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Enhancement of Shelf Life of Pasteurized Milk Using Nisplin® Essential Oils Emulsion

N.F. Nasr^{1*} and F.M.F. Elshagabee²

¹Department of Microbiology, ²Department of Dairy Science
Faculty of Agriculture, Cairo University, 12613 Giza, Egypt

*Corresponding author

ABSTRACT

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Pasteurized milk has suitable nutrition value for daily use, but its shelf life is only two weeks under refrigeration at 4°C. This study investigated the synergistic effect of Nisplin® combinations in emulsion with natural essential oils to extend the shelf life of pasteurized milk under poor refrigeration at 10°C. Total viable counts of pasteurized milk supplemented by low concentrations of nisin (5 IU/ml) combined in emulsion with clove, ginger or jojoba essential oils (25 µg/ml) treatments did not exceed 4.3 log CFU/ml and titratable acidity as lactic acid did not exceed 0.18 % after 20 days storage, which reflected as high score 9 in sensory acceptability test. Nisplin® combinations in emulsion with clove, ginger or jojoba essential oils were effective at low concentrations to extend the shelf life of pasteurized milk for 20 days at 10°C. Nisplin®-Jojoba emulsion has promising effect as natural preservatives for pasteurized milk under inefficient cold

Introduction

Pasteurized milk at 72°C for 15sec has suitable nutrition value for daily use, because higher temperatures of heat treatment reduce nutrition value of milk. But shelf life of pasteurized milk is only two weeks under refrigeration at 4°C during transportation and storage which is difficult in some regions. Therefore, extended shelf life of pasteurized milk under poor refrigeration becomes strongly needed.

Nisin is a polypeptide bacteriocin has bactericidal effect against vegetative Gram

positive bacteria and bacteriostatic effect against spore-forming bacteria (Delves-Broughton, 1990). Furthermore, Nisin is a non-allergenic naturally produced by *Lactococcus lactis* subsp *lactis* thus, considered safe food preservative for human, Nisin is commercially called Nisplin®, and used around 50 countries in the world (Jung *et al.*, 1992; Jay, 2000).

Added nisin for preservation of foods is partially exhausted because of interactions with food composition (Zhang *et al.*, 2001), which needs excessive amount of nisin for effective inhibition activity when added alone.

Therefore, effective bioactive additives mixtures of nisin and other natural agents such as plant essential oils are needed to reduce and inhibit pathogenic and spoilage microorganisms in ready to eat food products with low amount of nisin (Enan *et al.*, 2012). Many plant essential oils have antimicrobial activity against food-borne pathogenic bacteria and food spoilage bacteria and yeasts in food products (Dorman and Deans, 2000).

Safety, stability, sensory and nutritional quality of most foods is based on application of combined preservative methods (Ettayebi *et al.*, 2000; Leistner, 2000). This study aims to evaluate the synergistic effect of Nisplin[®] combination in emulsion with natural essential oils extracted from plant such as cinnamon, clove, ginger and jojoba as safe food additives to extend the shelf life of pasteurized milk under poor refrigeration conditions at 10°C, to achieve commercial and social benefits for our community.

Materials and Methods

Preparation of Nisplin[®]- essential oil emulsion

Nisplin[®] (10⁶ IU/g) was generously gifted from MIFAD Co., Cairo, Egypt. Cinnamon, clove, ginger and jojoba essential oils were purchased from EL Hawag, Cairo, Egypt. One gram of Nisplin[®] was dissolved in one liter of sterilized water, then Tween 80 (Merck, Darmstadt, Germany) was added at final concentration 0.2%. The oil / water emulsion was prepared by mixing each essential oil at final concentration 0.2 and 0.5% using a magnetic stirrer at 1500 rpm for 10 min at room temperature.

Indicator microorganisms

Indicator bacterial strains (Table 1) were obtained from faculty of agriculture, Cairo University and were propagated using nutrient

broth medium and incubated at 32 °C for 24 h. The viable counts were ranged between 6.40±0.53 and 6.74±0.61 Log CFU/mL.

Antimicrobial activity

Antimicrobial activity of nisin and emulsions of nisin with different essential oils were tested using disc diffusion assay (Bauer *et al.*, 1966) against pathogenic and spoilage bacterial strains of test microorganisms. The disc (6 mm in diameter) was saturated by 15 µl of tested emulsion. Positive reference standards; polymyxin (130 units/disc) and kanamycin (30 µg/disc) were tested for comparison. Table 1 shows tested microorganisms, incubation conditions and culture media.

