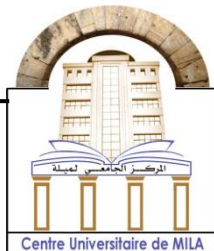


الجمهورية الجزائرية الديمقراطية الشعبية

République Algérienne Démocratique et Populaire

وزارة التعليم العالي و البحث العلمي

Ministère de L'Enseignement Supérieur et de la Recherche Scientifique



N° Ref :.....

Centre Universitaire Abdelhafid BOUSSOUF- Mila

Institut des Sciences de la Nature et de la Vie

Département des Sciences Biologiques et Agricoles

Mémoire préparé en vue de l'obtention du diplôme de

Master

Domaine : Sciences de la Nature et de la Vie

Filière : Sciences Biologiques

Spécialité : Biochimie Appliquée

Thème :

Psychrotrophic bacteria in milk and their negative effects on dairy products quality: A review

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Acknowledgement

And He is Allah: There is no god but He. To Him be praise, at the first and at the last: for Him is the Command, and to Him shall ye (all) be brought back | surah Al-Qasas.

First and foremost, I would like to praise Allah the Almighty, the Most Gracious, and the Most Merciful for His blessing given to me during my study and in completing this thesis.

May Allah blessing goes to His final Prophet Muhammad

(peace be up on him), his family and his companions.

I would like to express my gratitude and sincere thanks to my supervisor

Prof. abdelhafid BOUBENDIR, who has given me his valuable guidance, advice, corrections, comments, and suggestion and encouragement so I could complete this thesis in time.

Furthermore, I also express my deepest gratitude to my committee members,

Dr.CHEKROUD.K and Dr.BOUCHERJIT

My great honor is also bestowed upon all lecturers and staffs of biology Department.

My ultimate thanks is dedicated to my beloved Mother,

for their endless support, love, and prayer.

My special thanks go to my beloved family; my wife, who always supports me in every condition, has fought with me from the first letter of this thesis was written. Finally,

The authors apologize for the many colleagues who are not referenced in this work

due to space limitations I have a great expectation that my study will be beneficial and useful

for anyone who is interested in reading this final project

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Abstract

The autochthonous microbiota of raw milk immediately after milking generally comprises lactic acid bacteria such as *Lactococcus*, *Lactobacillus*, *Streptococcus*, and *Leuconostoc* species, which are technologically important for the dairy industry. Raw milk is usually stored at cold temperatures (about 4°C) before processing to reduce the growth of most bacteria. However, psychrotrophic bacteria can proliferate and contribute to spoilage of ultrahigh temperature (UHT) treated and sterilized milk and other dairy products during cold storage, due to their ability to produce extracellular heat resistant enzymes such as proteases and lipase. These enzymes are responsible for spoilage caused by visible or non-visible defects and lead to significant food waste and economic losses. Psychrotrophic bacteria have developed diverse ways of adaptation to cold environment, from cell envelope and enzyme adaptation, to cryoprotectant and chaperone production, and novel metabolic capabilities. Several methods are implemented to prevent and control such contaminations. Prevention methods include good manufacturing and hygiene practices, air filtration, and decontamination systems, while control methods include inactivation treatments, temperature control, biopreservation and refrigeration.

Key words: psychrotrophic bacteria, cold adaptation, spoilage, pathogenic bacteria, control

Résumé

Le microbiote autochtone du lait cru immédiatement après la traite comprend généralement des bactéries lactiques telles que *Lactococcus* , *Lactobacillus* , *Streptococcus* et Espèces de *Leuconostoc* , qui sont technologiquement importantes pour l'industrie laitière. Le lait cru est généralement stocké à des températures froides (environ 4 °C) avant d'être transformé afin de réduire la croissance de la plupart des bactéries. Cependant, les bactéries psychrotrophes peuvent proliférer et contribuer à la détérioration du lait traité et stérilisé à ultra haute température (UHT) et d'autres produits laitiers pendant le stockage au froid. , en raison de leur capacité à produire des enzymes extracellulaires résistantes à la chaleur telles que les aspeptidases et les lipases, ces enzymes sont responsables de détériorations causées par des défauts visibles ou non visibles et entraînent un gaspillage alimentaire et des pertes économiques importants. Les bactéries psychrotrophes ont développé diverses méthodes d'adaptation à un environnement froid, depuis l'enveloppe cellulaire et l'adaptation enzymatique, jusqu'à la production de cryoprotecteurs et de chaperons, ainsi que de nouvelles capacités métaboliques. Plusieurs méthodes sont mises en œuvre pour prévenir et contrôler de telles contaminations. Les méthodes de prévention comprennent de bonnes pratiques de fabrication et d'hygiène, la filtration de l'air et les systèmes de décontamination, tandis que les méthodes de contrôle comprennent les traitements d'inactivation, le contrôle de la température, la biopréservation et la réfrigération .

Mots clés : bactéries psychrotrophes, adaptation au froid, altération, bactéries pathogènes, contrôle

خلاصة

تشتمل الكائنات الحية الدقيقة الأصلية للحليب الخام مباشرة بعد الحلب عمومًا على بكتيريا حمض اللاكتيك مثل *المكورات اللبنية*، *والعصية اللبنية*، *والمكورات العقدية*، والتي تعتبر ذات أهمية تكنولوجية لصناعة الألبان. عادة ما يتم تخزين الحليب الخام في درجات حرارة باردة (حوالي 4 درجات مئوية) قبل المعالجة لتقليل نمو معظم البكتيريا. ومع ذلك، يمكن للبكتيريا ذات التغذية النفسية أن تتكاثر وتساهم في إفساد الحليب المعالج والمعقم ومنتجات الألبان الأخرى أثناء التخزين البارد. نظرًا لقدرتها على إنتاج إنزيمات مقاومة للحرارة خارج الخلية مثل الأسبيبتيداز والليباز، فإن هذه الإنزيمات مسؤولة عن التلف الناتج عن عيوب مرئية أو غير مرئية وتؤدي إلى هدر طعام كبير وخسائر اقتصادية. لقد طورت البكتيريا ذات التغذية النفسية طرقًا متنوعة للتكيف مع البيئة الباردة، بدءًا من غلاف الخلية والتكيف مع الإنزيمات، وحتى إنتاج المادة الواقية من البرد والمركبة، وقدرات التمثيل الغذائي الجديدة. يتم تنفيذ عدة طرق لمنع ومكافحة مثل هذه التلوثات. تشمل طرق الوقاية ممارسات التصنيع والنظافة الجيدة، وتنقية الهواء، وأنظمة إزالة التلوث، بينما تشمل طرق التحكم معالجات التعطيل، والتحكم في درجة الحرارة، والحفظ الحيوي، والتبريد.

الكلمات المفتاحية: البكتيريا الدهانية، التكيف مع البرد، التلف، البكتيريا المسببة للأمراض، السيطرة

Introduction

Milk is a fluid secreted by the female of the mammalian species and fulfills the nutritional requirements of the neonate, for instance: the energetic part (provided by lipids, lactose, and in excess by proteins), essential amino acids, and amino groups necessary for the biosynthesis of non-essential amino acids (provided by proteins), essential fatty acids, vitamins, inorganic elements, and water (**O'Mahony and Fox., 2014**).

Global milk production has increased by about 20% in the last decade, from 694 million tons in 2008 to 843 million tons in 2018 (**FAO 2020**). As a result, bovine milk is the most consumed food product representing about 48% of the total milk consumed globally, the European Union (EU), Australia, and New Zealand being the most important producers, followed by the United States and India (**McAuley et al., 2016**).

Data on milk production and consumption show increase in the production and consumption in the Maghreb countries. In the year of 2022, the production reached 2433986.67 in Algeria; 2060000 tons in Morocco and 1377333.33 tons in Tunisia (**FAO 2024**).

Milk is an excellent growth medium for microorganisms since it has a pH close to neutral, high water activity, and rich nutrient content. Therefore, the larger the population of microorganisms in raw milk, the lower the physical, chemical, microbiological, and sensory qualities of processed fluid milk and dairy products (**Singh et al., 2011**).

The number and types of microorganisms present in milk are influenced by season, farm hygiene, feed, and efficiency of cooling. The number of bacteria in milk ranges from a few hundred to thousands per milliliter of freshly drawn milk from healthy cows. The main criteria for high quality raw milk are the presence of a low number of spoilage microorganisms and the absence of animal pathogens (**Hassan and Frank, 2011**).

The most important deteriorating microorganisms in milk belong to two large groups, the mesophiles and psychrotrophs (**Becker et al., 2010**). Mesophiles are mostly found in situations of poor sanitary conditions during milking, and in the absence of

milk refrigeration grow well at 20°C and 40°C, and can ferment lactose, which is the main sugar in milk, to produce lactic acid, which increases the acidity of milk and causes clotting of casein, thus, separating the milk into whey and curd and compromising the industrial processing and quality of the final products **(Mcauley et al., 2016)**. Psychrotrophs are part of the refrigerated milk microbiota since they can grow at refrigeration temperatures (7°C or below), irrespective of their optimal growth temperature **(Hassan and Frank, 2011)**.

Storage of raw milk under refrigeration conditions is globally practiced to control mesophilic and thermophilic bacteria in raw milk, but refrigeration also provides ideal conditions for the growth of psychrotrophic bacteria **(Yuan et al., 2019a)**.

The dairy industry is facing issues related to technological problems caused by hydrolytic enzymes produced by psychrotrophic bacteria during cold storage. Although these bacteria can be eliminated by pasteurization or ultrahigh-temperature (UHT) treatment, the thermotolerant enzymes produced by these bacteria during milk storage and transportation under refrigerated conditions can survive thermal treatments applied during the manufacture of most dairy products **(Machado et al., 2017)**. Lipases and proteases are the main spoilage-causing enzymes that affect the quality of dairy products. Lipases hydrolyze triglycerides in milk, resulting in lipolytic rancidity characterized by off-flavors associated with soap and butyric acid **(Yuan et al., 2019a)**. Proteases hydrolyze casein fractions, produce off-flavors described as bitter, and result in age gelation **(Stoeckel et al., 2016)**. To meet increasing demands for dairy products of high quality, much attention has been paid to the diversity and spoilage potential of psychrotrophic bacteria and their negative impact on the quality of dairy products **(Yuan et al., 2019a)**.

The aim of this review is to highlight recent literature on the diversity of psychrotrophic bacteria in raw milk, and to describe the effects of psychrotrophs on sanitary, organoleptic, sensory and nutritional quality of milk and dairy products. Also the effects of psychrotrophs on yield, texture, and flavor of dairy products, the spoilage potential of these bacteria, and the specific technological problems caused by the biofilms and thermotolerant enzymes produced by these bacteria. Moreover, the means of preventing their growth are described. The potential strategies for controlling dairy spoilage caused by psychrotrophic bacteria starting at the farm level are also discussed.

1. Psychrotrophs

The term psychrotrophs (also denominated psychrotolerant) refers to microorganisms that have the ability to grow at low temperatures but have optimal and maximal growth temperatures above 15°C and 20°C, respectively (**Moyer, 2007**).

The term psychrophile (Gk psychros meaning cold and philes meaning loving, Syn: cryophiles) was first used by Schmidt-Nielsen in 1902 (**Ingraham and Stokes, 1959**).

Despite being described as “cold-loving” most of them grow above 20 °C but not above 30 °C. Hence they are actually cold-tolerant rather than “cold-loving”.

For quite some time cold-tolerant bacteria were categorized in two subdivisions: psychrotrophs (organisms capable of growing between 0 to 30 °C) and psychrophiles (organisms growing between 1 to 20 °C with an optima around 16 °C). However this distinction is ignored at present and all the cold-tolerant bacteria are dubbed psychrophiles (Figure 1).

A recent report on growth characteristics of bacteria isolated from different types of Antarctic biotopes (soil, grass, moss, biofilm formed on vertical rocks) reveals that 40-70% were capable of growing in the temperature range of 1-30 °C. Hence they were designated as psychrotolerant (**Romanovaskaia et al., 2012**).

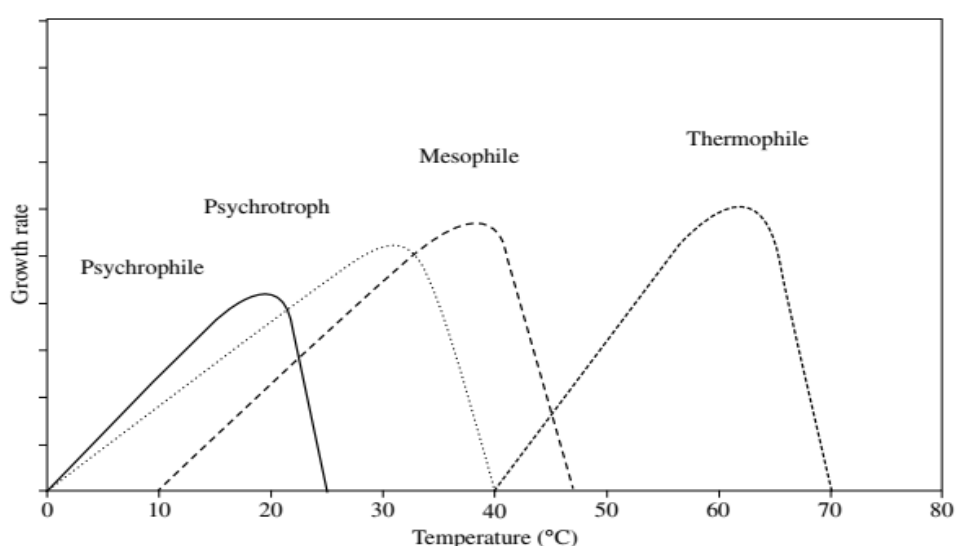


Figure 1. Typical temperature growth profiles for psychrophilic, psychrotrophic, mesophilic and thermophilic bacteria (**Knight, 2015**).

Psychrotrophs account for less than 10 % and in raw milk stored at low temperatures, they can become the predominant microorganisms, constituting up to 70% to 90% of the microbial population **(Cousin, 1982); (Sørhaug and Stepaniak, 1997).**

According to **Sørhaug and Stepaniak. (1997)** psychrotrophic bacteria present in raw cow's milk include the Gram-negative, and Gram positive genera (Table 01).

Table 01 Psychrotrophic bacteria present in raw cow's milk **(Sørhaug and Stepaniak, 1997)**

Gram-negative genera	Gram-positive genera
Pseudomonas	Aeromonas
Alcaligenes	Bacillus
Achromobacter	Clostridium
Serratia	Corynebacterium
Chromobacterium	Streptococcus
Flavobacterium	Lactobacillus
	Microbacterium

The presence and subsequent replication of populations of psychrotrophs may lead to the spoilage of milk **(Beales, 2004); (Pinto and al., 2006) ;(Nörnberg et al., 2010).**

Because the economic impact of this group of microbes upon the global dairy industry is substantial, psychrotrophic bacteria have been and continue to be studied extensively with the main objectives of developing effective control measures and establishing regulations to ensure the quality and safety of milk and dairy products **(Mcphee and Griffiths, 2011).**

Among psychrotrophic bacteria, *Pseudomonas* spp. predominate in refrigerated raw milk since they have great genetic diversity, metabolic versatility, and can efficiently adapt to cold temperatures due to the large amount of unsaturated lipids present in the cell membranes **(Özer and Yaman, 2014).**

2. Biodiversity of psychrotrophic microorganisms

2.1. Biodiversity psychrotrophic microbes from cold environments worldwide

Before molecular techniques were developed for microbial taxonomy, the psychrophiles were originally assigned to the following genera (Figure 2): *Brevibacterium*, *Microbacterium* and *Micrococcus* (Division: Actinobacteria); *Flavobacterium* (Division: Bacteroidetes); *Bacillus* and *Clostridium* (Division: Firmicutes); *Alcaligenes*, *Achromobacter*, *Pseudomonas* and *Vibrio* (Division: Proteobacteria). Other genera that have been described more recently are: *Agreia*, *Arthrobacter* and *Cryobacterium* (Division: Actinobacteria); *Phormidium* (Division: Cyanobacteria); *Algoriphagus*, *Bacteroides*, *Cytophaga*, *Gelidibacter*, *Polaribacter*, *Psychroflexus* and *Psychroserpens* (Division: Bacteroidetes); *Acetobacterium*, *Carnobacterium*, *Exiguobacterium*, *Planococcus* and *Planomicrobium* (Division: Firmicutes); *Octadecabacter*, *Aquaspirillum*, *Polaromonas*, *Desulfotalea*, *Alteromonas*, *Marinobacter*, *Marinomonas*, *Methylosphaera*, *Pseudoalteromonas*, *Psychrobacter*, *Colwellia*, *Moritella*, *Photobacterium*, *Psychromonas* and *Shewanella* (Division: Proteobacteria; those that also include barophilic isolates are designated with an asterisk). It therefore appears that the psychrophiles are widespread among the domain Bacteria with the majority of isolates coming from the Gram-negative Divisions Bacteroidetes and Proteobacteria. Thus, psychrophiles are autotrophic or heterotrophic, aerobic or anaerobic, spore-formers and nonspore-formers, phototrophs and nonphototrophs (DeLong et al., 1997).

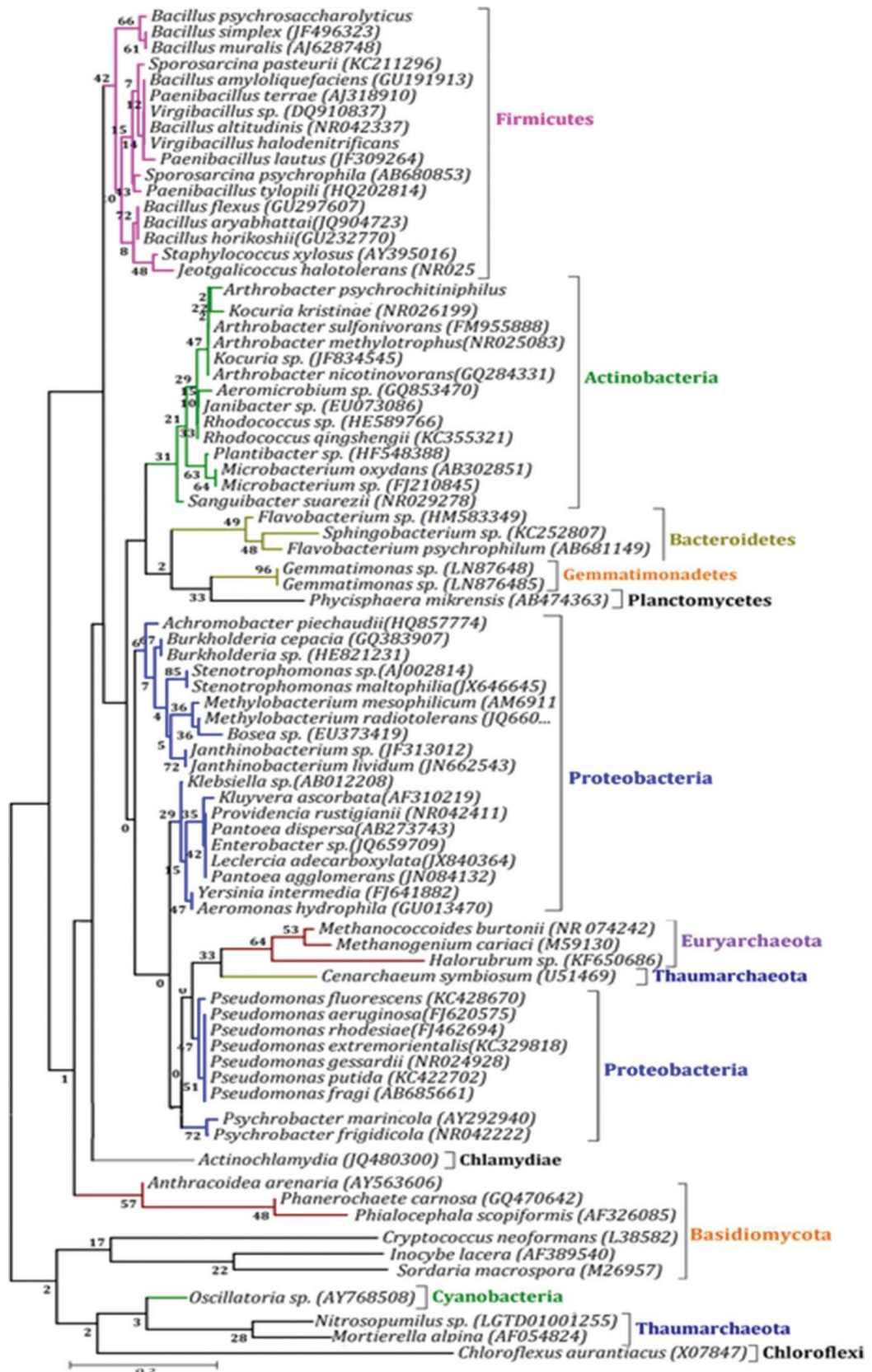


Figure 2. Phylogenetic tree showing the relationship among psychrotrophs, isolated from diverse cold habitats worldwide (Yuan et al., 2019b).

2.2. Psychrotrophic bacteria in raw milk

To gain a better understanding of the dairy microbiota, numerous techniques have been applied to explore the diversity of psychrotrophic bacteria in raw milk (Table 2).

Table 2. Predominant bacterial groups found in raw milk samples in different countries using culture-dependent and culture-independent methods after cold storage (Machado et al., 2017).

Country	Predominant groups		Storage conditions
	Culture-dependent methods	Culture-independent methods	
Algeria	<i>Stenotrophomonas rhizophila</i>		4°C for 7 days
	<i>Stenotrophomonas maltophilia</i>		
	<i>Chryseobacterium indologenes</i>		
	<i>Lactobacillus pentosus</i>		4°C for 10 days
	<i>Lactobacillus plantarum</i>		
	<i>Acinetobacter guillouiae</i>		4°C for 21 days
Australia	<i>pseudomonas fluorescens</i>		2°C for 10 days
	<i>Bacillus cereus</i>		
	<i>Bacillus weihenstephanensis</i>		
	<i>Bacillus circulans</i>		
	<i>Pseudomonas</i>		
	<i>Acinetobacte</i>		
	<i>Hafnia</i>		
	<i>Bacillus</i>		
	<i>Lactococcus</i>		
	<i>Microbacterium</i>		4–10°C for 10 day

Brazil	<i>P. fluorescens</i> <i>Pseudomonas putida</i> <i>Pseudomonas stutzeri</i> <i>Serratia liquefaciens</i> <i>Serratia odorifera</i> <i>Bacillus amyloliquefaciens</i> <i>Bacillus subtilis</i> <i>Bacillus spp</i> <i>Paenibacillus alvei</i> <i>Paenibacillus macerans</i> <i>Lactococcus lactis subsp. cremoris</i> <i>Lactococcus spp.</i> <i>Enterococcus faecali</i> <i>Enterococcus faecium</i> <i>P. fluorescens</i> <i>Acinetobacter spp</i> <i>Aeromonas hydrophila</i>		4°C for 2 days
Canada		<i>Acinetobacter sp.</i> <i>Acinetobacter calcoaceticus</i> <i>Staphylococcus aureus</i> <i>Staphylococcus equorum</i> <i>Facklamia tabacinasalis</i>	4°C for 3 days

		<i>Staphylococcus</i>	
The United States	<i>Pseudomonas</i> <i>Klebsiella</i> <i>Enterobacter</i> <i>Escherichia</i>		
Tunisia	<i>Pseudomonas</i> <i>Aeromonas</i>	4°C for 24–96 h	

2.2.1. Factors affecting the levels and diversity of psychrotrophic bacteria in raw milk

Levels of psychrotrophic bacteria differ in raw milk samples collected from different regions and in different seasons, from 10^2 to 10^7 CFU/mL (**Kim et al., 2017**); (**Ribeiro et al., 2018**).

The levels of microbial contamination in raw milk are tightly linked to the environment, the microbiological quality of water used for drinking or cleaning, animal feed, and the overall hygiene of milking equipment and milking procedures (**Lan et al., 2017**).

3. Sources of raw milk contamination

There are several hazards of cow's milk contamination, ranging from biological to chemical compounds. The risk of biological contamination of cow's milk derives mainly from cattle milking due to the exposure of udders to the environment, equipment, storage, dirty pipes, and others (Tamime, 2009). Chemical contamination of cow's milk comes from several sources: application of agrochemicals (Bedi et al., 2015), use of legal or illegal veterinary products (Kantiani et al., 2009), feed and forages contaminated with natural toxins (Hoogenboom et al., 2011), or through the improper use of chemicals during milk production, processing and packaging stages (Schettler, 2006). The Figure 3 and Table 3 show the direct and indirect pathways for contaminants entry into bovine milk (Rodríguez-Díaz et al., 2022).

