ASSESSMENT OF ANTIBACTERIAL SUSCEPTIBILITY PATTERN OF BACTERIA PRESENT IN DIFFERENT YOGHURT SAMPLES

Abstract

The main aim concerning this study was to identify the antibiotic resistance in commercially available probiotic *Lactobacilli.* The probiotics that are available commercially are usually considered safe to consume but due to their widespread use LAB acts as a reservoir of antibacterial resistance genes that can be passed vertically. Due to external genetic elements, there is also a possibility of horizontal transfer of resistance genes to pathogens and human gut microbiota, which can be harmful to the host.

This study evaluated the antibacterial susceptibility pattern of *Lactobacillus sp.* isolated from six different yoghurt samples. Among these six samples five samples were commercially available in local market and one sample was homemade yoghurt sample. Pure colonies of *Lactobacillus sp.* were isolated and confirmed using Gram staining, Endospore staining and Biochemical tests such as IMViC and Catalase test. Antibacterial susceptibility was screened for resistance and susceptibility of *Lactobacillus sp.* against 12 antibiotic discs of A1 Axiom multidisc ring for Gram positive isolates by using disc diffusion method. *Lactobacillus sp.* of six different yoghurt samples showed difference in resistance and susceptibility pattern. All the responses of *Lactobacillus sp.* of six different yoghurt samples in terms of resistance and susceptibility pattern were recorded. Among six different yoghurt samples, the *Lactobacillus sp.* of Milky Mist sample showed highest resistance profile which was followed by Karimnagar yoghurt sample. It was observed that all the *Lactobacillus sp.* of six different yoghurt samples showed resistance against Ampiclox (ACX20) antibiotic disc. Since there is rise in antibiotic resistance, these probiotic strains which were susceptible to certain antibiotics may become resistant in future. Therefore, antibiotic susceptibility should be considered as a vital tool for safety assessment of the probiotics. The zone of inhibitions of *Lactobacillus sp.* of six different yoghurt samples were statistically analysed using

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One- way ANOVA. One- way ANOVA was employed to test the significance and p-value <0.05 was considered as significant. The p- value obtained through experimental data was found to be 0.943257. This implies that the datasets were non-significant. Sample variance determines the spread of the values of different counts obtained, the samples variance in the study was found to be an average of 78.10732 respectively.

As a result, additional research on the isolation and characterization of probiotic bacteria from local dairy products, as well as their growth optimization may be required for the development of probiotic enriched food supplements and human health benefits through bacterial infection, prevention and control.

Key words: LAB, Human gut microbiota, Antibacterial susceptibility, Zone of inhibition, ANOVA.

I. INTRODUCTION

Background Study

Milk is an important part of many people's traditional diet around the world. The vast majority of milk produced is consumed at home and is rarely sold. However, high temperatures and lack of refrigeration facilities have made it impossible to process and preserve fresh milk. Hence, traditionally leftover liquid milk is converted into partially shelf stable goods such as yoghurt, cheese, acidified milk, butter and ghee at the home level. Acidification of milk through fermentation is an ancient method of milk preservation. (Ogwaro, 2002)

In different parts of the world, different methods of fermentation are used, which results in a variety of fermented milk products such as kumiss, kefir, acidophilus milk and yoghurt. (Tamime, 1980)

Product quality and consumer satisfaction are critical factors in boosting demand for various types of yoghurt. The increase in per capita annual yoghurt consumption in the majority of countries has been allocated to improved knowledge about the health advantages of yoghurt, the rising availability of fruit or flavoured yoghurt, and the variety of product presentations. (Küçüköner)

Yoghurt's healthy food profile is attributed to the probiotic impact of yoghurt microorganisms according to (Guarner) yoghurt bacteria are probiotic living microorganisms that give a health advantage to the host when administered in sufficient concentrations the health- promoting properties of live lactic acid bacteria in yoghurt include protection against gastrointestinal upsets improved lactose digestion by mal digesters, a lower risk of cancer, lower blood cholesterol, an improved immune response and the ability to help the body assimilate protein, calcium and iron. (Zubeir) (Owiah, 2017)

