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Antibacterial and Sensory Impact of β-carotene and Riboflavin on *Pseudomonas* Biofilm in Raw Milk

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ABSTRACT

Milk is a highly nutritious yet perishable food that is susceptible to microbial contamination and spoilage, particularly by *Pseudomonas* species. The present study aimed to assess the antimicrobial and sensory effects of β-carotene and riboflavin as natural additives for preserving raw milk, with a specific emphasis on controlling biofilm-forming *Pseudomonas* strains. A total of 100 raw milk samples were collected from different markets in Gharbia, Egypt, and tested for *Pseudomonas* spp. Biofilm formation was assessed in isolates, followed by the detection of the quorum-sensing transcriptional regulator *lasR*, pellicle formation protein A (polysaccharide biosynthesis protein A) *pelA*, and polysaccharide synthesis locus protein A *pslA* genes. An *in vitro* trial was performed on eight groups of raw milk to evaluate the effects of β-carotene and riboflavin at concentrations of 1 µg/ml and 4 µg/ml, respectively, on *Pseudomonas* counts and sensory properties. The groups include positive control (G1), raw milk inoculated with *Pseudomonas* and fortified with β-carotene (G2), raw milk inoculated with *Pseudomonas* and fortified with riboflavin (G3), raw milk inoculated with *Pseudomonas* and fortified with a combination of β-carotene and riboflavin (G4), negative control **(**G5), milk fortified with β-carotene only (G6), milk fortified with riboflavin only (G7) and milk fortified with both β-carotene and riboflavin (G8). Reverse transcription polymerase chain reaction (RT-PCR) was performed to assess the impact of these additives on *Pseudomonas*, specifically on *16S rRNA* and *pslA* gene expression. *Pseudomonas* spp. were found in 20% of samples, with 75% forming biofilms. The *pslA* gene was detected in 83.33% of these samples. β-carotene and riboflavin indicated dose-dependent antibacterial effects, with minimum inhibitory concentration values, and *minimum bactericidal concentration* values. Milk fortified with these compounds, particularly in G4, demonstrated the most significant reduction in *Pseudomonas* during a 6-day storage period. Additionally, β-carotene maintained a preferable sensory quality compared to other groups. RT-PCR confirmed the highest *pslA* gene suppression with the combined treatment. The current findings indicated that β-carotene at a concentration of 1 µg/ml, whether used independently or in combination with riboflavin, can serve as an effective natural preservative in dairy products.

Keywords: Beta-Carotene, Biofilm, *Pseudomonas*, *PslA* Expression, Riboflavin

INTRODUCTION

Milk is considered one of nature's most nutrient-rich foods, owing to its high levels of bioavailable proteins, lactose, lipids, calcium, and a wide range of bioactive constituents, including peptides, enzymes, immunological agents, growth factors, and hormones (Li et al., 2018). Nevertheless, this nutritional abundance creates a favorable environment for microbial proliferation, potentially resulting in rapid spoilage, decline in quality, shorter shelf life, and a higher risk of foodborne diseases spreading (Dash et al., 2022).

Pseudomonas, an important Gram-negative opportunistic pathogen**,** is considered among the most common psychotropic bacteria associated with milk spoilage (Zhang et al., 2019). These bacteria can grow at refrigeration temperatures (7°C or lower), resulting in putrefaction, fermentation, and the emergence of off-flavors such as fruity or bitter notes due to their capacity to metabolize fats and proteins (Atia et al., 2022). Although psychrotrophic bacteria are not typically part of the native microbiota of the bovine udder (Porcellato et al., 2020), their presence in chilled raw milk is primarily attributed to post-harvest contamination, particularly during or after milking. Milking equipment is frequently identified as a major source of this contamination (Oliveira et al., 2015). These spoilage organisms pose a considerable challenge to the dairy industry (Dogan and Boor, 2003), as they are capable of growing at refrigeration temperatures and producing heat-resistant proteolytic enzymes. Proteolytic enzymes can remain active even after pasteurization, thereby accelerating milk spoilage (Dogan and Boor, 2003; Xin et al., 2017). In addition, *Pseudomonas* spp. possess multiple virulence factors such as biofilm formation, exopolysaccharides, and quorum sensing that enhance their survival under adverse conditions (Zhao et al., 2019). Furthermore, *Pseudomonas* spp. can form biofilms and increase their resistance to disinfectants and antibiotics, making them difficult to eliminate (Drenkard, 2003).

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Biofilms formed by *Pseudomonas* spp. pose a significant challenge in dairy processing, as these bacteria can colonize surfaces such as bulk milk tanks and processing equipment, using their polar flagella to aid in motility and surface attachment (Jurado-Marein et al., 2021). These biofilms are composed of structured microbial communities that are embedded in a matrix of extracellular polymeric substances (EPS), which is made up of polysaccharides, proteins, extracellular DNA, and vesicles (Fulaz et al., 2019). This matrix acts as a protective barrier, shielding the bacteria from environmental stresses and antimicrobial agents, thereby promoting contamination and spoilage through the production of degradative enzymes. Thus, controlling biofilm formation is essential to maintaining milk safety and quality (Yuan et al., 2017). Two critical genes involved in biofilm development in *Pseudomonas* are *pslA* and *lasR*. The *pslA* gene encodes a glycosyltransferase responsible for the synthesis of *Psl* polysaccharide, which facilitates initial surface attachment and cellular aggregation. Meanwhile, *lasR* encodes a quorum-sensing transcriptional regulator that governs the expression of genes involved in bacterial communication and EPS production (Abootaleb et al., 2020).

Growing concern about the health hazards and quality deterioration linked to chemical preservatives and thermal processing has generated heightened interest in natural, environmentally sustainable antimicrobial alternatives. Traditional additives such as nitrites and hydrogen peroxide may pose safety risks, while high-temperature treatments can negatively affect the nutritional value and sensory qualities of food (Garcia-Fuentes et al., 2015). In contrast, phytochemicals have demonstrated effective antimicrobial properties against foodborne pathogens, highlighting their potential as viable options for preserving dairy products (Elafify et al., 2020).

Among these compounds, riboflavin (Vitamin B2) and carotenoids serve dual functional roles as natural additives (Farah et al., 2022; Naisi et al., 2023). Carotenoids, such as β-carotene, are naturally occurring pigments found in food sources. β-carotene possesses appropriate antioxidant and anti-inflammatory properties (Fiedor and Burda, 2014) and contributes to improving milk quality by preventing protein oxidation and enhancing flavor stability (Fiedor and Burda, 2014; Yabuzaki, 2017). Additionally, β-carotene exhibits selective antimicrobial activity, targeting harmful pathogens while preserving beneficial lactic acid bacteria, which is advantageous in dairy processing (Tao NengGuo et al., 2010).

