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Milk Production, Processing and Marketing

Edited by Khalid Javed

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Meet the editor

Dr. Khalid Javed is a professor of animal breeding and genetics at the University of Veterinary and Animal Sciences, Lahore, Pakistan. He graduated in Animal Husbandry from the University of Agriculture, Faisalabad, in 1982. He earned his Master's and Doctorate degrees in Animal Breeding and Genetics from the same University. He is engaged in research in different capacities at different livestock research stations in Punjab. He has vast experience in research, training, and teaching in the field of Animal Production. His research focus is on the selection and breeding of large and small ruminants. He has published a large number of scientific articles (N = 160) to disseminate knowledge to researchers and livestock producers in the areas of animal husbandry for the productivity enhancement of livestock.

Contents

Preface

It is an established nutritional fact that animal proteins are superior to vegetable proteins for the supply of essential amino acids. Milk is considered as a complete diet for an infant and contains essential nutrients for the development of young mammals. The substances in milk provide energy and antibodies that help protect against infection. Dairy animals of the present era are the outcome of thousands of years of breeding of undomesticated animals living in different parts of the world at different altitudes and latitudes, exposed to severe and extreme weather conditions. The present techniques used to produce milk from cows, goats, sheep, camels, and buffaloes started around 6000 years ago. The same species of animals are being maintained as dairy animals today. Most farmers are paid on the quality and composition of their milk. Whole milk, once approved for use, is pumped into storage silos where it undergoes pasteurization, homogenization, separation, and further processing. Milk is a highly perishable commodity because it is an excellent medium for the growth of microorganisms—particularly bacterial pathogens—that can cause spoilage as well as diseases in consumers. Milk processing allows the preservation of milk for days, weeks, or months and helps to reduce food-borne illness. An efficient milk marketing chain is one that enables farmers to receive at least 50% of the retail price of milk. The price of a product in the market is an important factor influencing consumer demand. When marketing a dairy product, the most important aspect of the strategy is determining the competition and audience. Through establishing these parameters, milk producers can help to flush out what aspect of a dairy business to highlight and where will be the most effective place to advertise to capture the target audience.

A large number of publications on milk production, processing, and marketing are available in the world market. The book under discussion has not been compiled to add yet another to such publications, rather it has been written using a novice format to meet the requirements of students, researchers, and policy makers working in different parts of the world in different environments in the fields of milk production, processing, and marketing.

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Chapter 1

Current Standing and Future Challenges of Dairying in Pakistan: A Status Update

Muhammad Naeem Tahir, Roshan Riaz, Muhammad Bilal and Hafiz Muhammad Nouman

Abstract

Pakistan is considered among the leading raw milk producing countries. Unlike the production systems in the developed countries, milk production systems in Pakistan represent smallholding with subsistence- or market-oriented-level farming followed by peri-urban or commercial-level farming. Historically, dairy sector has been owned and managed by the private sector. During the past two decades, new initiatives have been taken because of the active involvement of corporate private sector. These efforts have resulted in improvements like enlargement of herds and import of high-quality milk germ plasm, the productivity per animal, milk collection, processing and marketing, the supply of dairy inputs (machinery, equipment, feeds, semen, and elite dairy animals), and farmers knowledge, and skills on modern management practices. Conclusively, the dairy sector is performing at some sustainable level to meet the food requirements of the growing population and helping save a handful of foreign exchange. Yet, challenges like local replacements of high genetic potential dairy animals, health hazards of β-casein proteins, antibiotics and aflatoxins, and uneconomical operational costs facing the dairy industry in the near future need to be addressed. The main objective of this chapter is to identify the current trends in dairy industry of Pakistan and describe those factors, which can influence the sustainability and profitability of dairying in the near future.

Keywords: collection and processing, dairy inputs, food legislation, large peri-urban dairy farming, profitability, sustainability

1. Introduction

Pakistan is considered among the leading raw milk producing countries. Unlike the production systems in the developed countries like United States of America and most of Europe, milk production systems in Pakistan have similar characteristics to the most developing countries of subcontinent. Characteristically, smallholding with subsistence- or market-oriented-level farming keeps the major share (about 90%) followed by peri-urban or commercial-level farming [1].

Current dairying in Pakistan is a combination of both traditional and commercial methods of raising dairy animals, and producing milk and milk products. During last two decades, commercial farming with imported dairy inputs has increased and reached roughly about 1% of the total raw milk production. With the

changing human needs and urbanization, the traditional system are on the edge of converting from subsistence level to more commercial and large-scale production systems during the said period [2]. In addition, policy made at government level in 2007 (Pakistan's first-ever Livestock Policy) placed considerable focus on dairy sector development. This has invited several private stake holders to invest in the farming, procurement, and processing of dairy and dairy products.

Despite the fact that the government has identified the dairy sector as one of the key priority sectors for development, the farmers being the main stakeholders are still facing constraints of inherent nature. They find limited opportunities to access consumers or industry directly and, therefore, have little control on the price or quality of milk owning to small-sized enterprises and limited resource inputs.

This chapter reviews last 10 years data and reports those significant changes that have been brought about in dairying in the country. The main objective of this chapter is to identify the current trends in dairy industry of Pakistan and describe those factors, which can influence the sustainability and profitability of dairying in the near future.

2. Milk production systems: general characteristics

Unlike the production systems in the developed countries like United States of America, milk production systems in Pakistan have similar characteristics to the most developing countries of subcontinent; smallholders with subsistence- or market-oriented-level farming keeping the major share followed by peri-urban or commercial-level farming [1]. Dairy farming in Pakistan is practiced mainly by the private sector on various scales, in both urban and rural settings. However, the sector is generally characterized as fragmented and subsistence. With the exception of some peri-urban units, most dairy farming is practiced in mixed crop-livestock systems.

Classically, dairy production systems in Pakistan fall into five main systems of milk production based on location, herd size, and level of management. These are smallholder subsistence, smallholder market-oriented, rural commercial, peri-urban, and large peri-urban. **Figure 1** shows percentage contribution of different milk production systems in total annual milk production. These systems are explained in the following subsections.

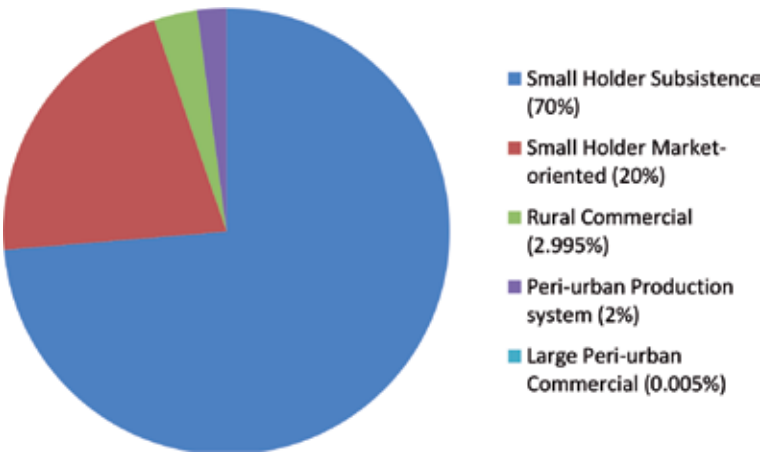

Figure 1.
Annual milk production from various milk production systems in Pakistan in 2016, based on Livestock Censes [4] and annual growth trends [5].

2.1 Smallholder subsistence production system

Smallholders produce milk to meet family requirements at minimal cost and have limited access to substantial milk market. The average subsistence unit consists of about three buffaloes, with at least two in milking. Average milk yields per animal are 3 L/day. The main inputs into these households' dairy production are often noncash resources, such as family-owned land and labor. Some 70% of smallholder milk producers fall into this category [3]. Most of the milk produced is utilized as fluid for fulfilling the family needs, and the rest is converted into butter and ghee. This system implies the use of household labor and therefore high labor-intensive occupation. Almost 50–60% of the feed requirements of these animals are fulfilled from grazing along with wheat straw and some green fodder. No purchased concentrates are offered. The proportional contribution of this system is declining and is replaced by smallholder market-oriented production system.

2.2 Smallholder market-oriented production system

As presented earlier, rural subsistence production system is changing into rural market-oriented smallholder production system with passage of years. This system is identified by its typical number of animal holdings and surplus milk production than the family requirements. There are usually 5–7 animals per household, both cows and buffaloes. Of these, there are usually 3–4 adult lactating animals along with one or two heifers and one or two male calves. Breeding bulls are normally absent. Feeding requirements of lactating animals are fulfilled from fodder along with wheat straw and seed cake. More than 70% of milk produced is sold either directly to retail shops or through intermediaries. This system is practiced by those smallholders who have access to nearby livestock markets, and they are encouraged to produce in excess of family requirements [3, 4].

2.3 Rural commercial production system

In 2006, dairy sector in Pakistan moved toward commercial side and this encouraged some progressive farmers to invest in milk production. A typical rural commercial dairy farm running on commercial basis consists of about 30 animals of which 70% are females, including some cows. Approximately 40% of these adult females are in milk during most of the year. Fodder crops provided 50% and straws about 35% of the feed requirements and concentrates made the rest of it. More than 90% of the milk produced at the farm is sold. Average milk yields per animal are 10 L/day. Potential channels for the marketing of milk in this system have changed from traditional system to selling to the commercial milk collection companies. This system presents the second largest source of milk collection by commercial dairy companies after large peri-urban commercial farming.

2.4 Peri-urban production system

Peri-urban production occurs in commercial-scale units located on the peripheries of major urban centers. With growing demand for milk in urban areas, rural commercial dairy farming moved toward peri-urban areas. These maintain herd sizes ranging from 20 to 200 (small) and from 200 to 2000 (large) head, and averaging 50 animals; 90% buffaloes and 10 % cows with nearly 90% of adult females in production [6]. These units employ family and hired labor, the latter being paid at local urban rates. Milk is delivered to the market twice a day. Major overheads in this system include hired labor costs, animal shelter, veterinary care, feed, water

and electricity bills, and milk transport. Milk is usually sold through direct sale to retail shops in the city after decreaming with the target to sell almost total produced milk. Male calves are disposed off within first 2 weeks of birth. These animals are fed chopped green fodder and wheat straw and concentrate mixture with target to sell almost total milk produced. The current number of dairy farms falling into this category accounts for 200 units situated across the country.

2.5 Large peri-urban commercial dairy farming (key farms, mega farms, corporate farms, etc.)

A rapid increase in urbanization during the last two to three decades has encouraged shifting of peri-urban dairy farming to large peri-urban commercial dairy farming (corporate farming). The owners of these farms aim at getting maximum milk production with economical and quality feeding and good management. These farms are categorized as high inputs-high outputs production systems with no limits on provision of feeding (good quality green fodder or silage along with concentrate mixture) as well as other inputs (medicine, machinery, mechanization, etc.). Dairy animals maintained at these farms are considered elite animals from pure Holstein Friesian and crosses of Holstein Friesian and Jersey breeds; and their yields per day are considerably higher (25 L/day) than those maintained under other production systems. These farms are usually coupled with small-level milk processing (chilling, pasteurization, and packaging), and finished product is disposed of through outlets or departmental stores or supplied to dairy companies (chilled, unprocessed). These modern dairy farms represent less than 1% of total dairy animals and milk production in the country; however, peri-urban (Sections 2.4) and large peri-urban collectively make about 1% of the total. These farms are mostly located in the cultivated areas of the country especially central Punjab province (14) and Sindh (1) (data provided by the sector). The average number of exotic animals kept at these farms is between 2000 and 5000, and the farms with more than 5000 animals also exist. The farms produce large quantities of fluid milk ranging from 0.02 to 0.1 million L/day.