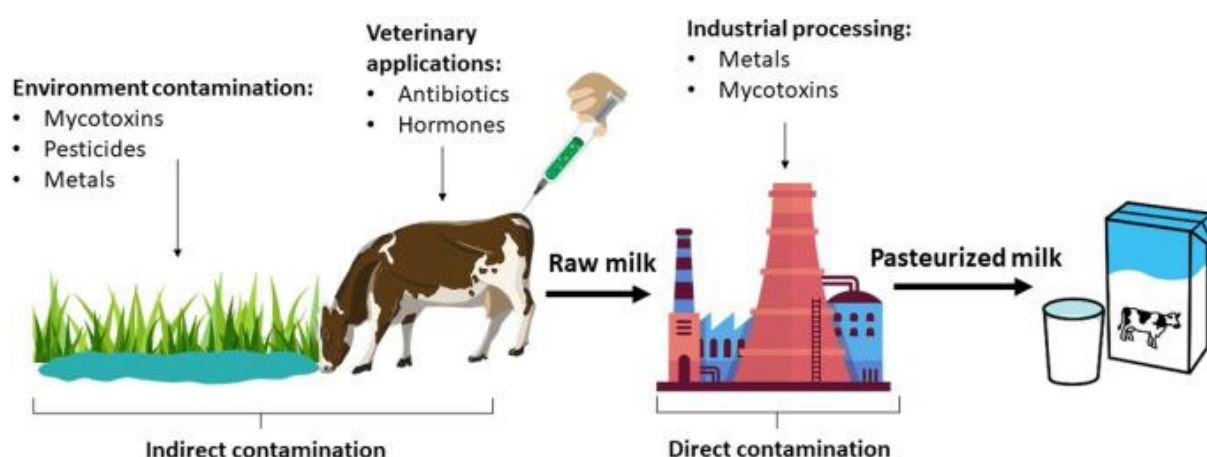


Figure 3. Sources of Milk contamination (Rodríguez-Díaz et al., 2022).

Table 3. Main categories of food safety hazards associated with milk and dairy products (adopted from the Food and Agricultural Organization (FAO, 2006).

Biological Hazards		Chemical Hazards		Physical Hazards	
i.	Pathogenic bacteria (including toxins produced by bacteria)	i.	Naturally occurring toxins	i.	Metal fragments
ii.	Toxigenic moulds/fungi	ii.	Direct and indirect food additives	ii.	Bone fragments
iii.	Parasites	iii.	Pesticide residues	iii.	Glass pieces
iv.	Viruses	iv.	Veterinary drug residues	iv.	Insects or their parts
v.	Other biological hazards	v.	Heavy metals	v.	Jewellery
		vi.	Environmental contaminants	vi.	Stones/soil/dust
		vii.	Chemicals from packaging material	vii.	Hair/fur

3.1. Microbial contaminants

The presence of several pathogenic microorganisms has been reported in raw and pasteurized cow's milk (Figure 4). Microbial contamination of raw milk can be due to diseases such as mastitis, improper handling on production farms, milking equipment, water sources, and feeding of cattle, utensils, and equipment used for milk storage on the farm or during transport (Yadav et al., 2014). Likewise, poor hygienic practices within the dairy industry can lead to the formation of biofilms on the sprinklers of cooling systems, pipes, cooling tanks, storage, and transport tanks. The contact of pasteurized milk with these surfaces increases the risk of contamination with pathogenic microorganisms, posing a danger to the consumer and the quality of the product (Weber et al., 2019).

The main types of microorganisms present in milk are bacteria, yeasts, and molds, which represent the different types of microorganisms present in cow's milk (Braem et al., 2012).

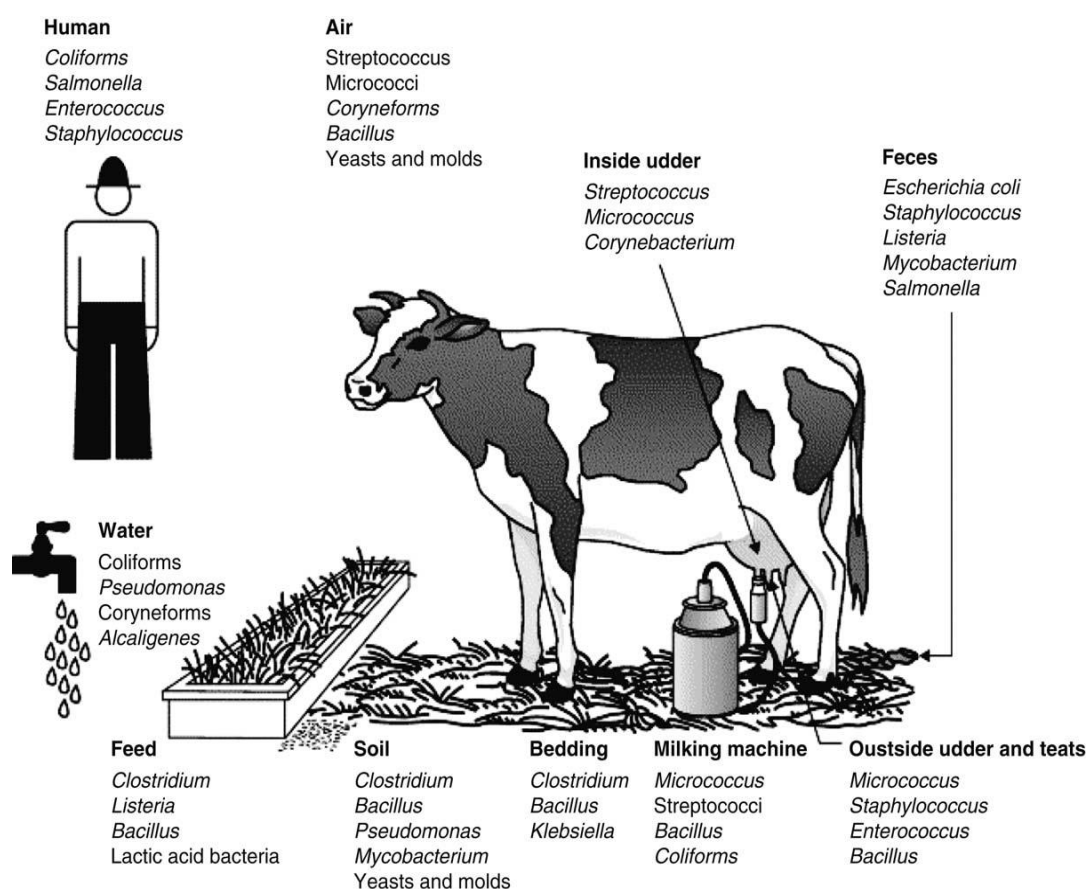


Figure 4. Sources of microbial milk contamination at the dairy farm (Hassan and Frank, 2011).

Consumption of milk contaminated by pathogenic microorganisms such as *Campylobacter*, *Salmonella*, *Yersinia*, *E. coli*, *Listeria*, and *S. aureus* can cause muscle and stomach pain, gastrointestinal diseases with diarrhea, fever, and nausea (Artursson et al., 2018). The Table 4 shows pathogenic microorganisms associated with raw milk and the diseases they cause (O'zer and Yaman, 2014).

Table 4. Pathogenic microorganisms associated with raw milk and the diseases they cause (O'zer and Yaman, 2014).

Enterobacteriaceae <i>Escherichia coli</i> , including 0157:H7 <i>Salmonella</i> <i>Yersinia enterocolitica</i> (psychrotrophic)	Gastroenteritis, hemolytic uremic syndrome Gastroenteritis, typhoid fever Gastroenteritis
Other Gram-negative bacteria <i>Aeromonas hydrophila</i> (psychrotrophic) <i>Brucella</i> spp. <i>Campylobacter jejuni</i> <i>Pseudomonas aeruginosa</i>	Gastroenteritis Brucellosis Gastroenteritis Gastroenteritis
Gram-positive spore-formers <i>Bacillus cereus</i> <i>Bacillus anthracis</i> <i>Clostridium perfringens</i> <i>Clostridium botulinum</i> (Type E is psychrotrophic)	Gastroenteritis Anthrax Gastroenteritis Botulism
Gram-positive cocci <i>Staphylococcus aureus</i> <i>Streptococcus agalactiae</i> <i>Streptococcus pyogenes</i> <i>Streptococcus zooepidemicus</i>	Emetic intoxication Sore throat Scarlet fever/sore throat Pharyngitis, nephritic sequelae
Miscellaneous Gram-positive bacteria <i>Corynebacterium</i> spp. <i>Listeria monocytogenes</i>	Diphtheria Listeriosis Tuberculosis

<i>Mycobacterium bovis</i> <i>Mycobacterium tuberculosis</i> <i>Mycobacterium paratuberculosis</i>	Tuberculosis Johne's disease
<i>Rickettsia</i> <i>Coxiella burnetii</i>	Q fever

3.2. Chemical contaminants

For a more detailed analysis, the chemical contaminants found in cow's milk have been classified into five groups: pesticides (22.05%), metals (22.18%), antibiotics (22.18%), mycotoxins (9.97%), and hormones (**Mollayusefian et al., 2021**).

3.2.1. Pesticides

A variety of pesticide residues in detectable amounts in raw milk, pasteurized, and UHT (ultra-high temperature) milk has been reported by several authors. This is due, among other factors, to the lipophilic properties and resistance to biodegradation of these types of contaminants. There are three possible forms in which pesticides can enter the animal's body: through contaminated water, through the pores of the skin when the animal is sprayed or soaked to treat ectoparasites, and through contaminated feed and forage, the latter being the main source of entry (**Fischer et al., 2016**); (**Rodríguez et al., 2022**).

3.2.2. Metals

Although metals are found in the environment either naturally or due to industrial and/or agricultural activities, there are several routes by which they reach the milk. Namely, ingestion of contaminated food, fodder, and/or contaminated drinking water. In the soil, they are absorbed by many crop plant species, which, when ingested by animals, are transferred to the lactating glands and finally excreted in milk (**Nag, 2010**).

Equipment used in the dairy industry is another source of contamination directly to milk with metals such as chromium and nickel. Heavy metals such as cadmium, lead, mercury, and arsenic reach milk by indirect contact through feed consumed by cattle (**Fischer et al., 2002**).

3.2.3. Antibiotics

Antibiotics are used in livestock activities in three basic ways: therapeutic, prophylactic, and growth promoters. About 80% of dairy cattle are subjected to antibiotic treatments on at least one occasion throughout their lives, mostly used as growth promoters and for the treatment of various diseases such as mastitis, arthritis, respiratory diseases, gastrointestinal diseases, and bacterial infections **(Bacanli and Başaran, 2019)**.

Cows eliminate antibiotics and their metabolites through milk, depending on the dose and route of application, level of milk production, type and degree of mammary disease, and time between treatment and milking. On the other hand, oral, intramuscular, or intravenous administration is less important from the point of view of milk hygiene than intramammary application. However, intramammary antibiotics are easy to apply and generally cheaper, so they are preferred in dairy farms. The most common disease in dairy cows is mastitis, whose treatment includes the wide use of tetracyclines, β -lactams, oxytetracycline, difloxacin, among others, being the β -lactams of greater application **(Fischer et al., 2002)**.

Within the latter group, the most employed are penicillin, ampicillin, and amoxicillin **(Oliveira et al., 2020)**.

According to the literature, the presence of antibiotics in milk has been evidenced, highlighting tetracycline, oxytetracycline, penicillin, and amoxicillin. While other antibiotics less reported in milk were rifamixin, gatifloxacin, spiramycin, and lomeflaxacin, with no indication in the studies of the purpose of their application in cattle **(Rodríguez et al., 2022)**.

3.2.4. Mycotoxins

The quality of food products is commonly affected by toxin contamination, of which 60 to 80 % are caused by mycotoxins **(Eskola et al., 2020)**. This means a risk for human health and great economic losses in the industrial sector. Mycotoxins are natural contaminants produced by *Aspergillus*, *Penicillium*, and *Fusarium* fungi **(Jiang et al., 2018)**.

The most prominent being Aflatoxin M1 (AFM1), which results from the metabolism of aflatoxin B1 in the liver of contaminated animals **(Ruangwises and Ruangwises, 2010);(Mollayusefian et al., 2021).**

3.2.5. Hormones

The use of hormones in the livestock industry increases production yields and medical treatments. Their fat-soluble characteristics favor their high persistence and presence in cow's milk due to the high-fat contents **(Baumrucker and Macrina, 2020).**

4. Cold-adaptive mechanism of psychrophilic bacteria

Introduction

Habitats of permanently low temperature dominate the Earth's biosphere and have been successfully colonised by a wide variety of organisms collectively termed psychrophiles or cold-adapted organisms. Microorganisms prevail in these cold habitats, especially bacteria, archaea, yeasts, cyanobacteria and protists, but microalgae and viruses are also common, and the ability of psychrophilic microorganisms to thrive in such environments reveals an adaptation to their habitat **(Margesin and Collins., 2019)**. It demonstrates a capacity to surmount the constraints inherent to life at low temperatures, which can only be achieved via a complex range of structural and functional adaptations of all cellular components, from the level of single molecules up to whole cells and even complete ecosystems **(D'Amico and al., 2006); (Morgan-Kiss et al., 2006); (Siddiqui et al., 2013); (De Maayer and al., 2014)**.

In the present paragraphs, we will discuss various adaptation strategies used by cold-adapted microorganisms to enable life in the harsh environmental conditions of their habitat and show how an understanding of these different strategies is leading to the development of various novel tools of commercial interest.

4.1. Stresses facing bacterial growth:

Life in the cold is characterised by a multitude of stresses in addition to low temperatures. Indeed, besides a reduced thermal energy, low temperatures also provoke further physicochemical constraints such as an increase in solvent viscosity and solubility of gases, namely an increased solubility of oxygen and reactive oxygen species (ROS), a decrease in the solubility of solutes and nutrients, reduced diffusion, increased osmotic stress, desiccation and ice formation **(Fonseca et al., 2016)**.

Furthermore, many cold ecosystems are often also characterised by fluctuating environmental conditions and/or multiple additional ecological limiting factors, including low nutrient levels, high salinity, oxidative stress, freeze–thaw cycles and low water activity **(Fonseca et al., 2016)**.

Sub-zero temperatures provoke ice formation which can lead to cryoinjury, osmotic stress, dehydration and even cell rupture and death. In the natural environment, onset of ice formation is usually delayed in the cell interior as compared to the cell

exterior due to the lower volume and densely packed, highly crowded nature of the former. Intracellular ice crystal formation, which is normally lethal to cells, can indeed occur when temperatures decrease at high rates, but in the natural environment, cooling is usually relatively slow and ice formation is thus mostly restricted to the extracellular space (**Fonseca et al., 2016**).

Extracellular ice formation, which can lead to physically damaging membrane fracturing, occurs with exclusion of solutes and removal of available liquid water. This leads to elevated extracellular solute concentrations and provokes intra/extracellular osmotic imbalances. In turn, this leads to stresses related to osmotic shrinkage and dehydration of the cell interior, not only negatively affecting cell function and survival but also preventing intracellular ice formation and instead leading to a non-crystalline amorphous (colloidal glassy) state with inhibition of cell metabolism. In addition, at relatively high sub-zero temperatures, in partially frozen environments subjected to freeze–thaw cycles and during temperature fluctuations, cells can also be subjected to harmful ice recrystallization stress, a thermodynamically driven process causing ice crystal coalescence and growth of large, fatally damaging ice crystals at the expense of smaller crystals (**Dolev and al., 2016**).

Microorganisms do not always make use of all tools in their cold adaptation toolbox, and in fact, each specific organism will use its own strategy, or combination of strategies, depending on its own specific requirements and on the environmental parameters and microbial community structure (**Collins and Margesin, 2019**).

4.2. Adaptation strategies used by cold adapted bacteria

The aim of the previous paragraphs is to summarize what we know about the cold adaptation of psychrophilic microorganisms. Obvious targets of the deleterious effects of low temperatures are cytoplasmic membranes and enzymes that tend to rigidify when the temperature drops. This affects membrane permeability, and hence the transport of nutrients and waste products, and catalysis, because enzymes require a certain flexibility to function (**Goodchild et al., 2004**); (**Ratkowsky et al., 2005**). Impaired protein folding and protein cold-denaturation can also cause problems at low temperatures, in particular for bacterial strains that sustain biological activities at temperatures as low as

–20 °C and resist freezing. Cold-shock proteins have also been described. What are their roles in this adaptation? Key biological activities that involve nucleic acids—such as DNA replication, transcription and translation can also suffer from exposure to low temperatures through the formation of secondary structures or super-coiled structures; how do psychrophilic cope with these phenomena? (Jin et al., 2022). Figure 5 summarise some of these mechanisms.

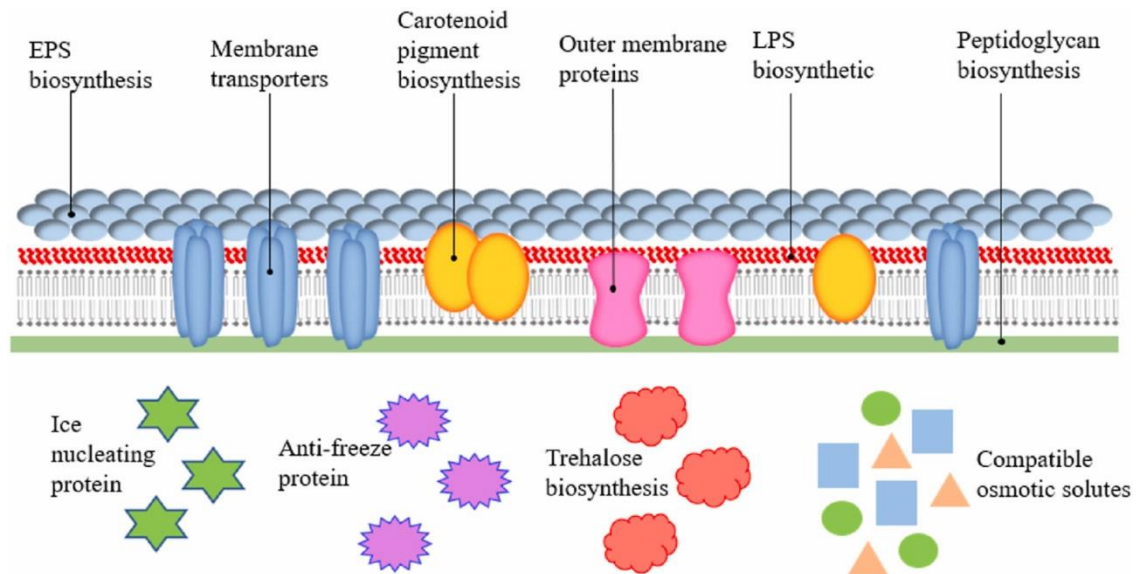


Figure 5. Adaptation strategies used by cold-adapted bacteria (Jin et al., 2022).

4.2.1. Cell envelope:

4.2.1.1. Cell membrane:

4.2.1.1.1. Increase the content of unsaturated fatty acids

Home viscous adaptation of cell membranes to low temperatures is accomplished in cold-adapted microorganisms via modification of the fatty acid composition of the lipid bilayer (D’Amico et al., 2001); (Siddiqui et al., 2013).

Cells mainly increase the content of unsaturated fatty acids, but increases in the content of short chain, methyl branched and/or cisomeric fatty acids are also common (Chintalapati et al., 2004); (Russell., 1997, 2008).

These fatty acids disrupt the packing order and reduce the packing density of the phospholipid bilayer, leading to a lowering of the liquid phase to gel phase transition temperature and maintenance of functional fluid bilayers even at low

temperatures. Indeed, in agreement with this cold adaptation strategy, an over representation and up regulation of genes encoding various proteins involved in membrane biogenesis and fatty acid synthesis as well as in fatty acid desaturation (desaturases, which simultaneously also protect against ROS) (**Medigue and al., 2005**); (**Méthé and al., 2005**); (**De Maayer and al., 2014**); (**He and al., 2015**); (**Goordial and al., 2016**).

4.2.1.1.2. Production of branched and cis-isomerisation fatty acid:

Production of branched chain fatty acids (**KAS-II, KAS-III**) and cis-isomerisation (fatty acid cis/trans isomerases) have been reported for numerous cold-adapted organisms) (**Medigue and al., 2005**); (**Méthé and al., 2005**); (**De Maayer and al., 2014**); (**He and al., 2015**); (**Goordial and al., 2016**).

4.2.1.1.3. The proteins embedded in the lipid bilayer

Finally, in relation to the other major components of the cell membrane, i.e. the proteins embedded in the lipid bilayer, an up regulation of membrane transport proteins has been observed in some psychrophiles and is believed to act in counteracting the reduced diffusion rates and transport inherent to low temperatures (**Bakermans and al., 2007**); (**De Maayer and al., 2014**).

4.2.1.2. Membrane pigments

Pigments, and especially carotenoids (pigmented polyisoprenoid hydrocarbons), have also been suggested to play a role in the modulation of cell membrane fluidity. Pigment production is common in psychrophilic microorganisms, being reported in isolates from ice cores and glaciers (**Shen et al., 2018**). Marine surface waters (**Dieser and al., 2010**). And high altitude soils (**Pandey et al., 2018**).

4.2.1.3. Peptidoglycan layer

Cold adaptation of other cell envelope components has been much less investigated than the cell membrane, but recent studies have begun to unveil possible strategies. In relation to adaptation of the cell wall, an upregulation of peptidoglycan biosynthesis genes and a thickened peptidoglycan layer at low temperatures has been

reported in some cold-adapted bacteria (Mykytczuk et al., 2013); (Rodrigues et al., 2008).

Similarly, *Planococcus halocryophilus* Or1 also displays a thickened outer cell surface, but this is achieved by a rather unique mechanism involving extracellular cell wall associated hydrophobic encrustations composed of peptidoglycan, calcium carbonate and choline (Mykytczuk and al., 2013).

4.2.2. Cryoprotection

Cold adapted microorganisms respond to multiple freezing related detrimental challenges by production of a variety of novel tools including, compatible solutes, ice-binding proteins (antifreeze and ice-nucleating proteins), extracellular polymeric substances (EPS) and/or biosurfactants (Mykytczuk et al., 2013); (Ghobakhlou et al., 2015);(Goordial et al., 2016).

4.2.2.1. Compatible solutes

Compatible solutes are low molecular mass, non-toxic organic osmolytes. Many cold-adapted microorganisms have an increased genome content of compatible solute biosynthesis, uptake and degradation genes and often accumulate up to molar concentrations of various different compatible solutes, with glycine betaine, trehalose, glycerol, sucrose, sarcosine, mannitol and sorbitol being commonly reported (Mykytczuk et al., 2013); (Ghobakhlou et al., 2015);(Goordial et al., 2016).

Accumulation of these organic osmolytes contributes to restoring osmotic balance and thereby counteracts water loss and cell shrinkage during freezing. In addition, they depress the freezing point of solution and, importantly also, the intracellular colloidal glass transition temperature (T_g) (Fonseca et al., 2016).

Figure 6 Simplified schematic of a composite cold-adapted bacterium showing strategies for tolerating salinity changes driven by temperature changes in sea ice. *Colwellia psychrerythraea* 34H is represented on the left half; *Psychrobacter arctica* on the right half. Cellular response to an increase in external salinity (S) is shown in the upper half; and response to a decrease in S in the lower half. When S increases, cells use transport proteins (aqua and purple shapes) to import compatible solutes (e.g., betaine, choline, proline; green dots) and maintain cellular integrity and function; when S

decreases, cells rapidly export salt ions (red dots) for the same purpose, through large-conductance (orange tubes) and small-conductance (red tubes) mechanosensitive ion channels, and then metabolize stored compatible solutes (interior boxes) no longer needed for osmotolerance. Circled numbers indicate the known number of copies, in the genomes of each organism, for genes putatively involved in each of these steps (based on data in (Ewert, 2013); (Ewert and Deming, 2014)).

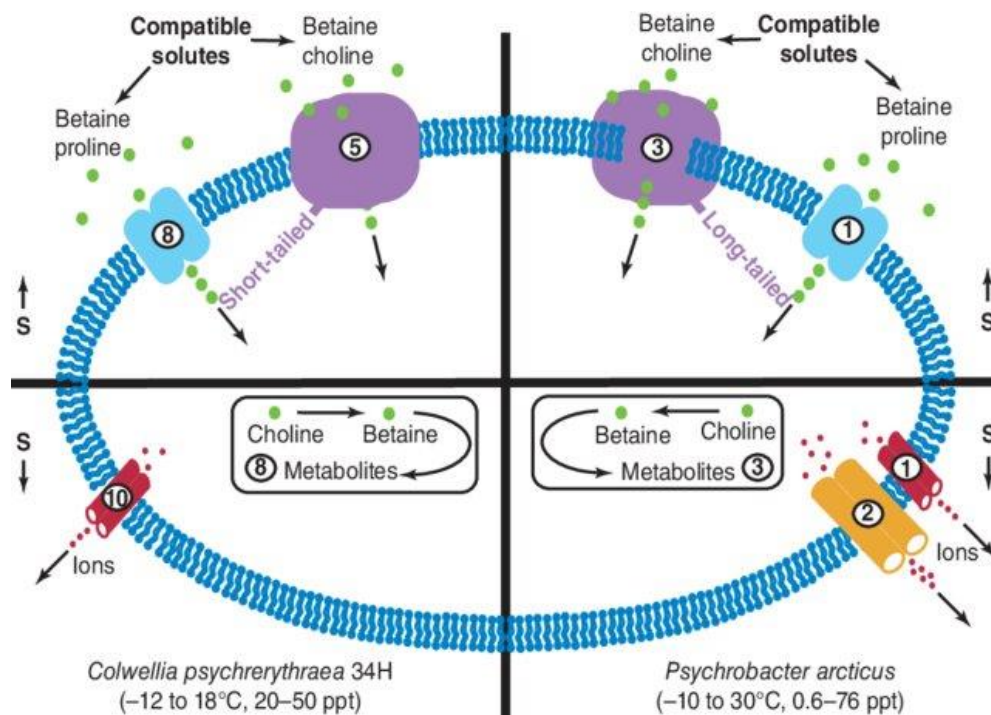


Figure 6. Composite cold-adapted bacterium showing strategies for tolerating salinity changes driven by temperature changes in sea ice (Ewert and Deming, 2014).

