

# Effect of Nisin on Technological and Microbiological Characteristics of Stirred Yoghurt

**Keywords:** Nisin; Yoghurt; Antibacterial activity

## Abstract

**Purpose of this paper:** Growing worldwide inclination towards healthful foods has compelled food processors to search for natural preservatives to enhance the shelf-life and extend the market reach. Limited shelf-life of cultured milk products arising from post-acidification could be enhanced with the application of bacteriocins such as nisin. Application of nisin in stirred yoghurt may retain its desirable properties during extended shelf-life.

**Design/Methodology/Approach:** Stirred yoghurt containing nisin was obtained by culturing cow milk, heated at 95 °C / 5 min with 3% Yoghurt-YH-3 incubated at 42 ± 1 °C for 5 h and then stirred for incorporating 25 RU/ml nisin, followed by further 1 h incubation. Effect of incorporation of nisin on technological (titratable acidity, diacetyl and acetoin content, volatile acidity and extent of proteolysis and microbiological (total viable microbial contents, coliform count, yeast and mould, lipolytic organism and proteolytic organisms) and antibacterial characteristics of stirred yoghurt were assessed during 40 days of storage at 10-15 °C.

**Findings:** Incorporation of nisin in yoghurt induced lower acid and volatile acid development, but a significantly higher proteolytic activity as well as diacetyl + acetoin production. Nisin exhibited antagonism against viable population but ineffective to yeast, mould and proteolytic organisms in yoghurt. Nisin containing yoghurt exhibited lower degree of antagonism against various pathogenic test organisms, which progressively increased during storage.

## Introduction

Growing consumer's inclination towards health foods has attracted yoghurt owing to its prophylactic properties and its consumption has increased significantly throughout the world including UK and USA [1,2,3]. To extend the market reach, shelf-life of yoghurt and other cultured milk products could be enhanced by adopting diverse techniques such as bacteriocins (nisin, Microgard™, natamycin), LP-system, high pressure treatment, post-production heat-treatments (thermization, microwave heating), UV irradiation, carbonization etc. [4].

Lactic acid bacteria (LAB) and their metabolites may have an important role towards microbiological quality and shelf-life of various fermented milk products [5]. With the objective of enhancing the shelf-life, inclusion of nisin producing strains of *Lactococcus lactis* subsp. *lactis* during the manufacture of cultured milk products, may not be advantageous due to their slower rate of acid production, limited proteolytic activity, inability to ferment sucrose and more proneness to bacteriophage [6,7].

Nisin has more than one mechanisms of antimicrobial action depending on several factors such as structural properties of target



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bacteria and exhibits antimicrobial activity against many species of Gram positive bacteria but not Gram negative bacteria due to their outer membrane barriers. Antibacterial mechanisms of nisin may be due to passage through cell wall and interaction with lipid II [8,9]. Nisin was inhibitory to both *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* and storage studies revealed prolonged inhibitory action towards the former organism [10].

It has been identified that nisin in pure form is not used for food processing owing to its very high specific activity and consequently JECFA (Joint FAO/WHO Expert Committee on Food Additives) specified that commercial nisin concentrates should possess at least 2.25% active nisin [11]. Nisaplin, a commercial preparation of bacteriocin nisin, is widely being used in dairy industries to attain three main objectives such as to combat an existing spoilage problem, permitting low heat-processing conditions and formulating new products [12-16].

Toxicological studies have revealed that nisin is safe for human consumption as it is inactivated by pancreatin and alpha-chymotrypsin, not detected in human saliva after 10 min of consumption, does not interfere with intestinal microbiota and has been designated as GRAS (Generally Recognised As Safe) for use as an anti-microbial [17-21]. Scientific Committee on Food (SCF) have approved an average daily intake of 0.13 mg/kg body weight and has been permitted for diverse food products in USA, Australia, South Africa, Russia and India [19,22]. In the present investigation an attempt has been made to evaluate the effect of inclusion of nisin on technological and microbiological characteristics of stirred yoghurt.

## Materials and Methods

### Type of milk

Autoclaved reconstituted skim milk (0.5% fat and 7.64% SNF)

was used for starter propagation as well as for evaluating their metabolic activities. For yoghurt manufacture fresh cow milk (3.0% fat, 8.5% SNF), obtained from local herds situated in Nadia district, West Bengal, India were used.