2.6 Trends in production systems

During the last 10 years, significant changes have occurred in dairy sector of Pakistan, and due to these changes, this sector is on the way to become an industry. A large number of modern dairy farms have been established in different areas. Such farms have adopted most modern management and feeding practices and well-trained man power. Milk produced on these farms is either sold out in processed/fresh form through outlets/departmental stores, etc. or supplied to dairy companies (data provided by the sector).

2.7 Trends in annual milk production and consumption

Approximately, 91% of milk is produced in rural areas, with peri-urban areas accounting for 19% now compared to previous corresponding figures of 80% (rural areas), 15% (peri-urban), and 5% (urban areas) [2] as presented in **Figure 2**. Annual milk production from 2006 to 2016 is presented in **Figure 3**, which shows that it increased by an average of 3.21% per year, or by an average of 1520 million L/year. Annual milk consumption increased at the same rate to that of milk production or by an average of 1216 million L/year. The extra volumes are a result of increased herd size, and cannot be attributed to enhanced animal productivity, which has remained constant.

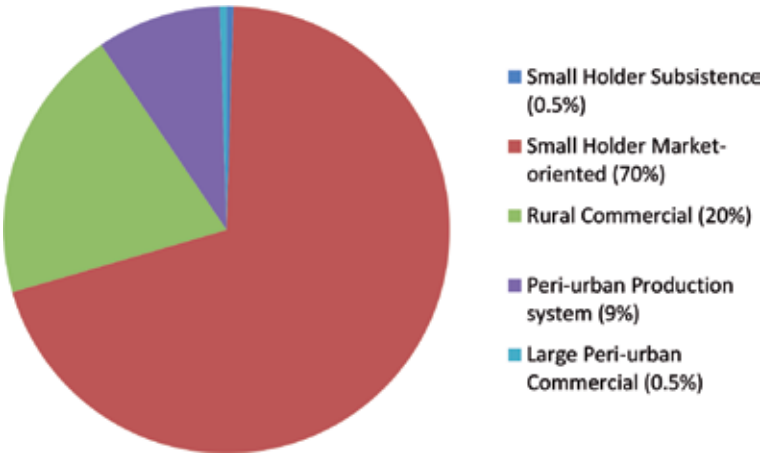

Figure 2.
Annual saleable milk production from various milk production systems in Pakistan in 2016, based on [6, 7] and the data provided by the sector.

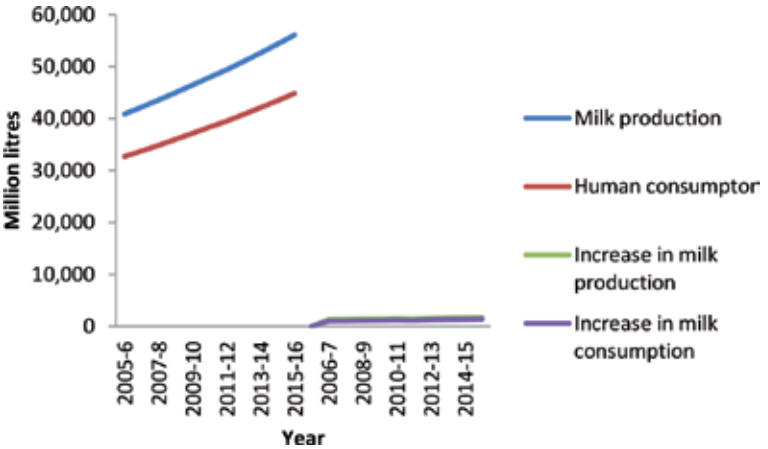

Figure 3.
Total milk production in the country and consumption by humans from 2006 to 2016. Source: Economic survey of Pakistan (2016–2017).

3. Population of major milk producing species of the animals

Pakistan has 44.4 million cattle and 37.7 million buffaloes, producing 20 and 34 billion L of milk, respectively [5]. The indigenous breeds of buffalo and cows are considered as poor producers with lactation yields of 1800 and 1195 L [8] that remained constant across years. The population of major milk producing animals is increasing at a constant rate of 3.3% per annum (**Figure 4**).

3.1 Buffaloes

Buffaloes are the major milk producing animals in Pakistan, representing about 46% of the total dairy herd and providing 62% of total milk production [5]. The three principal breeds are Nili, Ravi and Kundi. The Nili and Ravi breeds have originated from within a large tract evolved in between the great rivers of Ravi, Sutluj, and Chenab, indicating deltas of Nili- and Sandal-bars. Most famous cities of this tract are Faisalabad, Jhang, Lahore, Sahiwal, Okara, and Sheikupura. The Kundi

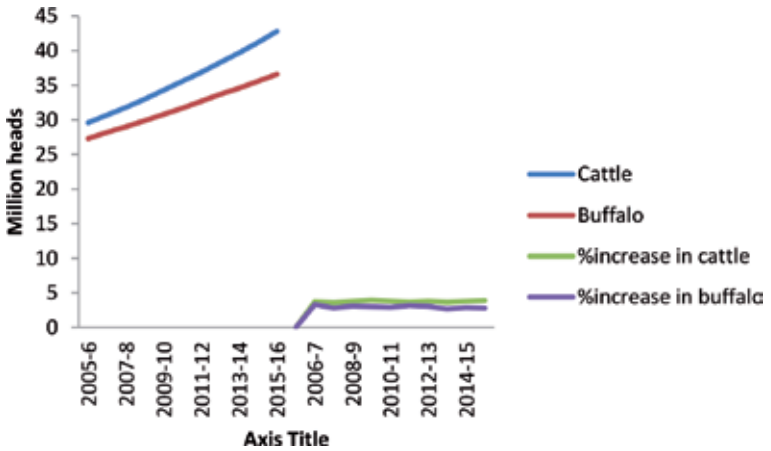

Figure 4.
Population dynamics of major milk producing species of large ruminants with percentage increase per year from 2006 to 2016. Source: Economic survey of Pakistan (2016–2017).

breed has been found all over Sindh province especially on the both side river Indus from Kashmore in the north to Shah Bandar in the south [9]. The Nili Ravi breed has evolved as a result of crossbreeding between the breeds: Nili and Ravi. The animals are massive and comparable to large imported breeds regarding feeding requirements. Their milk contains high contents of fat (**Table 1**; [10, 11]), which makes it possible to compare energy outputs in milk between buffalo and imported large dairy breeds of cows. There are currently breeding and performance evaluation programs established at various livestock research centers under the funding and supervision of the government, but farmers are largely excluded from the results of this research, thereby limiting its benefits. Buffalo farming at commercial levels is not common in the country because of less feed efficiency and other reproductive as well as managemental issues associated with the buffaloes. Therefore, the few set ups which were established in the past, e.g., Landhi Cattle Colony, Karachi and Fazal Dairy Farms, Muzaffargarh, are gradually shifting from buffalo to imported cattle.

3.2 Cattle

3.2.1 Indigenous dairy cattle

The cattle population is slightly larger than that of buffaloes, but cows produce on average only about 58% of the yield of buffaloes. All Pakistan's indigenous cattle

Parameters	Buffalo	Indigenous cow	Exotic cow (Holstein Friesian)
Total solids	15.6	12.1	12.2
Solids not fat	10.2	8.3	8.7
Fat	5.40	3.88	3.5
Crude protein	4.16	3.73	3.1
Lactose	5.30	3.84	4.9
Total ash	0.75	0.69	0.70

[1]*Jensen et al. [10] and Tahir et al. [11].*

Table 1.
Proximate parametric composition of raw milk from bubaline and bovine species.[1]

are Zebu (humped type, *Bos indicus*). There are 15 recognized breeds in the country, of which Red Sindhi and Sahiwal are well known internationally as tropical dairy cattle breeds. The home tract of Sahiwal cattle includes, Faisalabad, Jhang, Okara, and Sahiwal districts of central Punjab and Multan district of southern Punjab whereas that of Red Sindhi includes Dadu, Hyderabad, Karachi, and Thatta districts of province Sindh and Lasbela district of Baluchistan [9]. Cattle have traditionally been bred to produce bullocks for plowing and on-farm operations. Pure breeds account for 43% and nondescript for 44% [12].

3.2.2 Crossbred dairy cattle

A sizable population of cattle crossbreeds has recently emerged, and now represent 13% of Pakistan's total cattle population. Breeding policy allows the crossbreeding of nondescript cattle with Holstein, Friesian, and Jersey breeds, with the desired level of exotic inheritance being between 50 and 75%.

The productivity of dairy cattle crossbreeds is far higher than that of local nondescript or pure breeds, with longer lactation periods, higher milk production per lactation, and shorter calving intervals. These advantages make crossbred cattle highly preferred for intensive and semi-intensive dairy farming systems. Semen for crossbreeding programs is imported from countries such as the United States of America, the Netherlands, Germany, and Australia by private sector firms.

3.2.3 Imported dairy cattle

More recently, because of the involvement of private sector and policies made by the Government of Pakistan, Australian, Dutch, German and American Holstein Friesian and Holstein Friesian and Jersey crossbred cows have been imported and kept under specific management conditions by the commercial farmers. A sizeable (about 0.058 million heads to date) population of these elite cows is present in the country at large peri-urban dairy farms. These animals cost very high and require highly specific, most modern management and feeding practices and well-trained man power. These cattle represent less than 1% of total dairy animals and milk production (235 million L per annum to date) in the country (**Figure 5a** and **b**).

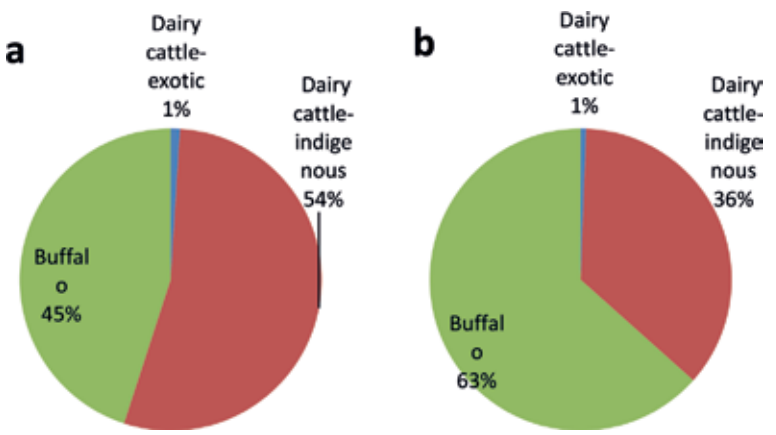

Figure 5.
Percentage contribution of imported dairy cattle in total population of large milk producing animals (a) and milk production (b), based on the data provided by the sector.

