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# Food stabilizing potential of nisin Z produced by wild *Lactococcus lactis* subsp. *lactis* from raw milk and some fermented products

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ABSTRACT

In this study, we evaluated food stabilizing potential of nisin Z produced by wild *Lactococcus* (*Lc.*) *lactis* subsp. *lactis* from a total of 114 raw milk and naturally fermented products. After microbiological cultivation of lactic acid bacteria, *Lc. lactis* subsp. *lactis* strains identified by mass spectrometry were screened for the nisin Z gene by PCR. Nisin Z-production activity was studied under batch fermentation conditions, followed by testing food stabilizing potential of nisin Z extracted. Of 35 *Lc. lactis* subsp. *lactis* identified, 3 were nisin *Z*-producer, and one strain (e69) of natural cheese origin produced 2350 IU/mL at 63 °C for 30 min and 80 °C for 10 min, at pH 2.0. This nisin Z purified was moderately inhibitory on Gram (−) bacteria, stable at −20 °C and 4 °C during 6 weeks, influential on the shelf life of UHT milk and fruit juice at 30 °C from 2 to 6 weeks, and susceptible to lipase (89%), pepsin (92%) and catalase (84%) at 37 °C for 60 min, respectively. Overall, our study revealed that wild *Lc. lactis* subsp. *lactis* exhibited good activity in nisin Z production and native "clean-label" bio preservative potential for consumers and the food industry without biotechnological adaptation.

## 1. Introduction

Minimally processed foods for consumer satisfaction have gained importance in the last decades. This situation resulted in today's food manufacturers reducing or removing additives, including preservatives (Elsser-Gravesen & Elsser-Gravesen, 2014). Some of the most promising solutions to this end are the naturally-occurring antimicrobial agents, also so-called bio preservatives, namely lactoperoxidase, lysozyme, saponins, flavonoids, bacteriocins, and chitosan. They are produced by food-grade lactic acid bacteria, usually heat stable, can improve the quality of foodstuffs, for example, in cheese production, to control the growth of starter cultures and enhance ripening, and can inhibit the growth of the microorganisms that cause problems in minimally processed foods. For example, the bacteriocin nisin is already widely used in food preservation (Cotter, Hill, & Ross, 2005).

Due to non-toxic, non-immunogenic, thermo-resistance characteristics and broad bactericidal activity, LAB bacteriocins can be considered good bio preservative agents. The increasing trend of limiting the use of chemical food preservatives has triggered research in the field of bio preservation to find an alternative approach to chemical preservatives due to globalization of food market, introduction of novel foods, innovations of new technologies (Huang et al., 2016; Abbasiliasi et al., 2017; Singh, 2018). These natural bio preservatives present an option for other preservation techniques that may negatively influence the nutritional status and sensory attributes of food or consumer health (Dušková, Španová, Dráb, & Rittich, 2009; de Oliveira Junior, Silva de Araújo Couto, Barbosa, Carnelossi, & de Moura, 2015; Baptista, Horita, & Sant'Ana, 2020). Within the food chain, natural bio preservatives are suitable for organic food production, thereby promoting an environmental and customer-friendly food industry (García, Rodríguez, Rodríguez, & Martínez, 2010). Furthermore, bacteriocins based food grade markers (immunity proteins) offer the possibility to replace antibiotic selective markers for genetic engineering of food-related bacteria (Singh, 2018).

Among naturally occurring bio preservatives, nisin is the most well-known member of all the class I lantibiotics (Egan et al., 2016; Wayah & Philip, 2018). Nisin, first marketed in England in 1953 to inhibit *Clostridium tyrobutyricum* in cheese, has been approved for use in over 48 countries as a GRAS status food bio-preservative (Singh, 2018), hence offering important benefits to the food industry (Shin et al., 2008; de Souza de Azevedo et al., 2020). It is synthesized through post-translational processing of ribosomally synthesized precursors by

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certain strains of *Lactococcus* (*Lc.*) *lactis* subsp. *lactis*. For the two decades, *Lc. lactis* has widely extended its utilization from food to being a successful microbial cell factory (Song, In, Lim, & Rahim, 2017). There are six variants of nisin designated as A–E, and Z. Nisin A was discovered in 1928 in fermenting milk cultures, and nisin Z was then isolated from *L. lactis* NIZO 22186 from a dairy product (de Kwaadsteniet, Ten Doeschate, & Dicks, 2008; Piper, Hill, Cotter, & Ross, 2011; Barbosa, Mantovani, & Jain, 2017; Clemens, Zaschke-Kriesche, Khosa, & Smits, 2018). Nisin A and Z differ by a single amino acid substituting histidine at position 27 in nisin A and asparagines in nisin Z. The structural modification does not affect antimicrobial activity. Still, it gives nisin Z important characteristics for food applications such as higher solubility and diffusivity than nisin A (Gharsallaoui, Oulahal, Joly, & Degraeve, 2016).

