ELSEVIER

Int. Dairy Journal **6** (1996) 33–42

0958-6946/96/$15.00 + 0.00

0958-6946(94)00053-0

Lactoperoxidase Suppresses Acid Production in Yoghurt During Storage Under Refrigeration

Mayumi Nakada,* Shun'ichi Dosako, Ryogo Hirano, Minoru Oooka & Ichiro Nakajima

Technical Research Institue, Snow Brand Milk Products Co., Ltd, 1-2, Minamidai 1-chome, Kawagoe, Saitama 350, Japan

(Received 8 June 1994; revised version accepted 30 November 1994)

ABSTRACT

Three types of fermented milk were prepared using either Lactobacillus bulgaricus, Streptococcus thermophilus, *or their mixed culture. Five mgkg*$^{-1}$ *of lactoperoxidase (LPO) suppressed acid production in the yoghurts during refrigerated storage. The most effective suppression was observed with the mixed starter culture. Addition of LPO did not affect the incubation time at 41°C except for the* L. bulgaricus *single culture. While the viable cell counts of the single cultures were constant during storage, with or without the addition of LPO, the viable cell counts of* L. bulgaricus *in the mixed culture decreased in the presence of LPO. A significant decrease in LPO activity was observed during incubation. The concentration of thiocyanate in LPO-treated yoghurt was lower than that in the control by 2–3 mg kg*$^{-1}$*. The addition of LPO produced a new type of yoghurt which retains acceptable quality during storage for at least two weeks.*

INTRODUCTION

Lactoperoxidase (E.C.1.11.1.7; LPO) is an enzyme present in milk, with a molecular weight of approximately 77.5kDa (Sievers, 1981). Its well-known function is an antibacterial effect in the presence of both hydrogen peroxide and thiocyanate (LP system)(Reiter, 1985). According to IDF (1988), 30 mg kg^{-1} of sodium percarbonate ($2Na_2CO_3 \times 3H_2O_2$) and 14 mg kg^{-1} of sodium thiocyanate (NaSCN) are needed to activate the LPO system in order to preserve raw milk. Attempts have been reported to preserve the quality of raw milk in areas where

*Author to whom correspondence should be addressed.

refrigeration facilities are unavailable, by adding thiocyanate and H_2O_2 to milk containing naturally occurring LPO (Björck, 1982), thus activating the LPO system. Bacterial growth in raw milk was delayed for 7–8 h at 30°C and 24–26 h at 15°C, by adding thiocyanate and H_2O_2 to raw milk (Björck et al., 1979).

Several researchers attempted to prepare yoghurt from raw milk in which the LPO system had been activated (Zall et al., 1983; Kumar & Mathur, 1989; Mehanna & Hefway, 1988). They added thiocyanate and H_2O_2 to raw milk, and then prepared yoghurt from this milk, using a mixed starter consisting of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. The production of lactic acid during fermentation was affected by the concentrations of thiocyanate and H_2O_2 added to the raw milk (Kumar & Mathur, 1989). These studies aimed at preserving raw milk by the LPO system and the application of such preserved milk.

The addition of neither H_2O_2 nor thiocyanate to food is permitted in Japan (Kishi, 1993). In yoghurt, however, lactic acid bacteria produce H_2O_2 (IDF, 1985) and milk contains a certain amount of thiocyanate (IDF, 1988).

Therefore, the LPO system would be activated when yoghurt is prepared from pasteurized milk to which only LPO is added. The activated LPO system would suppress the growth of lactic acid bacteria and consequently acid production, leading to the development of a new type of yoghurt in which the titratable acidity would remain stable at a certain level during storage.

However, there will be many problems with LPO-treated yoghurt. For example, activation of the LPO system may prolong the fermentation required for the gelation of casein. Also, the viable cell count of lactic acid bacteria in yoghurt, which might be reduced by the activated LPO system, is directed to be higher than 10^7 cfu g^{-1} by law in Japan (Namba, 1990).

