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Effect of Sodium Chloride (NaCl) Concentrations and Temperature on Antimicrobial Activity of Bacteriocins Produced by *Bacillus spp* on Soft Cheese

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This study investigated the influence of sodium chloride concentrations (0.1-0.5%) and temperatures (ambient (26-28°C); refrigerator(4-11°C) on antimicrobial activity of bacteriocin from *Bacillus* spp. Three bacteriocins from *Bacillus* spp were tested against *Staphylococcus aureus*[2], *Salmonella enteritidis*[3] and *Micrococcus luteus*[1]. Zones of inhibition observed at various salt concentrations were not significantly different at $p<0.05$ level, however bacteriocin produced by strain (In5a) showed inhibition (12.00±0.000) mm against *Micrococcus luteus* at 0.5% salt concentration. The other 2 bacteriocins had inhibition of less than 6.0mm. Bacteriocin produced by strain (Oe2a) was used alone and in combination with sodium chloride at 6.5 and 13% concentrations for cheese storage at ambient and refrigerator temperatures, Cheese kept at ambient temperature were mouldy by the fifth day of storage, while those kept at refrigerator temperature lasted two weeks. It was observed that the bacteriocin was not effective at ambient temperature, in addition, sodium chloride dramatically decreased bacteriocin sensitivity by an increase in microbial counts for samples kept at ambient temperature. Cheese kept at refrigerator temperature, had a decrease in microbial counts that was significant ($P<0.05$) at higher concentration (13%) than at lower concentration (6.5%) for aerobic counts. The reverse was the case for Enterobacteriacea, coliform, yeasts and moulds counts.

Key words: *Bacillus* spp, Bacteriocins, Sodium chloride, Temperature, Cheese.

Several factors have been observed to potentiate the production of bacteriocins. For instance, a decrease in pH results in decrease adsorption of bacteriocin molecules to the producer cells and hence an increase bio-availability, also temperatures (De vuyst, *et al.*, 1996) as well as nutrient availability play a crucial role in bacteriocin production whereas the effect of Sodium chloride on the production level of bacteriocins is still unclear.

Supplementation of culture media with growth enhancing factors, such as; salt, sugars, vitamins and nitrogen sources, by regulating pH or by choosing the best–adapted culture medium (Vingnolo *et al.*, 1995) have been observed to bring about maximal bacteriocin production.

Also the possibility of suppression of bacteriocin synthesis by sodium chloride (NaCl) is of particular interest, because of its wide spread used in a number of dairy products (e.g. cheese). However, how factors, such as the presence of salt and curing agents, influence bacteriocin titers still remains unclear.

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Previously, the effect of salt on production and/or activity of bacteriocins produced by lactic acid bacteria have been reported to be beneficial (Ganzle *et al.*, 1996; Uguen *et al.*, 1999) or harmful (De Vuyst *et al.*, 1996). Abee *et al*. (1994) studied the mode of effect of Nisin Z against *L. monocytogene*s and found that NaCl slightly increases Nisin's inhibitory activity. The salt concentrations used were of 0.5% and 2% weight per volume. In addition, Parente *et al*. (1998) demonstrated that increasing NaCl concentration from 1% to 4.2% slightly increased the effect of Nisin at 50IU/ml. Thomas and Wimpenny (1996), asserted to a synergistic effect of NaCl concentration on Nisin. Mazzotta *et al*. (1997) also observed that combination of Nisin and of 3% NaCl concentration has synergistic effects on resistant mutants of *C. botulinum.*

In contrast, Peykov *et al*. (2008) in his study on the effect of salt on bacteriocin production showed that sodium chloride (NaCl) had a suppressing activity upon bacteriocin production by three strains of *Enterococus species* isolated from dairy products, a fact which was more clearly observed in conditions of higher salt concentrations. This study is aimed at evaluating the effect of sodium chloride concentrations and storage temperatures on the antimicrobial activity of bacteriocins from *Bacillus* spp.

MATERIAL AND METHODS

Methods

Bacterial strains

Six laboratory cultures of indicator organisms viz. *Staphylococcus aureus*[2], *Salmonella enteritidis*[3] and *Micrococcus luteus*[1]. were used in this study.

