

## Use of Chemical & Bio-protective Factors for Preservation of Raw Milk

### Introduction

- Milk is an easily perishable raw material.
- Contaminating bacteria may multiply rapidly and render it unsuitable for processing and/or unfit for human consumption.
- Bacterial growth can be retarded by refrigeration and/or heat treatment thereby slowing down the rate of deterioration.
- Under certain conditions refrigeration may not be feasible due to economical and/or technical reasons.
- In India milk is produced in small quantities (2-4) liters by approx. 70 million farmers spread around 5 lakh villages
- Difficulties in applying refrigeration are specially a problem for certain areas in the country for setting up or expanding milk production.
- In these situations, it would be beneficial to have access to a method, other than refrigeration, for retarding bacterial growth in raw milk during collection and transportation to the dairy processing plant.

### Preservation of Milk with $H_2O_2$

- Initially some chemicals e.g., Mercuric chloride, Formalin, Potassium dichromate were tried
- In 1967 the FAO/WHO Expert Panel on Milk Quality concluded that the use of hydrogen peroxide might be an acceptable alternative in the early stages of development of an organized dairy industry
- $H_2O_2$  is a strong oxidizing, bleaching and germicidal agent
- Concentration of  $H_2O_2$  ranging between 0.02-0.50% resulted in preservation of milk for a very short period
- Addition of  $H_2O_2$  @ 1% (w/v) preserved freshness of raw milk upto 12-24 hours at room temperature
- Storage of milk at 5°C extended upto 100days

### Preservative action of $H_2O_2$

- Antimicrobial action of  $H_2O_2$  is not due to its oxidative properties as a molecule
- Production of other powerful oxidants e.g., singlet oxygen, superoxide radicals, and hydroxyl (OH) radical
- The reactive oxygen species cause irreversible damage to a host of cell components such as enzymes, membrane constituents & DNA
- OH<sup>-</sup> plays the largest role in the toxicity of  $H_2O_2$
- When produced adjacent to the DNA, OH radicals are unique in that they can both add to the DNA bases & abstract H-atoms from the DNA helix
- OH radicals also damage cell membranes – by increasing lipid peroxidation as well and ion permeability
- Effective against: *S. aureus*, *E. Coli*, *Bacillus subtilis*, *A. Niger*, *C. parapsilosis*

### ***Requirements of chemical preservative***

- No effect on organoleptic quality
- No reaction with the milk constituents
- Eliminated before consumption
- No toxic effect

### **Removal of residual $H_2O_2$**

- Could be removed by heat treatment or by addition of a catalase enzyme

### **Influence of $H_2O_2$ on milk flavour**

- High concentration impart 'metallic' and/or 'utensil' flavour to milk
- Milk containing high amount of undecomposed  $H_2O_2$  developed oxidized flavour

### Influence of $H_2O_2$ on proteins

- In higher concentration  $H_2O_2$  oxidizes proteins especially  $\beta$ -lactoglobulin, casein, and some enzymes *e.g.*, pepsin, trypsin and chymotrypsin
- Addition of  $H_2O_2 \geq 0.02\%$  reduce rennet coagulation properties and thus produce soft curd
- $H_2O_2$  causes complete breakdown of  $\beta$ -lactoglobulin
- Some essential and non-essential amino acids *e.g.*, tyrosine, tryptophan, cysteine, cystine, lysine and methionine are very sensitive to  $H_2O_2$

### Influence of $H_2O_2$ on fat

- It increases the oxidative rancidity of fat rich dairy products

### Other Issues of using $H_2O_2$

- Difficulty of controlling its use
- It may be misused to disguise milk of basic hygienic quality produced under poor hygienic conditions.
- The toxicological aspects of the use of relatively high concentrations of hydrogen peroxide in milk have also been questioned.