4.2.2.2. Ice-binding proteins

4.2.2.2.1. Antifreeze proteins (AFPs)

Antifreeze proteins (AFPs), also known as ice structuring or thermal hysteresis proteins, are noncolligative biological antifreezes which can bind to ice and inhibit ice growth and recrystallisation (Lorv et al., 2014); (Dolev et al., 2016b); (Voets., 2017). They were first identified in the blood of Antarctic fish but have since been reported in various bacteria, fungi, diatoms, plants, insects and crustaceans. Various types of AFPs of diverse structure exist in nature, being frequently glycosylated and/or lipidated and varying in size from 2 to 50 kDa, with a 1.5 MDa multidomain ice adhesion AFP being reported for the Antarctic bacterium *Marinomonas primoryensis* (Dolev et al., 2016a).

4.2.2.2. ice-nucleating proteins (INPs)

Ice-nucleating proteins (INPs) are large membrane bound proteins that facilitate ice formation. They initiate heterogeneous ice crystallisation at high sub-zero temperatures and are proposed to act by providing a template for the ordering and stabilisation of water molecules in an ice-like structure (Lorv et al., 2014); (Pummer et al., 2015); (Pandey et al., 2016). This lowers the activation energy barrier for freezing and nucleates ice growth at temperatures as high as -2°C (Li et al., 1997). The Figure 7 show biological functions of ice-binding proteins (IBPs) in :

- (a) Antifreeze (glyco) proteins in 'freeze-avoiding' species lower the freezing point of bodily fluids to halt further growth of internalized ice crystals.
- (b) Secreted IBPs from microorganisms like algae create a liquid habitat for their host in sea ice.
- (c) The terminal ice-binding domain of adhesins on the surface of the bacterium *Marinomas primoryensis* facilitates attachment to ice on Antarctic lakes for access to oxygen and nutrients.
- (d) Ice nucleation proteins in freeze-tolerant species of e.g. plants locally trigger ice nucleation at high subzero temperatures.
- (e) Many IBPs inhibit ice recrystallization during which ice crystals grow, merge, or change shape (Oude Vrielink et al 2016).

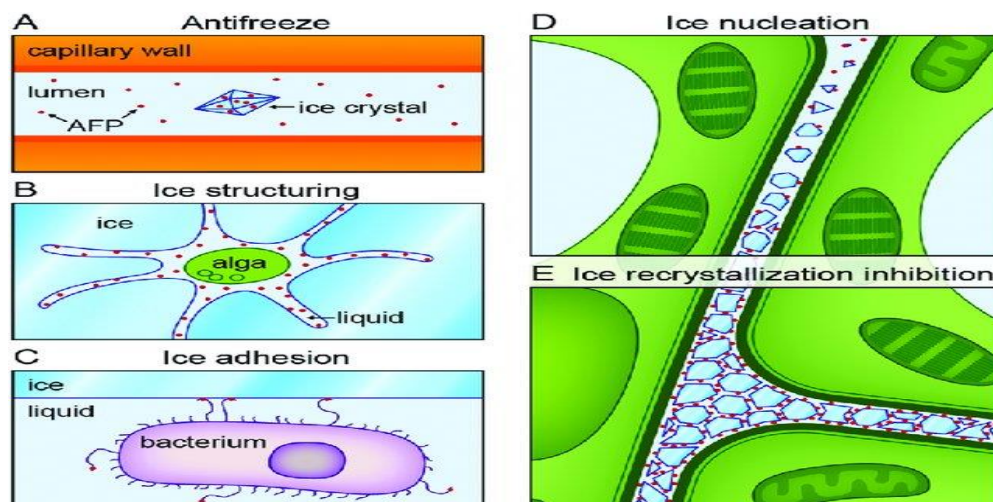


Figure 7. Biological functions of ice-binding proteins (Oude Vrielink et al.,2016).

4.2.2.3. Extracellular polymeric substance (EPS)

Extracellular polymeric substances (EPS) are multifunctional, high molecular weight biopolymer complexes secreted by various organisms into their local environment. They are large, complex, highly diverse architectural structures composed principally of carbohydrates (homo or hetero-polysaccharides), but also proteins and lower concentrations of nucleic acids, lipids, phenols and humic substances. EPS are produced by a wide variety of organisms and are found either attached to the cells outer surface or released into the surrounding environment (Figure 8). They form hydrated gels that play an important role in the formation of biofilms and in the modification of the physical, chemical and biological characteristics of the cell environment. Indeed, they are believed to have multiple functions, including in cell adhesion and nutrient scavenging, but are also thought to be important in protective functions such as osmoprotection, ROS scavenging, extracellular protein protection and even in cryoprotection (Ewert and Deming., 2013); (Deming and Young., 2017).

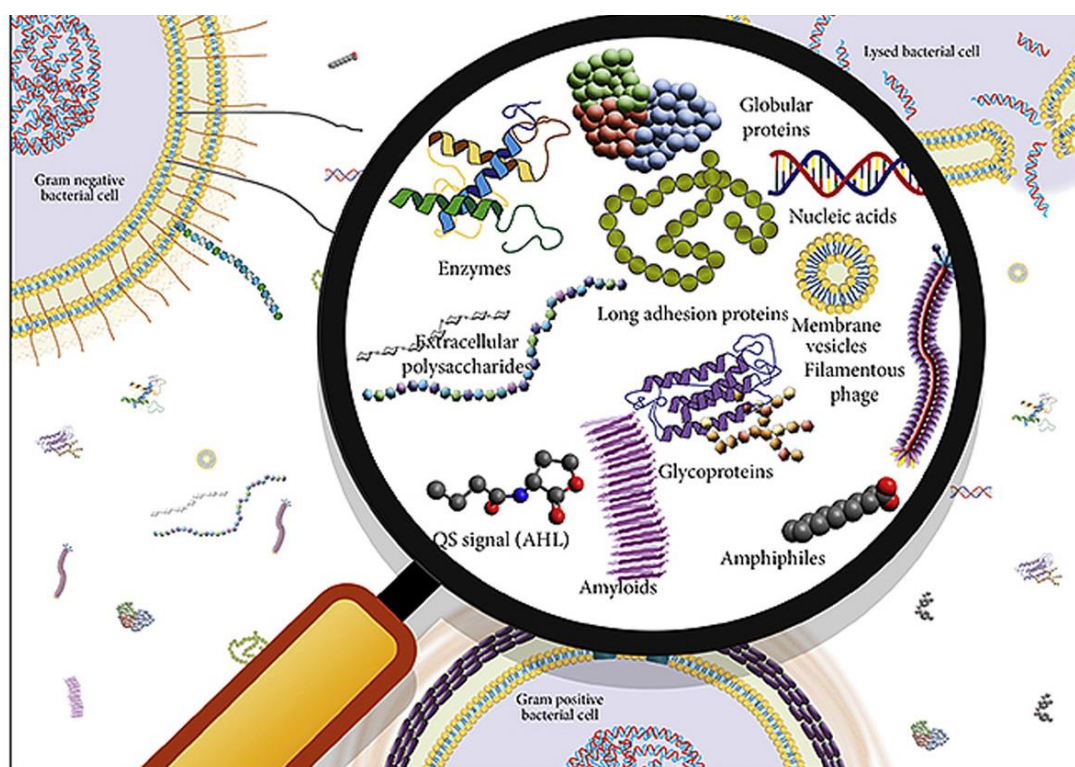


Figure 8. The extracellular polymeric substances (EPS) responsible for the establishment and function of biofilms (Seviour et al., 2019).

4.2.2.4. Biosurfactants

Biosurfactants are surface active amphiphilic compounds of microbial origin which reduce surface and interfacial tension between liquids, solids and gasses.

Generally, they are of low molecular weight, in contrast to bioemulsifiers, such as for example EPS, which tend to be of high molecular weight, and can be composed of sugars, amino acids, fatty acids and/or functional groups such as carboxylic acids. They are structurally diverse compounds but the most commonly reported are glycolipids (rhamnolipids, sophorolipids, trehalose lipids and mannosylerythritol lipids, etc.), lipopeptides (surfactin, iturin, fengycin, viscosin, etc.), phospholipids, fatty acids and neutral lipids (Figure 9). In nature, they are believed to play roles in enhancing the bioavailability of poorly soluble hydrophobic substrates, in regulating biofilm structure and surface attachment/detachment, in bacterial pathogenesis and quorum sensing, as well as acting as antibacterial and antifungal agents. In relation to cold adaptation, a glycolipid biosurfactant isolated from an Antarctic yeast was shown to have IRI Ice Recrystallization Inhibitors activity (**Kitamoto et al., 2001**), while a role for biosurfactants as osmolytes has also been suggested (**Perfumo et al., 2018**).

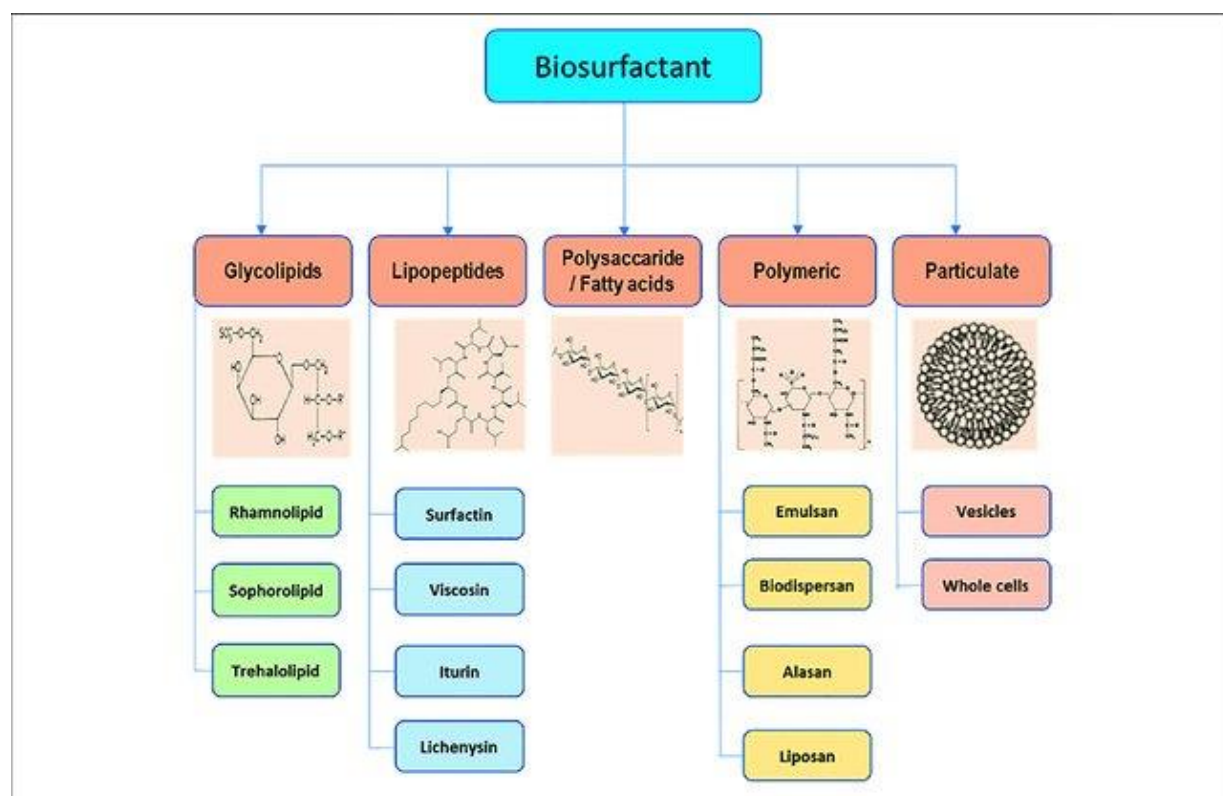


Figure 9. Main classes of biosurfactants and their structures.(Sharma et al., 2021).

4.2.3. Enzymes:

Cold-adapted enzymes, also known as psychrophilic or cold-adaptive enzymes, are enzymes with high catalytic activity and high thermolability under long-term low temperature conditions (Cavicchioli et al., 2002). Typically, the specific activity of these cold enzymes is higher than that of their mesophilic counterparts at temperatures of approximately 0-30 °C (Gerday et al., 2000). The molecular basis of cold-adapted enzymes has received attention regarding to their ability to cause spoilage of food or potential applications in a wide range of biotechnological fields.

The catalytic activity (k_{cat}) of cold-adapted enzymes is always higher than their mesophilic counterparts at low or room temperatures (Xia et al., 2020). In another word, the activity of the enzyme is less dependent on temperature (lower $\Delta H^\ddagger$) than the mesophilic counterparts (higher $\Delta H^\ddagger$) (Siddiqui et al., 2006).

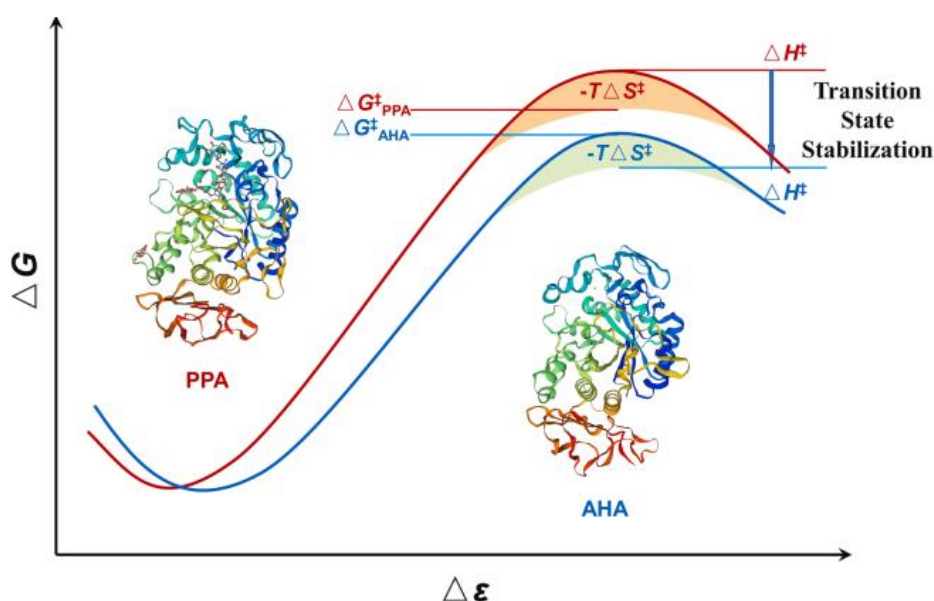


Figure 10. Thermodynamic activation profiles between different temperature-adapted homologous enzymes (psychrophilic α -amylases AHA and its closest structural homologue mesophilic α -amylases, PPA (D'Amico et al., 2000) (D'Amico et al., 2003)

Generally, these cold-adapted enzymes are sensitive to heat, and will be unstable and heat-labile at temperatures that are not detrimental for their mesophilic counterparts (Gerday et al., 2000); (Feller and Gerday, 2003); (Duarte et al., 2018).

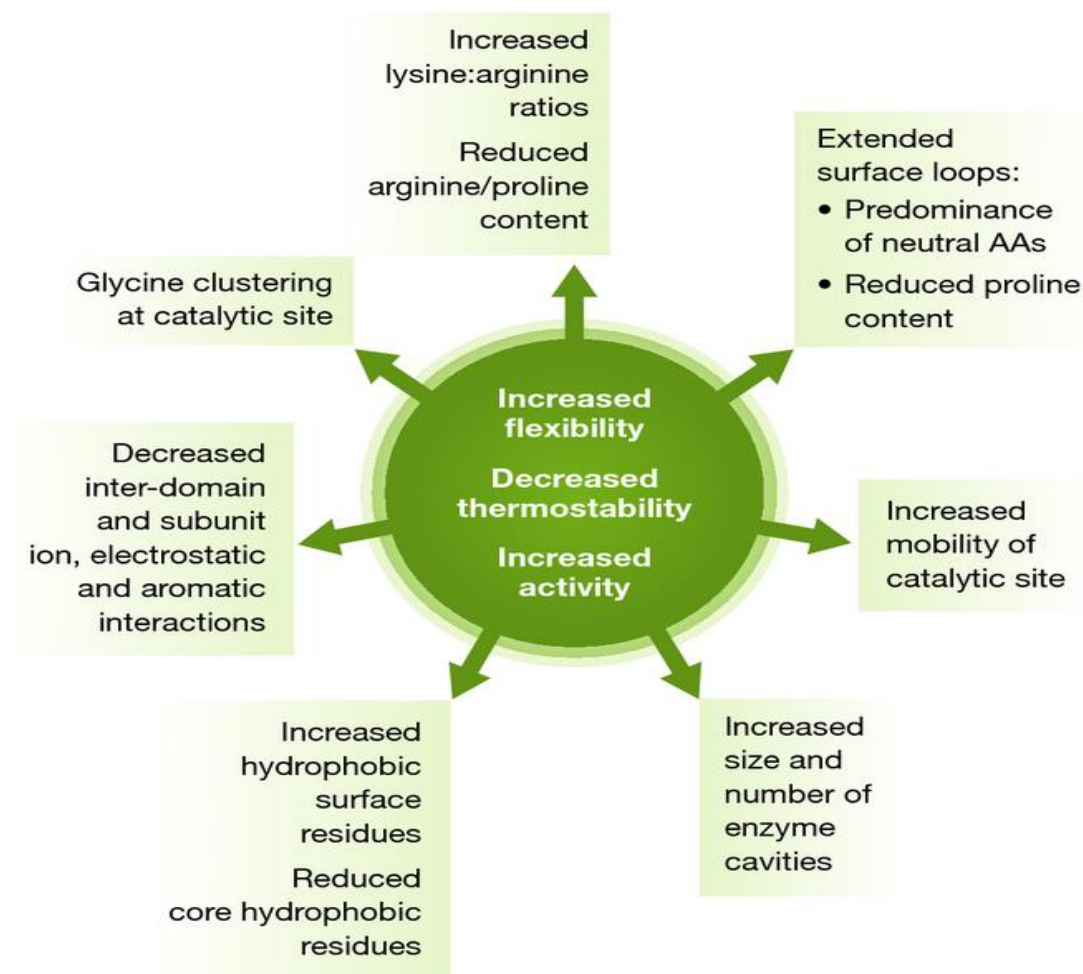


Figure 12. Common structural modifications of psychrophilic enzymes resulting in decreased thermostability, increased flexibility and increased specific activity

(De Maayer et al., 2014).

4.2.4. Chaperones

Protein and RNA/DNA chaperones, which facilitate efficient protein and RNA/DNA folding, respectively, play important roles in counteracting protein misfolding and aggregation as well as low-temperature stabilisation of RNA and DNA secondary structures. DNA and RNA chaperones are important in maintaining efficient transcription, translation and DNA replication. They are transiently produced as part of the coldshock response in mesophilic and thermophilic microorganisms but are often continuously overexpressed as coldacclimation proteins or upregulated at low temperatures in psychrophiles (Lim et al., 2000).

4.2.5 Metabolic adjustments:

Recent studies making use of modern omics approaches such as genomics, transcriptomics and proteomics have revealed a number of additional traits common to various cold adapted microorganisms (**Tribelli and Lopez., 2018**).

In particular, studies have indicated various metabolic adjustments at low temperatures, including a downregulation of primary metabolism pathways and substitution with abridged or alternative secondary pathways, as well as accumulation and metabolism of reserve compounds. Oxidative metabolic processes, namely, glycolysis, the pentose phosphate pathway, the TCA cycle and/or the electron transport chain, as well as pathways involving metal ions and molybdopterin metabolism are reported to be downregulated at low temperatures in some psychrophiles (**Medigue and al., 2005**); (**Piette and al., 2011**); (**Tribelli et al., 2015**).

4.3. Genetic support of cold adaptation

4.3.1. The role of genome sequencing

The sequencing and analysis of whole genomes (genomics) is a powerful tool that is being applied to many microorganisms in order to identify the distinguishing molecular features and gene content of those microorganisms. Genomic analyses allow the detection of trends that may only be apparent at the genome level rather than at the level of individual genes, due to differences resulting from genetic drift. For example, biases in amino acid abundance of the genomes of hyperthermophiles have been reported, and reflect adaptations to living at high temperatures (**Singer and Hickey, 2003**).

In addition, examination of gene content has been used to better understand the metabolic capabilities of the smallest microorganisms such as *Mycoplasma genitalium* and *Chlamydia* (**Fraser et al., 1995**); (**Read et al., 2000**). Similarly, genomics can be used to investigate cold adaptation of psychrophiles at the molecular level by analyzing amino acid composition, codon usage, and nucleotide content, and at the level of genes by examining gene content and other unique features. To date, only ten cold-adapted microorganisms have been completely sequenced (Table 05), accounting for a mere 2.5% of all microbial genomes sequenced (10 of 398). All of these cold-adapted

organisms have been isolated from polar regions and have provided valuable information about cold adaptation. Comparative studies of cold adaptations in these organisms should reveal which adaptations are common to all psychrophiles and which are specific to the particular environment each psychrophile inhabits, or to the particular family of organisms they represent **(Rivkina et al., 2000); (Bock and Eicken, 2005).**

Table 5. Psychrophilic microorganisms whose genomes have been sequenced **(Bakermans et al., 2009).**

<i>Colwellia psychrerythraea</i>	Arctic sea ice
<i>Desulfotalea psychrophila</i>	Arctic marine sediment
<i>Methanococcoides burtonii</i> , <i>Methanogenium frigidum</i>	Ace Lake, Antarctica (salinity close to sea water)
<i>Polaribacter filamentus</i>	Arctic surface sea water
<i>Polaribacter irgensii</i>	Antarctic sea water
<i>Pseudoalteromonas haloplanktis</i> <i>TAC125</i>	Antarctic sea water
<i>Psychrobacter arcticus</i> 273-4, <i>Psychrobacter cryohalolentis</i> K5	Siberian permafrost
<i>Psychromonas ingrahamii</i>	Arctic sea ice

4.3.2. Review of a gene sequencing protein cold adaptation

Bacteria respond to a rapid temperature drop (cold shock) by a transient induction of cold induced proteins (Cips) **(Graumann and Marahiel, 1996); (Phadtare, 2004).**

In *Escherichia coli* numerous Cips have been identified so far including, e.g., cold shock protein (Csp) family **(Yamanaka et al., 1998)**, RNA helicase csdA **(Charollais et al., 2004)**, exoribonucleases PNPase and RNaseR **(Phadtare, 2012)**, initiation factors 2 α and 2 β , NusA and RecA **(Jones et al., 1987).**

Cold shock proteins are small nucleic acid-binding proteins ranging from 65 to 75 amino acids in length **(Graumann and Marahiel, 1996); (Czapski and Trun, 2014)** CspA was first described in *E. coli* **(Goldstein et al., 1990).**

Xia et al. (2001) discovered that in *E. coli* four out of nine csp genes (cspA, cspB, cspE, and cspG)

Figure 13 and Table 6 showing details about cold shock protein CspA gene.