Isolation of bacilli strains

Bacilli strains were isolated from common food products namely; 'Wara' (cheese), 'Fura' (fermented millet), 'Elubo' (yam flour) and 'Kulikuli' (groundnut cake).

Bacterial identification

Bacterial isolates were identified by microscopic examination after gram staining and staining for presence of endospores which is a characteristic of the genus *Bacillus*. Biochemical tests; catalase, oxidase and coagulase tests were also carried out.

Identification of the bacteriocinogenic isolates:

Pure isolates selected as potential bacteriocin-producer were identified on the basis of their cultural, morphological, physiological and biochemical characteristics (Schillinger and Lûcke, 1987).

Screening for Bacteriocin-Producing Bacilli Detection of Antimicrobial Activity

The antimicrobial activity of the bacillus strains isolated was observed by using them to inhibit indicator organisms. Indicator organisms used were *Salmonella enteritidis*[3], *Staph. aureus*[2] and *Micrococcus luteus*[1].

Harvesting of Bacteriocin

The 3 strains of bacilli showed antimicrobial activity against indicator organisms (17-27mm diameter zones of clearance on modified Muller-Hinton agar against salmonella and yeast strains. *Bacillus spp* isolates were grown in 10mls of nutrient broth and incubated at 30-37°C temperature overnight. The broth was centrifuged at 3500 revolutions for 15 minutes after which the supernatant was drawn out using a pipette. The supernatant were neutralized using 1M NaOH and glacial acetic acid. An uninoculated nutrient broth was used as control.

Sensitivity of bacteriocin harvested to Indicator Organism using Agar Well Diffusion (AGW) Assay

The supernatant from the 3 bacilli strains were tested against 3 indicator organisms selected from the previous group. A strain of *Salmonella enteritidis*, one strain of *Staphylococcus aureus* and *Micrococcus luteus* using Agar Well Diffusion (AWD) method (Lasta *et al.*, 2008).

Modified Muller-Hinton agar plates was prepared and allowed to cool to about 45°C. Prior to pouring, the indicator organisms of 10^4cfuml cell concentrations were incorporated into the agar using appropriate jars. Modified media thereafter was dispensed into petri dishes. Wells, of about 5mm in diameter were bored into the agar underplayed with Modified Muller-Hinton agar and filled with 50µl of the harvested. The plates were pre-incubated at 4^0C for 30minutes to allow for diffusion of any inhibitory metabolite into the surrounding agar, and then incubated at 37°C for 18-24 hours. Clear zones of inhibition were examined for in the agar surrounding the wells. This was done in two replicates.

Effect of Salt (Sodium Chloride) at different Concentration on bacteriocin from *Bacillus* spp

Three strains recording high zones of inhibition (17-27mm) was selected for this phase of the experiment. The bacilli test strains (Oe2a, Ok2a, and In5a) were grown in nutrient broth to which sodium chloride was added at concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5% and incubated at 37°C temperature overnight. The broth was centrifuged at 3500 revolutions for 15 minutes after which the potency of the bacteriocin was evaluated against indicators organisms using AWD assay.

Preservation of local soft cheese using bacteriocins in combination with salt concentrations at ambient (26-28°C) and refrigeration temperature (4- 11°C).

Bacteriocin obtained from one of the three selected test strain (Oe2a) was used in preserving cheese alone and in combination with different salt concentrations (6.5 and 13%) and subjected to two storage temperatures (ambient (26-28°C) and refrigeration temperature (4- 11°C). Sixty gram (60g) each of freshly prepared cheese was aseptically cut out and placed in four sterile jars. To each was added 50ml of whey. The jars were labeled A, C, D and E. Jar A contained cheese alone (control) , jar C contained the cheese to which bacteriocin alone was added, jar D contained cheese to which was 6.5% salt concentration was added to the bacteriocin, while jar E contained cheese to which 13% salt was added to the bacteriocin.