Pasteurized milk preparation

Buffalo's milk was obtained from Dairy plant of faculty of Agriculture, Cairo University. It was pasteurized at 72 °C for 15 sec using laboratory water bath (Jenway, Staffordshire, UK). Different Nisplin[®]-essential oils emulsions were added after membrane filter sterilization using 0.45µsyringe filter (Chrom Tech., Minnesota, USA). Each emulsion was added at concentration 0.5% of pasteurized milk. Final concentrations in pasteurized milk were 5 IU/ml of nisin and 25µg/ml (ppm) of essential oil. Titratable acidity (APHA, 1978) and total bacterial counts using standard plate count agar (Oxoid) at 32 °C for 48h (APHA, 1976) of pasteurized milk samples were measured during twenty days of cold storage at 10 ±1 °C.

Sensory evaluation of pasteurized milk mixed with Nisplin[®]-essential oil emulsions

A panel of eight assessors was selected and trained as described in ISO (1993) standard. The Hedonic scale (1: dislike very much, 9: like very much) for evaluation the overall acceptability of pasteurized milk mixed with

different emulsions was used (Lawless and Heymann, 1998; Gandy *et al.*, 2008).

Data analysis

The data were expressed as means with standard deviation (SD) of three replicates using Excel 2010 (Microsoft, Redmond, WA, USA).

Results and Discussion

Antimicrobial activity of Nisplin[®]-essential oil emulsions against pathogenic and spoilage bacteria

Data in Table 2 show the antimicrobial activity of different Nisplin[®]-essential oil emulsions. The concentration of 0.5% of Nisplin[®]-essential oil emulsions was more effective to inhibit test organisms. Combinations of Nisplin[®] with essential oils have higher inhibition effect than Nisplin[®] alone particularly at 0.5% concentration. Results obtained by Solomakos *et al.*, (2008) showed that combination of essential oils and nisin revealed higher antimicrobial activity more than each of them alone against *Escherichia coli* O157:H7 in tryptic soy broth.

Diameters of inhibition zone were varied with different treatments, and ranged between 7 and 11 mm. *Listeria monocytogenes* was the most sensitive bacterial strain, where it inhibited with inhibition zone diameters 11, 11, 10 and 10 mm by cinnamon, clove, ginger and jojoba, respectively, in combinations with Nisplin[®] by 0.5% concentration. Addition of thyme essential oil (0.8%) in combination with nisin (500 IU/g) for minced fish meat could reduce the viable count of *Listeria monocytogenes* from 4.9 to 2.3 log CFU/g after 2 days and less than 2 log CFU/g after 4 days of storage at 4°C (Abdollahzadeh *et al.*, 2014). Synergistic anti-listerial effect was found between nisin and the active

components of essential oils including carvacrol, thymol and eugenol (Yamazaki *et al.*, 2004).

Spore forming bacterium (*B. cereus*) was the most resistant bacteria strain; it was not inhibited by nisin alone or nisin combination emulsion with cinnamon, clove and ginger in 0.2% concentration. Also, *B. cereus* gave the least inhibition zone diameters using 0.5% concentration of nisin combination emulsions with all essential oils comparing with other microbial strains. In the same direction, Ultee *et al.*, (1998) reported that *B. cereus* was 2.3-fold resistant against essential oil fraction carvacrol than vegetative cells.

Except *Bacillus cereus*, 0.5% concentration of nisin combination emulsions with all essential oils could effectively inhibit all Gram positive and Gram negative bacterial strains with inhibition zone diameters ranged between 8 and 11 mm (Table 2). Similarly, Singh *et al.*, (2001) reported that combination of nisin with plant essential oils could activate antimicrobial effect of nisin. Moreover could overcome resistance of Gram negative bacteria (Helander *et al.*, 1998).

Tested essential oils have antimicrobial substances; ginger essential oil contains β -sesquiphellandrene, caryophyllene and zingiberene (El-Baroty *et al.*, 2010). Cinnamon oil contains cinnamaldehyde and eugenol which were active antibacterial components (Gende *et al.*, 2008; El-Baroty *et al.*, 2010). Also, eugenol (79%) was the main antimicrobial component of clove essential oil (Ranasinghe *et al.*, 2002; Ayoola *et al.*, 2008).

Latex of jojoba plant contained slight steroids and rich tannins and revealed a broad spectrum inhibition effect against G⁺ and G⁻ bacteria and fungi strains (Abu-Salem and Ibrahim, 2014). Jojoba seeds oil contains mixture of saturated and unsaturated fatty

acids, alcohols and phenolic compounds (Al-Qizwini *et al.*, 2014; Al-Ghamdi *et al.*, 2017).

