RESEARCH

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Psychrophiles: Defects triggering organisms in pasteurized milk

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General Note
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ABSTRACT

A study was conducted to determine microbial activity in pasteurized milk under prolonged storage at 4°C temperature for 20 days. Psychrophilic count, mesophilic count, coliform count, yeast and mold count along with MBRT tests were conducted each day at the interval of 24 hours. Each day physical defects and sensory evaluation has been also examined. Psychrophiles rose abundantly and grow at very faster rate after 6th day and reached up to 7 crores after 20 days upon storage of pasteurized milk at 4°C temperature. Coliform, Total Mesophilic count and Total fungal isolates were also affected at 4°C temperature, as they multiplies at very slow rate due to slow functioning of enzymatic and metabolic regulations. MBRT time was affected by microbial activity, on 0 day MBRT was 96 hours which reduced up to 5:15 hours on 20th day. Pure, partial or combined influence of total microbial load at 4°C temperature in pasteurized milk leads to development of numerous defects. The dominant defects examined were off flavours like fruity, stale, bitter, rancid and putrid developed progressively along with physical defects including ropiness and curd formation upon prolonged incubation of pasteurized milk at 4°C temperature.

Key words: Pasteurized Milk, Psychrophilic bacteria, Microbial activity, Defects development.

Abbreviations: C-Centigrade, MBRT- Methylene Blue Reduction Test, O-R- Oxidation Reduction, CFU-Colony Forming Unit, ml- Milliliter.

1. INTRODUCTION

In the early days of milk production, the little cooling practices were accomplished with water (Barber et al.1942). Progressively with improvement in cooling system and over all dairy practices, the system came in to force to castoff the raw milk delivered with perceptible defects. Now days the innovative protocols

for chilling of milk attended by bulk coolers and clean milk production practices. In current years majority of dairy industries smearing GMP protocols, the workforces are particularly trained for better quality of raw and pasteurized milk production. Cold chain is sustained by work forces during transportation and distribution of milk and milk products. Although protracted cooling of milk may raise ropiness in milk. This ropiness and some other stowing defects such as fruity flavour, bitter taste is accredited to psychrophilic bacteria announced after pasteurization process. Any considerable microbial count after the refrigerator storage designates the presence of psychrophilic bacteria that may diminish shelf life of the milk. Numerous types of psychrophilic bacteria are imperative in milk. Conceivably the most notorious are those that produce fruity odour and ropiness. These defects develop from the growth of organisms which can be determined by three factors: 1) The dominant type or types of organisms. 2) The temperature of incubation. 3) Competition or lack of competition among the organisms (Barber et al.1942). Psychrophiles are extremophiles those are susceptible for the growth and reproduction in cold temperatures ranging from 5 to 10°C.They can be contrasted with thermophiles, which thrives at bizarrely hot temperatures. The environments in which Psychrophiles dwell are abundant on earth, as a large fraction of our planetary surfaces. It has been recommended that the term" Psychrotrophic" be applied to those organisms able to grow relatively rapidly at 7° C and below (Hubble et al. 1960). Species of *Pseudomonas, Flavobacterium, Alcaligenes, Acinetobacter* and *Bacillus* are often countered among Psychrotrophic bacteria (Mossel et al. 1960).

2. SCOPE OF THE STUDY

The objective of carrying out this study is to enumerate psychrophilic bacteria, their protagonist possessions in defects development and Influence of less temperature (4°C±0.5°C) on O-R potential (Eh value)of methylene blue dye in pasteurized milk under lengthy incubation at 4°C±0.5°C temperature. An effort was also made to study the impact of less temperature (4°C±0.5°C) on the growth of Coliform, Total Mesophilic count and Total fungal load along with undesirable physical changes in pasteurized milk under refrigerated conditions. The study was undertaken specifically at 4°C±0.5 °C, as in India mostly the cold chain is maintaining at 4 to 5 °C temperature for pasteurized milk pouches from milk producers to milk consumers.