The jars were stored at ambient temperature *(26-28°C) for 5days and refrigeration temperature* (4-11°*C*) for 13days. Days of sampling and serial dilutions were has follows 0 (10^3), 3 (10^7), 5 (10^9), 7(10^9), 9 (10^5), 11(10^6) and 13(10^6). On each sampling day 5g cheese sample was homogenized and serially diluted in 0.1% sterile peptone water. Enumeration for aerobes was done on plate count agar, enterobactericeae and coliforms on maCconkey agar, yeasts and moulds on sabourand agar. All plates were incubated at 37 °C for 24hrs.

RESULTS

Screening and Identification of Bacteriocinogenic Strains

Forty-five isolates were obtained and screened for antimicrobial spectrum against gram-positive and gram- negative bacteria using the surface diffusion method. The average diameter of the inhibition zones measured was from (5.90±0.000-30.00±0.000) mm (data not shown). Among the isolates, eight strains showing maximum zones of inhibition were selected.

Detection of antimicrobial spectrum

Results of the surface diffusion method showed that the majority of the isolates contain antimicrobial metabolites, with a wide spectrum that inhibited the growth of the indicator organism used (data not shown). Inhibition zones of 5.90 – 25.0mm were detected .

Sensitivity of Bacteriocin Harvested to Indicator Organisms

Bacteriocin harvested from the eight strains selected examined for inhibition against the three indicator organisms (*Salmonella enteritidis, Staphylococcus aureus and Micrococcus luteus)* used, showed that the bacteriocin posses a wide range antimicrobial spectrum against the indicator organisms. This was observed by zones of inhibition measuring about (5.90±0.000-25.00±0.000) mm (Table 1).

Effect of salt concentrations on the microbial activity of bacteriocin

The effect of salt on bacteriocin was only observed at 0.5% salt concentration with strain In5a showing an inhibiting of (12.00±0.000) mm against *Micrococcus luteus* (ML). Salt concentrations, indicator organisms and the test strains were not significantly ($P<0.05$) different from one another (Table 2).

Effect of bacteriocin and salt concentrations on microbial quality of cheese at ambient temperature

By the end of the fifth day the samples were spoilt, there was an increase in the microbial counts obtained in the samples along the days of storage except for the coliform counts.

On day three of storage, sample C(cheese in bacteriocin alone) was not different from sample D (cheese in bacteriocin+6.5% salt) in microbial floral except for the yeast and mould counts, while in sample E (cheese in bacteriocin+13% salt) the counts were higher significantly ($P<0.05$) for aerobic, coliforms, molds and yeasts, except for enterobacteriaceae counts

Also there was a reduction in the microbial count obtained for sample with bacteriocin alone (C) as compared with the control

(sample A), except for the values obtained for enterobactericeae counts on the 3rd day and yeast and mould counts on the fifth day. Comparing sample C with sample D, the decrease was only observed on day three with the aerobic count while other counts increased throughout the storage days. Sample E with a higher salt concentration (13%) as compared to sample D, (6.5% salt concentration) had an increase in the microbial counts all throughout the five storage days (Table 3).

Effect of bacteriocin and salt concentrations on microbial quality of cheese at refrigeration temperature

Samples kept at refrigerator temperature lasted for two weeks of storage. The cheese samples were also observed to retain its freshness after the storage days. For the control sample (B) the microbial counts increase along the days until the 7th day when there was a sharp decline in the counts. This was also observed for samples; containing bacteriocin alone (sample F), with salt concentration of 6.5% (sample G) and that with salt concentration of 13% (sample H).

On the third day of storage, all the samples were not significantly different from one another for the microbes evaluated. By the fifth day counts in the control sample (B), had increase counts for all the microbes, while that in sample stored with bacteriocin alone (sample F) reduced, however comparing counts in sample F with that from sample G and H, they were higher. Nevertheless, counts in sample H (cheese with bacteriocin+13% salt), was higher than sample G (cheese with bacteriocin+6.5%salt). The coliform counts were generally low for all the samples as compared to other microbes. By the eleventh day, yeast and mould counts in all the samples was <1 log (0.99±0.000), revealing the effectiveness of all these treatments for yeast and mould inhibition.