4. Safety of milk and dairy products

The demand for safe, high-quality foods with a long shelf-life is increasing day by day in the country. This reflects an improvement in the income as well as knowledge and awareness level of common masses. However, milk and milk products are biochemically unstable; i.e., they deteriorate very quickly and they accept foreign odors and materials very easily. Hence, maintaining the quality of milk poses a great challenge to the producers, collectors, and/or processors until it reached to the final destination. This is a fact that the dairy industry is highly unregulated in Pakistan, and the marketing chain is exclusively in the private sector. Generally, the milk is produced under compromised hygienic conditions that results in poor quality. Adulteration has been very common to increase milk volumes at farmer and intermediaries level in the past. The quality of milk is ensured by boiling at high temperatures during household consumption. Lack of hygiene, adulteration by various agents, and absence of a cold chain were identified as the primary contributors to low-quality milk in the past [2].

4.1 Measures being taken

Maintaining a high standard of hygiene is one of today's most important milk production objectives. The hygiene level directly influences the production's economical result, and dairies are enforcing this by steadily raising their quality requirements for raw milk. More importantly though, consumers are concerned about the safety of dairy products and the conditions under which these are produced. It is critically important to ensure the high quality at each step of this chain. It is, therefore, required that raw milk should be produced from healthy animals under good hygienic conditions and all control measures be applied from production to consumption to protect human health.

Several dairy development programs for the production, distribution, and processing of hygienic milk have been started during the last two decades at private sector. These programs seek to ensure the production of hygienic milk by providing farmer education, implementing strict quality tests, and establishing cold chain collection and supply systems.

4.1.1 Quality tests and hygienic measures

The corporate private sector has implemented various strategies to ensure milk quality and safety at collection. At the first place, various milk qualitative and quantitative tests at village (VMC) and regional milk collection centers (RMC) are performed. These include organoleptic, temperature, clot on boiling, fat%, solids not fat, total solids, and specific gravity. Tests for aflatoxins, antibiotics, and physiochemical characteristics are performed at RMC to ensure product processing quality and safety. A complete list of the tests performed at dairies is presented in **Table 2**.

At the second place during processing or intermediate steps, various systems for quality and safety management, e.g., ISO 9000, FSMS 22000, total quality management (TQM), hazard analysis and critical control point (HACCP), and many other ISO certificates are adopted [13].

4.1.2 Farmers' support

The large dairy organizations like Nestle and Engro have provided farmers with the dairy inputs that have facilitated enhance and good quality milk production. Nestlé Pakistan Ltd. through its Kisan Club (https://www.nestle.pk/asset-library/

Physical	Organoleptic	Chemical	Physiochemical	Ratios
Clot on boiling	Appearance	Acidity	Aflatoxin	Protein to SNF ratio
pH	Consistency	Ammonium sulfate	Antibiotics	Solids not fat
Specific gravity	Smell	Formalin	Alcohol precipitation test	
Temperature	Taste	Hydrogen	Butyro-refractometer value	
Total solids		Salt	Detergent	
		Sodium	Fat	
			Free sugars	
			Glucose	
			Methylene blue dye Reduction	
			Protein	
			Sorbitol	
			Starch	
			Total plate count	
			Urea	
			Whey protein	

[1]*Based on the data provided by the sector.*

Table 2.
List of physiochemical, chemical, and organoleptic tests performed at regional milk collection centers and processing plants in Pakistan.[1]

documents/financial_reports/csv_report_2016.pdf: accessed October 19, 2018) aimed for major improvements in dairy farm sustainability by helping farmers decrease farm input cost and increase productivity resulting in better economic returns. Kisan Club helps achieve that by providing access to subsidized farm supplies like chillers and farm machinery, financial support through milk advances, and bank loans and technical services about health, breeding, and management. The results of this project showed an increased hygienic milk production and ensured supply to the collectors.

4.1.3 Rewards and punishments

Many commercial milk collection organizations such as Engro Foods Ltd. and Nestle Pakistan Ltd. have adopted a reward and punishment system to ensure milk quality and wholesomeness. Engro Foods Ltd., for example, has adopted such a system, which is called as Incentive Systems, and they have introduced the following incentives:

1. Volume incentive will be paid to commercial dairy farmer (CDF; with 51–500 L/day milk production) and large farmers (LF; with more than 500 L/day) if either of these is supplying more than 51 L daily to the collection center. Different volume slabs per day above a minimum of 51 L/day corresponds to different incentives.

2. Loyalty incentive will be given @Rs. 1/L to either CDF or LF if he supplies milk to the company at least 28 days a month.

3. Total plate count (TPC) incentive will be given @Rs. 0.5/L to only CDF if his milk sample TPC falls under 200,000/mL of milk. The rate of this incentive is reduced to Rs. 0.2/L if TPC level falls between 200,000 and 300,000/mL of milk.

4.1.4 Establishment of cold chain

Under traditional system of milk collection and transportation, milk is transported over long distances, often in extreme weather conditions without cold storage facilities. Milk losses due to the lack of cold storage are estimated at about 15–20% of total milk production in some areas according to an Asian Development Bank report [14]. To minimize the effects of transportation on milk quality, the corporate private sector has maintained collection centers and established the cold chain.

4.2 Food safety legislation and regulation

Previously, food safety issues in Pakistan were dealt by the following laws [15]:

i. Pure Food Ordinance, 1960

ii. Pakistan Hotels and Restaurant Act, 1976

iii. Pakistan Standards and Quality Control Authority (PSQCA) Act, 1996.

These laws had the capacity to achieve at least a minimum level of food safety; however, they were very poorly enforced.

As presented earlier, use of growth promoters such as (Boostin®), milk let down facilitators (Oxytocin®), and addition of water to increase physical milk volumes have been very common among the suppliers other than supplying to the dairies.

However, these malpractices have been banned by law (Punjab Pure Food Act, 2011; accessed September 28, 2018) and declining by strict actions of Punjab Food Authority and quality control units of private sectors.

The Punjab Food Authority [16], formed under the Punjab Food Authority Act 2011 and the Pure Food Rules 2011, has been very active since its inception on July 2, 2012 in various districts of Punjab province. The authority aims to ensure food safety & quality in the entire food chain in collaboration with manufacturers, food business operators, consumers, government departments, autonomous bodies, and other stakeholders. The authority issues guideline for the stakeholders related to the food industry, regulates and monitors the food business and certifies food items to ensure compliance with the food standards [16]. The authority also arranges awareness programs and takes part in educating people related to food business. In recent years, remarkable improvements in the keeping quality of milk can be partially attributed to the role of PFA.

A brief overview of PFA is given in Annexure 1. The new regulations [16] and Punjab Pure Food Rules (Punjab Pure Food Act, 2011; accessed September 28, 2018) have clearly stated definitions of various food items including all forms of milk, and explicitly prohibit or limit the use of harmful preservatives, including bacteria inhibitors such as penicillin and formalin, and other substances such as urea, sugar, and glucose. The use of oxytocin or any growth promoters is also prohibited by law. These laws also obligate rules and regulations for dairy processing corporations to provide hygienic milk through regulated quality testing, packaging, storage, distribution, and recalling.

5. Environmental consequences of dairying

Livestock activities play a significant role in the maintenance of surrounding environment, including, air land, soil, and water. These may have direct impact on subsoil water, rivers, and lakes by adding solid waste and pollutants, which emits nutrients, organic matter, pathogens, and drug residues or indirect, in the form of competition for natural resources. Animals and their waste contribute to climate change by emitting greenhouse gases or by changing land use resulting from increased demand for feed grains and grazing. **Figure 6** shows a most recent estimate of greenhouse gas emissions from the livestock activities within agriculture sector of Pakistan.

5.1 Sources of environmental pollution

The production systems and large dairy facilities have been identified as the two major sources of environmental degradation in Pakistan's dairy sector [2].

5.1.1 Production systems

The data on the production systems indicate that there has been rapid growth in peri-urban and large peri-urban commercial dairy farming with intensive management conditions. This means that large numbers of animals are kept in small compounds. Under traditional management practices, the solid and liquid waste is often disposed of in major water bodies. Furthermore, the water used in farming operations is drained into main water resources. This not only contaminates public drinking water, but also leads to unchecked methane emissions. The second aspect of animal farming with respect to environmental consequences is the overgrazing and competition for food between animals and humans. The ever increasing livestock population [5] with poor level productivity poses serious threats on sustainability of range resources and cultivable lands for feed production [17].

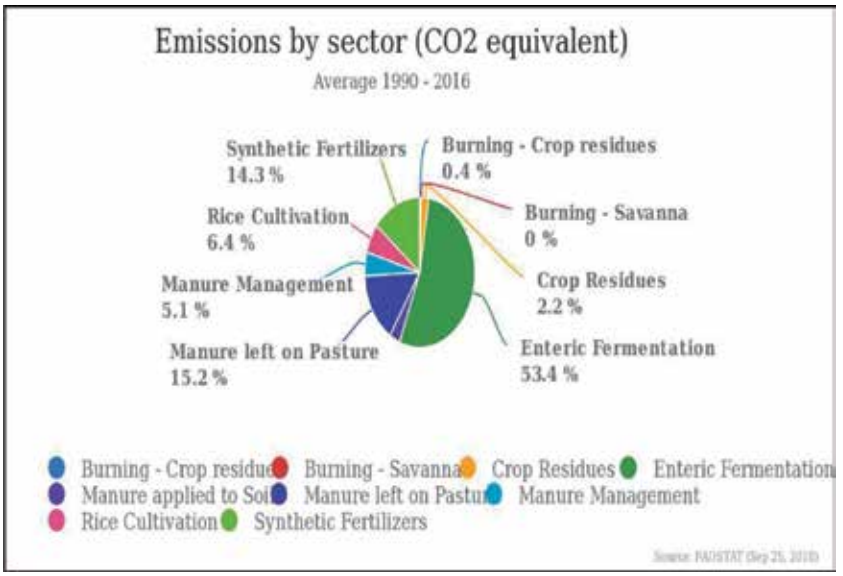

Figure 6.
Total greenhouse gas emissions from agriculture sector in Pakistan. Source: FAOSTAT 2018 (accessed: September 25, 2018).

5.1.2 Processing facilities

In the recent years, a sizeable number of milk processing plants is in operations. These plants pollute the surrounding environment and contribute to climate change by adding solid waste, liquid waste, soil pollution and noise, and air emissions. Of these, wastewater from washing and cleaning operations is the greatest pollutant, and is estimated to be between 5.5 and 30 cubic meter for every cubic meter of milk processed at a typical dairy unit [18, 19].

5.2 Awareness and understanding of environmental consequences

There is a lack of understanding for environmental consequences of livestock in developing countries and the situation in Pakistan is no different, as demonstrated by the Livestock Policy's (2007) failure to consider environment. Awareness and understanding of the environmental consequences of dairying are extremely limited in almost all sectors, especially the government sector and small-scale farming. The focus is on the more obvious contamination, such as flies and odor, rather than the serious threats of land degradation, water pollution, biodiversity erosion, and global climate change.

6. Improvements

Since last reviews in 2002 [1], 2011 [2], 2013 [20] and 2015 [21], various changes have been brought about in various subsectors of the dairy industry in Pakistan. Only significant improvements are being identified here and a summary is presented as Annexure 2.