Total viable bacterial count of pasteurized milk contained Nisplin®-essential oil emulsions during storage

Buffalo's milk was pasteurized at 72 °C for 15 sec, then Nisplin®-essential oils emulsions were added. Final concentrations in pasteurized milk were 5 IU/mL of nisin and 25µg/mL (ppm) of essential oil. Table 3 shows changes in total bacterial counts of pasteurized milk samples during twenty days of cold storage at 10 ±1 °C. The initial total bacterial count was around 3.6 log CFU/mL in all samples. During storage, total bacterial count of control sample increased gradually to reach 5.92 log CFU/mL, while Wirjntoro and Lewis (1996) considered pasteurized milk spoiled when total plate count exceeds 6.5 log CFU/mL, and reported that, addition of nisin (20-50 IU/ mL) to milk prior to pasteurization extended the shelf life of milk from 2 to 4 weeks at 10°C. In our results, addition of Nisplin® allowed total bacterial count to increase from 3.6 to 4.9 log CFU/mL in compare with count of control sample 5.92 log CFU/mL during 20 days at 10 ±1 °C. Total bacterial counts of pasteurized milk samples contained Nisplin®-essential oils emulsions increased slowly from 3.6 log CFU/mL to reach 4.25, 4.30, 3.95 and 3.92 log CFU/mL with cinnamon, clove, ginger and jojoba Nisplin®-essential oils emulsions, respectively, in compare with Nisplin® alone and control samples reached 4.90 and 5.92 log CFU/mL after 20 days at 10 ±1 °C. Similarly, other studies found that combination of essential oils (0.6%) and nisin (500 IU/g) revealed higher antimicrobial activity more than each of them alone against *Escherichia coli* O157:H7 in minced beef meat during storage at 10°C (Solomakos *et al.*, 2008). Also samples of minced sheep meat previously inoculated with 10⁴CFU/g *Salmonella*

enteritidis showed significantly lower counts after treatment with combination of oregano essential oil (0.6%) and nisin (500 IU/g) than each of them alone during storage at 10°C, which indicate that antimicrobial activity of combination was stronger (Govaris *et al.*, 2010).

Antibacterial effect of nisin due to hydrophobic interaction between amino acids residue of nisin and fatty acids of cell membrane phospholipids (Henning *et al.*, 1986). Furthermore, electrostatic attraction between nisin molecules and negative charge of phospholipids causes the antibacterial effect (Sahl and Bierbaum, 1998). Synergism effect of nisin and essential oils attributed to damage in structure of cell membrane (Helander *et al.*, 1998; Breukink *et al.*, 1999). Essential oils can increase the size or number of pores formed in cell membrane by nisin, which cause leakage of intracellular metabolites and dissipation of membrane potential which lead to reduction of viable cells count (Pol and Smid 1999; Ali *et al.*, 2008).

Titrateable acidity of pasteurized milk contained Nisplin®-essential oil emulsions during storage

Table 4 shows changes in titrateable acidity of samples of pasteurized milk supplemented with low concentrations of Nisplin®(5 IU/mL) combined in emulsion with clove, ginger or jojoba essential oils (25 µg/mL) during twenty days of cold storage at 10 ±1 °C. Level of acidity in control sample was 0.21% only after 10 days and reached 0.45% after 20 days, while acidity of pasteurized milk with Nisplin® alone reached 0.21% mean time 20 days. On the other hand, acidity after twenty days did not exceed 0.19% in pasteurized milk with Nisplin®-Cinnamon emulsion and 0.18% with Nisplin®-clove and Nisplin®- ginger emulsions, moreover acidity only reached 0.17% with Nisplin®-jojoba emulsion.

Therefore, Nisplin® combinations in emulsion with clove, ginger or jojoba essential oils were effective at low concentrations to extend the shelf life of pasteurized milk for 20 days under limited refrigeration conditions at 10°C. Wirjntoro and Lewis (1996) reported that addition of nisin solution to pasteurized milk could decrease the changes in acidity during cold storage period. Also, addition of Thymus essential oil in emulsion or non-emulsion form could decrease the development of acidity in UHT contaminated

milk with spoilage and pathogenic bacteria (Ben Jemaa *et al.*, 2017).

Sensory acceptability of pasteurized milk contained Nisplin®-essential oil emulsions

Results in Table 5 show the sensory acceptability of pasteurized milk samples contained Nisplin®-essential oils emulsions. All treatment obtained high score in Hedonic scale (9: like very much) at initial time.