Yoghurt is a popular fermented dairy product that is consumed all over the world. Lactic acid fermentation of milk is achieved through the action of starting culture containing *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Salama, 2022)

Conjugated linoleic acid has been demonstrated to be a powerful natural and anti-carcinogen that can also lower the risk of cardiovascular disease, fight inflammation, reduce body fat, particularly belly fat, lower cholesterol and triglycerides, raise metabolism, reduce insulin resistance, and improve the immune system. (Hartigh, 2019)

Yoghurt Culture Bacteria

The proteolytic activity of the two yoghurt bacteria is mild yet significant, resulting in symbiotic development of the two organisms and taste component generation. *Lactobacillus bulgaricus* can hydrolyse caseins, whereas *Streptococcus thermophilus* has low proteinase activity. (Tamime, Yoghurt: Technology and Biochemistry, 1980)

Antibacterial Susceptibility Testing

For both bactericidal and bacteriostatic drugs, antibacterial susceptibility testing determines the concentration of an antibiotic that inhibit bacterial growth. (Brown, 2016) (Sanguinetti) (AS, 2007) (Belkum, 2019) The importance of accurate antibacterial susceptibility testing in at least guiding antibiotic use in the clinical cannot be underestimated. (Doern, 1994) Antibacterial susceptibility testing is critical for the development of novel antibacterial since it allows us to (i) determine the preclinical activity of drug candidates and identify lead compounds, (ii) determine the possibility of resistance development and (iii) offer estimations of potential in vivo and more importantly, clinical efficacy when testing drugs in biological matrices reproducing infection sites, such as blood/plasma/ serum, lung bronchiolar lavage fluid/sputum, urine, biofilms, and so on. (Breteler, 2011)

II. MATERIALS AND METHODS

Sample Collection

Homemade yoghurt and five different yoghurt samples were collected randomly from the local marker under sterilized conditions to check the antibacterial susceptibility pattern in the laboratory.

Culturing on MRS Agar Medium

All the glassware and media were autoclaved at 121ºC and 15 lbs pressure, before following any kind of procedures for maintaining sterile conditions and to avoid contamination.

The six different yoghurt samples were serially diluted through a series of standard volumes of sterile diluent, to reduce the concentration of cells present in the sample. Small volumes of each dilution were used to make a series of spread plates on the MRS Agar. Following incubation, some of the dispersed cells form isolated colonies. A colony is a big group of bacterial cells on solid media that can be seen with the naked eye as a distinct entity. In this approach, it was assumed that a colony is formed from a single cell and hence represented as a clone of pure culture. After incubation, by looking down at the top of the colony, the general shape of the colony and the shape of the edge or margin was deciphered. The nature of the colony elevation was seen when viewed from the side with the plate held at the eye level. After identifying a well- isolated colony, it was picked up and streaked on to a new medium to obtain a pure culture. *Lactobacillus* pure colonies were obtained and validated using gram staining, endospore staining and biochemical tests such as biochemical assays such as IMViC tests and catalase tests.

Screening of Antibacterial Drug Resistance

Loop full of culture from the pure culture of the same morphology type were taken and inoculated into the sterile 2mL of nutrient broth for all the test isolates. Incubated the broth to produce bacterial suspension of moderate turbidity. The derived suspension of

bacterial cultures from the broth were further used in inoculation for further analysis. Sterile Nutrient agar plates were labelled according to the test cultures. The dry surface of the nutrient agar was inoculated using spread plate technique by adding 0.5mL of Nutrient broth. Dipped the spreader in alcohol and flamed it thoroughly, once cooled, gently spread the entire plate for uniform distribution of the sample. A1 Axiom multi discs were used for Gram positive bacterial isolates. These impregnated discs were carefully passed down to ensure contact with the agar surface. The disc must not be relocated once it came in contact with the agar surface. Incubated the plates for 24hrs in inverted position within 15mins after the discs were applied.

Reading and Interpretation of Results

After incubation the zone of inhibition was calculated using callipers and recorded down for observations. Zone of inhibition interpretation standard chart was used to classify the isolates based on sensitive, moderately sensitive and resistant respectively.

