Characterisation and bacteriophage sensitivity of Enterococci isolated from traditional fermented dairy product

.

ABSTRACT

Aims: The study aimed at detection and classification of Enterococci isolates from *Thayir*, a traditional fermented dairy product.

Study design: statistically significant number of *Thayir* samples were collected from across the study are to isolate and characterise Enterooccci.

Place and Duration of Study: The study was carried out in Wayanad District of Kerala, India during the period 2022-23.

Methodology:

Thayir samples were aseptically collected and bacteriologically cultured to detect enterococci. The isolates obtained were tested for the ability to form biofilms and hydrolyse gelatin. The antibiotic sensitivity pattern were studied using disk diffusion assay. Simple Kmeans clustering algorithm was applied to group the isolates. Phage sensitive and resistant isolates were identified among the safer isolates.

Results: eight isolates were obtained. None of the isolates hydrolysed gelatin, but four produced biofilm. Antibiogram revealed increased sensitivity to nitrofurantoin, but most isolates were sensitive to antimicrobials. Clustering analysis revealed a set of three safer

isolates, of which on was sensitive to the phage EFΦ91.

Conclusion: The studiy revelawed the presence of enterococcus fecalis and enterococcus faecium in thayir samples. Analyses revelaed that the isoaltes were diverse and continued monitoring for antimicrobial resitisanc eagainst Enterooccci in food samples is necessary.

Keywords: Enterococcus, antibiotic sensitivity profiling, clustering analysis, bacteriophage

1. INTRODUCTION

The gut microflora plays an important role in overall health and well-being. Symbiotic association of the host with its gut microflora begins in the early life of the host, and fermented food acts as an important source(1). Fermentation driven by communities of uncharacterized microflora in traditional foods can be a boon and sometimes a bane. These undefined microbes potentially carry virulence genes or harbour multiple antibiotic-resistance properties that can be transmitted horizontally to other invading bacteria, creating significant public health concerns(2).

Enterococci, classified as lactic acid bacteria, are part of the subdominant microbiota of many artisanal dairy products and often contribute to the development of sensorial characteristics, modulate the microbiota of the product and may play a role in controlling pathogenic as well as deterioration microorganisms(3). Enterococcus is evolutionarily adapted to become important opportunistic pathogens and can trade its genes by horizontal transfer(4). Enterococci of both clinical and food origin possess genomes of high malleability due to a wide host range and pheromone-responsive plasmids, enabling them to gather and spread their resistance and virulence genes, and are emerging as important nosocomial pathogens(5). These include the genes of vancomycin resistance, a glycopeptide antibiotic considered the final resort for severe Staphylococcus aureus and enterococcal infections(6) (7)

Despite much enquiry in recent years on the presence, technological properties, potential health benefits, antibiotic resistance and carriage of virulence factors, the verdict on the safety of Enterococci in food is shrouded in mystery and is an ongoing global debate. Due to this ambiguity, the bacteria is not considered to have Qualified Presumption of Safety (QPS)

in the European Union or is Generally Regarded As Safe (GRAS) in the USA(8). A better perception of their role in the dairy system is necessary to consider their commercial use as probiotics.

The sensitivity of bacteria against bacteriophages is critical in fermented milk production, as the attack of bacteriophages on starter cultures will potentially result in starter failure. At the same time, phages can function as a biocontrol strategy to eliminate enterococci possessing multidrug-resistant and virulent traits from fermented milk. The high host specificity of bacteriophages can be exploited for their use in the control of selected strains without affecting the starter cultures.

2. MATERIAL AND METHODS

2.1 Study area and Samples

"Thayir", a traditionally prepared fermented dairy product was considered for the study. Sampling was done by visiting 30 households of Wayanad District, Kerala , India. For the study, 100 ml each of Thayir samples were collected in sterile bottles were transported to the lab in an ice box at 4^{o}C. The samples were serially diluted and pour-plated on de Man Rogosa Sharpe (MRS) agar and incubated at 37^{o}C for 24 hours. Typical colonies were selected and subjected to Gram staining, catalase and oxidase tests. To isolate Enterococci from the colonies, Gram-positive cocci that were both catalase and oxidase negative were cultured on Bile aesculin agar to confirm aesculin hydrolysis evident by the formation of black hallowed colonies. Isolates of *Enterococci* confirmed by aesculin hydrolysis were further studied.

