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Identification of *Lactobacillus* from commercial yogurt drink products and determination of their resistance to hydrogen peroxide

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Abstract

The genus *Lactobacillus* contains 315 species, and several of these have been identified as probiotics which are found in yogurt and yogurt-based drinks. This study was conducted to identify *Lactobacillus* genus from yogurt drink products and to determine its resistance to H_2O_2. Five yogurt drink samples were cultured in MRS agar and subjected to biochemical tests such as gram staining, endospore staining, acid-fast staining and catalase test. Thereafter, H_2O_2 resistance was determined by H_2O_2 resistance assay and it was statistically analysed using One-Way ANOVA from the SPSS Statistical Software. Creamy colour circular colonies in MRS agar plates, purple colour rod-shaped gram-positive bacteria in Gram staining, red colour non spore forming bacteria in endospore staining, non-acid-fast blue colour bacteria in acid-fast staining and absence of oxygen bubbles during catalase test confirmed the presence of *Lactobacillus* in all five samples. In H_2O_2 resistance assay, when comparing the H_2O_2 resistance of *Lactobacillus* at 0 and 6 hours, three samples indicated resistant to H_2O_2 following the 6 hours incubation period. One-Way ANOVA analysis indicated there was a statistically significant difference between 0 and 6 hours in H_2O_2 resistance. The biochemical tests confirmed the presence of *Lactobacillus* in all five samples. The H_2O_2 resistance assay confirmed the resistance of *Lactobacillus* to H_2O_2 in certain samples. Findings of this study emphasize the importance of screening *Lactobacillus* for H_2O_2 resistance to achieve its health benefits.

Keywords: *Lactobacillus,* probiotics, yogurt drink, H_2O_2 resistance

1. Introduction

The concept of probiotics evolved during the 20th century. It was based on a hypothesis presented by Elie-Metchnikoff, a Nobel Prize-winning scientist from Russia, claimed that consuming fermented yogurt products can lead to a long and healthy life. He believed that this activity could help decrease the harmful microbial population in the gut.[1] The term probiotic was first introduced by Stillwell and Lilly in 1965 to describe substances that are produced by a single microorganism and stimulate the growth of another.[2] They were then refined by Fuller in 1989 as microbial cultures that are beneficial to the host and can improve the balance of the intestinal microbial population.[3] In 2001, an expert committee of the World Health Organization and Food and Agriculture Organization of the United Nations adopted a definition of probiotic that stated they are live microorganisms that provide health benefit to the host when consumed in appropriate quantities.[4] A probiotic strain is expected to possess characteristics such as tolerance to gastric acid and bile, which is important during oral administration,[5] adherence to mucosal and epithelial surfaces (a crucial feature for effective immune modulation, pathogen competitive exclusion, and suppressing pathogen adhesion and colonization),

antimicrobial activity against pathogenic bacteria and to exhibit bile salt hydrolase activity.[6]

The microbes must be regarded as Generally Recognized as Safe (GRAS) status against pathogens in order to be used as probiotics.[7] The probiotic microorganisms that are most commonly used in human nutrition are *Lactobacillus* and *Bifidobacterium*.[8] However this research is focused on *Lactobacillus*, which falls under the GRAS criteria.[9]

The genus *Lactobacillus* is classified under phylum Firmicutes, class Bacilli, order II Lactobacillales, and family Lactobacillaceae.[10] They use carbohydrate in a fermentation process, with lactic-acid being the key product.[11] *Lactobacillus* are characterized as facultatively-anaerobic, catalase-negative, Gram-positive, non-spore-forming rods that often grow better under microaerophilic conditions.[12] *Lactobacilli* are chosen as probiotics as they exhibit a number of vital characteristics, including survival through the gastrointestinal-tract by being high tolerant to acid and bile-salts, ability to adhere to gastrointestinal surfaces to competitively exclude pathogens,[13] withstanding low acidic pH, inhibiting potentially pathogenic species (antimicrobial activity), resisting antibiotics, producing exopolysaccharides, normalizing cholesterol levels and withstand commercial manufacturing processes.[14-16]

Lactobacilli have been used in food-fermentation such as in yogurt, cheese, sour dough and pickles.[17] Yogurt drink is categorized as stirred yogurt with a low viscosity.[18] It supplies the body with liquid and nutrition in an easily absorbing form and it is high in protein, magnesium, potassium, vitamin B12 and calcium.[19-20] Kownacki *et al*[19] indicates yogurt drink kept the volunteers hydrated, reduces body temperature, sweat rate and hormonal stress. Kang, Kim and Kim, [21] observed the presence of *Lactobacillus* in yogurt drink. This research is focused in the identification of *Lactobacillus* from yogurt drink products and determination of their resistance to H_2O_2.