Riboflavin plays a vital role in human metabolism as a cofactor in redox reactions (Farah et al., 2022). Furthermore, it exhibits potent antimicrobial activity upon photoactivation, where light exposure generates reactive oxygen species (ROS) that induce lethal oxidative stress in microbial cells (Farah et al., 2022). This property makes riboflavin an effective natural photosensitizer, with proven activity against *Pseudomonas aeruginosa (P. aeruginosa)* (Najari et al., 2024). Riboflavin supports immune function by enhancing pathogen clearance and reducing inflammation (Farah et al., 2022).

The present study aimed to evaluate the antimicrobial and sensory effects of β-carotene and riboflavin, both individually and in combination, as natural preservatives in raw milk, focusing on their influence on *Pseudomonas* spp. survival, inhibition zones, *pslA* gene expression, and sensory attributes during cold storage.

MATERIALS AND METHODS

Ethical approval

The present study was approved by the Research Committee of the Animal Health Research Institute and was authorized by the Institutional Animal Care and Use Committee (IACUC) of the Agricultural Research Center (ARC) under approval number ARC-AHRI 444429.1/24.

Sample collection and preparation

A total of 100 random milk samples were collected from different markets in Gharbia Governorate, Egypt. The collected samples were immediately transported to the Microbiology Department at the Animal Health Research Institute (Tanta Branch) in a chilled container, maintaining strict aseptic conditions, to be prepared and analyzed promptly for detecting *Pseudomonas*.

Isolation and identification of *Pseudomonas*

One mL of the milk sample was inoculated into 9 mL of nutrient broth and incubated at 37°C for 24 hours. Subsequently, a loopful of the enriched culture was streaked onto *Pseudomonas* cetrimide selective agar supplemented with King's B agar, uniformly spread, and incubated at 37°C for 18-24 hours. Colonies exhibiting characteristic blue-green or green-yellow pigmentation with a distinct sweet, grape-like odor were presumptively identified as *Pseudomonas* and selected for further analysis. Biochemical characterization was performed according to ISO (2004) guidelines, including oxidase and catalase tests, gelatin liquefaction and hydrolysis, sugar fermentation assays, and pigment production. Isolates showing biochemical profiles consistent with *Pseudomonas* spp. were subjected to further examination by polymerase chain reaction (PCR) for biofilm-related virulence genes detection (*lasR, pelA, and pslA*).

Detection and evaluation of biofilm production

Tube method

The tube method (TM) is a qualitative method for detecting biofilm formation (Christensen et al., 1985). Bacteria were grown in Trypticase soy broth for 24 hours at 37°C, then the tubes were washed with phosphate-buffered saline (pH 7.2), air dried, stained with 1% crystal violet for 10 minutes, and washed with deionized water. Biofilm formation was assessed utilizing the control strains. The presence of biofilm was identified by a visible film on the walls and base of the tube. The formation of a ring at the liquid interface was indicative of a non-biofilm producer. The results were evaluated visually and categorized as negative (1), weak positive (2), moderate positive (3), and strong positive (4; Figure 1).

Tissue culture plate method

The tissue culture plate (TCP) method was used to assess biofilm formation by *Pseudomonas* isolates (Vasudevan et al., 2003). Biofilm formation was assessed using the microtiter plate method. Bacterial cultures (10^6 CFU/mL) were grown in tryptic soy broth (TSB) supplemented with 1% glucose and incubated at 37°C for 24 hours. The cultures were then diluted 1:100 in fresh TSB, and 200 µL of the diluted suspension was added to each well of a 96-well microtiter plate. After 24 hours of incubation at 37°C, the wells were gently washed three times with phosphate-buffered saline (PBS), air-dried, and stained with 50 µL of crystal violet 1% for 15 minutes. Excess stain was removed by washing the wells three times with 200 µL of sterile distilled water, followed by air drying at 45°C for 20 minutes. To quantify biofilm, the stained biofilm was solubilized with 200 µL of ethanol 95% per well for 30 minutes. The optical density (OD) was measured at 630 nm using an ELISA microplate reader (MR-96, CLINDIAG, China) at the Microanalysis Unit, Faculty of Science, Tanta University, Tanta, Egypt.

Absorbance

Each strain was tested three times, and the average OD readings were calculated. The final OD value for each strain's biofilm activity was obtained by subtracting the cut-off value (ODc) from the average OD. Biofilm production was then classified based on the criteria including $OD \leq ODc$ (Non-biofilm producer), $ODc < OD \leq 2 \times ODc$ (Weak biofilm producer), $2 \times ODc < OD \leq 4 \times ODc$ (Moderate biofilm producer), and $OD > 4 \times ODc$ (Strong biofilm producer), as described by Stepanović et al. (2007). The positive reference strain for biofilm formation was *Pseudomonas aeruginosa* ATCC 27853.

Detection of biofilm virulence genes in *Pseudomonas* using polymerase chain reaction

Isolates exhibiting strong biofilm-forming ability were selected for PCR analysis to detect virulence genes associated with biofilm production (*lasR*, *pelA*, and *pslA*) using uniplex PCR. Genomic DNA was extracted from *Pseudomonas* isolates using the QIAamp DNA Mini Kit (Qiagen, Germany), with slight protocol modifications. Oligonucleotide primers and related analyses were provided by Metabion (Germany), as detailed in Table 1.

Table 1. The primers used for the detection of *Pseudomonas* and biofilm virulence genes

Gene	Sequence	Amplified product (bp)	Reference
Pseudomonas 16S rDNA	GACGGGTGAGTAATGCCTA	618	(Spilker et al., 2004)
	CACTGGTGTTCCTTCCTATA		
pelA	CATACCTTCAGCCATCCGTTCTTC	786	(Ghadaksaz et al., 2015)
	CGCATTCGCCGCACTCAG		
pslA	TCCCTACCTCAGCAGCAAGC	656	
	TGTTGTAGCCGTAGCGTTTCTG		
lasR	CTGTGGATGCTCAAGGACTAC	133	(Saleh et al., 2019)
	AACTGGTCTTGCCGATGG		

Preparation of β-carotene and riboflavin stock solution

A 1 mg/mL riboflavin solution (Sigma-Aldrich) was prepared under dark conditions by dissolving 1 mg of riboflavin in 1 mL of distilled water. Similarly, a 1 mg/mL β-carotene solution (Sigma-Aldrich) was prepared under light-protected conditions by dissolving 1 mg of β-carotene powder in 1 mL of dimethyl sulfoxide (DMSO), the concentration of which did not exceed 1% (v/v). Both solutions were stored in light-protective tubes and used within 30 minutes of preparation to prevent degradation.