However by the end of the storage days, sample F was effective for inhibiting Aerobic counts as compared to other microbes in relation to the control sample. Comparing samples F, G and H for the aerobic and enterobactericeae counts, higher salt concentration only helped in lowering the aerobic counts obtained in comparison to the other samples. While for coliform and enterobactericeae

Table 1. Mean zones of inhibition (mm)) exhibited by the bacteriocins against indicator microorganisms

Indicator organism Bacillus strains	*Micrococcus luteus*	*Salmonella enteritidis*	*Staphylococcus aureus*
Bf4b	6.00±0.000	8.95±4.313	5.90±0.000
Bk4b	6.00±0.000	5.90±0.000	5.90±0.000
In5a	6.00±0.000	20.00±0.000	5.90±0.000
In5c	6.00±0.000	5.90±0.000	5.90±0.000
Mk1a	6.00±0.000	20.00±0.000	5.90±0.000
Oe2a	6.00±0.000	25.00±0.000	5.90±0.000
Ok2a	6.00±0.000	17.00±0.000	5.90±0.000
Sw3b1	6.00±0.000	5.90±0.000	5.90±0.000

Values are means ± standard deviation in millimeters
Keys: - Mw1a –bacteriocin from Mw1a strain
Oe2a –bacteriocin from Oe2a strain
Ok2a – bacteriocin from Ok2a strain
Sw3b1- bacteriocin from Swb1 strain
Bf4b – bacteriocin from Bf4b strain
Bk4b – bacteriocin from Bf4b strain
In5a – bacteriocin from In5a strain
In5c – bacteriocin from In5c strain
Indicator organisms: ML –Micrococcus luteus
Sal – Salmonella spp , Sta – Staphylococcus spp
Y2 - Yeast strain 2
Y3 – Yeast strain 3
NB: 5.9- no zone of inhibition observed; 6.0- observed zones of inhibition were not prominent.

Table 2. Effects of salt (Sodium chloride) at different concentration on bacteriocin sensitivity

Indicator organisms	Salt concentration	Sal					ML					Sta				
		0.1	0.2	0.3	0.4	0.5	0.1	0.2	0.3	0.4	0.5	0.1	0.2	0.3	0.4	0.5
Strains	Ok 2a	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00
	Oe 2a	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00
	In5a	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	12.00±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00	5.90±0.00

Values are means ± standard deviation in millimeters
Keys: Indicator organisms: ML –*Micrococcus luteus* Sal – *Salmonella spp*
Sta – *Staphylococcus spp* 5.9:- no zone of inhibition observed.

Table 3. Microbial counts of cheese preserved with bacteriocin from *Bacillus* spp and salt at different concentrations at ambient temperature (26-28°C)

Day		0				3				5			
Counts		Aerobic	Enterobacteriaceae	Coliform	Yeast & mould	Aerobic	Enterobacteriaceae	Coliform	Yeast & mould	Aerobic	Enterobacteriaceae	Coliform	Yeast & mould
samples	A	6.68±0.040[a]	6.68±0.040[a]	0.99±0.000[a]	6.77±0.130[a]	10.42±0.025[a]	9.59±0.015[a]	0.99±0.000[a]	10.39±0.055[a]	11.80±0.075[a]	11.33±0.055[a]	0.99±0.000[a]	10.96±0.060[a]
	C					045[b]9.97±0.	100[b]9.87±0.	0.000[a]0.99±	000[b]9.00±0.	0.040[b]1.11±	000[ab]11.03±0.	000[a]0.99±0.	035[a]11.33±0.
	D					9.72±0.160[b]	9.95±0.040[b]	0.99±0.000[a]	9.84±0.0005[c]	11.34±0.070[c]	11.62±0.000[b]	0.99±0.000[a]	6.75±5.76[b]
	E					10.95±0.045[ab]	9.89±0.035[b]	0.99±0.000[a]	10.82±0.075[a]	11.06±0.045[b]	10.92±0.045[bc]	0.99±0.000[a]	11.51±0.070[a]

Results are given in means ± standard error of mean (log_{10} CFU/mil)
means ± standard error of mean with similar superscript are not significantly different from one another in counts for the microbe evaluated

