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Biochemical and Molecular Characterization of Different Bacterial Isolates From Milk and Milk Products

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ABSTRACT

Milk is foremost constituent of the diet; its quality assurance is considered essential to the welfare of a community. Usually milk is contaminated with different kinds of microorganisms at milk collecting places. Various bacterial colonies in milk were analyzed by performing morphological, biochemical and molecular characterization. We identified variable bacterial species in different milk products like fresh curd, Buffalo milk, Chocolate, which were collected from local market of Lucknow. *Staphylococcus aureus, Micrococcus luteus, Shigella sonnei, Klebsiella pneumoniae, Lactobacillus fermenti and Pseudomonas* morphologically and biochemically were identified where bacteria were especially gram negative. Furthermore, 16SrDNA molecular characterization was also done in this study. It was also observed that multiple bacterial species were responsible for single milk and milk product spoilage. The isolates could be used as indicators of microbial quality. Further study will be open a new area for the researcher working in this direction. Milk microflora includes spoilage and pathogenic microorganisms. Many milk borne diseases such as tuberculosis, brucellosis and typhoid fever are caused due to spoilage of milk and milk products. In the processing of milk, some of them may produce undesirable effects and some micro-organisms produce food infections which can either carry the pathogens that will increase the likelihood of infection of the consumers food. The smooth colonies with entire edge contamination of milk and milk products are largely due to human factor and unhygienic conditions.

Keywords: *Staphylococcus aureus, Micrococcus luteus, Shigella sonnei, Klebsiella pneumoniae, Lactobacillus fermenti , Pseudomonas,* spoilage.

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INTRODUCTION

Milk is an essential part of daily diet for the growing children and expectant mothers. Milk is a major constituent of the diet, its quality assurance is considered essential to the welfare of a community. Milk is nutritious food for human beings, also serves as a good medium for the growth of many microorganisms, especially *Lactobacillus, Streptococcus, Staphylococcus and Micrococcus sp.* Bacterial contamination of raw milk can originate from different sources animals such as from animals, air, milking equipment, feed, soil, feaces and grass (Torkar & Teger, 2008). Milk microflora includes spoilage and pathogenic microorganisms. Many milk borne diseases such as tuberculosis, brucellosis and typhoid fever are known (Horst, 1995). Milk is spoiled by a wide range of microorganisms some of which are pathogenic and are responsible for milk borne diseases. The milk is very easily contaminated if collected unhygeinically and handled carelessly leading to quick spoilage (Chatterjee*et.al.* 2006) and is often contaminated by *Escherichia coli* bacteria under poor sanitary conditions which can affect public health. The coliform group of bacteria is defined as the indicator (faecal coliform) of suitability of milk for consumption. The quality of milk is determined by aspects of composition and hygiene. Due to its complex biochemical composition and high water activity milk serves as an excellent culture medium for the growth and multiplication of many kinds of microorganisms. Therefore in the processing of milk, some of them may produce undesirable effects and some micro-organisms produce food infections which can either carry the pathogens that will increase the likelihood of infection of the consumers food. The smooth colonies with entire edge contamination of milk and milk products are largely due to human factor and unhygienic conditions. Usually milk is contaminated with different kinds of microorganisms at milk collecting places.

Milk is an ideal medium for microbial growth because of its high water content and the large variety of available nutrients which can be used by microorganisms as energy source. The main components of whole milk are 87.3% water, 4.8% carbohydrates (mainly lactose), 3.7% fat, 3.2% proteins, and 1% non-protein nitrogenous compounds, minerals and vitamins. Furthermore, its pH is almost neutral ranging from 6.5 to 6.7 making it an ideal growth environment for bacteria. Temperature also has a part to play as spoilage microorganisms can become active at temperatures between 2 and 30 Within this temperature range the growth of one particular bacterial species, the Gram negative *Pseudomonas*, is dramatic. The other major members of the spoilage flora on pasteurized milk include the endospore-forming bacteria of the *Bacillus* genera, and other Gram positive rods and cocci such as *Lactobacillus*, *Corynebacterium* and *Lactococcus* species. Bacterial spoilage causes significant economic losses for the food industry. Product contamination with

psychrotrophic microorganisms is a particular concern for the dairy industry as dairy products are distributed at temperatures permissive for the growth of these organisms. The diverse microbes that may be categorized as psychrotrophic are ubiquitous in nature and can be isolated from soil, water, and vegetation (Dogan, Belgin;et.al.2003

MATERIAL AND METHOD

Sample collection-

Three commonly used milk and milk product sample were taken for the study. The sample were Buffalo milk, Chocolate and fresh curd. The samples were bought from street vendors of local market of Lucknow (U.P.). Each sample was placed separately in sterile plastic bags and immediately transported to the laboratory for processing within 1 h of collection. They were brought to the laboratory for microbiological analysis. Included in the analysis performed were isolation, biochemical characterization and screening.

