The antifungal effect of di-acetyl produced by isolated lactobacilli from traditional dairy products

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Abstract

Filamentous fungi, commonly called as molds, represent a significant category of spoilage microorganisms that contribute to substantial economic losses and pose serious health threats within the food supply chain. This study evaluated the antifungal effect of di-acetyl produced by *Lactobacillus* species isolated from traditional fermented products against *Penicillium* sp. Thirty-three samples of traditional yoghurt, dough, and kefir were cultured, and the isolates were identified using molecular approaches (specific PCR and 16S rRNA sequence analysis) and a biochemical test. The effects of different media on biomass and di-acetyl production were analyzed. Antifungal activity of di-acetyl was assessed against *Penicillium sp*. Out of 16 isolates from kefir, yoghurt, and dough, ten lactic acid bacteria (LAB) isolates were identified as *Lactobacillus casei* by a biochemical test and molecular methods. Results showed that the MR-VP medium exhibited the best effect on biomass and di-acetyl production by isolates. GC analysis showed that yoghurt-LABs producing high di-acetyl have a considerable inhibitory effect on *Penicillium sp.* The antifungal properties of di-acetyl generated by novel *L. casei* isolates seem to present a promising advantage, indicating the potential of di-acetyl as a bio-conservative in food and dairy commerce. Therefore, we can recommend applying the di-acetyl to the surface paper of fermented products to prevent mildew caused by *Penicillium sp.*

1. Introduction

Food spoilage by yeasts and filamentous molds is responsible for widespread food waste and has significant economic repercussions. In the agri-food industry, fermented dairy products such as cheese and yogurt can be affected by fungal spoilers. Mycotoxins produced by these organisms represent a significant health risk and are a major food safety threat that requires careful monitoring for their presence in food (Tropcheva *et al*., 2014; Salas *et al*., 2018; Awuchi *et al*., 2022).

The most common genera involved in the spoilage of dairy are Penicillium, Mucor, Fusarium, Aspergillus, Cladosporium, Candida, Meyerozyma, and Yarrowia (Oliveira *et al.,* 2014; Salas *et al*., 2018; Lipińska *et al.,* 2018). Agri-food industries usually protect products against

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microbial contamination through various chemical and physical techniques. A growing consumer aversion to chemical additives in products has led to the introduction of foods that are free from preservatives but still ensure safety, minimal processing, and extended shelf life (Tropcheva *et al*., 2014; Salas *et al*., 2018; Lipińska *et al*., 2018). Additionally, the issue of antibiotic-resistant organisms is on the rise, and recent legislation has imposed restrictions on the use of certain preservatives currently approved for use in food products (Brul *et al*., 1999). These problems raise the need for an alternative way to preserve foods (Tropcheva *et al*., 2014).

Recently, new advancements in technology have sparked increased interest in bio-preservation, a technique aimed at combating food spoilage while producing more natural food products that align with consumer preferences. This method emphasizes the elimination of artificial preservatives and minimizes processing. The primary microorganisms employed in this preservation process are bio-protective cultures, notably lactic acid bacteria (LAB) and Propionibacterium, which play a crucial role in extending the shelf life of food items (Pawlowska *et al*., 2012; Russo *et al*., 2016; Erfani *et al*., 2024; Lipińska *et al.,* 2018). Lactic acid bacteria (LAB) are a large heterogeneous group of non-spore-forming, Gram-positive bacteria that have been classified based on various morphological, metabolic, and physiological characteristics (Morelli *et al.*, 2011). LAB is naturally found in dairy products and used as starter cultures in the fermentation process. Applying LAB to reduce levels or replace the antifungal traditional preservation is an attractive alternative as these microorganisms are generally recognized as safe (GRAS) (Salas *et al*., 2018). In this context, several reports on the antifungal properties of LAB and their possible use as bio-protective agents have been published in recent years (Oliveira *et al*., 2014; Dali *et al*., 2010; Delavenne *et al*., 2012; Raman et al., 2022; Marcelli et al., 2024).