Table.1 Incubation conditions and culture media of tested microorganisms for antimicrobial activity test

Positive reference standard	Microbial Type		Incubation conditions	Culture medium
Polymyxin (130 units/disc)	Gram negative bacteria	<i>Escherichia coli</i> O:157 (ATCC 9311)	37°C for 24-48 hr	Mueller-Hinton Agar (Bauer <i>et al.</i> , 1966)
		<i>Salmonella typhimurium</i> (ATCC 14028)		
		<i>Pseudomonas fluorescens</i> (NRRL-B-253)		
Gram positive bacteria	<i>Staphylococcus aureus</i> (MRSA) (ATCC 43300)	30°C for 24-48 hr		
	<i>Listeria monocytogenes</i> (ATCC 13932)			
Gram positive Spore forming bacteria	<i>Bacillus cereus</i> (ATCC 33018)		30°C for 24-48 hr	
	<i>Bacillus subtilis</i> (NRRL-B-354)			

Table.2 Antimicrobial activity of Nisplin[®] and different Nisplin[®]-essential oil emulsions against food borne pathogens and spoilage bacteria

Treatments	Inhibition zone diameter (mm) of pathogenic and spoilage bacteria						
	<i>Bacillus cereus</i>	<i>Bacillus subtilis</i>	<i>Listeria monocyrogene</i>	<i>Staphylococcus aureus</i>	<i>Pseudomonas fluorescens</i>	<i>Salmonella typhi</i>	<i>Escherichia coli</i>
Nisplin [®]	-	7.0 ±0.0	7.3 ±0.6	7.0 ±0.0	7.5 ±0.3	7.0 ±0.0	7.0 ±0.0
Nisplin [®] -0.2% Cinnamone	-	7.5 ±0.0	7.5 ±0.5	7.2 ±0.3	7.0 ±0.0	7.7 ±0.6	7.5 ±0.9
Nisplin [®] -0.5% Cinnamone	7.1 ±0.2	8.0 ±0.0	11.0 ±0.0	8.0 ±0.0	8.0 ±0.0	9.0 ±0.0	8.0 ±0.0
Nisplin [®] -0.2% Clove	-	8.1 ±0.0	8.7 ±0.6	7.0 ±0.0	8.0 ±0.6	7.0 ±0.0	7.0 ±0.0
Nisplin [®] -0.5% Clove	8.0 ±0.0	8.5 ±0.0	11.0 ±0.0	9.0 ±0.0	9.0 ±0.4	8.3 ±0.3	9.0 ±0.0
Nisplin [®] -0.2% Ginger	-	8.5 ±0.0	7.0 ±0.0	7.0 ±0.0	7.5 ±0.0	7.0 ±0.0	7.0 ±0.0
Nisplin [®] -0.5% Ginger	7.0 ±0.0	9.0 ±0.0	10.0 ±0.0	8.0 ±0.0	9.0 ±0.0	8.0 ±0.0	8.0 ±0.0
Nisplin [®] -0.2% Jojoba	7.0 ±0.0	10.5 ±0.0	7.0 ±0.0	7.0 ±0.0	9.2 ±0.0	7.0 ±0.0	7.0 ±0.0
Nisplin [®] -0.5% Jojoba	8.0 ±0.0	11.0 ±0.0	10.0 ±0.0	8.0 ±0.0	9.5 ±0.0	8.0 ±0.0	8.0 ±0.0
Positive reference standard	21.0 ±0.5	22 ±0.0	15.0 ±0.5	20.0 ±0.0	15.0 ±0.5	13.7 ±0.6	16.0 ±0.5

±: Standard deviation
 No inhibition effect

Table.3. Mean values of total bacterial count (Log CFU/mL) in pasteurized buffalo milk (72°C / 15 Sec) mixed with Nisplin[®]-essential oil emulsions during cold storage period (10 ±1 °C / 20 d)

Treatments	Storage period, days at 10 ±1 °C				
	0	5	10	15	20
Control	3.65±0.52	4.21±0.53	4.95±0.62	5.30±0.65	5.92±0.71
Nisplin [®]	3.60±0.61	3.90±0.51	4.25±0.52	4.50±0.62	4.90±0.50
Nisplin [®] -Cinnamom	3.70±0.65	3.80±0.55	3.85±0.63	3.95±0.70	4.25±0.65
Nisplin [®] -clove	3.52±0.69	3.61±0.57	3.80±0.51	3.90±0.61	4.30±0.54
Nisplin [®] -ginger	3.65±0.72	3.68±0.71	3.72±0.81	3.86±0.50	3.95±0.43
Nisplin [®] -jojoba	3.61±0.62	3.65±0.78	3.70±0.45	3.81±0.45	3.92±0.58