3. MATERIALS AND APPARATUS

- Sterile Petridishes (Dishes-Culture, Petri, BOROSIL).
- Sterile 2.2 ml Pipettes (Graduated BOROSIL, 7056).
- Violet Red Bile Agar(M049, Himedia):- Prepared according to Supplier's direction.
- Potato Dextrose Agar(M096, Himedia):-Prepared according to Supplier's direction.
- Standard Plate Count Agar (M091A, Himedia):-Prepared according to Supplier's direction.
- Standard tubes for Methylene Blue Reduction Test (Tubes, 15 X 150, 9800, BOROSIL)
- Phosphate Buffer: The buffer was prepared by dissolving 34 grams of Potassium Dihydrogen Orthophosphate (Qualigens, 13405) in 1000 ml of distilled water with final pH 7.2 used as the stock phosphate buffer and auxiliary diluted it by taking 1.25 ml of stock solution of phosphate buffer in 1000 ml of distilled water in volumetric flask (Flask Volumetric,BOROSIL,5640).9.2 ml of similar buffer distributed in dry test tubes (25 x 150), stoppered with non-absorbent cotton, followed by autoclaved at 121 °C for 15 lbs for 15 minutes.
- Methylene Blue Dye: The Dye solution was set according to supplier's direction (PROLABO, 330733). The methylene blue tablet (1 No.) crushed by mortar and pestle under aseptic condition followed by addition of dye powder in 900ml sterile distilled water. The dye solution was placed in amber coloured bottle for overnight in a dark and cool place.

Table 1 Results

Day	MBRT (Hours)	Total Psychrophilic Count (cfu/ml)	Coliform Count (cfu/ml)	Total Mesophilic Count (cfu/ml)	Total Yeast & Mold Count (cfu/ml)	Sensory Analysis/ Organoleptic Taste
0 Day	96	4,00	0	9,000	0	Fresh
1st Day	97	5,00	0	10,000	0	Fresh
2nd Day	94	5,00	0	12,000	0	Fresh
3rd Day	93	6,00	0	14,000	2	Fresh
4th Day	90	8,00	0	17,000	3	Fresh
5th Day	82	9,00	2	19,000	5	Fresh
6th Day	72	1,100	3	13,000	4	No Freshness
7th Day	70	11,100	7	21,000	3	Slight Acidic
8th Day	67	22,300	9	31,000	7	Slight Acidic
9th Day	58	34,000	11	33,000	11	Acidic + Ropiness
10th Day	39	81,000	23	36,000	9	Fruity+ Ropiness + Curd
11th Day	30	1,09,000	60	41,000	8	Fruity+ Ropiness + Curd
12th Day	21	2,77,000	110	83,000	10	Fruity+ Ropiness + Curd
13th Day	18	5,90,000	140	1,02,000	12	Fruity+Stale+ Ropiness + Curd
14th Day	11	11,50,000	330	2,03,000	16	Fruity+Stale+Bitter+ Rancid Putrid + Ropiness + Curd
15th Day	9	26,90,000	540	7,00,000	21	Fruity+Stale+Bitter+ Rancid+ Putrid + Ropiness+ Curd
16th Day	7	50,00,000	720	5,80,000	27	Fruity+Stale+Bitter+ Rancid+ Putrid + Ropiness+ Curd
17th Day	6	1,20,00,000	880	10,00,000	31	Fruity+Stale+Bitter+ Rancid+ Putrid + Ropiness+ Curd
18th Day	5:30	3,00,00,000	910	26,00,000	47	Fruity+Stale+Bitter+ Rancid + Putrid + Ropiness+ Curd
19th Day	5:00	5,30,00,000	1,020	40,00,000	89	Intolerable foul smell and Taste + Highly Ropiness + Curd
20th Day	5:15	7,00,00,000	1,500	83,00,000	110	Intolerable foul smell and Taste+ Highly Ropiness + Curd

Figure 1
Storage of Milk Pouches at 4ºC for 20 days

Figure 2
Methylene Blue Reduction Test for Psychrophiles (Initial & After)

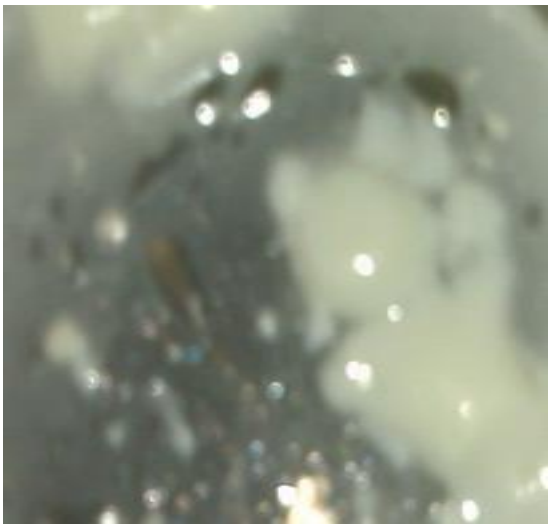


Figure 3
Physical Defects (Curd formation after 10th Day)