***In vitro* evaluation for antibacterial activity of β-carotene and riboflavin**

The well diffusion method was employed to estimate the antibacterial activity of β-carotene and riboflavin against biofilm-forming *Pseudomonas*. After culturing the target bacteria in broth and adjusting the concentration to 10^6 CFU/mL, 100 µL of the bacterial suspension was spread evenly onto agar plates under aerobic conditions. Wells were

aseptically punched into the agar plate using a sterile cork borer with a diameter of 6-8 mm. Freshly prepared solutions of β-carotene, which was dissolved in DMSO, and riboflavin (dissolved in PBS) at concentrations of 0.5, 0.25, and 0.125 mg/mL were dispensed into the wells (20 µL per well). Each compound was tested on separate plates to avoid cross-interference. The plates were then incubated at 37°C for 24 hours to observe zones of inhibition (CLSI, 2020). Positive controls included 10 µg of imipenem, whereas negative controls utilized DMSO for β-carotene and distilled water for riboflavin. Antimicrobial activity was assessed by measuring the diameter of the inhibition zones (mm) around the wells. The inhibition zones were categorized as greater than 15 mm, indicating pronounced antimicrobial activity, 13-15 mm, representing moderate activity, and less than 12 mm, signifying weak or no antimicrobial activity. These criteria were derived from the studies conducted by Ahgilan et al. (2016) on riboflavin and Muntean et al. (2021) on β-carotene.

Determination of minimum inhibitory concentration and minimum bactericidal concentration of β-carotene and riboflavin

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of β-carotene and riboflavin against *Pseudomonas* were determined using a 96-well microtiter plate (CLSI, 2020). Briefly, one *Pseudomonas* strain identified as a strong biofilm producer was selected for the assay. To each well of a microtiter plate, 100 µL of freshly prepared Mueller-Hinton broth was added, followed by 100 µL of bacterial suspension standardized to 0.5 McFarland turbidity. Subsequently, 100 µL of freshly prepared β-carotene and riboflavin solution at a concentration of 0.5 mg/mL was added to the first well and thoroughly mixed. Two-fold serial dilution was done to estimate the MIC of β-carotene and riboflavin. The positive control well contained only bacterial culture without β-carotene and riboflavin, while the negative control well included DMSO and sterile distilled water for β-carotene and riboflavin, respectively.

The microtiter plates were incubated for an additional 24 hours at 37°C. Bacterial growth was assessed by observing changes in turbidity. The MIC was defined as the lowest concentration of β-carotene and riboflavin at which no visible bacterial growth occurred. For determining the MBC, samples from wells with no visible growth were streaked onto antibiotic-free agar plates and then incubated for 24 hours. The MBC was identified as the lowest concentration of β-carotene and riboflavin at which no bacterial colonies were detected.

Evaluation of the addition of β-carotene and riboflavin to milk

Preparation of bacterial cultures

A *Pseudomonas* strain, which was confirmed as a biofilm producer by PCR, was selected for the present study. The bacterial culture was prepared following the United States Food and Drug Administration guidelines (FDA, 2001). The bacterial suspension was then standardized to a final concentration of 10^6 CFU/mL for use in the following process.

Milk sample collection and grouping

The milk samples were divided into eight groups, each consisting of 100 mL, classified according to two main objectives including bacterial proliferation assessment and sensory analysis. The negative control group was employed in both procedure assessments.

Bacterial growth analysis

Five groups were designed to evaluate the antimicrobial effect of β-carotene and riboflavin including positive control (G1), raw milk inoculated with *Pseudomonas* and fortified with β-carotene (G2), raw milk inoculated with *Pseudomonas* and fortified with riboflavin (G3), raw milk inoculated with *Pseudomonas* and fortified with a combination of β-carotene and riboflavin (G4), and the negative control group (G5). The concentrations of riboflavin (4 µg/ml) and β-carotene (1 µg/ml) applied to the milk were derived from their pre-determined MICs. Treated milk samples were stored at 4 ± 1°C, and *Pseudomonas* counts were monitored over 6 days in accordance with the APHA (2004) protocol.

Sensory evaluation

To assess the impact of β-carotene and riboflavin on the sensory properties of milk, the following groups were evaluated. Negative control (Untreated raw milk, G5), milk fortified with β-carotene only (G6), milk fortified with riboflavin only (G7), and milk fortified with both β-carotene and riboflavin (G8). Sensory evaluation included the assessment of appearance, odor, flavor, and overall acceptability, following the method outlined by Clark et al. (2009), which was performed by a trained panel consisting of scientists and students. To monitor temporal changes in milk quality, sensory assessments were carried out daily during the six-day refrigerated storage period. Each attribute was evaluated on a 10-point hedonic scale using the classifications including excellent (9-10), good (8-9), fair to good (7-8), and poor (6). This evaluation aimed to determine the impact of natural additive fortification on sensory characteristics and to estimate shelf life based on consumer acceptability.

Molecular identification

Expression of 16S rDNA and pslA

Milk samples from the different experimental groups were analyzed using reverse transcription quantitative polymerase chain reaction (RT-qPCR) to determine whether the presence of β-carotene and riboflavin in co-culture with *Pseudomonas* influenced the expression of the *16S rDNA* and *pslA* genes. The primers used in RT-qPCR are presented in Table 2. The cycle threshold (Ct) values of each sample were compared to those of the control group using the delta delta Ct (ΔΔCt) method to quantify the relative changes in gene expression among the samples (Spilker et al., 2004; Ghadaksaz et al., 2015).

Table 2. Primers, sequences, and target genes for SYBR green reverse transcription quantitative polymerase chain reaction

Target gene	Primers sequences	Reference
Pseudomonas 16S rDNA	F-GACGGGTGAGTAATGCCTA	(Spilker et al., 2004)
	R-CACTGGTGTTCCTTCCTATA	
PslA	F-TCCCTACCTCAGCAGCAAGC	(Ghadaksaz et al., 2015)
	R- TGTTGTAGCCGTAGCGTTTCTG	

Statistical analysis

The data are presented as the mean ± standard error (SE). The mean values of the different groups were compared using Duncan's post hoc test in a one-way analysis of variance (ANOVA) at a significance level of $p \leq 0.05$. The method cited by Petrie and Watson (1999) was employed to conduct the statistical analysis, which was then computerized using SPSS version 20 (2011).

RESULTS

Incidence of *Pseudomonas* isolated from milk samples

Based on microbiological and biochemical characterization, *Pseudomonas* was isolated from the total collected raw milk samples with an isolation rate of 20% (20/100).

Evaluation of biofilm production

The isolated *Pseudomonas* strains were tested for biofilm production. The current findings revealed that 15 out of 20 isolates (75%) were characterized as biofilm producers with diverse capabilities utilizing the TCP method. Specifically, eight isolates (40%) exhibited strong biofilm-forming capacity, five isolates (25%) demonstrated moderate capacity, and two isolates (10%) were classified as weak. The TM identified 12 isolates (60%) as biofilm producers, with six (30%) classified as strong, four (20%) as moderate, and two (10%) as weak producers (Table 3, Figure 1).