**Source and maintenance of starter cultures**

Freeze dried cultures of Yoghurt-YH-3, *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus* W and *Bifidobacterium bifidum* NDRI were obtained from National Collection of Dairy Cultures, National Dairy Research Institute, Karnal, India. All the cultures were maintained in plain sterile skim milk, except for *B.bifidum* NDRI in sterile skim milk fortified with 1% dextrose and 1% yeast extract [23].

**Source and maintenance of pathogens**

Virulent pathogenic strains of *Bacillus cereus*, *Shigella dysenteriae* (National Collection of Dairy Cultures, National Dairy Research Institute, Karnal, India), *Salmonella typhi*, three strains (03, 018, 078) of *Escherichia coli* and two strains (P<sub>1</sub>, P<sub>3</sub>) of *Salmonella typhimurium* (Department of Veterinary Microbiology, West Bengal University of Animal and Fishery Sciences, Nadia, India) were maintained on nutrient agar slants (Hi-Media, Bombay, India) by weekly propagations and were activated by three successive transfers at 24 h

intervals in nutrient broth (Hi-Media, Bombay, India).

**Source of Nisaplin**

Nisaplin brand nisin with an activity of 1,000,000 IU/ g, obtained from Aplin and Barrett Ltd., England was added to inoculated milk after 3 h and 5 h of incubation at recommended concentration 25 RU/ml [24].

**Preparation of stirred yoghurt**

Stirred yoghurt was obtained by culturing cow milk, heated at 95 °C/5 min with 3% Yoghurt-YH-3 incubated at 42 ± 1 °C for 5 h and then stirred for incorporating 25 RU/ml nisin, followed by further 1 h incubation. Stirred yoghurt, stored at 15 ± 1 °C was subjected to various technological as well as microbiological attributes at intervals of 0, 5, 10, 15, 20, 25, 30, 35 and 40 days.

**Analytical Techniques**

**Technological attributes**

Metabolic activities of various starter combinations and technological attributes of stirred yoghurt were evaluated on the basis of titratable acidity, diacetyl and acetoin content, volatile acidity and extent of proteolysis [25-28].

**Table 1:** Effect of nisin on the metabolic activities of yoghurt cultures during incubation and storage.

Starter combinations	Time of incorporation of nisin	Titratable acidity (% lactic acid)					Volatile acidity (ml 0.1 N NaOH/50 mg of curd after 6 h of incubation at 42 ± 1 °C (h))
		During incubation at 42 ± 1 °C (h)			During storage at 15 ± 1 °C (h)		
		4	5	6	96	120	
YH-3 (3%)	-	0.54	0.64	0.69	0.73	0.83	2.4
YH-3 (3%) + N	3	0.52	0.55	0.67	0.69	0.79	2.2
	5	-	-	0.66	0.69	0.69	2.3
YH-3 (6%)	-	0.56	0.67	0.70	0.76	0.92	2.4
YH-3 (6%) + N	3	0.54	0.58	0.68	0.70	0.92	2.0
	5	-	-	0.68	0.70	0.89	2.1
YH-3 (3%) + BB (1%)	-	0.55	0.65	0.71	0.74	0.85	2.3
YH-3 (3%) + BB (1%) + N	3	0.53	0.58	0.69	0.71	0.91	2.0
	5	-	-	0.68	0.70	0.79	2.2
YH-3 (6%) + BB (2%)	-	0.58	0.70	0.73	0.78	1.0	2.0
YH-3 (6%) + BB (2%) + N	3	0.55	0.64	0.73	0.77	0.98	1.4
	5	-	-	0.72	0.74	0.93	1.9
ST (1.5%) + LB (1.5%)	-	0.52	0.59	0.67	0.80	0.90	2.2
ST (1.5%) + LB (1.5%) + N	3	0.46	0.47	0.58	0.69	0.78	2.0
	5	-	-	0.58	0.67	0.73	2.1
ST (3.0%) + LB (3.0%)	-	0.53	0.60	0.68	0.90	1.02	2.1
ST (3.0%) + LB (3.0%) + N	3	0.50	0.56	0.61	0.84	0.99	1.5
	5	-	-	0.60	0.68	0.92	1.6
ST (1.5%) + LB (1.5%) + BB (1%)	-	0.52	0.59	0.67	0.85	0.93	2.1
ST (1.5%) + LB (1.5%) + BB (1%) + N	3	0.46	0.51	0.62	0.83	0.91	1.8
	5	-	-	0.62	0.69	0.84	2.0
ST (3.0%) + LB (3.0%) + BB (2%)	-	0.54	0.60	0.69	0.92	1.42	2.0
ST (3.0%) + LB (3.0%) + BB (2%) + N	3	0.53	0.59	0.64	0.88	1.25	1.7
	5	-	-	0.64	0.71	1.02	1.9