1.1 Importance of Lactobacilli resistance to H_2O_2. In the mammalian colon, a part of gastrointestinal-tract,[22] a single cell-thick continuous layer of epithelial cells forms the physical barrier between the body and the gut lumen, a compartment that is highly populated with environmental microbes and other foreign and potentially harmful substances. Thereby, colonic epithelial cells generate extracellular H_2O_2 in response to injury or gut microbes.[23] H_2O_2 production is also a capacity of several *Lactobacilli* such as *Lactobacillus acidophilus*, *Lactobacillus gasseri* and *Lactobacilli johnsonii*.[24] Unsuccessful colonization of *Lactobacillus* in gastrointestinal-tract and accumulation of H_2O_2 may occur as *Lactobacillus* lacks the H_2O_2 scavenging enzymes, such as catalase.[25] H_2O_2 is harmful as it can be easily converted into the highly reactive free hydroxyl radical by interacting with trace iron in the cell through Fenton reaction[26] which leads to protein, DNA, and lipid damage as well as cell death.[27] However, *Lactobacilli* has alternative mechanisms to prevent and resist H_2O_2 toxicity and reduce the occurrence of Fenton reaction to protect the cell against such failures.[26-28] Manganese containing pseudocatalase (Mn-Kat) has been discovered in *Lactobacillus plantarum*, which serves as the catalytic active site to catalyze the disproportionation of the toxic oxygen metabolite H_2O_2, into oxygen and water.[29-30] Heme dependent catalase (Heme-Kat) enzyme identified in *Lactobacillus sakei*, is able to incorporate iron atoms, thereby reducing the iron available to Fenton reaction thus preventing the formation of hydroxyl radical.[31-32] The Glutathione system in *Lactobacillus fermentum*, oxidizes Glutathione to a disulfide (by glutathione peroxidase) which then rapidly reduces back to glutathione by Gluathione reductase (GSH-r) in order to maintain a redox environment and to detoxify H_2O_2.[33]

Additionally, in *Lactobacillus plantarum* TrxB1 gene encodes for Thioredoxin Reductase (TrxR). The thioredoxin system is composed of NADPH, TrxR, and thioredoxin.[34] Thioredoxin system provides electrons to peroxidises to remove reactive oxygen species from H_2O_2.[35] Furthermore, Hydrogen peroxide resistance gene (hprA1) in *Lactobacillus casei* and *Lactobacillus paracasei* encodes hprA1 protein which binds to iron in the cell, thus preventing the formation of a hydroxyl radical (through the fenton reaction).[36] Moreover, NADH peroxidase gene (npr) encodes for NADH peroxidase which is a major H_2O_2 degrading enzyme in *Lactobacillus casei*. It reduces H_2O_2, to water and oxygen.[37] Thereby, using such alternative mechanisms *Lactobacillus* ensures its colonization and viability under oxidative stress environmental conditions such as in Gastro Intestinal Tract (GIT) and provides complete health benefits. The purpose of this study was to identify *Lactobacillus* in yogurt drink samples and analyze their H_2O_2 resistance and therefore its survival in the GIT can be identified. Further, the resistant *Lactobacillus* strains can be incorporated in yogurt drink manufacturing processes to achieve its health benefits.

2. Methodology

2.1 Sample preparation. Five different commercial yogurt drink samples were purchased from local market.

2.2 Culturing of samples. A sterile loopful of sample was cultured on De Man Rogosa and Sharpe (MRS) agar using quadrant streaking method and placed in the incubator at 37°C for 48 hours to visualize colonies.