Detection of biofilm-related virulence genes in *Pseudomonas* using polymerase chain reaction

A total of six *Pseudomonas* isolates were confirmed positive for *16S rDNA*. All isolates tested negative for the *pelA* and *lasR* genes, while 83.33% were positive for the *pslA* gene (Figures 2 and 3).

Diameter of the inhibition zone of β-carotene and riboflavin against *Pseudomonas*

The combination of β-carotene and riboflavin exhibited notable antibacterial activity against *Pseudomonas*, as evidenced by the inhibition zone diameters. The diameters demonstrated a proportional increase relative to the concentrations employed (0.5, 1, and 2 mg/ml; Figure 4). Specifically, the inhibition zone diameter for β-carotene exhibited a significant increase ($p < 0.05$), rising from 16 ± 0.5 to 18.5 ± 0.29. Similarly, riboflavin increased from 15.30 ± 0.17 to 16 ± 0.57. Furthermore, the combination of β-carotene and riboflavin illustrated a substantial increase, rising from 17 ± 0.57 to 19.93 ± 0.06 ($p < 0.05$).

Determination of minimum inhibitory concentration and minimum bactericidal concentration of β-carotene and riboflavin

The current results revealed that the MIC of β-carotene and riboflavin was 1 µg/mL and 4 µg/mL, respectively. The MBC was determined to be 2 µg/mL for β-carotene and 8 µg/mL for riboflavin.

Impact of β-Carotene and riboflavin on *Pseudomonas* survival in refrigerated raw milk

The current findings demonstrated that *Pseudomonas* counts progressively increased in the positive control group (G1) over the six-day storage period (Table 4). While milk samples treated with β-carotene (G2), riboflavin (G3), or both combinations (G4) indicated significantly reduced bacterial growth ($p < 0.05$), G4 demonstrated the greatest antimicrobial effect during refrigeration storage at 4 ± 1°c.

Sensory evaluation of raw milk during refrigeration storage

Table 5 illustrates the evaluation of the sensory characteristics of raw milk over six days of refrigerated storage. The current results indicated that the β-carotene group (G6) maintained the highest sensory scores throughout storage, followed by groups fortified with riboflavin (G7) and a mix of β-carotene and riboflavin(G8), while the negative control group (G5) resulted in lower sensory scores.

Effect of β-Carotene and riboflavin on *pslA* gene expression

The current findings demonstrated that the expression of the *pslA* gene in G4 was decreased to a 0.07-fold change compared to the control group (G1). Additionally, groups G2 and G3 exhibited reduced expression levels of the *pslA* gene, with fold changes of 0.31 and 0.54, respectively. These findings suggested a decrease in *pslA* gene expression under the influence of β-carotene and riboflavin (Figures 5 and 6).

Table 3. Degree of biofilm-forming ability of *Pseudomona*s strains isolated from the examined samples

Tested organism	Biofilm producer						Total biofilm producer		Non-biofilm producer	
	Strong positive		Moderate positive		Weak positive					
Pseudomonas	**TCP**	**TM**	**TCP**	**TM**	**TCP**	**TM**	**TCP**	**TM**	**TCP**	**TM**
	8(40%)	6(30%)	5(25%)	4(20%)	2(10%)	2(10%)	15(75%)	12(60%)	5(25%)	8(40%)

Percentage (%) is calculated in relation to the total number of isolated strains. TM: Tube method, TCP: Tissue culture plate method.

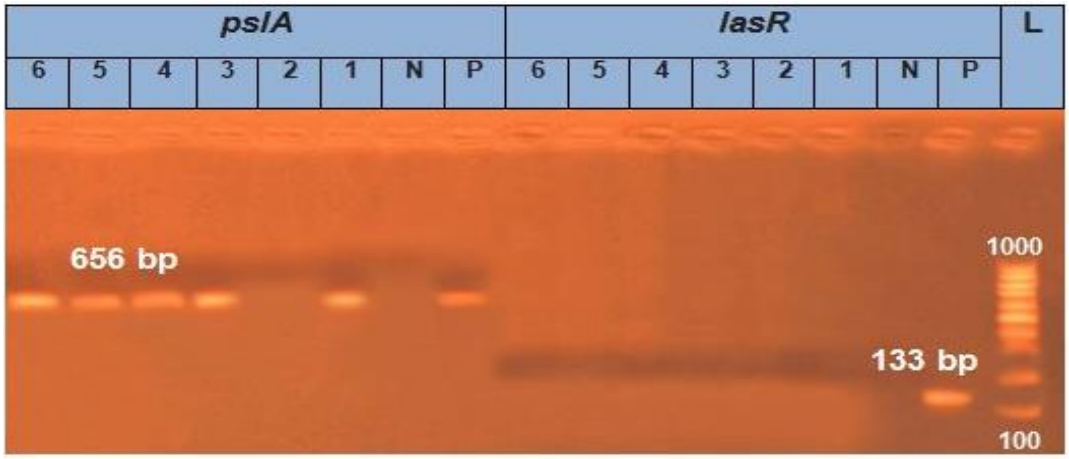

Figure 1. Biofilm formation by the tube method. **A**: Strong biofilm producer, **B**: Moderate biofilm producer, **C**: Weak biofilm producer, **D**: Non-biofilm producer.

Figure 2. Detection of *pslA* and *lasR* genes in 6 *Pseudomonas* isolates at 656 bp and 133 bp, respectively, using agarose gel electrophoresis. L (Ladder): DNA ladder (100-1000bp). Lan (1,3,4,5,6) Positive amplification of the *pslA* gene at 656 bp. Lan (1,2,3,4,5,6) Negative amplification of the *lasR* gene at 133 bp P: Positive control; N: Negative control