Preparation of stock solution:

After suitable spoilage the stock solution was prepared either by squeezing or crushing of each spoiled milk sample and a homogenous suspension was obtained by thoroughly vortexing the mixture. The stock solution was prepared in sterile falcon tubes separately.

pH Measurement:

pH of all undiluted samples was measured by pH meter immediately after collection. It is important to determine the pH of the milk sample before undertaking microbiological examination as this can influence the colony count and organisms sought.

Serial dilution:

The technique involved the removal of a small amount of an original solution to another container that is then brought up to a predetermined volume using the working solution (i.e. ddH_2O). To make a 1:100 dilution (10^{-2}), remove 10µl and place this volume in a tube containing 990µl of ddH_2O. This is often represented as 1:100 or 10^{-2}.

To dilute this by a factor of 1:1000, remove 1µl of the 1:100 dilutions and place it in a tube containing 999µl of ddH_2O or media. The secondary concentration (1:100) has been diluted by a factor of 1,000 and the original solution has been diluted by a factor of 100,000 (the dilution factor). Same process was repeated to obtain 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions.

MEDIA-MEDIA PREPARATION

Nutrient agar was the main medium used for the isolation of bacteria, some other important selective media were also used in the bacterial isolation process such as:Eosin-Methylene blue

EMB), Mac-Conkey agar, Mannitol Salt Agar, MRS

Nutrient Agar

It can be used for the cultivation and enumeration of bacteria which are not particularly fastidious.

Composition:

Peptic digest of animal tissue (5.000)gm/L, Sodium chloride (5.000)gm/LBeef extract(1.500)gm/L, Yeast extract (1.500)gm/L, Agar (15.000)gm/L, Final pH (at 25°C) 7.4±0.2 .Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mixed well before pouring. (www.google.com)

SELECTIVE MEDIA-

Mannitol salt agar, EMB Media ,De Man, Rogosa, and Sharpe (MRS) agar medium.

Isolation of bacteria

The milk and milk product sample were diluted quantitatively so that the total number of colonies on a plate ranged between 30 and 300. For few samples plates suitable for counting were obtained by planting 1ml and 0.1ml of undiluted sample and 1ml of sample diluted (ratio, 1:100). The cover of the sterile Petri dish was lifted just high enough to insert the pipet. The pipette was removed without retouching it to the plate; then 15 to 20ml of the melted culture medium between 44^0C was added. The plate was gently rotated for thorough distribution of inoculum through the medium. The plate was incubated in inverted position in an incubator for 24 hours. The number of colonies counted on plate multiplied by dilution of sample gives the number of bacteria per ml.Representative colonies on the culture plates were successively sub cultured onto fresh agar plates of the same medium until pure cultures were obtained. Stock cultures were grown on nutrient agar and stored in the refrigerator.

Identification of isolated bacteria

The isolated organisms were identified by their colony morphology, microscopic examination, motility study and relevant biochemical tests according to standard laboratory methods.

Morphological characterization:

Cell morphology, cell arrangement, cell size, motility, presence of spores, and Presence of cell granules were determined by phase microscopy. Stained mounts were used for determining the Gram reaction and spore formation.

BIOCHEMICAL TESTS

Oxidase test, Catalase Test, Triple Sugar Iron Agar ,Sugar fermentation, Gas production, Hydrogen sulfide production ,Dimethyl Red and Voges Proskauer (MR-VP) **,** Citrate Test **.**

Concentration of DNA Samples:

Mainly DNA samples are concentrated by ethanol precipitation. In the presence of salt (mainly monovalent cations) and at low temperature, absolute ethanol precipitates nucleic acid. The acetate group replaces the hydroxyl group of water and DNA is able to make hydrogen bonds and becomes heavier. It can be easily separated by centrifugation or by spooling.