### Natural preservatives in milk

- Immunoglobulins
- Lactoferrin
- Lysozyme

### Immunoglobulins

- Antibodies synthesized in response to stimulation by specific antigens
- Offers non-specific humoral immunity response to Gram –ve enteric & respiratory bacteria

Name	Types	Description
IgA	2	Prevents colonization of bacteria
IgD	1	Activate basophils and mast cells to produce antimicrobial factors
IgE	1	Binds to allergens and triggers histamine release from cells and basophiles that involved in allergenic reactions
IgG	4	Provides the majority of antibody-based immunity against invading pathogens. The only antibody capable of crossing the placenta to give passive immunity to fetus.
IgM	1	Eliminates pathogens in the early stages of B cell mediated (humoral) immunity

- Buffalo colostrum: 76.2  $\mu\text{g}$ / 100 g whey protein, Buffalo milk: 43.4  $\mu\text{g}$ /100g WP
- Cow colostrum: 40.1  $\mu\text{g}$ / 100 g whey protein, Cow milk: 0.45 $\mu\text{g}$ /100g WP
- Confers a high degree of immunity as antibodies especially to the neonates

## Lactoferrin

- Lactoferrin is a Fe-binding globular glycoprotein is one of the components of the immune system of the body
- It has antimicrobial activity e.g., bacteriostatic, bacteriocidal and fungicide
- Antimicrobial activity results from its unsaturated Fe character, as it is only 5-30% saturated with Fe and removes Fe from the microbes
- Shown to inhibit the grown of food borne pathogens e.g., *E. coli* & *S. aureus*

Product	mg/ml
Cow milk	0.10
Cow colostrum	0.70
Buffalo coloustrum	0.75
Buffalo milk	0.32
Human milk	10.0
Human colostrum	1.0

## Lysozyme

- Lysozyme, also known as muramidase or N-acetylmuramide glycanhydrolase, are glycoside hydrolase enzymes
- It damages bacterial cell walls by catalyzing hydrolysis of 1,4- $\beta$ -linkages between N- acetylmuramic acid & N-acetyl-D-glucosamine in a peptidoglycan and between N-acetyl-D-glucosamines in chitodextrins
- Lysozyme is abundant in a number of secretions e.g., tears, saliva, human milk and mucus

## Lysozyme

- Bovine milk: 13 µg/100ml
- Human milk 10-39 mg/100ml (3000 times more activity than bovine milk)
- Egg albumin is the richest source
- Lysozyme is a natural form of protection from pathogens like *Salmonella*, *E.coli*, and *Pseudomonas*
- Egg white lysozyme is used to prevent “late blowing of cheese” because it inhibits the growth of *Clostridium tyrobutyricum* spores

## Effect of Pasteurization on antimicrobial components of milk

Milk Component	Role in Milk	Effect of Pasteurization
Bovine immunoglobulins	Transfers immunity against bovine pathogens to calves; may provide some lactogenic immunity to the gut. Most immunoglobulins are carried in colostrum	No loss in activity during batch pasteurization (62.7°C/30min), retains 60-70% activity after HTST pasteurization
Lactoferrin	An Fe-binding protein, scavenger of Fe, thereby providing anti-bacterial effects by limiting the availability of free Fe required for bacterial proliferation	Unheated and pasteurized lactoferrin have similar antibacterial properties
Lysozyme	Active primarily against Gram +ve bacteria. In conjunction with lactoferrin has bactericidal effects	Greater than 75% of activity retained after heating at 80°C/15 sec

### Limitations of Practical Application of Natural Preservatives

- None of the three natural preservatives could be used in practical conditions for mass preservation of milk:
- Present in very small quantities in milk
- Unavailability in large quantities
- Expensive fractionation methods
- Specific against bacterial action
- Ineffective when at high bacterial load
  
- During the last decades research was concentrated to combine chemical preservation with naturally occurring bio-protective factors
- Lactoperoxidase/ thiocyanate/ hydrogen peroxide system (LP-s)

### Introduction

- An alternative method for preserving milk would still be of great advantage in certain situations. The search for such a method has therefore continued.
- Interest was focused on combining an indigenous antibacterial systems with very low dose use of chemicals to determine if these could be applied practically to preserve raw milk.
- During the last decade research has demonstrated that one of these systems, the **lactoperoxidase/ thiocyanate/ hydrogen peroxide system (LP-system)** can be used successfully for this purpose.



