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Study on the Acetaldehyde and Diacetyl Producing Abilities of Enterococcus and Lactobacillus Strains Isolated from Yogurt

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Abstract

Background and Objective: This study aimed to assess the ability of lactic acid bacteria isolated from yogurt to produce acetaldehyde and diacetyl using solid-phase microextraction gas chromatography-mass spectrometry method. Two species of lactic acid bacteria, Lactobacillus and Enterococcus, were isolated from Iranian traditional yogurts. Enterococcus strains showed distinct biochemical characteristics, including lipolytic activity, citrate metabolism and aromatic compound synthesis, which significantly affected the sensory characteristics of various cheeses during ripening.

Material and Methods: The study investigated ability of these strains to produce acetaldehyde and diacetyl as single starters and co-cultures. The biochemical characteristics of the strains were assessed, including their chemical profiles, acid production ability, yogurt sensory evaluation and antimicrobial susceptibility for Enterococcus strains.

Results and Conclusion: Lactobacillus strains showed the highest rate of acetaldehyde production. Acetaldehyde production ranged 0.45-8.33 $mg.kg^{-1}$ and diacetyl production ranged 2.00-13.20 $mg.kg^{-1}$ in growth of Enterococcus. In contrast, acetaldehyde production in Lactobacillus strains ranged 2.23-25.59 $mg.kg^{-1}$ and diacetyl production ranged 0.42-5.96 $mg.kg^{-1}$ when the bacteria were incubated at 5 °C for 14 d. In co-culture with Enterococcus, presence of Enterococcus slightly increased production of acetaldehyde and diacetyl. Additionally, presence of Enterococcus strains positively affected taste, color and texture of the yogurt samples. No previous studies have specifically assessed production of acetaldehyde and diacetyl by Enterococcus strains in yogurts.

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1. Introduction

Yogurt is one of the most widely consumed dairy products due to its rich nutritional values and numerous health benefits [1]. Yogurt production relies on the fermentation activity of lactic acid bacteria (LAB), which play a critical role in milk coagulation and texture formation [2]. The LAB species are preferred in the food industry due to their probiotic characteristics and their ability to enhance the nutritional and sensory qualities of fermented products. Additionally, their biochemical activity contributes to pH regulation and the production of secondary metabolites such as hydrogen peroxide, diacetyl and bacteriocins, making them excellent candidates as starter cultures [3]. Starter cultures consist of selected microbial strains that affect the organoleptic characteristics of dairy products, including texture, flavor, aroma and appearance. From LAB, enterococci are commonly detected in various dairy products, including yogurt and cheese [4]. These bacteria contribute to the characteristic sensory attributes of dairy products by producing aromatic compounds through biochemical processes such as lipolysis, proteolysis and citrate metabolism [5]. Several studies have demonstrated the positive effects of enterococci on cheese quality, improving

its structure, consistency, texture, taste and color [6]. Due to their natural preservative characteristics and produced aromatic compounds, these bacteria have been considered appropriate candidates for processing dairy products [7]. In recent years, interests in natural preservatives have increased, with research highlighting the ability of enterococci to produce bacteriocins, particularly enterocins [8]. Typically classified as class II bacteriocins, enterocins are small, heat-stable non-lantibiotic peptides that inhibit a specific range of bacteria, including foodborne pathogens such as *Clostridium* spp., *Vibrio cholerae*, *Listeria* spp. and *Staphylococcus aureus*. Enterocins have been shown to extend the shelf life of dairy products and their technological uses have led to the suggestion of enterococci as additional starters or protective cultures in cheese production. Additionally, enterococcal strains, particularly combinations of *Streptococcus thermophiles* and *Enterococcus faecium*, have been assessed as probiotics in clinical settings and suggested as potential alternatives to antibiotic treatments [9]. Despite their potential benefits, the use of enterococci in food production has increased concerns due to reports of antibiotic-resistant strains and their association with human infections [10]. While enterococci detected in traditional dairy products are often considered natural contaminants or part of the fermentation process, their presence has been linked to possible fecal contamination, increasing safety concerns in dairy processing [11,12]. These bacteria are naturally present in milk and play a significant role in the microbiota of fermented foods, particularly meats and cheeses. Their ability to tolerate heat and adapt to various environmental conditions allows them to survive Pasteurization and persist in refrigerated products [8]. However, enterococcal strains generally show weak acidification capabilities, limiting their effectiveness as primary starter cultures [11]. Studies have shown that dairy-originated enterococci decrease the pH slightly after 16-24 h of incubation at 37 °C, with few strains reaching pH less than 5.00-5.20 [12]. Research on *E. faecalis* from traditional Italian cheeses suggests that the strain is a more potent acidifier than *E. faecium*, capable of decreasing the pH of skim milk to approximately 4.5 within 24 h of fermentation [13]. Despite the well-established roles of *S. thermophilus* and *L. bulgaricus* in yogurt fermentation, their precise mechanisms in aromatic compound production and additionally functional characteristics are insufficiently understood. Enterococci, commonly detected in fermented dairy products, have been suggested as potential contributors to flavor and preservation, still their direct role in acetaldehyde and diacetyl production as single strains in yogurt is not systematically investigated. This study aimed to address this gap by assessing the ability of enterococcal strains isolated from Iranian traditional yogurts to produce acetaldehyde and diacetyl independently. Furthermore, this study investigated their potential for generating aromatic compounds and their microbial characteristics, with the goal of identifying novel candidates for enhancing yogurt flavor and stability. Regarding their flexibility to extreme environmental conditions such as pH fluctuations, temperature variations and salinity, enterococci may offer a robust alternative or complement to conventional starter cultures. Additionally, their potential probiotic benefits warrant further investigation for uses in functional dairy products. This study addressed the question of if the presence of enterococci contributed to the development of desirable and acceptable flavors in dairy products such as yogurts and cheeses. By elucidating the role of enterococci in yogurt fermentation, this study aimed to provide a detection for developing innovative starter cultures with improved sensory and preservative characteristics.