The objective of this study was to examine the effect of LPO on the growth and acid production by single strains of *L. bulgaricus*, *S. thermophilus* and a mixed culture of *L. bulgaricus* and *Str. thermophilus*.

MATERIALS AND METHODS

Lactoperoxidase (LPO)

Lactoperoxidase (DMV, the Netherlands, 180U mg^{-1}) was dissolved in water at 5% (w/v), and then passed through a 0.45 μm filter (GL SCIENCE, Tokyo, Japan).

Preparation of starter

Lb. delbrueckii subsp. bulgaricus, *Str. salivarius subsp. thermophilus* (both isolated from a mixed starter culture B-37, CHR. HANSEN, Denmark) or a mixed culture of *L. bulgaricus* and *S. thermophilus* (B-37) were inoculated into reconstituted skim milk (12%, w/w, pasteurized at 90°C for 10 min) at a concentration of 3%, then incubated at 32°C for 16 h. After incubation, a 5% LPO solution was added to each starter at a concentration of 0.33%; the concentration of LPO added to the starter was 167 mg kg^{-1}. As a control, pasteurized water was added in place of the LPO solution to each starter.

Preparation of yoghurt

Commercial milk (3.5% fat, 8.3% SNF) was heated at 90°C for 10 min and then cooled to 32 ± 1°C for the two single cultures or 42 ± 1°C for the mixed culture. Each starter was inoculated into the milk at a concentration of 3%. Since the LPO concentration in the starter was 167 mg kg^{-1}, the final LPO concentration in the yoghurt was 5 mg kg^{-1}.

The two single cultures were incubated at 30°C for approximately 16 h until the titratable acidity reached 0.7% (*L. bulgaricus*) or 0.6% (*S. thermophilus*). The mixed culture was incubated at 41°C for approximately 3 h until the titratable acidity reached 0.8%.

The yoghurts were then promptly transferred to a refrigerator and kept at 5°C overnight. After 24 h, the yoghurts were transferred to another refrigerator and stored at 10°C for 14 days.

Measurements

The titratable acidity (% lactic acid) was determined by titration with 0.1M NaOH. Viable cell counts were determined by counting colonies on BL Agar "Nissui" (Nissui seiyaku Co., Ltd., Tokyo). The activity of LPO in yoghurt was determined by an enzyme-linked immunosorbent assay (ELISA), following the method of Sato et al. (1992). The concentration of thiocyanate was determined spectrophotometrically using ferric nitrate (IDF, 1988). H_2O_2 was determined with an oxygen electrode (ORITECTOR MODEL-V; Oriental Electric Co., Ltd., Niiza, Japan). Formic acid was determined spectrophotometrically using 1-ethyl-3-(3-dimethyl amino propyl)-carbodiimide hydrochloride with a Carboxylic Acid Analyzer S-14 (Tokyo Rikakikai Co., Ltd., Tokyo).

RESULTS

Effect of LPO on incubation time

Incubation times required to reach given acidities are listed in Table 1. To achieve the same acidity level, the *L. bulgaricus* culture required 3 h longer in the LPO-treated samples compared to control samples, but LPO did not affect the rate of acid production by the two other cultures. The results for the mixed culture were the same as reported by Mehanna & Hefnawy (1988), who found that the acidity of LPO-activated milk increased at the same rate as that of control milk.

Effect of LPO on the acidity and viable cell counts of the single culture *L. bulgaricus* and *S. thermophilus* during storage at 10°C

The viable cell counts of both cultures remained constant during the storage period, independent of the addition of LPO (Fig. 1). However, acid production by the single cultures in the presence of added LPO was clearly lower than that in controls (Fig. 2). These results imply that the addition of LPO suppressed the rate of acid production but not bacterial growth.

TABLE 1
Incubation Time of Cultures to Reach Given Acidities

	Incubation temp. °C	*Final acidity*, %	*Incubation time*, h	
			Control	*LPO treated*[a]
L. bulgaricus	30	0·6	17·92	20·92
S. thermophilus	30	0·5	15·92	15·67
Mixed culture[b]	41	0·8	3·25	3·18

[a] 5 mg kg^{-1} Lactoperoxidase.
[b] *L. bulgaricus:S. thermophilus* = 1:1.