Table 1: Standard chart of A1 Axiom Antibiotic multidrug for Gram Positive Isolates

Antimicrobial Agent	Code	Content	Resistant mm or less	Intermediate mm	Susceptible mm or less
Amikacin	AK	30mcg	14	15-16	17
Ampiclox	ACX	20mcg	22	23-27	28
Ciproflaxacin	CIP	5mcg	15	16-20	21
Clarithromycin	CLR	15mcg	13	14-17	18
Cefotaxame	CF	30mcg	14	15-22	23
Sparfloxacin	SF	5mcg	15	16-18	19
Cefuroxime	CR	30mcg	14	15-17	18
Cefoperazone	CFP	75mcg	14	15-18	19
Gentamicin	G	10mcg	12	13-14	15
Roxythromycin	RX	15mcg	13	14-17	18
Cefadroil	CD	30mcg	14	15-17	18
Azithromycin	AZ	15mcg	13	14-17	18

mcg= micro grams

Statistical Analysis

All results were analysed using descriptive statistical techniques such as mean and standard deviation. One way ANOVA was employed to test the significance and p-value <0.05 was considered as significant. All statistical analysis were performed by Microsoft Excel sheet.

III. RESULTS AND DISCUSSION

Morphological, Cultural and Biochemical Analysis of Isolated *Lactobacillus sp.* of Different Yoghurt Samples

Primary identification of all the six different yoghurt samples was done by gram staining and endospore staining procedures. Morphological and various selected biochemical tests had been advocated as per the Bergey's manual of Systematic Bacteriology. After performing these procedures, it was observed that all the bacterial isolates of six different yoghurt samples were gram positive rods and they were non- endospore producers **(*Table 2*).** By performing biochemical tests like IMViC and Catalase, the bacterial isolates from six different yoghurt samples were confirmed as *Lactobacillus sp.* **(*Table 2*)**

Screening for Multidrug Resistance for Gram Positive Isolates

Antibacterial susceptibility pattern was screened for the resistance and susceptibility of *Lactobacillus sp.* against A1 Axiom multidisc ring for Gram positive bacterial isolates by using disc diffusion method. *Lactobacillus sp.* of six different yoghurt samples showed difference in resistance and susceptibility pattern against 12 antibiotics of the A1 Axiom multidisc ring for Gram positive bacterial isolates. The diameters of the zone of inhibitions around the discs were measured to the nearest milli meters using callipers or ruler accordingly. The isolates were classified as sensitive, moderately sensitive or intermediate and resistant according to the interpretative standard zone of inhibition chart (***Table 1*).** All the responses of *Lactobacillus sp.* of six different yoghurt samples in terms of resistance and susceptibility pattern against antibiotics were recorded in the tabular form (***Table 3) (Figure 1*).** Among all the *Lactobacillus sp.* of six different yoghurt samples, the *Lactobacillus sp.* of Milky mist yoghurt sample showed highest resistance profile by showing resistance against six antibiotic discs of A1 Axiom multidisc ring for Gram positive isolates such as ACX20 (21mm), CF30 (12mm), LE5 (15mm), G10 (10mm), AN30 (11mm) and CFP75 (14mm). This was followed by Karimnagar yoghurt sample, which showed resistance against five antibiotics of A1 Axiom multidisc ring for Gram positive bacterial isolates such as ACX (10mm), CF30 (0mm), BA25 (15mm), CR30 (0mm) and RX15 (11mm). All the *Lactobacillus sp.* of six different yoghurt samples showed resistance against the Ampiclox (ACX20) antibiotic. ***(Table 3).***

Table 2: Morphological, cultural and biochemical characteristics of bacterial isolates from different yoghurt samples