Table 4. Microbial counts of cheese preserved with bacteriocin from *Bacillus* spp and salt at different concentrations at refrigeration temperature (4-11°C)

Day		E				3				5			
Counts		Aerobic	Enterobacteriaceae	Coliform	Yeast & mould	Aerobic	Enterobacteriaceae	Coliform	Yeast & mould	Aerobic	Enterobacteriaceae	Coliform	Yeast & mould
sample	B	6.68± 0.040[a]	6.39± 0.080[a]	0.99± .000[a]	6.77± 0.130[a]	10.29± 0.030[a]	9.56± 0.080[a]	0.99± 0.000[a]	10.64± 0.055[a]	11.66± 0.035[a]	11.41± 0.025[a]	0.99± 0.000[a]	11.66± 0.025[a]
	F					10.29± 0.030[a]	9.56± 0.080[a]	0.99± 0.000[a]	10.64± 0.055[a]	10.34± 0.000[b]	10.60± 0.275[b]	10.76± 0.000[b]	10.16± 0.045[b]
	G					10.29± 0.030[a]	9.56± 0.080[a]	0.99± 0.000[a]	10.64± 0.055[a]	10.48± 0.030[e]	10.86± 0.200[bc]	10.75± 0.000[b]	0.99± 0.000[c]
	H					10.29± 0.030[a] 0.045[ab]	9.56± 0.080[a] 035[b]	0.99± 0.000[a] 0.000[a]	10.64± 0.055[a] 0.075[a]	11.54± 0.130[ad] 0.045[b]	11.31± 0.035[a] 045[bc]	10.78± 0.000[b] 000[a]	10.48± 0.015[d] 070[a]

Table 4. Cont.

Day		7				9				11			
Counts		Aerobic	Enterobacteriaceae	Coliform	Yeast & mould	Aerobic	Enterobacteriaceae	Coliform	Yeast & mould	Aerobic	Enterobacteriaceae	Coliform	Yeast & mould
Samples	B	12.67± 0.020[a]	10.69± 0.000[a]	5.84± 4.850[ab]	0.99± 0.000[a]	7.70± 0.021[a]	7.93± 0.010[a]	7.36± 0.00[a]	0.99± 0.00[a]	9.03± 0.040[a]	9.00± 0.045[a]	0.99± 0.000a	0.99± 0.000[b]
	F	11.63± 0.010[b]	11.93± 0.000[b]	0.99± 0.000[a]	0.99± 0.000[a]	8.11± 0.085[b]	7.92± 0.020[a]	7.08± 0.00[b]	0.99± 0.00[a]	8.40± 0.100[b]	9.09± 0.090[a]	0.99± 0.000a	0.99± 0.000[b]
	G	10.30± 0.000[c]	10.78± 0.000[c]	0.99± 0.000[a]	0.99± 0.000[a]	8.58± 0.007[c]	8.22± 0.030[b]	7.56± 0.03[c]	7.99± 0.035[b]	8.20± 0.035[c]	9.34± 0.040[b]	8.98± 0.030b	0.99± 0.000[b]
	H	10.40± 0.095[c]	10.00± 0.000[d]	0.99± 0.000[a]	0.99± 0.000[a]	7.86± 0.120[a]	8.26± 0.030[cb]	0.99± 0.00[d]	0.99± 0.00[a]	8.28± 0.045[c]	9.38± 0.030[b]	8.34± 0.040c	0.99± 0.000[b]

Table 4. Cont.

Day	13			
Counts	Aerobic	Enterobacteriaceae	Coliform	Yeast and mould
Sample B	9.43±0.040[a]	8.34±0.045[a]	7.98±0.025[a]	0.99±0.000[a]
F	9.19±0.025[b]	8.99±0.020[b]	8.59±0.010[b]	0.99±0.000[a]
G	9.40±0.070[a]	8.29±0.030[a]	7.69±0.030[c]	0.99±0.000[a]
H	9.09±0.025[b]	8.77±0.020[c]	8.08±0.000[d]	0.99±0.000[a]

Values represent; Mean microbial count ± standard error of mean (log10 CFU/mil). Values with the same superscript(s) along the rows are not significantly different from one another at $p<0.05$ level for the microbe evaluated from the cheese sample.

counts, the lower salt concentration decreased the counts obtained as compared with the other samples (Table 4).