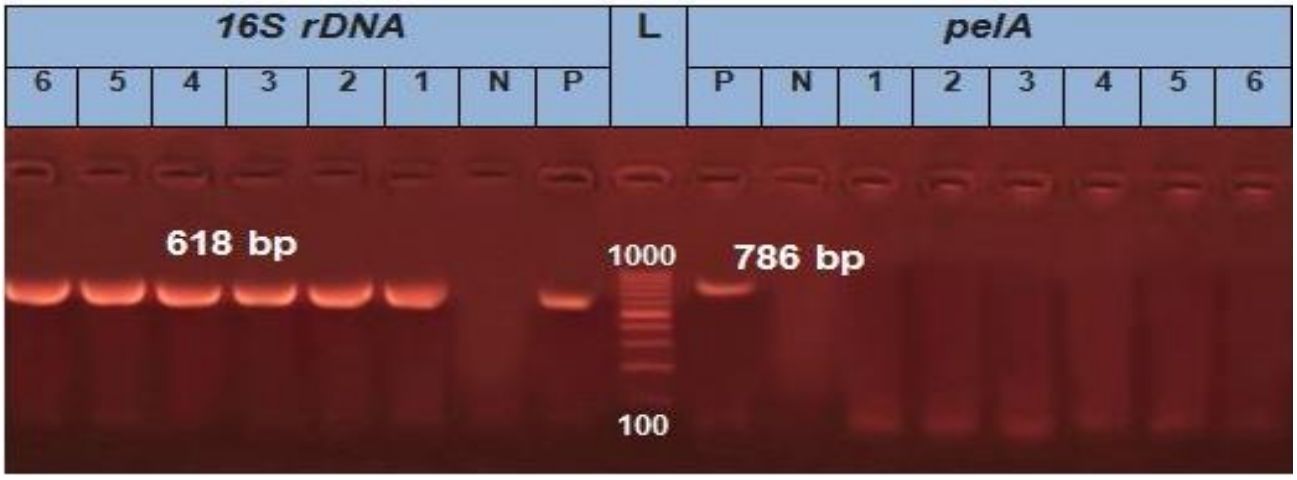

Figure 3. Detection of *16S rDNA* and pel*A* genes in 6 *Pseudomonas* isolates at 618 bp and 786 bp, respectively, using agarose gel electrophoresis. L (Ladder): DNA ladder (100-1000bp). Lan (1,2,3,4,5,6) Positive amplification of the *16S rDNA* gene at 618 bp. Lan (1,2,3,4,5,6) Negative amplification of pel*A* gene at 786 bp P: Positive control; N: Negative control.

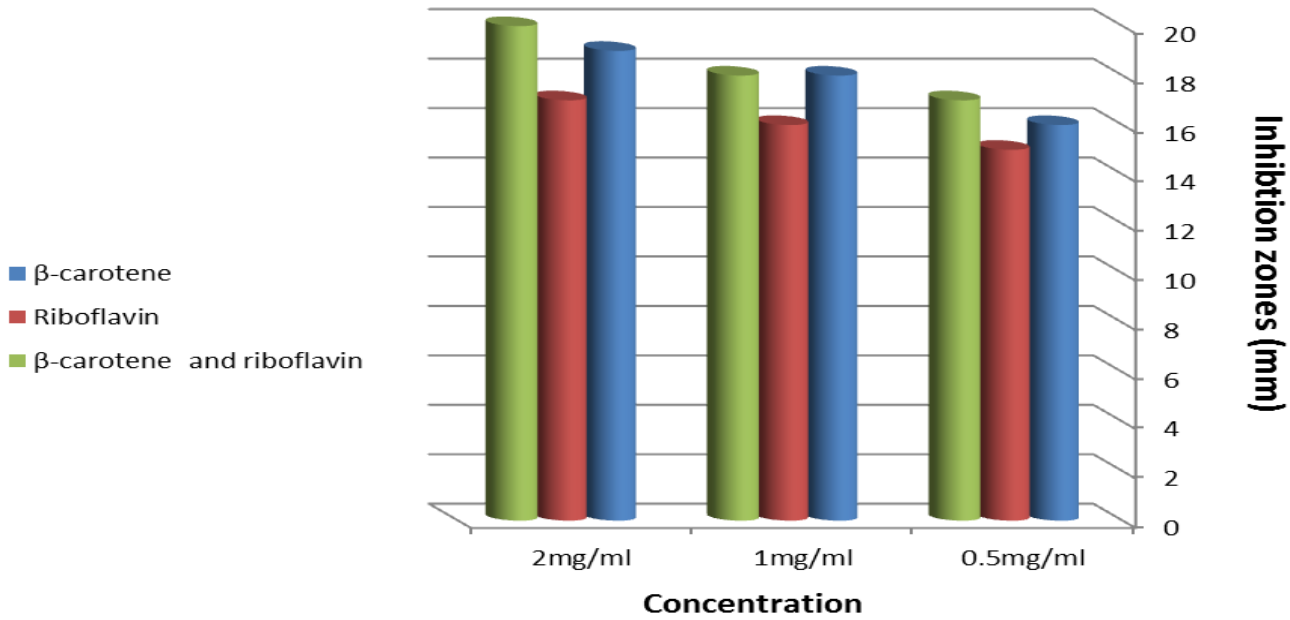

Figure 4. Inhibition zones of β-carotene and riboflavin by agar well diffusion method increased proportionally with the concentrations used.

Table 4. Impact of β-carotene and riboflavin on the survival rate of *Pseudomonas* as log cfu/g in raw milk during refrigeration storage

Groups / Days	G1	G2	G3	G4
Day 0	6.43 ± 0.01^{a}	6.40 ± 0.01^{a}	6.41 ± 0.01^{a}	6.39 ± 0.01^{a}
Day 1	6.92 ± 0.01^{a}	6.25 ± 0.02^{c}	6.39 ± 0.01^{b}	5.92 ± 0.02^{d}
Day 2	7.13 ± 0.01^{a}	5.90 ± 0.02^{c}	5.98± 0.01^{b}	5.34 ± 0.01^{d}
Day 3	7.59 ± 0.06^{a}	5.39 ± 0.01^{c}	5.68 ± 0.02^{b}	4.84 ± 0.01^{d}
Day 4	7.61 ± 0.02^{a}	5.10 ± 0.04^{b}	4.99 ± 0.01^{b}	4.55 ± 0.03^{c}
Day 5	7.81 ± 0.02^{a}	4.83 ± 0.04^{b}	4.98 ± 0.01^{b}	4.35 ± 0.03^{c}
Day 6	7.97 ± 0.02^{a}	4.56 ± 0.06^{c}	4.81 ± 0.01^{b}	3.92 ± 0.01^{d}

G1: Negative control, raw milk without *Pseudomonas*, G2: Raw milk fortified with beta-carotene, G3: Raw milk fortified with riboflavin, G4: Raw milk fortified with a mix of beta-carotene and riboflavin. The values represent as mean ± Standard error. ^{a,b,c,d} Means within a row followed by different superscript letters are significantly different ($p < 0.05$).