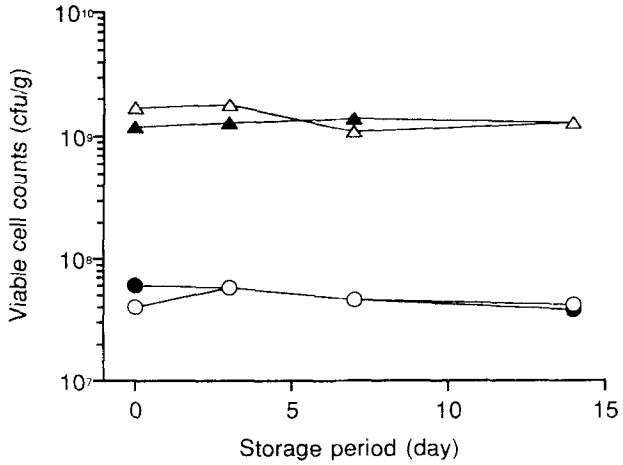

Fig. 1. Changes in viable cell counts of single cultures during storage at 10 C. *L. bulgaricus* with 5 mg kg^{-1} of LPO added (●) and control (○), and *S. thermophilus* with 5 mg kg^{-1} of LPO added (▲) and control (△).

Effect of LPO on the acidity and viable cell counts of the mixed culture of *L. bulgaricus* and *S. thermophilus* during storage at 10°C

Changes in the viable cell count of *L. bulgaricus* in the mixed culture, with or without added LPO, are shown in Fig. 3. The viable cell count of the control remained unchanged during storage at 10°C, whereas that of the culture containing LPO decreased to 5×10^7 cfu g^{-1} after 14 days. However, the viable cell count of *S. thermophilus* in the mixed culture containing added LPO was maintained at 5×10^8 cfu g^{-1}, which was the same as that of the control (data not shown). Therefore, the total viable cell counts exceeded 10^8 cfu g^{-1} and met the legal requirement (10^7 cfu g^{-1}).

The titratable acidity of the mixed culture free of LPO increased from 0.8 to 1.1% during storage at 10°C (Fig. 4), but the increase was strongly suppressed in the presence of LPO; suppression was not enhanced at higher LPO concentrations.

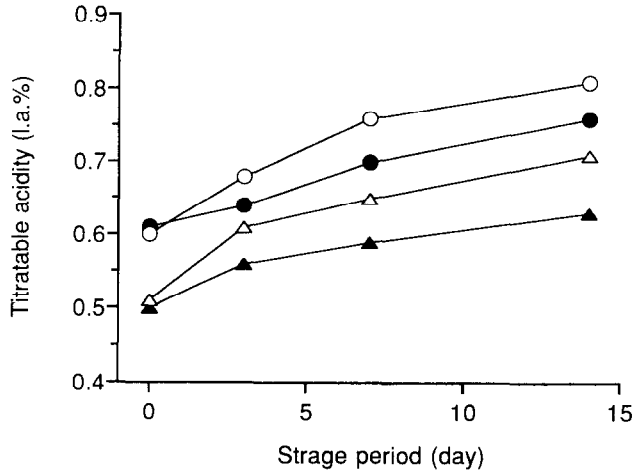

Fig. 2. Changes in titratable acidity (% lactic acid) of single cultures during storage at 10°C. *L. bulgaricus* with 5 mg kg^{-1} of LPO added (●) and control (○), and *S. thermophilus* with 5 mg kg^{-1} of LPO added (▲) and control (△).

