Brownish pink colored (no endospores) rod-shaped bacteria were observed in sample A, B, C, and E. Green coloured spores were not observed.

Acid-fast staining

The Acid-fast staining results are shown below (Figure 5).

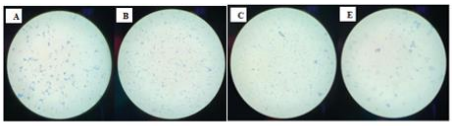

Figure 5: Acid-fast staining results.

The bacterial cells were appeared in pale blue colour (acid-fast negative) in sample A, B, C, and E.

According to the biochemical assay results, all samples except sample D were assumed to contain Lactobacillus.

Determination of the Antibacterial activity against Escherichia coli

The inhibition zones of Escherichia coli (Figure 6) are shown below

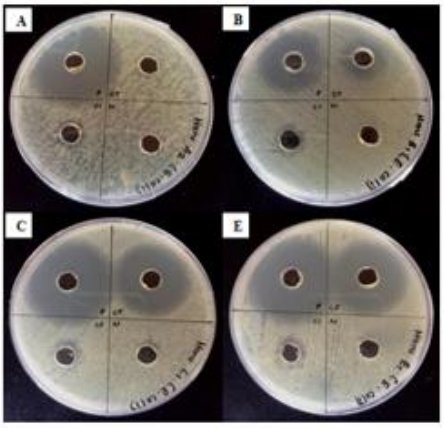

Figure 6: Antibacterial activity test results against *Escherichia coli*.

The inhibition zones were observed for positive control and cell-intact for all samples, but inhibitions zones were observed for cell-free suspensions for all samples except sample A. Zones were not observed for any negative controls and also contaminations were not observed in any samples.

Table 1: Inhibition zones values against Escherichia coli.

Samples	Positive control (mm)	Cell Intact (mm)	Cell Free (mm)	Negative control (mm)
A	40.00±01.00	09.00±00.00	00.00±00.00	00.00±00.00
B	39.50±00.50	10.67±00.60	26.67±00.50	00.00±00.00
C	39.33±00.60	11.60±00.10	31.70±00.60	00.00±00.00
E	39.67±01.10	12.23±00.10	32.03±00.10	00.00±00.00

Highest inhibition zone diameters were observed for cell-free than cell-intact in all four samples except sample A. Highest diameter was observed in sample E for both cell-intact and cell-free. Inhibition zones were not observed for any negative controls (Table 1).

Determination of the Antibacterial activity against Staphylococcus aureus

The inhibition zones of Staphylococcus aureus are shown below (Figure 7).

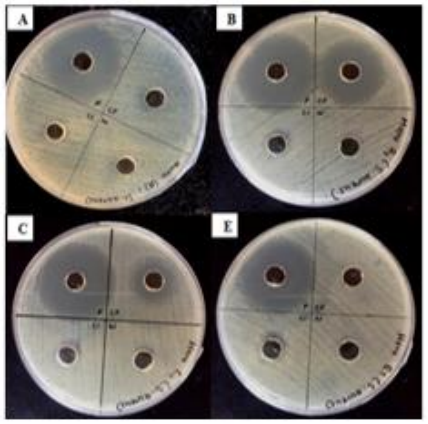

Figure 7: Antibacterial activity test results against *Staphylococcus aureus*.

The inhibition zones were observed for positive control and cell-intact for all samples, but inhibitions zones were observed for cell-free suspensions for

only sample B and C. Zones were not observed for any negative controls and also contaminations were not observed in any samples.

Table 2: Inhibition zones values against Staphylococcus aureus.