Realizing the positive impact that development funding can have on raw milk production, the private sector has recently started to implement projects aiming at organizing farmers for milk collection and marketing, while providing them with information and access to market channels resulting in enhanced productivity. These projects, started under public-private partnership, reach large number of beneficiaries, and show relatively quick results. The rapidly increasing demand and high margins are additional driving forces behind private sector investments.

6.1 Enlargement of herds and import of high-quality milk germ plasm

To date, various companies are supplying elite class pedigreed dairy animals and high-quality semen imported from various countries including the United States of America, Australia, Germany, and the Netherlands. A total of about 0.058 million heads exotic dairy cattle have been imported during the last two decades and are being maintained at large peri-urban commercial dairy farms. These companies supply 3–7 months pregnant heifers with a farm gate price range of 2500–4700 US$ per imported animal (based on the data provided by the sector). The animals are usually ensured against any accidental injuries or death during the transportation and afterward. The semen doses from elite class pedigreed bulls are also available with a price range of 40–80 US$/dose.

6.2 Focus on improving the productivity per animal rather than improving their number

While looking at the statistical data for the last 10 years (2006–2016) or even before, it is clear that policy focus was mainly put on increasing the number of

animals per year rather than on increasing the productivity per animal (**Figure 4**). This indicates that any improvements in raw milk production are mainly caused by increase in number of animals every year. As presented previously, the indigenous dairy animals are characterized as poor producers with low daily milk yields (4.78 vs. 6.0 L), lactation yields (1195 vs. 1800 L), and less days in milk (250 vs. 300) [8, 11]. With the introduction of crossbreeding, daily milk, and lactation yields are far better now (12 and 3600 L, respectively) with compromised milk fat content (6 vs. 3.6% for buffaloes and crossbred cattle, respectively) under rural commercial and peri-urban dairy farming. This provides some evidence that there has been improved productivity per animal; however, crossbred cattle represent a small proportion of total population [7, 12].

6.3 Improvements in milk collection, processing, and marketing

In the past 10 years (2006–2016), the private sector dairy organizations namely Engro Foods Ltd./Frieslandcampina & Nestle Pakistan Ltd. played a vital role in milk collection and marketing. They mainly aimed at getting high-quality, safe, and secure milk. To ensure this, they have installed milk chillers at village level so that milk can be preserved safely on immediate basis after collection from individual farmers and maintained a cold supply chain, thus, providing with means for securing quality and wholesomeness of the product and successful marketing at the doorsteps of the farmers. According to a recent update, Nestle Pakistan Ltd. has installed about 2100, Engro Foods Ltd. installed 1250, whereas Nurpur installed 300 chillers with about 500 chillers installed by other dairy companies. **Figure 7** shows trends in milk collection by private dairy sector (both traditional and commercial) in two base years of 2006 and 2016.

Similarly, there has been a significant increase in the processing capacity of various dairies during this period of 10 years (2006–2016). **Figure 8** shows that the total processing capacity for pasteurized and ultrahigh-treated fluid milk and milk products from all dairy processing was estimated to be 32 million L/year in 2006 [2], which reached to a corresponding value of 2326 million L/year in 2016. Some recent figures indicate that a total of about 15 dairy processing plants are functional in the

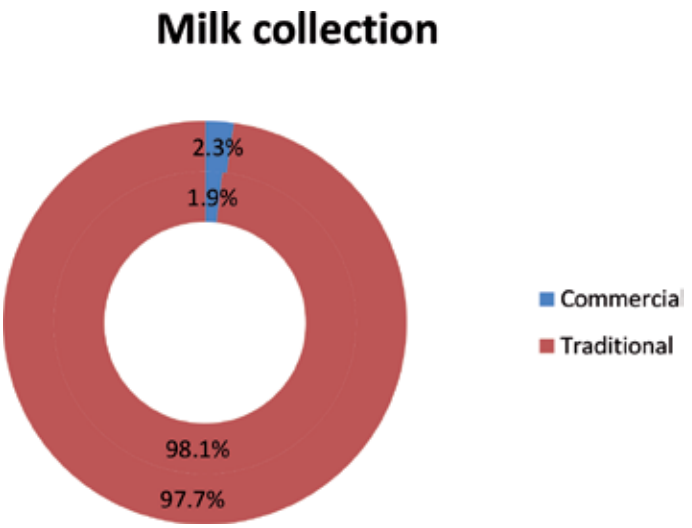

Figure 7.
Milk collection by traditional and commercial methods in two base years (2006 inner circle and 2016 outer circle). Based on data provided by the sector.

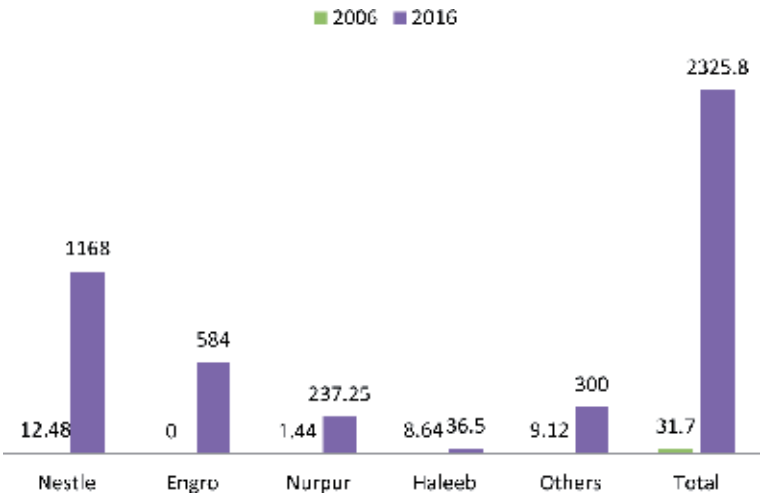

Figure 8.
Milk processing (fluid milk and dairy products) by large dairies in two base years (2006 and 2016), based on [2] and the data provided by the sector.

country with varying capacity for product volume and assortment. Three big dairy companies, e.g., Nestle Pakistan Ltd., Engro Foods Ltd., and Nurpur Ltd. occupying the first, second, and third position, are processing about 1168, 584, and 237 million L/year, respectively. In addition to the fluid milk collected from inside the country, a large amount of dry milk is also imported. The large differences between collection and processing of fluid milk by dairies are explained through the import of dry milk, which had an estimated amount of 284 million L/year during 2016–2017. The dry milk is used after reconstitution for various purposes, during flush and lean periods.

6.4 Improvements in the supply of dairy inputs

As the trend from traditional to commercial dairy farming is gaining fame and acceptance among the farmers and corporate stake holders, the demand for dairy inputs is on an increase on daily or monthly basis. These inputs range from seed stock dairy animals, modern dairy housing fixtures and equipment, commercial feeds including concentrate, silage and hay to medicine, vaccines, and semen. A significant number of multinational and local companies to supply dairy inputs has emerged in the recent past and continues to grow in the future. These firms are mostly located in Lahore or Karachi, and the surrounding of these cities are known as the hubs for peri-urban and large peri-urban dairy farming.

6.4.1 Establishment of commercial feed units and silage making

Little is known about the actual number of commercial feed units supplying concentrate, hay, or silage in 2006, as there number was so small to be included in the counting. Importantly, Ghazi Brothers and ICI, Pakistan are considered as two main companies who started their sale operations of animals inputs of medicine and supplements as early as 2000s. Then, the era came when more players started their business for supplying complete range of animal farming inputs. These companies were either sister companies of some most famous brands or were off-shoots of some international groups (Bovitech®). The animal inputs of feeding (commercial concentrate, bailed hay and silage, vitamin and mineral premixes, and growth promoters), treatment (de-wormers, vaccines, and medicines), and reproductive management are examples of year-round supplies by these companies (based on data provided by the sector).

6.4.2 Ensured supply of dairy farm machinery

The most updated dairy farm machinery and equipment are available in the country now. Many commercial companies are either preparing the required equipment at local market level or import from abroad. These companies not only provide inputs but also services and consultancy. The important farm machinery and farm structures may include, but not limited to, dairy sheds and layouts, milking machines, lines and parlors, milk chillers and utensils, forage harvesters, choppers and inoculants mixtures, and hay and silage bailers (based on the data provided by the sector).

6.5 Farmers' education on modern management practices

As mentioned earlier, several farmers education programs related to dairy farming are in place now. In the recent past, Engro Foods Ltd. started and completed a large farmers and extension worker training program titled "Big Push," in which they trained a total of 12,000 Basic Livestock Workers for basic dairy farming, keeping in hygienic, and clean milk production (http://www.engrofoods.com/bigpush.html: accessed October 15, 2018). Among those trained, 750 persons got special training as livestock extension workers (LEW), artificial insemination technicians, and farm supervisors and village milk collectors. This program was completed in collaboration with Punjab Skills Development Foundation during a period of one and half year (February, 2017–June, 2018).

Similarly, Nestle Pakistan Ltd. has taken several initiatives to rural development by taking several steps to increase the knowledge and skill level of farmers for enhanced and good quality milk production and quality of life (https://www.nestle.pk/asset-library/documents/financial_reports/csv_report_2016.pdf: accessed October 19, 2018). The company's initiatives positively impacted the lives of small dairy farmers by imparting training to around 77,000 farmers through farmer help camps. Nestle, through its famous Dairy and Rural Development Foundation (DRDF) trained 48,600 dairy farmers and 500 commercial farmers and farm managers on best dairy farm management practices, trained and established 2450 AITs, trained and established 7000 Women LEWs as entrepreneurs serving farmers in 10,000 villages in South Punjab. They started a street theater and mass awareness campaign, which reached approximately 3 million dairy households to increase knowledge of best dairy farm practices. They upgraded 118 local farms to model farms to meet rural communities' requirements and now serving as service and supply hubs for small farmers.

7. Future challenges

Conclusively, the dairy sector is performing at some sustainable level to meet the food requirements of the growing population and helping save a handful of foreign exchange to be expended on the import of milk and milk products, yet some challenges facing the industry in the near future need to be addressed.

7.1 Replacements considerations of high genetic potential dairy animals

Dairy heifer replacement raises significant attention among the stakeholders, and this issue should be taken as challenge if we want to keep the pace of commercial dairy farming. To the present day, in the absence of specific breeding plans adopted, the corporate and mega farms established in various parts of the country are importing their seed stock from various technological advanced countries. The first seed

stock has completed their productive life or near to completion at many of these farms. To maintain the commercial activity and profitability, the older and spent seed stock needs to be replaced with the younger ones of same genetic potential for milk.

Import of dairy animals every time from the exporting countries requires high input costs and extensive paper work. It is also an indicator of poor sustainability of farm economics. Furthermore, many political situations around the globe may delay or cut off the supplies of these important dairy inputs. It is, therefore, required that the replacement stock should be raised locally. However, the production of high genetic potential heifers at low costs is challenging and requires huge resources to be spared for research and propagation of research outcomes.