### Three components of LP-system

1. Lactoperoxidase enzyme
  - Lactoperoxidase is an enzyme which is found in most mammalian tissues
  - It is present in human saliva and also in bovine and buffalo milk in relatively high concentrations.
  - The average values of Lactoperoxidase content:
    - Cow milk 0.452 U/ml
    - Buffalo milk 0.90 U/ml
  - These values are 50-100 times above than what is required to operate the LP-system
  - Lactoperoxidase is quite heat stable

### Three components of LP-system

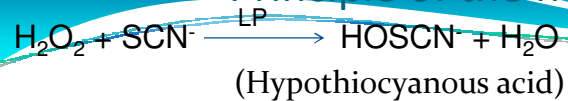
2. Thiocyanate ( $\text{SCN}^-$ )
  - Some thiocyanate is present in milk although the level is variable i.e., 1-15 mg/l (5 ppm on average)
  - Thiocyanate content (ppm)
    - Beetroot 100
    - Cabbage 88.5
    - Cauliflower 31.5
    - Spinach 5
    - Tomatoes 2
    - Beans 1.4

### Three components of LP system

#### 3. Hydrogen peroxide ( $\text{H}_2\text{O}_2$ )

- Present in milk is very small concentration
- There is evidence of in vivo production of  $\text{H}_2\text{O}_2$  by LAB:
  - *L. bulgaricus* 6-12 ppm
  - *L. Lactis* 6-12 ppm
  - *L. plantarum* 3-13 ppm
  - *L. acidophilus* 55 ppm

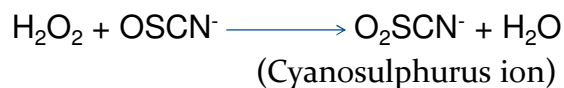
### Principle of the Method



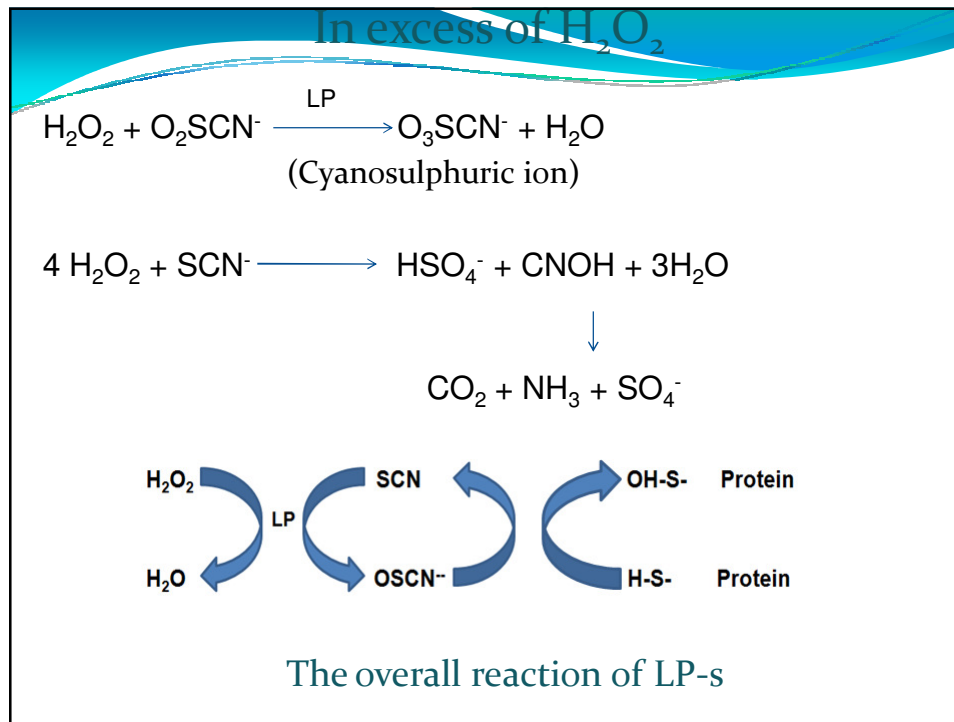
At milk pH (6.5-6.8 dissociates) ↓



(Hypothiocyanate ions)



- These agents react specifically with free sulphhydryl groups, thereby inactivating several vital metabolic bacterial enzymes (e.g., hexocinase, glyceraldehyde-3-phosphate dehydrogenase) consequently blocking their metabolism and ability to multiply.