Figure 13. Cold shock protein CspA gene **(NCBI, 2024).**

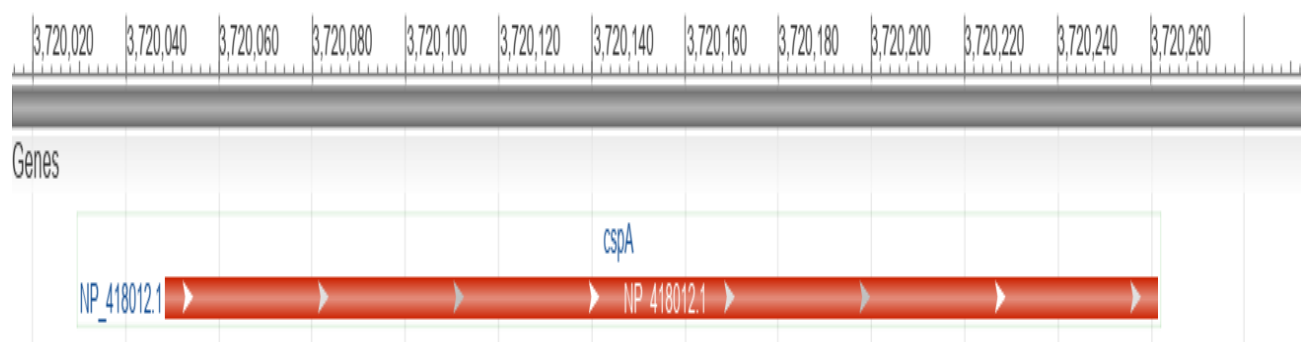


Table 6. Cold shock protein CspA gene details **(NCBI, 2024).**

Gene symbol	cspA
Gene description	cold shock protein CspA
Gene type	protein coding
Also known as	ECK3543
Sequence	NC_000913.3 (3720049..3720261)

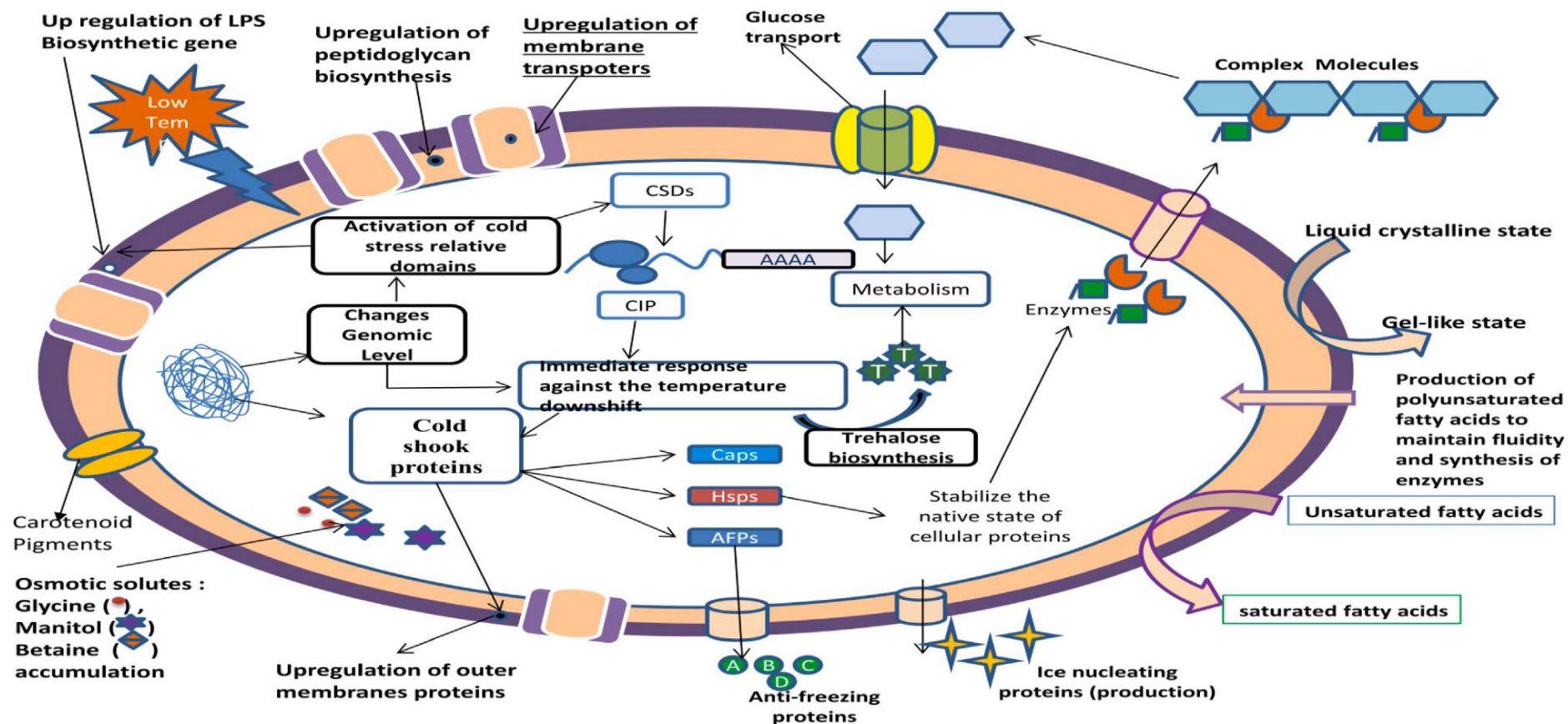


Figure 14. An overall view of adaptive modification in DNA, proteins and lipids of the bacterial 1592 cell during cold shock response (Cavicchioli et al., 2002); (Maayer et al., 2014).

5. Effects of psychrotrophs on dairy products

5. 1. Milk composition

Milk is a fluid secreted by the female of all mammalian species, of which there are about 4500 extant species (about 80% of mammalian species are extinct), and it meets the complete nutritional requirements of the neonate. The principal requirements are for energy (supplied by lipids and lactose and, when in excess, by proteins), essential amino acids and amino groups for the biosynthesis of nonessential amino acids (supplied by proteins), essential fatty acids, vitamins, inorganic elements, and water. The nutritional requirements of the neonate depend on its maturity at birth, its growth rate, and its energy requirements, which in turn depend mainly on environmental temperature. Therefore, the gross composition of milk shows large interspecies differences, which reflect these requirements **(Fox and McSweeney, 1998); (Fuquay et al., 2011); (McSweeney and Fox, 2013).**

The milk of certain domesticated animals and dairy products derived from them are major components of the human diet in many parts of the world. Domesticated goats, sheep, and cattle have been used for milk production since about 8000 BC **(Kindstedt, 2012).**

Milk is a very complex fluid containing several hundred molecular species (several thousand if all triglycerides are counted individually). The principal constituents are water, lipids, sugar (lactose), and proteins. In addition, there are numerous minor constituents, mostly at trace levels for example, minerals, vitamins, hormones, enzymes, and miscellaneous **(O'Mahony and Fox., 2014).**

In the following sections, the chemistry of milk carbohydrates, lipids, proteins, salts, and some minor constituents are described.

5.1.1. Carbohydrates

5.1.1.1. Lactose

The principal carbohydrate in the milk of most species is the reducing disaccharide, lactose, which is composed of galactose and glucose linked by a beta 1-4 glycosidic bond. Its concentration varies from 0 to 10% and milk is the only known source of lactose **(Fox and McSweeney., 1998); (McSweeney and Fox., 2009).**

5.1.1.2. Oligosaccharides

In addition to lactose, the milk of most, and probably all, species contains other free saccharides, mainly oligosaccharides (OSs), the concentration, proportions and types of which show large interspecies differences. The concentration of OSs is higher in colostrum than in milk (**Newburg and Newbauer., 1995**); (**Urashima et al., 2001**); (**Mehra and Kelly., 2006**); (**Urashima et al., 2009**); (**Urashima et al., 2011**).

5.1.2. Lipids

Lipids (commonly called oils or fats, which are liquid or solid, respectively, at ambient temperature) are those constituents of tissues, biological fluids, or foods that are soluble in an apolar solvent (**Fox and McSweeney., 1998**); (**Fox and McSweeney., 2006**).

5.1.3. Fatty Acids

Fatty acids (FAs) are carboxylic acids with the general formula $R-COOH$, where the alkyl group, R, is a hydrocarbon chain containing 3 to 25 carbons (total number of carbons, 4 to 26), which may be saturated or unsaturated (one to six double bonds), and is usually straight (normal), with small amounts of branched chain, hydroxyl, and keto (oxo) acids. The vast majority of FAs have an even number of carbon atoms because they are synthesized from, and elongated by adding, a 2-C compound, acetyl CoA, on each cycle of the multienzyme fatty acid synthetase (FAS) (**O'Mahony and Fox., 2014**).

5.1.4. Milk proteins

Milk proteins contribute ~25% of the daily protein intake of the U.S. diet, suggesting the importance of milk proteins in human nutrition. Milk proteins include caseins (CN), whey proteins, milk fat globule membrane (MFGM) proteins and enzymes that naturally occur in milk (**Farrell et al., 2004**).

5.1.4.1. Casein

The main protein found in milk is casein, especially in cow's milk. Due to casein, milk is white and opaque appearance. This protein is combined with calcium and phosphorus as clusters of casein molecules, called micelles / calcium phospho-

caseinate. Casein protein consists of different proteins, α -casein, β -casein and κ -casein (Southward, 2002); (Sawale, 2017).

Except for κ -casein, all caseins are phosphoproteins, mainly serine and threonine. α -casein is the main casein, which contains 8-10 phosphate groups and is difficult to dissolve in water. β -casein contains 5 phosphate residues, which is more hydrophobic than the other two residues and hardly soluble in water (Figure 15). Kappa-casein is a glycoprotein containing negatively charged carbohydrate moieties. Since, α -casein and β -casein are highly phosphorylated, they are very sensitive to the concentration of calcium salts, and that is, they will precipitate with excess Ca^{2+} ions. In milk, κ -casein combines with α -casein and β -casein to form micelles, so it will not precipitate in the presence of calcium ions (De, 2002); (Bhat, 2016).

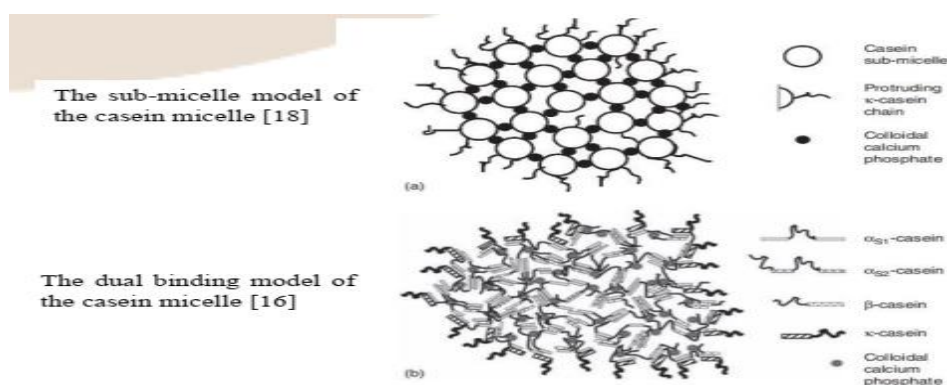


Figure 15. The structure of casein micelle (Fox and O'Mahony 2019).

5.1.4.2. Whey protein

The by-product whey is obtained from cheese and casein manufacturing containing serum proteins called as whey proteins (Anema, 2020). It is more beneficial as it doesn't contain any antinutrients as that of soy proteins (Geiser, 2003). It consists of α -lactalbumin, β -lactalbumin, lactoglobulin, glycomacropeptides, IgG, bovine serum albumin and lactoferrin (minor protein) in the proportions 11.3–16.5%, 37.9–49%, 15–20%, 5.0–8.0%, 3.0–5.0% and 1.3–1.8%, respectively. Other Minor Protein present in whey: lactoferrin, glycoproteins, lactoperoxidase and transferrin. In addition, whey protein is rich in amino acids, less carbohydrates, and less fat. It contains cysteine which can make glutathione (Gangurde, 2011).

5. 2. Psychrotrophs enzymes

In general, the majority of psychrotrophic microorganisms isolated from milk have the ability to produce hydrolytic enzymes that break down the major milk constituents such as protein, fat and lecithin **(Sørhaug and Stepaniak, 1997); (Baur et al., 2015a); (von Neubeck et al., 2015)**. Several peptidases, lipases and phospholipases produced by psychrotrophic bacteria isolated from milk have been described in the literature **(Sørhaug and Stepaniak, 1997); (Chen et al., 2003); (Samaržija et al., 2012)**.

Many of these hydrolytic enzymes are heat resistant and consequently retain part of their activity after conventional heat treatment applied in dairy industries such as pasteurization and UHT treatment. Regarding quality and economic aspects, the thermostable hydrolytic enzymes have the most significant effect in dairies since these enzymes lead to flavor defects and technological problems such as sedimentation and gelation in UHT milk, rancidity and flavor defects in milk powder and cheese during their shelf life **(Sørhaug and Stepaniak, 1997)**.

The indigenous milk enzymes are minor constituents of milk and are secreted in milk from following sources **(Fox and Kelly, 2006a)**.

1. From blood plasma through defective mammary cells.
2. From secretory cell cytoplasm, some of which may possibly be entrapped within some fat globules by the surrounding milk fat globule membrane (MFGM) at the time of secretion from the cell.
3. From the milk fat globule membrane (MFGM) itself, the outer layer of which is obtained from the apical membrane of the mammary cell and which, in turn, originates from the Golgi membranes; this may be the most possible source of secretion of indigenous enzymes in milk.
4. Somatic cells (leukocytes), which may enter the mammary gland from the blood to fight bacterial infection (mastitis), and therefrom enter milk.
5. From contaminating microorganisms which may secrete extracellular enzymes or release intracellular enzymes after the cells have died and lysed **(Nikam et al., 2023)**.

5. 2.1. The main proteolytic enzymes found in raw milk

The term proteolytic enzyme includes all the hydrolases that act on proteins, or further degrade the fragments of them. A few synonyms of proteolytic enzymes such as peptide-bond hydrolase, peptidase or protease could be found in the literature albeit the International Union of Biochemistry and Molecular Biology (IUBMB) recommend the term peptidase (**Barrett, 2001**).

The main problem of the peptidases secreted by psychrotrophic bacteria is that they are heat-stable, which means that they resist at least pasteurization but it is not always described to what extent these enzymes also resist the higher temperatures of UHT. Milk proteolysis can occur through the action of a natural enzyme, plasmin, or through enzymes produced by psychrotrophs (**Nielsen, 2002**).

5. 2.1.1 Plasmin

Plasmin is the main proteolytic enzyme found naturally in fresh milk (**Nielsen, 2002**). It mainly hydrolyzes β - and α S2-casein and, to a lesser extent, α S1-casein, while κ -casein is resistant to hydrolysis by this enzyme (**Recio et al., 2000**).

Enzymatic hydrolysis that occurs during heat treatments at elevated temperatures or cheese processing causes the removal or dissociation of κ -casein, thus, affecting the electrostatic and steric stabilization of the micellar surface and promoting the aggregation of micelles, which leads to clot formation (**Fox and McSweeney, 2004**).

5.2.2. Lipolytic enzymes

Undesirable lipolysis of milk and dairy products has not been studied as much as undesirable proteolysis. Exogenous lipases produced by psychrotrophic bacteria can hydrolyze milk fat and release free fatty acids (FFAs), mono- and di-acylglycerols and glycerol. The lipolytic activity of psychrotrophs is species-specific.

According to **Decimo et al. (2016)** bacterial triacylglycerol hydrolysis may occur to a greater or lesser extent, but the type and amount of released FFAs are not easily predictable. The lipolytic activity generates undesirable product flavors such as

rancid, unclean, soapy, or bitter, making the product barely acceptable to the consumer **(Deeth, 2006)**.

Among the lipolytic psychrotrophic bacteria, *Pseudomonas* spp. is the predominant Gram-negative group found whereas *Bacillus* spp. is the predominant Gram-positive group **(Chen et al., 2003); (Decimo et al., 2014); (Vithanage et al., 2016)**.

5.2.2.1 Lipase

Lipase (EC 3.1.1.3) breaks down triglycerides into free fatty acids and glycerol (Figure 16). From technological point of view, lipase is the most significant indigenous milk enzyme to dairy industry as it causes undesirable rancid flavor in milk due to hydrolytic rancidity while in certain varieties of ripened cheeses, it contributes desirable flavor too. Usually, the substrate (soluble esters) required for enzyme activity is not accessible inherently. But, during the rapid cooling of milk, enzyme dissociates from the casein micelles and interacts to fat globules imparting in “spontaneous lipolysis” and mild mechanical treatments, such as agitation, foaming, freezing or homogenization, cooling/warming, to unpasteurized milk leads to disruption of MFGM resulting in interaction between the casein micelles and its associated lipase **(Corbin, 1965)**.

Apart from to contribute off-flavors in milk and dairy products, lipolysis causes reduction in the surface activity of milk due to liberation of free fatty acids, and as a result, decrease in milk foaming capacity, for example, in cappuccino coffee, and also decrease in its whipping characteristics and churning time **(Deeth and Fitz-Gerald, 2009)**.

Milk contains multiple forms of lipase which are due to self-association of lipase or its association with other milk proteins and 90% of lipase in milk is interlinked with the micellar casein. It is sensitive to heat and light **(Fox and Kelly, 2006a)**.

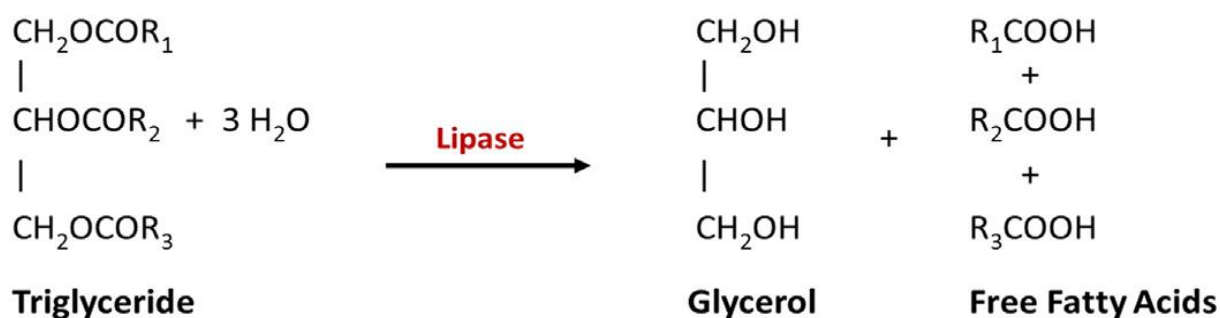


Figure 16. Reaction catalysed by lipase (Nikam et al., 2023).

5.2.2.2 Lipoprotein lipase (LPL)

Lipoprotein lipase LPL (EC 3.1.1.34) is a 90 kDa homodimer wherein each monomer contains 450 amino acid and 8% carbohydrates. The enzyme mainly originates from the vascular endothelial surfaces bounded with heparin sulfate chains playing an important role in lipid synthesis in the mammary gland. The optimum pH and temperature for LPL are 9.0 and 37°C. The catalytic activity k_{cat} of LPL is 3000/s under optimum conditions and milk has adequate lipase (12 mg/ L; 1020 nM) to cause hydrolytic rancidity in short period, that is, within 10 s. However, LPL causes hydrolytic rancidity in most of the milk samples only if the MFGM is damaged, for example, by agitation, foaming, cooling/warming, freezing, or homogenization. But, some bovine milk samples undergo spontaneous lipolysis, with no activation step. Such milk sample contains high level of apolipoprotein C-II, which activates LPL. Normal milk has a higher level of proteose peptone-8 (PP-8), which mainly inhibits LPL (Girardet et al., 1993); (He et al., 2012); (Fox et al., 2015).

5.2.2.3 Esterases

In addition to the well-documented lipase system, bovine milk contains several other carboxyl ester hydrolases, collectively referred to as esterases. Esterases differ from lipases by their substrate preference for soluble rather than emulsified esters form as well as their preferences for hydrolysing esters of short rather than long-chain acids.

According to Kitchen. (1985), milk possesses several esterases, mainly significant are arylersterases (EC 3.1.1.7), carboxylesterase (EC 3.1.1.1), and cholinesterase (EC 3.1.1.8). Arylersterase (also known as solalase/A-esterases) has received considerable attention because of its elevated levels in colostrum and mastitic

milk. Since its level in mastitic milk correlates well with other indices of mastitis, it has been suggested as a sensitive indicator of the disease. The enzyme originates from blood, where its activity is up to 2000 times that in milk. Carboxylesterase (B-esterases) activity is elevated in mastitic milk and colostrum. **(Kitchen, 1981).**

5.2.2.4. Phospholipase C

The production of different phospholipases has been reported for Gram-negative and Gram-positive psychrotrophs **(Sørhaug and Stepaniak, 1997).**

The phospholipase most studied is phospholipase C, which can be either hemolytic or nonhemolytic. Phospholipase C activity has been detected in the genera *Pseudomonas*, *Bacillus*, *Serratia*, *Hafnia*, *Acinetobacter*, and *Microbacterium* in raw milk by **(Vithanageetal, 2016).**

According to **De Jonghe et al. (2010)**, in the genus *Bacillus* only the species *B. cereus* is able to produce the phospholipase C. The presence of this enzyme was not observed for the species *B. licheniformis* or *B. subtilis*. This enzyme is particularly heat stable **(Sørhaug and Stepaniak, 1997)** and disrupts the integrity of the MFG membrane **(Craven and Macauley, 1992); (Shah, 1994).**

The phospholipase C of *P. fluorescens* is well known as a heat-stable enzyme, presenting high residual activity after pasteurization and UHT treatment **(Sørhaug and Stepaniak, 1997).**

Vithanage et al. (2016) observed that about 25% of *Bacillus* and *Pseudomonas* strains isolated from raw milk presented 50 to 75% of residual phospholipase C activity after heat-treatment of 140°C during 4 s.

5.2.2. Example of peptidase from psychrotrophic bacteria

AprX peptidase is the most studied heat-stable peptidase produced by the microbiota found in raw milk, although other species isolated from milk samples may also produce peptidases different from AprX, such as *Klebsiella oxytoca* **(Tondo et al., 2004)** or *Serratia liquefaciens* **(Decimo et al., 2014).** *Bacillus* spp. show more diverse proteolytic activity than *Pseudomonas* spp., and many species may secrete more than one type of extracellular and intracellular peptidase **(Nabrdalik et al., 2010).**

AprX is a peptidase of 45 to 50 kDa encoded by the *aprX* gene located on the *aprX-lipA* operon, which contains eight genes and spans 14 kb (McCarthy et al., 2004). In general, AprX is rich in alanine and glycine residues and poor in cysteine and methionine residues (Dufour et al., 2008).

The AprX protein is highly conserved within *Pseudomonas* species (76–99% similarity for AprX of *P. fluorescens* group), but is more heterogeneous between species (57–69% similarity for AprX between strains of *P. fluorescens* and *P. fragi*) (Marchand et al., 2009b); (Matéos et al., 2015).

In addition to the four AprX sequence groups (with one group split into two subgroups) identified within *Pseudomonas* raw milk isolates by (Marchand et al., 2009b). A fifth group was added recently including Mozzarella isolates (Caldera et al., 2016).

AprX exhibits activity in a large range of pH (4.5–10) with an optimum activity between 7.5 and 9, which proves that AprX is an alkaline peptidase. AprX generally exhibits activity in a large range of temperatures (0–55°C) with optimal activity between 37 and 47°C (Dufour et al., 2008); (Martins et al., 2015); (Matéos et al., 2015).

Inhibition studies revealed that AprX was inhibited by typical divalent-ion chelators such as EDTA (Ca²⁺ and Zn²⁺ chelator), EGTA (Ca²⁺ chelator), *o*-phenanthroline (Zn²⁺ chelator) while serine peptidase inhibitors (PMSF and leupeptin) did not affect activity of the enzyme (Liao and McCallus, 1998); (Dufour et al., 2008); (Matéos et al., 2015).

AprX may hydrolyze the four types of casein (as1, as2, b, and k) with a large activity spectrum (Baglinière et al., 2013).

Matéos et al. (2015) have shown that cleavage sites are mainly found in hydrophobic areas of casein.

5.4. Spoilage

Since people began producing and storing food products, spoilage and food loss and waste became important issues for human with regards to food safety and security. Nowadays, up to one third of all food is spoiled or squandered before consumption, which represents about 1.3 billion tons per year. These losses are the results of one or

more problems occurring in the supply chain, from initial agricultural production down to the consumer level (**Sangoyomi et al., 2010**).

Concerning food spoilage, a food product can be physically, chemically, or microbiologically spoiled. Parasites, bacteria, and/or fungi are the main agents causing microbial spoilage. Certain parasites and bacteria are of public health concern because they are common contaminants of foods and are often responsible for food outbreaks. Nonetheless, being ubiquitous in nature, fungi are also very common in food. For a long time, besides altering food properties, they were not regarded as particularly detrimental to human health, although *Claviceps purpurea* has been related to ergotism outbreaks in the Middle Ages more than 200 years ago (**Torkar et al., 2008**).

It is only in recent times that several mycotoxins produced by certain fungal species have been regarded as a major threat to human and animal health, especially in developing countries, being responsible for different adverse health effects (**Jay, 1992**).

5.4.1. Definition of spoilage

Spoilage is defined as becoming unfit for human consumption due to changes in the food's texture, colour, odour or flavour. These changes can occur through chemical or microbiological mechanisms (**Rawat, 2015**).

5.4.2. Spoilage of milk and milk products

Milk spoilage encompasses a range of undesirable quality changes that can be identified by both producers and consumers through various indicators such as abnormal flavours, colour shifts, and inconsistent textures. Terms such as "acidic aftertaste," "chalky mouthfeel," and "sourness" are often associated with spoilage (**Bandler and Barnard, 1984**).

Due to its complex metabolic structure and high-water content, milk provides a favourable environment for harmful bacteria to grow and spread. Both primary and secondary factors can contribute to milk spoilage (**Lu and Wang, 2017**).

Dairy products may be spoiled due to the action of physical agents including moisture absorption and desorption, chemical reactions like lipid oxidation, browning, etc., and most importantly due to the action of microbes in form of vegetative cell and

even due to the production of toxic metabolites in to milk environment. Additionally, some aspects of food, like moisture and oxygen levels can encourage the growth of microorganisms (**Saha et al., 2024**). The types of spoilage found in milk and milk products are shown in Figure 17.