The LAB from the Lactobacillus species demonstrated an inhibitory effect against various molds and yeasts (Lipińska *et al*., 2018; Salas *et al*., 2018). Though LABs are promising sources of active bio-protective compounds, some of their habitats have not been widely studied. Screening of these organisms' different habitats is required to find active LAB compounds. Bacterial fermentation induces the production of acetic acids and lactic acid (primary metabolites), which play a role in fermented food bio-preservation, and secondary metabolites, including carbon dioxide, ethanol, hydrogen peroxide, fatty acids, acetoin, di-acetyl, and bacteriocins (Crowley *et al*., 2013; Salas *et al*., 2018). Diacetyl (2,3-butanedione, di-acetyl) is produced by several bacteria, including Lactobacillus, Streptococcus, Leuconostoc, and Pediococcus as a by-product. This product is of considerable value, characterized by a pronounced buttery scent, and is frequently utilized as a fundamental ingredient in various everyday chemicals, including food, tobacco, and fragrance. Its production occurs through the nonenzymatic oxidative decarboxylation of α-acetolactate during the process of bacterial fermentation, subsequently transforming into acetoin and 2,3-butanediol via the action of 2,3-butanediol dehydrogenase. Di-acetyl stands out as one of the key compounds generated through fermentation by lactic acid bacteria (LAB), playing a crucial role in determining fermented products' quality and consumer appeal (Xinxin *et al*., 2023).

Although its antibacterial effects were reported against *Bacillus globigii* and *Bacillus subtilis* (Jay, 1982), research on fungi that inhibit diacetyl remains limited. Previous studies have indicated that di-acetyl inhibits the mycelial growth of pathogenic fungi isolated from wolfberry fruits, specifically, Mucor circinelloides LB1, Fusarium arcuatisporum LB5, Alternaria iridiaustralis LB7, and Colletotrichum fioriniae LB8 (Ling *et al*., 2021). Furthermore, investigations into the antibacterial mechanisms of di-acetyl have been relatively scarce. Notably, it has been reported that diacetyl causes damage to the cell membranes of various Penicillium strains (Calov *et al*., 2020).

Our main objective in this study was to develop a bio-protective compound of *Lactobacillus* spp. with antifungal activity that could represent a natural alternative used in the food chain.

Therefore, this study evaluated isolating LAB strains from traditional dairy products, identifying them, and determining their ability to produce di-acetyl. In addition, the antifungal activity of di-acetyl against Penicillium sp. growth in vitro was investigated.

2. Materials and Methods

2.1. Sample collection (Yoghurt, Dough, and Kefir)

Twenty different traditional yoghurts, eleven samples of traditional dough, and two samples of traditional kefir were gathered from three locations in Kerman province (Iran). All the samples were collected in a sterile tube, and then the specimens were transported aseptically to the laboratory, where homogenization was carried out.

2.2. Isolation of Lactic Acid Bacteria from Yoghurt, Dough, and Kefir

2.2.1. Pre-enrichment method

After blending to obtain uniformity of the samples, approximately 0.2 gr of each sample was added to Man, Rogosa, and Sharpe (MRS) broth (Merck, Germany) and Tryptic Soy Broth (TSB) (Merck, Germany) at 30°C for 24 -48 hours under anaerobic conditions. After incubation, single colonies were subcultured on MRS agar plates for purification.

Regarding the presence of yeast in non-pasteurized traditional Yoghurt, Dough, and Kefir that interfered with the separation of Lactobacillus, formic acid (0.1%) was added to TSA and MRS mediums. The cultures were investigated after incubation at 30ºC for 3-5 days.

2.3. Identification of Lactic Acid Bacteria Isolates

An approach combining phenotypic and genetic parameters was applied to identify the Yogurt, Dough, and Kefir isolate species.

2.3.1. Phenotypic characterization of LAB strains

Lactic acid bacteria were characterized using morphological and biochemical tests, including Gram-stain, catalase, oxidase, (mobility examination) SIM, indole production, and carbohydrate fermentation patterns (Lactose-Sucrose-Arabinose-Trehalose-Salissin) (Jyoti *et al*., 2003).