N: Nisin was incorporated in autoclaved reconstituted skim milk @ 25 RU/ml during incubation at 42 ± 1 °C; YH-3: Yoghurt-YH-3; LB: *Lactobacillus delbrueckii* subsp. *bulgaricus* W; ST: *Streptococcus thermophilus*; BB: *Bifidobacterium bifidum* NDRI

**Table 2:** Effect of nisin on the technological characteristics of stirred yoghurt during storage.

Chemical characteristics	Product type	Days of storage at 10 - 15 °C								
		0	5	10	15	20	25	30	35	40
Titratable acidity (% lactic acid)	CY	0.47	0.49	0.67	0.70	0.86	0.98	1.12	-	-
	NY	0.41	0.42	0.46	0.47	0.48	0.59	0.68	0.86	0.95
Volatile acidity (ml 0.1 N NaOH/50 mg of curd)	CY	2.00	1.80	1.50	1.04	1.02	0.96	0.95	-	-
	NY	2.00	1.80	1.56	1.08	1.04	1.02	0.98	0.88	0.75
Proteolytic activity (mg tyrosine / ml)	CY	0.01	0.01	0.02	0.02	0.02	0.02	0.02	-	-
	NY	0.02	0.03	0.04	0.04	0.04	0.05	0.05	0.06	0.06
Diacetyl and acetoin (ppm)	CY	10.0	16.2	16.8	17.0	18.9	21.0	29.2	-	-
	NY	17.0	29.2	37.8	40.0	42.0	45.0	51.0	57.0	62.0

CY: Yoghurt prepared from cow milk employing 3% Yoghurt-YH 3;

NY: Yoghurt prepared from cow milk employing 3% Yoghurt-YH 3 containing 25 RU/ml nisin added after 5 h of incubation at 42 ± 1 °C

**Table 3:** Effect of nisin on the microbiological characteristics of stirred yoghurt during storage.

Microbiological characteristics	Product type	Days of storage at 10 - 15 °C								
		0	5	10	15	20	25	30	35	40
Standard plate count* × 10 <sup>5</sup>	CY	9	17	85	89	105	110	119	127	-
	NY	3	11	42	53	35	27	23	17	15
Yeast & mould count* × 10	CY	0	2	4	5	7	9	9	13	-
	NY	0	1	5	6	6	8	9	9	10
Proteolytic count* × 10	CY	0	1	2	2	3	5	8	-	-
	NY	0	0	2	2	2	0	0	0	0
Lipolytic count* × 10	CY	0	0	0	0	0	0	0	-	-
	NY	0	0	0	0	0	0	0	0	0
Coliform count* × 10	CY	0	0	0	0	0	0	0	-	-
	NY	0	0	0	0	0	0	0	0	0

\*Counts are expressed as cfu/ml

CY: Yoghurt prepared from cow milk employing 3% Yoghurt-YH 3 ;

NY: Yoghurt prepared from cow milk employing 3% Yoghurt-YH 3 containing 25 RU/ml nisin added after 5 h of incubation at 42 ± 1 °C

### Microbiological attributes

Microbiological attributes of stirred yoghurt were evaluated on the basis of total viable microbial contents using nutrient agar (Hi-Media, Bombay, India), coliform count using violet red bile agar (Hi-Media, Bombay, India), yeast and mould using potato dextrose agar (Hi-Media, Bombay, India), lipolytic organism using tributyrin agar (Hi-Media, Bombay, India) and proteolytic organisms using milk agar (Hi-Media, Bombay, India) adopting the methods of Bureau of Indian standards [25]. Antibacterial spectrum of the product was estimated by modified cup agar assay technique [29].