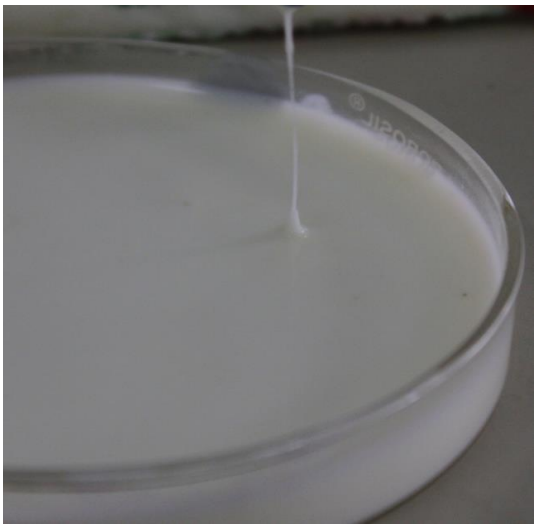


Figure 4
Physical Defect (Ropiness in Milk)

4. METHODOLOGY

4.1. Collection and sampling of Pasteurized Milk Samples

A total 20 pouches of Standard Pasteurized Milk(Fat:4.5 %, SNF:8.5 %, Pack Size:-200ml) of one specific lot was nominated for the study purpose from the cold storage(Temperature 5.0ºC) of Vidya Dairy, Anand, Gujarat, India. Samples were brought to microbiological laboratory for evaluation of microbial quality characteristics under protracted holding of pasteurized milk at 4ºC±0.5ºCtemperature. The samples were kept in adistinctive type of refrigerator (Figure 1), and removed for a few minutes each day for sampling at the interval of 24 hours. The samples were examined each day for different parameters including psychrophilic count, Total Mesophilic count, Coliform count, Total fungal count, Methylene Blue reduction Test and for sensory evaluation.

4.2. Enumeration of Psychrophilic Counts

From the collected sample, 1 ml of pasteurized milk transferred in 9 ml of phosphate buffer to make primary dilution (10^{-1}). Then a series up to 10^{-7} dilution was prepared by transferring primarydilution (1ml) into test tube containing sterile phosphate buffer (9ml) to obtain 10^{-2}dilution and repeating the operations with sterile diluents (9 ml) using the 10^{-2} and further dilutions to obtain 10^{-3}, 10^{-4},10^{-5},10^{-6},10^{-7} dilutions. Inoculum (1 ml) of 10^{-1},$10^{-2}$$10^{-3}$ 10^{-4},10^{-5},10^{-6},and 10^{-7} were transferred in to petridishes(in duplicate) through sterile pipettes (2.2 ml) and warmed (42º±0.5ºC) sterile plate count agar (15 ml) was evenly mixed with the inoculum. The plates were allowed to get solidified at 25ºC for 10 minutes followed by incubation (In inverted position) at 4ºC ±0.5ºC for 12 days. Parallel to these plates, control plates ofPlate Count Agar were also tracked without inoculum to check sterility of respective medium.

4.3. Enumeration of Coliform, Total Mesophilic Count and Total Fungi Count

Identical sampling, dilution and plating protocol (as 4.2) was employed for coliform count using Violet Red Bile Agar, Total Mesophilic count using Plate Count Agar and for Total fungal Load using Potato Dextrose Agar.The plates were allowed to get solidified at 25ºC for 10 minutes. Petridishes containing Violet Red Bile Agar and Plate Count Agar were incubated at 37º±0.5ºC (In inverted position) for 48 hours while petridishes containing Potato Dextrose Agar were incubated at 25 ±0.5ºC (In inverted position) for 6 days. Parallel to these plates, control plates of respective media were also run without inoculum to check sterility of respective medium.