2.2 Detection of virulence factors

Isolates were tested for the ability to form biofilms and the ability to hydrolyse gelatin. Biofilm formation was qualitatively evaluated based on colony characteristics when streaked on Congo red agar (9). The formation of slimy and shining black colonies within 24 h of incubation indicated biofilm formation. For the detection of gelatinase activity, isolates were stabbed on nutrient gelatin agar plates and incubated at 37°C for 24 h. The formation of clear

zones around the colonies was visualised by flooding the plates with saturated ammonium sulphate solution.

2.3 Antibiogram

The antibiotic susceptibility of the isolate was evaluated by the disk diffusion assay (Bauer et al., 1966). The optical density of overnight grown bacterial cultures was adjusted to 0.5 McFarland unit and swabbed to make a lawn culture in Muller Hinton Agar. Antibiotic discs were placed equidistant and incubated at 37℃ for 24 hours. At the end of incubation, measured the zone diameters and the results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) (2020) guidelines. The antibacterials used were Penicillin (P- 10 units), Ampicillin (AMP- 10 mcg), Linezolid (LIN- 30 mcg), Vancomycin (VA- 30 mcg), Tetracycline (TE- 30 mcg), Chloramphenicol (C- 30 mcg), Erythromycin (E-15 mcg), Ciprofloxacin (CIP- 5mcg), Nitrofurantoin (NIT- 300ug) and Gentamicin (GEN- 120mcg).

2.4 Molecular characterisation

The 16s rRNA gene was sequenced to characterise the three least virulent isolates obtained. Total DNA was isolated using a Bacterial DNA isolation Kit (GenEluteTM Bacterial Genomic DNA Kit, Sigma- Aldrich Cat. No.....) following the manufacturer's instructions and used as the template. The universal primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′)(10) was employed.

The reaction was carried out with an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 45 s and extension at 72°C for 2 min and a final extension step at 72°C for 10 min. Amplification products were separated on a 1.5 % agarose gel and visualised under UV light after staining with ethidium bromide (1 mg/mL). The sequencing of PCR products was done at AgriGenome Labs Pvt. Ltd., Ernakulam, India and compared with the GenBank database using the Basic Local Alignment Search Tool (BLAST).

2.5 Isolation and purification of bacteriophages

Phages were isolated from fermented milk samples collected from households by multiple host enrichment methods (11). The phages were detected by spot tests and plaque assays. Two to three phage plaques were picked and grown through double-layer agar. The phage lysates thus obtained were propagated on *E. faceium* cultures to obtain high titer stocks. Centrifuged these crude phage lysates at 4000 × *g* (Neuation, Gujarat) for 20 min, and the supernatants were filtered through a 0.45-µm pore-size syringe filter (Merck, Ireland). The phage stocks were purified (Ackermann 2009) using Polyethylene glycol and stored at 4oC.

2.6 Morphological characterization of phages

The morphology of purified phages was examined by transmission electron microscope. 1 mL of pure phage suspension was centrifuged at 20,400 × *g* for 1.5 h at 4 °C and washed twice using phage buffer (SM buffer, Himedia). Discarded the supernatant and the pellet was re-suspended in 20 µL of sterile SM buffer, 5 µL of the re-suspended phages were carried out on a 200-mesh Formvar carbon-coated copper grid and negatively stained with 2% (w/v) phosphotungstic acid (PTA), pH 7.0. The grid was dried for 5 min and then observed at 80 kV using a transmission electron microscope at STIC, CUSAT, Cochin, Kerala.

2.7 Statistical analysis

To interpret the antibiotic sensitivity patterns, box-and-whisker plots and stacked density plots of the diameter of the zone of inhibition were created, providing visual insights into the distribution and variability of antibiotic resistance among the isolates. Further, the isolates were clustered into different groups based on the presence of virulence factors and antibiotic sensitivity profiles. K-means clustering with Euclidean distance of the normalised data was employed to group the isolates based on their antibiotic profiles and properties of gelatinase activity and biofilm formation. A silhouette plot and an elbow plot were prepared to assess the clustering quality. Further, the density-based spatial clustering of applications with noise (DBSCAN) of the normalised data was performed to validate the clustering. Hierarchical clustering was then applied to the calculated distance matrix using a complete linkage algorithm.