Nisin, produced by several strains of *Lc. lactis* subsp. *lactis* is also industrially manufactured and commercialized by DuPont Danisco under the trade name Nisaplin. Nisaplin has a broad spectrum of action against Gram (+) bacteria, including LAB, *Bacillus cereus*, *Clostridium botulinum*, *Staphylococcus aureus*, *Listeria innocua*, and *Listeria monocytogenes*, but, generally, it is hardly effective against Gram-(−) bacteria, molds and yeasts (Hwanhlem, Ivanova, Haertlé, Jaffrès, & Dousset, 2017; de Souza de Azevedo et al., 2020). In contrast to Gram-positive, Gram (−) bacteria are much more tolerant to nisin due to their less permeable cell walls (Gong et al., 2018). Nisin is manufactured via fermentation of milk or whey by strains of *Lc. lactis* subsp. *lactis*. The resulting fermentate is concentrated, separated, spray dried, milled to yield a final powdered product, and purchased commercially from different suppliers. Nisin commercial preparations are not completely soluble and generally contain only 2.5% of pure nisin in 25 g. Thus, a high cost of US$770, with an activity of $1 \times 10^6$ IU/g, whereas 1 g pure nisin contains $40 \times 10^6$ IU. A biological activity of 40 IU thus corresponds to 1 μg of pure nisin (de Arauz, Jozala, Mazzola, & Vessoni Penna, 2009).

In recent years, the production of nisin has been improved through genetic modifications to nisin producing strains (Özel, Şimşek, Akçelik, & Saris, 2018). However, naturally occurring nisin also may present broad antimicrobial mechanisms, which may contribute to the maintenance of food (Baptista et al., 2020). Therefore, the studies have focused on its synthesis from native microbiological sources such as traditional fermented foods, dairy products, and sometimes from non-food matrices since it is easily accepted by consumers because of its natural origin (de Souza de Azevedo et al., 2020). Among the potential microbiological sources, lactic acid bacteria (LAB) has gained significant attention to producing nisin instead of synthetic preservatives (Kaškonienė et al., 2017). However, LAB growth for nisin industrial production requires complex nutrition conditions (Gharsallaoui et al., 2016), with some disadvantages such as poor stability and short duration of antimicrobial activity. In addition, it is also affected by producer strain, media composition, pH, temperature, agitation and aeration, substrate and product inhibition, adsorption of nisin onto producer cells, and enzymatic degradation (Gong et al., 2018). Therefore, over nisin Z-producers have been screened, and nisin Z has been widely found in naturally occurring nisin-producing *Lc. lactis* subsp. *lactis* strains (de Vos, Mulders, Siezen, Hugenholtz, & Kuipers, 1993).

The objective of this study was to evaluate the food stabilizing potential of nisin Z produced by wild *Lc. lactis* subsp. *lactis* from raw milk and some traditional fermented dairy products, namely cheese, boza, and kefir. In this sense, we first characterized nisin Z producing *Lc. lactis* subsp. *lactis* strains, determined the best nisin Z producer under different batch fermenter and growth conditions, purified the nisin Z, exploited for its antimicrobial and stability activities, aiming to ensure the quality and safety of some selected food products, including UHT milk and fruit juice.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Sampling

From May 2018 to July 2019, 114 native food samples (60 raw milk, 32 cheese, 14 kefir, and 8 boza) were collected from 40 villages in Marmara, Central, and Eastern Anatolia regions of Turkey. All the samples were put into sterile sampling bags using sterile hand gloves and appropriate sampling tools to prevent cross-contamination (EC Samancta SAM-110, 2012) and transported to the laboratory in a thermobox container at four °C until sample preparation and analysis.

#### 2.1.2. Reference strains

*Lactobacillus* (*L.*) *sakei* ATCC®15521 (WDCM, Japan) as a negative control for bacteriocin production, and *Lc. lactis* subsp. *lactis* ATCC®19435 (WDCM) as a positive control for determining antimicrobial spectra were used as the reference strains for testing.

### 2.2. Methods

#### 2.2.1. Sample preparation, cultivation, and identification of Lc. lactis subsp. lactis strains

Sample preparation and isolation of *Lc. lactis* subsp. *lactis strains* were conducted according to ISO 11133 (2014). The samples were homogenized and serially diluted in ¼ Ringers solution (Sigma-Aldrich 96724, USA) and directly streaked onto DeMan, Rogosa, and Sharpe agar (MRS) (Merck 1.10660, Germany). The pH was adjusted to 5.4 with acetic acid (Merck 100063) and M17 agar (Merck 1.15108) supplemented with 0.5% lactose (Medium 17 containing lactose LM17) (Merck 107661) to isolate LAB. To inhibit yeast and molds, natamax®antimicrobial (Danisco, USA) was used at 20 mg (l:1 concentration). The plates were incubated at 30 °C and 45 °C for 48 h under anaerobic conditions using an anaerobic jar (Oxoid BR38, UK). After incubation, each colony having a different morphology was picked and streaked on LM17 or MRS agar plates again. Purified isolates were stored in MRS broth (Merck 1.15029) containing 20% (v/v) glycerol at −20 °C (Karakas-Sen & Karakas, 2018). Finally, each isolate was identified by Vitek® MS mass spectrometry microbial identification system (bioMérieux, France).