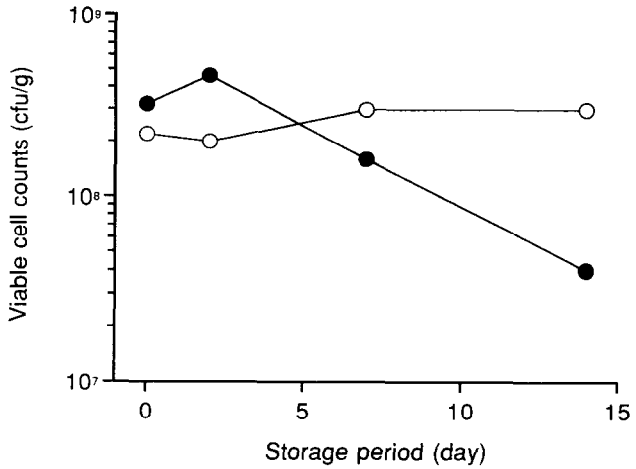

Fig. 3. Changes in viable cell counts of *L. bulgaricus* in a mixed (*L. bulgaricus* : *S. thermophilus* = 1:1) culture during storage at 10°C, and 5 mg kg^{-1} of LPO added (●) and control (○).

In a mixed culture, *L. bulgaricus* and *S. thermophilus* stimulate each other by the production of growth factors (Driessen, 1981). *S. thermophilus* is stimulated by free amino acids and peptides which *L. bulgaricus* liberates from milk proteins, while *L. bulgaricus* is stimulated by formic acid and/or CO_2 produced by *S. thermophilus* (Veringa et al., 1968; Driessen et al., 1982).

We determined the concentration of formic acid in the mixed culture, with or without added LPO. At day 8, the LPO-free culture contained 1.4 mg formic acid

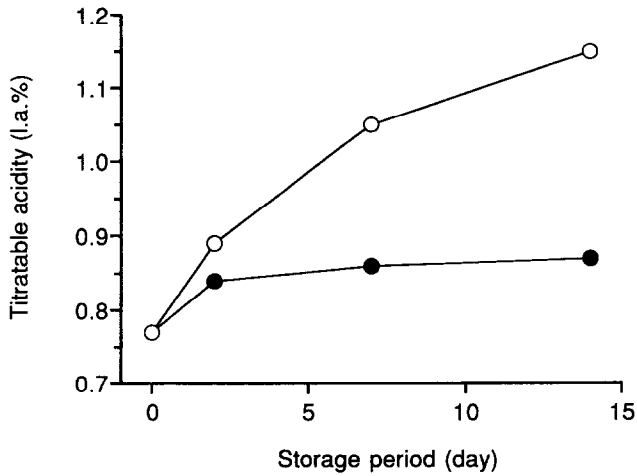

Fig. 4. Changes in titratable acidity (% lactic acid) of a mixed (*L. bulgaricus* : *S. thermophilus* = 1:1) culture during storage at 10°C, and 5 mg kg^{-1} of LPO added (●) and control (○).

$100g^{-1}$, whereas the LPO-treated culture contained only 0.1mg $100g^{-1}$. This indicated that the LPO system suppressed not only the formation of lactic acid but also the production of formic acid by *S. thermophilus*. Thus, the insufficient supply of formic acid from *S. thermophilus* probably retarded the growth inhibition of *L. bulgaricus* during storage at 10°C.

Concentration of hydrogen peroxide

Hydrogen peroxide is of particular importance for the LPO system. The concentrations of H_2O_2 in the *L. bulgaricus*, *S. thermophilus* and the mixed cultures at day 1 were found to be approximately 3, 1 and 5 mg kg^{-1}, respectively. In the cultures containing 5 mg kg^{-1} of LPO, H_2O_2 was not detected throughout the storage period, since LPO decomposed H_2O_2 produced by the lactic acid bacteria.

Concentration of thiocyanate

When the LPO system is activated, the concentration of thiocyanate (SCN^-) is expected to decrease, since it is converted to hypothiocyanate ($OSCN^-$) by the LPO system (Aune & Thomas, 1977). Changes in the concentration of thiocyanate in the *L. bulgaricus* culture during storage are shown in Fig. 5. While 8–8.5 mg kg^{-1} of thiocyanate were detected in the LPO-free culture, the culture with added LPO contained 7.6 mg kg^{-1} at day 0 and this value decreased during storage. The *S. thermophilus* single culture and the mixed culture showed the same tendency as the *L. bulgaricus* culture (data not shown).