3. RESULTS AND DISCUSSION

3.1 Enterococcus isolates and their virulence factors

Thirty samples of Thayir were collected from the Wayanad District and inoculated in MRS agar (Fig 1.A). Among the total isolates obtained, eight were Gram-positive cocci. These isolates were both catalase and oxidase negative, and they hydrolyzed bile esculin (Fig 1B). Based on these characteristics, the isolates were identified as Enterococcus spp. This identification process confirms the presence of Enterococcus spp. in the Thayir samples from this region. Gram staining, catalase, and oxidase tests, along with bile esculin hydrolysis, provided a reliable method for identifying Enterococcus spp. This finding aligns with previous studies that have reported the presence of Enterococci in various artisanal dairy products (12). Four out of the eight Enterococcus isolates were capable of producing biofilms (Fig 1C), while isolates ADMT 64, M2, M11, and M12 were not. Additionally, all isolates tested negative for gelatin hydrolysis. These findings suggest variability in the virulence factors of Enterococcus present in thayir from the Wayanad District. Biofilm formation can be both beneficial and detrimental. Biofilms, constituted of exopolysaccharides, can improve the texture and consistency of fermented products and can be advantageous in enhancing the sensory qualities of Thayir. However, biofilms can also be considered as a virulence factor, as it enhances bacterial survival and resistance to environmental pressures, including resistance against antibiotic therapy (9). All the isolates tested negative for gelatin hydrolysis, indicating a lack of gelatinase activity, indicating that the isolates were incapable of tissue invasion and damage (13)).

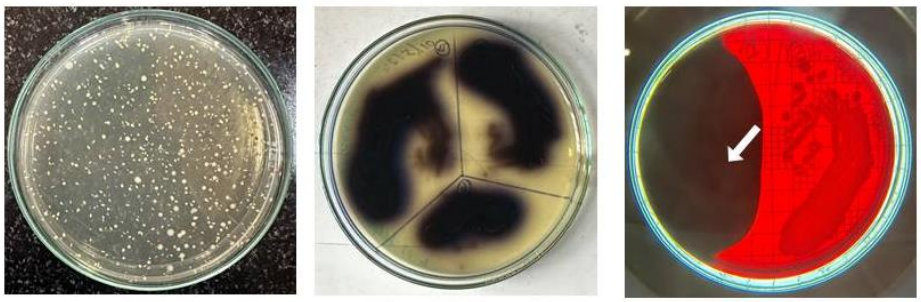

Fig. 1. Isolation and cultural characteristics of Enterococci form Thayir samples

A: Culturing on MRS agar B: Colonies with black halo in bile aesculine agar C: Biofilm forming colonies on Congo Red agar (Arrow)

3.2 Antibiotic sensitivity profile

Antibiotic sensitivity profiling revealed the average antibiotic sensitivity index of the enterococcal isolates to be 0.416, indicating most isolates were resistant to the antibiotics tested. This finding is concerning, as it suggests that Enterococci in Thayir could act as reservoirs of antibiotic-resistance genes, which may be transferred to other bacteria, posing a public health risk (5). Notably, three isolates were sensitive to most antibiotics tested, indicating variability in resistance profiles.

Among the isolates studied, the isolate ADMH91 exhibited the highest susceptibility index (0.82) and was sensitive to nine antibiotics tested. The isolate M12 was resistant to all antimicrobials except gentamicin (120ug). All the isolates except M12 were sensitive to nitrofurantoin, While five were sensitive to chloramphenicol. Linezolid was effective against four isolates. High-level aminoglycoside resistance was observed in the isolate M13. The median diameter of the zone of inhibition was higher than the CLSI cut-off point for chloramphenicol, nitrofurantoin, and gentamicin. The median zone size for linezolid was equal to the cut-off point. All other antibiotics had a lower median zone diameter than the cut-off value. (TABLE). Enterococci is known to be intrinsically resistant to beta-lactams and aminoglycosides (14). The high resistance to ampicillin and penicillin observed in most isolates is thus consistent with the known resistance patterns of Enterococci. Enterococcus faecium, in particular is often resistant to beta-lactam antibiotics (15). Enterococci are known to be sensitive to nitrofurantoin. In th represent study, all the isolates except M12 was sensitive to nitorfurantoin, and M12 showed intermediate sensitivity to the drug. Linezolid, chloramphenicol and tetracycline displayed higher zone sizes against most of the isolates, suggesting effective sensitivity. However, the presence of high-level aminoglycoside resistance in one isolate (M13) underscores the need for ongoing surveillance and careful antibiotic stewardship. Enterococci are among the earliest bacteria to acquire vancomycin resistance. This constitutive resistance is encoded by the vanC ligase genes. In the present

study, three isolates ADMH 91, ADMT 29 and ADMT 64 were sensitive to both gentamicin and vancomycin. These findings suggest that some Enterococcus isolates in Thayir may possess beneficial properties. However, the enterococcal genome is associated with increased plasticity in acquiring, sharing, and transmitting genetic traits, which could potentially lead to the spread of resistance (16)