DISCUSSION

The use of bacteriocin for preservation of foods may contribute to more uniform and safer products. However, the bacteriocin activity levels in food matrix are less than the expected activity levels. This is due to the specific conditions in the food environment (Stiles.*et al.,* 1996). For this reason it is necessary to select strains synthesising bacteriocin in an environment adapted for effective bacteriocin production.

It has been suggested that the decrease in bacteriocin production in the presence of salt is due to interference of sodium chloride molecules with binding of the induction factor (Nilsen, *et al.,* 1998), which is essential for bacteriocin production, to its receptor. Hence, addition of salt may be one of the major causes of the reduced efficacy of bacteriocin-producing starter cultures in food environments, however, research is in progress to examine the roles of other compounds in food matrix in this regards.

The possibilities of bacteriocin synthesis suppression by sodium chloride are a point of particular interest, because this substance is widespread and often used in a number of dairy products (e.g. cheese).

Inhibition was only observed with bacteriocin from (In5a) against *Micrococcus luteus* at salt concentration of 0.5% (table1), other indicator organismswere able to maintain their growth in the presence of this salt concentration, thus making the bacteriocin ineffective. At salt concentrations lower than 0.5% no zones of inhibitions were observed. This reveals that there was a reduction in growth of the bacteria at low salt concentration, thus a reduction in bacteriocin production at these salt concentrations. This is in contrast to reports; by (Ganzle, *et al.*,1999; ,Korkeala, *et al.,* 1992,Vingnolo, *et al.,* 1995; Uguen, *et al.,* 1999) that low salt concentrations of salt can enhance bacterial growth and reports from (De Vuyst, *et al.,* 1996; Rozes, *et al.,* 1996; Uguen, *et al.,* 1999), where a strong negative effect was observed at high salt concentrations on the growth of LAB.

In addition, salt concentrations of 0.1-0.5% did not potentiate the antimicrobial activity of the bacteriocin from the three strains tested. Unlike, bacteriocins from *Lactococcus brevis* that produced increase activity when the medium was supplemented with 1-2% salt concentration in an earlier study by Ogunbanwo *et al.,* 2003ab.

Also, the difference observed in bacteriocin activity at the different storage temperatures can be due to the fact that at ambient temperature, the temperature is high and fermentation is enhanced thus, affects the bacteriocin producing *Bacilli strain* resulting into the decrease bacteriocin efficacy, hence an increased microbial counts. This is similar to observations by (Diep, *et al.,* 2000; Messens, *et al.,* 2002) where curvacin A produced by *L. curvatus* LTH 1174 was observed to be abolished at an elevated temperature and fermentation. However, the reduction in yeast, mould and coliform counts at this temperature can be due to a drop in pH during fermentation which will render some species more susceptible to the bacteriocin that is produced in situ (Verluyten, *et al.,* 2004).This also explains, the results obtained from samples kept at refrigerator temperature, where temperature is lower and fermentation reduced.

CONCLUSION

Our findings showed a decrease in the efficacy of bacteriocin under conditions of increasing NaCl concentrations, especially at ambient temperature. The results obtained from the use of 6.5 and 13% salt concentrations in combination with bacteriocin in cheese storage is similar to reports by El-molla *et al.,* 1981 where *S. entericia Subsp entericia Serotype typhinurium* survived with 5% salt concentratrion added to the cheese milk, This is of important consideration in food processing and preservation involving a combination of salt and bacterioicin e.g. Cheese.

It can therefore be suggested from this study that, sodium chloride has suppressing activity upon the efficacy of bacteriocin; this finding was potentiated in conditions of higher salt concentrations. The effect of sodium chloride on bacteriocin efficacy should be taken into account in food industry because of the possibilities of the future application of bacteriocins from *Bacillus* spp. The chemical composition and the physical condition of food can also have a significant influence on the activity of bacteriocins; therefore, salt concentrations added to dairy products should be kept as low as possible.

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