#### 2.2.2. Screening of nisin Z gene by PCR

A total DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega Corporation, USA). The primers for screening of *the nisin Z* gene were designed according to Olasupo, Schillinger, Narbad, Dodd, and Holzapfel (1999) and Rattanachaikunsopon and Phumkhachorn (2008) as nisin $Z_f$ (5′-GTTCGAAGGAACTACAAAATAAATT-3′) and nisin $Z_r$ (5′-ACAGACCAGCATTATATTTCTGC-3′). Amplification was carried out with C1000 Touch™ Thermal Cycler (BioRad, USA) with one cycle for 2 min at 94 °C, 35 cycles for 1 min at 94 °C, 30 s at 40 °C, 1 min at 72 °C, and final elongation at 72 °C for 7 min. Gel-electrophoresis of the amplicons was performed on a 1.5% agarose gel, photographed by Biorad GelDoc 2000 imaging system, and analyzed by Biorad Quantity One 4.6.3 GelDoc XR Software. An 835-bp fragment was amplified from the genomic DNA of *Lc. lactis* subsp. *lactis* strains.

#### 2.2.3. Determination of nisin production activity

Nisin production activity of each nisin Z positive strain was evaluated by a well-cut diffusion technique (Dodd, Horn, & Gasson, 1990). First, Nisaplin (Sigma-Aldrich N5764) was diluted in the range of 0.5–10000 IU/mL at pH 2.5 to obtain a standard nisin curve of zone diameters after overnight incubation at 37 °C. Then, after incubating the strains in M17 broth for 22 h at 30 °C and centrifugation at 8000 rpm for 5 min, the supernatant was transferred into a tube, its pH adjusted to 2.5 with hydrochloric acid (HCl) (Merck 100317), and filtered through a 0.22 μm filter paper (Advantec, Japan). The amount of nisin in the filtrate was calculated using the standard nisin curve. Following that,

active *L. sakei* ATCC®15521, adjusted to 0.5 mc Farland in M17 broth, was inoculated onto M17 agar with a sterile cotton swab. Next, Wells was punched out using a 7 mm cork borer in the plate, and 100 μL of each nisin solution was instilled on it. The plates were then exposed to incubation at 30 °C for 12 h and 24 h. Finally, the inhibition zone diameter of each well was measured in mm, and nisin production activity was determined by comparing each measurement with the standard nisin curve. A zone diameter greater than 5 mm was considered as the best performing nisin Z producer.

*2.2.4. Batch fermentation and growth conditions*

Fermentation runs were monitored in a 5 L batch-cut fermenter (New Brunswick® BioFlo Fermentor, USA). Ten different growth mediums designated as A, B, C, D, E, F, G, H, I and J were prepared with milk powder (Enka Süt, Turkey), whey powder (Enka Süt), monopotassium phosphate ($KH_2PO_4$) (Sigma-Aldrich P5665), yeast extract (Sigma-Aldrich Y1625), and peptone (Sigma-Aldrich 70179) (Table 1) for nisin Z-producing strains. Five percent of the medium and 30 mL of bacterial inoculum were filled into the fermenter. The fermenter was run at 200 rpm, pH 6, and 30 °C throughout 33 h, and the bacterial viability count (CFU/mL) was monitored at 3-h intervals by taking a sample. Each sample was diluted by 1/6, and optical density (OD) was read at 600 nm, centrifuged at 10000 rpm, and washed with deionized water. The cell mass was determined by drying at 70 °C. The standard curves for OD and cell mass were obtained, and the equations for each standard curve were determined. The dry cell mass for each run was calculated later according to Singh et al. (2016) and Jozala, de Lencastre Novaes, Cholewa, Moraes, and Penna (2005).

*2.2.5. Final product extraction and stabilization*

The fermentation liquid was subjected to lyophilization (SP Scientific Genesis Pilot Lyophilizer, USA) at −45 °C for 2 h. Then, the and vacuum drying (Keissen Products Labplant Basic Spray Dryer, UK) by centrifugation at 6500 rpm for 20 min. After the drying process, the activity of nisin Z was determined (Matsusaki, Endo, Sonomoto, & Ishizaki, 1996).

*2.2.6. Determination of food stabilizing activities of nisin Z*

*2.2.6.1. Thermal and pH stability.* The thermal stability of the nisin Z was tested to assess the influence of high temperature (in the range of 63 °C, 80 °C, 100 °C, and 121 °C) on its antimicrobial activity two durations (10 min and 30 min). Regarding pH stability, the pH of the nisin Z producing strain was adjusted to 2, 4, 6, 8, 10, and 12 using HCl and NaOH. After standing at room temperature for 4 h, the indicator microorganism *M. luteus* was inoculated on MHA, followed by incubation for 24 h at 30 °C. After that, zone diameter differences were calculated as % change inactivation.

*2.2.6.2. Antimicrobial activity on Gram (−) bacteria.* Antimicrobial activity of nisin Z on Gram (−) bacteria was determined by agar-well diffusion method, using *Escherichia (E.) coli* ATCC®35218, *Staphylococcus (S.) aureus* ATCC®25923, and *Enterococcus (E.) faecalis* ATCC®29212 as the indicator microorganism. First, an aliquot of 25 mL bacterial culture ($10^8$ CFU) was applied with a sterile swab on the Mueller Hinton Agar (MHA) plate. Then, the test samples were prepared in deionized water so that the starting concentration of nisin Z corresponded to 25 μg/mL. Afterward, the solutions were transferred into the bored wells of 8 mm in diameter (250 μl/well) and incubated for 24 h at 35 °C. Finally, the inhibition zone diameter was measured (Blay, Lacroix, Zihler, & Fliss, 2007; Holcapkova et al., 2017).