Table 1. Diameter of zone of inhibition of isolates against various antibiotics

Isolates	LIN (23)	C (18)	CIP (21)	P (15)	E (23)	VA (17)	TE (19)	NIT (17)	GEN (10)	AMP (17)
ADMH 91	27	23	24	25	15	24	24	25	21	18
M13	0	22	0	0	0	0	11	26	0	0
ADMT 64	35	31	20	38	11	22	26	23	35	29
M 12	0	0	0	0	0	0	19	13	18	15
M 2	0	0	0	0	0	0	18	25	18	15
B 8	28	23	18	0	26	0	17	27	19	14
M 11	29	24	17	0	29	0	17	24	18	16
ADMT29	19	18	17	17	12	18	25	23	22	18

*Values in parentheses indicate the CLSI cut off diameter below which the drug is considered not to be sensitive

To understand about the inter quartile deviations (IQD) of antibiotic sensitivity pattern, a box and whiskers plot using the antibiogram data (Fig. 2) was constructed. Nitrofurantoin showed a the least IQD with a high median, indicating that the isolates were consistently sensitive to the drug. High susceptibility of enterococci to nitrofurantoin is documented [REF] and the findings of the present study aligns with the existing literature. However, the isolate M12 was found to be having only intermediate sensitivity against the drug. Tetracycline also displayed a narrow range and moderate median, indicating less variability in the zone size. Even though six isolates were sensitive to chloramphenicol, the IQD of the zone diameter was wider with a higher median. Linezolid also showed wide ranges and higher medians.

Although ampicillin showed a narrow range, the median zone size was lower than the cut off value, as most of the isolates were resistant. Ciprofloxacin, Erythromycin and Penicillin exhibited wide ranges and lower medians, with many outliers, depicting high variability in sensitivity among the isolates, with most of the isolates being resistant. On testing for high-level aminoglycoside resistance, Gentamicin (120µg) exhibited a narrow range with a relatively higher median, suggesting a consistent sensitivity profile, with one resistant isolate showing high-level aminoglycoside resistance. All but three isolates were resistant to vancomycin with zero zones of inhibition. The variation of the zone diameters of ciprofloxacin and erythromycin highlight the heterogeneity in resistance patterns, indicating that these antibiotics may not be consistently effective against Enterococci from Thayir. The detection of multi-drug resistant Enterococci in Thayir raises important public health concerns regarding traditional home made fermented dairy products. Enterococci are known for their ability to acquire and disseminate resistance genes, which can be transferred to other pathogenic bacteria, exacerbating the challenge of antibiotic resistance (17).