Samples	Positive control (mm)	Cell Intact (mm)	Cell Free (mm)	Negative control (mm)
A	34.47±00.11	09.00±00.50	00.00±00.00	00.00±00.00
B	33.67±00.11	09.33±00.50	11.33±00.60	00.00±00.00
C	34.50±00.09	10.00±00.00	14.34±00.50	00.00±00.00
E	34.33±00.06	10.33±00.60	00.00±00.00	00.00±00.00

Highest diameters were observed for cell-free than cell-intact in sample B and C. Highest diameter was observed in sample E for cell-intact and sample C for cell-free. Inhibition zones were not observed for any negative controls (Table 2). According to Table 1 and Table 2, cell-free suspensions were found to have larger inhibition zones for Escherichia coli compared to Staphylococcus aureus.

DATA ANALYSIS

The comparison of the mean inhibition zones of is shown below.

Table 3: Comparison of the inhibition zones of cell-intact and cell-free samples against Escherichia coli.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig
Between Groups	4.351	1	4.351	13.998	.010
Within Groups	1.865	6	.311		
Total	6.216	7			

In *Table 3,* the P-value was observed <0.05 that indicates a significant difference between the inhibition zones of cell-intact and cell-free against *Escherichia coli.*

Table 4: Comparison of the inhibition zones of cell-intact and cell-free samples against *Staphylococcus aureus.*

ANOVA

	Sum of Squares	df	Mean Square	F	Sig
Between Groups	.151	1	.151	12.381	.013
Within Groups	.073	6	.012		
Total	.225	7			

In Table 4, the P-value was observed <0.05 that indicates a significant difference between the inhibition zones of cell-intact and cell-free against Staphylococcus aureus.

Table 5: Comparison of the mean inhibition zones of cell-intact samples.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig
Between Groups	.080	1	.080	22.018	.003
Within Groups	.022	6	.004		
Total	.102	7			

In Table 5, the P-value was observed <0.05 that indicates a significant difference between the inhibition zones of cell-intact against Escherichia coli and Staphylococcus aureus.

Table 6: Comparison of the mean inhibition zones of cell-free samples.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig
Between Groups	4.651	1	4.651	28.503	.002
Within Groups	.979	6	.163		
Total	5.630	7			

In Table 6, the P-value was observed <0.05 that indicates a significant difference between the inhibition zones of cell-free against Escherichia coli and Staphylococcus aureus.

DISCUSSION

Lactobacillus is the main type of probiotic bacteria found in dairy products with tremendous amount of health benefits. Yoghurt drink is a probiotic rich food that helps to reduce disease conditions (Brito et al., 2012). This study was carried out to isolate Lactobacillus from yoghurt drink samples and to evaluate the antibacterial activity of Lactobacillus bacteria.

MRS agar was used for the isolation of Lactobacillus from yoghurt drink samples that provides nutrition by peptones, glucose, and beef extract whereas magnesium and manganese sulfates stimulates the growth. Especially, ammonium citrate allows the Lactobacillus growth by inhibiting the cultivation of other organisms (Tharmarajah and Shah, 2003). The study carried by Kumar and Kumar (2014) and confirmed the creamy coloured, round in form, entire in margin and raised in elevation colonies of Lactobacillus on MRS agar. Similarly, the Figure 1 showed the same morphologies in all five samples (A, B, C, D, and E) that indicated the growth of probiotics. Also a contamination was observed in sample D because MRS agar is a glucose rich agar that facilitates the growth of both yeast and fungal contaminations (Pereira et al., 2012).

Biochemical tests were performed for the detection of pure Lactobacillus colony and to subculture it for further studies. Gram's staining is a differential staining method that helps to differentiate Gram positive and Gram negative bacteria. Gram positive bacteria contain a thick cell wall made up of 90% of peptidoglycan layer. The study by Matheson (1999) showed that the peptidoglyacan layer consists of teichoic acid that prevents the decolourization that stains the Gram-positive bacteria in purple colour due to crystal violet-iodine complex. According to the Figure 2, Gram-positive bacilli was observed in sample A, B, C, and E whereas Gram-positive cocci was observed in sample D. The Lactobacillus is a Gram-positive bacillus that confirms the presence of Lactobacillus in sample A, B, C, and E. Gram-positive cocci could be the presence of Streptococcus, Enterococcus or Lactococcus (Abouloifa et al., 2019).