### LP- s basics

- As milk proteins contain very few sulphhydryl groups and those that are present are relatively inaccessible to OSCN<sup>-</sup> (masked), the reaction of this compound is in milk quite specific and is directed against the bacteria present in the milk.
- End products of the intermediates are sulphate, ammonium ions and carbon dioxide
- At higher levels of  $H_2O_2$ , LP gets inactivated and bactericidal effect is due to toxicity of  $H_2O_2$  itself

### LP- s basics

- The antibacterial effect of the LP-system is proportional to the thiocyanate concentration in the milk
- The activation of the lactoperoxidase has a bacteriostatic effect on the raw milk and effectively extends the shelf life of raw milk for 7–8 hours under ambient temperatures of around 30°C or longer at lower temperatures.
- This allows adequate time for the milk to be transported from the collection point to a processing centre without refrigeration.

- The antibacterial oxidation products of thiocyanate are not stable at neutral pH. Any surplus of these Decomposes spontaneously to thiocyanate.
- The velocity of this reaction is temperature dependent, i.e. more rapid at higher temperatures. Pasteurisation of the milk will ensure a complete removal of any residual concentrations of the active oxidation products.
- Effect against bacteria is species & strain dependent. Against mixed raw milk flora dominated by mesophilic bacteria – bacteriostatic; Against some Gram –ve bacteria (*pseudomonas*, *E. Coli*) bacteriocidal
- Due to the mainly bacteriostatic effect of the system it is not possible to disguise poor quality milk, which originally contained a high bacterial population, by applying this method

### Practical Application of the Method (as prescribed by CAC)

- 14 mg of NaSCN is added per litre of milk. The milk should then be mixed to ensure an even distribution of the SCN-. Plunging for about 1 minute with a clean plunger is normally satisfactory.
- This causes increase the overall level of thiocyanate to 15 ppm (around 5 ppm is naturally present).
- Secondly, 30 mg of sodium percarbonate ( $2\text{Na}_2\text{CO}_3 \cdot 3\text{H}_2\text{O}_2$ ) is added per litre of milk. The milk is then stirred for another 2-3 minutes to ensure that the sodium percarbonate is completely dissolved and the hydrogen peroxide is evenly distributed in the milk
- This provides an equimolar amount (8.5 ppm) of hydrogen peroxide in the system
- It is essential that the sodium thiocyanate and sodium percarbonate are added in the order stated above.

### Practical Application of the Method

The enzymatic reaction is started in the milk when the hydrogen peroxide (sodium percarbonate) is added. It is completed within about 5 minutes from the addition of  $\text{H}_2\text{O}_2$ ; thereafter, no hydrogen peroxide is present in the milk.

- The activation of the lactoperoxidase system should be carried out within 2-3 hours from the time of milking.
- Quantities of sodium thiocyanate and sodium percarbonate needed for the treatment of a certain volume of milk, for example 40 or 50 litre, should be distributed to the collecting centre/point in prepacked amounts lasting for a few weeks at a time.

### Effect of LP-s on Acidity Development

Interval after milking (hr)	TA (%LA)	
	Control	LP-treated
4	0.128	0.126
5	0.135	0.126
6	0.135	0.126
7	0.135	0.128
8	0.137	0.128
9	0.160 (Sl. Sour)	0.122
10		0.122
11		0.122
12		0.153 (Sl. Sour)

- Extension of three hours in keeping quality achieved

### Studies on use of LP-s

- Cows milked at 4-6 AM; samples stabilized at 7:30-8:00 AM; Temperature 30-32°C

Test	Treat ment	Percentage sample acceptance at				
		10 AM	12 AM	2 PM	4PM	6PM
Ten min resazurin	LP	100	100	70	50	30
	C	80	60	10	0	0
Acidity*	LP	100	100	80	60	50
	C	70	60	20	0	0
Alcohol stability	LP	100	100	90	60	50
	C	70	60	30	10	10
COB	LP	100	100	100	100	80
	C	100	100	90	30	30