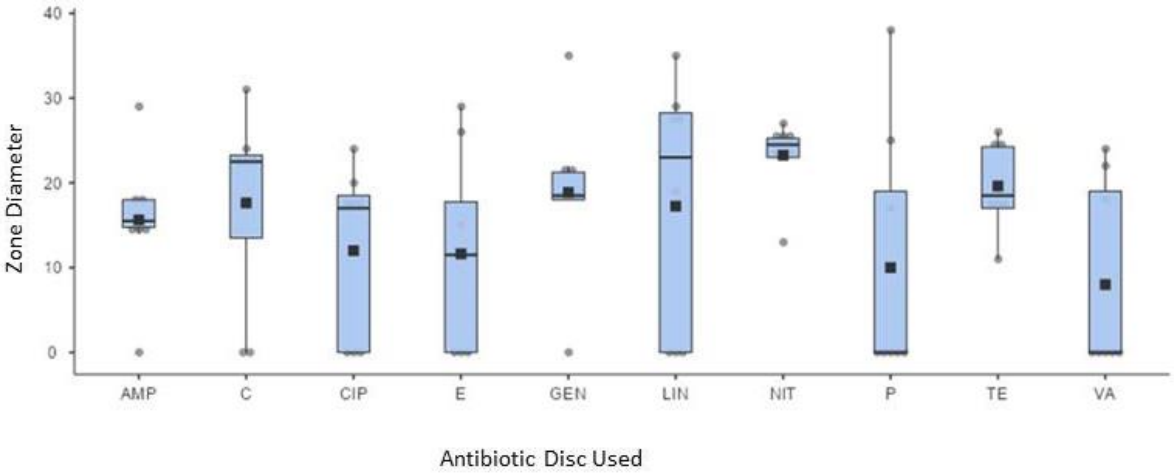

Fig. 2. Box and whiskers plot showing the diameter of zone of inhibition against different antibiotics by the isolates. Y axis indicates the zone diameter in mm.

The density plots for the zone sizes of different antibiotics against Enterococcus isolates provided a visual summary of their sensitivity distribution (Fig 3). Nitrofurantoin exhibited a high peak at the larger zone size, indicating high sensitivity among the isolates. Similarly, Linezolid displays a strong peak at a high zone size, suggesting that most isolates were sensitive. Tetracycline had a distribution with a higher peak, indicating moderate to high

sensitivity among the majority of isolates. Chloramphenicol shows a broad peak, reflecting variability in sensitivity among the isolates. Ciprofloxacin presented double peaks, indicating the presence of subgroups within the isolates with different levels of sensitivity. Penicillin had a flatter distribution with multiple peaks, highlighting variability in sensitivity. Erythromycin showed a relatively flat distribution with multiple peaks, indicating high variability in response among the isolates. Finally, Ampicillin exhibited a single peak around a low zone size, signifying that most isolates had low sensitivity to this antibiotic. Gentamicin (120µg) showed a peak at a higher zone size, indicating the presence of high-level aminoglycoside resistance in only one isolate. Vancomycin also displayed multiple peaks, suggesting variable sensitivity among the isolates.

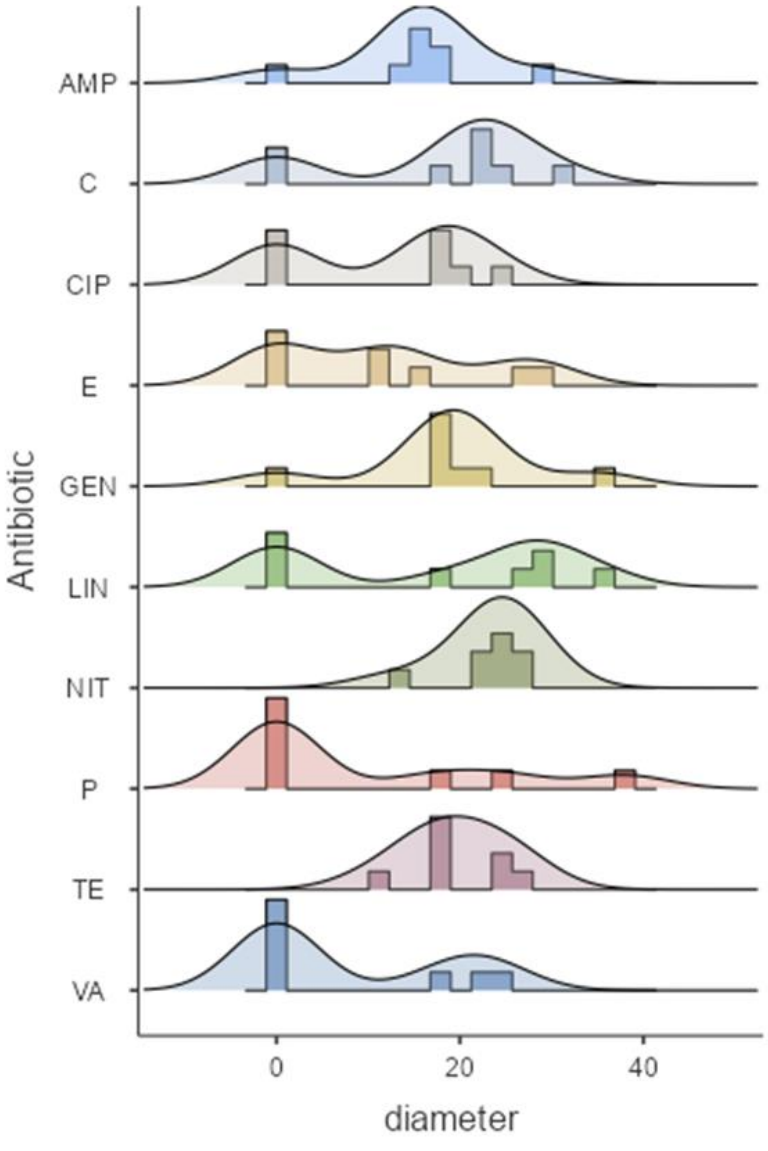

Fig. 3. Stacked density plot showing the diameter of zone of inhibition against different antibiotics by the isolates. X-axis indicates the zone diameter in mm.