2.3.2. Molecular characterization and genotypic analysis of LAB strains

Molecular identification and genotyping of strains were carried out based on species-specific PCR, 16S rDNA sequencing, and BLAST. DNA was extracted from strains that grew in MRS broth using a Gram-positive bacterial DNA extraction kit (Sinaclone, Iran), and then concentration and purity of DNA were determined by NanoDrop absorbance readings (260/280 nm) (NanoDrop, Thermo Scientific) (Danova *et al.,* 2005). In order to confirm the presence of the 16S rRNA gene, PCR amplification was conducted utilizing primer pairs specific to the target gene (27F: AGA GTT TGA TCC TGG CTC AG, 1492R: CGG TTA CCT TGT TAC GAC TT). Primers were used for amplification of the partial 16S rRNA gene and amplified a fragment length of 1500 bp. PCR was carried out in a total volume of 25µL and consisted of 2µL of genomic DNA from each strain, 1µL of each primer, 2.5µL of 10x PCR buffer (Takapouzist, Iran), 1.5µL of $MgCl_2$, 0.5 µL Taq polymerase, and 0.5 µL dNTP. The thermal cycler amplification protocol was established with the following parameters: an initial denaturation step at 94°C for 3 minutes, followed by 32 cycles consisting of denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute, and extension at 72°C for 1 minute. This was concluded with a final extension at 72°C for 10 minutes. The resulting PCR products were analyzed using agarose gel electrophoresis, which was subsequently stained with ethidium bromide (Qiagen, Germany) and visualized using the gel documentary (ProteinSimple, USA). The PCR products of 16S rRNA were purified utilizing a DNA Purification Kit (Denazist, Iran), and the resulting DNA was

subsequently employed in the sequencing process conducted by Macrogen in Seoul, Korea. The obtained sequences were analyzed against the sequence data available in the NCBI GenBank through the BLAST program (blast.ncbi.nlm.nih.gov). A similarity threshold of greater than 98% between the 16S rRNA sequence of the unidentified isolate and that of a reference strain was established as the criterion for identification. Additionally, a phylogenetic tree was constructed using the neighbor-joining method with the aid of Chromas software (Ver 1.41) and MEGA 5.1 (Gouy *et al*., 2010).

2.4. Evaluation of di-acetyl production

The *Lactobacilli* isolates were cultured in MR-VP medium (Merck, Germany) and were incubated in aerobic conditions at 30ºC for 72 h under agitation at 150 rpm for 10 minutes. Di-acetyl production was assessed using Barritt's reagent as follows: Following incubation, 5.2 mL of MR-VP medium was combined with 0.6 mL of reagent A and 0.6 mL of reagent B. After 15 minutes, the appearance of a red color indicated the presence of di-acetyl, while a yellow color signified a negative result.

2.5. Evaluation of MRS, Elliker, and MR-VP mediums in biomass and di-acetyl production

A bacterial suspension of 1 ml was calibrated to a turbidity of 0.5 McFarland (1.5×10^8 CFU/mL) and subsequently utilized to inoculate 50 ml of MRS, Elliker, and MR-VP media (Merck, Germany). The cultures were incubated at 30°C for 3 to 5 days with continuous agitation (150 rpm) and then analyzed for bacterial growth and biomass production.

For evaluation of diacetyl production, isolates were cultured in MRS, Elliker, and MR-VP mediums and incubated in aerobic conditions at 30ºC for 72 h under agitation at 150 rpm for 10 minutes. Di-acetyl production was confirmed using Alpha-Naphthol and potassium hydroxide reagents and spectrophotometry. The LAB isolates that produced higher levels of di-acetyl were sub-cultured in MR-VP medium at 30ºC under agitation for 7 days and then analyzed.

2.6. Extraction of di-acetyl

Two-step extraction and silica-gel assays were used for di-acetyl extraction from LAB cultures. In the two-step extraction method, the LAB culture was first mixed with di-ethyl ether and acetate solutions, and then, the organic phase was separated from the watery phase using a Separator funnel. After distillation at 60ºC temperature, the solution was analysed using a hydroxylamine colorimetric assay to determine di-acetyl production.

In the silica-gel assay, the suspension is passed through a column involving silica-gel and a filter. Then, the silica-gel was washed using ethanol and analyzed using a hydroxylamine Colorimetric assay. The samples were further analyzed using gas chromatography.

2.7. *In vitro* antifungal effect of di-acetyl

The antimicrobial effects of di-acetyl against *Penicillium* isolates were evaluated using well-diffusion and macro-broth dilution assays. *Penicillium* strains were isolated from moldy cheese samples.

2.7.1. Well-diffusion agar

Penicillium isolates were cultivated on Sabouraud dextrose agar (Merck, Germany) at 25 °C for 3 to 5 days. The mold spores were then suspended in sterilized normal saline and adjusted to a turbidity equivalent to the 0.5 McFarland standard (1×10^6 CFU/ml). These spore suspensions were subsequently inoculated onto Sabouraud dextrose agar, adding 0.1 µg of di-acetyl to each well. Miconazole served as the positive control in this experiment. The plates were incubated aerobically at a temperature of 25±2 °C for a period ranging from 24 to 72 hours. After the incubation period, the diameters of the inhibition zones were measured. All experiments were conducted in triplicate.