Sl. No	Yoghurt Samples	Morphological and cultural characteristics	Gram's staining	Endospore staining	Motility test	Biochemical Characterization of Bacterial Isolates				
						Indole	MR	VP	Citrate	Catalase
1.	**Homemade**	1mm, White, shiny smooth, round	**Gram Positive bacilli**	Non Endosporic	**Non motile**	-	+	-	-	-
2.	**Heritage**	Small, 0.1-0.5mm, roughdull and round	**Gram Positive bacilli**	Non Endosporic	**Non motile**	-	+	-	-	-
3.	**Jersey**	Small, 0.1-0.5mm, rough dull and round	**Gram Positive bacilli**	Non Endosporic	**Non motile**	-	+	-	-	-
4.	**Milkymist**	Small, 0.1-0.5mm, rough dull and round	**Gram Positive bacilli**	Non Endosporic	**Non motile**	-	+	-	-	-
5.	**Nandini**	1.0 mm white, rough, irregular and round	**Gram Positive bacilli**	Non Endosporic	**Non motile**	-	+	-	-	-
6.	**Karimnagar**	1mm, White, shiny smooth, round	**Gram Positive bacilli**	Non Endosporic	**Non motile**	-	+	-	-	-

Table 3: Determination of diameter of zone of inhibition of Lactobacillus sp. of different yoghurt samples in mm

Antibiotic Code	***Homemade***	***Heritage***	***Jersey***	***Milky mist***	***Nandini***	***Karimnagar***
ACX20	***19(R)***	***19(R)***	***20(R)***	***21(R)***	***0(R)***	***10(R)***
CIP5	***19(I)***	***22(S)***	***18(I)***	***24(S)***	***30(S)***	***35(S)***
CLR15	***18(S)***	***15(I)***	***21(S)***	***19(S)***	***24(S)***	***30(S)***

CF30	***0(R)***	***12(R)***	***12(R)***	***12(R)***	***15(I)***	***0(R)***
BA25	***0(R)***	***22(I)***	***19(I)***	***21(I)***	***11(R)***	***15(R)***
LE5	***19(I)***	***19(I)***	***21(I)***	***15(R)***	***40(S)***	***41(S)***
CR30	***30(S)***	***12(R)***	***11(R)***	***16(I)***	***0(R)***	***0(R)***
AZ15	***17(I)***	***15(I)***	***15(I)***	***15(I)***	***17(I)***	***15(I)***
G10	***29(S)***	***18(S)***	***18(S)***	***10(R)***	***19(S)***	***14(I)***
AN30	***30(S)***	***17(I)***	***17(I)***	***11(R)***	***10(R)***	***30(S)***
CFP75	***28(S)***	***1(R)***	***14(R)***	***14(R)***	***15(I)***	***18(I)***
RX15	***17(I)***	***15(I)***	***16(I)***	***16(I)***	***18(S)***	***11(R)***
AVG=	***18.83***	***15.58***	***16.83***	***16.166***	***16.58***	***18.25***