*2.2.6.3. Storage and shelf-life activity.* The storage and shelf-life activity of the nisin Z was determined based on bacteriocin activities up to 10 weeks at three different temperatures (−20 °C, 4 °C, and 37 °C) in UHT fruit juice and UHT milk, as the testing food products. To do this, an amount of 11.25 mg/kg final purified nisin Z product was added to the UHT milk and fruit juice products. Drop in the activity value after storage was followed up regularly (Siroli et al., 2020).

*2.2.6.4. Sensitivity of nisin Z to various enzymes.* The sensitivity of nisin Z to various enzymes, including lipase (Sigma L-1754 Type 1; 8.6 U/mg in 0.05 $V_M$ Tris HCI at pH 8.0), pepsin (Merck 7189 Type 3; 2001 U/mL in 0.2 $V_M$ citrate at pH 6.0), and catalase (2000 U/mg in 10 $mV_M$ potassium phosphate at pH 7.0) was tested based on enzymatic degradation percent by incubation for 60 min at 37 °C (Brink, Minekus, van der Vossen, Leer, & Veld, 1994; (Olasupo et al., 1999).

## 3. Results and discussion

In this study, we evaluated the food stabilizing potential of the bio preservative nisin Z produced by *Lc. lactis* subsp. *lactis* strains of raw milk and some fermented product origin. In this sense, we first cultivated LAB identified *Lc. lactis* subsp. *lactis* strains by mass spectrometry, characterized nisin Z producers, determined the best nisin Z producer under different batch fermenter conditions, purified the nisin Z extracted, exploited for its antimicrobial and stability activities, aiming to ensure the quality and safety of UHT milk and fruit juice, respectively. Furthermore, this work revealed that one *Lc. lactis* subsp. *lactis* strain of natural cheese origin exhibited good activity in nisin Z production and native bio preservatives potential in fermentation challenge without biotechnological adaptation.

### 3.1. Culture-based results

In this study, 161 LAB isolates were cultured microbiologically from the samples. Among them, 35 (15 from raw milk, 13 from natural cheese, 5 from kefir, and 2 from boza) were identified as *Lc. lactis* subsp. *lactis* by mass spectrometry. Industrially, some enzymes and compounds are manufactured from *Lc. lactis* strains, including lactic acid, L-alanine, hyaluronic acid, riboflavin, bacteriocin, ethanol, nisin Z, bile salt hydrolase, etc. (Song, In, Lim, & Rahim, 2017). Among them, nisin as a natural antimicrobial food preservative may be present in milk (cow, ewe, and goat), dairy products (cheese), canned vegetables, flour products, fermented products (kefir and boza), pasteurized liquid eggs, some alcoholic beverages and salad dressings (Hwanhlem et al. 2017), and as well as in plant material, river water and human milk (Beasley & Saris, 2004). Lc produces this bacteriocin. *lactis* strains, the most extensively examined lactococcal species in the food industry (Koral &

**Table 1**
Different growth media (g/1000 mL).

| Amount (g/1000 mL) | Media Formula | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J |
| Milk powder | 100 | 75 | 50 | 25 | 0 | 20 | 20 | 20 | 20 | 20 |
| Whey | 20 | 20 | 20 | 20 | 20 | 100 | 75 | 50 | 25 | 0 |
| $KH_2PO_4$ | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Yeast extract | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Peptone | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Total | 150 | 125 | 100 | 75 | 50 | 150 | 125 | 100 | 75 | 50 |

Tuncer, 2012). Within *Lactococcal* species, Lc. lactis subsp. lactis has been extensively used in starter cultures for milk fermentation, applied as bio-preservation or co-starter cultures to produce bacteriocins in situ, with nisin (Parapouli et al., 2013). Our study showed that raw milk, traditionally manufactured cheese, kefir, and boza from different regions of Turkey were good sources of *lactococcal* species, in particular, *Lc. lactis* subsp. *lactis*.

### 3.2. Molecular screening results

In this study, 35 strains identified as wild *Lc. lactis subs. lactis*, only three strains (1 from raw milk and 2 from natural cheese) (8.6%) were found to be positive for the native nisin Z gene by PCR screening (Fig. 1). Numerous food ecology studies have identified wild *Lc. lactis* subsp. *lactis* were carrying nisin Z-encoding genes (Parapouli et al., 2013). However, Nisin Z has been restricted to being used as a bio-preservation (E234) in cheeses and some egg-based preparations by some regulations. This situation has forced the food industry to search for nisin-producing *Lc. lactis* strains as a part of the biopreservation strategy in the fermentation process (Bukvicki et al., 2020; Fusieger, Perin, Teixeira, de Carvalho, & Nero, 2019). On the other hand, particular attention has been focused on developing bacterial strains exhibiting overexpression of bacteriocins by genetic manipulation (Park, Itoh, Kikuchi, Niwa, & Fujisawa, 2003). However, this approach is still limited due to restrictive regulations and lack of acceptance by the consumers (Bravo, Rodríguez, & Medina, 2009). In addition, the nisin Z gene was detected in *Lc. lactis* strains from a variety of national and private collections. For instance, de Vos et al. (1993) determined 14 of the 26 strains as positive for the nisin Z gene. Therefore, our study seems to contribute to the biopreservation strategy in the fermentation challenges.