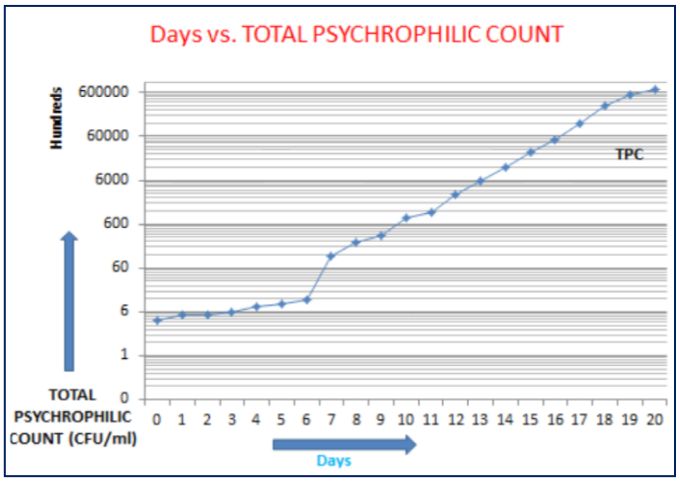
4.4. Methylene Blue Reduction Test

In pre-sterilized test tube (15 x 150), 10 ml of milk sample was taken followed by addition of 1.0 ml of Methylene blue dye. The air tight rubber cock was placed at the opening of the test tube to avoid entrance of atmospheric oxygen. Tubes were gently inverted three times for even distribution of dye content in milk. The tubes were positioned in a water bath at 4ºC ±0.5ºC. At the interval of half an hour, each tube was scrutinized for discoloration of dye (Figure 2).

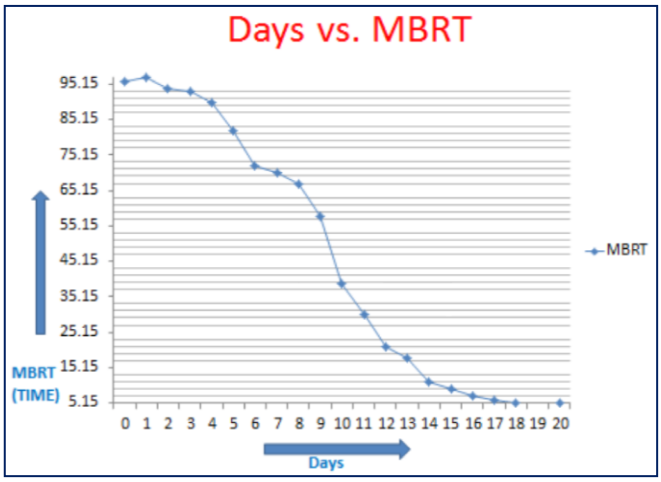
5. RESULTS & DISCUSSION

Psychrophilic count of pasteurized milk was assessed and results are presented in Graph 1. Anextensive variation was witnessed in psychrophilic count upon protracted incubation at 4ºC ±0.5ºC for 12 days. At 0 day, the psychrophilic load was 400 cfu/ml which was extended up to 900 after 5 days upon incubation at the refrigerated conditions. The drastic rise in psychrophilic count was observed from 6th day 1,100cfu/ml to 10th day 81,000 cfu/ml.Psychrophilic counts were further increased after 10th day and reached up to 7,00,00,000 cfu/ml at the end of 20th day. Freshness and pleasant flavour of pasteurized milk was partially inexpressive after 6th day but after 9th day, some defects like ropiness and fruity flavour were examined. The pleasant flavour of milk was majorly deteriorated between 11th to 15th day as would be expected merely by looking at the plate counts of psychrophiles. The general trend in most samples examined was for the count to become great by 8th, 9th and 10th day. Defects frequentlyexamined were curd formation (Figure 3) and ropiness (Figure 4) along with fruity flavour

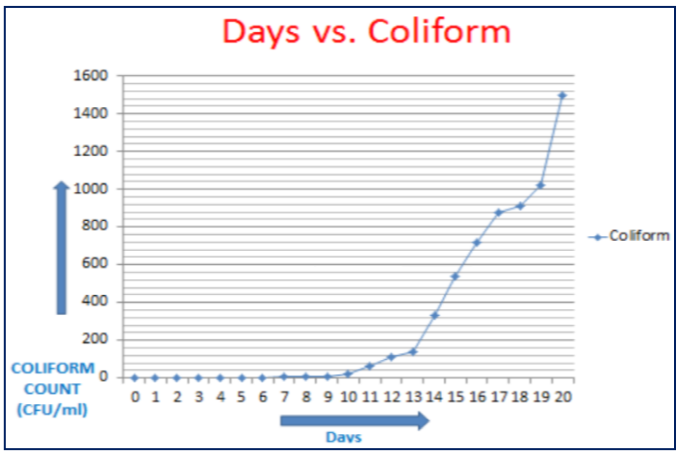
after about 10 days then after picked up. For the sample showing psychrophilic count on the 1st day, high counts and off flavours like fruity, stale, bitter, rancid and putrid developed progressivelyup to the end of 20th day (Table 1).