The concentrations of thiocyanate in the yoghurts were similar to those in other reports (2–7mg kg^{-1})(IDF, 1988).

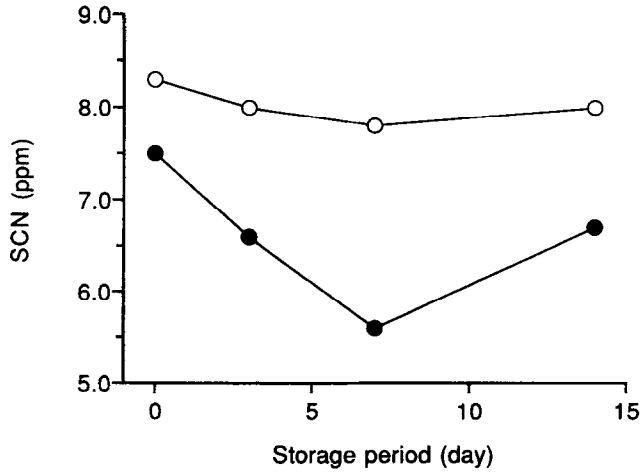

Fig. 5. Changes of thiocyanate (SCN^-) concentrations in the *L. bulgaricus* culture during storage at 10°C ; 5 mg kg^{-1} of LPO added (●) and control (○).

Activity of LPO

LPO activity decreased rapidly during incubation, down to 10% of the initial activity after 3 h at 41°C (Fig. 6). During storage at 10°C, LPO activity was found to be below the detection limit (0.6% of the initial activity) in the *L. bulgaricus* culture and the mixed culture, but in the *S. thermophilus* culture, a low level of LPO (10%) remained at day 14. High concentrations of H_2O_2 inactivate LPO irreversibely (Jenzer et al., 1986). We also found that LPO was inactivated at 5 mg

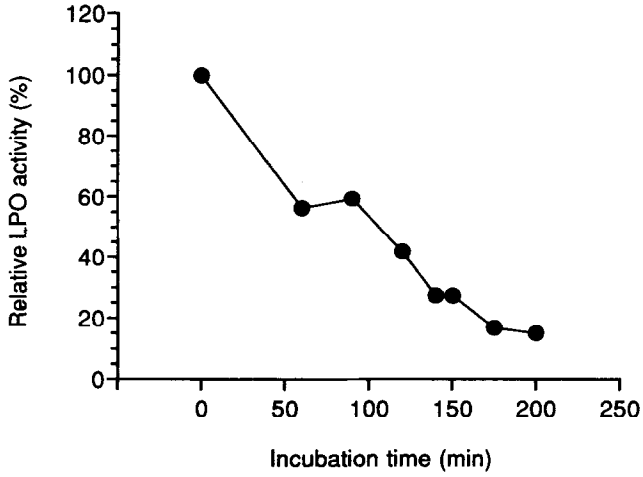

Fig. 6. Decrease in LPO activity in a mixed culture (*L. bulgaricus* : *S. thermophilus* = 1:1). During incubation at 41°C, LPO activity was measured by ELISA.

kg^{-1} H_2O_2 (data not shown). Thus, it is likely that the lower production of H_2O_2 in the *S. thermophilus* culture was responsible for the prolongation of LPO activity.

DISCUSSION

The LPO system is activated in the presence of thiocyanate and H_2O_2 (Reiter, 1985). The thiocyanate is then oxidized to hypothiocyanate which blocks glycolysis in bacteria by inhibiting metabolic enzymes such as glyceraldehyde 3-phosphate dehydrogenase (Carlsson et al., 1983) and hexokinase (Adamson & Pruitt, 1981).