### 3.3. Results of nisin production activity

The nisin production activity of three nisin Z positive *L. lactis* subsp. *lactis* strains were evaluated by a well-cut diffusion technique, as previously described by Dodd et al. (1990). The nisin Z as the control indicator reached an inhibition zone diameter of 7.0 mm at 10000 IU/mL nisin Z. In contrast, the inhibition zone diameters of three nisin Z producers were 3.2 mm, 2.1 mm, and 5.5 mm, respectively. Among them, one strain (e69) isolated from a natural cheese sample with a zone diameter of 5.5 mm was selected as the best performer as nisin Z producer for further analyses (Table 2). Lalpuria et al. (2013) reported that the inhibition zones versus nisin concentration range from 2.0 to 6.0 mm. In another study, the nisin production activity was determined to be from 9.09 to 9.23 versus a nisin standard solution of 5 IU/mL (Pongtharangkul & Demirci, 2004). In our study, we obtained the nisin production activity at higher nisin Z standard solutions. Therefore, we can say that our isolates exhibited a performance better than the previously conducted studies.

### 3.4. Results of batch fermentation and growth

In this study, the best performer strain (e69) was monitored in a batch fermenter at pH 6 and 30 °C for 33 h. Optimal bacteriocin production for technological applications in some food products, as in dairy products, remains a challenge. Higher bacteriocin levels are produced in the absence of growth-stimulating nutrients or at temperatures and pH conditions lower than required for optimal growth (Furtado, Todorov, Landgraf, Destro, & Franco, 2014). Bacteriocin production in fermentation systems is affected by the type of carbohydrate, nitrogen source, pH, temperature, and other nutritional and physicochemical properties (Saraiva et al., 2020). According to the previously conducted studies, production of nisin Z was optimal at 30 °C and pH 5.0–5.5 (Matsusaki et al., 1996), at 30 °C and pH 5.5 in batch culture (Carvajal-Zarrabal, Nolasco-Hipólito, Bujang, & Ishizaki, 2009), and 25 °C and 30 °C and pH 6.0 or 6.5 under aerobic than anaerobic condition (Saraiva et al., 2020). Overall, our fermentation conditions were closely similar to the conditions described by the previous studies.

In our work, the best performer strain e69 can produce nisin Z in ten different medium formula containing milk powder, whey powder, $KH_2PO_4$, yeast extract, and peptone content was carried out in a batch fermentation process. The mediums usually include an excess of protein (tryptone, peptone, meat extract, and yeast extract), leading to the nonconsumption of a substantial proportion and unnecessary costs and difficulties to the nisin purification processes (Vázquez, González, & Murado, 2006; Senan et al., 2016). Bacterial viability counts of the strain e69 are given in Table 3. They were found as $5 \times 10^{10}$ CFU/mL at 33rd h in the medium D, with a composition of 25 g milk powder, 20 g whey, 10 g $KH_2PO_4$, 10 g yeast extract, and 10 g peptone, followed by formulas B and A as $3 \times 10^{10}$ and $2 \times 10^{10}$ CFU/mL, respectively. Saraiva et al. (2020) reported that optimal nisin Z production was achieved in tryptone and casein peptone at pH 6.0 or 6.5. As given in our medium formulas, whey has been used in some research to produce nisin (Guerra, Rua, & Pastrana, 2001).

Regarding the milk, it was utilized as a substrate for growing some LAB strains belonging to the genera *Lactococcus* undergoing fermentation process in 10% and 30% (Mathur, Beresford, & Cotter, 2020). According to the literature, good carbon (C) substrate is necessary for optimal production of nisin by LAB, and lactose is one of the possible C sources. In the whey, the lactose content is around 4.5–6.0%. Some works evaluated the effect of fermentation media fortification by lactose on nisin production, and optimal nisin production was reported when the medium contained 30 g/L lactose. It indicates that the composition of media s essential, as much as the bacterial strains used and fermentation conditions (Temperature, pH, and time). For instance, Holcapkova et al. (2017) reported the optimum level of lactose fortification as 0–20% (w/v). In addition, other studies said that the addition of $Ca^{2+}$ to the medium showed a stimulating effect on the production of nisin Z, which 3150 IU/mL nisin z was obtained in the medium supplemented with 0.1 $V_M$ $CaCl_2$ (Matsusaki et al., 1996), and tryptone and casein peptone (Saraiva et al., 2020). Saraiva et al. (2020) indicated that lactose was also a good energy source for nisin Z production, whereas


**Fig. 1.** 1.5% agarose-gel, Ethidium bromide-stained 1.5% (w/v) agarose gel showing PCR products of the nisin Z photographed by Biorad GelDoc 2000 imaging system. Lane 1 was loaded with a 100-bp-ladder DNA marker, and a DNA fragment was amplified at the expected size of 835 bp.