7.2 Considerations for β-casein proteins

Among the milk protein, the casein constitute 80% and the whey proteins constitute the rest. There are several types of casein in milk, and β-casein is the second most common. β-casein exists in at least 13 different forms [22]. Two major forms of β-casein protein exist in the form of A1 and A2. A2 is the original β-casein protein in all dairy animals. Breeds like the Holstein, Friesian, Ayrshire, and British Shorthorn that originated in northern Europe produce milk that is generally high in A1 β-casein. Other breeds that originated in the Channel Islands and Southern France, and the rest of the world (*Bos indicus*) produce milk that is high in A2 β-casein. This includes breeds like the Guernsey, Jersey, Charolais, and Limousin in Europe and Sahiwal, Red-Sindhi, etc. in subcontinent [23].

Several health hazards are associated with β-casein A1 type protein. During processing and digestion of milk, several peptides are released due to enzymatic cleavage and have various beneficial effects on the body. These peptides are called bioactive peptides. Bioactive peptides vary due to the genetic polymorphism for β-casein protein. β-Casomorphin7 (BCM7), having opioid like properties, can be released easily from A1 type β-casein [24, 25]. BCM7 has potential negative effect on the opioid receptors of nervous system, endocrine system, and immune system of the human body. BCM7 is associated with diabetes type 1, coronary heart disease, Autism, schizophrenia, and sudden infant death syndrome [26–29]. However, Truswell [23] in his review on the A1 and A2 hypothesis, negated any association between the A1 type β-casein protein in milk and mentioned health issues.

A1 and A2 debate is still an open challenge to the dairy industry and for human health. Several techniques are available to quantify the A1 and A2 in individual milk, bulk milk, dairy products, and different breed milk. More precise and accurate research on the association of the A1 with different diseases and syndromes, and their tolerance levels adjustments is needed. The research should expand to include the milk from other species of the animals too. If the scientific studies rule out hazardous effects of A1 β-casein on the human health, a great shift in the current dairy farming practices is expected in the country and the world afterward.

7.3 Presence of antibiotics and aflatoxins

7.3.1 Antibiotics

Presence of antibiotics in milk is a worldwide issue. In countries like Pakistan with poor hygienic conditions, prevalence of various diseases is common. Antibiotic residues are regarded as the unacceptable antibiotic levels or their active metabolites in tissues or products from the treated animals. Over the last few decades, antibiotics residues and antibiotic resistance are posing the biggest challenge to the public health. The potential hazards of antibiotic residues can be classified as: those who

directly affect human health by consuming animal products (cause allergic reaction to the sensitive persons, ototoxicity, carcinogenicity, reproductive effects, and teratogenicity), those which are excreted in animal feces and urine and pollute water and other land resources, and those which hinder the process of culturing during the production of dairy products.

In order to minimize the residues of the antibiotics in the milk, multiple international agencies like FAO, WHO, CAC, and EEC are working and they have set the standard maximum residual levels (MRLS) for animals and their products. Products containing residues more than these levels are considered illegal. Several awareness programs should be addressed at the public level to minimize antibiotic residue. These may include: improved hygienic management practices at farm, minimum use of antibiotics after the laboratory procedures and sufficient withdrawal period, grading of milk according to the presence of antibiotic residues, and rejection of milk with unacceptable antibiotics levels.

7.3.2 Mycotoxins

Mycotoxins have a great range of the diversity, but aflatoxin (AF) is the abundant toxic compound found in various food and feeds. Aflatoxins are the dangerous toxic chemical compound produced by the *Aspergillus spp*. of fungi predominantly *A. flavus* and *A. parasiticus*. The aflatoxin problem is worldwide even in the temperate zones where the temperature, humidity, and harvesting conditions favor the growth of this fungus. More than 20 different AF derivatives like B1, B2, G1, G2, M1, etc. are identified as the dominating derivatives. AF after ingestion or after entry through skin disseminate within the body and have serious health hazards like carcinogenicity, mutagenicity, retarded growth, impaired liver functions, and allergic reactions [30]. The public health problems depend upon the severity of exposure, duration of exposure and type of AF exposure, and on the basis of this, aflatoxicosis is considered as acute and chronic.

Large number of fatalities occurs due to acute aflatoxicosis, but due to chronic exposure, most of the animals and humans got infected. Annually, 4.5 billion of human population is presented to the chronic exposure of AF [30] causing immune suppression, decreased food intake, susceptibility to the other infections like plasmodium and HIV, and reduced production.

Aflatoxin M1 (AFM1) is the major (about 95%) excreted AF metabolite in the milk and is related to severe health issues. Trace amounts of AFM2, AFL, AFM4, and AFQ1 are also detected in milk but have less public health importance [31]. Long-term feeding of AFB1-contaminated feed results in the appearance of AFM1 in the milk [32]. Studies suggest that the highly producing animals secrete more AFM1 in milk due to the more consumption of highly concentrated feed. Rate of carryover of AFM1 in the milk from the dairy cows ranges from 0.3 to 6.5% [33].

There is no standard procedure to control carryover of AFB1 from feed to the AFM1 in the milk. However, numerous strategies have been described [34–37] and they are listed as follows:

- Pasteurization decreases 7.62% of AFM1 from the milk
- Milk concentration can reduce AFM1 by a factor of 60–70%
- Development of proper standards and rules for AFS
- Interaction with the international organizations like FAO and WHO for the adoption of standardization

- Setting a standardized upper limit of the AF in food chain
- Development of precise, specific, and economical innovative technologies for the detection of multiple AF in the feed, milk, and milk products
- Development of resistant plants to the fungus growth.
- Development of the breeds that have the genetic resistance to that biotransformation from AFB1 to AFM1 in the milk
- Research on gene regulation of mycotoxin producing organisms.

7.4 Considerations for competitive operational costs

The current day dairy operations like starting the enterprise, feeding the animals, maintaining a high level of hygiene and cleanliness at farm, and disposal of milk are performed at relatively high costs because of high costs of various dairy input (elite dairy animals, feed ingredients, preventive medication, electricity bills, etc.). These high costs directly control the product price and reduce the profit margins, and are variable among different systems of milk production. As per estimates of collected data from farmers maintaining herds in different production systems, the farm-gate price for 1 kg milk production of cows ranges from 42 (rural subsistence and rural market oriented) to 52 PKR (all other cow milk production systems; the prices are usually discounted for dairy organizations as a reward of the dairy inputs provided by them to the farmers) and that of buffaloes ranges from 55 to 65 PKR. These prices are relatively higher than those incurred in the more developed countries of the world. This situation prevents the investors to invest in the dairy business and causes the import of milk and milk products to fulfill country's requirements of milk. It is, therefore, suggested that farmers should be encouraged to produce milk at relatively low prices to make dairy sector of Pakistan to be more competitive with rest of the world. It is further suggested that a system for price determination of per kg milk production based on differences arising from species and system of milk production to propagate buffalo dairy farming and reduce malpractices in milk marketing may be introduced.

7.5 Research

7.5.1 To save environment and water resources

Pakistan has been included among the countries to face severe shortage of clean and hygiene water in the near future. The underground water table is getting deeper, and the available surface water is facing a merciless run-off. As discussed earlier, milk production and processing activities require large quantities of clean and hygienic water. No resources are committed to research on and discussion of the environmental effects of the dairy sector either by private or public sector, although there has been some recent interest in the development of treatment plants of wastewater and biogas by the private dairy organizations [19]. Therefore, sincere efforts are required with focused strategies to: (1) accurately estimate the current emissions of greenhouse gases and waste water from the agricultural sector, especially livestock and (2) mitigate these emissions through available resources.

8. Conclusions

The dairy industry in Pakistan represents smallholding with subsistence- or market-oriented farming followed by peri-urban or commercial-level farming. Historically, dairy sector has been owned and managed by the private sector. The population of dairy animals as well as milk production from these animals is increasing at a constant steady rate every year. The per animal productivity of the local dairy cows and buffaloes remained the same over the years; however, crossbreeding and import of elite dairy cows tended to increase in pursuance of increased productivity per animal. During the past two decades, various changes have been brought about in various subsectors as a result of new initiatives taken by the corporate private sector. These efforts have resulted in enlargement in the size of dairy units, improvements in milk collection, processing and marketing, increased supply of dairy inputs (machinery, equipment, feeds, semen, and elite dairy animals), and enhanced farmers knowledge and skills on modern management practices.

Conclusively, the dairy sector is performing at some sustainable level to meet the food requirements of the growing population. Yet, challenges like local replacements of high genetic potential dairy animals, health hazards of β-casein proteins, antibiotics and aflatoxins, and uneconomical operational costs facing the dairy industry in the near future need to be addressed.

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Conflict of interest

No conflict of interest is declared.

1	Punjab Food Authority regulates and monitors the food business in order to ensure compliance by farmers, manufacturers, distributors, importers, and other stake holders in order to provide safe food
2	Formulate standards, procedures, processes, and guidelines in relation to any aspect of food including food business, food labeling, food additive, and specify appropriate enforcement systems
3	Enforcement of food safety and quality standards
4	Specify procedures and guidelines for setting up and up-gradation of food laboratories
5	Specify licensing, prohibition orders, recall procedures, improvement notices, and prosecution in the court of law
6	Provides scientific advice and technical support to the government in matters relating to food safety
7	Establishment of food laboratories
8	Organize training program in food safety and standards
9	Promote general awareness regarding food safety and standards

10	Certify food products/items for export
11	Forward and backward traceability of food items
12	Surveillance including collection, integration, analysis, interpretation, and dissemination of data related to food and nutrient intakes

[1]*Punjab Pure Food Rules (2011).*

Annexure 1.
Responsibilities of Punjab Food Authority, 2011.[1]

1	Milk procurement markets in remote areas with heavy investments
2	Clear milk purchasing norms (introduced 13 total solids system, previously people were purchasing milk at fat/gross system only)
3	Quality norms, 28 tests have been introduced to market
4	Cold chain in the milk value chain
5	Efficient mode of payment (milk automation networking, weekly payment system through banks to make the payment structure safe)
6	Introduced exotic breeds—proved to be a turning point in dairy farm developments and in farm mechanization
7	Encouraged, motivated farmers and investors to invest in dairy farming
8	Made demonstration farms
9	Provide free veterinary & agricultural services
10	Offered premium incentives for farm development and milk quality
11	Encourage financial institutions to extend loans for dairy farming
12	Brought global expertise and resources of dairy farming in Pakistan
13	Attract Foreign aid and developed entrepreneurships among small livestock holder through trainings & workshops
14	Developed institutes like DRDF to develop AIT/livestock extension workers to help the dairy farmers
15	Offered incentives to commercial dairy farmers to produce aflatoxin, antibiotic-free healthy milk
16	Offered low-interest loans to newly developed farmers by involving different banks like Alfalah, JS Bank
17	Aflatoxin-free feed has been introduced by involving the investors
18	Low cost feed pattern has been introduced like beet pulp feeding and sugarcane mud in the areas where animals were facing feed deficiency due to less resources of dairy farmers
19	Provision of hybrid fodder seed to farmers at relatively low price to tackle the feed shortage during lean months

[1]*Based on data provided by the sector.*

Annexure 2.
A summary of dairy initiatives taken by private dairy sector in collaboration with public sector partners.[1]

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Chapter 2

Reconnoitering Milk Constituents of Different Species, Probing and Soliciting Factors to Its Soundness

Amjad Aqib, Muhammad Ijaz, Aftab Anjum, Muhammad Kulyar, Muhammad Shoaib and Shahid Farooqi

Abstract

Milk composition and production varies from species to species, reflecting its diversified benefits on health. Lipids from caprine and ovine milk are anti-obesity and anti-atherogenic while prebiotic in the case of caprine. Higher contents of selenium from caprine and iron from camel milk play a role in immune system and oxygen transport system, respectively, whereas enriched vitamins like riboflavin, folic acid, B6, vitamin A of bovine, and foliate of cattle are effective in the synthesis of hemoglobin, and high niacin content of caprine is anti-cancerous. Camel milk is found to have characteristics of anti-carcinogenic, antidiabetic, and autoimmune therapeutic. Various processing techniques like pasteurization, skim milk powder processing, and ultra-high temperature processing are necessary for safe provision of milk to meet consumers' demand. Change in flavor, loss of micronutrients, biofilm production, and spore-forming bacteria are prominent challenges during processing. Antimicrobial resistance and disease conditions are exaggerating factors of milk deterioration with respect to quality and quantity. Preclinical trials like somatic cell count, California mastitis test, proteomic analysis, Raman spectroscopy-based analysis, and X-ray fluorescence analysis are helpful in avoiding the spread of disease and controlling of economic losses. This chapter focuses differential functions of bioactive of milk, issues arising during processing techniques, and preclinical studies of milk for safer production and consumption of milk.