±: Standard deviation

Table.4. Mean values of titratable acidity (TA% as lactic acid) in pasteurized buffalo milk (72°C / 15 Sec) mixed with Nisplin®-essential oil emulsions during cold storage period (10 ±1 °C / 20 d)

Treatments	Storage period, days at 10 ±1 °C				
	0	5	10	15	20
Control	0.16±0.02	0.16±0.03	0.21±0.06	0.29±0.04	0.45±0.03
Nisplin®	0.16±0.03	0.16±0.01	0.17±0.02	0.18±0.04	0.21±0.01
Nisplin®-Cinnamon	0.16±0.03	0.16±0.05	0.17±0.01	0.18±0.03	0.19±0.05
Nisplin®-clove	0.16±0.01	0.16±0.02	0.16±0.04	0.17±0.04	0.18±0.06
Nisplin® - ginger	0.16±0.05	0.16±0.01	0.16±0.03	0.17±0.05	0.18±0.08
Nisplin®-jojoba	0.16±0.04	0.16±0.03	0.16±0.02	0.17±0.03	0.17±0.06

±: Standard deviation

Table.5 Mean values of Sensory Hedonic scale (1 to 9) of pasteurized buffalo milk (72°C / 15 Sec.) Mixed with Nisplin®-Essential oil emulsions during storage period (10 ±1 °C for 20 d)

Treatments*	Storage period, days at 10 ±1 °C		
	0	10	20
Control	9.0±0.00	7.5±0.51	1.0±0.00
Nisplin®	9.0±0.00	9.0±0.00	7.5±0.60
Nisplin® - Cinnamon	9.0±0.00	9.0±0.00	8.0±0.00
Nisplin®-clove	9.0±0.00	9.0±0.00	9.0±0.00
Nisplin® - ginger	9.0±0.00	9.0±0.00	9.0±0.00
Nisplin®-jojoba	9.0±0.00	9.0±0.00	9.0±0.00

±: Standard deviation

During storage, the Hedonic scale of control treatment decreased to 7.5 after 10 days and reached lowest level (1: dislike very much) after 20 days. Sensory Hedonic scale of treatment with Nisplin® alone decreased to 7.5 and treatment with Nisplin®-Cinnamon emulsion decreased to 8.0 after 20 days, while pasteurized milk supplemented with Nisplin®-clove, Nisplin®-ginger and Nisplin®-jojoba emulsions had high score in Hedonic scale (9: like very much) after twenty days of storage under limited refrigeration conditions at 10°C indicating the sensory acceptability during storage reflects increasing of acidity due to microbial activity and reflects spoilage level of pasteurized milk samples. Leistner and Gorris (1995) reported that food preservation

using multiple preservatives in small amounts was more effective than preservation by a large amount of a single preservative because both ensure microbial stability and safety and maintain the sensory, nutritive and economic properties of food products. Wirjntoro and Lewis (1996) did not report any changes in sensory evaluation of pasteurized milk after addition of nisin solution. Also, addition of cinnamon essential oil at MIC had not any negative effect on the sensory evaluation of pasteurized milk (Cava *et al.*, 2007).

Collective results of present study induced that, total viable counts (Table 3) did not exceeded 4.3 log CFU/ml after 20 days storage at 10°C in pasteurized milk

supplemented by low concentrations of Nisplin® (5 IU/mL) combined in emulsion with clove, ginger or jojoba essential oils (25 µg/mL). In the same trend titratable acidity as lactic acid (Table 4) did not exceeded 0.18 %, which reflected in sensory acceptability test (Table 5), where these treatments were marked by high score (9: like very much). It can be deduced that, clove, ginger or jojoba essential oils emulsions in combination with Nisplin® were effective treatments to extend the shelf life of pasteurized milk. Finally, addition of Nisplin®-jojoba emulsion to pasteurized milk resulted in enhanced its shelf life whereas the lowest total viable count (3.92 log CFU/ml) and the lowest acidity (0.17%) were achieved.

In conclusion, Nisplin® combinations in emulsion with clove, ginger or jojoba essential oils were effective to extend the shelf life of pasteurized milk for 20 days without any negative effect on its sensory evaluation under limited refrigeration conditions at 10°C. Also, jojoba essential oil has better and promising effect for commercial use as an effective natural bio-preservative product especially at levels of small holder farmers and local dairy markets.

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