**Table 2**
Inhibition zone diameter results of nisin Z positive strains.

| | Nisin concentration (IU/mL) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1000 | 2000 | 3000 | 4000 | 5000 | 6000 | 7000 | 8000 | 9000 | 10000 |
| | Inhibition zone (mm) | | | | | | | | | |
| nisin Z (control) | 4.7 | 5.1 | 5.9 | 6.2 | 6.3 | 6.4 | 6.4 | 6.6 | 6.7 | 7.0 |
| A | 1.7 | 2.0 | 2.2 | 2.3 | 2.3 | 2.3 | 3.0 | 3.1 | 3.1 | 3.2 |
| B | 0.9 | 0.9 | 1.0 | 1.2 | 1.3 | 1.3 | 2.0 | 2.1 | 2.1 | 2.1 |
| C (e69) | 3.4 | 3.9 | 4.1 | 4.3 | 4.6 | 4.8 | 5.1 | 5.2 | 5.4 | 5.5 |

**Table 3**
Bacterial viability counts of the strains e69 in all media used (CFU/mL).

| Media | Duration (h) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 3 | 6 | 9 | 12 | 15 | 18 | 21 | 24 | 27 | 30 | 33 |
| A | <10 | 10 | $4.10^2$ | $6.10^4$ | $6.10^5$ | $3.10^7$ | $3.10^8$ | $7.10^{10}$ | $8.10^{10}$ | $7.10^{10}$ | $6.10^{10}$ | $2.10^{10}$ |
| B | <10 | 10 | $2.10^2$ | $3.10^4$ | $1.10^6$ | $5.10^7$ | $3.10^8$ | $6.10^{10}$ | $9.10^{10}$ | $6.10^{10}$ | $4.10^{10}$ | $3.10^{10}$ |
| C | <10 | 10 | $3.10^2$ | $3.10^4$ | $7.10^5$ | $2.10^8$ | $2.10^9$ | $7.10^{10}$ | $8.10^{11}$ | $8.10^{11}$ | $4.10^10$ | $6.10^9$ |
| D | <10 | 10 | $4.10^2$ | $2.10^4$ | $2.10^5$ | $4.10^8$ | $5.10^8$ | $2.10^{10}$ | $5.10^{10}$ | $2.10^{11}$ | $6.10^{10}$ | $5.10^{10}$ |
| E | <10 | 10 | 10 | $5.10^2$ | $8.10^3$ | $3.10^5$ | $3.10^6$ | $6.10^7$ | $8.10^8$ | $9.10^8$ | $5.10^8$ | $2.10^7$ |
| F | <10 | 10 | 10 | $2.10^2$ | $5.10^3$ | $7.10^5$ | $2.10^6$ | $6.10^8$ | $9.10^7$ | $3.10^9$ | $2.10^8$ | $8.10^7$ |
| G | <10 | 10 | 10 | $6.10^2$ | $3.10^3$ | $2.10^5$ | $1.10^7$ | $2.10^8$ | $6.10^7$ | $5.10^8$ | $6.10^8$ | $9.10^6$ |
| H | <10 | 10 | 10 | $2.10^2$ | $8.10^3$ | $6.10^5$ | $4.10^5$ | $4.10^6$ | $5.10^7$ | $2.10^9$ | $5.10^8$ | $4.10^9$ |
| I | <10 | 10 | 10 | 10 | $810^3$ | $4.10^4$ | $4.10^5$ | $3.10^5$ | $8.10^8$ | $4.10^9$ | $8.10^8$ | $1.10^8$ |
| J | <10 | 10 | 10 | $4.10^2$ | $7.10^3$ | $2.10^5$ | $8.10^6$ | $3.10^5$ | $5.10^7$ | $9.10^7$ | $7.10^7$ | $7.10^8$ |

glucose was an imperfect carbon source for nisin Z production. In our study, the ability to produce fermenter output nisin Z of the strain e69 was determined to 8.8 mm for *L. sakei* on MRS agar and 21.1 mm M. *luteus* on MHA agar in formula D, respectively.

On the other hand, the actual highest nisin Z output was detected in Medium A as 2350 IU/mL, followed by 1158 IU/mL in the Medium C and 522 IU/mL in Medium F. The lowest produced amount of nisin Z was derived as 13.6 IU/mL in the Medium E and as 19.7 IU/mL in the Medium J. Similarly, Mitra, Mukhopadhyay, and Biswas (2009) reported 4000 IU/mL nisin in skim milk and 2400 AU/mL in fat milk. Overall, our findings indicated that the composition of the media used is essential, but the bacterial strain used and fermentation conditions (Temperature, pH, and time) are also significant factors influencing nisin Z production. Biotechnology has improved nisin production by genetically modified strains. Still, these techniques should develop the antimicrobial activity of nisin or its stability at elevated temperature and under neutral or alkaline conditions, as Jozala, Novaes, and Pessoa (2015) pointed out.

#### 3.4.1. Results of final product extraction and stabilization

In our study, an amount of 2350 IU/mL fermentation-derived nisin Z was obtained as the final product by lyophilization and vacuum drying. Our findings showed that lyophilization recovered 98% (2305 IU/mL), and protein content was found as 868.6 mg/L. On the other hand, bacteriocin solutions can also be concentrated with organic solvents and instrumental approaches. Because bacteriocins are secreted into the culture medium, most strategies focus on bacteriocins from the culture supernatant. Although these procedures can reduce the working volume, they do not provide a high degree of purification (Parada, Caron, Medeiros, & Soccol, 2007). For many LAB bacteriocins, the lyophilized form is more stable and retains its activity during storage at −20 °C and −80 °C for three months (Şahingil, İşleroğlu, Yıldırım, Akçelik, & Yıldırım, 2011). Overall, lyophilization enabled us to take the vast majority of the produced nisin Z effectively under suitable conditions.