2.3 Gram staining. Gram staining was carried out on heat fixed bacterial smear prepared from cultivated colonies from MRS agar. Few drops of crystal violet were added onto the slide and air dried for 60 seconds and the excess was washed off. Drops of Grams iodine was added and was air dried for 60 seconds. The excess was washed out. Thereafter, drops of decolourizer were added to wash the excess stain. Then it was rinsed with distilled water. Drops of Safranin were added and air dried for 60 seconds. The slide was then rinsed with distilled water, left to air dry and it was observed under the microscope at 100x with immersion oil.

2.4 Acid fast staining. A thin smear was prepared and heat fixed. The smeared slide was then flooded with carbolfuchsin and heated it using a spirit lamp until a visible steam was rising. The slide then rinsed with distilled water. The slide then was flooded with acid alcohol for 15 seconds, methylene blue for 60 seconds and rinsed with distilled water after each step. The slide was observed under microscope at 100x magnification.

2.5 Endospore staining. A thin smear was prepared and heat fixed. The smear was covered with a piece of filter paper and placed over a small beaker of boiling water. The slide was flooded with malachite green for 5 minutes. Slide then moved from the beaker, allowed to cool and the filter paper was removed. Drops of safranin were added to the slide and it was rinsed with distilled water after each step. The slide was then observed under microscope at 100x magnification.

2.6 Catalase test. Using a sterile inoculation loop a drop of autoclaved distilled water and same colony was picked, and placed onto labelled glass slide. Then, few drops of 3% H_2O_2 were added to slide, and observations were noted.

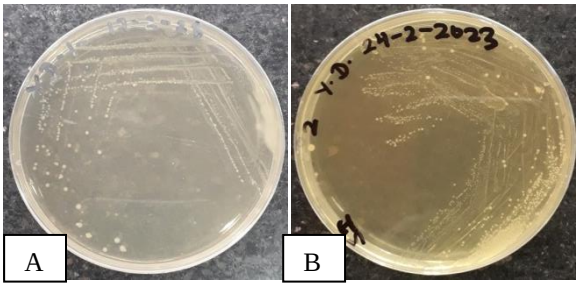
2.7 Sub-culturing. A loopful of isolated *Lactobacillus* colony from the MRS agar plates were sub-cultured in 30ml of MRS broth in a falcon tube. The sub-cultures were incubated at 37°C for 48 hours.

2.8 Hydrogen peroxide resistance assay Two centrifuge tubes were labelled for each sample as control and test. To these tubes, 3ml of MRS broth sample was added. Tubes were centrifuged for 10 minutes at 3000rpm. The supernatant was discarded and the pellet was then re-suspended in 6ml of saline solution. Cell density was checked at 600mn in UV spectrophotometer. The samples were diluted accordingly to obtain an absorbance of 0.2nm. It was centrifuged at 3000rpm for 1 minute. Saline was discarded and 10ml of fresh MRS broth was added. 36.28µL of 32mM H_2O_2 was added to the test tubes and 36.28µL of MRS broth was added to the control tubes. 3ml of the sample was added to a plastic cuvette and triplicates of absorbance reading at 600nm were recorded at 0 hours. The tubes were then placed in incubator for 6 hours and the UV spectrophotometer readings at 600nm were obtained again. [38]

2.9 Statistical/data analysis. 0 hours absorbance readings and 6 hours absorbance readings were statistically compared using one-way ANOVA in SPSS software. P value < 0.05 was considered statistically significant.