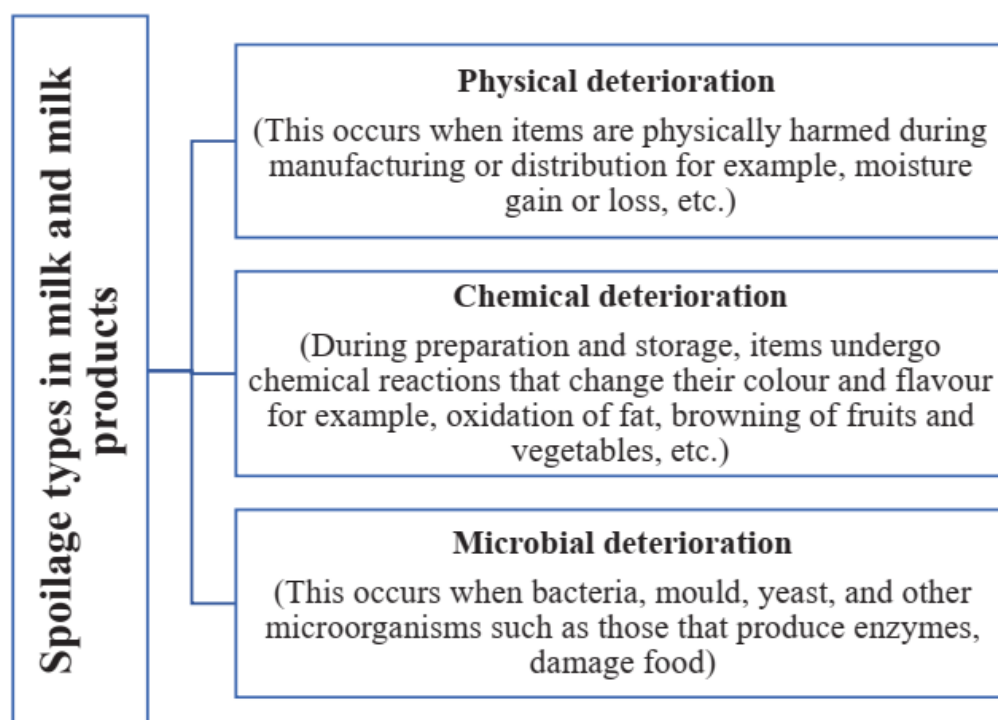


Figure 17. Types of spoilage of milk and milk products (**Saha et al., 2024**).

5.4.3. Causes of spoilage of milk and milk products

Milk obtained from animals such as cow, buffalo, sheep, and goat milk are sterile when they are inside udder, but immediately after milking, and it loses its sterility and can be contaminated in a number of ways. Contamination sources in farm environments encompass various factors, including the animals themselves, comprising both internal and external aspects such as udders and coats, along with elements like air quality and feed storage for livestock. Various potential sources of contamination exist in dairy processing facilities, such as the quality of water utilised, inadequate storage temperatures, prolonged storage durations, insufficient ventilation, cross-contamination risks, condition of milk handling equipment and storage tanks, presence of biofilms in plant pipes, and prolonged delays between milk reception and storage (**Lu and Wang, 2017**).

5.4.4. Forms of microbial spoilages

There may be several forms of microbiological spoilage. From the very natural souring of milk to discolouration of milk products. Majorly, gas production, ropiness, off flavour development is considered as the parameter for microbial spoilage of food materials. The Table 7 comprised almost all the possible microbial spoilage with the causative microorganisms (Erkmen and Bozoglu, 2016).

Table 7. Types of microbial spoilage in milk and milk products (Saha et al., 2024).

Nature of spoilage	Sign of spoilage	Causative organism
Natural souring or curdling	Sour milk, Curd formation	<i>Lactobacillus</i> spp., <i>Streptococcus</i> spp
Gas production	Explosion of curd	<i>Clostridium</i> spp., <i>Bacillus</i> spp., Coliform
Ropiness/Sliminess	Rope like structure	<i>Alcaligenes</i> spp., <i>Klebsiella</i> spp., <i>Enterobacter</i> spp.
Proteolysis	Putrid off-flavours bitterness in UHT processing	<i>Alcaligenes</i> , <i>Flavobacterium</i> , <i>Bacillus</i> , <i>Micrococcus</i> , <i>Pseudomonas</i> spp.
Sweet curdling	pH becomes alkaline and leads to curd formation	<i>Bacillus</i> spp., <i>Proteus</i> spp., <i>Micrococcus</i> spp.
Lipolysis	Rancid flavour Off flavours	<i>Pseudomonas</i> , <i>Staphylococcus aureus</i> , <i>Bacillus</i> spp., <i>Penicillium</i> spp.
Bitty cream	Cooked flavour in sterilised milk	<i>Bacillus cereus</i> , <i>Bacillus cereus</i> var. <i>mycoides</i>

Off-flavours	Malty flavour Fruity flavour Musty potato flavour Phenolic flavour Medicinal flavour Acid flavour	<i>Streptococcus lactis</i> var. <i>Pseudomonas fragi</i> <i>Pseudomonas muciodolens</i> <i>Maltigenes</i> <i>Bacillus circulans</i> <i>Enterobacter aerogens</i> <i>Streptococcus lactis</i>
Discolouration	Blue colour Yellow colour Red colour Brown colour Grey colour	<i>Pseudomonas syncyanea</i> <i>Pseudomonas synxantha</i> <i>Micrococcus roseus</i> <i>Pseudomonas putreficans</i> <i>Clostridium</i> spp

5.4.5. Classification of spoilage organisms

Based on optimum growth temperature, the spoilage organisms can be classified into four groups (Figure 18). The main reason behind the survival of different category of bacteria at different temperature is the difference in the cell membrane composition of different bacteria. As milk is perishable commodity, and it is expected to maintain proper cold chain while producing milk and its derivatives, psychrotrophs are the matter of concern as it can survive at refrigerated conditions (**Saha et al., 2024**).

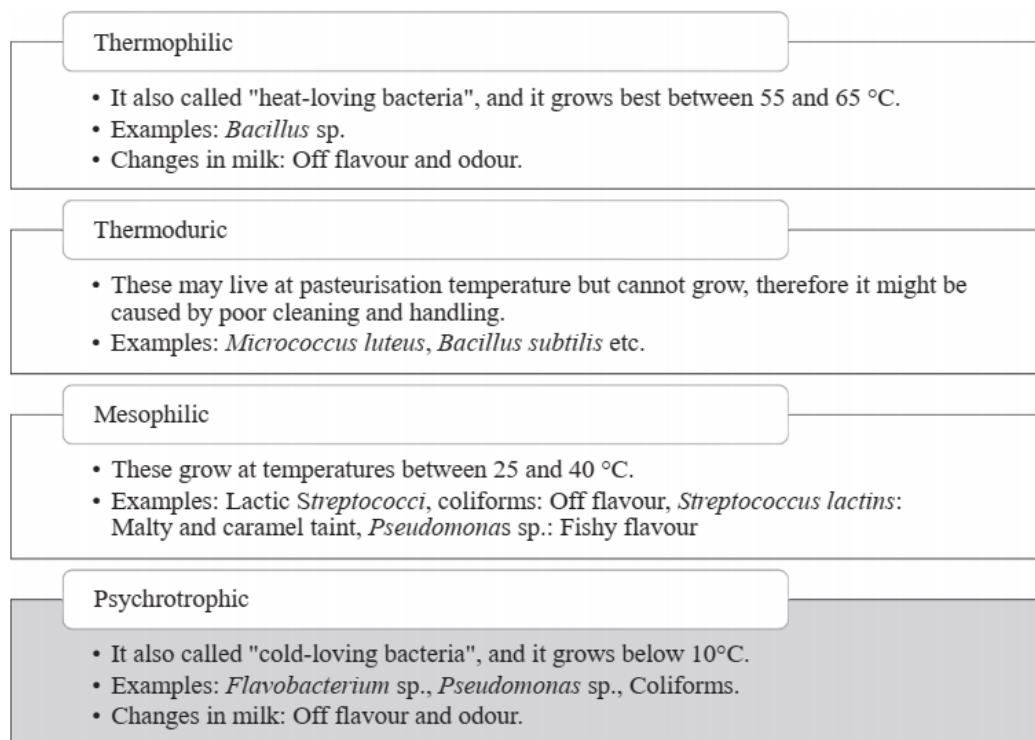


Figure 18. Classifications of spoilage microorganisms (Saha et al., 2024).

5.4.6. Psychrotrophic bacteria

A subset of microorganisms known as psychrotrophs or psychrotolerant strains are able to endure under conditions that are normally just 7°C in temperature (Moyer and Morita, 2007).

Although these microorganisms are uncommon in the air, they are widely distributed in water, soil, and plants across the globe. Their maximal metabolic activity occurs between 20 and 30°C (Schinik, 1999).

This feature is critical for guaranteeing food safety and preservation because many food products are exposed to freezing temperatures during manufacture, transit, processing, and storage (Russell, 2002); (Beales, 2004).

Refrigeration is the most widely used food preservation method, which also applies to milk and milk products. As stated by Beales. (2004), it can be utilised either on its own or in conjunction with other preservation techniques such chemical application. From the time the milk is harvested on the farm until it is processed, it is refrigerated for a lengthy time (O'Brien and Guinee, 2011).

Chilling raw milk often reduces its mesophilic and thermophilic bacterial content, but it also feeds the growth of psychrotrophic bacteria **(Barbano et al., 2006); (De Jonghe et al., 2011); (Samaržija et al., 2012).**

According to **Lafarge et al. (2004)** and **Xin et al. (2017)**, raw milk's microbial community shifts towards psychrotrophic bacteria during storage, which lowers the milk's quality. Most cultivable psychrotrophic bacteria found in milk belong to gram-negative genera, including *Pseudomonas*, *Achromobacter*, *Aeromonas*, *Serratia*, *Alcaligenes*, *Chromobacterium*, and *Flavobacterium* spp. Although they are found in much lesser quantities, other Gram-positive taxa include *Bacillus*, *Clostridium*, *Corynebacterium*, *Streptococcus*, *Lactobacillus*, and *Mycobacterium* spp. **(Sørhaug and Stepianiak, 1997); (Mcphee and Griffiths, 2011).**

The next section provides a brief discussion of the two most common psychrotrophic organisms that cause spoilage: *Pseudomonas* species and *Bacillus* species

5.4.6.1. Psychrotrophic spoilage by *Pseudomonas* spp.

The most common psychrotrophic bacterium found in milk and milk products belongs to the *Pseudomonas* genus, which is essential to the dairy sector **(Marchand et al., 2009).**

Milk and milk products can spoil because of some psychrotrophic *Pseudomonas* species, including *P. fluorescens*, *P. fragi*, *P. putida*, and *P. putrefaciens*. Their optimal growth temperature is over 200°C, and they can survive in a temperature range of 4 to 420°C **(Chakravarty and Gregory, 2015).**

Pseudomonas species are the most common microorganisms in both raw and pasteurised milk during milk deterioration. *Pseudomonas* is an enormously varied and important bacterial genus that can be found in a variety of natural environments, including soil, household dust, fresh water, clouds, aseptic solutions, cosmetics, pharmaceutical items, and medical equipment, etc. **(Franzetti and Scarpellini, 2007).**

Therefore, it is not surprising that *Pseudomonas* species are frequently linked to spoiling, especially in refrigerated milk. Surfaces of milking, storage, and transportation equipment that have not been properly cleaned result in significant contamination by

Pseudomonas. Additionally, contamination can occur during the filling process following pasteurisation (**Oliveira et al., 2015**).

Pseudomonas spp. hydrolytic enzyme activity in cold-stored raw milk is 100%. However, after pasteurisation and 30-40% following milk sterilisation, several of these enzymes can still function at levels of 60-70% and 30-40%, respectively. This has led to a great deal of research and documentation on these bacterial enzymes (**Fox and Stepainak, 1983**); (**Sørhaug and Stepianiak, 1997**); (**Braun et al., 1999**); (**Koka and Weimer, 2001**); (**Oliveira et al., 2015**).

Proteinase, lipase, and phospholipase are the three hydrolytic enzymes that *Pseudomonas* spp. may make; however, certain strains can only manufacture one or the other (**Matta and Punj, 1999**). The heat-stable extracellular peptidases that *Pseudomonas* releases during cold storage can continue to function even after pasteurisation or treatment at extremely high temperatures (UHT) (**Marchand et al., 2009**); (**Glück et al., 2016**).

Proteases contribute to the development of bitterness in milk, gelation of UHT sterilised milk, and reduced yield of soft cheese. *Pseudomonas* spp. proteinases are capable of degrading κ , α s1, and β -casein present in milk, leading to the physical breakdown of the colloidal system. The production of coagulation and the development of a very bitter flavour in the finished product are frequent symptoms of this deterioration (**Hu et al., 2018**).

Gram-negative psychrotrophic bacteria, such as *pseudomonas* spp., produce lipases and phospholipases that catalyse the hydrolysis of milk fat and lecithin, resulting in the liberation of free fatty acids (**Hu et al., 2018**).

The quantities of free fatty acids in milk fat often exhibit a range of 0.5 to 1.2 mmol per 100 g. The development of a rancid taste in dairy products may be attributed to an elevation in the concentration of free short-chain fatty acids (C4-C8). Medium chain fatty acids are the primary cause of soapy, unclean, or bitter odours in the products (**Hu et al., 2018**).

According to **Samarzija et al. (2012)**, there is a prevailing belief that free long-chain fatty acids within the range of C14-C18 have no significant effect on the flavour characteristics of milk and milk products.

5.4.6.2. Psychrotrophic spoilage by *Bacillus* spp.

Gram-positive rods known as *Bacillus* spp. can either generate endospores and be motile or not. They include a vast variety of bacteria with different nutritional needs and the capacity to endure a variety of temperatures, pH levels, and osmotic pressures. Due to their varied physiological traits, it is difficult to standardise methods for removing these bacteria from milk and dairy products and to define the circumstances under which they should be inactivated (**McGuiggan et al., 1994**); (**Francis et al., 1998**).

The spore content of milk was strongly associated with the degree of contamination of the teats with soil. High water content of soil, low evaporation of water and dirty access alleys were the most important factors correlating with high spore concentrations (**Christiansson et al., 1999**).

In the genus *Bacillus*, *B. stearothermophilus*, *B. licheniformis*, *B. coagulans*, *B. cereus*, *B. subtilis*, and *B. circulans* are the most frequently isolated species from raw and heat-treated milk and dairy products. These facultatively anaerobic or aerobic thermoresistant bacteria have spores that activate and change into vegetative forms when milk is heated. In comparison to *Pseudomonas* species, *Bacillus* species' vegetative cells are known to produce a wider range of thermostable extracellular and intracellular hydrolytic enzymes (**Chen et al., 2003**); (**Chen et al., 2004**).

Particularly *B. cereus*, a *Bacillus* species isolated from milk, exhibits proteolytic and lipolytic activities, as well as phospholipolytic activities in about 80% of instances (**Matta and Punj, 1999**); (**Haryani, 2003**). It has been found that raw milk tends to contain more Gram-negative psychrotrophic bacteria than Gram-positive spore-forming bacteria (**Samarzija et al., 2012**).

However, in certain technical environments or when stored at temperatures below 10°C for extended periods of time, such as in supermarkets, Gram-positive spore-forming bacteria tend to become more prevalent. Gram-positive spore-forming bacteria become predominate. It is because gram-negative bacteria are killed during heat treatment, while Gram-positive bacteria, such as *Bacillus cereus*, survive as spores and thus become the main contaminating bacteria. Studies have shown that *Bacillus cereus*

was present in 52% of ice cream samples, 29% of powdered milk samples, 17% of fermented milk samples, and 2% of pasteurised milk samples **(Wong et al., 1988)**.

Some *Bacillus* spp. strains have even been found to thrive in temperatures as low as 2°C **(Griffiths and Phillips, 1990)**.

Unfortunately, the leading cause of milk and milk product degradation, which can result in significant financial losses for the dairy industry, is generally attributed to *Bacillus* species **(Meer et al., 1991);(Brown, 2000)**.

What's more concerning is that certain *Bacillus* species and their enzymes have been linked to harmful foodborne toxins. *B. cereus*, in particular, is a common contaminant in milk and dairy products and can produce several enterotoxins that can cause illness in humans. Haemolytic BL (HBL), non-haemolytic enterotoxin (Nhe), and cytotoxin K (CytK) are a few examples of enterotoxins that have been associated to systemic infections and gastrointestinal disorders in people. After ingesting a contaminated product, these enterotoxins are released into the small intestine. In addition, emetic enterotoxins such cereulids, which *B. cereus* directly secretes into food produce poisoning and result in symptoms including nausea and vomiting within 1-6 h of consumption of the product **(Brown, 2000); (Senesi and Ghelardi, 2010)**.

Compared to *Pseudomonas* species, *Bacillus* species display a wider spectrum of proteolytic activity, with many of them being able to produce different kinds of extracellular and intracellular proteinases **(Samarzija et al., 2012)**.

However, the precise species and strain of *Bacillus*, as well as the temperature, affect how much proteolytic alterations occur. At temperatures over 4 °C, the hydrolysis of the casein occurs most quickly, forming the para-casein complex. At the same temperatures, the breakdown of α - and β -casein is slightly less pronounced and often not seen at 4 °C **(Janstova et al., 2004)**.

Significant lipolytic activity is also present in *Bacillus* species above 4°C, and milk-derived lipases typically indicate selectivity for the hydrolysis of mono- and diacylglycerols. For instance, *B. licheniformis* and *B. cereus* have been linked to significant lipolytic alterations that increase the amount of free fatty acids in milk **(Chen et al., 2004); (Janstov a et al., 2006)**.

Bacillus species generate lipases, which are more thermostable than proteinases. However, both kinds of enzymes are sufficiently thermostable to continue to function even at high milk heat treatment temperatures in milk powder and all other dairy products (Samarzija et al., 2012). The Table 8 summarizes kind of defect caused by psychrotrophic bacteria (Saha et al., 2024).

Table 8. Kinds of psychrotrophic spoilage in different milk and milk products (Saha et al., 2024)

Product	Kind of defect	Cause
Raw milk	Rancid flavour	Increased by the activity of the phospholipase enzyme as a result of lipase synthesis.
	Unclean soapy flavour	fatty acids are formed, and the flavour is rancid (C10-C12 chain)
	Metallic flavour	Released unsaturated fatty acids are converted to aldehydes and ketones by the action of lipases.
Pasteurised milk	Precipitation when milk added to hot beverage (bitty cream) Gelation	Activity of Bacillus spp phospholipases and proteinases, fat destabilisation Thermoresistant proteinases [Gram-positive and Gram-negative of psychrotrophic bacteria]
	Fruity flavour	Synthesis of esters; <i>Pseudomonas fragi</i>
	Sweet curdle	Protein hydrolysis

	Fouling in heat exchangers	Proteases decrease heat stability of proteins
UHT milk	Rancidity (1 to 7 month storage)	Due to the production of heat resistant lipases
	Gelation	Due to the production of heat resistant proteases
Sterilised milk	Rancidity, Off-flavour, Bitterness, Soapy	Due to the action of thermostable lipases and the heat stabilised proteinases, there is a high concentration of free fatty acids and protein breakdown.
	Gelation after 1 week	Thermostable proteinases [Grampositive and Gram-negative psychrotrophic bacteria]
	Sweet curdling after 1-2 weeks	Thermostable proteinases [Grampositive and Gram-negative psychrotrophic bacteria]
Fermented milk	Firm gel, Higher viscosity Bitter, Rancid, Unclean and Fruity	Hydrolysis of κ -casein in raw milk Lipolytic changes caused by the lipases of psychrotrophic bacteria
Cream, butter	Rancidity, Off-flavour, Fruity, Bitterness, Soapy	Prior to pasteurisation, milk (cream) contained a high concentration of lipases and proteinases; postpasteurisation contamination

		(Pseudomonas spp., Bacillus spp.). High levels of free fatty acid (C4–C6 and C10–C12) concentrations
Ice cream	Off-flavours	Because of enzymatic activity, the cream is of poor quality.
Powder	Rancidity	Milk or whey powders are used as components to confections that include fat and are meant to be kept for a long time; whole milk powder undergoes some lipolysis during prolonged storage (i.e. >1 year)
Cheese	Rancidity, Off-flavour	During continuous hydrological
	Lower yields	Proteinases from Pseudomonas spp. induce proteolysis.
	Shorter coagulating time	higher levels of free amino acids (bacterial proteinases) that promote starting culture development.
	Longer coagulation time	A higher level of free fatty acid concentration (bacterial lipases) prevents starting culture development.

5.3. Biofilms

Milk obtained from the udder of a healthy milk animal is almost in sterile condition but gets contaminated during milking, transportation, storage and processing and also due to the entry of microbes through many other sources. Insufficient sanitization and cleaning causes contaminants to accumulate in milk processing

equipment which subsequently form biofilm that further become significant source of contamination of dairy product **(Bhosale, 2021)**.

Biofilm is aggregations of microbial cells interconnected by extracellular polymeric substances which accelerate growth on different material surfaces adversely affect the dairy industry. Biofilm formation possesses profound implications and throws a major challenge to the dairy sector where they act as the principal reservoir of microbial contamination. These lead to financial crisis by impairment of raw material and its products. It is emphasized that good manufacturing practice, good hygienic practice and hazard analysis and critical control point should be implement in dairy industry to prevent the contamination of dairy products. There are different methods to detect the biofilm forming colonies. Different common processes involved in the control of biofilm forming colonies. i.e. Physical control, cleaning and disinfection, ultrasonication, steel coatings, High Hydrostatic Pressure, Non-thermal Plasma, Chemical Control, Mechanical Control **(Bhosale, 2021)**.

The existence of biofilm has been explored for several years in the food industry. The first documentation was done roughly 75 years back in 1943 **(Singh et al., 2019)**.

5.3.1. Definition of biofilms

Biofilms are three dimensional aggregations of microorganisms attached to surfaces. Bacteria in the biofilm join together and form a protective matrix around each other. It is estimated that up to 90% of microbial populations exist as biofilms, rather than as discrete organisms (planktonic cells) floating around in the environment. **(Singh et al., 2019)**.

Biofilms are sessile microbial communities where microbes live together in association with each other on biotic or abiotic substrates which are bounded by extracellular polysaccharides, proteins, lipids and DNA. In other words, simply, biofilms represent an important mode of bacterial life colonizing most of the surfaces in nature **(Singh et al., 2019)**.

In 2012, the term biofilm was defined by the International Union of Pure and Applied cells that are frequently embedded within a self-produced matrix of extracellular

polymeric substances (EPS) adhere to each other and/or to a surface (**Richter et al., 2015**).

5.3.2. Chemical composition of biofilm matrix

In biofilms, microorganisms live in a self-produced matrix of EPS that form their immediate environment. In most biofilms, less than 10% of the dry mass is composed of microbial cells, whereas more than 90% accounts for the matrix (**Flemming and Wingender, 2010**); (**Meena et al., 2015**).

The architecture of microbial communities and their maintenance depends significantly on the production and composition of EPS (**Limoli et al., 2015**).

The chemical configuration of extracellular polymeric substances varies depending on microorganisms type, shear forces, nutrient availability, or temperature. Biofilm matrix contains various classes of macromolecule like polysaccharides, nucleic acids, proteins, lipids, and peptidoglycan (**Sharma et al., 2022**). The role of each component in the biofilm matrix is described in Table 9.

Table 9. Chemical composition of biofilm matrix and their functional roles. (**Sharma et al., 2022**).

Example	Examples	Functions
Polysaccharides	Alginate, Pel, Psl (polysaccharide synthesis locus), levan, and cellulose	●Alginate is involved in the mechanical stability of biofilms, increases EPS hydration, and regulates the interaction between the cells and the substrate surface ● Pel is involved in the structural framework of the matrix

		and essential for the interactions of microbial cells, especially during the initial stages of biofilm formation ● Psl is required for the maintenance of biofilm architecture and involved in the adherence of biofilms to abiotic and biotic surface ● Levan is associated mainly with storage benefit rather than structural role to biofilm polysaccharide. It has been hypothesized the possible role of levan in the resistance of <i>P. brassicacearum</i> biofilms ● Cellulose is the major polysaccharide component and plays an important role in maintaining biofilm stability. In <i>P. fluorescens</i>, cellulose is required for biofilm formation,
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		especially at the air/liquid interface
Proteins	Dextran, N-acetyl- β -hexosaminidase (encoded by dspB), biofilm associated surface protein (bap), Accumulation associated protein (Aap), protein A, Embp	Proteins play an essential role in biofilm structure and stability. They enhance the thermostability and resistance of microbial communities to survive better in the environment. Some enzymes promote the detachment of microorganisms via degradation of structural EPS
Nucleic acids	Extracellular DNA (eDNA)	eDNA is an integral part of the matrix, plays an important role as an intercellular connector, and induces antibiotic resistance. Some microbial strains such as <i>P. aeruginosa</i> and <i>Bacillus cereus</i> use DNA as an adhesin during formation of biofilm
Lipids	Lipopolysaccharides, rhamnolipids, surfactin, emulsan, and viscosin	Lipids are crucial for the adhering of microorganisms to surfaces. They also act as surfactants and may be useful for bioremediation of oil spills and enhance oil recovery microbially. Lipids play a

		part in dispersion of biofilm
Water		• Water is by far the largest component of the matrix (approximately 97%). Water in the matrix keeps the environment hydrated, thus buffers the microbial cells against fluctuations in water potential

5. 3.3. Role of biofilm to microorganisms

According to **Bhosale. (2021)**, microorganisms residing in biofilms get many advantages as compared to freely swimming planktonic stage, and that's the reason for them to prefer biofilm mode of living. Some of these potential advantages are:

- Microorganisms in biofilms exhibit elevated antimicrobial tolerance and also get protected from environmental stresses such as extreme pH, oxygen, osmotic shock, heat, freezing, UV radiation, predators.
- Extracellular polymeric matrix formed from the secreted exopolysaccharides (EPS) increases the binding of water resulting in decreased chance of dehydration (desiccation) of the bacterial cells, which is a common stress condition experienced by planktonic cells.
- The adherent nature of microbial cells in biofilms allows rapid exchange of nutrients, metabolites, and genetic material.

5.3.4. Biofilm forming microbes of dairy industry

Microorganisms occurring in the food industry could be a source of secondary contamination in food products. The other important biofilm forming genera of dairy industry are *Bacillus*, *Pseudomonas*, *Listeria*, *Lactobacillus*, *Staphylococcus*, *Streptococcus*, *Salmonella typhimurium* and *Coronobacter sakazakii* etc. (**Singh et al., 2019**).