2. Materials and Methods

2.1. Microbiological Analyses

Traditional yogurt samples were collected from various regions in western Iran. Lactobacillus strains were cultured on MRS (de Man, Rogosa and Sharp) agar at 37 °C for 48 h using CO_2 incubator, while Enterococcus strains were cultured on M17 agar (Merck, Darmstadt, Germany) at similar temperature and time using shaking incubator at 200 rpm. For long-term storage, the cultured strains were preserved in liquid media with 20% sterile glycerol at -80 °C. The molecular identification of Enterococcus and Lactobacillus strains was carried out based on genetic databases [14].

2.2. Chemical Analyses

In the carbohydrate fermentation experiments, the isolated strains were assessed with sugars such as lactose, sucrose and sorbitol [15]. The growth rate of these strains was assessed at various NaCl concentrations to assess their tolerance and adaptability to salt. Specifically, NaCl concentrations of 0-6.5% were used in the culture media. To assess growth, 50 µL of the bacterial culture was inoculated into 5 ml of the media (Merck, Darmstadt, Germany) containing 4 and 6.5% NaCl. After 24 h of incubation at 30 °C, the growth rate of the strains was assessed via turbidity at 620 nm [16].

2.3. Antimicrobial Susceptibility of the Enterococcus Isolates

The antimicrobial susceptibility of Enterococcus strains was assessed against antibiotics that targeted various bacterial mechanisms, including inhibitors of cell envelope synthesis (ampicillin 5µg, vancomycin 5µg, kanamycin 10µg, imipenem 10µg), gentamicin 30 µg, tetracycline 10µg, chloramphenicol 5µg, erythromycin 10µg, clindamycin 15µg) and ciprofloxacin 5µg). Minimum inhibitory

concentration was assessed using microdilution method as described by the clinical and laboratory standards institute. In this method, after assessing bacterial concentration in standard and physiological media after 18 h of culture, a series of dilutions were prepared using stock solution of antibiotics. The bacteria were incubated at 37 °C for 24 h and the MIC values were then assessed.

2.4. Preparation of Yogurts

The selected bacterial strains were added to 100 mL of pasteurized milk at a concentration of 10^8 CFU.mL^{-1} after cooling down the milk to the optimal incubation temperature of 42-44 °C. The mixture was incubated at 42 °C. After the gel pH decreased to nearly 4.50 ±0.02 and clot formation was observed, the yogurt was rapidly cooled to 20 °C, followed by storage at 5 °C [17]. This two-stage cooling process served to stabilize the curd structure, decrease whey separation (syneresis), minimize thermal stress on the microorganisms and regulate the flavor by preventing excessive acidity.

2.5. Sensory Evaluation of Yogurts

Sensory evaluation of the yogurts was carried out by a panel of fifteen trained individuals using scoring method based on the criteria by Tamime and Robinson. The sensory attributes included appearance, color, aroma, taste, texture and overall acceptability.

2.6. Acid Production Ability

To assess the acid production ability, yogurt was prepared by fermenting pasteurized milk at 42-44 °C with an Enterococcus strain at a concentration of 10^8 CFU.mL^{-1}. The milk was incubated for 14 h and the pH was assessed once clot formation occurred. Moreover, pH was assessed using EDT353 pH meter (London, UK) calibrated with pH 7 and pH 4 buffers.