However, the concentration of thiocyanate in milk (8–8.5 mg kg^{-1}, Fig. 5) is less than that required to exert an optimal antibacterial effect (12–15 mg kg^{-1})(IDF, 1988; Zall et al., 1983; de Valdez et al., 1988). Additionally, the decrease in the concentration of thiocyanate in the culture, by added LPO, was approximately 3 mg kg^{-1} at maximum (Fig. 5). Probably, this was due to the conversion of intracellular hypothiocyanate to harmless thiocyanate by the action of NADH-OSCN or NADPH-OSCN oxidoreductases (Carlsson et al., 1983). Thus, the bacteriostatic effect was not exerted, but acid production was partially inhibited.

While acid production was suppressed during storage at 10°C, acid production was not affected at 41°C. We speculate that the temperature dependence is attributable to the activity of the enzyme that converts intracellular hypothiocyanate to thiocyanate. At 41°C, the activity may be high enough to eliminate intracellular hypothiocyanate; thus, no appreciable changes in acid production occur. The activity of the enzyme decreases at low temperature; a small amount of hypothiocyanate remains within the cell, which partially inhibits acid production.

LPO activity decreased considerably during incubation at 41°C. However, residual activity seems to be sufficient to suppress acid production, since the thiocyanate level in the mixed culture continued to decrease up to day 7 (Fig. 5); H_2O_2 was not detected throughout the 14 day storage period; higher LPO concentration did not enhance the suppression (data not shown). It is thus likely that the suppression of acid production requires only a low level of LPO activity when incubation at 41°C is terminated at a desirable titratable acidity.

The increase in the acidity of yoghurt during storage under refrigeration, due to the production of lactic acid by viable lactic acid bacteria (so-called after-acidification), has been a major problem for many decades, since the yoghurt becomes sour during storage. Many attempts have been made to solve the problem, such as "heat shock" treatment (Waes, 1987), use of low-lactose skim milk (Richter et al., 1973), and application of a diffusion membrane-reactor system (Nakajima, 1990) or hydrostatic pressure (Tanaka & Hatanaka, 1992). However, none of these has been successful from a practical point of view.

The addition of LPO is, on the other hand, promising for the development of yoghurt that maintains a favourable balance between sweetness and sourness during storage for at least two weeks. Advantages over conventional yoghurt are: (1) nearly complete suppression of after-acidification; (2) the incubation time required to develop the necessary acidity during production is unaffected by the LPO system; (3) the total viable cell count was higher than 10^8 cfu g^{-1}, which

satisfies the legal requirement for yoghurt in many countries (Puhan & Zambrini, 1992). Although commercially available LPO is expensive, the increase in the cost of yoghurt associated with the addition of LPO is negligible, due to very low level of LPO required.

The LPO-treated yoghurt retains a fresh taste for at least two weeks and reduces sugar consumption for those who dislike sourness and add sugar to yoghurt.

REFERENCES

Adamson, M. & Pruitt, K. M. (1981). Lactoperoxidase-catalyzed inactivation of hexokinase. *Biochim. Biophys. Acta*, **658,** 238–47.

Aune, T. M. & Thomas, E. L. (1977). Accumulation of hypothiocyanite ion during peroxidase-catalysed oxidation of thiocyanate ion. *Eur. J. Biochem.*, **80,** 209–14.

Björck, L. (1982). Activation of the lactoperoxidase system as a means of preventing bacterial deterioration of raw milk. *Kieler Milchwirtschaftliche Forchungsberichte*, **34,** 5–11.

Björck, L., Claesson, O. & Schulthers, W. (1979). The lactoperoxidase/ thiocyanate/H_2O_2 system as a temporary preservative for raw milk in developing countries. *Milchwissenschaft*, **34,** 726–29.

Carlsson, J., Iwami, Y. & Yamada, T. (1983). H_2O_2 excretion by oral streptococci and effect of lactoperoxidase-thiocyanate-H_2O_2. *Infect. Immun.*, **40,** 70–80.