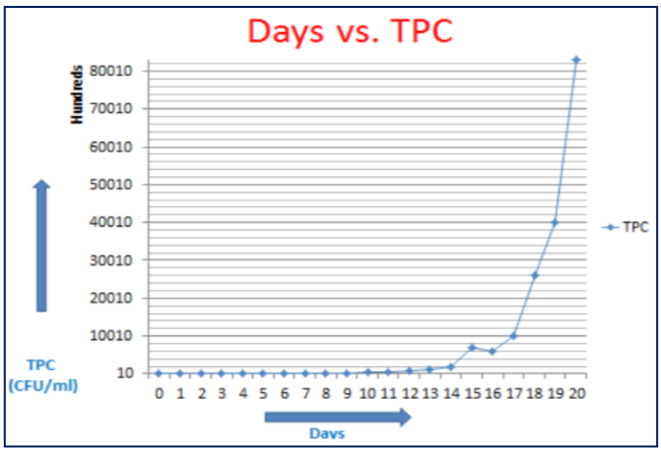
Graph 1
Day wise growth pattern of Total Psychrophilic bacteria in Pasteurized Milk



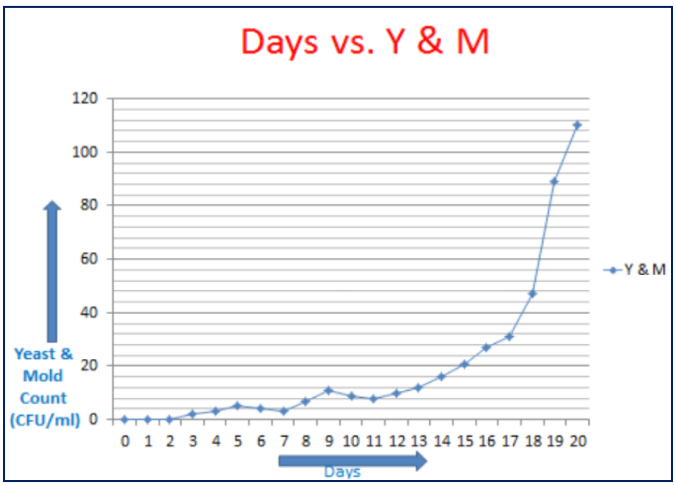
Graph 2
Influence of Psychrophilic bacteria on Methylene Blue Reduction Test



Graph 3
Day wise growth pattern of Total Coliform bacteria in Pasteurized Milk



Graph 4
Day wise growth pattern of Total Plate Count in Pasteurized Milk



Graph 5
Day wise growth pattern of Total Yeast and Moldcount in Pasteurized Milk

Psychrophiles customs a wide diversity of metabolic pathways including photosynthesis, chemoautotrophy and heterotrophy thus forming it robust, diverse communities. Under favorable condition of lesser temperature laid themselves for bio-chemical activities resulting in to accumulation of their metabolites in pasteurized milk. Buchanan and Hammer (1915) have described ropiness in milk as a change from its normal consistency to a condition in which long threads of viscous masses are observed. They attributed this condition to the production of carbohydrate (polysaccharide) derivatives. The organisms which usually produce ropiness in milk are those possessing a large capsule, such as *Alcaligenes viscosus* and members of the *Escherichia-Aerobacter* group

(Buchanan et al, 1915). The capsular material produced by many bacteria consists of complex polysaccharides identified as polymers of simple monosaccharide. Although these bacteria are generally non-pathogenic and majority killed by pasteurization except *Bacillus* spores (Grosskopf et al.1969). They may be introducing in pasteurized milk as post pasteurization contamination from Silo, Horizontal Milk Storage Tanks and from pipe lines of the plant. The numbers of psychrophilic bacteria in pasteurized milk depends on the sanitary practices prevailing during production, processing, and aseptic packing along with time and temperature control of the plant. The influence of psychrophiles on shelf life of pasteurized milk may also depends on temperature control, packing process, rate of growth, the storage period and metabolic regulations of the organisms. Effect of psychrophiles on Methylene Blue Reduction Time is presented in Graph 2. It was witnessed that under refrigerated condition at 4°C ± 0.5°C, the MBRT was 96:00 hours on 0 day which was gradually reduced up to 5:15 hours at 20th day (Figure 2). It may be due to purely, partially or combined effect of microflora which affects Eh value (Oxidation-Reduction Potential) of Methylene Blue dye.