2.7. Assessment of Acetaldehyde and Diacetyl Productions by the Strains

After a 14-h incubation at 42 °C, milk was fermented using the strain with a concentration of 10^8 CFU.mL^{-1}. Aroma compounds in the yogurt samples were analyzed using quadrupole mass spectrometer coupled with an Agilent 7890 USA-made gas chromatography-mass spectrometry (SPME-GC-MS) system. The samples were stored at 5 °C for 14 d before analysis. Separation was carried out using polydimethylsiloxane (PDMS) capillary column with an internal diameter (I.D.) of 0.25 mm and a film thickness of 30 µm. Sample injection was carried out using split/splitless inlet with a 2:1 split ratio. Chromatographic separation was achieved using HP-5 MS capillary column (5% phenyl, 95% dimethylpolysiloxane) with specifications of 30 m length, 0.25 mm I.D. and 0.25 µm film thickness, made of silica. The chromatographic conditions included injection volume of 1.00 mL.min^{-1}, split injection mode (2:1), inlet temperature of 270 °C, initial oven temperature of 40 °C (held for 5 min), increased to 250 °C at a rate of 8 °C.min^{-1} and held for 2 min, carrier gas flow rate of 1.00 mL.min^{-1} (constant flow) and interface temperature of 290 °C. The assessment protocol for PAH-hydroxy compounds in sensory samples involved allowing the sample to equilibrate to room temperature (RT), weighing 1 g of the sample, adding 1 mL of water for homogenization, shaking the mixture for 2 min, heating to 80 °C for 20 min while inserting an SPME fiber and then using SPME syringe to inject the extracted vapors into the GC-MS system. The SPME fiber used in this study included PDMS with an 80 µm coating.

2.8. Molecular Verification of the Isolated Strains Using 16S rRNA Gene

To verify the identity of the isolated strains, the 16S rRNA gene was amplified using cetyl trimethyl ammonium bromide method for DNA extraction. The primers included forward primer (F): AGAGTTTGATCMTGGCTCAG and reverse primer (R): GGTTACCTTGTTACGACTT, amplifying 1500-bp fragments of the 16S rRNA gene. The PCR was carried out with an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 35 s and extension at 72 °C for 45 s. The products were visualized on a 1.5% agarose gel stained with Rima Sight DNA stain. The sequencing was carried out using 630R and 616V primers and the sequences were analyzed using the highest similarity to available gene sequences in NCBI [18].

2.9. Statistical Analysis

Statistical analysis was carried out using one-way ANOVA to compare the growth rates and sensory evaluation scores. Post-hoc Tukey's test was used for multiple comparisons, with significance at $p < 0.05$. All exper-iments were carried out in triplicate. Standard deviation (SD) was used to assess the variation within the dataset. For antimicrobial susceptibility, MIC values were compared to standard breakpoints. All analyses were carried out using SPSS software.

3. Results and Discussion

3.1. Chemical Analyses

As members of the LAB group, Enterococcus strains can coagulate skim milk when cultured as a single strain. All of the isolated strains demonstrated the ability to ferment lactose, as shown in Table 1. However, further characterizations of this characteristic are uninvestigated. The capacity to ferment sucrose belonged to two strains of Enterococcus and four strains of Lactobacillus were able to ferment sorbitol. Some LAB include metabolic pathways for sorbitol that are encoded by genes organized in operons. These pathways include sorbitol transport system, sorbitol 6-phosphate dehydrogenase (S6PD) and regulatory

proteins. The sorbitol metabolism operons of *L. casei* and *L. plantarum* have been previously characterized [19]. Strains that are capable of fermenting a variety of sugars show better growth and higher rate of acid production.

Table.1. Sugar fermentation by enterococcal strains

	lactose	sucrose	sorbitol
N1	+	-	-
N2	+	+	-
N3	+	+	-
N4	+	-	+
N5	+	+	-
N6	+	-	-
N7	+	-	-
N8	+	-	+
L1	+	-	+
L2	+	-	
L3	+	+	+
L4	+	-	-
L5	+	+	+
L6	+	-	+
L7	+	-	-
L8	+	-	-

L= *lactobacillus*. N=*Enterococcus*

Findings showed that certain strains such as N2, N3, N5, N6 and N7 (Figure 1) grew well and were able to tolerate sodium chloride concentrations ranging 0-6.5% ($p < 0.05$). This is consistent with previous studies on the high salt tolerance of enterococcal strains [20]. This ability enables them to grow well in salty food products such as cheeses, fermented meats and pickles. In contrast, other strains did not grow as well and likely required longer incubation periods. Lactobacilli generally show moderate salt tolerance, though this could vary by species and strain. Many lactobacilli can tolerate 4% sodium chloride concentrations, though it might partially inhibit their growth. Species such as *L. plantarum* and *L. casei* show higher salt tolerance and can grow at concentrations as high as 6-8% [21]. Based on the results from Figure 2, a significant difference was seen in optical density measurements for the Lactobacillus strains before and after incubation at salt concentrations of 0 and 4% ($p < 0.01$). In contrast to previous studies, the Lactobacillus strains not only tolerated 4% salts but also showed improved growth, particularly L7 and L8 strains.