Given the wide variations in antimicrobial resistance, K-means clustering was performed based on virulence factors and antibiotic resistance profiles. This analysis revealed distinct groups of isolates with varying levels of resistance and virulence, providing a deeper understanding of the diversity among the Enterococcus isolates.

3.3 Clustering analysis

The data regarding antibiotic sensitivity profiles, biofilm formation, and gelatinase activity were normalied and subjected to clustering analysis using simple K-Means clustering. The analysis resulted in two to eight clusters with Within-Cluster Sum of Squares (WCSS) ranging form 9.7 to 0.31. Plotting the WCSS values against the number of clusters indicated two 'elbows' at two and four clusters (FIG 4A). The silhouette scores ranged from 0.140 to 0.293, with the highest score of 0.394 at four clusters. Since the silhouette scores were modest, a DBSCAN plot was created with data items sorted by score on the x-axis and distance to the Kth nearest neighbour on the y-axis, revealing the first 'valley' at two cluster levels with the neighbourhood distance of 6.92 (FIG 4B). Hence, hierarchical clustering based on the Euclidean distance of cluster sizes was applied at two cluster levels. One cluster (C1) containing three isolates (ADMT 29, ADMH 91, and ADMT 64) grouped together, while all other isolates formed a single cluster (C2) (FIG 5). Further analysis confirmed that the data was normally distributed, and the variance between the two groups was equal ($p>0.05$). An independent sample t-test showed that the antibiotic sensitivity index of C1 was significantly higher than that of C2 ($p=0.05$).

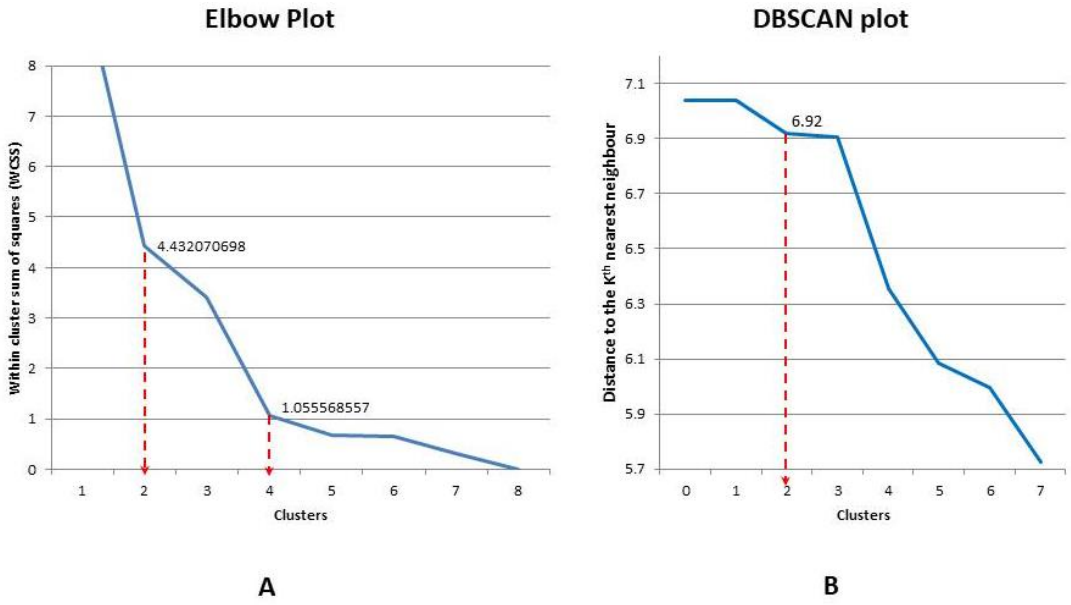

Fig. 4. Validation of clustering by Elbow plot (A) and DBSCAN plot (B)