2.7.2. Macro-broth dilution method

The macro-broth dilution assay was performed by a two-fold serial dilution of the di-acetyl in distilled water at concentrations ranging from 0.125- 32 µl/mL. The mold spore suspension was diluted to 1×10^5 CFU/ml, then 0.1 ml of standardized suspension was added to each tube containing various concentrations of di-acetyl in broth medium. The tubes were incubated at 25°C for 24-72 h, and the lowest concentration of di-acetyl with an inhibitory effect on the growth of *Penicillium* was considered as minimal inhibitory concentration (MIC) (CLSI, 2012). A tube containing broth medium and inoculum without di-acetyl was taken as the negative control, while broth containing inoculum and miconazole was used as the positive control.

2.8. Statistical analysis

All experiments were conducted in triplicate. The significant difference was determined by SPSS software using an ANOVA test at a level of 0.05.

3. Results and Discussion

3.1. Isolation, identification, and molecular typing of the LAB isolates

The findings from both classical and microbiological analyses indicated that out of 16 LAB isolates derived from the yoghurt, dough, and kefir, 10 were classified within the genus Lactobacillus (Table 1). These isolates exhibited characteristics typical of Gram-positive, catalase-negative, small rod-shaped, non-motile, and non-spore-forming bacteria that can produce gas from glucose under anaerobic conditions. The addition of formic acid to the selective mediums was effective in inhibiting yeast growth. Of the ten isolates, 8 were from yoghurt, 2 were from the dough, and the yeast species were isolated only from kefir.

Table1: Identification of Newly Isolated Lactobacilli from Dairy Products was Conducted Using Classical Biochemical Assays

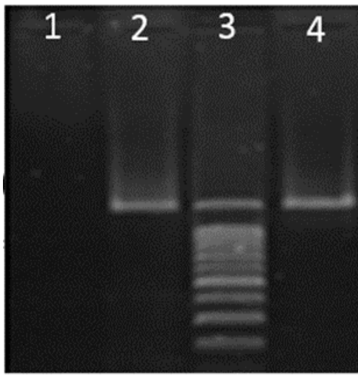
Parameters	1	2	3	4	5	6	7	8	9	10
Gram stain	+	+	+	+	+	+	+	+	+	+
Cellular morphology	Rod	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Catlalase	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-
Sugar fermentation tests										
Glucose	+	+	+	+	+	+	+	+	+	+
Arabinose	-	-	+	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+	-	+	+
Galactose	+	+	+	+	-	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+
Gas from glucose	-	-	-	-	-	-	-	-	-	-
Most probably organism	*L. casei*	*L. casei*	*L. casei*	*L. casei*	*L. casei*	*L. casei*	*L. casei*	*L. casei*	*L. casei*	*L. casei*

The PCR products, which were species-specific and approximately 1500 bp in size, confirmed that three strains were classified as belonging to the species *L. casei*. The identification process was completed through the analysis of 16S rRNA gene sequences. Subsequent BLAST analyses of these sequences indicated that all isolates exhibited a 99% similarity to *L. casei*. (Table 2 and Fig 1).

Table 2: Identification of Newly Isolated Lactobacilli from Dairy Products was Conducted by 16s Rdna Sequence Analysis and Yields of Diacetyl

Isolate NO.	Identified as	Probablity (%) according to APLAB identification	Probablity (%) based on the BLAST analysis of the sequences	Isolation source	Production of Di-acetyl
1	*L. casei*	99.4 (very good)	100	Yoghurt	-
2	*L. casei*	99.6 (very good)	100	Dough	++
3	*L. casei*	87.8(low)	100	Yoghurt	+
4	*L. casei*	99.9 (excellent)	100	Yoghurt	++++
5	*L. casei*	99.9 (excellent)	100	Yoghurt	++++
6	*L. casei*	86.5(low)	100	Dough	++
7	*L. casei*	99.2 (very good)	100	Yoghurt	-
8	*L. casei*	99.9 (excellent)	100	Yoghurt	+++
9	*L. casei*	99.7 (very good)	100	Yoghurt	++
10	*L. casei*	72.5 (low)	100	Yoghurt	-

Figure 1: Agarose Gel Electrophoresis (1% w/v) was Conducted on the PCR Products Generated using Species-specific Primers for L. cacei



Note. 1: PCR negative control (no DNA added); 2*: L. cacei* ATCC 393; 3: Ladder 100 bp; and 4: *Lactobacilii* isolate.