Table 10. Biofilm forming microbes of Dairy Industry (**Singh et al., 2019**).

Biofilm forming genera in Dairy Industry and example	Why they form biofilms?
1. Bacillus Example <i>B.subtilis</i>	Present in raw and even pasteurized milk, (It requires mainly carbon and energy to make the biofilm and use a number of sugars, organic acids and different organic compounds for this task).
2. Pseudomonas Example – <i>P. fluorescens</i>	(It is well-known for this cause because of its high heat resistance and short generation time and these characteristics make it a successful biofilm former)
3. Listeria Example- <i>Listeria monocytogenes</i>	(It is mainly affected by temperature, strain origin and nutrient level and also has the property of attachment to surfaces passively and its biofilms are primarily comprised of teichoic acids which can grow on polypropylene, steel, rubber and/or glass surfaces)
4. Staphylococcus Example- <i>Staphylococcus epidermidis</i>	(In the process of staphylococcal biofilm formation, the accumulation and development of a mature stage depend mainly on the polysaccharide intercellular adhesions (PIA) that promote bacterial accumulation, especially polysaccharide poly-N-succinylb- 1-6 glucosamine (PNAG))
5. Streptococcus Example- <i>Streptococcus Thermophilus</i>	(Affects mainly cheese and pasteurized milk – In the heating chamber of the section where temperature remains within 0 to 730C lies, the maximum degree of biofilm formation occurs. As a result the defects in milk and cheese quality like acidic flavour and undesirable texture are spotted.)
6. Lactobacillus Example- <i>L. rhamnosus</i>	(Biofilm formation by Lactobacillus spp. Is relatively beneficial because of its property of colonization and longer mucosal permanence of the host as these help in avoiding pathogenic bacterial colonization)
6. E. coli Example- <i>E. coli</i>	(The autoinducer- 2 (AI-2) of <i>E. coli</i> O157:H7 act as supplementary force for biofilm production as AI-2 signals

	regulate chemotaxis, flagellar synthesis and motility of genes. The <i>E. coli</i> O157:H7 yields exopolysaccharides (EPS) which helps in cell attachment and formation of 3D structures of biofilms.)
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5.3.5. Biofilm formation by psychrotrophic bacteria

Bacteria frequently adhere to surfaces and form spatially organized communities known as biofilms within a self-produced matrix composed of extracellular polymeric substances (EPSs) **(Flemming and Wingender, 2010)**.

Biofilms pose serious challenges to the dairy industry because they allow bacteria to adhere to various kinds of surfaces, such as stainless steel, plastic, rubber, and polypropylene, within a few minutes followed by formation of mature biofilms within several days or even hours **(Marchand et al., 2012)**.

This sessile biofilm is a survival strategy for microorganisms because it protects them against stressful conditions (e.g., disinfections) commonly encountered in dairy processing environments. Thus, biofilms have been recognized as sources of contamination by pathogenic and spoilage microorganisms in the dairy industry, where these biofilms are responsible for equipment damage, increased energy costs, product spoilage, and foodborne illnesses and infections through cross-contamination **(Møretrø and Langsrud, 2017); (Yuan et al., 2019a)**.

Low temperatures favor EPS production and increase the possibility of biofilm formation **(Tribelli and Lopez , 2011)**. A study conducted to explore the biofilm-forming capacity of an extensive collection of psychrotrophic bacterial isolates revealed a very high tendency of these bacteria to form biofilms on both polystyrene and stainless surfaces **(Yuan et al., 2018)**.

Some evidence suggests that persistence of certain strains may be enhanced by low-temperature adaptation mechanisms **(Cabrita et al., 2015); (Puga et al., 2015)**. These findings could explain the extensive biofilm formation by psychrotrophic bacteria on various milk contact surfaces, such as storage tanks, transportation tankers, milk pipelines, and heat exchangers **(Marchand et al., 2015)**.

Figure 19 Shows biofilm of *L. monocytogenes* by Scanning Electron Microscopy (SEM) Scratched on the Surface of the Bottom Cover of Milk.

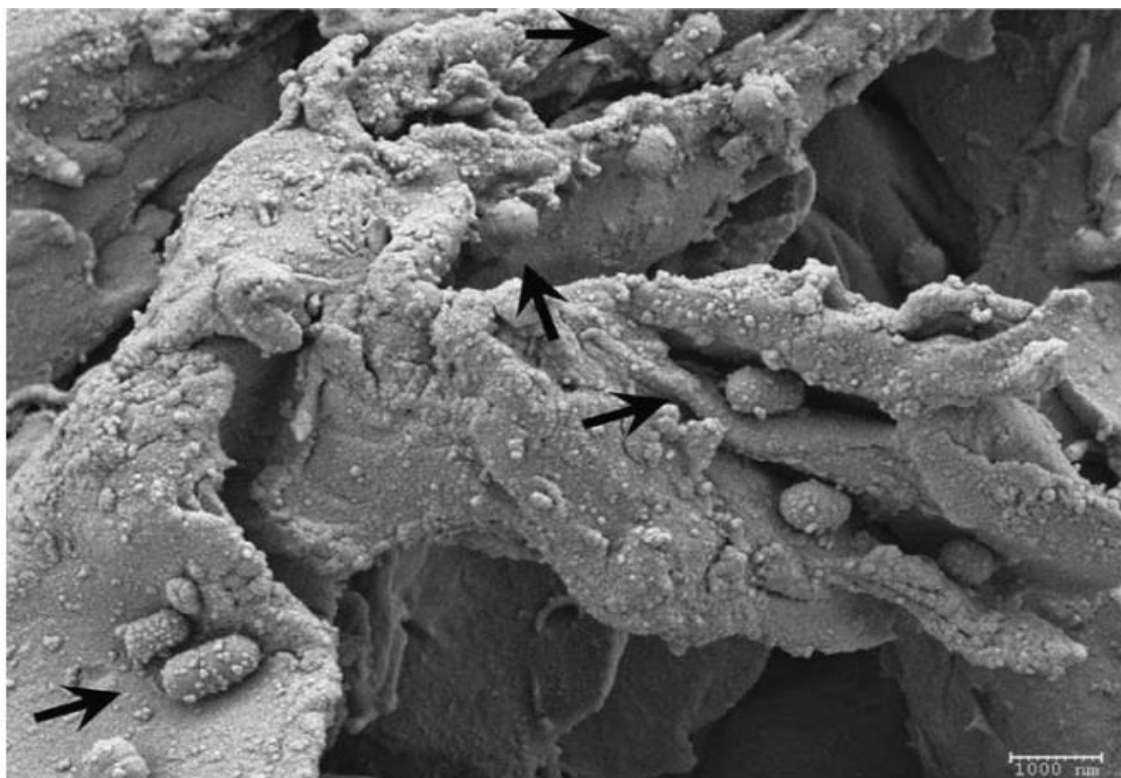


Figure 19. Scanning Electron Microscopy (SEM) biofilm of *L. monocytogenes* Scratched on the Surface of the Bottom Cover of Milk (**Latorre et al., 2010**).

5. 3.6. Common process for biofilms control

5. 3.6.1. Physical Control

5. 3.6.1.1. Cleaning and disinfection

In the dairy industry the classical operations of cleaning and disinfection are essential parts of milk production. The efficiency with which these operations are performed greatly affects the final product quality. Generally, disinfectants do not penetrate the biofilm matrix left on a surface after an ineffective cleaning procedure, and thus do not destroy all the biofilm living cells. Therefore, cleaning is the first step and of utmost importance to improve the sanitation of the processing equipment it is important to effectively remove food debris and other residues that may contain microorganisms or promote microbial growth (**Menon, 2016**).

The use of high temperature can reduce the need for the application of physical forces such as water turbulence and scrubbing. Chemical products commonly used for cleaning are surfactants or alkali products, used to suspend and dissolve food residues by decreasing surface tension, emulsifying fats, and denaturing proteins. Cleaning in place (CIP) procedures are usually employed in milk processing lines. An independent quality control system to monitor the cleaning results for a dairy plant can be integrated in the Hazard Analysis Critical Control Points (HACCP) program **(Menon, 2016)**.

Evaluation of biofilm sanitation should be part of the HACCP development plan in order to control those biofilms prevalent in the processing areas. Moreover, impairing the formation of biofilms can be achieved through a better knowledge of the mechanisms that contribute to their formation, development and maintenance **(Menon, 2016)**.

5. 3.6.1.2. Ultrasonication

Combined effect of power ultrasound and heat (thermosonication) has proved to be more efficient method of microbial inactivation than either of the two methods alone. Microbial inactivation of ultrasound treatment accounts for generation of acoustic cavitations, resulting in increased permeability of membranes, selectivity loss, cell membrane **(Bhosale, 2021)**.

Thinning confined heating, singlet electron transfer in cooling phase, and hydroxyl radical formation. High-frequency ultrasound method, patented as sonoxide, has more than 600 applications and provided best results in inhibiting bacterial and algal growth in industrial waters. Ultrasonic-treated cells were found to lack internal content when viewed under transmission electron microscopy, but disintegration was not affirmed to be main reason of cell death **(Bhosale, 2021)**.

Ultrasonication has achieved the FDA requirement of a 5-log reduction in microbial population. Exploitation of ultrasound as means of inhibiting and killing micro-organisms came from the observation that sonar used for anti-submarine warfare resulted in killing of fishes. Ultrasound frequency of 20 kHz and power of 12.8 W was used on 50 cm³ water contaminated with *Streptococcus* mutants for a period of 15 min and 97% microbial reduction was achieved. Ultrasonic power of around 100 W was found to be optimal for maximum microbial inactivation and ultrasonication has been

found to be effective method for microbial inactivation in *Escherichia coli*, *Listeria monocytogenes*, and other pathogens. Efficiency of ultrasonic treatment as antimicrobial tool depends on the physical (size, hydrophobicity) and biological (gram-status, growth phase) characteristics of the micro-organisms. It has been demonstrated that micro-organisms with “soft” and thicker capsule are extremely resistant to ultrasonic treatment (Bhosale, 2021).

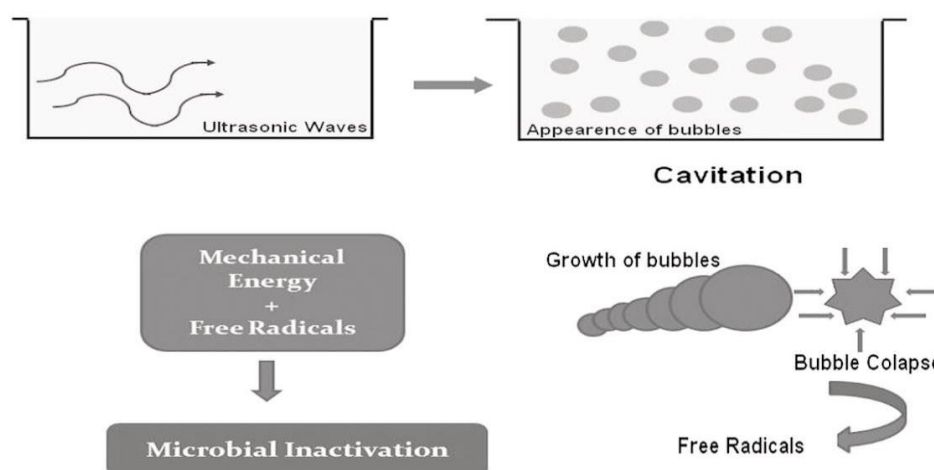


Figure 20. Cavitation phenomenon and microbial inactivation by ultrasonic waves (Bhosale, 2021).

5.3.6.1.3. Steel coatings

One promising approach focuses on nanotechnology agents. The unique properties of nanoparticles (NPs) distinguish them from their bulk chemical counterparts. Mechanism One such property is their large surface area to volume ratio, which creates a higher number of functional sites and can enhance the influence of NPs on a given microorganism. Since the antibacterial properties of some NPs are mediated mainly by direct contact with the bacterial cell wall and do not require penetration, most bacterial antibiotic resistance mechanisms are not relevant when dealing with NPs. This favorable property has stimulated extensive research on the antibacterial effects of diverse NPs types, such as carbon-based materials (fullerenes and carbon nanotubes), dendrimers that provide cavities for other molecules, nanocomposites, natural NPs and metal-based NPs, including silver, gold, metal oxides. Due to their potent antimicrobial effects, silver compounds have been used since ancient times to prevent microbial infections associated for example to water consumption. Currently, silver NPs

are the most widely studied. However, metal oxide NPs are more commonly used within industry. They include iron oxide (Fe₃O₄), titanium oxide (TiO₂), zinc oxide (ZnO), copper oxide (CuO) and magnesium oxide (MgO). These NPs show antimicrobial properties and can be applied in diverse industrial environments. [REF](#)

5.3.6.1.4. High Hydrostatic Pressure

High hydrostatic pressure (HHP) 300-900 MPa is able to destroy or inactivate vegetative bacterial cells. However, this technology is not effective in the case of endospores (such as those in the case of *B. cereus*), unless a pre-treatment is carried out at lower pressures (300-400 MPa) in order to allow germination of existing spores. Anyway, some non-germinating spores could remain in the food matrix after HHP treatments, and therefore, at industrial level, HHP is usually combined with thermal treatments (50°C to 100°C), or in some cases with essential oil components. One important advantage of HHP treatments is that they do not alter the organoleptic and nutritional properties of the food matrixes (taste, vitamins, etc.), a great advantage with respect to high temperature methods ([Galle et al., 2018](#)).

5.3.6.1.4. Non-thermal Plasma

Non-thermal plasma is a partially ionized gas with low temperature and interesting antimicrobial properties. It is produced at atmospheric pressure by mixing UV light with oxygen, nitrogen, ozone, and water and helium, under an electrical discharge. It is able to destroy bacterial biofilms of Gram-negative (*Pseudomonas spp*, *S. enterica*) or Grampositive (*Bacillus spp*) species in just 10 min, However, its use is still restricted to some laboratory applications, due to its high cost ([Galle et al., 2018](#)).

5. 3.6.2. Chemical Control

Biofilms can be control by the use of biocides, antibiotics, and ion coatings. Studies show that use of amino glycosides in combination with iron-chelating compounds is important in the disruption of *Pseudomonas aeruginosa* biofilms. Application of sodium citrate can also inhibit biofilm formation of Staphylococci species in vitro. Antimicrobia agent such as N-alkylpyridinium bromide attaches to a

poly (4-vinyl-N-hexylpyridine) is capable of inactivating about 99% of *E. coli*, *S. epidermidis*, and *P. aeruginosa* bacteria. Peroxyacetic acid (PAA) is a sanitizer with high oxidizing potential is effective against bacteria, fungi and spores in the dairy industry because it is not inactivated by catalase or peroxides. Studies show that use of ozone in Europe was effective in disinfecting drinking water and it is also a better oxidizer than chlorine and hence effective in inactivating *Pseudomonas fluorescents* on glass slides (Kabwanga et al., 2018).

5. 3.6.3. Mechanical control

The mechanical biofilm control methods aim at the disturblization of bacteria from surface attachment, surface charge and hydrophobicity through the application of compounds that can prevent the biofilm formation and their spread on surface. The use of smooth surfaces equipment is more preferred as they are less susceptible to biofilm adhesion. Modification of the surface charge of polymers also enables the prevention of biofilm. Positively-charged polycationic chains enable the molecule to stretch out and generate bactericidal activity (Kabwanga et al., 2018).

5. 3.6.4. Bacteriophages control of biofilms

Bacteriophages are a numerous group of viruses which are easily manipulated, and they have various functions in biotechnology, bacterial control, and therapeutics. Bacteriophages are ubiquitous in nature that infects bacteria naturally and may provide a natural, highly specific, nontoxic, feasible approach for controlling biofilm formation. They may either coexist with their host by inserting themselves into the bacterial genome (lysogenic bacteriophages) or destroy them. Phage T4 and E27 help in the control of *E. coli* and *Pseudomonas aeruginosa* biofilms. *Enterobacter agglomerans* type of biofilms can be destroyed through cell lysed by bacteriophage. Many studies show that phages alone disrupt *Staphylococcus epidermidis* growing biofilm colonies on silicon catheters. Phages are also effective in the removal of biofilms in their early stages of development about 5 days old biofilms of *P. fluorescens*. A bacteriophage such as *L. monocytogenes* phage ATCC 23074-B1 helps to inactivate *L. monocytogenes* (Kabwanga et al., 2018).

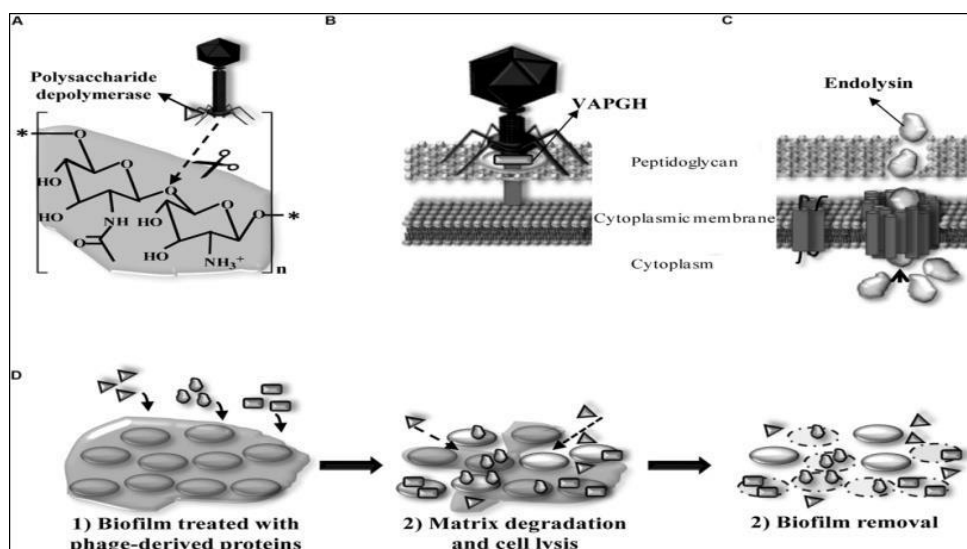


Figure 21. Bacteriophages control of biofilms (Kabwanga et al., 2018).

5. 3.6.5. Enzymatic control of biofilm

Biofilm in the dairy industry are formed by; *Lactobacillus bulgaricus*, *Lactobacillus lactis*, *Streptococcus thermophiles* are the most common microorganism that form biofilms. Enzymes like α -amylase, β -glucanase and protease were proved effective in the cleaning of adhered industrial biofilm formed during paper pulp production. Exopolysaccharide degrading enzymes more so the colanic acid degrading enzymes derived from a *Streptomyces* isolate was reported for the removal and prevention of biofilm formation. Biofilms control with proteases such as Proteinase K and Trypsin, ensure the destruction of biofilm formation and biofilm removal and can disrupt biofilms formed by *S. aureus*. Synergistic action of enzymes in combination with surfactant and phenolic antimicrobials are important in the control of biofilms although the application of enzymes in biofilm control is still limited. Enzymes like lipases and proteases are often selected as complementary cleaning agents when simple chemicals such as alkaline and acid are not enough for cleaning and recovering the membrane capacity. However, most of the studies using enzyme cleaners focus on the removal of protein fouling, but did not aren't effective on biofilms (Kabwanga et al., 2018).

5. 3.6.6. Novel safe approaches for the control of biofilm formations

Novel safe approaches for the control of biofilm formations are shown in Table 11

Table 11. Novel safe approaches for the control of biofilm formations (**Meltem, 2015**).

1. Polysaccharides	Can inhibit the biofilm formation of bacteria, possibly by modifying the physical properties of both abiotic and biotic surfaces. It was shown that <i>E. coli</i> exopolysaccharides can alter the abiotic surface properties such as increase the hydrophobicity of glass surfaces and also can prevent cell-to cell auto aggregation via adhesions of bacteria
2. Enzymes	Serine proteases were efficiently reducing <i>Bacillus</i> biofilms whereas polysaccharides remove more efficiently <i>Pseudomonas fluorescens</i> than serine proteases. Polysaccharide polymerases and esterase can also control biofilm formations.
3. Nisin- produced by some strains of <i>Lactococcus lactis</i> and has been employed as an antibiofilm agent	Nisin has a mode of action that results in the formation of pores in the cell membrane of the bacteria. Pore formation leads to cell lysis and death. The bactericidal activity of nisin has been shown to target other Gram positive bacteria closely related to <i>Lactococcus lactis</i> and some Gram positive pathogens, such as <i>Listeria monocytogenes</i>. Nisin is effective against planktonic cells of multi-drug resistant staphylococci
4. Citric acid- alternative disinfectant in controlling biofilm formation in the dairy industry.	The prevention and removal of biofilm formation of <i>S. aureus</i> strains isolated from raw milk by citric acid treatments (2% and 10%) for 20 min were assessed for comparison with peracetic acid treatment (0.3%) on both on microtitration plate and stainless steel coupons. The prevention and removal of biofilm formation ratios and the numbers of prevented or removed <i>S. aureus</i> strains were observed to be higher by using citric acid treatments compared with peracetic acid treatment on both surfaces. Moreover, the prevention and removal of biofilm formation were substantially higher when the concentration of citric acid treatment increased from 2% to 10% and the

	stainless coupons were used.
5. Gallic acid- phenolic products found in plants such as tea leaves, fruits and flowers	It has been shown that gallic acid has strong antimicrobial activity against several bacterial strain.also reported antibiofilm activity of gallic acid for the prevention and removal of <i>E.coli</i> , <i>P. aeruginosa</i> , <i>S. aureus</i> and <i>L. monocytogenes</i> biofilms. The researchers found that gallic acid can prevent and remove these pathogens by promoting reductions in biofilm activity >70% of all tested microorganisms.
6. Malic acid	The antimicrobial action of malic acid is to lower the pH value or cause the significant damage to the cytoplasm of bacteria also found that malic acid was also effective in food industry for complete inhibition of Salmonella Typhimurium biofilm in carrot and other food contact surfaces
1. Polysaccharides	Can inhibit the biofilm formation of bacteria, possibly by modifying the physical properties of both abiotic and biotic surfaces. It was shown that <i>E. coli</i> exopolysaccharides can alter the abiotic surface properties such as increase the hydrophobicity of glass surfaces and also can prevent cell-to cell auto aggregation via adhesions of bacteria

6. Bacterial Strands

Introduction

Milk is one of the most valuable foods regularly consumed among people. Due to high nutrient composition, milk production has been popular and played an important role in global food security. However, milk is highly vulnerable to bacterial contamination and can be the source of some food-borne diseases (**Jay, 2000**). Raw milk can be contaminated with pathogens originated from dairy cows or farm environment. Bacteria can be transferred into milk during milking or at any stage of milk handling, through dirty udders, improperly sanitized milking equipment, and cows with subclinical mastitis (**Kessel et al., 2011**) ; (**Amagliani et al., 2012**).

Many milk borne human diseases are spread through the consumption of contaminated milk (**Parekh and Subhash, 2008**). Bacteriological safety of milk continues to be a topic of concern in the dairy industry and public health domain. In general, the microbial content of milk is a major factor that determines the quality of milk. (**Oliver et al., 2009**).

6.1. Raw milk quality parameters

6.1. 1. Definition of raw milk

Raw milk is not very well defined. One definition is given by Council Directive 92/46/EEC of 16 June 1992 in which under Article 2 it is stated: Milk produced by secretion of the mammary glands of one or more cows, ewes, goats or buffaloes, which has not been heated beyond 40°C or undergone any treatment that has an equivalent effect . Thus, all milk extracted from the mammary glands that is not heated above 40°C can be characterized as raw milk (**Wiesner and Ribbeck, 2000**).

6.1. 2. Hygienic quality of raw milk

Milk of good hygienic quality shows the following criteria (**Ruegg and Tabone, 2000**):

- Low or very low numbers of saprophytic spoilage microorganisms.
- Absence or low numbers of pathogenic microbes.

- Absence of chemotherapeutic residues.
- Reduction or minimization of chemical contaminants.

A variety of microbiological count methods, including the total bacterial count (TBC) ,coliform count (CC) and somatic cell count (SCC) is available for monitoring the hygienic quality of raw milk (**Jayarao et al., 2004**).

6.1. 2.1.Total Bacterial Count

The Total Bacterial Count (TBC) is the most common method used for evaluating the hygienic quality of raw milk, which estimates the total number of bacteria present in milk (**Ruegg and Reinemann, 2002**).

Standard plate count (SPC), Petrifilm aerobic count (PAC), and plate loop count (PLC) are methods used to estimate The Total Bacterial Count in milk (**Laird et al., 2004**)

Increased The Total Bacterial Count (TBC) can be caused by growth of bacteria on unsanitary milking equipment, contamination from soiled cow udders, inadequately cooled milk, and occasionally by milking of mastitic cows (**Murphy and Boor, 2000**); (**Hayes et al., 2001**); (**Chambers, 2002**); (**Costello et al., 2003**).

6.1. 2.2.Coliform Count

The Coliform Count (CC) measures the number of coliform bacteria in milk primarily originating from cow's environment. The elevation of CC in milk is an indicator of poor sanitary practices in farm (**Reinemann et al., 2000**).

Coliform Count (CC) is a nonregulated test that has been used historically to assess milk production practices such as milk refrigeration, milking machine sanitation, and premilking udder hygiene (**Guterbock and Blackmer, 1984**); (**Reinemann et al., 1997**); (**Murphy and Boor, 2000**); (**Davidson et al., 2004**).