### 3.5. Results of food stabilizing activities of nisin Z

#### 3.5.1. Thermal and pH sensitivity

This study evaluated the inhibitory effect of the strain e69 on *M. luteus* at different temperatures and durations (Table 4). The diameters of the inhibition zone and the activity values were detected as

**Table 4**
Results of some bio-preservative activities of the strains e69 and nisin Z.

| Inhibitory effect on *M. luteus* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control (e69) | | | 120 °C/10 min. | | 100 °C/10 min. | | 80 °C/10 min. | | 63 °C/30 min. | |
| Inhibition zone (mm) | 17.1 | | | 13.8 | | 14.8 | | 15.9 | | 16.1 | |
| nisin Z activity (IU/mL) | 2350 | | | 1862 | | 1997 | | 2146 | | 2137 | |
| **pH and antimicrobial activity on *M. luteus*** | | | | | | | | | | | |
| pH | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| nisin Z activation (%) | – | +5 | −16 | −38 | −30 | −22 | −50 | −88 | −95 | −100 | −100 |
| Zone diameter (mm) | 17.2 | 18.0 | 14.4 | 10.6 | 12.0 | 13.4 | 8.6 | 2.06 | 0,86 | – | – |
| **Antimicrobial activity on some Gram (−) bacteria** | | | | | | | | | | | |
| Gram (−) bacteria | | | *E. coli* ATCC 35218 | | | *S. aureus* ATCC 25923 | | | *E. faecalis* ATCC 29212 | | |
| Inhibition zone (mm) | | | 3.16 | | | 2.36 | | | 2.95 | | |
| **Enzymatic sensitivity** | | | | | | | | | | | |
| Enzyme | | | Zone diameter (mm) | | | Difference (mm) | | | Activity (%) | | |
| Control | | | 16.84 | | | – | | | 100 | | |
| Lipase | | | 14.99 | | | 1.85 | | | 89 | | |
| Pepsin | | | 15.49 | | | 1.35 | | | 92 | | |
| Catalyze | | | 14.14 | | | 2.70 | | | 84 | | |

13.8 mm and 1862 IU/mL at 120 °C for 10 min, 14.8 mm and 1997 IU/mL at 100 °C for 10 min, 15.9 mm, and 2146 IU/mL at 80 °C for 10 min, and 16.1 mm and 2173 IU/mL 63 °C for 30 min, respectively. Overall, the nisin z produced by the best performer e69 strain exhibited the best thermal activity at 63 °C, 30 min, and 80 °C, 10 min. In addition to that, we assessed the pH activity of the strain e69 at different pH values 2, 4, 6, 8, 10, and 12. We assayed for its antimicrobial activity against *M. luteus* on MHA based on the activation percentage (Table 4). The findings were determined to be 100% at pH 12, 95% at pH 10, 50% at pH 8, 30% at pH 6 and 16% at pH 4, whereas it was 0% at pH 2. The pH values below and above pH 3 showed a significant decrease in the activity, especially those furthest removed from pH 2. In the literature, some studies reported that nisin lost its activity by 5% at 115 °C for 20 min and 15% at 121 °C for 15 min (Davies et al., 1998), a sharp reduction in activity was observed at pH values both lower and higher than 3.0 (Rollema, Kuipers, Both, de Vos, & Siezen, 1995), pH values and heating (65 °C for 30 min, 75 °C for 15 s) had no apparent effect on the antimicrobial activity (Perin et al., 2013). Optimal stability was observed at pH 2.0 & 25 °C (Tan, Luo, Liu, Zhang, & Jia, 2015). Heating nisin to 115 °C for 20 min at pH 3 results in less than 5% loss in activity, making it suitable for high-heat processing (Gough et al., 2017). Overall, our results on pH sensitivity of our native nisin Z and wild strain e69 exhibited very similar results around pH 2 and 3, below pH 4, following the previously conducted studies.

### 3.5.2. Antimicrobial activity on Gram (−) bacteria

In this study, the inhibitory effect of the strain e69 on *E. coli* ATCC®35218, *S. aureus* ATCC®25923, and *E. faecalis* ATCC®29212 was tested with the agar-disc diffusion methods. Our results revealed that the diameters of the inhibition zone of the nisin z on the indicator microorganisms were measured as 3.16 mm for *E. coli* ATCC®35218, 2.95 mm for *E. faecalis* ATCC®29212 and 2.36 mm for *S. aureus* ATCC®25923, respectively (Table 4). It is well-known that purified nisin Z shows antibacterial activity both against Gram (+) and Gram (−) bacteria through two distinct mechanisms: (1) a high-salt-sensitive mechanism for *E. coli* and (2) a high-salt-insensitive mechanism for *S. aureus*. On the other hand, recent studies have indicated that nisin exerts significant antibacterial activity against Gram (+) but not Gram (−) bacteria. The insensitivity of Gram (−) bacteria to nisin could be due to its large size (1.8–4.6 kDa), which restricts its passage across the outer membrane of Gram-negative bacteria (Kuwano et al., 2005). Many studies investigate the bio-control characteristics of nisin Z on Gram (−) bacteria in the literature. For example, Blay et al. (2007) reported that nisin had no antibacterial effect on *E. coli*, effective against *S. aureus* (Ellis, Ross, & Hill, 2019), combined use of nisin z and leucocin was effective against *L. monocytogenes* and *S. aureus* and moderately against *E. coli* (Fu et al., 2018), whereas practically insignificant for *S. aureus* as reported by Malinowska-Pańczyk and Kołodziejska (2009). Overall, our results showed that nisin z had a moderate effect on the selected indicator Gram (−) bacteria in food preservation.