The polyphasic approach was applied to the identification of *Lactobacillus* isolates. Of 16 LAB isolates from yoghurt, dough, and kefir, 10 belonged to the Lactobacillus gen*us.* In this study, the media that was more related to dairy products, which contained formic acid, was chosen to avoid yeast growth. Biochemical tests confirmed the isolates to be *L. casei* with varying degrees of probability. Therefore, further molecular detection methods were also needed. Species-specific PCR is one method that has proven useful for identifying bacteria that are difficult to distinguish using classical microbiology tests and for distinguishing between phenotypically similar species. (Quere *et al*., 1997). The PCR results revealed that all of the isolates belong to *L. casei* species. Sequence analysis of the 16S rRNA gene uniquely identified the strains as *L. casei* with a probability of 100%. To be classified within the species, a minimum of 98% identity to the consensus sequence of the 16S rRNA gene is required (Wang *et al*., 2008). Phylogenetic analysis demonstrated a significant sequence similarity with the type strain of *L. casei* ATCC393, alongside an evolutionary divergence from other Lactobacillus species.

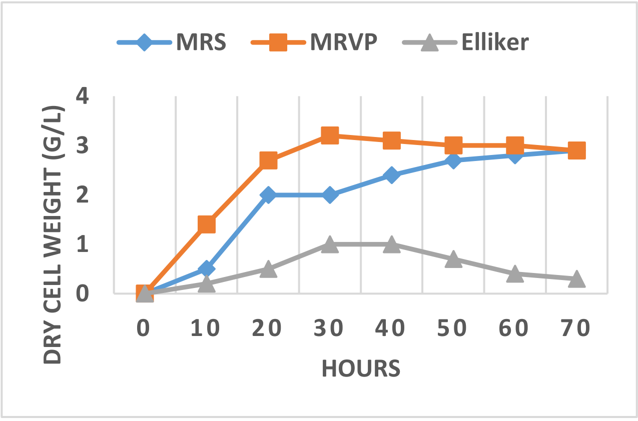
3.2. Determination of di-acetyl production

The results of culturing different isolates during the screening stage in MRS, Elliker, and MR-VP media indicated that MR-VP media has better results than the other media in terms of growth and organolytic biomass. The di-acetyl production ability in ten isolates was analysed, and results exhibited that only 7 isolates could produce di-acetyl using MR-VP medium and spectrophotometry (530 wavelength) (Fig 2). These results showed that isolates 4 and 5 have more ability to produce di-acetyl than those isolated from yoghurt, so isolates that were able to create more biomass also produced more diacetyl.

Lactobacillus species can synthesize antibacterial agents, including biosurfactants, bacteriocins, hydrogen peroxide, diacetyl, and various organic acids. Furthermore, their ability to

generate hydrogen peroxide and their role in suppressing the growth of pathogens have also been documented (Szczerbiec *et al.*, 2022).

Figure 2: Productivity as a Percentage of the Maximum Diacetyl on Various Carbon Sources, Medium Containing Glucose (MR-VP), Dextrose+citrate (MRS), and Lactose+sucrose (Elliker medium)



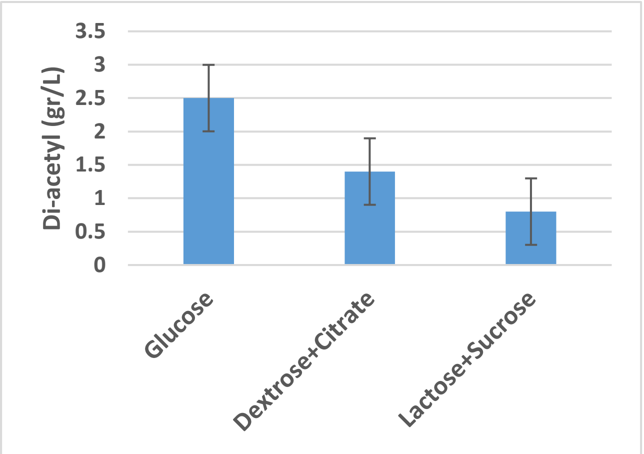
Di-acetyl is predominantly present in low concentrations across various dairy products. Its production by specific lactic acid bacteria species occurs through the fermentation of citric acid and the breakdown of α-acetolactate during the fermentation process of 2,3-butanediol in dairy products. In this context, 2,3-butanediol serves as a glucose overflow metabolite generated by the biological fermentation activities of diverse microorganisms (Jay, 1982; Li *et al.*, 2023).