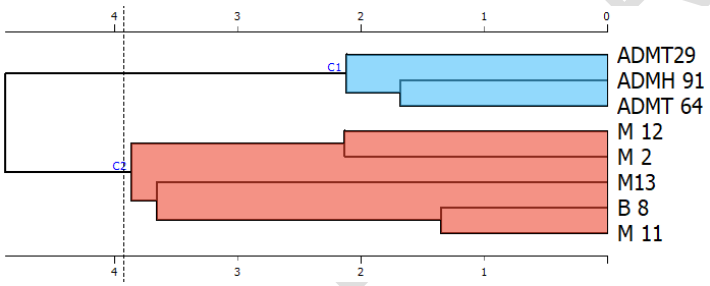

Fig. 5. Hierarchical clustering of isolates showing the two clusters of isolates

The clustering analysis of Enterococcus isolates based on antibiotic sensitivity profiles, biofilm formation, and gelatinase activity shed light on the heterogeneity and potential risks associated with these bacteria in Thayir(18). The "elbow method," which involves plotting the WCSS values against the number of clusters, revealed two significant 'elbows' at two and four clusters. These points suggested potential optimal cluster numbers(19). However, the relatively low silhouette scores, with a maximum of only 0.293 for four clusters, indicated moderate clustering quality, potentially due to the small sample size. This implied that although the clusters were somewhat distinct, the overall separation was not strong enough. To further evaluate the clustering quality and ensure a more robust grouping, a DBSCAN plot was employed(20). This plot sorted data items by score and distance to the Kth nearest neighbour, revealing the first 'valley' at a two-cluster level. The 'valley' suggested that a two-

cluster grouping might provide more meaningful separation among the isolates. Consequently, hierarchical clustering based on the Euclidean distance of cluster sizes was applied at the two-cluster level. This hierarchical approach identified two distinct groups: Cluster 1 (C1), which included three isolates (ADMT 29, ADMH 91, and ADMT 64), and Cluster 2 (C2), which encompassed all other isolates. The clear separation into these two clusters suggests that the isolates in C1 shared similar characteristics distinctly different from those in C2.

Cluster C1, which contained three isolates, had a significantly higher antibiotic sensitivity index compared to C2. This finding is particularly noteworthy as it suggests that the isolates in C1, despite being part of the same sample set, exhibited markedly different resistance profiles. The independent sample t-test confirmed that the antibiotic sensitivity index of C1 was significantly higher than that of C2 ($p<0.05$). This statistical validation supports the clustering analysis and highlights the distinct differences in antibiotic resistance profiles between the two clusters.The presence of isolates with lower resistance in C1 is promising, suggesting potential beneficial properties of certain Enterococci in Thayir. These findings point to the need for further investigation into the specific characteristics and potential functional benefits of these less resistant isolates.

3.4 Molecular characterisation and phage typing

On molecular characterization, the isolates were identified as two E. faecalis strains and one E. faecium strain based on 16S rRNA gene sequencing. The two E. faecalis isolates, designated as ADMT 64 and ADMT 29, exhibited 100% similarity to their respective type strains. The E. faecium isolate demonstrated a 98.94% similarity to the complete genome of E. faecium (Accession no. CP043484). The sequences obtained from the isolates were deposited in the NCBI database with accession numbers MW647897 for E. faecalis ADMT 64, OK576649 for E. faecalis ADMT 29, and OM648281 for E. faecium. Phylogenetic analysis revealed that E. faecalis ADMT 29 and E. faecium ADMH 91 were more closely related to each other compared to E. faecalis ADMT 64. It was observed that the phylogenetically similar strains, E. faecalis ADMT 29 and E. faecium ADMH 91, were biofilm producers, whereas the other E. faecalis strain, ADMT 64, was a non-biofilm producer.