### Statistical analysis

Results obtained in the present investigation were analysed statistically by the method of Snedecor and Cochran [30].

### Result and Discussion

#### Effect of nisin on metabolic activities of yoghurt cultures

Results related to effect of incorporation of nisin (25 RU/ml) at the end of 3 h and 5 h of incubation at 42 ± 1 °C on rate of acid development, volatile acidity and post-acidification during storage at 15 ± 1 °C for 96 - 120 h of Yoghurt-YH-3 and *S. thermophilus* + *L.*

*delbrueckii* subsp. *bulgaricus* W in association with *B. bifidum* NDRI have been proclaimed in Table 1.

For both sets of yoghurt cultures, an increase in inoculum from (3 - 6%) did not exhibited any significant increase ( $P > 0.05$ ) in rate of acid production and volatile acidity up to 6 h of incubation, however higher ( $P < 0.05$ ) post-acidification being noted for higher inoculum. Addition of nisin induced slower rate of acid production as well as volatile acid production, which may be attributed to inhibition of yoghurt cultures by nisin. However, Shahani demonstrated higher degree of acidification by both *S. thermophilus* (0.92 vs. 0.94% lactic acid) and *L. delbrueckii* subsp. *bulgaricus* (1.88 vs. 1.95% lactic acid) in presence of nisin [31]. Inhibitory activity of nisin on *S. Thermophilus* and *L. delbrueckii* subsp. *Bulgaricus* have already been demonstrated [32,33]. Time of inclusion of nisin did not revealed any significant effect ( $P > 0.05$ ) on extent of acidification up to 6 h of incubation, but late addition of nisin (after 5 h) exhibited pronounced retarded acid development during subsequent prolonged storage (120 h), indicating preservative effect of residual nisin. Fowler and McCann registered retention of 28.57% nisin activity after 24 h of storage at 22 °C [34]. Higher volatile acid production by all culture combinations denoted with late inclusion of nisin after 5 h may be ascribed to better metabolic activities of yoghurt cultures prior to addition of nisin.

**Table 4:** Effect of nisin on the antibacterial properties of stirred yoghurt during storage.

Pathogenic test organisms	Product type	Diameter of zone* of inhibition during storage at 10 - 15 °C								
		0	5	10	15	20	25	30	35	40
<i>S. typhimurium</i> P <sub>1</sub>	CY	7.5	7.5	7.5	8.0	8.5	8.5	9.0	-	-
	NY	0.0	0.0	0.0	5.0	6.0	7.0	8.0	8.5	9.0
<i>S. typhimurium</i> P <sub>3</sub>	CY	5.5	5.5	5.5	6.0	6.5	7.5	8.5	-	-
	NY	0.0	0.0	6.5	8.5	8.5	9.0	9.0	9.5	10.0
<i>S. dysenteriae</i>	CY	5.5	5.5	5.5	6.0	6.0	6.5	7.0	-	-
	NY	5.5	5.5	6.0	6.0	6.5	7.0	7.5	7.5	8.0
<i>B. cereus</i>	CY	0.0	7.0	7.5	8.5	8.5	9.0	9.0	-	-
	NY	0.0	0.0	0.0	0.0	5.5	5.5	5.5	6.0	6.0
<i>S. typhi</i>	CY	5.5	5.5	6.0	7.5	8.5	8.0	7.0	-	-
	NY	0.0	0.0	5.5	6.5	7.0	8.5	8.0	7.5	6.5
<i>E. coli</i> 018	CY	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	-
	NY	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>E. coli</i> 078	CY	0.0	0.0	0.0	5.5	5.5	5.5	5.5	-	-
	NY	6.5	6.5	6.5	6.5	6.5	0.0	0.0	0.0	0.0
<i>E. coli</i> 03	CY	6.0	6.0	6.0	6.5	6.5	7.0	7.5	-	-
	NY	0.0	0.0	5.5	5.5	5.5	6.0	7.0	7.5	8.0