Gel Electrophoresis:

Agarose solution (50ml) was poured on a presealed glass plate. The gel was allowed to set and then comb were removed carefully. 1X TAE (Running) buffer was poured into chamber to cover the gel and fill the wells. Prepare DNA sample by adding 8µl loading dye (bromophenol blue) and 5µl DNA and mixed well and then load the prepared DNA sample in wells. Connected the electrode to power plug at constant voltage of 50V for 2-3 hrs and allowed the sample to move under the influence of electric field. The mobility of sample was tracked by movement of bromophenolblue and electrophoresis was allowed to continue till the tracking dye reached 3/4th of the gel length and finally examined the gel under gel doc.

Identification of bacterial isolates by 16S rDNA amplification and sequencing of the amplified products:

The DNA isolated, as above, was amplified with the PCR System (Bio-Rad, USA) using 16S rDNA universal primers and sequenced for the identification of bacterial strain at molecular level. The 16S rDNA genes were amplified by polymerase chain reaction. The PCR thermal cycling programme used was as follows: Initial denaturation at 95^{o}C for 5min; 30 cycles of denaturation, annealing and extension at 94^{o}C, 52^{o}C and 72^{o}C for 30s, 30s and 1min 25s respectively, followed by a final extension at 72^{o}C for 10min and kept at a hold temperature of 4^{o}C. All the amplifications were carried out in triplicates. Amplification of the PCR products of expected size was confirmed by electrophoresis. The sequence of the 16S rDNA was determined by Amnion Biotech Inc, Bangalore, India. The gene sequences of each isolate obtained in this study were compared with known 16s rRNA gene sequences in the GenBank database.

RESULTS AND DISCUSSION

The study was aimed to isolate and characterize different bacteria from different sample like Buffalo milk, Chocolate, fresh curd sample.

ISOLATION OF BACTERIA

Microorganisms were isolated on N.A. by pouring the serially diluted samples directly on nutrient agar plates. After 24hrs of incubation at 37^{0}C, we observed bacteria of different texture, elevation, edges, colony size and shape.

(A) **(B)** **(C)**

Figure 1(a) Showing Buffalo milk sample of 10^{-7} dilutions spreaded on nutrient agar medium.

Figure 1 (b) Showing fresh curd sample of 10^{-7} dilutions spreaded on nutrient agar medium.

Figure 1 (c) Showing chocolate sample of 10^{-7} dilutions spreaded on nutrient agar medium.

Table 1 CFU counts: The no. of CFU counts was found to be dependent upon dilution factor

Sample	No.of colonies	Dilution factor	CFU/ml
Buffalo milk	11	10^7	$2.2x10^9$
Fresh curd	20	10^5	$4x10^7$
chocolate	10	10^5	$2x10^7$

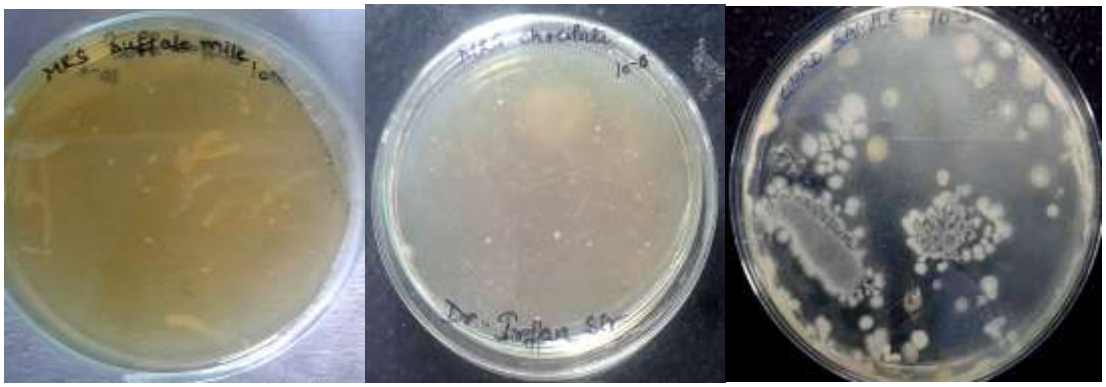
Further more, when sample on NA plate was observed for their texture, size, shape and colour. We found that colonies of microorganism were Round, clear, opaque, and elevated with entire and irregular edges.