3.2. Antibiotics Affecting Growth of the Enterococcus Strains

Enterococci are naturally abundant in various environments, including dairy products. All strains assessed in this study showed no hemolytic activity and were negative for catalase. Assessing antibiotic resistance is essential for assessing safety of Enterococcus strains. A similar antimicrobial susceptibility profile was observed in a previous study [22]. The MIC results (Table 2) revealed that all isolated strains were susceptible to imipenem, gentamicin, tetracycline and chloramphenicol. Eighty-seven percent of the Enterococcus strains were sensitive to erythromycin, ampicillin and kanamycin. Furthermore, 62.5% of the strains were susceptible to clindamycin and 75% were susceptible to vancomycin and ciprofloxacin. Vancomycin resistance is particularly important as it is the last line of defense against multiple-resistant Enterococcus infections. Resistance to glycopeptides is a critical factor in assessing safety of these strains.

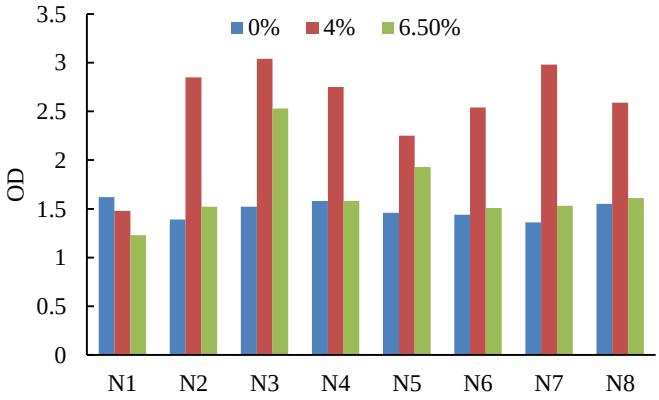

Figure. 1. The Enterococcus strain's tolerance to various NaCl concentrations by optical absorption (620 nm) 1OD 620≈10^8 CFU.ml^{-1}

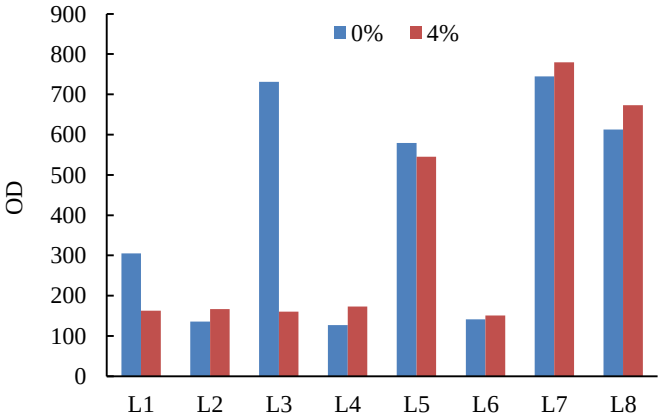

Figure. 2. The Lactobacillus strain's tolerance to various NaCl concentrations by optical absorption (620nm) 1OD620≈10^8 CFU.ml^{-1}

In addition to vancomycin resistance, certain Enterococcus strains are resistant to other antibiotics commonly used in veterinary and human medicines. Some of these strains are addressed as pathogens for humans and animals. The virulence factors of Enterococci include antibiotic resistance, colonization, adhesion to host tissues [with pheromone-responsive plasmids encoding the adhesin aggregation substance and the chromosomally encoded enterococcal surface protein (Esp)], invasion of tissues and resistance to host defense mechanisms virulence factors. One well-known enterococcal virulence factor is hemolysin. Eaton and Gasson (2001) demonstrated the presence of these virulence factors in Enterococcus strains isolated from foods and medical sources as well as those used as starter cultures. Particularly, medical strains showed the highest prevalence of virulence factors, while starter cultures showed the lowest prevalence. Pathogenic Enterococcus strains induce pathological changes either directly through toxin production or indirectly via inflammation. It is strongly recommended to use strains free from any virulence factors or determinants in food production. Selecting specific Enterococcus strains for use as adjunct starters must be carried out with extreme caution thorough assessments to ensure safety.