\*Cut off  $\geq 0.16\%$  LA

### Microbiological Effects & Performance of LP-s

Effectiveness of LP-s depends on three factors:  
storage temperatures of milk, bacterial cell density  
& type of organism

Temperature (°C)	Time (hrs)
31-35	4-7
30	7-8
25	11-12
20	16-17
15	24-26
4	5-6 days

- LP-s persists only for limited time-period which decreases as the ambient temperature increases
- LP-s relevant to low temperature storage due to the growth of *Pseudomonas* spp. & *Lysteria monocytogenes*
- The efficacy is low at high bacterial concentrations

### Performance of LP-s at Different bacterial loads

- The efficacy is low at high bacterial concentrations
- NaSCN : 15 ppm, H<sub>2</sub>O<sub>2</sub> 10 ppm
- Addition after 3 hrs of milking
- Incubation temperature 37°C

Bacterial count (SPC/ml)	MBR Time (hrs)
$6.7 \times 10^6$	8.5
$6.4 \times 10^7$	5.0
$8.2 \times 10^7$	3.0

## Microbiological Effects & Performance of LP-s

- Antimicrobial activity of LP-s has been demonstrated against a wide range of microorganisms, e.g., bacteria, HIV-1 virus, moulds, yeasts, mycoplasma & protozoa
- Depending upon the bacterial species or even the strain of the microorganism the effect can be bacteriostatic or bactericidal
- LAB: sometimes self-inhibitory, Catalase +ve are not

## Impact of LP-s on Some Common Milk-borne Pathogens

Pathogen	Effect of LP-s	Demonstrated in
<i>E. Coli</i> including <i>E. Coli</i> 0157:H	Bactericidal  Reduced gastrointestinal colonization rate of coliform bacteria	Raw cow milk  Raw cow, goat & camel milk, culture medium and infant formula
<i>Salmonella typhimurium</i>  <i>Salmonella typhimurium</i> & other <i>Salmonella</i> spp.	Bactericidal & Bactriostatic (depending on No. of mos)  Bactericidal	Raw milk  Culture medium, infant formula and fresh cheese
<i>Camphylobactor jejuni</i>	Bactericidal	Cow milk
<i>Staphylococcus aureus</i>	Bactericidal & Bactriostatic	Cow, goat & camel milk
<i>Lysteria monocytogenous</i>	Bactericidal & Bactriostatic (Depending on temp. , time & strain)	Raw cow & goat milk, UHT milk, soft cheese
<i>Yersinia enterocolitica</i>	Bactericidal	Cow milk
<i>Brucella melitensis</i>	Bactericidal	Goat milk



### Health Issues Associates with the use of LP-s

- The levels of  $H_2O_2$  introduced into the milk via sodium percarbonate is very low and not of concern
- As there is no change to the enzyme concentrations naturally present in milk, this component is not considered of toxicological significance
- Thiocyanate interferes with iodine metabolism and uptake by the thyroid, especially in Iodine deficient subjects hence a potential goitrogenic substance

### Effect of LP-s on serum thiocyanate level

- 400 ml milk daily containing 20 ppm NaSCN (equivalent to 8 mg of thiocyanate daily)

Time interval (weeks)	Serum thiocyanate level (mg/L) [Non-smokers]	Serum thiocyanate level (mg/L) [Smokers]
0	4.0	8.4
4	7.8*	10.7
8	6.9	10.1
12	7.0*	8.9
*Significant increase over initial		
Level >18 cause impaired thyroid function		

### Effect of LP-s on Milk Processing & Technology

- No effect on sensorial attributes of milk
- The flavour of fermented goat's milk & cheese improves as a result of action of lactoperoxidase
- Interaction of lactoperoxidase with SH groups of proteins could alter the texture of gelled products
- Evidence of the last phenomena is mixed
- Some evidence of slower rennet clotting and weaker gels in cheese, and lower acid production in yoghurt
- If the LP-s treated milk has been subjected to adequate heat-treatment no effects on the quality of cheese and fermented milk products

### Effect of LP-s on Milk Processing & Technology

- LP-s has a clear potential for inhibition of lactic starter
- Susceptibility of lactic acid starter bacteria can be categorized into three groups as follows:
  1. The most sensitive group of organisms which generate hydrogen peroxide, e.g., *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*
  2. Organisms that are sensitive but do not have the ability to generate hydrogen peroxide and thus require an exogenous source of hydrogen peroxide e.g., *Lactobacillus helveticus*, *S. thermophilus*
  3. Organisms resistant to inhibition e.g., *Lactococcus lactis*.