Keywords: milk composition, bovine, camel, caprine, ovine, mare, differential functions, processing techniques, preclinical milk tests

1. Introduction

Milk, according to USDA, is a sterile lacteal secretion from mammary glands by full milking of one or more animals and considered free of colostrum. Basically, milk is composed of significant components that may be categorized into macro and micro milk components. The former category is comprised of protein, lipids, and oligosaccharides mainly lactose, whereas the latter contains minerals and vitamins [1]. Utilization of milk from various species of animals depends upon likelihood of people and access to the dairy animals. In such situations, some of dairy animals are overlooked due to their limited population in specific regions of the world. Camel and caprine milk is specifically medicinal in nature that is limitedly utilized as

staple use. The production systems are fewer than needed which is a grave situation. It is a dire need to explore bioactive components of milk from various animals and to investigate alternative resources to feed the hungry and to be benefited by pharmacological aspects.

The likelihood of food consumption stresses it to be natural, free from chemical preservatives, and microbiologically safe with extended shelf life [2]. The rapid development of our society in the past few decades and the careless use of large amount of agricultural services are appearing to be a burden over human health. The hunger of the increasing population cannot be satisfied with fresh milk due to unequal production and utilization system. Previous 15 years have noticed dairy industry emerging as technology revolution in product processing [3]. But there are several harms to the soundness of milk associated with processing techniques in terms of quality and quantity losses. The current chapter encompasses bioactive components of milk from different milk-producing animals and their chances of being deteriorated by processing techniques.

2. Dairy milk bioactive components and their role on health and diseases

2.1 Bovine milk

Bovine milk as whole milk and its products are serving an easy way of achieving good nutrition. Bovine milk contains the nutrients needed for growth and development of the calf and is a resource of lipids, proteins, amino acids, vitamins, and minerals. The milk is also blessed with many substances like hormones, growth factors, immunoglobulins, peptides, cytokines, polyamines, bioactive peptides, and many enzymes that play different roles in our body [4]. Milk composition has dynamic properties and its composition varies depending on lactation, diet, age, breed, energy balance, and udder health. Colostrum is very different from milk, but the most important difference is the concentration of milk protein. It will be twice in colostrum compared with milk in late lactation. Lipids in bovine milk are suspended or emulsified in the form of fat globules covered with membranes. Lipids are mainly composed of different types of fatty acids having different fraction. Milk contains about 32 g of protein per liter. Milk protein is a good source of essential amino acids. In addition, milk contains various biologically active proteins, ranging from antimicrobial drugs and ending with nutritionally enriched proteins, as well as growth factors, hormones, enzymes, antibodies, and immune-stimulants. Nitrogen in milk is distributed in casein, serum, and nonprotein nitrogen. The content of casein in milk is about 80% of milk protein. Whey protein is a globular protein that is more soluble in water than casein. The main components are β-lacto globulin, α-lactalbumin, bovine serum albumin, and immunoglobulin. Milk also contains many minerals, vitamins, and antioxidants. Antioxidants prevent the oxidation of milk and also provide protection to cells which involve in milk production and to udder also. The most important antioxidants in milk are mineral selenium and vitamins E and A [5]. However, cow milk (CM) differs from buffalo milk (BM) composition of different milk bioactive components. Buffalo milk has lower cholesterol but more calories and fat compared with cow's milk. Cow milk has higher cholesterol level than BM, but higher fat contents are present in BM with higher calorie percentage. Buffalo milk has a higher content of fat, lactose, casein, whey proteins, and minerals than cow milk. All of the casein in buffalo milk is present in the micellar form, while in the CM, only 90–95% of the casein is in the micellar state and the rest is present in serum phase. The calcium content is higher in BM

than in milk from cow, and it contains more colloidal calcium and phosphorus. The BM is richer in fat than milk from cattle, and absence of b-carotene in BM, which is present in CM, is another notable characteristic [6].

2.2 Camel milk

Camel is a blessing from God as narrated in Muslim's Holy Quran [7]. The total population of camels in the world accounts 23.9 million. Among the countries, India has 1.9% population of camels over the total world camel population [8]. They are playing a crucial part in the lifestyle of numerous communities, especially those living in arid regions of the Middle East and the Arabian region since many centuries [9]. Total CM production is 1.3 × 106 tons [10], and the annual trade volume in the world is $ 10 billion which is expected to be increased in the near future [11]. Among various types of camels such as Bactrian camel (two humped), dromedary camel (single humped), wild Bactrian (true camels), plus llama, alpaca, guanaco, and vicuna camels [12], the dromedary camel is a resident of desert and dry land environment and accounts 94% of the total world population [13]. Camel milk being a good source of fats occupies opaque white color having salty taste due to high vitamin C content and good odor [14]. The overall constituents of camel milk account 3.4% protein, 3.5% fat, 4.4% lactose, 0.79% ash, and 87% water [15]. Mineral contents are important enriching constituents of milk which in the case of camel accounts for 0.60–1.0% [16] vis-a-vis Ca, P, Mg, Fe, Na, Zn, K, and Cu [17]. Many vitamins like A, C, D, E, and B groups are present in dromedary species of camel. High amount of vitamin C, fatty acids, and fructose and lack of beta-lactoglobulin are the most significant health promoting properties [18].

2.3 Caprine milk

Goats were first domesticated by ancient peoples in the Middle East 10,000 years ago [19]. Goat farming has been increased from 751.63 million (year 2000) to 1006.79 million (year 2016) and is being ranked third after cattle and sheep in total population of animals in all over the world according to FAO [20]. However according to the Economic Survey of Pakistan, goat population has been also increased from 70.3 to 72.2 million in Pakistan [21]. The salient differential bioactive components account for total solids (13.20%), fat content (4.50%), oligosaccharides especially lactose (4.3%), protein contents (3.60%), minerals (0.80%), and vitamins [22].

2.4 Equine milk

Horses can live in many different environments and develop in different ways. The first ancestor of Hyracotherium lived about 60 million years ago. They have four toes on the front paw and three toes on their hind legs [23]. Currently, there are hundreds of breeds of equine present all over the world. However, dairy breeds are predominately found in Mongolia and USSR. Among the dairy breeds, Haflinger horses are the most important milk breed of adults weighing 500 kg, known for their milk production capacities in European countries. [24]. The lactation of mare starts almost 7 days after birth and lasts almost up to 5–8 months of foal age [25]. Due to small mammary gland, mare requires multiple milking (5–7/day) with 2–3 hour intervals [26]. However, the gross milk composition of different breeds varies with an average of fat (1.25%), protein (2.15%), lactose (6.40%), and small amount of minerals 0.4% [25].

Bioactive name	Species name	General characters/ functions	General composition	Differential composition (%)	Differential functions
Lipids	Buffalo	Anti-cancer, antiviral, antibacterial, anti-plaque, anticaries, anti-inflammatory, anti-atherogenic, antihypertensive, prevent CHD [32, 35]	Triglycerides 98% (SFA, USFA, SCFA, MCFA, LCFA), CLA [5] Fat globule membrane 1–2% (diglycerides, monogly-cerides, phospholipids, sterols, FFA), peptides [5]	Fat (8.30%) [28] >50% SFA	Increases HDL and cholesterol level due to high SFA
	Cow			Fat (4.88%) [28] CLA (15 mg/100 mL) [33] FG > 5 μm [33]	
	Caprine			Fat (3.84%) [28] High MCFA, SCFA, and CLA (35 mg/100 mL) [22] FG < 5 μm [33]	Reduce cholesterol and LDL, rapidly digested, anti-obesity, treatment of malabsorption patients [22]
	Ovine			7.1% [43]	Anti-atherogenic, decrease LDL cholesterol [39]
				High caproic, caprylic, and capric acids [37] and low butyric acid [38], high oleic acid [39] FG < 3 μm [34]	
	Camel			3.5% [36]	
	Mare		Low triglycerides (80%) High phospholipids (5%) and FFA (9%) [25]	1.25% [25] FG = 2–3 μm [25] High level of MCFA, higher contents of LA and ALA [25]	
Proteins	Buffalo	Iron carrier, lactose synthesis, retinol binding activity, immunomodulator, anticarcinogenic, antioxidant, antimicrobial, anti-inflammatory	Caseins 80% (αs-1, αs-2, β,k) Whey proteins 20% (α-La, β-Lg, Ig, LF, Lyz, growth factors) [40]	4.48% [28]	
	Cow			3.49% [28]	
	Caprine			3.42% Low αs1 casein, high lactoferrin [33]	Low αs1 casein helps easily tolerated by Childs, treatment of CMA, increased iron absorption [33]
	Ovine	Antihypertensive, antitumor, ACE inhibitory activity [41, 42]		5.7% [43]	
	Camel			3.4% [15, 18] High whey proteins (high Ig, lactoferrin, lysozyme), no β-Lg, high PGRP [15, 18]	Anti-cancerous activity especially breast cancer, antidiabetic, treatment of autoimmune diseases [15, 18]
	Mare		Caseins 50% (αs1, αs2, β, k), whey proteins 39% (less β-Lg, more α-La & Ig) [25]	2.15% [25] High β-casein (50%), low kappa casein, αs1 and αs2 (40%), also high gamma casein (10%) [29] High lactoferrin (>10 times) [30]	Rich source of essential AA and source of nutrition, easily digestible due to high whey proteins