3.3. Sensory Evaluation Scores of Yogurt

Flavor is one of the key factors affecting the acceptability and preference of food products. Table 3 presents the sensory analysis of the yogurt samples. Of the Enterococcus strains, N3, N6 and N7 samples showed the highest production of acetaldehyde and diacetyl. Previous studies on Enterococcus strains introduced into cheeses have demonstrated their positive effects on ripening, flavor, aroma, color, texture and the overall sensory profile of fully matured cheeses [23]. In yogurt samples, the presence of Lactobacillus cultures resulted in increased acidity and decreased hydration, which improved the texture and ultimately contributed to higher sensory scores. This process effectively enhanced the sensory characteristics of the yogurt samples. The current findings indicated that the incorporation of Enterococcus strains into fermentation cultures facilitated the production of diacetyl, contributing to a richer buttery taste in the yogurt.

3.4. Acid Production Ability

Lactobacillus strains include the ability to produce lactic acid at concentrations ranging 1.5-2% in culture media. The extent of acid production is affected by factors such as strain type, composition of the culture media, temperature and the duration of fermentation. Optimal acid production typically occurs within a temperature range of 37-45 °C. Acid production plays a vital role in yogurt quality by enhancing its shelf life (through pH decrease, inhibiting the growth of undesirable bacteria), contributing to flavor development (giving yogurt its characteristic tartness) and helping in texture formation (as milk proteins coagulate at low pH to form the typical yogurt structure).

Table.2. Antibiotic resistance profiles of enterococcal

	MIC breakpoint *Enterococcus* spp. [§] MIC ($mg.ml^{-1}$)	*E. faecium* N_1	*E. faecium* N_2	*E. faecium* N_3	*E. faecium* N_4	*E. faecium* N_5	*E. faecium* N_6	*E. faecium* N_7	*E. faecium* N_8
Vancomycin	S≤4≥ R, 4<R	S	S	R	R	S	S	S	S
Ciprofloxacin	S≤4≥ R, NS	S	S	2	R	4	S	4	R
Clindamycin	NS, 4<R	S	S	4	4	16	8	16	16
Ampicillin	S≤4, R ≥8, 2<R	S	S	S	S	S	R	S	S
Gentamicin	NS, 32<R	S	S	S	S	S	S	S	S
Tetracycline	NS, 4<R	S	S	S	S	S	S	S	S
Chloramphenicol	NS, 16<R	S	S	S	S	8	S	S	S
Kanamycin	NS, 1024<R	1024	S	1024	1024	1024	1024	S	R
Imipenem	S≤4, R≥8, NS	S	4	S	S	S	S	S	S
Erythromycin	NS, 4<R	S	4	S	S	S	4	16	4

NS = not specified by cited documents; R = resistant; S = sensitive

[§] Recommendation of EUCAST (2019) and EFSA (2012), EUCAST($mg.l^{-1}$), EFSA ($mg.l^{-1}$)

Table.3. Sensory evaluation scores in produced yoghurt

	Color (0-20)	Texture (0-30)	Taset Aroma (0-50)	Total (0-100)
N1	18	24	30	72
N2	20	27	45	92
N3	19	29	48	96
N4	20	25	45	90
N5	20	27	48	95
N6	20	28	48	96
N7	20	30	50	100
N8	20	30	40	90
L1	13	8	40	61
L2	15	30	26	71
L3	13	28	36	77
L4	15	30	27	72
L5	15	30	50	95
L6	15	30	45	90
L7	10	30	25	65
L8	15	24	27	66
L1N1	20	30	40	90
L2N2	15	30	50	95
L3N3	15	20	50	95
L4N4	20	28	47	95
L5N5	20	30	50	98
L6N6	20	30	50	99
L7N7	15	20	50	100
L8N8	15	30	40	85

L= lactobacillus. N=Enterococcus

In contrast, Enterococcus strains generally show limited acidification potentials in milks. A recent study on dairy-derived Enterococcus strains revealed that only a small proportion were able to decrease the pH to 5.00–5.2 after 16–24 h of incubation at 37 °C. However, *E. faecalis* strains isolated from tradi-tional Italian cheeses demon-strated a significant acidifica-tion capacity in skim milk, decreasing pH to nearly 4.5 after 24 h of fermentation. Particularly, *E. faecalis* showed a greater acidification potential, compared to *E. faecium*. Due to their relatively low acidification and proteolytic activities, Enterococcus strains are generally not reported as primary starter cultures in cheese production. Although acidification and proteolytic activities are not directly correlated, strains that are more acidifying often show higher proteolytic activities [15]. An effective acid-producing starter culture, when inoculated at 10%, should decrease pH of milk from 6.6 to 5.3 within 6 h. In contrast, Enterococcus strains are majorly used as adjunct cultures in cheese production for purposes other than acidification such as using as probiotics, accelerating ripening and enhancing flavor. As shown in Figure 3, certain Enterococcus strains could decrease the pH of milk to nearly 4 after 14 h. Compared to other LAB, these generally need a longer time to achieve a similar pH decrease.