(R) = Resistant, (I) = Intermediate, (S) = Susceptible

I. The *Lactobacillus sp*. isolated from the homemade yoghurt has found to be resistant against three antibiotics used, **ACX 20** (19mm), **CF 30** (0mm) and **BA 25** (0 mm) While recorded to be moderately sensitive towards **CIP 5** (19mm), **LE 5** (19mm), **AZ 15** (17mm) and **RX 15** (17mm). The most efficient zones and also the maximum was towards **CLR 15** (18mm), **CR 30** (30mm), **G10** (29mm), **CFP 75** (28mm) and **AN30** (30mm). The average inhibitory affect is found to be 18.83mm.
II. The *Lactobacillus sp*. isolated from the Heritage yoghurt sample has found to be resistant against four antibiotics used, **ACX 20** (19mm), **CF30** (12mm), **CFP75** (1mm) and **CR30** (12mm) while recorded to be moderately sensitive towards **CLR15** (15mm), **BA25** (22mm), **LE 5** (19mm), **AZ15** (15mm), **AN 30** (17mm) and **RX 15** (15mm). The most efficient zones and also the maximum was towards **CIP 5** (22mm) and **G 10** (18mm). The average inhibitory affect is found to be 15.58mm.
III. The *Lactobacillus sp.* isolated from the Jersey yoghurt sample has found to be resistant against four antibiotics used, **ACX 20** (20mm), **CF 30** (12mm), **CR 30** (11mm) and **CFP 75** (14mm). While recorded to be moderately sensitive towards **CIP 5** (18mm), **BA 25** (19mm), **LE 5** (21mm), **AZ 15** (15mm), **AN 30** (17mm) and **RX 15** (16mm). The most efficient zones and also the maximum was towards **CLR 15** (21mm) and **G 10** (18mm). The average inhibitory affect is foundto be 16.83mm.
IV. The *Lactobacillus sp.* isolated from the Milky Mist yoghurt sample has found to be resistant against six antibiotics used **ACX20** (21mm), **CF30** (12mm), **LE5** (15mm), **G10** (10mm), **AN30** (11mm) and **CFP75** (14mm). While recorded to be moderately sensitive towards **BA 25** (21mm), **CR 30** (16mm), **AZ 15** (15mm) and **RX15** (16mm). The most efficient zones and also the maximum were towards **CIP5** (24mm) and **CLR15** (19mm). The average inhibitory affect is found to be 16.166mm.
V. The *Lactobacillus sp.* isolated from the Nandini yoghurt sample has found to be resistant against four antibiotics used ACX20 (0 mm), BA25 (11mm), CR30 (0 mm) and **AN30** (10mm). While recorded to be moderately sensitive towards **CF30** (15mm), **AZ15** (17mm) and **CFP75** (15mm). The most efficient zones and also the maximum was towards **CIP5** (30mm), **CLR15** (24mm), **LE5** (40mm), **G10** (19mm) and **RX15** (18mm). The average inhibitory affect is found to be 16.58mm.
VI. The *Lactobacillus sp*. isolated from the Karimnagar yoghurt sample has found to be resistant against five antibiotics used **ACX20** (10mm), **BA25** (15mm), **CR30** (0 mm),

CF30 (0 mm) and **RX15** (11mm). While recorded to be moderately sensitive towards **AZ 15** (15mm), **G10** (14mm) and **CFP75** (18mm). The most efficient zones and also the maximum was towards **CIP5** (35mm), **CLR30** (30mm), **LE5** (41mm) and **AN30** (30mm). The average inhibitory affect is found to be 18.25mm.

Figure 1: Determination of diameter of zone of inhibitions of Lactobacillus sp. from six different yoghurt samples

Zone of inhibitions of *Lactobacillus sp.* which was measured in mm by comparing with the standard chart of A1 Axiom multidisc ring of gram-positive isolates were graphically represented by using Microsoft Excel sheet ***(Figure 2).***

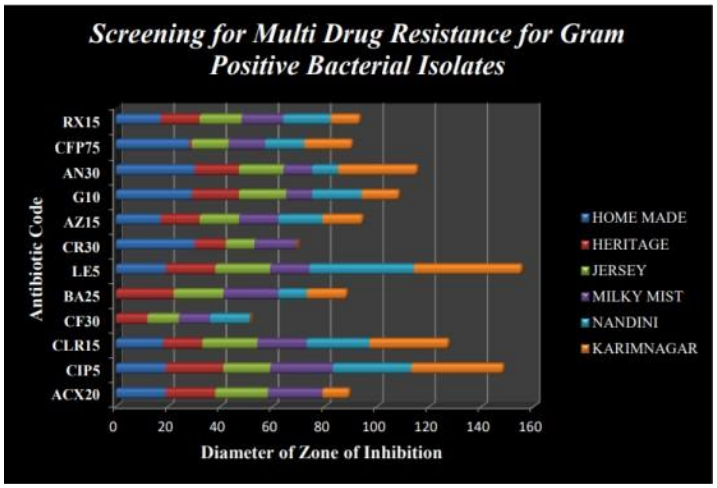

Figure 2: Graphical representation of multidrug resistance for Lactobacillus sp. from six different yoghurt samples using Microsoft Excel sheet

Statistical Analysis

Statistical analysis was carried out by using Microsoft Excel sheet and the data summary of *Lactobacillus sp.* of six different yoghurt samples in the form of mean, standard deviation, standard error and sample variance were tabulated ***(Table 4)***