In the current study, the production of di-acetyl varied among the LAB isolates. Two LAB isolates isolated from traditional yogurt exhibited the greatest di-acetyl production.

3.3. Evaluation of MRS, Ellikler, and MR-VP mediums in biomass and di-acetyl production

After evaluating the growth of isolated LAB in different mediums containing different carbon sources, the maximum biomass yield was shown in the MR-VP medium (Fig 3).

Figure 3: Concentration of Biomass for the Growth of L. casei on Glucose, Dextrose+citrate, and Lactose+sucrose after 72 Hours



Different levels of di-acetyl were produced during the growth of LAB isolates on three mediums containing various carbon sources. The maximum production of di-acetyl was 2.5 g from 1 ml of growth culture when the MR-VP medium was used as the growing medium. This suggests that di-acetyl is produced in the presence of glucose as a carbon source.

The effects of culture medium and carbon source on specific growth rate and biomass production of LAB bacteria, as well as the production of di-acetyl, were analyzed. The results showed that L. casei could grow on various carbon sources, including glucose, dextrose with citrate, and lactose with sucrose. However, the MR-VP medium has the most significant effect on biomass and di-acetyl (as antifungal metabolite) production. This medium contains glucose as a carbon source, suggesting that this carbon source was better in biomass and di-acetyl production in *L. casei* than MRS medium, which has dextrose and citrate, and Elliker medium, which contains lactose and sucrose. The maximum level of di-acetyl production was 2.5 mg from 1 ml of MR-VP growth culture.

In the study by Nielsen et al. (1994), glucose and citrate were equally preferred substrates for *L. rhamnosus*, which showed the same growth rate (0.28 h), which was in agreement with our results. Other organisms usually prefer glucose as a

substrate with a maximum growth rate (Jyoti *et al.,* 2003). Several studies reported that the selection of the screening medium is a critical step, as the number of potential antifungal components may decrease gradually (Santra *et al*., 2024; Hossain et al., 2024; Salas *et al*., 2018). In another study, the produced Di-acetyl yields increased when bacteria were grown on media containing glucose+pyruvate and glucose+citrate, in which glucose+citrate had a greater effect than citrate alone (Jyoti *et al.,* 2003). Lipin *et al*. (2018) reported maximum concentration of di-acetyl production in the medium containing citrate as the carbon source, which differs from our findings. The antifungal metabolites of *Lactobacillus sp*. were highly dependent on the compounds present in the medium. In our study, *L. casei* isolated from yoghurt showed a higher level of di-acetyl production than the others.

3.4. Extraction of di-acetyl

Di-acetyl was extracted from LAB cultures using a two-step extraction assay. The production of pink color in the solution showed the extraction of di-acetyl (hydroxylamine colorimetric assay). The results of gas chromatography also confirmed the extraction of di-acetyl.

The silica-gel assay using hydroxylamine colorimetric assay did not reveal the extraction of di-acetyl, which indicated that this method is unable to differentiate di-acetyl.

Analysis of the gas chromatography confirmed these results (Fig 4).

The results of di-acetyl extraction using two assays revealed that the two-step extraction method was the better way to extract the produced di-acetyl in the culture than the silica-gel method. The extracted di-acetyl was confirmed by gas chromatography.

Figure 4: Results from Gas Chromatography Confirmed the Successful Extraction of Diacetyl

The growth rate of glucose consumption was reflected in higher production of di-acetyl. In a study by Adeyemo *et al*. (2018), *Lactobacillus plantarum* followed by *L. acidophilus* had the highest production of antimicrobial compounds. According to the literature, the most active antifungal strains found in fermented dairy products are associated with the *L. casei* group (Delavenne *et al*., 2012; Chen *et al*., 2021). The isolated strain's di-acetyl production was monitored for up to seven days, and results indicated various degrees of production.