Table 5. Sensory evaluation of raw milk during refrigeration storage

Storage period	G5	G6	G7	G8
Appearance scores (10-point scale)				
Day 0	9.85 ± 0.03^{a}	9.80 ± 0.02^{a}	9.70 ± 0.04^{ab}	9.65 ± 0.03^{ab}
Day 1	9.30 ± 0.04^{b}	9.70 ± 0.02^{a}	9.50 ± 0.03^{ab}	9.45 ± 0.05^{ab}
Day 2	8.70 ± 0.05^{b}	9.50 ± 0.02^{a}	9.10 ± 0.04^{ab}	9.05 ± 0.04^{ab}
Day 3	7.90 ± 0.06^{bc}	9.20 ± 0.03^{a}	8.70 ± 0.05^{ab}	8.60 ± 0.05^{ab}
Day 4	7.10 ± 0.07^{c}	8.90 ± 0.04^{a}	8.20 ± 0.04^{ab}	8.10 ± 0.05^{ab}
Day 5	6.40 ± 0.08^{c}	8.60 ± 0.04^{a}	7.60 ± 0.05^{ab}	7.50 ± 0.05^{ab}
Day 6	6.00 ± 0.08^{c}	8.50 ± 0.04^{a}	7.10 ± 0.05^{ab}	7.00 ± 0.05^{ab}
Flavor scores (10-point scale)				
Day 0	9.80 ± 0.03^{a}	9.65 ± 0.02^{a}	9.50 ± 0.04^{ab}	9.45 ± 0.03^{ab}
Day 1	9.00 ± 0.04^{ab}	9.60 ± 0.02^{a}	9.20 ± 0.03^{ab}	9.10 ±0.05^{ab}
Day 2	8.30 ± 0.05^{b}	9.40 ± 0.02^{a}	8.80 ± 0.04^{ab}	8.75 ± 0.04^{ab}
Day 3	7.50 ± 0.06^{bc}	9.10 ± 0.03^{a}	8.40 ± 0.05^{ab}	8.30 ± 0.05^{ab}
Day 4	6.80 ± 0.07^{c}	8.80 ± 0.04^{a}	8.00 ± 0.04^{ab}	7.95 ± 0.05^{ab}
Day 5	6.10 ± 0.08^{c}	8.50 ± 0.04^{a}	7.40 ± 0.05^{ab}	7.30 ± 0.05^{ab}
Day 6	5.70 ± 0.08^{c}	8.00 ± 0.04^{a}	7.20 ± 0.05^{ab}	7.10 ± 0.05^{ab}
Odor scores (10-point scale)				
Day 0	9.80 ± 0.03^{a}	9.82 ± 0.02^{a}	9.75 ± 0.04^{ab}	9.71 ± 0.03^{ab}
Day 1	9.00 ± 0.04^{ab}	9.50 ± 0.02^{a}	9.10 ± 0.03^{ab}	9.05 ± 0.05^{ab}
Day 2	8.40 ± 0.05^{b}	9.30 ± 0.02^{a}	8.70 ± 0.04^{ab}	8.65 ± 0.04^{ab}
Day 3	7.40 ± 0.06^{bc}	9.00 ± 0.03^{a}	8.30 ± 0.05^{ab}	8.25 ± 0.05^{ab}
Day 4	6.60 ± 0.07^{c}	8.70 ± 0.04^{a}	7.90 ± 0.04^{ab}	7.85 ± 0.05^{ab}
Day 5	5.90 ± 0.08^{c}	8.40 ± 0.04^{a}	7.30 ± 0.05^{ab}	7.20 ± 0.05^{ab}
Day 6	6.00 ± 0.02^{c}	7.99 ± 0.03^{a}	7.10 ± 0.02^{ab}	7.00 ± 0.03^{ab}
Overall acceptability scores (10-point scale)				
Day 0	9.81 ± 0.03^{a}	9.75 ± 0.02^{a}	9.60 ± 0.04^{ab}	9.55 ± 0.03^{ab}
Day 1	9.00 ± 0.04^{b}	9.70 ± 0.02^{a}	9.30 ± 0.03^{ab}	9.25 ± 0.05^{ab}
Day 2	8.40 ± 0.05^{b}	9.50 ± 0.02^{a}	8.90 ± 0.04^{ab}	8.85 ± 0.04^{ab}
Day 3	7.60 ± 0.06^{bc}	9.20 ± 0.03^{a}	8.50 ± 0.05^{ab}	8.45 ± 0.05^{ab}
Day 4	6.90 ± 0.07^{c}	8.90 ± 0.04^{a}	8.10 ± 0.04^{ab}	8.05 ± 0.05^{ab}
Day 5	6.20 ± 0.08^{c}	8.60 ± 0.04^{a}	7.50 ± 0.05^{ab}	7.45 ± 0.05^{ab}
Day 6	5.90 ± 0.03^{c}	8.30 ± 0.04^{a}	7.15 ± 0.05^{ab}	7.05 ± 0.05^{ab}

G5: Negative control, raw milk, G6: Raw milk fortified with β-carotene, G7: Raw milk fortified with riboflavin, G8: Raw milk fortified with a mix of β-carotene and riboflavin. The values represent as mean ± Standard error. ^{a,b,c,d} Means within a row followed by different superscript letters are significantly different ($p < 0.05$).

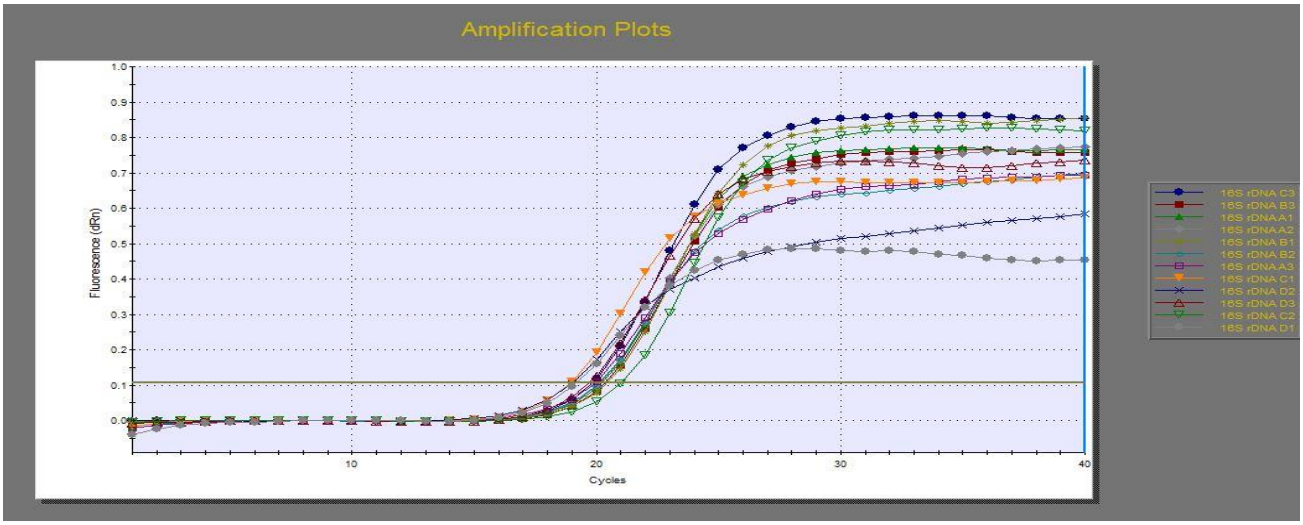

Figure 5. Down-regulation of the *16S rDNA* gene in the *Pseudomonas*-treated groups compared to the non-treated control group