### 3.5.3. Storage and shelf-life activity

In this study, we evaluated the stability of the nisin Z throughout storage by determining bacteriocin activities at different temperatures −20 °C, 4 °C, and 37 °C for up to 10 weeks. Our results indicated that the purified nisin Z was stable at both −20 °C and 4 °C during a period of 6 weeks, whereas it lost 96% of its activity at 37 °C up to 4 weeks. "*Natural, healthy and safe*" is the main criteria raised for food by the consumers. Consumers prefer eating minimally processed foods with no additives and requiring longer food shelf life. This could be achieved by incorporating bacteriocins or bacteriocin-producing strains (Kondrotienė et al., 2018). Thus, nisin z producing *Lc. lactis* subsp. *lactis* would be suitable for producing high-quality food products endowed with a longer shelf-life (Siroli et al., 2020). A study by Holcapkova et al. (2017) reported that nisin z storage at 25 °C resulted in a significant drop in its activity value after 55 days. In contrast, residual activity levels observed in a milk-based pudding indicated high revel retention, with only 25–50% loss in nisin activity for up to 27 days at 15 °C (Oshima et al., 2014). In our study, UHT fruit juice and UHT milk were used for determining the effect of nisin Z on the shelf life, up to 10 weeks with 11.25 mg/kg nisin addition. Our results revealed that the purified nisin Z was effective on UHT fruit juice up to 6 weeks, while it was effective on UHT milk up to 2 weeks, both at 30 °C. In the literature, several pieces of research showed that nisin Z extended the shelf-life of milk to 2 months under refrigeration (Mitra, Mukhopadhyay, & Biswas, 2011), up to 13 days in the milk-based pudding (Oshima et al., 2014), up to 15 days at cold storage in freshly pasteurized milk (Kuo et al., 2017), in the flavored soft drinks at 20 °C up to 120 days (Garavaglia et al., 2019), and even five days in the chilled sturgeon fillet (Gui, Zhang, Gao, & Li, 2021). It is a well-known fact that nisin activity generally increases at low pH and low initial microbial loads. Our nisin-Z producing straine69 in food protection continues on the natural trend of adding food-derived ingredients and providing more extended shelf life benefits. Another benefit that our wild nisin Z producing wild strain e69 is against food wastage for different food materials because fruits are among the least expensive and fastest spoiling foods (42%), followed by milk and dairy products (26%) (Sridhar, Ponnuchamy, Kumar, & Kapoor, 2020).

### 3.5.4. Sensitivity to nisin Z various enzymes

In this study, our findings indicated that the susceptibility of our nisin Z to lipase, pepsin, and catalase was determined to be 89%, 92%, and 84%, at 37 °C for 60 min (Table 4). The susceptibility of nisin Z to enzymatic degradation may be advantageous for its applications in food because it may be more quickly digested than other nisins and does not affect the intestinal flora, as explained by Park et al. (2003). In the literature, Zhao, Duan, Yang, Niu, and Wang (2015) reported that a decrease in the inhibitory activity of nisin Z was 33.3% in pepsin. Overall, the characterization of the sensitivity of wild-type nisin Z to enzymes, in particular, proteolytic enzymes, needs further investigation to provide much information for its commercial application and effective production in food because bacteriocins are highly sensitive to the action of proteolytic enzymes (Cleveland et al., 2001; Nogina, Diachkova, Tikhonov, & Tikhonova, 2020). According to a study by Park et al. (2003), the inhibitory activity of *Lc. lactis* subsp. *lactis* was inactivated by proteolytic enzymes but not affected by lipase and catalase. Another study by Perin, Moraes, Viçosa, Silva Júnior, and Nero (2012) showed that some *Lc. lactis* subsp. *lactis* strains produced substances sensitive to lipase, suggesting the presence of lipids in their structures, as observed in some bacteriocins. Overall, our findings on the sensitivity of our nisin Z to various enzymes matched with other previous results across the world and exhibited an excellent susceptibility to lipase, catalase, and pepsin, indicating efficient solubility characteristics in the food applications.

## 4. Conclusions

This work was undertaken to elucidate the subspecies identity of wild Lc. lactis subsp. lactis *strain*, possessing nisin Z-mediated bio preservative activities, from a native raw milk and cheese pool. Therefore, our findings contributed to the efforts to exploit the microbiota environments for the wild strains of technological interest and provided insight into the natural microbiota and fermented foods to manufacture the nisin Z compound. Furthermore, our study revealed that raw milk and traditional cheese harbored nisin Z producing strains, one *Lc. lactis* subsp. *lactis* strain showed promising activity in nisin Z production, and it possessed biopreservation potential in fermentation challenges. Overall, the wild biodiversity may provide "clean label" ingredients and safer products for both consumers and the food industry without further biotechnological adaptation strategy.

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## CRediT authorship contribution statement

**Doğan Murat:** Investigation, Methodology, Project administration, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Tekiner İsmail Hakkı:** Conceptualization, Methodology, Writing – review & editing.

## Declaration of competing interest

None to declare.

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