3.5. *In vitro* tests for molds' growth inhibition

Controlling spoilage microorganisms in food and extending its shelf life still poses significant challenges today. Food poisoning with disparate types of toxin-producing molds is a serious complication, and mold growth leads to significant economic losses; for instance, postharvest diseases caused by fungai contribute significantly to postharvest losses of fruits, some fungal pathogens produce mycotoxins, which affect the quality and safety of fruit (GodanaQiya *et al*., 2023). The current study focused on the depiction of *lactobacilli* isolated from common dairy products and the antifungal activity of their produced di-acetyl.

The antifungal efficacy of extracted di-acetyl against *Penicillium*, evaluated using the well diffusion agar method, demonstrated no inhibition zones for mold growth. Conversely, the micro-broth dilution method revealed a broad spectrum of activity for di-acetyl, effectively inhibiting *Penicillium* by a minimum inhibitory concentration (MIC) of 0.64 μg/ml.

Dairy products are susceptible to spoilage pathogens, and their low pH levels create an ideal environment for fungal growth. It is evident that molds associated with dairy decay exhibit significant diversity at both the class and species levels. As many as 100 species have been identified as responsible for the spoilage of dairy items (Tropcheva *et al*., 2014; Shi *et al*., 2022). The Penicillium strains selected in this study were derived from contaminated cheese, which is known to be a common contaminant in dairy products (Kavková *et al*., 2021). We evaluated the antifungal activity of extracted di-acetyl. The mechanism of antimicrobial action often is not defined due to a complex interaction between different compounds. In other studies, the LAB strain or its culture supernatant has been used for assessing antifungal activity because it showed the effect of the combination and synergy of all of the antimicrobial agents, but in this research, we extracted di-acetyl from the cultures and investigated its antifungal effects.

In one study, Tuma *et al*. (2007) reported the antifungal activity of *L. paracasei* against *Penicillium* species, *Fusarium proliferatum,* and *Aspergillus niger*. Gerez *et al*. (2013) observed the good antifungal effect of *L. plantarum* isolated from various food sources. Another study showed that the antifungal activity of *Lactobacillus plantarum* and *Lactobacillus fermentum* strains against spoilage bacteria such as *Aspergillus niger, Rhizopus stolonifer*, and Mucor species was effective (Temitope *et al*., 2015). A recent investigation revealed that LAB ZZUA493 exhibits wide-ranging antifungal efficacy against various species, including *Trichoderma longibrachiatum*, *Aspergillus oryzae*, *Aspergillus niger*, *Fusarium graminearum,* and *Aspergillus flavus*. The organic acids generated by ZZUA493 seem to play a significant role in the suppression of fungal growth (Zhao *et al*., 2022). Additionally, the antibacterial properties of di-acetyl have been documented against *Bacillus subtilis* and *Bacillus globigii* (Jay *et al.,* 1982).

Our findings showed a broad spectrum of antifungal activity of di-acetyl extracted from 10 newly identified *L. cacei* strains, which exhibited antifungal activity against *Penicillium* sp by macro-broth dilution method. Di-acetyl exhibited a very good antifungal effect against *Penicillium* sp., isolated from contaminated cheese sources. However, the well diffusion method results did not show zones of inhibition, and any inhibitory activity against Penicillium sp. suggested that di-acetyl may not be diffusible into the agar. This may be the reason for the difference in the results of the two methods.

4. Conclusion

As the resistance of microbial food spoilage to current preservatives is increasing, food safety can be enhanced by developing new organic antimicrobial agents from natural sources, particularly LAB, which are sources of bio-preservative compounds. In this study, ten lactobacilli strains were identified as *L. cacei* isolated from traditional dairy products, and their antimicrobial di-acetyl was analyzed. *L. cacei* isolates were grown on various carbon sources.

The inhibitory activity of lactobacilli against spoilage fungi by di-acetyl production is dependent on the cultivated medium. The results of this study are promising and highlight the important role of di-acetyl from *L. cacei* isolates as an antifungal biopreservative that can improve food quality and safety in various food technologies. Such experiments are a starting point for solving food spoilage problems and extending shelf life in the home.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgment

This study was approved by the Islamic Azad University, Neyshabur Branch, as an MS thesis

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Conflict of Interest

The authors declare no conflict of interest.

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None declared.

Authors' Contributions

Samaneh Dolatabadi designed the study, wrote the protocol, and the manuscript draft. Azin Latifi contributed to sampling and performed the microbiological tests. Mohammad Mehdi Motaghi performed the statistical analysis. Bita Behboodian contributed to writing the paper.

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