Coliform counts in pasteurized milk, occasionally used as an index of milk quality are expected to increase with the standard plate count. High coliform counts confirm the poor sanitation practices. The genera comprises *Escherichia coli, Enterobacter aerogenes, Klebsiella* spp. Relevant to coliform load, it was inspected that on 0 day, coliform count was not detected but gradually they were raised up to 11 cfu/ml at the end of day 9th day which was further increased to 1,500 cfu/ml on 20th day (Graph 3). Similar trend was scrutinized for Total Mesophilic count which was9,000 cfu/ml on 0 day which extended up to 83,00,000 cfu/ml at the end of 20th day (Graph 4). At low temperature, enzymes functions very slowly or halts functioning (Patel R et al, 2004). This is because of low temperature mostly increase the viscosity of fluids and hardening of lipids which results in to growth obstruction, retardation or inhibition for mesophiles as looking at the growth pattern of Mesophilic counts. In the case of Yeast and Mold Count, at 0 day counts were 0 cfu/ml which was further extended to 110 cfu/ml at the end of day 20 (Graph 5). It is the clear case effect of less temperature on metabolic and enzymatic regulations of total microflora. The enzymatic regulation abridged at certain level at lesser temperature 4°C ± 0.5°C, but still the metabolic activity is going on at very slow rate resulting in to the high count of microflora at the end of day 20 which results in to defects development in pasteurized milk.

7. CONCLUSION

From the results, convinced evidence derivedwhich exhibits the influence of less temperature (4°C ± 0.5°C) on the quality of pasteurized milk. The psychrophilic load may be augmented upon prolonged holding of pasteurized milk at the refrigeration temperature. Impact of psychrophiles and total microbes present in pasteurized milk on O-R potential is least up to 4 days but longer storage of pasteurized milk under refrigerated conditions may affect the O-R potential and thus MBRT may be reduced. In parallel to this, metabolic regulation plus enzymatic activity of coliform, Mesophilic bacteria and fungi may reduceat certain level but longer storage at 4°C ± 0.5°C of pasteurized milk may rise in total microbial load. Longer storage of pasteurized milk i.e. 10 to 20 days may results in to development of off flavours like fruity flavour and off taste i.e. sour, stale, bitterrancid, putrid along withphysical defects i.e. Ropiness and curdling of milk. It might be purely, partially or combined effect of total microbial flora existing in pasteurized milk.

SUMMARY OF RESEARCH

The study highlights effect of microbial activity at 4°C temperature upon prolonged storage of pasteurized milk for 20 days. Metabolic regulations of psychrophilic population are extremely increased at 4°C temperature while microbial flora other than psychrophilic organisms cultivates at very slow rate due to effect of less temperature on enzymatic regulations. The extreme high microbial population leads to off flavours like fruity flavour and off taste i.e. sour, stale, bitter, rancid, putrid along with physical defects i.e. Ropiness and curdling of milk upon prolonged storage of pasteurized milk at 4°C.

FUTURE ISSUES

From the findings it deduced that longer storage of pasteurized milk may rise to defects development in pasteurized milk at4°C Knowledge relevant to numbers and types of psychrophiles is useful to determine degree of cold chain maintenance and marketing strategies to distribute safe, wholesome and high quality of fluid milk.

DISCLOSURE STATEMENT

The experimental work described here was begun, continued and completed under the financial assistance of Vidya Dairy, Anand Agricultural University Campus, Anand-388 001, Gujarat, India.

PHOTO GALLERY

To the casual spectator, photographs displayed in this research work may appear to be magnitude work. To exhibit photographs at this degree requires a network of solid support and it was delivered by Sunny Kharwa. We are indebted grateful to him for his unusual aptitude and boost us with his special attention and care.

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