### Intended Utilization of the Method

The method should primarily be used to **prevent undue bacterial multiplication in raw milk** during collection and transportation to the dairy processing plant

- This method should only be used in situations when **technical, economical and/or practical reasons do not allow the use of cooling** facilities for maintaining the quality of raw milk.
- Use of the LP-system in areas which currently **lack an adequate infrastructure for collection of liquid milk**, would ensure the production of milk as a safe and wholesome food, which otherwise would be virtually impossible.
- The **method should not be used by the individual milk producer** but at a suitable collecting point/centre. These centres must be equipped with proper facilities for cleaning and sanitizing the vessels used to hold and transport milk.

### Current status of use of LP-s

Current worldwide milk output 660 MMT (approx.)

- Around 40% is contributed by developing world – with small dairy farmers contributing about 70% of the total
- Though the cost of milk production is low in developing countries they are net milk importers
- The World Bank estimates that 20% of milk in developing countries is wasted due to high capital investment in refrigeration and can incur high running and maintenance costs
- Use of LP-s is a reliable & economical method of preserving raw milk as compared to refrigeration in small-scale dairy enterprises, coupled with good hygiene and sanitation.

### Using LP-s for preserving evening milk

- Five times increase in SCN & H<sub>2</sub>O<sub>2</sub> (75:50 ppm) increase the keeping quality of raw milk up to 15 hrs
- Upon addition of second dose of H<sub>2</sub>O<sub>2</sub> on the 10<sup>th</sup> hr the keeping quality was increased up to 18 hrs
- The 18 hrs shelf life would permit the collection of evening milk with LP treatment along with untreated morning milk
- Mixing of these two milks at the dairy would bring down the SCN level to the CAC recommended level 15ppm

### Cuba & Latin America Example

- In Cuba, more than 50% of the milk is not refrigerated due to high cost of cooling equipment and lack of electricity
- Use of the LP-s has allowed significant quantities (50,000 MT) of milk, valued at US\$100m (over 13 yrs), which would otherwise have been lost, to enter the food chain
- In Latin America, 30m litres of milk was activated using the LP-s in last five years
- Fifty percent of the milk that would otherwise be lost is saved through the LP-s, amounting to a value of around US\$3 m

### Current status

- There is a codex provision that the LP-s should not be used for products intended for international trade
- The LP-s is not adopted in the first place in some countries because of a fear of being excluded from international markets.
- If products treated with the LP-s are not considered suitable for international trade then this raises doubts as to whether it is appropriate and safe to use for milk and dairy products in the domestic market

### Indian Scenario

- NaSCN : 15 ppm, H<sub>2</sub>O<sub>2</sub> 10 ppm, after 3 hrs of milking, incubation temperature 37°C
- Samples were taken after one hour of activation of the LP-s

Trial No.	SPC/ml		MBRT (hrs)	
	Control	Treated	Control	Treated
1	6.8 x 10 <sup>6</sup>	4.8 x 10 <sup>5</sup>	6.50	8.5
2	6.4 x 10 <sup>7</sup>	9.5 x 10 <sup>5</sup>	3.50	5.0
3	8.2 x 10 <sup>7</sup>	2.2 x 10 <sup>7</sup>	1.75	3.0
4	1.4 x 10 <sup>7</sup>	7.5 x 10 <sup>6</sup>	2.0	4.5
Average	2.6 x 10 <sup>7</sup>	2.9 x 10 <sup>6</sup>	3.25	5.25

## Indian Scenario

- LP-s have not been approved:
  - Dairy development policy is based on “Clean milk production”
  - Monitoring is a problem
  - Confused with pure chemical preservation
  - Concern about SCN
  - Long term implication – development of LP-s or anti body resistance stain
- In India use of any chemical preservative for raw milk to pre processed/used for human consumption is not permitted