3. Results

3.1 Colony morphology. Yogurt drink samples streaked on MRS agar and incubated at 37˚C for 48 hours.



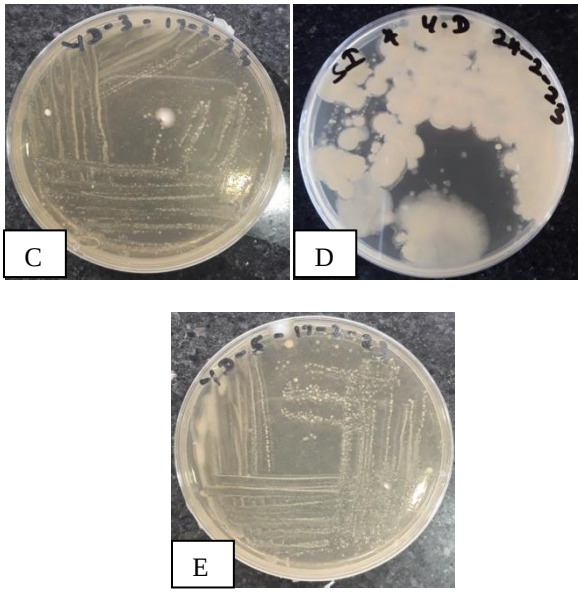
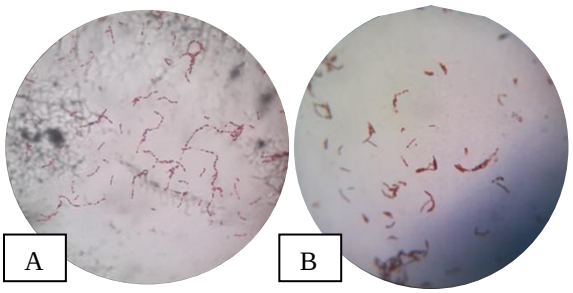


Figure 1. Bacterial growth of samples A - E on MRS agar after 48 hours incubation.

As shown in Figure 1 creamy colour, circular, entire, relatively small colonies were observed in MRS agar. Overgrowth was observed in sample D.

3.2 Gram staining. The colonies from MRS agar were subjected to Gram staining. As shown in the Figure 2 isolated bacteria were found to show morphology of rod shape and purple in colour.



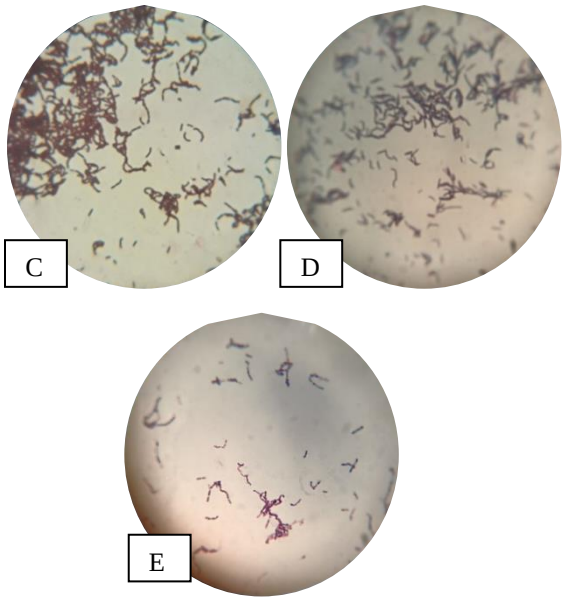
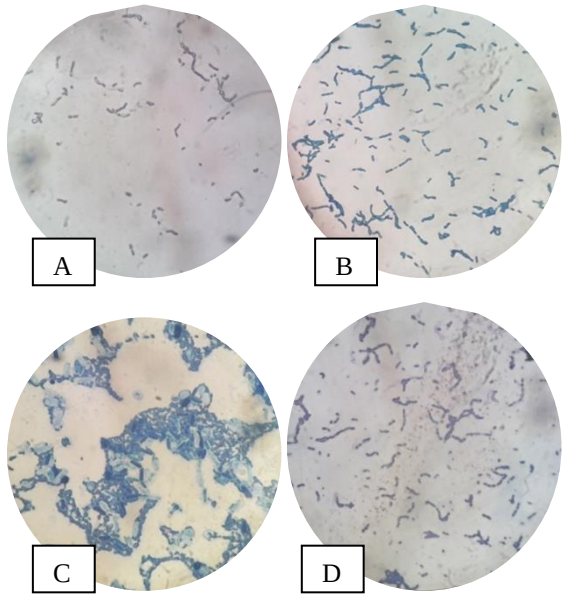


Figure 2. Bacterial identification by Gram staining images samples A - E under compound light microscope at 100 × magnification.