Catalase test indicates the presence of catalase enzyme that produces oxygen and H2O from hydrogen peroxide (H2O2) that produces bubbles of oxygen when a small inoculum is introduced to H2O2. Lactobacillus species do not produce catalase enzyme and release oxygen from H2O2 (Aryan, 2019). The catalase test showed the presence of bubble formation in only sample D that confirms the presence of catalase-positive bacteria in sample D. The bubble formation was not observed in sample A, B, C, and E that confirms the presence of catalase-negative bacteria Lactobacillus (Figure 3).

Endospore staining indicates the endospore formation in the bacteria. Malachite green mainly binds to the spore wall firmly than the cell wall. Therefore the study by Petti et al., 2008 confirmed that when washing the smear with water, the stained cell wall gets decolourized and gets stained by safranin in brownish pink colour but the spore remains in green colour. According to Figure 4, pink colored rod-shaped bacteria were observed in sample A, B, C, and E and green coloured spores were not observed in any sample. Therefore the results confirmed that the samples were only containing non spore-forming vegetative bacteria which are Lactobacillus.

Acid-fast staining differentiates acid-fast bacteria which consist of a waxy mycolic acid cell wall. These cells can be only stained by aniline dyes with the heat application (Bayot and Sharma, 2018). According to this study, pale blue coloured bacteria were observed in all four samples that confirm that the presence of non-acid fast Lactobacillus in sample A, B, C, and E (Figure 5). All the above biochemical tests confirmed the presence of Lactobacillus in all the samples except sample D.

The probiotic compounds such as lactic acid, acetic acid, H2O2, and antimicrobial peptides produced by these bacteria influence the survival of other pathogens (Chang et al., 2019). According to Dasari et al. (2014), the cell-free suspension of Lactobacillus had a high antibacterial activity than the cell-intact. Similarly in this study, cell-free suspension against Escherichia coli and Staphylococcus aureus of all the samples were observed with a larger inhibition zone compared to the cell-intact (Figure 6 and Figure 7). The cell-free was prepared by breaking the bacterial cell. Therefore, testing the antibiotic compounds mainly lactic acid directly on the harmful pathogen is more effective than introducing the whole bacterial cell (Dasari et al., 2014). The cell-free of sample A did not inhibit Escherichia coli whereas sample A and E did not inhibit Staphylococcus aureus. Therefore, it showed that the pathogens are resistant to antibacterial compounds in sample A or have no antibacterial activity against both pathogens (Chang et al., 2019).

According to the one-way ANOVA results, a significant difference ($P<0.05$) between the antibacterial activity of cell-

intact and cell-free against Escherichia coli was observed in Table 3. The Table 4 showed a significant difference between the antibacterial activity of cell-intact and cell-free against Staphylococcus aureus. The Table 5 showed a significant difference between the antibacterial activity of cell-intact samples against Escherichia coli and Staphylococcus aureus. The Table 6 showed a significant different between the antibacterial activity of cell-free samples against Escherichia coli and Staphylococcus aureus. Especially, the cell-free suspension of sample C and E confirmed with a highest antibacterial activity against Escherichia coli whereas sample B showed against Staphylococcus aureus.

CONCLUSION

In conclusion, out of all five yogurt drink samples, only four samples were confirmed with the presence of Lactobacillus. Sample C and E reported to have the highest antibacterial activity against Escherichia coli whereas sample B showed against Staphylococcus aureus. Cell-free suspension of Lactobacillus showed a high antibacterial activity than the cell-intact against both Escherichia coli and Staphylococcus aureus. This study helps to understand the importance of the antibacterial activity of Lactobacillus in yogurt drink samples that reduces the infections causes by Escherichia coli and Staphylococcus aureus species. Therefore, the results confirm that the cell-free suspension of Lactobacillus is a great option to use as a therapeutic compound against infectious diseases.

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