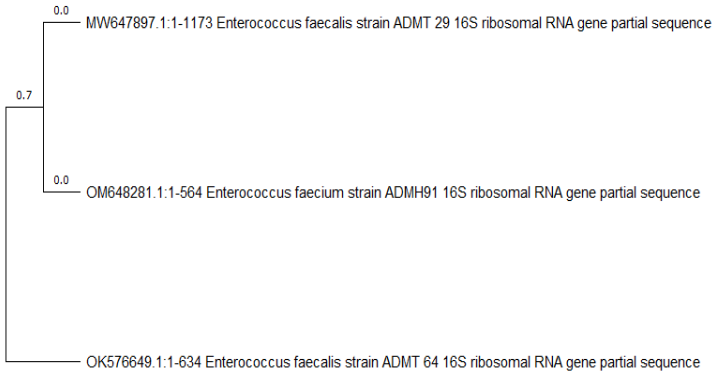

Fig. 6. Phylogenetic tree of isolates belonging to cluster C1

Since the three isolates of C1 did not posess the virulence factors ad were sensitive to antimicrobials, the phage typing was carried out. Among the three host strains (ADMT 29, ADMT 64 and ADMH 91), ADMH 91 showed sensitivity towards the phage in the fermented milk sample by giving a clearance zone in spot assay. On double layer agar assay single plaques of similar pattern were observed. The phage was named E.faecium phage EFΦ91. Morphological examination by TEM revealed the phage with an isometric head (around 52.96 nm), a contracted tail sheath length of 78.69 nm suggestive of myovirus morphology(21).

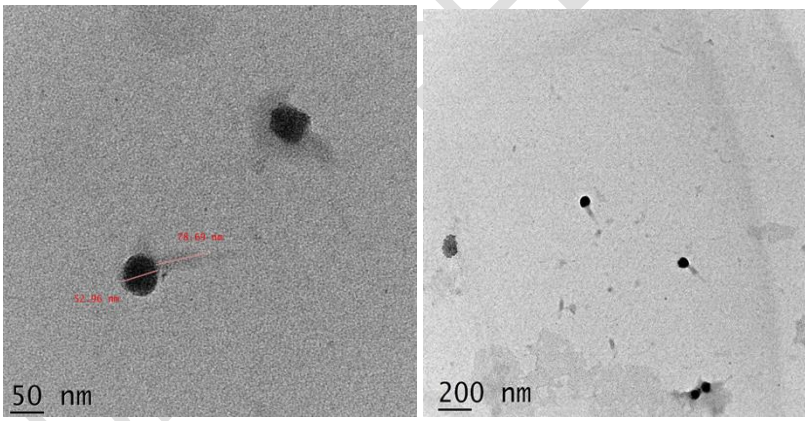

Fig. 7. Morphology of EFΦ91 phage under Transmission electron microscope

The discovery of phage-sensitive bacteria among the Enterococcus isolates indicates significant diversity in phage sensitivity within this group. Selecting Enterococci sensitive to specific phages can enhance the safety and quality of fermented dairy products. Phage sensitivity testing should thus be an integral part of the selection process for beneficial Enterococci in dairy technology(22).

Phage sensitivity testing allows for the identification of Enterococcus isolates that are not only beneficial for fermentation but also safe for consumption. By selecting phage-sensitive isolates, we can ensure that these bacteria do not pose a risk of pathogenicity, thereby improving the overall safety of fermented dairy products. Additionally, specific phages can help control unwanted bacterial populations during the fermentation process, maintaining the desired microbial balance.

The identification of phages such as EFΦ91 that can specifically target and lyse pathogenic Enterococci offers significant potential for food safety. These phages can be used to treat or prevent contamination by pathogenic Enterococci in food products. The ability to target specific bacterial strains without affecting beneficial microbiota makes phage therapy a promising approach for enhancing the safety of fermented foods.

4. CONCLUSION

In this study, thirty Thayir samples were collected from different households in the Wayanad District to isolate and characterize Enterococcus species. Eight isolates were obtained, none of which could hydrolyse gelatin. However, four isolates were identified as biofilm producers. Antibiotic sensitivity profiling revealed that seven out of eight isolates were sensitive to nitrofurantoin, though most isolates exhibited resistance to multiple antibiotics. Notably, one isolate showed high-level gentamicin resistance. Three isolates demonstrated resistance to multiple antibiotics, including vancomycin, to which Enterococci are considered intrinsically resistant. A simple K-means clustering of the isolates, based on their antibiotic sensitivity profiles and the presence of virulence factors, revealed moderate clustering into two groups. Sequencing of the 16S rRNA gene of the three isolates with the highest antimicrobial sensitivity indices identified two as E. faecalis and one as E. faecium. Furthermore, a bacteriophage, EFΦ91, presumptively belonging to the family Myoviridae, was found to be capable of infecting the isolate ADMH91. This finding highlights the potential for using specific bacteriophages to target and control pathogenic Enterococci in fermented dairy products.

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