Graphical representation of data summary of statistical analysis was depicted in the form of graph using Microsoft Excel sheet ***(Figure 3)***

Yoghurt Samples	N	Count	Mean	Standard Deviation	Standard Error	Sample Variance
Homemade	12	226	18.8333333	10.2410345	2.95633202	104.8788
Heritage	12	187	15.8333333	5.664215156	1.63411807	32.08333
Jersey	12	202	16.8333333	3.32574895	0.906006102	11.06061
Milky Mist	12	194	16.1666667	4.32399927	1.24823107	18.69697
Nandini	12	199	16.5833333	11.3654847	3.28093282	129.1742
Karimnagar	12	219	18.25	13.143439	3.7941841	172.75

N= Number of antibiotic discs in A1 Axiom multidisc for Gram positive isolates

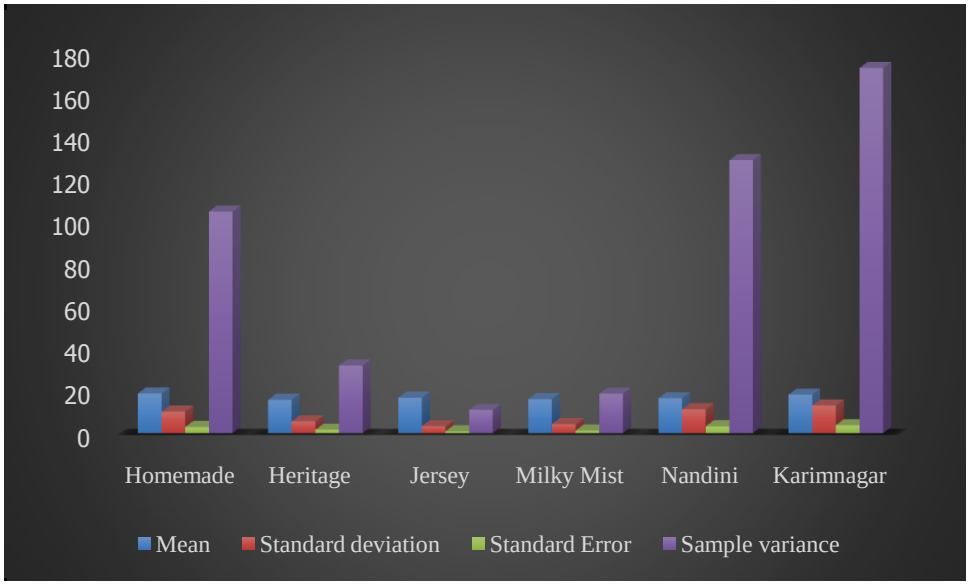

Figure 3: Graphical representation of data summary of Lactobacillus sp. from six different yoghurt samples

To determine the values of zone of inhibition obtained were statistically analyzed by using One- way ANOVA. ANOVA test was the starting step in analyzing variables that affect a given data set. This test permits a comparison of more than two groups at the same time to check in case a relationship exists between them. The result of the ANOVA equation, the F statistic, permits for the analysis of multiple groups of data to determine the variability between samples and within samples. If no real difference exists between the tested groups,

which is called the null hypothesis, the result of the ANOVA's F-ratio statistic will be near to 1. The significance was tested using One-way ANOVA, with p- value <0.05 being significant. The experimental data showed a p-value of 0.943257. This means that the datasets were not statistically significant ***(Table 5)***

Standard error of the regression is the average distance that the quantified counts fall from the regression line. In our case, the observed values fall an average of 2.3033007 units from the regression line. These standard errors are useful in precise predictions of the sampling. Sample variance determines the spread of the values of different counts obtained, the sample variance in the study was found to be an average of 78.10732 respectively ***(Table 5)***

- The P-value was found to be 0.943257
- The F-stat score was 0.240161
- The average standard error was 2.3033007
- The average standard deviation observed was 8.0106536
- The average sample variance was 78.10732