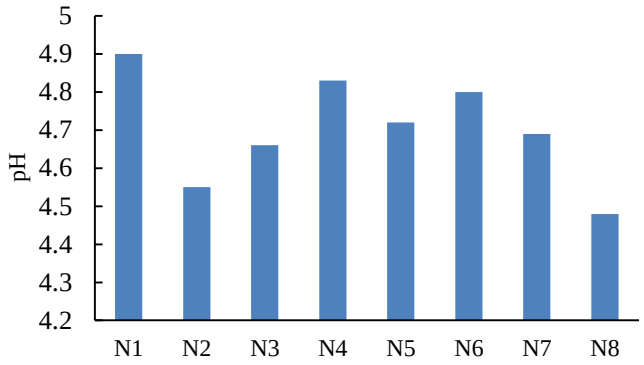

Figure.3. pH changes in evaluation scores in produced yoghurt (N=Enterococcus)

3.5. Assessment of Acetaldehyde and Diacetyl Produced by Enterococcus and Lactobacillus Strains

The primary aromatic compounds in yogurts are carbonyl compounds, with acetaldehyde as the major contributor to its characteristic flavor. While fatty acids and carbohydrates can play a role in aroma formation, casein is the major precursor of aromatic compounds in milks. The proteolytic system of LAB breaks down casein into amino acids, which are converted into aromatic compounds [21]. The LAB ability to metabolize citrate and pyruvate is critical for aroma formation as many LAB species convert citrate into aromatic compounds such as acetate, acetaldehyde and diacetyl [22]. Research suggests that yogurt products with low acetaldehyde concentrations can preserve the characteristic yogurt aroma, indicating that acetaldehyde is one of the important aroma components. Diacetyl, another key aromatic compound, significantly contributes to yogurt buttery flavor and overall aroma, especially in products with low acetaldehyde levels. In commercial yogurt production, *S. thermophilus* and *L. bulgaricus* are typically used in co-cultures, which enhance yogurt flavor, aroma, pH and texture. Co-culturing these species significantly increases acetaldehyde production, compared to use of *L. bulgaricus* alone. This study assessed the effects of Lactobacillus and Enterococcus strains on the production of acetaldehyde and diacetyl, comparing them with samples made with Enterococcus as the sole starter culture. Studies have suggested that *S. thermophiles* is the unique species capable of producing diacetyl [22]; however, limited information on citrate metabolism in Enterococcus strains are available. Research by Freitas et al. showed that *E. faecalis* and *E. faecium* strains from Picante cheeses could metabolize citrate in milks, with *E. faecium* showing a lower rate of citrate metabolism, compared to that *E. faecalis* was [23]. The Advisory

Committee on Novel Foods and Processes has approved *E. faecium* strain K77D as a starter culture for fermented dairy products [16]. The present study detected that Enterococcus strains were capable of producing acetal-dehyde and diacetyl. As shown in Figure 4, these strains produced more diacetyl than acetaldehyde. This study was the first to assess and quantify these aromatic compounds in Enterococcus strains individually, providing novel insights into their role in flavor development of dairy products. While previous studies have focused on starter cultures in cheese production, specific contribution of Enterococcus to aroma formation in yogurts has largely been uninvestigated [20]. In this study, N3 and N6 strains produced significant quantities of acetaldehyde, reaching 5 and 8.33 (mg.kg^{-1}), respectively. Moreover, N5 and N8 strains showed the highest diacetyl production with concentrations of 13.2 and 12 mg.kg^{-1}, respectively. In comparison, *Lacto-bacillus* strains played a major role in acetaldehyde production. As shown in Figure 5, Lactobacillus isolates of L5 and L7 produced high levels of acetaldehyde (25.59 and 19.2 mg.kg^{-1}, respectively), while isolates of L6 and L8 generated the highest diacetyl concentrations (5.96 and 5.50 mg.kg^{-1}, respectively). The combination of L4N4 showed increased diacetyl production, while acetaldehyde levels increased in samples containing L1N1, L4N4 and L8N8. These findings highlighted the promising potential of Enterococcus strains for diacetyl production in yogurt fermentation. Particularly, no previous studies have specifically quantified acetaldehyde and diacetyl productions by Enterococcus strains in yogurt, underscoring the novelty of the present study. The L5 strain, which showed the highest acetaldehyde production and received a high score in sensory evaluation, demonstrated the ability to metabolize all sugars (lactose, sucrose and sorbitol). The N5 strain, which could hydrolyze lactose and sucrose, produced the highest diacetyl levels and received a high score in sensory evaluation. Strains nos. L6 and L8 demonstrated high diacetyl production from hydrolysis and received favorable sensory evaluation scores. The two strains showed the ability to metabolize lactose and sorbitol as well. The L6 strain demonstrated the ability to metabolize lactose and sucrose, received a relatively high sensory evaluation score and showed high capacity for diacetyl production.