3.3 Acid fast staining. The colonies from MRS agar were subjected to Acid-fast staining.



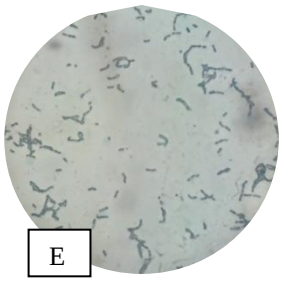


Figure 3. Bacterial identification by Acid fast staining images samples A – E under compound light microscope at 100× magnification.

The bacterial isolates appeared in blue colour rod shaped as shown in Figure 3.

3.4 Endospore staining. The colonies from MRS agar were subjected to Endospore staining.

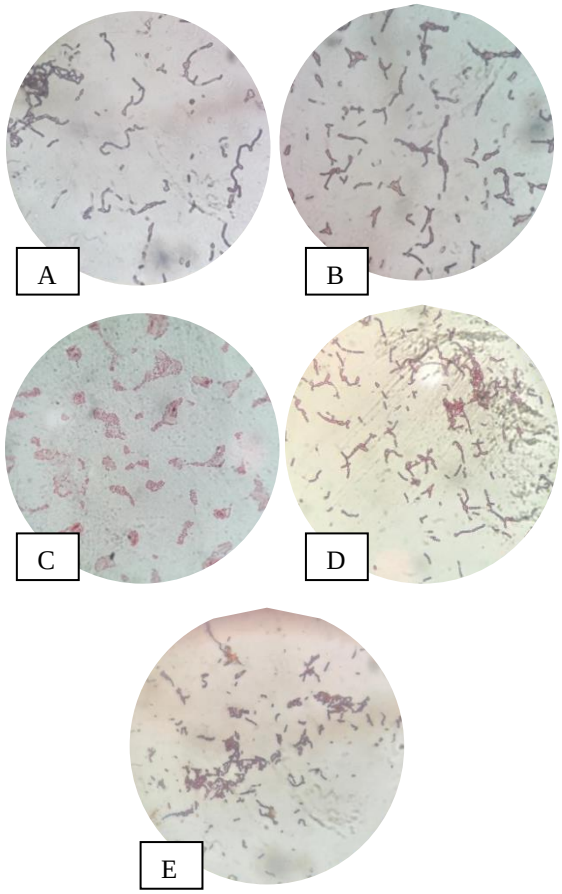


Figure 4. Bacterial identification by endospore staining images samples A – E under compound light microscope at 100× magnification.

The isolated bacteria were non spore forming, thereby appeared in red colour rod shaped as shown in Figure 4.

3.5 Catalase test. The colonies from MRS agar were subjected to catalase test detect presence or the absence of catalase enzyme.

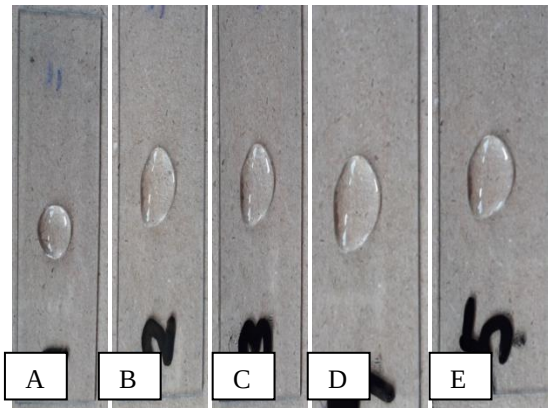


Figure 5. Catalase test results of samples A – E

As shown in Figure 5, by the absence of bubbles and effervescence, negative catalase test was observed in all the five samples.

3.6 Sub culturing. Colonies from MRS agar were sub cultured for further use.

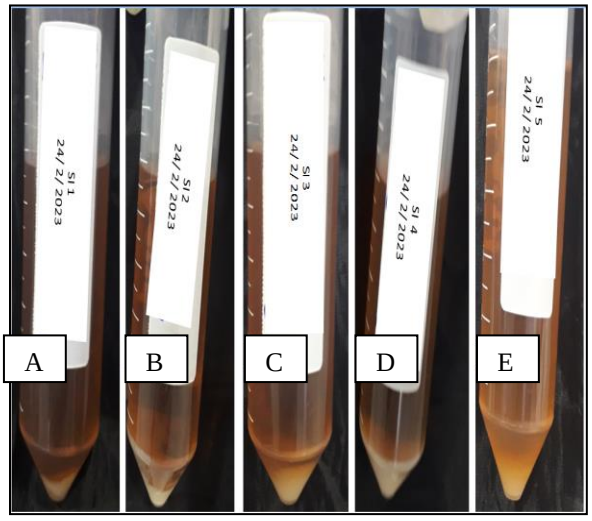


Figure 6. Bacterial sub-cultures of samples A – E on MRS broth after 48 hours incubation at 37˚C