Coliform count is a practical indicator of milking hygiene because it is easy and inexpensive to perform (the test can be performed on the farm), and it is often correlated with the population of other bacteria in bacteria count of bulk tank milk BTM (**Jayarao et al., 2004**); (**Pantoja et al., 2009**).

However, because coliform bacteria populations can increase rapidly under some conditions, it is important to distinguish between the level of initial contamination and increased Coliform count (CC) that may be the result of incubation in the milk handling system after milk harvest.

Coliform bacteria are gram-negative non-spore-forming rods and can be found in feces, aquatic environments, soil, and on vegetation. Coliforms are important mastitis pathogens **(Hogan and Smith, 2003)** and are widely distributed in the farm environment **(Hogan et al., 1989); (McKinnon et al., 1990); (Sanderson et al., 2005)**.

When the total bacterial count of bulk tank milk (BTM) is within regulatory limits, coliforms are mostly destroyed by pasteurization. However, in some circumstances (e.g., pasteurization failures and consumption of unpasteurized milk or dairy products), the presence of coliforms in bulk milk can result in spoilage and severe human disease **(Chambers, 2002); (Mandell et al., 2005)**.

The enumeration of coliform bacteria in raw milk has been used as an indication of fecal contamination **(Davidson et al., 2004)**. Soiled udders and teats are common sources of fecal contamination and often indicate inadequate premilking cow preparation. Increased numbers of coliforms in bulk milk can also occur when coliforms grow on residual milk left on milk contact surfaces or in poorly sanitized milking equipment **(Guterbock and Blackmer, 1984);(McKinnon et al., 1990); (Chambers, 2002)**.

6.1. 2.3.Somatic cell counts

Somatic cell counts (SCC) have been widely used as an indicator for monitoring the mammary health status of cows, the suitability of milk for human consumption and basis to provide incentives to the producers based on quality of milk. In a healthy quarter, the somatic cell population primarily consists of macrophages (approximately 60% of cells), lymphocytes (approximately 30%), polymorphonuclear (PMNs) cells (approximately 10%) and sloughed epithelial cells from lining of the mammary gland **(Burvenich et al., 1995); (Paape and Contreras, 1997)**.

6.2. Posturized milk quality parameters

6.2. 1 definition of Pasteurization

Pasteurization is a thermal process widely used in the food and dairy industry with the objective of minimizing health hazards from pathogenic microorganisms and to prolong product shelf-life. There are several temperature-time combinations to pasteurize milk that range from 63°C (145°F)/30 minutes or 72°C (161°F)/15 seconds to 100°C (212°F)/0.01 seconds (**Alvarez and Parada-Rabell, 2005**).

6.2. 2 Laboratory pasteurization count

Laboratory pasteurization count (LPC) estimates the number of thermoduric bacteria present in milk after pasteurization (**Frank and Yousef, 2004**).

Thermoduric bacteria can multiply in biofilms present on milking equipment surfaces; thus, LPC has been used as an indicator of milking-equipment sanitation (**Guterbock and Blackmer, 1984**); (**Murphy and Boor, 2000**).

6.3. Regulation of milk in Industry Standard Milk

In many countries, processors and cooperatives have established price incentives or premium payment for raw milk with a low bacteriological load. Countries have developed their own standards that are considered in premium payment for milk. All these standards mandate the production of milk with as low bacteriological load as possible. Both farmers and processors need accurate information about the total bacterial count in raw milk to determine premium allocation. The microbial counts to be expected or desired in samples of raw milk obviously are dependent on the extent of processing (**Özer and Yaman, 2014a**).

6.3.1. Algeria

Milk and its derivatives are regulated by the Interministerial order of **2 Moharram 1438** corresponding to **4 October 2016** setting the microbiological criteria for foodstuffs (**JORA 2017**). All criteria are summarised in Table 12

Table 12. Microbial criteria applicable to dairy products (JORDAP N°39, 2017).

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ANNEX I

Microbiological criteria applicable to foodstuffs

1- Milks and dairy products

Categories of foodstuffs	Microorganisms/ metabolites	Sampling Plan		Microbiological limits (cfu (1)/ g or cfu/ml)	
		n	c	m	M
Raw milk	Aerobic germs 30 C	5	2	3.105	3.106
	Staphylococci . coagulase +	5	2	102	103
	Thermotolerant coliforms	5	2	5.102	5.103
	Salmonella	5	0	Absence in 25 ml	
	Antibiotics	1	.	Absence in 1 ml	
	Listeria monocytogenes	5	0	100	
Pasteurized milk and other pasteurized liquid dairy products	Aerobic germs 30 C	5	2	104	105
	Enterobacteriaceae	5	0	10	
	Salmonella	5	0	Absence in 25 ml	
UHT milk and sterilized milk	Aerobic germs ± 30 C	5	0	10/0.1ml	
Powdered milk and powdered whey	Enterobacteriaceae	5	2	10	102
	Staphylococci ± coagulase +	5	2	10	102
		5	0	Absence in 25 g	
Raw milk cheeses	Salmonella Escherichia coli	5	2	104	105
	Staphylococci coagulase +	5	2	103	104
		5	0	Absence in 25 g	
		5	0	100	
Cheeses made from milk which have undergone a less severe heat treatment than pasteurization and ripened cheeses made from pasteurized milk or whey or which have undergone a heat treatment more severe than pasteurization	Salmonella Listeria monocytogenes Escherichia coli	5	2	102	103
	Staphylococci ± coagulase +	5	2	102	103
		5	0	Absence in 25 g	
		5	0	100	
Unripened soft cheeses (fresh cheeses) made from pasteurized milk or whey or having undergone a heat treatment stronger than pasteurization	Salmonella Listeria monocytogenes Escherichia coli	5	2	102	103
	Staphylococci coagulase +	5	2	10	102
		5	0	Absence in 25 g	
		5	0	100	
Raw milk cream	Salmonella Listeria monocytogenes Escherichia coli	5	2	102	103
	Staphylococci . coagulase +	5	2	103	104
	Salmonella	5	0	Absence in 25 g	
	Listeria monocytogenes	5	0	100	

1- Milks and dairy products (continued)

Categories of foodstuffs	Microorganisms/ metabolites	Plan		Microbiological Sampling	
		n	c	m	M
Pasteurized cream	Enterobacteriaceae	5	2	10	102
	Staphylococci coagulase +	5	2	10	102
		5	0	Absence in 25 g	
	Salmonella Listeria monocytogenes	5	0	100	
Ice creams and frozen dairy desserts	Aerobic germs $\pm$ 30 C	5	2	105	106
	Staphylococci $\pm$ coagulase +	5	2	10	102
	Enterobacteriaceae	5	2	10	102
	Enterobacteriaceae (2)	5	2	50	5.102
		5	0	Absence in 25 g	
		5	0	100	
Raw butter	Salmonella Listeria monocytogenes Escherichia coli	5	2	10	102
	Staphylococci coagulase +	5	2	102	103
		5	0	Absence in 25 g	
	Salmonella Listeria monocytogenes	5	0	100	
Pasteurized butter	Enterobacteriaceae	5	2	10	102
	Staphylococci $\pm$ coagulase +	5	2	10	102
		5	0	Absence in 25 g	
	Salmonella Listeria monocytogenes	5	0	100	
Concentrated butter	Aerobic germs 30 C	5	2	5.102	5.103
	Staphylococci coagulase +	5	0	Absence	
	Total coliforms	5	0	Absence	
	Salmonella	5	0	Absence in 25 g	
Fermented milks (Lben, RaibÖ)	Total coliforms	5	2	3.104	3.105
	Thermotolerant coliforms	5	2	30	3.102
	Staphylococci $\pm$ coagulase +	5	2	3.102	3.103
		5	0	Absence in 25 g	
	Salmonella Listeria monocytogenes	5	0	100	
Yogurts or yogurts and dairy desserts	Enterobacteriaceae	5	2	10	102
	Staphylococci $\pm$ coagulase +	5	2	10	102
		5	0	Absence in 25 g	
	Salmonella Listeria monocytogenes	5	0	100	
Caseins-caseinates	Aerobic germs 30 C	5	2	3.104	3.105
	Staphylococci coagulase +	5	0	Absence	
	Total coliforms	5	0	Absence in 0.1 g	
	Salmonella	5	0	Absence in 25 g	

(1) CFU: colony forming unit.

(2) This criterion applies at the portioning stage in retail trade, i.e. during splitting or handling with a view to direct sale to the end consumer.

6.3.2. The United States

In the United States, Grade A milk is the only milk regulated under federal orders. The Grade A Pasteurized Milk Ordinance PMO dictates the proper standards for Grade A milk from production to distribution. Dairy farms producing Grade A milk must have proper licensure and sanitary conditions for production. During processing, Grade A milk must go through rigorous standards including required bacterial counts, somatic cell counts, cooling checks, and sampling must be followed before being cleared for distribution (FDA and US PHS, 2013).

While regulations vary from state to state, the FDA Pasteurized Milk Ordinance dictates specific chemical, physical, bacteriological, and temperature standards seen in Table 13.

According to the US Food and Drug Administration (FDA) guidelines, the raw milk must have total bacteria less than 100 000 cfu ml⁻¹, and this figure must be lower than 20 000 cfu ml⁻¹ after pasteurization.

Table 13. Grade “A” Pasteurized Milk Ordinance (FDA, 2013).

Milk Grade	Quality Standards	
Grade A pasteurized milk	Temperature	• Cooled to 7°C (45°F) or less and maintained thereat
	Bacterial limits	• Not to exceed 20,000 mL ⁻¹ , or g
	Coliform	• Not to exceed 10 mL ⁻¹ • Provided, that in the case of bulk milk transport tank shipments, shall not exceed 100 mL ⁻¹
	Phosphatase	• Less than 350 mU/L for fluid products and other milk products
	Drugs	• No positive results on drug residue detection methods
Grade A ultrapasteurized (UP) milk	Temperature	• Cooled to 7°C (45°F) or less and maintained thereat
	Bacterial limits	• Not to exceed 20,000 mL ⁻¹ , or g
	Coliform	• Not to exceed 10 ML ⁻¹ . Provided, that in the case of bulk milk transport tank shipments, shall not exceed 100 mL ⁻¹

	Drugs	• No positive results on drug residue detection methods
Grade A raw milk and milk products for pasteurization, ultra-pasteurization, or aseptic processing	Temperature	• Cooled to 10°C (50°F) or less within 4 hours or less, of the commencement of the first milking, and to 7°C (45°F) or less within 2 hours after the completion of milking
	Bacterial limits	• Individual producer milk not to exceed 100,000 mL⁻¹ prior to commingling with other producer milk • Not to exceed 300,000 mL⁻¹ as commingled milk prior to pasteurization
	Drugs	• No positive results on drug residue detection methods
	Somatic cell count	• Individual producer milk not to exceed 750,000 mL⁻¹

6.3.3. European Community

With increasing global trade in milk products and European trade in milk and milk products, nearly all countries have tried to maintain the principally excellent image of milk products. Especially during recent years a series of new food laws, regulations and standards have been established with the primary goal of improving the prevention of any potential impairment of consumer health due to milk consumption (consumer protection) **(Hamann, 2010)**.

For the European Community some Regulation **(EC) No. 178/2002** of the European Parliament and the Council of **28 January 2002** laying down the general principles and requirements of food law, establishing the European Food Safety Authority and defining procedures in matters of food safety. This is the so-called basic regulation that especially states that the producer (i.e. farmer, dairy farmer, etc.) has the responsibility for quality and safety of the products he or she is bringing to the market **(Hamann, 2010)**.

- Regulation **(EC) No. 852/2004** of the European Parliament and the Council of 29 April 2004 on the hygiene of foodstuffs.
- Regulation **(EC) No. 853/2004** of the European Parliament and the Council of

29 April 2004 laying down specific hygiene rules for food of animal origin.

- Regulation (EC) No. 854/2004 of the European Parliament and the Council of

29 April 2004 laying down specific rules for the organization of official

According to the European Union regulation (**Council Directive 92/46/EEC**), raw cow's milk intended for the production of heat treated milk, fermented milk, junket, jellies, or flavored milk and cream must meet the following standards:

Plate count at 30°C 100 000 cfu ml⁻¹ Somatic cell count 400 000 ml⁻¹ Although from a risk analysis point of view, there appears to be no substantial differences between the EU and US standards, there are some differences between the EU and US regulations regarding the limits on bacterial levels in milk. These differences are presented in Table 14. (**Özer and Yaman, 2014**).

Table 14. Comparison of the EU and US regulations regarding the limits on bacterial levels in milk (cfu ml⁻¹) (**Özer and Yaman, 2014**).

Raw milk for production	European Union	Union United States
Bacteria (SPC)	<100 000	<100 000 _a / _b <300 000
Drugs/ml	<0.004 mg	None detectable
Pasteurized milk		
Bacteria (SPC)	5000/50 000	<20 000
Enterobacteriaceae	5	
Coliforms	5	<10
_a Individual producer. _b Commingled milk.		

7. Controlling dairy spoilage caused by psychrotrophic

7.1. Strategies to aid in reducing dairy product spoilage and waste

Strategies for preventing or reducing dairy food waste due to spoilage should include a variety of approaches throughout the production process, including: **(Martin et al., 2021)**.

- (1) Reducing transmission of spoilage microbes from environmental sources on the farm and from the processing facility into dairy products;
- (2) Applying processing technologies to reduce or eliminate the presence of microbial contaminants;
- (3) Controlling the outgrowth of spoilage microorganisms through biocontrol strategies;
- (4) Preventing contamination through proactively monitoring and controlling spoilage organisms in raw ingredients and the environment; and
- (5) Using data-driven decision making tools to optimize quality and reduce spoilage.

Strategies to reduce dairy product spoilage have long focused on preventing recontamination of processed products with spoilage microorganisms through a focus on cleaning and sanitation in the processing environment. For example, adequate cleaning and sanitation is necessary to prevent the development of biofilms in dairy processing equipment. Biofilms are a major source of dairy product contamination during processing **(Knight, 2015)**, and once biofilms are established in processing equipment, they are a considerable challenge to eliminate **(Bremer et al., 2015)**.

Reducing microbial contamination during processing through cleaning and sanitization has been shown to have a dramatic effect of dairy product shelf life. For example, the shelf life (i.e., the number of days for the bacterial concentration to reach 20,000 cfu/mL) of fluid milk in a study by **Gruetmacher and Bradley, (1999)** was increased by more than 11 d (from 9 d to more than 21 d) following proper cleaning and sanitization with chlorine, and changing the sanitizing agent from chlorine to peroxyacetic acid increased the shelf life to more than 30 d **(Gruetmacher and Bradley, 1999)**.

7.1.1 On-farm intervention strategies

Bacterial spores are found ubiquitously in natural environments, specifically in the dairy farm environment, and enter raw milk primarily during milking (**Martin et al., 2019**).

Several studies have investigated the role of farm practices and sources in the transmission of spores from environmental niches into raw milk, with bedding, feed, and parlor practices reportedly playing important roles. The use of simple, low-cost on-farm interventions, such as those discussed later, to reduce the concentration of spores in bulk tank raw milk is a viable strategy for reducing dairy food waste due to microbial spoilage (**Martin et al., 2021**).

Magnusson et al. (2006) investigated the effect of premilking cleaning methods on teats experimentally contaminated with spores of *Clostridium tyrobutyricum* and *Bacillus cereus*. The authors found that cleaning teats with a moist towel followed by drying with a dry towel significantly reduced spore counts in raw milk.

Similarly, **Evanowski et al. (2020)** applied a combination of interventions to reduce spore levels in bulk tank raw milk, including training milking parlor employees on enhanced teat-end cleaning and implementing a standard laundered towel cleaning protocol (i.e., laundering with detergent and chlorine bleach and fully drying).

7.1.2. Processing technologies and practices

7.1.2.1. Heat treatment of milk

The first attempts to preserve milk by heat treatment were made by Nicolas Appert in 1810. These were unsuccessful because the milk coagulated during heat treatment. About 1860, Louis Pasteur applied a low-heat treatment (a process now known as pasteurization) to preserve wine and beer. This process was first applied in the dairy industry in 1885 by N. Fjord to improve the microbiological quality of cream for butter making. In 1886, Franz Soxhlet suggested that milk fed to children should be heat-treated and in 1895 it became a legal requirement in Denmark to pasteurize milk at creameries to prevent the spread of tuberculosis among farm animals through skimmed milk returned to farmer (**Connell and Fox, 2022**).

Table 15. Major heat treatments used in the dairy industry (Deeth and Smithers, 2018).

<i>Heat treatments (temperature –time conditions)</i>	<i>Applications</i>	<i>Bacteria destroyed</i>	<i>Significant chemical effects</i>	<i>Comments</i>
Thermization (57–68 °C/30–5 s)	Extending shelf-life of raw milk prior to further processing Manufacture of some cheeses	Some non-spore-forming pathogens and psychrotrophic spoilage bacteria	Minor effects	Product not suitable for drinking as some pathogens may remain viable
Pasteurization (72–80 °C/15–30 s or 63–65 °C/15–30 min)	Drinking milk with shelf life up to 20 days; stored under refrigeration Manufacture of most cheeses	Non-spore-forming pathogens and psychrotrophic spoilage bacteria	Small effect on vitamins, 5%–10% whey protein denaturation, inactivation of alkaline phosphatase and lipoprotein lipase	Standard minimum conditions in most countries are usually 72 °C/15 s; an alternative process is batch or holder pasteurization at 63–65 °C/15–30 min
Extended-shelf-life (ESL) processing (125–140 °C/1–10 s)	Drinking milk with shelf life of more than 20 days; stored under refrigeration;	All non-spore-forming bacteria and most psychrotrophic and mesophilic spores	Depends on mode and severity of heating; significant but variable denaturation of whey proteins (25%–85% of β -lactoglobulin)	No standard heat processing conditions; minimal alteration of flavor at higher temperatures for shorter times.
Ultra-high-temperature (UHT) processing (135–150 °C/1–10 s)	Drinking milk with long shelf-life (6–9 months); stored at ambient temperature	All non-spore-forming bacteria and all spores except highly heat-resistant spores	Depends on mode and severity of heating: Effects less with direct than indirect heating systems; high level of whey protein denaturation (70%–95% of β -lactoglobulin)	Produces mild heated/cooked/sulfurous flavor; further chemical changes occur during storage; shelf life limited by gelation
In-container sterilization (110–120 °C/10–20 min or 125 °C/5 min)	Evaporated/condensed milk, drinking milk with long shelf-life (up to 12 months); stored at ambient temperature	Destroys all non-spore-forming bacteria and all spores except highly heat-resistant ones	Complete denaturation of whey proteins, extensive Maillard reaction	Causes strong cooked flavor and light brown discoloration; more stable than UHT milk
90–95 °C/5–10 min	Yogurt manufacture	Most non-spore-forming bacteria	Almost total denaturation of whey proteins (WP)	Causes increased viscosity of yogurt through formation of WP– κ -casein complexes and enhanced water binding capacity
72–80 °C/15–30 s)	Low-heat skim milk powder (SMP)	Non-spore-forming pathogens and psychrotrophic spoilage bacteria	As for pasteurization; little whey protein denaturation—WPNI > 6.0 g/L	Product used for recombined milk, milk, standardization, cheese manufacture
85 °C/1 min; 90 °C/30 s; 105 °C/30 s	Medium-heat SMP. Pre-heating in UHT processing Whole milk powder.	Non-spore-forming pathogens and psychrotrophic spoilage bacteria	Moderate to substantial whey protein denaturation—WPNI 1.5–6.0 g/L; inactivation of plasmin; exposure of some sulfhydryl groups and formation of some sulfhydryl compounds which act as antioxidants in whole milk powder	Product used for ice-cream, chocolate, confectionery Decreases fouling of UHT plants and reduces plasmin-catalyzed proteolysis during storage Improves storage stability of whole milk powder
90 °C/5 min; 120 °C/1 min; 135 °C/30 s	High-heat SMP. Forewarming for sterilization of concentrated milk	Non-spore-forming pathogens, psychrotrophic spoilage bacteria and most spores at the most severe conditions	Extensive whey protein denaturation—WPNI < 1.5/L;	SMP used for recombined evaporated milk
>120 °C/>40 s	High-high-heat SMP	Non-spore-forming pathogens, psychrotrophic spoilage bacteria and most spores	Almost complete denaturation of whey proteins—WPNI << 1.5 g/L	Product used for bakery products and recombined evaporated milk

7.1.2.2. Extended Shelf-Life and Ultra-High Temperature Treatments

Extended Shelf-Life (ESL) and Ultra-High Temperature (UHT) treatments are high-temperature processes which use similar heating equipment but differ primarily in the temperature of heating and conditions of storage of the products (**Deeth and Lewis, 2017**).

Several aspects of ESL and UHT processing and products are summarized in Table 16.

Table 16. Comparison of Extended Shelf-Life (ESL) and Ultra-High Temperature (UHT) processing and products (**Deeth, 2011**).

<i>Characteristic</i>	<i>ESL</i>	<i>UHT</i>
Heating conditions	Commonly 120–130 °C for <2–5 s	Commonly 138–142 °C for 2–5 s
Preheat holding	Not necessary	Advisable for inactivation of plasmin
Bacteria destroyed	All non-spore-forming bacteria including thermodurics, most spores	Almost all bacteria
Bacteria not destroyed	Spores with high heat-resistance	Very highly-heat-resistant spores
Cause of spoilage	Post-processing contaminants and psychrotrophic sporeformers	Rarely bacterial; gelation, sedimentation, fat separation, off-flavor
Packaging conditions	Ultra-clean or aseptic	Aseptic
Storage conditions for final product	Refrigeration	Ambient
Shelf life	30–60 days with ultra-clean packaging, more with aseptic packaging	6–12 months

A basic principle of high-temperature processing of milk, which applies to ESL and UHT processing, is that, for the same bactericidal effect, high-temperature, short-time heating causes less chemical change than low-temperature, long-time heating. Thus, UHT heating at 140°C for a few seconds causes much less chemical change than batch sterilization in retorts at 120°C for several minutes. The significance of this difference is that for UHT and ESL processing the least change in flavor (e.g., development of cooked flavor), is achieved by heating at a high temperature for a short time rather than heating for a longer time at a lower temperature (**Deeth and Lewis, 2017**).

7.1.2.3. Thermization (LTLT) and Pasteurization (HTST)

7.1.2.3.1. Thermization

Thermization is the generic term for a range of sub-pasteurization heat treatments of milk (**Deeth and Smithers, 2018**).

This process markedly reduces the number of spoilage bacteria with minimum collateral heat damage to milk components and it does not cause changes in flavour (Deeth, 2011).

Originally, thermization was developed as a heat treatment of milk before cheese making. Thermization at 57–68 °C for 10–20 s was aimed at destroying adventitious bacterial flora and providing a suitable environment for the multiplication of lactic acid bacteria introduced in starter cultures. In the 1970s, when the cold storage of raw milk became a widespread practice, a concept was developed to extend the keeping quality of raw milk by thermization on delivery to dairy plants or on farms between milk collections. The thermized and promptly cooled milk could be cold-stored for an additional 3 days because thermization markedly reduces the heat-labile psychrotrophic microflora responsible for spoilage at low temperatures (spoilage without souring). At low storage temperatures, those microorganisms can produce heat-stable proteinases, lipases, and phospholipases which cause flavor and texture defects in long-shelf-life products such as cheese and UHT milk. Extension of the keeping quality allowed dairies to be closed over weekends. Thermization at 62–68°C for 15 s is practiced widely; it can be conveniently performed in a plate heat exchanger designed for the pasteurization of milk using a standard 15 s holding time (Deeth, 2011).

The term thermization has also been applied to heat treatment of 55°C for 6 min. This treatment has been applied to cultured milk during the manufacture of quarg, (Zakrzewski et al., 1991).

7.1.2.3.2. Disadvantages of Thermization

While thermization reduces psychrotrophic bacterial growth, it has been shown to activate germination of *Bacillus cereus* spores (Stadhouders, 1982).

Thermization at 65°C for 10 s may be sufficient to stimulate the germination of *B. cereus* spores; activated, spores may germinate after 6 h at 10°C. (Deeth, 2022).

7.1.2.3.2. Pasteurization

Pasteurization of milk has been one of the most successful food safety technologies. It was first used on wine, prior to 1857 and then on beer. The first pasteurization systems for milk were introduced in Germany in 1895 and in the US in 1907. These were batch or holder or in-vat systems. They were widely used until the

1950s although by then the continuous process or the high-temperature, short-time (HTST) process was well established. Batch processes are still used in some small-scale processing operations (Deeth, 2011).

Pasteurization is the second lowest intensity treatment after thermization (Deeth, 2021). Whereas milk subjected to thermization is not considered a safe product, pasteurized milk is considered safe because the heating conditions used are designed to inactivate the most heat-resistant pathogenic non-sporeforming organisms, namely *Mycobacterium tuberculosis* and the slightly more heat-resistant *C. burnetii* (Deeth, 2011).

7.1.2.3.3. Pasteurization Conditions

It is generally accepted that the minimum conditions for pasteurization are 63°C for 30 min (batch) or 72°C for 15 s for the high-temperature, short-time (HTST) (Deeth, 2021).