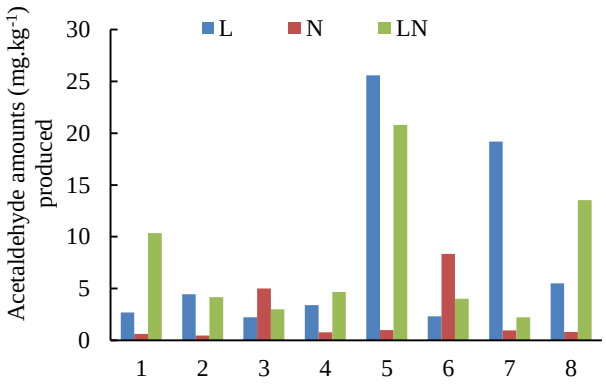

Figure.4. Acetaldehyde amounts (mg.kg^{-1}) produced by Enterococcus (N), Lactobacillus (L) and the combination of Lactobacillus and Enterococcus (LN)

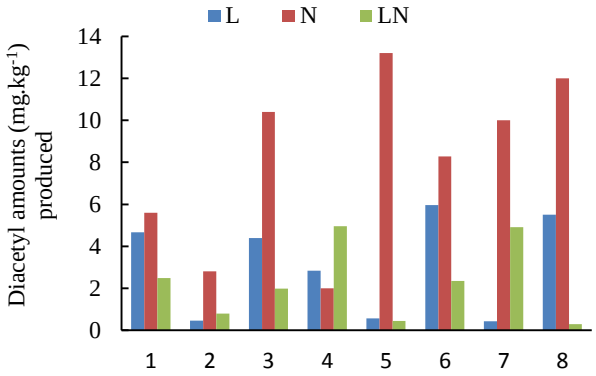

Figure.5. Diacetyl amounts (mg.kg^{-1}) produced by Enterococcus (N), Lactobacillus (L) and the combination of Lactobacillus and Enterococcus (LN)

3.6. Amplified 16S rDNA restriction analysis

The electrophoresis results (Figure 6) verified successful amplification of the 16S rRNA gene in the isolated strains. The gel image demonstrated distinct bands at 1500 bp for the samples cultured on MRS and M17 media, indicating accurate gene amplification. The lane distribution was as follows: the first lane contained the molecular weight marker (100-bp ladder), the second lane served as the negative control without DNA templates (showing no bands) and the lanes corresponded to PCR products of the isolated strains. The presence of clear 1500-bp bands in the samples and nucleotide sequence analysis using BLAST of NCBI verified the reported 99.09% identity of *L. helveticus* and 97.21% identity *E. faecium*. This molecular validation supports use of these strains in further biochemical and microbial analyses, reinforcing their suitability for uses in dairy fermentation.