As shown in Figure 6 high turbidity was observed in the broth and creamy colour colonies were observed in the bottom of the broth.

3.7 H_2O_2 resistance assay. The absorbance reading for H_2O_2 resistance assay is mentioned below in Table 1.

Table 1. H_2O_2 resistance of *Lactobacillus* at 0 hours and 6 hours

Sample	Absorbance readings for 0 hours (OD)	Absorbance readings for 6 hours (OD)
1	0.046 ± 0.0015	0.034 ± 0.0020
2	0.148 ± 0.0005	0.106 ± 0.0005
3	0.071 ± 0.0028	0.092 ± 0.0055
4	0.077 ± 0.0015	0.106 ± 0.0147
5	0.055 ± 0.0005	1.885 ± 0.0411

According to Table 1 sample 1 and 2 shows a decline in absorbance following 6 hours incubation period. Sample 3, 4, and 5 shows an increase in absorbance following the 6 hours incubation period.

3.8 Statistical/data analysis. Statistical significance was determined by one-way ANOVA analysis comparing 0 hours and 6 hours absorbance.

Table 2. Comparison of H_2O_2 resistance of *Lactobacillus* at 0 hours and 6 hours

ANOVA					
Hydrogen peroxide resistance					
	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	3.784	4	.946	4.700	.006
Within Groups	5.032	25	.201		
Total	8.816	29			

As shown in Table 2 when comparing the H_2O_2 resistance of *Lactobacillus* at 0 hours and 6 hours, it has a p – value of 0.006 which is less than 0.05. It indicates that there is a statistically

significant difference between 0 hours and 6 hours of H_2O_2 resistance.

4. Discussion

The genus *Lactobacillus* contains 315 species, and several of these bacteria have been identified as probiotics. These can be found in yogurt-based products.[39,40] The present study was focused to identify *Lactobacillus* from yogurt drink samples and to determine its resistance to H_2O_2, which aids its survival in gastrointestinal tract. As shown in Figure 1 isolates were subjected to grow on selective MRS agar media and produced round shape, off-white to cream color entire margin colonies those similar to the *Lactobacillus* grown on MRS agar media as previously reported by Chowdury *et al.*[41] Further biochemical tests such as gram staining, endospore staining, acid fast staining and catalase test were performed to confirm the presence of *Lactobacilli* as in Kumar and Kumar.[42]

As shown in Figure 2 upon Gram staining, isolates were found to be rod shaped, purple colour positive in Gram reaction which exhibited similar characteristics of *Lactobacillus* in Kumar and Kumar.[42] The principle of gram staining depends on the presence of thick peptidoglycan cell wall of *Lactobacillus* and its ability to bind to basic dyes (violet crystals) even after washing with decolourizer and counter stain safranin.[43] *Lactobacilli* are nonspore-forming.[44] Under the microscope, endospore appear light green in colour, and the vegetative cells in pink.[45]

In endospore staining in Figure 4 isolates were mainly pink stained rod shaped, the absence of green colour endospores confirms the presence of non-spore forming *Lactobacillus* in the sample.