IDF (1988). Code of practice for the preservation of raw milk by the lactoperoxidase system. (1988). Bulletin 234, International Dairy Federation, Brussels, pp. 2–14.

de Valdez, G. F., Bibi, W. & Bachmann, M. R. (1988). Antibacterial effect of the lactoperoxidase/thiocyanate/H_2O_2 (LP) system on the activity of thermophilic starter culture. *Milchwissenshaft*, **43,** 350–2.

Driessen, F. M. (1981). Modern trends in the manufacture of yoghurt. Bulletin 179, International Dairy Federation, Brussels, pp. 107–15.

Driessen, F. M., Kingma, F. & Stadhouders, J. (1982). Evidence that *Lactobacillus bulgaricus* in yoghurt is stimulated by carbon dioxide produced *Streptococcus thermophilus*. *Neth. Milk Dairy J.*, **36,** 135–44.

IDF (1985). Protective proteins in milk-biological significance and exploitation. Bulletin 191, International Dairy Federation, Brussels, pp. 2–35.

Jenzer, H., Jones, W. & Kohler, H. (1986). On the molecular mechanism of lactoperoxidase-catalysed H_2O_2 metabolism and irreversible enzyme inactivation. *J. Biol. Chem.*, **261,** 15550–6.

Kishi, S. (1993). Agents for manufacture, In *Syokuhin-Tenkabutsu-Binran*, eds. Syokuhin to Kagaku-sya, Osaka, pp. 331–402.

Kumar, S. & Mathur, B. N. (1989). Studies on the manufacture of yoghurt and Mozzarella cheese from milk preserved by LP-system. *Indian J. Dairy Sci.*, **42,** 194–7.

Mehanna, N. M. & Hefnawy, SH. A. (1988). Effect of thiocyanate-lactoperoxidase-H_2O_2 system on the manufacture and properties of yoghurt. *Egyptian J. Dairy Sci.*, **16,** 55–63.

Nakajima, M. (1990). New movement of bioreactor and membrane technique in Europe, *The Food Industry*, **33** (12), 31–7.

Namba, K. (1990). Compositional standards and standards of manufacturing and storing methods of milk products, In *Ministerial Ordinance Concerning Compositional Standards, Etc. for milk and milk products*, eds. Japan Food Hygiene Association, Tokyo, pp. 33–41.

Puhan, Z. & Zambrini, A. V. (1992). Overview of current availability and technology of fermented milks in IDF member countries. Bulltein 277, International Dairy Federation, Brussels, pp. 22–7.

Reiter, B. (1985). The biological significance of lactoperoxidase in milk. *Protides Biol. Fluids*, **32,** 111–4.

Richter, R. L., Reineccius, G. A. & McKay, L. L. (1973). Acid production by *Streptococcus lactis* in low-lactose skim milk. *J. Food Sci.*, **38,** 796–8.

Sato, K., Dosako, S., Nakajima, I. & Ido, K. (1992). Effects of ionic strength on thermostability of lactoperoxidase. *Biosci. Biotech. Biochem.*, **56,** 2054–5.

Sievers, G. (1981). Structure of milk lactoperoxidase. Evidence for a single polypeptide chain. *FEBS Lett.*, **127,** 253–6.

Tanaka, T. & Hatanaka, K. (1992). Application of hydrostatic pressure to yoghurt to prevent its after-acidification. *Nippon Shokuhin Kogyo Gakkaishi.*, **39,** 173–7.

Veringa, H. A., Galesloot, T. E. & Davelaar, H. (1968). Symbiosis and identification of a growth factor for *Lactobacillus bulgaricus* produced by *Streptococcus thermophilus*. *Neth. Milk Dairy J.*, **22,** 114–20.

Waes, G. (1987). Application of "heat shock" treatment on set yoghurt. *Milchwissenschaft*, **42,** 146–8.

Zall, R. R., Chen, J. H. & Dzurec, D. J. (1983). Effect of thiocyanate and H_2O_2 in cultured products. *Milchwissenschaft*, **38,** 264–6.