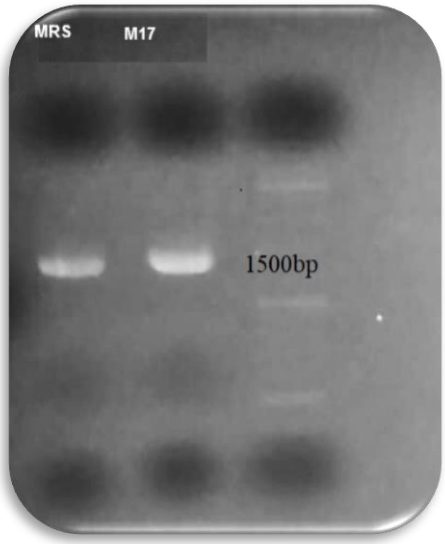

Figure.6. PCR result of the 16S rRNA gene in the isolated strains

4. Conclusion

This study was the first to investigate use of Enterococcus strains as starter cultures for yogurt fermentation, assessing their overall effects on yogurt quality as well as their ability to produce acetaldehyde and diacetyl. Reviews demonstrate that multiple strains of *E. faecalis* and *E. faecium*, isolated from dairy products, include the metabolic capacity to generate key flavor compounds, including acetoin, acetaldehyde, ethanol and diacetyl. These findings highlight the significant role of enterococci in enhancing the sensory attributes of dairy products, particularly in the development of cheese flavor and aroma. When used as single-strain starters, the enterococcal isolates successfully fermented yogurt samples, forming a stable gel structure. Furthermore, these strains showed efficient diacetyl production, contributing to the distinct buttery aroma of the final product. Sensory analysis revealed that the unique scent of the yogurt samples could be attributed to diacetyl-producing enterococcal strains, as recognized by the trained assessors. The findings of this study provided compelling evidence of the technological potential of enterococcal strains in dairy fermentation. Their ability to enhance flavor and aroma with their acidification capacity supported their uses as adjunct starter cultures in commercial yogurt production. These results facilitate further studies for optimizing enterococcal strains for industrial uses, potentially expanding their roles in development of high-quality fermented dairy products. Despite the potential uses of enterococci in food systems, significant concerns must be addressed before their safe uses can be ensured. A major issue is the increasing prevalence of enterococcal strains resistant to glycopeptides and other antibiotics, which increases public health concerns. Additionally, their capacity of producing biogenic amines in foods and the presence of virulence factors in clinical and food-derived isolates question their safety in food products. However, the high potency of enterococci to horizontally transfer genes-particularly antibiotic resistance genes- to pathogenic bacteria complicates the establishment of reliable selection criteria. Regarding these risks, safety of enterococci in foods is controversial, necessitating further clinical and epidemiological investigations.

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6. Conflict of Interest

The authors report no conflict of interest.

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بررسی توانایی تولید استالدئید و دی‌استیل توسط سویه‌های انتروکوکوس و لاکتوباسیلوس جدا شده از ماست

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چکیده

سابقه و هدف: این مطالعه با هدف ارزیابی توانایی باکتری‌های لاکتیک اسید جدا شده از ماست در تولید استالدئید و دی‌استیل با استفاده از روش ریزاستخراج فاز جامد کروماتوگرافی گازی- طیف سنجی جرمی انجام شد. دو سویه باکتری لاکتیک اسید، لاکتوباسیلوس و انتروکوکوس از ماست سنتی ایرانی جدا شد. سویه‌های انتروکوکوس ویژگی‌های بیوشیمیایی متمایزی از جمله فعالیت لیپولیتیک، متابولیسم سیترات و تولید ترکیبات معطر داشتند که به‌طور قابل‌توجه بر ویژگی‌های حسی پنیرهای گوناگون در طول رسیدن تأثیر گذاشت.

مواد و روش ها: این مطالعه توانایی این سویه‌ها در تولید استالدئید و دی‌استیل به‌عنوان تک آغازگر و کشت‌های همزمان را بررسی کرد. ویژگی‌های بیوشیمیایی سویه‌ها از جمله مشخصات شیمیایی، توانایی تولید اسید، ارزیابی حسی ماست و حساسیت ضدمیکروبی در برابر سویه‌های انتروکوکوس مورد ارزیابی قرار گرفت.

یافته‌ها و نتیجه‌گیری: سویه های لاکتوباسیلوس بیشترین میزان تولید استالدئید را داشتند. هنگام رشد انتروکوکوس محدوده تولید استالدئید $mg.kg^{-1}$ ۸/۳۳-۰/۴۵ و تولید دی‌استیل $mg.kg^{-1}$ ۲/۱۳-۰۰/۲۰ بود. در مقابل، در سویه‌های *لاکتوباسیلوس* تولید استالدئید $mg.kg^{-1}$ ۲۵/۵۹ – ۲/۲۳ و تولید دی‌استیل در محدوده $mg.kg^{-1}$ ۵/۹۶ – ۰/۴۲ هنگام گرمختانه‌گذاری باکتری‌ها در دمای $5^{\circ}C$ و به‌مدت ۱۴ روز بود. در کشت همزمان با انتروکوکوس، حضور انتروکوکوس تولید استالدئید و دی‌استیل را اندکی افزایش داد. علاوه بر این، وجود سویه‌های *انتروکوکوس* بر طعم، رنگ و بافت نمونه‌های ماست تأثیر مثبت داشت. هیچ مطالعه قبلی به‌طور خاص تولید استالدئید و دی‌استیل توسط سویه‌های *انتروکوکوس* در ماست را ارزیابی نکرده‌اند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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- انتروکوکوس
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- ماست

نویسنده مسئول

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