Bioactive name	Species name	General characters/ functions	General composition	Differential composition (%)	Differential functions
Carbohydrates	Buffalo	Probiotic, antioxidant, anti-inflammatory, Help in calcium	Lactose (lactulose, lactitol, lactobionic acid, galacto)	Lactose (4.86%) [28]	
	Cow			4.47% [28]	
	Caprine	Transport and absorption, beneficial bacteria growth promoter, source of fiber, treat constipation [31]	Oligosa-ccharides (galactose, glucose, NANA) [44]	Lactose (4.11%) [28] High amount of oligosaccharides (>10 times than cow)	Prebiotic
	Ovine			4.6% [43]	
	Camel			4.4% [15, 18] High lactose	
	Mare			6.40% [25]	
Minerals	Buffalo	Strengthening bones, avoid osteoporosis, antioxidant, antihypertensive, DNA synthesis and repair, anti-cancerous, immunomodulatory, avoid	High Ca, P, K, and Na and trace Mg, Zn, Fe, Cu, and Se [45, 46]	0.81% [28]	
	Cow			0.76% [28]	
	Caprine			0.89% [28] High selenium [45, 46]	Component of complement system, formation of interleukins by T cells [45, 46]
	Ovine			0.9% [43]	
	Camel	asthma, maintain fluid integrity [45, 46]		0.79% [15, 18] High chloride, low citrate, high Zn, Cu, Fe, and Mn [47]	High iron helps in oxygen transport, component of ETC [47]
	Mare			0.4% [25]	
Vitamins	Buffalo	Source of nutrition, antioxidant, anti-cancerous, anti-inflammatory, protect from osteoporosis, atherosclerosis [47, 48]	Fat soluble (A, D, E, K), water soluble (B complex) [47, 48]	High riboflavin, folic acid, B6, vitamin A	Immunity enhancer, treatment of CHD, prevention of megaloblastic anemia, role in morphogenesis [45, 46]
	Cow			High folate and vitamin B12 [45, 46]	Help in synthesis of hemoglobin [45, 46]
	Caprine			High niacin, vitamin B3, vitamin A [49]	Anti-cancerous activity [49]
	Ovine				
	Camel			High vitamin C and niacin, low vitamin A, low vitamin E [47, 48]	Antidiabetic, antioxidant, wound healing [47, 48]
	Mare			High vitamins A, D3, and K3 [25]	

SFA = saturated fatty acids; USFA = unsaturated fatty acids; SCFA = short-chain fatty acids; MCFA = medium-chain fatty acids; LCFA = long-chain fatty acids; FFA = free fatty acids; HDL = high-density lipids; LDL = low-density lipids; FG = fat globule; α-La = alpha lactalbumin; β-Lg = β-lactoglobulin; Ig = immunoglobulin; LF = lactoferrin; Lyz = lysozyme; A.A = amino acid; LA = linoleic acid; ALA = alpha linolenic acid; CLA = conjugated linoleic acid; ETC = electron transport chain; CMA = cow milk allergy; ACE = angiotensin-converting enzyme; CHD = congenital heart defect; PGRP = peptidoglycan recognition proteins; NANA = N-acetylneuraminic acid.

Table 1.
General and differential compositional-cum-functional physiology of bioactive components of milk from different animals.

2.5 Sheep milk

Unlike caprine and bovine milk, sheep milk is rich in total solids and major milk contents that supply energy. Ovine milk is highly enriched in bioactive components such as lipids, proteins, oligosaccharides, minerals, and vitamin contents (**Table 1**) [27].

3. Milk processing techniques and their harms to milk

Rapid development of our society in the past few decades and careless use of large amount of agricultural services are appearing to be a burden over human health [3]. The hunger of the increasing population cannot be satisfied with fresh milk due to unequal production and utilization system. In the past 15 years, the dairy industry has evolved with newer techniques of production, products, and processing [3]. But there are several harms to the soundness of milk associated with processing techniques in terms of quality and quantity losses.

3.1 Pasteurization

Diseases associated with the consumption of milk are common due to microbial contamination. To keep milk safe from such microbial contaminants, primarily large-scale techniques (like pasteurization) are adopted to every milk production system. For this purpose, collected milk from dairy farms is sent to a reservoir of processing units for processing, where a large amount of milk is stored [50]. Transportation of milk in such a way may cause a source of spreading viruses and bacteria. That milk is usually pasteurized and assuming that heat treatment has demolished appropriately [51]. However, some bacteria remain intact due to microbial biofilm within the distribution line and unhygienic behavior of employees [52]. While repeated or prolonged heat treatment causes protein denaturation and binding of denatured whey protein with casein micelles leads to migration of soluble calcium and phosphate to the colloidal stage and mollify of the enzymes. Research shows that the available amount of lysine, iodine, folate, and vitamins B12, C, B6, and B1 in milk decreases after pasteurization [53]. Heat treatment reduces α-la (α-lactalbumin) and PGRP (peptidoglycan recognition protein) in the case of camel milk [54]. Extreme pH, removal of bound Ca^{2+}, addition of denaturant agents, or cleavage of disulfide bridges can denature α-lactalbumin in several ways [55]. Among vitamins, vitamin C is the most important that can be quickly destroyed when milk is heated [56].

Treatment at elevated temperatures reduces the quality of milk supply, as many nutrients are thermally unstable [2]. The second most parameter is aroma of dairy products, which critically affects consumer acceptance, shelf life, and other attributes. When thermal treatment is employed to reduce or destroy the microbial load and enzyme activity to ensure safety and to increase shelf life, the aroma of the milk changes and differs from that of raw milk [57]. Ultra-pasteurization (UP) and ultra-high temperature (UHT), high temperature/short time (HTST), DSI-UP, or IND-UP are widely used thermal treatments for extended shelf life of milk. These processing techniques affect color due to various reactions during thermal processing or storage. These key changes in flavor during thermal processing of milk are associated with Maillard reactions [58].

3.2 Ultra-high temperature (UHT)

The contents of flavored milk are sweeteners such as natural sugar, sucrose, fructose, glucose syrup, or a sweetener without calories depending upon the

manufacturer and the consumer demand [59]. As a result of heat treatment, the basic constituents like proteins, carbohydrates, and vitamins of flavored milk undergo chemical and biochemical modifications [60]. Some of these modifications include lactulose and acid formation through lactose degradation. It promotes dehydroalanine development by side chains of amino acids through β-elimination. It is a compound that reacts readily with lysine yielding lysinoalanine and the denaturing of whey proteins [61].

3.3 Skim milk

A multistage processing technique (like skim milk development) involves wide use of heat treatment for milk preservation [62]. The formation of Maillard intermediates and glycation products during manufacture of dairy products has been studied [63]; the focus of these studies was the reduction in nutritional values, e.g., lysin [64].

3.3.1 Biofilm resistance of the bacteria in a milk powder processing factory

In the dairy industry, it is known to use a closed production system without removing or opening equipment using the CIP process. In terms of economic benefits, short cleaning procedures and long-term use of equipment in processing lines are common [65]. As a result, bacteria remain on the surface of the device and can accumulate in hard-to-reach places, such as dead ends, cracks, seals, and valves, where the complexity of cleaning and disinfection is difficult [66]. Undesirable biofilms on the surfaces of food processing have certain properties, such as increased tolerance to antimicrobial agents, increased secondary metabolites, etc. These are the potential cause of bacterial contamination [67]. In dairy industry, the presence of such biofilms leads to contamination after processing, shortens the shelf life, and promotes the transmission of diseases [68].

3.3.2 Presence of spore-forming bacteria in skim milk

The existence of spore-forming bacteria in milk is a critically important issue in the dairy industry. Bacterial endospores can survive in harsh environmental conditions such as high heat, low pH, desiccation, or cleaning and sanitizing chemicals [69]. Compared with vegetative cells, spores have also been found to attach more readily to stainless steel, leading to the formation of biofilms that can promote bacterial contamination within dairy processing plants. Spores present in final products can germinate and produce enzymes that decrease the quality and shelf life of dairy product; it causes the significant economic losses [70]. Additionally, some spore formers such as *Bacillus cereus* and *Bacillus subtilis* can produce toxins that are responsible for food poisoning [71]. *Thermophilic geobacillus* spp. and *Anoxybacillus* spp. are other spore formers of importance to the dairy industry, as they are commonly present in dairy powders and evaporated milk [70].

4. Factors affecting milk production and composition

The milk quality is influenced by many factors acting together and influences each other [72]. One of the most important factors is disease that adversely affects livestock systems that leads to decrease in yield, income, and survival of livelihood. The impact of livestock diseases is complicated and often exceeds the impact on the respective producers [73]. Selection of dairy animals, nutritional

management, advances in milking technology, and mammary gland of the dairy animals are the other factors that are also associated with milk yield as well as its composition [74].

4.1 Antimicrobial effects on quality of milk production and processing

Unadulterated high-quality milk that is free of antimicrobial residues is the most appropriate choice to farmers, consumers, and milk processing companies. Such milk enables the farmers to get a fair price [75]. Antibiotics are widely used in livestock production for therapeutics, growth promoters, and prophylactics since many years [76]. Such antimicrobial drugs affect the antibiotic-sensitive bacteria that involves in many fermentation processes. So, the presence of these antimicrobial drugs may affect the dairy products. This results in damage to the sensory properties and coagulation or ripening of the dairy products [77]. If the sale of raw milk is considered "unsuitable for consumption," due to the presence of antibiotics, transmission of milk may lead to ban by competent authorities. Costs of storage and subsequent disposal are the duties of farmers. So, farmers must incur large economic losses [78].

4.1.1 Assessment of antimicrobial coatings for packaged fresh milk

Packaging is an integrated technology that includes protection, ease of use, and communication. To protect the product, it is important to select and design the appropriate packaging materials [79]. Antimicrobial coatings are increasingly being used as a means of prolonging the shelf life of dairy products. This expansion helps consumers to reduce the amount of household waste milk [80]. The quality of the packaged milk depends on the internal properties of milk (oxidation-reduction potential, respiration rate, water activity, chemical structure, etc.) and the external factors (ambient composition, withholding temperature, relative humidity, etc.) [81]. Use of polymeric coating for maintaining the quality of milk is not possible due to many factors' involvement like design and development. Moreover, coating contributes to thermal and gas related mechanical properties due to its unique chemical structure [82]. But, due to the high cost of this process, small industries are unable to adopt it [83].

4.1.2 Diseases and disease conditions

Disease has a lot of impact, including a decline in productivity in livestock [84]. High infectious animal diseases, such as foot-and-mouth disease (FMD), hemorrhagic septicemia (HS), mastitis, peste des petits ruminant (PPR), and surra, cause irreparable economic losses for agricultural communities [85]. Ketosis is one of the diseases that causes lower milk production and an increased risk for developing other metabolic and infectious diseases which further affect milk properties [86]. There are many other factors especially environmental which affects production and properties of milk [87]. Mastitis is considered the most frequent health disorder in dairy farms. Decrease in milk components is one of the major origins of these economic losses both for clinical and subclinical infections [88]. These milk components are used as indicators of the metabolic status of cattle. The most relevant parameters of milk explain the balance of energy, protein, mineral, and acid-base balance and their standard concentrations and trends associated with various types of metabolic disorders. A comprehensive result of changes in the composition of milk can be used to identify early health problems. These changes in composition may help in protective cure of diseases [73].

5. Role of preclinical studies in safeguarding milk production and its properties

Dairy products are an important part of the human diet for more than 8000 years and are one of the official dietary recommendations for many countries in the world [89]. Daily intake of milk and dairy products has been identified as an important part of a balanced diet [90], because milk serves as a whole range nutrient consumed by humans (**Figure 1**).