The different microorganism isolates from different samples on NA plates, where further grown on selective media such as Mannitol plate, EMB plate and showing negative result.

For the further identification of microorganism, the result has been shown that we further wanted to find out the presence of some *Lactobacillus* Spp. In our sample like fresh curd, buffalo milk, chocolate. So we further performed spreading of serially diluted samples on MRS agar plate for lactobacilli and incubated for 24 hours at 37^{o}C. After 24 hours incubation, we observed the plates.

Isolation of microorganism from MRS plate-Lactobacillus MRS Agar was used for the cultivation of all Lactobacillus species. Lactobacilli MRS medium is based on the formulation of de Man, Rogosa and Sharpe with slight modification.

Sample-1 Sample-2 Sample-3



(a) **(b)** **(c)**

Figure 2 (a) Showing Buffalo milk sample of 10^{-7} dilutions spreaded on MRS medium.

Figure 2 (b)Showing Chocolate sample of 10^{-7} dilutions spreaded on MRS medium.

Figure2 (c)Showing fresh curd sample of 10^{-7} dilutions spreaded on MRS medium.

Isolated microorganisms were identified and their CFU has been counted.

Table 2CFU= no. of colonies x dilution factor/1000

Sample	No. of colonies	Dilution factor	CFU/ml
Buffalo milk	15	10^6	$3x10^8$
Fresh curd	12	10^6	$2.4x\ 10^8$
Chocolate	25	10^5	$5x10^7$

Gram staining:

For more precise identification, bacterial isolates were further stained with Gram staining technique to determine whether the bacterial isolates are cocci or bacilli as well as Gram positive or negative. Microscopic view of grams stained bacteria isolated from Chocolate sample showing (+) ve result of Bacillus spp.,bacteria isolated from fresh curd sample showing (+) ve result of Cocci sp.and bacteria from Buffalo milk sample showing (-) ve of Cocci bacillary spp.

Table 3: Morphological characterization

Sample name	Colony colour	Gram staining	Cell shape	Species name
Chocolate	White	Bacillus(+ve)	R	*Lactobacillus Fermenti*
Fresh Curd	Yellow	Cocci(+ve)	C	*Micrococcus Luteus*
Buffalo milk (10-7 dilution)	White(small)	Coccobacillary(-ve)	C	*Klebsiella spp.*
Buffalo milk (10-7 dilution)	White(large)	Bacillus (+)	R	*Lactobacillus casei*

BIOCHEMICAL TEST RESULT-

For proper identification of the unknown isolates, bacteria were characterized biochemically by performing several tests such as catalase, oxidase, IMViC (indole, MR-VP, Citrate) tests, TSI test

and sugar fermentation (glucose, lactose, mannitol). These tests confirmed the bacterial identity by thoroughly studying the results according to Bergey's Manual of Determinative Bacteriology.

Oxidase test

The oxidase test is a key test to differentiate between the families of Pseudomonadaceae (ox +) and Enterobactericeae (ox -).

Table 4: Oxidase test

Sample	Media	Oxidase test
Chocolate	NA	+ve
Buffalo milk	NA	+ve
Fresh curd	NA	-ve

Catalase test

The catalase test is used to differentiate staphylococci (catalase-positive) from streptococci (catalase-negative). The enzyme catalase" is produced by bacteria that respire using oxygen, and protects them from the toxic by-products of oxygen metabolism. Catalase test of milk samples shows Catalase (-) ve result for Chocolate sample, Catalase (+) ve for fresh curd sample , Catalase (-) ve for Buffalo milk sample. Positive test shows BUBBLE formation and negative test shows NO BUBBLE formation.

Triple Sugar Iron test

Triple sugar iron agar (TSI) is a differential medium that contains lactose, sucrose, a small amount of glucose (dextrose), ferrous sulfate, and the pH indicator phenol red. It is used to differentiate enteric based on the ability to reduce sulfur and ferment carbohydrates.