Acid fast organisms with a high concentration of mycolic acids in their cell walls can be distinguished using acid fast stains. Non-acid fast bacteria will stain blue/green with the counterstain, while acid fast bacteria will be red. This staining was important since the acid fast bacterial cell envelope is a specialised variation of the Gram-positive cell envelope.[46,47] Isolates in Figure 3 appeared to be red coloured and rod shaped which shared similar non acid-fast staining feature of *Lactobacillus* in Khalil and Anwar, 2016.[48]

As shown in Figure 5 catalase negative test reaction was characterized by the absence of oxygen bubbles formation, that indicate the *Lactobacillus* bacteria do not produce the catalase enzyme which converts H_2O_2 to water and oxygen.[49]

As in Figure 6 pure colony of *Lactobacillus* isolate was emulsified into MRS broth to sub culture and following incubation, turbidity with creamy pellet of *Lactobacillus* were observed similar to Agbankpe *et al.*[50] Thereby, through the morphological colony characteristics and biochemical tests the samples were confirmed to contain *Lactobacillus*.

The H_2O_2 resistance of *Lactobacillus* was observed using H_2O_2 resistance assay.[38] Population density is estimated from the turbidity of the culture and is typically expressed as OD (optical density), typically at a wavelength of 600 nm.[51] As shown in Table 1 the addition of H_2O_2, following 6 hours incubation has decreased the mean OD values in sample 1 and 2, which suggest that bacteria were not resistant to H_2O_2 and thereby decreased in population density. Sample 3, 4 and 5 had shown an increase in OD value which suggest that bacteria in those samples were not affected by the addition of H_2O_2 and continued growth in MRS broth. Despite the absence of H_2O_2 scavenging enzymes, such as catalase in *Lactobacillus*,[52] certain samples were found to resist H_2O_2. Similar results were obtained by Serata, Kiwaki and Lino 2016,[53] and the study showed that *Lactobacillus casei* strain was found to be resistant to H_2O_2 due to its

advantageous hpra1 gene, suggesting the samples 3,4 and 5 possibly carrying the *Lactobacillus casei* species. Resistance property of *Lactobacillus brevi* was examined by Fang *et al.,* 2018,[54] where it was observed that *Lactobacillus casei* and *Lactobacillus paracasei* showed less resistant than *Lactobacillus brevi* in the survival resistance assay.[55] Using plating technique, incubating *Lactobacillus* treated with different concentration of H_2O_2 has indicated *Lactobacillus acidophilus* and *Lactobacillus reuter* were more resistance to higher concentration (as $30gl^{-1}$) of H_2O_2 due to the presence of NADH peroxidise. *Lactobacillus salivarius* and *Lactobacillus casei* were found to be the least resistant with no growth at a low concentration (as $20gl^{-1}$) of H_2O_2.[56]

As different strains exhibit different level of resistance towards H_2O_2, this explains the difference in results obtained for samples A – E, which indicates the presence of different strains of *Lactobacillus* in all five yogurt drink samples. Hydrogen peroxide is a weak oxidant, but it may give rise to hydroxyl radical that causes oxidative damage to the cells.[27] Other anti-oxidant enzymes such as Heme-dependent catalase,[32] glutathione peroxidase,[33] thioredoxin reductase,[35] manganese containing pseudocatalase,[30] and NADH peroxidase[37] are responsible for the varied degrees of hydrogen peroxide resistance displayed by various samples containing *Lactobacillus*. According to the Table 2 the statistical analysis was performed using one way ANOVA test. P value of 0.006 was obtained which was less than 0.05 and considered there is statistically significant difference between 0 hours and 6 hours of H_2O_2 resistance. The study was aimed to isolate *Lactobacillus* from five yogurt drink samples and checked for H_2O_2 resistance for its successful survival in gastrointestinal tract.

However, further confirmatory tests can be carried out using wider sampling to analyse the diversity of *Lactobacillus* strains present in the samples and their H_2O_2 resistance can be determined. Thereby the successful resistant strains with the highest survival rate can be identified and incorporated into yogurt drink manufacturing to maximize the health benefits.

5. Conclusion

The purpose of this study was to identify *Lactobacillus* from commercial yogurt drink products and to determine their H_2O_2 resistance. All the five samples were examined for morphological and biochemical characterization. The study demonstrated that all five samples contained rod shaped Gram positive, non-acid fast, non-spore forming and catalase negative isolates which confirmed to be *Lactobacillus.* Furthermore, during H_2O_2 resistance assay it was observed that three of the samples contained H_2O_2 resistant *Lactobacillus.* The study emphasizes that the importance of screening *lactobacillus* for H_2O_2 resistance to achieve its health benefits.

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