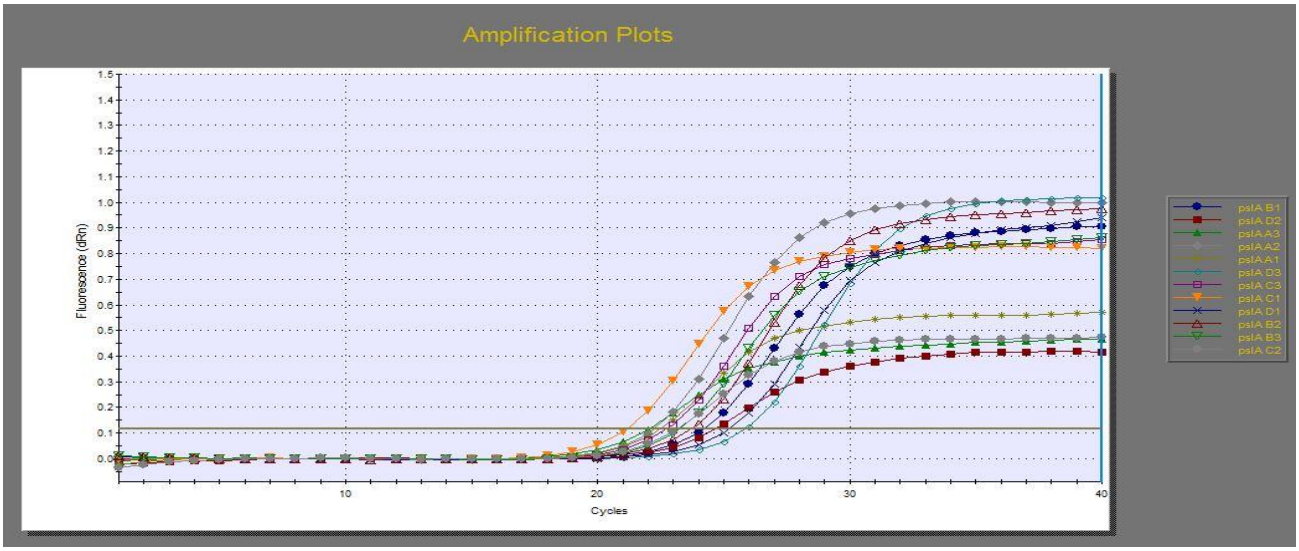

Figure 6. Down-regulation of the *pslA* gene in the *Pseudomonas*-treated groups compared to the non-treated control group

DISCUSSION

In the current study, *Pseudomonas* was isolated from 20% of the samples (20/100). Likewise, Garedew et al. (2012) documented an isolation rate of 18.5% in Gondar town, Ethiopia. Conversely, Atia et al. (2022) reported a higher prevalence of 56% from raw milk samples collected in Shebin El-Kom city, Egypt, whereas Deiab et al. (2023) identified a prevalence of 36% in bulk tank milk from Menofia Governorate, Egypt. The variation in the isolation rates observed in the present study may be attributable to a variety of factors, including hygiene practices, geographic location, disease prevention measures, management strategies, and the efficacy of the immune system.

Bacterial species capable of producing biofilm frequently demonstrate heightened virulence, thereby facilitating their attachment to solid surfaces and the formation of a protective matrix around the cells. This matrix protects bacteria against antibiotics and disinfectants designed to eradicate them (Wilson et al., 2017). During the present study, 75% of isolates were identified as biofilm producers by TCP, while only 60% were identified as biofilm producers by TM, which displayed different levels of biofilm-forming ability. The present findings were lower than those reported by Ngo et al. (2021), who observed biofilm formation in 100% of *Pseudomonas* isolates. Similarly, Deiab et al. (2023) reported a higher prevalence, with 78% of isolates forming biofilms, comprising 48.7% classified as strong, 23.07% as moderate, and 28.2% as weak producers. Conversely, lower rates were observed by Milivojević et al. (2018) and Banda et al. (2020), with biofilm production identified in only 16.3% and 15% of *Pseudomonas* isolates, respectively.

Molecular methods are increasingly preferred for pathogen detection, supplementing traditional culture-based plate enumeration techniques (Zhang et al., 2019). In the present study, 83.33% of the *Pseudomonas* isolates were found to carry the *pslA* gene, which was consistent with the findings of Al-Sheikhly et al. (2020), who observed a 100% prevalence of the *pslA* gene in isolates obtained from the Department of Biology, College of Science, University of Anbar, Iraq. Unlike Abdelraheem et al. (2020), who reported detection rates of 66.7% for *pelA* and 74.1% for *lasR* at Minia, Egypt, neither of these genes was detected in any of the isolates analyzed in the current study. Similarly, Al-Sheikhly et al. (2020) observed the presence of the *pelA* gene in all the isolates. Differences in the prevalence of biofilm-associated virulence genes (*pslA*, *pelA*, and *lasR*) across studies may be attributed to genetic diversity among isolates, differences in sample sources and environmental conditions, as well as different methods. β-carotene, widely recognized as a precursor of vitamin A and a powerful antioxidant, has recently garnered attention for its potential antibacterial properties. Although the precise mechanisms remain incompletely understood, several prospective modes of action have been identified. These mechanisms included disruption of bacterial cell membranes, as β-carotene is a lipophilic compound capable of integrating into and disturbing membrane structure (Hagaggi and Abdul-Raouf, 2023), and the generation of ROS, where β-carotene may act as a pro-oxidant under certain conditions, leading to increased ROS production within bacterial cells (Bae et al., 2021). Similarly, riboflavin (Vitamin B2), an essential nutrient present in both plants and animals, plays a crucial role in human health and has demonstrated antibacterial, antifungal, and anticancer activities (Farah et al., 2022).

The present study assessed the antibacterial activity of β-carotene and riboflavin against *Pseudomonas* spp., which demonstrated notable antibacterial effects, as indicated by clear inhibition zones. These results align with the findings of

Muntean et al. (2021), who reported inhibition zones for β-carotene ranging from 15.33 ± 0.58 mm to 16.0 ± 0.0 mm, and with those of Ahgilan et al. (2016), who observed a 15.7 ± 0.6 mm inhibition zone for riboflavin.

The present study demonstrated that the MIC of β-carotene was 1 µg/mL, and that of riboflavin was 4 µg/mL. The MBC was determined to be 2 µg/mL for β-carotene and 8 µg/mL for riboflavin. Comparatively, Muntean et al. (2021) reported a MIC of 6.25 µg/mL and an MBC of 25 µg/mL for β-carotene, suggesting that during the present study, β-carotene exhibited more potent antibacterial activity at significantly lower concentrations. Importantly, the antibacterial efficacy of β-carotene and riboflavin can be influenced by different factors, including the specific bacterial strain and experimental methodology (Tao NengGuo et al., 2010). Therefore, MIC values can vary across different studies and conditions.