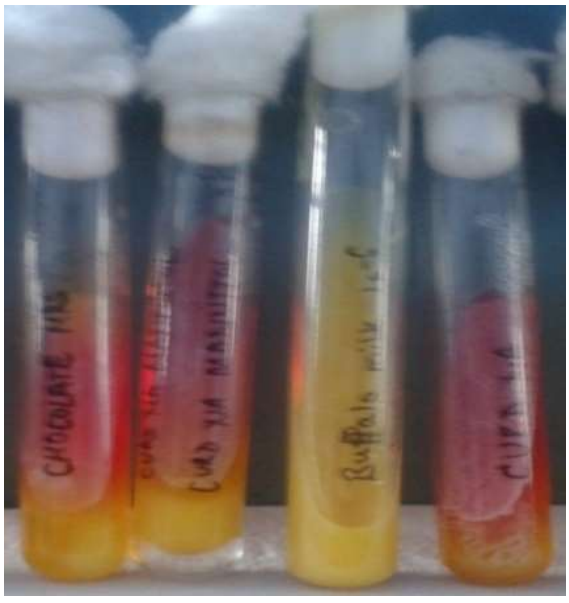


Figure 3: Showing no production of Hydrogen sulphide in Buffalo milk, Chocolate, fresh curd sample.

Table 5: TSI test

Sample	Slant	Butt	Hydrogen Sulphide	Suspected Microorganism
Chocolate	R	Y	-	*Shigella spp.*
fresh curd	R	Y	-	*Shigella spp.*
Buffalo milk	Y	Y	-	*Staphylococcus aureus*

Indole test

It is used as part of the IMViC procedures, a battery of tests designed to distinguish among members of the family Enterobactericeae.

Results: Positive (Cherry red coloration in oil layer on top of broth)and Negative (Not red in oil layer on top of broth).

(a) (b)

Figure 4(a) Showing indole (+) ve result for Buffalo milk, Chocolate, fresh curd sample.

Figure 4(b) Showing MR (+) ve for Chocolate, Buffalo milk, and fresh curd sample, and VP (-) ve for Chocolate , curd sample, but (+) ve for Buffalo milk sample.

MRVP test

All the bacterial isolates from fresh Curd, Chocolate and Buffalo milk sample using MRVP for result interpretation.

For methyl red test

Red color indicated positive test while negative reaction is showed by yellow color.

For Voges-Proskauer test

Positive test was indicated by eosin pink color within 2-5 minutes while negative test showed yellow color.

Citrate test

Positive test showed color change from green to blue whereas negative test remained green.

Table 6: Sugar Fermentation Test-

Sample	Glucose	Lactose	Mannitol
Chocolate	+	+	+
Fresh curd	+	+	_
Buffalo milk (10^{-6} dilution)	_	+	+
Buffalo milk (10^{-7} dilution)	+	+	+

Isolated bacteria were identified in this entire sample.

S. aureus, Shigella spp., Lactobacillus spp., Micrococcus sp., and Klebsiella sp. etc.

BACTERIAL TYPING BY 16SrDNA AMPLIFICATION:

In order to identify bacterial isolates at the species level we further aimed to analyse the 16s ribosomal DNA amplification for bacterial typing. In this scenario we first of all isolated the bacterial genomic DNA from different isolates by a following generalized protocol for the isolation of bacterial genomic DNA. The isolated bacterial DNA was visualized on agarose gel stained with Ethidium bromide (EtBr) as shown in following figure

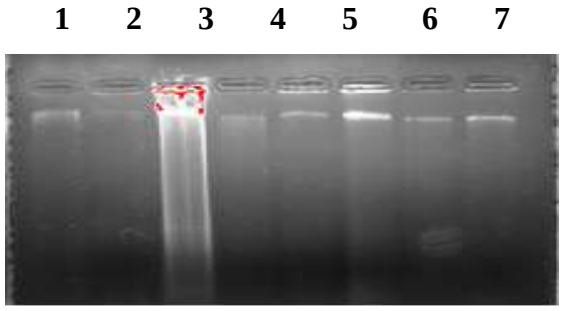


Figure.5:Showing DNA bands in Agarose Gel.