A One- way ANOVA is used to compare two means from two independent (unrelated) groups using the F- distribution. The null hypothesis for the test is that the two means are equal. Therefore, a significant result means that the two means are unequal. The mean values obtained through the analysis were

- Homemade (18.83333333± 10.2410345)
- Heritage (15.58333333± 5.664215156)
- Jersey (16.8333333± 3.32574895)
- Milky mist (16.16666667± 4.32399927)
- Nandini (16.5833333± 11.3654847)
- Karimnagar (18.25± 13.143439)

Table 4: Statistical analysis- ANOVA summary for bacterial isolates

Source of Variation	**SS**	**df**	**MS**	**F**	**p- value**	**F- crit**
Between Groups	93.79167	5	18.75833	0.240161	0.943257	2.353809
Within Groups	5155.083	66	78.10732			
Total	5248.875	71				

ss= sum of squares
ms= mean of squares

IV. CONCLUSION

The main aim of this study was to perform the antibacterial susceptibility testing of bacterial isolates from six different yoghurt samples. Primary identification of bacterial isolates of all the six different yoghurt samples were done by Gram staining and Endospore staining procedures. Morphological and various selected biochemical tests had been advocated as per the Bergey's manual of Systematic Bacteriology. After performing these procedures, it was confirmed that the bacterial isolates which were isolated from six different yoghurt samples were *Lactobacillus sp.* The antibiotic susceptibility was determined by using disc diffusion method, against 12 antibiotics of the A1 Axiom multidisc ring for Gram positive bacterial isolates. The resistance and susceptibility of *Lactobacillus sp.* of six different yoghurt samples were confirmed by comparing with standard chart of A1 Axiom multidisc for Gram positive isolates. Among all the *Lactobacillus sp.* of different yoghurt samples, the *Lactobacillus sp.* of Milky mist yoghurt sample showed the highest resistance profile by showing resistance against six antibiotic discs of A1 Axiom multidisc ring for Gram positive bacterial isolates such as ACX20 (21mm), CF30 (12mm), LE5 (15mm), G10 (10mm), AN30 (11mm) and CFP75 (14mm). This was followed by Karimnagar yoghurt sample, which showed resistance against five antibiotics of A1 Axiom multidisc ring for Gram positive bacterial isolates such as ACX (10mm), CF30 (0mm), BA25 (15mm), CR30 (0mm) and RX15 (11mm). All the *Lactobacillus sp.* of six different yoghurt samples showed resistance against the Ampiclox (ACX20) antibiotic. Graphical representation of the multidrug resistance of *Lactobacillus sp.* of six different yoghurt samples was carried out by using Excel sheet.

Statistical analysis of *Lactobacillus sp.* of six different yoghurt samples were carried out by One- way ANOVA and Excel sheet. To evaluate the values of zones of inhibition of *Lactobacillus sp.* against antibiotics in various yoghurt samples, one-way ANOVA was used statistically. ANOVA test was the starting step in analyzing variables that affect a given data set. This test permits a comparison of more than two groups at the same time to check in case a relationship exists between them. The result of the ANOVA equation, the F statistic, permits for the analysis of multiple groups of data to determine the variability between samples and within samples. If no real difference exists between the tested groups, which is called the null hypothesis, the result of the ANOVA's F-ratio statistic will be near to 1. The significance was tested using One-way ANOVA, with p- value <0.05 being significant. The experimental data showed a p-value of 0.943257. This means that the datasets were not statistically significant. The spread of the values of different counts obtained was determined by sample variance, which in the study was found to be an average of 78.10732 respectively. A One-way ANOVA was used to compare two means from two independent (unrelated) groups using the F-distribution. The null hypothesis for the test was that the two means were unequal.

Because antibiotic resistance is on the rise, the security of these probiotic strains is becoming increasingly important and it is impossible to ignore their capacity to spread antibiotic resistance genes to pathogenic or commensal bacteria because *Lactobacillus sp.* which are susceptible to specific antibiotics, may develop resistant in the future. As a result, antibiotic susceptibility should be regarded as a critical tool for evaluating the safety of probiotics.

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