\*Including the diameter of the well (5 mm)

CY: Yoghurt prepared from cow milk employing 3% Yoghurt-YH 3;

NY: Yoghurt prepared from cow milk employing 3% Yoghurt-YH 3 containing 25 RU/ml nisin added after 5 h of incubation at 42 ± 1 °C

No significant ( $P > 0.05$ ) change in extent of acidification and volatile acid production by either sets of yoghurt cultures could be noticed with the inclusion of *B. bifidum* NDRI, indicating no symbiotic effect between them, hence their inclusion during the manufacture of stirred yoghurt was not suggested. Titratable acidity produced by yoghurt cultures in association with *B. bifidum* (0.67 - 0.73% lactic acid) was higher (0.61% lactic acid) than those noted for freshly prepared probiotic yoghurt but lower than reported values (0.89 - 1.10% and 0.55% lactic acid, respectively) for bifidus yoghurt and dietetic yoghurt containing *B. bifidum* [35-38]. Inclusion of *B. bifidum* exhibited better acid production in association with *L. delbrueckii* subsp. *bulgaricus* than with *S. Thermophilus*, however no considerable deviation noted with either combined *L. delbrueckii* subsp. *bulgaricus* + *S. Thermophilus* or Yoghurt or Yoghurt -YH -3 [36,39]. Similarly, with regard to flavour profiles of yoghurt, associated growth of *B. bifidum* with *S. thermophilus* + *L. delbrueckii* subsp. *bulgaricus* induced lower contents of acetaldehyde, ethanol, diacetyl and volatile acidity, however slight improvement denoted in association with Yoghurt-YH-3 [39,40]. Due to least post-acidification (0.69% lactic acid after 120 h of storage at 15 ± 1 °C) and highest volatile acid production (2.3 ml 0.1 N NaOH/ 50 mg curd after 6 h of incubation at 42 ± 1 °C), 3% Yoghurt YH-3 and nisin incorporation time after 5 h of incubation were finalized for the manufacture of stirred yoghurt containing nisin.

### Effect of nisin on technological attributes of stirred yoghurt

Results pertaining to effect of incorporation of nisin (25 RU/ml) on the technological attributes of stirred yoghurt have been proclaimed in Table 2. Incorporation of nisin in yoghurt induced lower acid development in contrast to samples without nisin (0.41 - 0.68% vs. 0.47 - 1.12% lactic acid) during 30 days of storage at 10-15 °C due to inhibitory action of nisin on yoghurt cultures. Titratable acidity attained by nisin containing yoghurt up to 35 days of storage

(0.86% lactic acid) was within the suggested limits of 0.85 - 0.90% lactic acid [41]. Progressive decline in volatile acidity during storage were noticed in yoghurt, irrespective of presence of nisin. Incorporation of nisin into yoghurt induced no change ( $P > 0.05$ ) in volatile acidity up to 5 days, but subsequent storage up to 30 days showed higher content up to 30 days in nisin containing samples in contrast to those without nisin. Significantly higher ( $P < 0.05$ ) proteolytic activity (0.02 - 0.05 vs. 0.01 - 0.02 mg tyrosine/ml) as well as diacetyl + acetoin content (17.0 - 51.0 vs. 10.0 - 29.2 ppm) were denoted in nisin containing yoghurt than those without nisin during 30 days of storage. Sarkar and Misra noted volatile acidity and diacetyl and acetoin content of probiotic yoghurt to reach the peak value (2.0 - 2.9 ml of 0.1 N NaOH/50 mg curd and 40.0 - 101.0 ppm, respectively) after 3 days, followed by a decline during subsequent storage [35]. A decline in concentrations of acetaldehyde but an increase in diacetyl and diacetyl and acetoin during storage of yoghurt have been reported [42,43]. Proteolytic activity demonstrated in nisin containing yoghurt at the end of 35 days (0.06 mg tyrosine/ml) was within the limits of 0.05 - 0.10 mg tyrosine/g required for good quality yoghurt [44]. Enhanced proteolytic activity in nisin containing yoghurt may be due to breakdown of nisin by nisinase enzyme producing *S. thermophilus* [45].