The current results indicate that *Pseudomonas* survival increased over time in the control group, while all treated groups, β-carotene, riboflavin, and their combination, demonstrated antimicrobial effects. β-carotene demonstrated a more substantial reduction compared to riboflavin alone, and the combination of both had the most pronounced inhibitory effect on *Pseudomonas* growth during refrigerated storage of raw milk.

Recent studies suggested that riboflavin can suppress or inactivate a wide range of microorganisms, including bacteria, viruses, fungi, and parasites (Akompong et al., 2000; Tonnetti et al., 2013; Ahgilan et al., 2016). However, studies specifically focused on its antimicrobial properties remain limited. Some evidence indicated that riboflavin alone can inhibit the growth of several pathogenic microbes, such as *Staphylococcus aureus (S. aureus)*, *Enterococcus faecalis (E. faecalis)*, *Salmonella typhi (S. typhi)*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa (P. aeruginosa)*, *Candida albicans*, and *Plasmodium falciparum* (Kashiwabuchi et al., 2013; Ahgilan et al., 2016). Ahgilan et al. (2016) reported that riboflavin, at a concentration of 50 µL, was effective in inhibiting the growth of Gram-positive bacteria, including *S. aureus*, *E. faecalis*, *S. typhi*, and *P. aeruginosa*. During the present study, the group treated with β-carotene demonstrated a significant reduction in comparison to the control group, thereby supporting the hypothesis that β-carotene exhibits antibacterial properties. One proposed mechanism involves the disruption of bacterial cell membranes, where lipophilic characteristics of β-carotene enable it to integrate into the lipid bilayer, thereby increasing membrane permeability, causing leakage of intracellular contents, and ultimately decreasing bacterial viability (Hagaggi and Abdul-Raouf, 2023). Additionally, under specific conditions, β-carotene may act as a pro-oxidant, inducing the generation of ROS. These ROS can damage essential cellular components such as DNA, proteins, and lipids, resulting in bacterial cell death (Sinha et al., 2023).

The sensory evaluation results revealed a significant effect of β-carotene and riboflavin on the sensory attributes of raw milk during cold storage. Among all treatment groups, the milk samples fortified with β-carotene consistently received the highest scores for appearance, flavor, odor, and overall acceptability throughout the 6-day storage period, with values scored 8.50, 8.00, 7.99, and 8.30, respectively, on day 6. The current findings indicated that β-carotene not only enhances the visual appeal of the milk but may also contribute to preserving its organoleptic properties over time. This finding is attributed to the significant antioxidant role of β-carotene that reduces protein and lipid oxidation, stabilizes flavor, and thereby improves overall milk stability (Yabuzaki, 2017).

Numerous studies have indicated that β-carotene enhances sensory stability and shelf life in dairy products. Cakmakci et al. (2014) incorporated 15% carrot juice into set-type yoghurt and observed the highest sensory scores throughout a 21-day cold storage period. Similarly, Senarathne and Wickramasinghe (2019) developed a β-carotene-rich drinkable yoghurt containing 10% carrot pulp, which remained microbiologically stable and sensorially acceptable over 35 days. Overall, the incorporation of β-carotene has demonstrated potential as an effective natural strategy to improve the shelf life and sensory quality of raw milk, thereby offering promising applications in dairy preservation and functional food development.

The current results demonstrated that β-carotene and riboflavin can significantly reduce *Pseudomonas* virulence by targeting and downregulating the *pslA* gene, a key gene involved in biofilm formation. Treatment with β-carotene and riboflavin resulted in a significant decrease in *pslA* gene expression levels across all treated groups in comparison to the untreated control group (G1). Notably, the gene expression in Group G4 was reduced to 0.07-fold, indicating a dramatic suppression of *pslA* gene expression. Additionally, this effect was observed in groups G2 and G3, with fold changes of 0.31 and 0.54, respectively. These findings suggested that both β-carotene and riboflavin can interfere with the regulatory pathways involved in biofilm formation, possibly through their antimicrobial properties or through modulation of gene expression related to exopolysaccharide production. The significant reduction in *pslA* expression corresponds with a concurrent decline in biofilm formation, thereby corroborating the hypothesis that the suppression of this gene directly influences *Pseudomonas* pathogenicity. β-carotene and riboflavin exhibited antibacterial properties at minimal MIC and MBC values; their influence on cell viability may indirectly result in reduced expression of virulence-associated genes, including *pslA*. Alternatively, these compounds might act as signaling modulators, interfering with quorum sensing or other regulatory systems essential for biofilm development. The current results aligned with the

findings of Abdulhadi et al. (2023), who reported that β-carotene pigment exhibited the strongest antimicrobial activity at a concentration of 100 mg/mL, as well as with Ahgilan et al. (2016), who demonstrated the inhibitory effects of riboflavin against several bacterial and fungal species. These findings presented opportunities for developing new anti-virulence strategies that specifically target biofilm-associated mechanisms and pathways.

CONCLUSION

Pseudomonas spp. was detected in 20% of samples and 60% of the isolates were biofilm producers, with 83.33% carrying the *pslA* gene. β-carotene and riboflavin demonstrated vigorous antibacterial activity, low MIC/MBC values, and significantly reduced *Pseudomonas* counts during cold storage, especially when combined. The RT-qPCR revealed significant suppression of the *pslA* gene expression, thereby confirming its anti-biofilm activity effects. Sensory evaluation preferred milk treated with β-carotene, followed by milk with riboflavin, and the combinationβ-carotene and riboflavin demonstrated potential as natural additives to improve safety, prolong shelf life, and preserve milk quality. Future studies should focus on clarifying the molecular mechanisms by which β-carotene and riboflavin suppress biofilm-related genes, verifying their preservative efficacy under real milk processing and storage conditions, and confirming their impact on sensory attributes and nutritional quality to ensure both safety and consumer acceptance

DECLARATIONS

Ethical considerations

This manuscript is the authors' original study and has not been submitted or published elsewhere. The authors have verified the content for plagiarism and confirm that the article is based on their own original scientific findings.

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Availability of the data and materials

All data supporting the findings of the present study are available in the manuscript.

Author contributions

Heba E. El-Shora and Eman K. Fathalla conceived and supervised the overall study design and execution. Radwa A. Lela and Amal F. Elbanna were responsible for sample collection, data analysis, and interpretation of the results. Doaa M. Sharaf and Momen M. Aladl contributed to the experimental design and critically reviewed the manuscript. All authors contributed to manuscript writing and approved the final edition for submission and publication.

Competing interests

The authors declared no conflict of interest.

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