Quantification:

The amount of genomic DNA was quantified according to the formula as earlier discussed in material and methods and the purity of DNA was checked and was found to be between the ranges:

Table- 7(DNA Concentration= 50µg/ml × A_{260}× dilution factor)

Medium	Isolates	A_{260}	A_{280}	A260/A280 Ratio	DNA Concentration (µg/ml)
MRS	Buffalo small	0.164	0.088	1.86	82
	Buffalo large	0.092	0.047	1.95	46
	Curd	0.881	0.605	1.45	440
	Chocolate	0.074	0.047	1.57	37
NA	Buffalo large	0.068	0.040	1.70	34
	Buffalo small	0.174	0.086	2.02	87
	Curd	0.593	0.401	1.47	296
	Chocolate(white)	0.060	0.029	2.06	30

Chocolate(yellow)	0.565	0.356	1.58	282

DISCUSSION

A detailed study on the total microbial population of different milk products studied revealed that the highest bacterial population was found in the Buffalo milk, fresh curd sample. While least microbial population was recorded in the Chocolate sample. The literature reviewed in the present study provided evidence that *Staphylococcus aureus*is frequently occurring organism in fresh curd sample. The methods of production, transportation, handling and sale of milk are entirely unhygienic. The milk sold in raw form poses a great hazard to public health without adopting hygienic measures because of possibilities of contamination with *S. aureus*.

However, Milk products (fresh curd, Chocolate, Buffalo milk) available at Lucknow market were highly contaminated with *Lactobacillus fermenti.*Such system could pose favourable environment for bacterial contamination. The unclean hands of worker, poor quality of milk, unhygienic conditions of manufacturing unit, inferior quality of material used and water supplied for washing the utensils could be the source of accelerating the bacterial contamination of milk products and the post manufacturing contamination.

Psychrotrophic bacteria are not part of the natural microbial population of the udder, and therefore their presence in raw milk is exclusively the result of milk contamination after milking (Gaunt, 1986; Suhren, 1989; Munsch-Alatossava et al., 2005). The most commonly stated sources of Gram-negative psychrotrophic bacteria are: residual water in milking machines, milk pipelines or coolers, dirty udders and teats, inadequate cleaning of surfaces of dairy equipment for reception, transport and storage of milk, and biofilm (Santana et al., 2004; Simõoes et al., 2010). In terms of the sources of psychrotrophic spore-forming bacteria, such as those from the genus *Bacillus*, there is no universally accepted opinion. In general, the appearance of *Bacillus* spp. in raw milk is usually attributed to seasonal effects (Sutherland and Murdoch, 1994). Hay and dust are considered to be sources of these bacteria during winter months, while teats dirtied by soil are sources during the humid summer months. Christiansson et al. (1999) confirmed that the number of spores in milk is significantly correlated to the degree of soil contamination of teats. On the contrary, Lukas Eva et al. (2001) and Foltys and Kirchenerová (2006) did not confirm a significant seasonal influence on the presence of *Bacillus* spp. in raw milk, based on a one-month analysis of raw milk samples collected over the course of one year from different farms. A high correlation with incidence of *Bacillus* spp. in raw milk was confirmed in August and October by Lukas Eva et al. (2001), who explained this as being due to changes in meals and poor udder hygiene during milking, and not

due to a seasonal influence. The bulk milk storage tanks, pipelines and filling machines are a significant source of contamination of milk and dairy products with *Bacillus* species (Most Eller and Bishop, 1993; Eneroth et al., 1998; Sillankorva et al., 2008; Simõoes et al., 2010). Wijman et al. (2007) confirmed that within 24 hours, *B. cereus* forms a biofilm in all systems that are partially filled during technological operations or where residual liquid are reminded after the end of the process. Depending on the conditions, biofilm can consist of up to 90 % spores, and probably is the favourable site for the formation of spores. Among psychrotrophic bacteria that are associated with milk and dairy products, *Pseudomonas* spp. and *Bacillus* spp. are the most common isolated organisms in raw or heat treated milk at the time of spoilage (Mc Phee and Griffiths, 2002).

CONCLUSION

Bacteria are contaminants of all fresh foods. In order to avoid excessive spoilage, various measures can be employed to kill bacteria or to retard bacterial growth. These include keeping foods cold (or frozen), boiling (as is done for canned foods), salting (pickling), dehydrating (as in beef jerky), and adding anti-bacterial preservatives. In the particular case of milk, pasteurization combined with refrigeration is the most common technique used. Pasteurization does not kill all the bacteria (or spores) in milk, but does eliminate most of the pathogenic bacteria that have been historically associated with milk, such as tuberculosis, brucellosis, and typhoid. Pasteurization was first developed in order to kill these pathogens, but it was soon discovered that this process also improved the keeping quality of the milk without sacrificing the taste.

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