### Effect of nisin on microbiological attributes of stirred yoghurt

Results pertaining to effect of incorporation of nisin (25 RU/ml) on the microbiological attributes of stirred yoghurt have been proclaimed in Table 3. Total viable population (TVC) was significantly ( $P < 0.05$ ) lower in nisin containing yoghurt than those without nisin ( $3 \times 10^5$  -  $170 \times 10^5$  vs.  $9 \times 10^5$  -  $127 \times 10^5$  cfu/ml). It was further pointed out that there was a slight increase in TVC of nisin containing yoghurt up to 15 days ( $3 \times 10^5$  -  $53 \times 10^5$  cfu/ml), followed by a gradual decline ( $53 \times 10^5$  -  $15 \times 10^5$  cfu/ml) after 40 days. Reasons attributable to such

behaviour of yoghurt cultures are synergistic action of nisin, other elaborated antimicrobial compounds and developed lactic acid in the product. Nisin is capable of binding the anionic phospholipids of the cell membrane which results in efflux of intracellular components and depolarisation of cytoplasmic membrane resulting in cessation of all biosynthetic processes [46,47]. No significant difference ( $P > 0.05$ ) in counts of yeast and mould and proteolytic organisms in yoghurt up to 30 and 20 days, respectively were encountered due to application of nisin. Delves-Broughton has also mentioned non-effectiveness of nisin on yeasts and moulds. However, none of the yoghurt samples had lipolytic or coliforms [48].

### Effect of nisin on antibacterial properties of stirred yoghurt

Exhibition of variable degree of antagonism against various pathogenic test organisms by yoghurt with or without nisin have been demonstrated, lower being observed in most of the samples containing nisin (Table 4). Reason attributable to reduced antagonism in nisin containing yoghurt is due to lower production of antimicrobial compounds resulting from inhibition of yoghurt cultures by nisin. During storage up to 40 days, a progressive increase in antagonistic activity of yoghurt against all test organisms, irrespective of presence of nisin were denoted, except against *S. typhi* for which a declining trend noted after 35 days. However, none of the yoghurt samples could exhibit antagonism against *E. Coli* O18. Maximum antibacterial activity was exhibited by nisin containing yoghurt against *S. typhimurium* P<sub>3</sub> (10mm), followed by *S. typhimurium* P<sub>1</sub> (9.5 mm), *S. dysenteriae* (8.0 mm) and *E. Coli* O3 after 40 days of storage. Inhibitory activity of yoghurt cultures against *B. Cereus*, *S. typhimurium*, *S. Dysenteriae*, *Shigella* spp. and *E. Coli* has been registered [39,49-51]. Mel'nikova and Koreleva denoted greater inhibitory activity of *L. delbrueckii* subsp. *bulgaricus* than *S. Thermophilus* [50].

### Conclusion

Due to least post-acidification (0.69% lactic acid after 120 h of storage at  $15 \pm 1$  °C) and highest volatile acid production (2.3 ml 0.1 N NaOH/50 mg curd after 6 h of incubation at  $42 \pm 1$  °C), 3% Yoghurt YH-3 and nisin (25 RU/ml) incorporation time after 5 h of incubation were recommended for the manufacture of stirred yoghurt containing nisin. Inclusion of *B. bifidum* NDRI during the manufacture of yoghurt was not suggested. Stirred yoghurt containing 25 RU/ml nisin retained desirable technological, microbiological as well as antibacterial characteristics during 40 days of storage at 10 - 15 °C. Application of nisin for shelf-life extension of stirred yoghurt made from cow milk is suggested. Though the difference in quality of curd obtained employing cow milk and full cream milk is beyond the scope of this research, however no significant is expected as the average fat content of cow milk (3.50%) produced in West Bengal, India is similar with those in full cream milk (3.50%).

Greater inclination of health conscious consumers towards yoghurt owing to its diverse nutritional and therapeutic has been noted. Manufactures could not satisfy their consumers due to few technical problems such as high post acidification, thereby reducing the viability of starter cultures and antibacterial activity towards pathogens or undesirable flora. Application of nisin in yoghurt will overcome all the technological problems and extend market reach.

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