According to **Codex Alimentarius, (2003)**, these conditions are designed to achieve at least a 5-log reduction of *C. burnetii* in whole milk. Pasteurization inactivates pathogens that might be present in raw milk with the exception of spore-formers such as *B. cereus* of which some strains can be toxigenic (Juffs and Deeth, 2007). Table 17 showing the temperatures at which the six most common non-spore forming pathogens are sufficiently inactivated with 15 s hold times. All six are inactivated at well below

72°C (Pearce et al., 2012).

Table 17. Temperatures of inactivation of pathogens during heat treatment for 15 s (Pearce et al., 2012).

Pathogen	Temperature of inactivation for 15 s hold time (C)	log reduction at temperature of inactivation
<i>Listeria monocytogenes</i>	65.5 >	>6.9
<i>E. coli</i> 0157:H42	65	>6.8
<i>Yersinia enterocolitica</i>	62.5	>6.8
<i>Cronobactersakazakii</i>	67.5	>6.7
<i>Staphylococcus aureus</i>	66.5	>6.7
<i>Salmonella typhimurium</i>	61.5	>6.9

7.1.2.3.4. Other methods

Other nonthermal processing technologies have also been explored to inactivate microorganisms in dairy products, including pulsed electric fields ultrasound, irradiation and others (**Martin et al., 2021**).

7. Milk and dairy products biopreservation

Some compounds having medical properties like spices, for example, black pepper, coriander, cassia bark, cinnamon, cumin, and cardamom. In the earliest time, the human has used spices for a valuable purpose. A hundred varieties of spices were used to create the preserved food. In different industries, some spices are used for the wide applications which are beneficial for living organisms such as the type of spices are good for digestion and metabolism similar to cinnamon and ginger. Hence, material are using for production of perfumes, a color of food, and its flavor. Turmeric, ginger, red chili, garlic and small cardamom are antimicrobial effects, not all types of spices and herbs are safe for us (**Hill al., 2017**).

In earlier times, milk was used for a longer time due to its vast process of preservation as it was completed in three steps: pasteurization, sterilization, dehydration These processes convert raw milk into other forms of dairy products for the consumption of mankind. This method was adopted on a small scale level and other level for milk processing. The main purpose for the preservation is that to control the growth of spoilage microorganisms that cause contamination in storage and to increase the shelf-life of products (**Thorning et al., 2016**).

Biopreservatives play an important role due to their advantageous effects on health as compared to chemical preservatives. Different types of microorganisms are classified and studied by researchers to estimate beneficial uses for preservation. (**Kerr, 2003**); (**Donovan al., 2005**).

8.1.Plant extracts as natural antimicrobials and Mode of action

Antimicrobials are compounds used for food preservation by controlling the growth of spoilage causing and pathogenic microorganisms. There are a range of natural compounds with antimicrobial activity that have been identified from various sources (plants, animals, or microbes). However due to the structural differences between Gram-negative and Gram positive bacteria, the efficacy of antimicrobial agents may vary (**Stan et al., 2021**).

In plants, natural compounds exerting antimicrobial activity are phenolic compounds, alkaloids, sulfur-containing compounds, and terpenoids, as well as essential oils and their constituents (**Diao et al., 2013**); (**Tajkarimet al., 2020**).

Generally, natural compounds with different chemical groups can permeate or disrupt the cytoplasmic membrane, allowing the passage or release of nonspecific compounds. Increased cell membrane permeability leads to the release of intracellular compounds, especially potassium, calcium, and sodium ions, causing irreversible damage (**Zhang al., 2016**); (**Khameneh al., 2019**). Natural compounds may also inhibit the ATPase enzyme responsible for the energy generation of the cell, which leads to cell death (**dos Santos et al., 2017**); (**Ritota et al., 2020**).

In a study conducted by **Gonelimali et al.**, the plant extracts significantly affected the cell membranes of Gram positive and Gram negative bacteria, as demonstrated by the decline in pH and the hyperpolarization of the cell membrane. Some fatty acids, in particular, have the ability to interfere with the structure of the cell membrane, displacing phospholipids and increasing their permeability. Five of them are acetylenic: 6-hexadecinoic, 6-heptadecinoic, 6-octadecinoic, 6-nonadecinoic, and 6-eicosinoic acids, whereas the other three are saturated: palmitic, heptadecanoic, and stearic acids (**Vila et al., 2013**).

The activity of polyphenols depends on the number and position of hydroxyl groups. Polyphenols may inhibit the enzymes of microorganisms, possibly through interactions with sulfhydryl groups or through less specific interactions with proteins (**Arif al., 2009**).

Mikłasińska-Majdanik et al, (2018) reported that phenolic compounds partially damaged the bacterial membrane, inhibited virulence factors such as enzymes and toxins, and suppressed bacterial biofilm formation.

In addition, some natural polyphenols, aside from their direct antibacterial activity, exert a synergistic effect when combined with common chemotherapeutics. Essential oils also have antibacterial, antioxidant, and antimutagenic properties, as well as possible health benefits. These natural compounds, which are generally recognized as safe (GRAS), reduce lipid oxidation in foods and hence hold potential as natural food additives (**Zengin et al., 2014**).

There are many types of compounds in essential oils which have proven antimicrobial properties. They include phenolic monoterpenes (thymol and carvacrol), phenylpropanoids (eugenol), alcoholic monocyclic monoterpenes (α -terpineol and terpinen-4-ol), as well as bicyclic monoterpene hydrocarbons (α -pinene) and ketones (camphor). The acidic nature of the hydroxyl group of phenols facilitates a hydrogen bond with the enzyme active center, which is responsible for their high activity (**Tampieri et al., 2005**).

Terpenoids can rupture the cell membrane due to their lipophilic nature. Challenges of using plant extracts as antimicrobials in the dairy industry. In vitro, several studies have demonstrated that plant extracts and essential oils of aromatic and medicinal plants have antimicrobial activity against pathogens and spoilage microorganisms associated with food contamination (**Hassanien et al., 2014**); (**Moro et al., 2015**).

However, these results cannot be strictly valid due to the complexity of the food matrix. Moreover, many factors interfere with the activity of these compounds, such as proteins, lipids, packaging, storage temperature, type of microorganism, and compound stability (**Calo et al., 2015**); (**dos Santos et al., 2017**).

Proteins and lipids, for example, can wrap around the surface of the microorganism, forming a physical barrier that prevents the bioactive compound from coming into contact with the microorganism, thus reducing its efficacy (**dos Santos et al., 2017**). In a similar report, the antimicrobial activities of cinnamon and clove essential oils were lower in the high-fat milk samples than in the skim milk samples (**Cava et al., 2007**).

Dairy products are foods with a high content of proteins, lipids, minerals, and vitamins. So, when adding extracts and essential oils of aromatic plants to dairy products as antimicrobials, we must take into account the following factors:

1. Natural compounds added as antimicrobials should be in greater concentrations than those tested in vitro.

According to **Gammariello et al. (2008)** the concentration of active compounds used to inhibit the growth of pathogenic microorganisms in Fior di Latte cheese was significantly higher than the level tested in vitro.

The minimum inhibiting concentration of pomegranate essential oil against *Listeria monocytogenes* and *Staphylococcus aureus* (105 CFU/mL each) was higher than 2.5 mg/mL in a culture medium, while its concentration of 40 mg/mL in Cheddar cheese failed to inhibit the same population of those microorganisms (**Shan et al., 2011**).

Hassanien et al., (2014) also mentioned that the 0.1% concentration of black cumin essential oil reduced the growth of *L. monocytogenes*, *S. aureus*, *Escherichia coli*, and *Salmonella enteritidis* in a culture medium, while in cheese, such concentrations were not effective against *S. aureus* and *L. monocytogenes*.

2. In some cases, mixing some plant components at low concentrations has a higher antimicrobial effect than adding them separately, which proves their synergistic effect (**dos Santos et al., 2017**);

3. Dairy products contain all the nutrients necessary for the microbial growth of cultured cells, allowing for a faster recovery of cells damaged by natural antimicrobials (**Smith-Palmer et al., 2004**);

4. The contents of natural compounds can decrease during processing and storage. **Libran et al. (2013)** reported a decrease in the content of compounds from basil and tansy essential oils added during cheese production. In another study, rosemary essential oil added to sheep milk during cheese manufacturing had a loss of 37.49% (**Moro et al., 2015**);

5. Microencapsulation can improve the stability of natural substances throughout processing and storage (**Caleja et al., 2016**). Nanoemulsions of plant extracts can decrease the quantity of a required effective dose and enhance the material's bioactivity against bacteria by allowing them to penetrate the cell membrane and thus destabilize its lipid bilayers (**Salvia-Trujillo et al., 2013**). For example, a nanoemulsion of anise extract performed better than bulk extract as an antimicrobial agent against some foodborne pathogenic bacteria (**Ghazy et al., 2021**).

8.2. Plant extracts as antibacterial in dairy products

Several plants in various forms (powder, essential oils, extracts, etc.) have been successfully used in dairy foods. Plant extracts of cinnamon, garlic, lemongrass, cress,

rosemary, sage, and oregano individually inhibited the population of *L. monocytogenes* in processed cheeses (Wahba et al., 2020).

According to Shan et al.() all the extracts of cinnamon stick, pomegranate peel, grape seed, oregano, and clove inhibited the growth of *S. aureus*, *L. monocytogenes*, and *S. enterica* in cheese (Shan al., 2011). As a result, these extracts, especially clove, have the potential to be employed as natural food preservatives. Cayenne and green pepper extracts also reduced the *S. aureus* population in Egyptian Kareish cheese (Mahajan al., 2016).

Mahajan et al (). Reported that the aqueous extracts of pine needles improved the microbiological properties of low-fat Kalari, an Indian hard cheese, due to their antioxidant and antimicrobial properties.

Sulfurcontaining compounds are credited with antimicrobial activity in plant-based compounds, particularly diallyl sulphides in *Allium* species, terpenoids (carvone and limonene) in spearmint essential oil, eugenol in clove oil, and thymol in thyme oil. Ginger's antimicrobial activity is attributed to several compounds, including gingerols, gingerdiols, and shogaols (Vazquez al., 2001);(Al-Snafi al., 2015).

Table 18 lists bioactive components and their functional qualities in plants used to manufacture functional dairy products.

Table 18. Plants and their bioactive compounds with antimicrobial and antioxidant activities in some dairy products

Plants	Scientific name	Bioactive components	Applications
Thyme	<i>Thymus vulgaris</i> L.	Thymol (phenolic monoterpenes)	Ricotta cheese, Coalho cheese, Mimicking models, Fior di Latte cheese, Feta cheese, Labneh, Butter
Basil	<i>Ocimum basilicum</i> L.	Carvacrol (phenylpropanoids)	Ricotta cheese, Serra da Estrela cheese, Ice cream
Cloves	<i>Eugenia caryophyllata</i> L.	Eugenol (α -terpineol and terpinen-4-ol)	Yoghurt, Paneer cheese, ArzaUlloa cheese

Cinnamon	<i>Cinnamomum zeylanicum</i>	Cinnamaldehyde	Cheddar-based media, Spreadable cheese, Appenzeller cheese, processed cheeses
Ginger	<i>Zingiber officinale</i> L.	Gingerols, Gingerdiols, Shogaols	Fortified cheese, UF-soft cheese
Oregano	<i>Origanum vulgare</i> L.	Carvacrol, Thymol, γ -terpinene, <i>p</i> -cymene, Carvacrol methyl ethers	Cheddar-based media, Feta cheese
Black cumn	<i>Nigella sativa</i> L.	Thymoquinone, Thymol, α -hederin	Iranian white cheese, Feta cheese, Domiati cheese
Roselle	<i>Hibiscus Sabdariffa</i> L.	Calyx, Chlorhexidine, Amoxicillin-clavulanic acid, Tetracycline, Metronidazole	Yoghurt
Garlic	<i>Allium sativum</i> L.	Oil-soluble organosulfur compounds: include Allicin, Ajoenes, Allyl sulfides, Actericidal, Antibiofilm, Antitoxin, and Anti-quorum	UF soft cheese, soft cheese, processed cheeses
Sage	<i>Salvia officinalis</i> L.	Geraniol, Pinene, Limonene, Carnosol, Saponin, Catechins, Apigenin, Luteolin, Rosmarinic, Carnosine, Vanillic, Caffeic acids	Sour cream, Fior di Latte cheese, Cheddar cheese, Yoghurt, Ghee, Butter oil
Rosemary	<i>Rosmarinus officinalis</i> L.	1,8-cineole, Borneol, Camphor, Caffeic acid, Rosmarinic acid, Luteolin-7-O glucoside,	Ghee, Butter oil, Sour cream, Yoghurt, Sheep's cheese, Cheddar-based media, Cottage cheese, Herbed cottage

		Carnosic acid, Ursolic acid, Carnosol, di- and triterpenes	cheese, Flavored yoghurt
Lemongrass	<i>Cymbopogon</i> L.	Myrcene, limonene, citral, geraniol, citronellol, geranyl acetate, neral, and nerol	Indian soft cheese, Yoghurt, Coalho cheese, processed cheeses
Dill	<i>Anethum graveolens</i> L.	Quercetin, Kaempferol , Myricetin, Catechins, Isorhamneti n, Carvone, Limonene	Milk

In addition, the essential oils of aromatic plants also showed anti-bacterial activity in food preservation, even with Gram-negative bacteria. Gram-negative bacteria have an effective permeability barrier consisting of a thin lipopolysaccharide exterior membrane, which could restrict the penetration by the extruding plant extracts. Gram-positive bacteria have a mesh-like peptidoglycan layer which is more accessible to permeation by plant extracts (Zgurskaya al., 2015).

In Feta cheese and Iranian white cheese, oregano (0.1%) and thyme (0.1%), salvia (0.1%), basil (1%), and black cumin essential oils had antimicrobial activity against *L. monocytogenes* (Govaris al., 2011) ;(Ehsani al., 2016).

In Iranian white cheese inoculated with *E. coli* O157:H7 and treated with black cumin essential oil, the pathogen growth was significantly lower compared to the control during storage (Azizkhani et al., 2016).

Adding clove essential oil to Paneer cheese increased its shelf life to 10 days in the treated cheese compared to 5 days in the control sample. Furthermore, the control samples had a higher microbial count compared to the treated cheese. Clove essential oil added at concentrations of 0.5 and 1% dramatically reduced the growth rate of *L. monocytogenes* in cheese at 30 and 7°C. However, high concentrations of clove oil may adversely affect the sensory properties of food. Thus, small concentrations may

be enough to ensure low bacterial load and, therefore, food safety (**Vrinda Menon et al., 2001**) .

The addition of aqueous licorice and cinnamon extracts to yoghurt exhibited the strongest inhibitory effect on *Helicobacter pylori* development when compared to the control yoghurt (**Behrad et al., 2009**).

According to **Mahgoub et al.** adding 0.2% *Nigella sativa* essential oil to the cheese improved its physicochemical and sensory qualities. In addition, it provided the most effective antibacterial capability against *S. aureus*, *S. enteritidis*, and *E. coli* (**Mahgoub et al., 2013**).

In goat milk-based yoghurt containing *Lactobacillus acidophilus* and roselle extract, higher antimicrobial activities were observed against *Bacillus cereus*, *E. coli*, *S. aureus*, and *Salmonella typhi*. This could be attributed to the production of higher antimicrobial compounds such as antimicrobial peptides and organic acids (**Hanifah et al., 2016**).

09. Applications of cold-adaptation tools in industry

9.1. Applications of cold-adapted enzymes

The ultimate goal of biotechnology is the delivery and usage of developed processes or technology in different industries. Cold Adapted Enzymes CAEs are well suited for various industries due to their natural properties like high catalytic activity at low temperatures, cost efficiency, limited undesirable product formation, and many more **(Bruno et al. 2019)**.

Primarily, they require lesser energy for activity as compared to their mesophilic and thermophilic counterparts. This property makes them well suited in current biotechnology industries where the prime goal is reducing the energy needs of a process.**(Kumar et al., 2021)**

A brief overview of the application of cold-adapted enzymes in various industries is also shown in Figure 22 and Table 19.

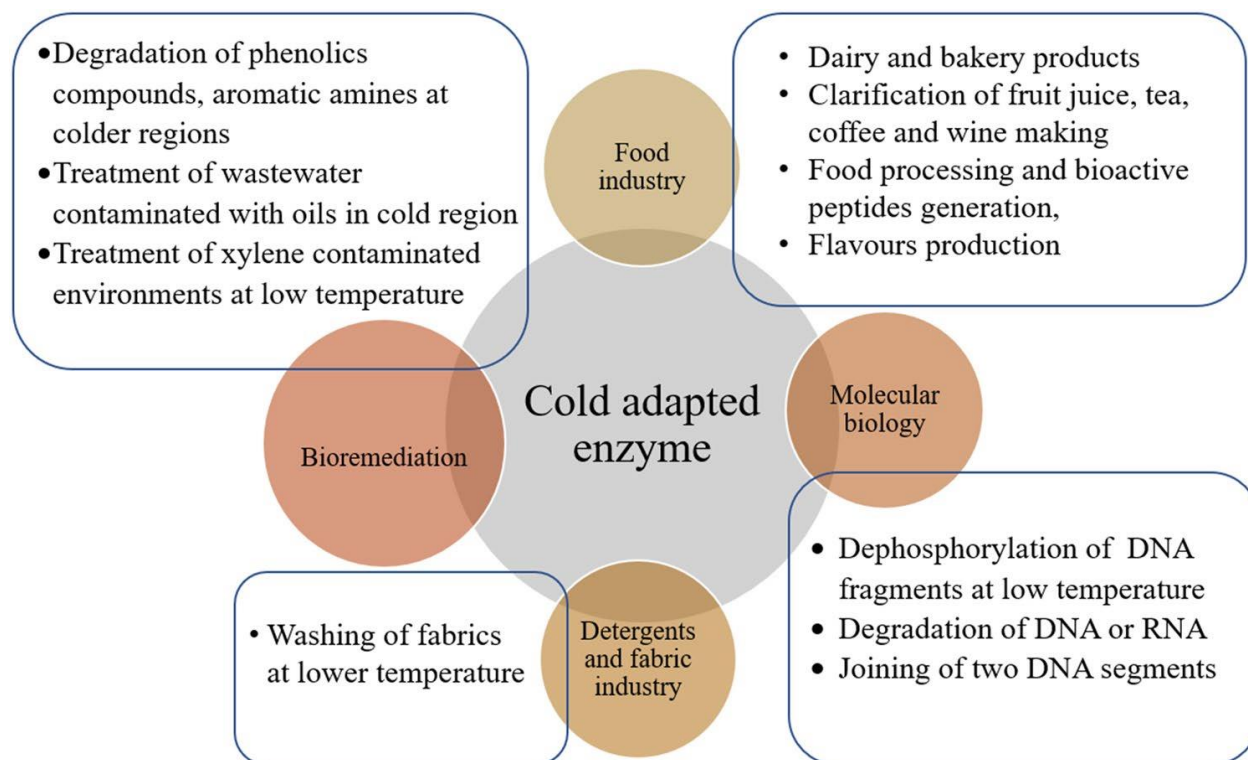


Figure 22. A summary of the applications of cold-adapted enzymes in various industries **(Kumar et al., 2021)**.

Table 19.The applications of cold-adapted enzymes in various industries (**Kumar et al., 2021**).

Industry	Cold-adaptive enzyme	Application
Food	α -Amylase	Beverages, bread, and various dairy products
	β -Galactosidase	Production of lactose-free milk products, increasing sweetness of milk products
	Pectinase	Clarification of fruit juice, natural oils extraction, wine, coffee, and tea making
	Protease	Food processing (tenderizing meat at low temperatures), bioactive peptides generation
	Xylanase	Bread and baking industry to help in increasing softness of the bread
	Lipase	Improving flavours of various food products
Detergent and fabric	Lipase	Washing of fabrics at lower temperature
	Amylase	
	Protease	
Molecular biology	Alkaline phosphatase	Dephosphorylation of the 5' end of linearized DNA fragments at reduced temperature
	Nucleases	Degradation of DNA or RNA to remove contaminating nucleic acids

	DNA ligase	Joining of two DNA segments
Bioremediation	Laccase	Degradation of phenolics compounds, aromatic amines, and other organic compounds at colder regions
	Lipase	Bioremediation of wastewater contaminated with oils in cold regions
	Xylene monooxygenase, Catechol 2,3-dioxygenase	Bioremediation of xylene contaminated environments at low temperature
Mics	Endo- β -1,4-glucanase	Biofuel production from seaweeds, cellulosic bio-ethanol production at low temperature
	Cellulase	Cellulose processing industry requiring low temperature, saccharification of switchgrass and coffee grounds
	β -Glucosidase	Hydrolysis of cellobiose to ethanol
	Lipase	Biodiesel production at low temperature
	Superoxide dismutase	Antioxidative and antiapoptosis
	Cephalosporin C acylase	Low-temperature biosynthesis of 7-aminocephalosporanic acid
	N-Acetylneuraminate lyase	Synthesis of sialic acids

	S-Formylglutathione hydrolase	Synthesis of glutathione and formate
	Transaminase	Development of stereoselective amination at low temperatures

9.2. Applications of cold-adaptation tools in industry

Table 20 showing an overview of cold-adaptation tools with commercial potential.

Table 20. An overview of cold-adaptation tools with commercial potential (Collins and Margesin, 2019) .

Bioproduct	Proposed cold-adaptation functions	Potential applications
Membrane fatty acids: unsaturated fatty acids, long-chain polyunsaturated fatty acids (LC-PUFA)	Unsaturated fatty acids: maintenance of membrane fluidity LC-PUFA: maintenance of membrane fluidity	Nutrition and health
Membrane pigments: carotenoids	Maintenance of membrane fluidity Cryoprotection	Nutrition and health
Compatible solutes	Osmoprotection: against freezing induced osmotic stress Desiccation protection: against freezing induced desiccation Freezing point depression Colloidal glass transition temperature depression Protein and membrane stabilisation	Cryopreservation Stabilisation of biological materials Food stability and freshness Cosmetics and skin care Plant resistance enhancement
Antifreeze proteins	Ice growth inhibition (thermal hysteresis)	Frozen foods preservation and quality enhancement

	Ice recrystallisation inhibition Membrane stabilisation? Ice adhesion?	Cryopreservation Cryosurgery Freeze tolerance enhancement, e.g. crops, fish Gas hydrate inhibition ‘Ice-prevention’ materials
Ice-nucleating proteins	Extracellular ice crystal nucleation - Prevention/reduction of damaging intracellular ice formation - Small ice crystals? - Release of latent heat of crystallisation?	Artificial snow production Frozen foods and beverages industries Microfluidic devices: freeze–thaw valves Cell surface display

		Climate control
Extracellular polymeric substances	Ice growth inhibition Osmoprotection: against freezing induced osmotic stress Desiccation protection: against freezing induced desiccation Ice-recrystallisation inhibition	Biopolymers Biofloculants Bioabsorbants Bioemulsifiers Bioadhesives Thickening agents Cryopreservation
Biosurfactants	Ice-recrystallisation inhibition? Osmoprotection?	Biosurfactants
Cold-adapted enzymes	Maintenance of adequate metabolic flux	Enzymes markets: low to moderate temperature processes, processes where

		simplified inactivation (e.g. by mild heat treatment) is beneficial
Chaperones	Promotion of protein folding and stability Destabilisation of RNA/DNA secondary structures	Low temperature recombinant protein production
Storage compounds: polyhydroxyalkanoates, cyanophycins	Overcoming carbon and nitrogen uptake deficiencies	Biopolymers Fine chemical synthesis Biofuels Stress tolerance enhancement: plants

Conclusion

The consumption of fresh raw milk or naturally fermented milk without any artificial additives or thermal pretreatment is gaining popularity worldwide as it has many advantages including enhanced nutritional value, digestibility, therapeutic benefits and safety against pathogens.

However, milk is an excellent culture medium for the growth and reproduction of microorganisms. It is known that raw milk harbor a complex microbial ecosystem encompassing numerous strains. Microorganisms are originate from different sources: air, milking equipment, feed, soil, excrements, grass, water, skin, and hair of the animals, utensils or from the milk handlers.

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. These microbes may account for only a small fraction of the initial flora of processed milk. Bacterial spoilage ensues when growth conditions during refrigerated storage allow psychrotrophic microbes to increase in number and to become the dominant microflora.

Psychrotrophic bacteria from numerous genera have been isolated from milk, both Gram negative (*Pseudomonas*, *Stenotrophomonas*, *Aeromonas*, *Serratia*, *Acinetobacter*, *Alcaligenes*, *Achromobacter*, *Enterobacter*, *Proteus*, *Yersinia*, *Klebsiella* and *Flavobacterium*); and Gram positive (*Bacillus*, *Listeria*, *Clostridium*, *Corynebacterium*, *Microbacterium*, *Micrococcus*, *Enterococcus*, *Streptococcus*, *Staphylococcus*, and *Lactobacillus*). Of these, *Pseudomonas* is the most frequently reported psychrotroph in raw milk.

Refrigeration create harsh conditions, to overcome the multiple challenges inherent to life in their cold habitats, the psychrotrophic bacteria have developed a diverse array of highly sophisticated synergistic adaptations at all levels within their cells: from cell envelope and enzyme adaptation, to cryoprotectant and chaperone production, and novel metabolic capabilities.

Several methods are implemented to prevent and control such contaminations. Prevention methods include good manufacturing and hygiene practices, air filtration, and decontamination systems, while control methods include inactivation treatments, temperature control, biopreservation and refrigeration.

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