5.1 Preclinical tests for milk analysis

5.1.1 X-ray fluorescence analysis

Recently, this technology has become widespread. The XRF method makes it possible to carry out analyses without sample separation. It helps in the quantification of minerals, trace elements, and volatiles which are difficult to determine in other analytical methods [92]. X-ray fluorescence spectroscopy (XRF) is an extension of the milk component analysis domain. Various configurations of XRF spectrometers are commercially available and are designed to provide economical and rapid analysis of milk. XRF is an excellent tool for daily analysis of the milk in dairy industries and research institutes. The results of the analysis can be used to assess nutritional value and evaluate the milk and dairy products [93].

5.1.2 Raman spectroscopy-based analysis

In this method, different types of milk quantity samples are used to classify several classes using reduction techniques in combination with random forest classifiers (RF). Quantitative and experimental analyses are based on locally collected milk samples from various species, including cow, buffalo, goat, and human milk samples. This classification is based on changes in the intensity of Raman peaks in a milk sample. The analysis of principal components (PCA) was used as a reduction technology in combination with RF to emphasize changes in Raman spectra that can differentiate milk samples from different species. The proposed method shows a sufficient opportunity to distinguish samples of cow milk from different species due to an average accuracy of about 94%, a specificity of about 97%, and a sensitivity of about 93% [94].

5.1.3 Somatic cell count (SCC) test

Mastitis is mostly caused by bacterial pathogens invading the mammary gland. Typical pathogens, namely, *Escherichia coli*, a gram-negative bacterium usually associated with acute, clinical mastitis, and *Staphylococcus aureus*, a gram-positive bacterium often associated with chronic mastitis, can cause differential activation of the immune system [95]. Somatic cell count (SCC) is used as key indicator in mastitis screening programs typically applied in the frame of dairy herd improvement (DHI) testing programs [96]. Direct microscopic somatic cell count (DMSCC) is one of the approved methods by FDA (Foss, Hillerød, Denmark). Flow cytometry and Ekomilk Scan® are also used to check the somatic cell count (**Figures 2** and **3**) [97].

5.1.4 California mastitis test (CMT)

The technique, invented in 1957 by Schalm and Noorlander, is used to detect intramammary infection caused by a major mastitis pathogen in early lactation cows

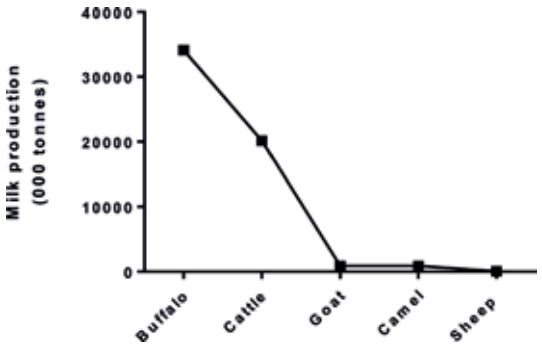

Figure 1.
Gross milk production of different milk-producing species [21].

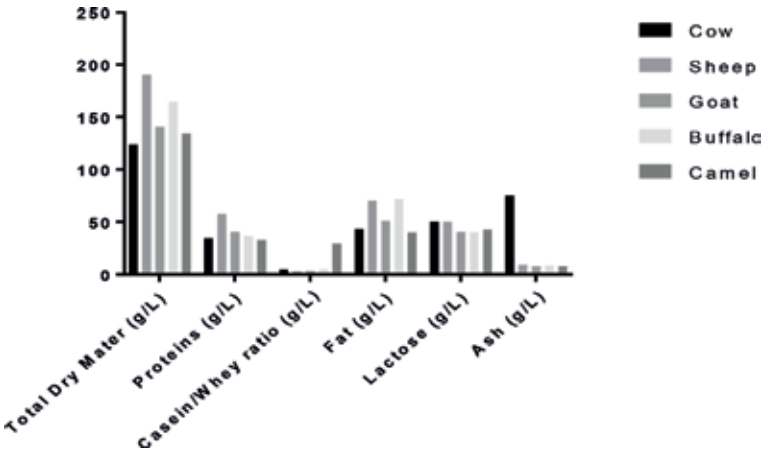

Figure 2.
General composition of milk from different dairy animals [91].

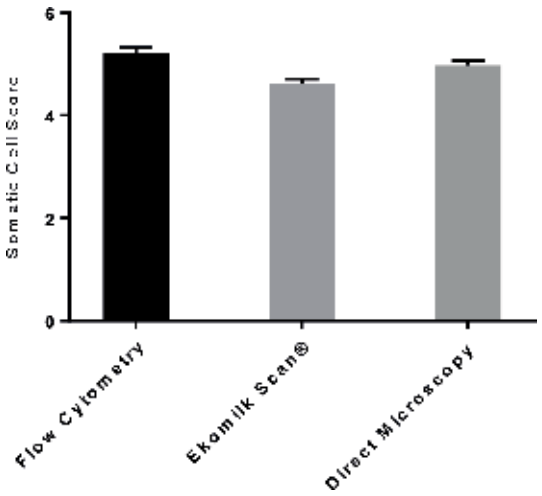

Figure 3.
Comparison of the somatic cell score (SCS) using different methods.

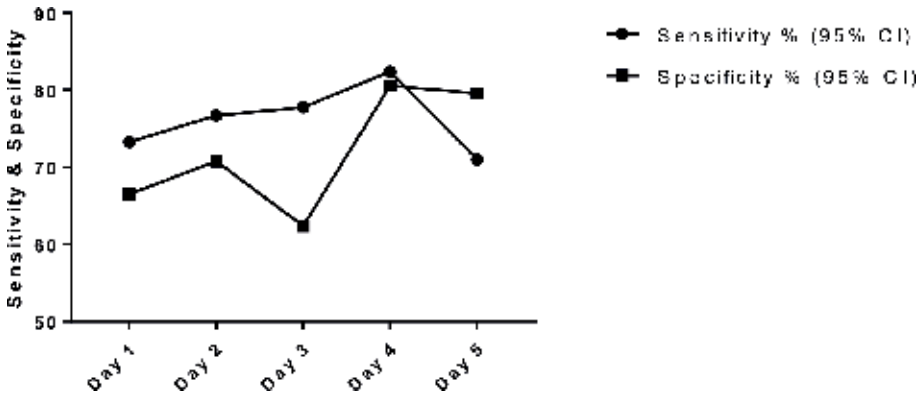

Figure 4.
Sensitivity and specificity within first week of calving through CMT [100].

[98]. They indicated that the degree of precipitation and gel formed by a mixture of the reagent and milk reflected the somatic cell count of the milk (**Figure 4**) [99].

5.1.5 Proteomics techniques for mastitis control

Early detection of mastitis and related pathogenic factors improves animal health status through timely and effective treatment. With the development of related technologies of proteomics, such as 2D-gel electrophoresis (2D-GE) and mass spectrometry (MS), several new proteins associated with mastitis have been identified [101]. The evolution of proteomic profiles of pathogens can help to identify the existing information on enzymes, toxins, and metabolites. However, the successful use of these new biomarkers for detection devices remains a challenge [102].

6. Conclusions

Fat is higher in bovine specie as compared to others and it is the main source of HDL and cholesterol enhancement in blood. Protein of ovine is higher than other milk-producing animals. Protein from camel milk (lactoferrin, immunoglobulin, lysozyme) is very useful in diabetes, cancer, and autoimmune diseases. High selenium found in caprine milk fortifies immune system, while higher contents of zinc, iron, and manganese in camel milk speak of greater oxygen carrying capacity by helping ion transport exchange. Higher riboflavin, folic acid, B6, and vitamin A in buffalo milk are blessings to enhance immunity and decrease of megaloblastic anemia. Antidiabetic, antioxidant, high vitamin C and niacin, low vitamin A and E are more defined properties that refer as wound healer agent. Heat treatment protocols result in denaturation of lysine, iodine, folate, and vitamins B12, B6, B1, and C, inactivation of enzymes, and change in flavor. Skim milk production often favors increase in biofilm resistance and spread of presence of spore-forming bacteria. Adding to this are the diseases or disease conditions exacerbating compromised soundness of milk. Preclinical studies are effective approaches to avoid deterioration of milk. X-ray fluorescence analysis is effective in evaluation of nutritive values of milk and milk products without decomposition of milk. Raman spectroscopy-based analysis successfully differentiate between milk of different species with higher sensitivity and specificity. Somatic cell count and California mastitis tests are

fruitful in estimation of intramammary infection. Latest techniques like proteomic protocols are explorable approaches as an effective preclinical study of milk.

Conflict of interest

Authors declare no conflict of interest.

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Chapter 3

Composition and Properties of Camel Milk

Rita Rahmeh, Husam Alomirah, Abrar Akbar and Jiwan Sidhu

Abstract

Camel is considered as one of the most important and ecologically harmless domesticated animals in the dry region of Asia and Africa. Camels have considerable economic importance not only as a draught animal, but also for their milk and its by-products. They can produce a significant amount of milk from poor feed as compared to any other dairy species. This characteristic, in addition to the growing recognition of the economic value, and health benefits of camel milk make it a center of attention for people, particularly in arid- and semi-arid areas. Moreover, camel milk is a highly nutritious medium permissive for the growth of many diverse bacterial species. These bacterial populations are mainly grouped into pathogenic, spoilage, and technologically relevant bacteria. This chapter reviews the existing knowledge on the composition, nutritional value, health-promoting properties, and economic value of camel milk and its by-products. Furthermore, the relevant studies describing the microbiota of camel milk are included.

Keywords: camel milk, pathogens, probiotics, economic value, human health

1. Introduction

Camels are domestic animals exceptionally well-adapted to arid lands. They can survive extended dry periods and heat and reproduce under harsh conditions, intolerable to other domestic animals. According to the Food and Agriculture Organization of the United Nations, 28 million camels were enumerated worldwide in 2016 [1]. There are two species of camels, one-humped Arabian camels or dromedaries (*Camelus dromedarius*) found in the Arab peninsula mostly in the Horn of Africa, the Sahel, Maghreb, Middle East, and South Asia. The second species is the two-humped Bactrian camels (*Camelus bactrianus*) domesticated in China and Mongolia [2]. The economic value and other benefits of camels make them the focus of attention of numerous scientific studies pinpointing the anatomic characteristics, a physiological adaptation of camels to adverse climates, and the bioactive molecules present in camel milk [3]. Their humps consist of stored fat that can be metabolized when food and water are inaccessible beside the ability of their organs to release water when needed. As additional ways for adaptation to their environment, camels have a third, clear eyelid protecting their eyes from sand and flat broader feet for walking in the desert. Camels are multi-purpose animals raised for riding, carrying loads, and producing milk, wool, hair, and meat (**Figure 1**). Milk is the most valuable camel product and it is known as 'white gold of the desert' [4, 5]. It is mainly consumed raw

by the Bedouins (people who inhabited the desert) where access to green vegetables and fruits is limited, thus providing, in that case, a significant nutritional relevance. Although camel milk is linked to the culture identity of Bedouins for a long time, small-scale and large-scale farms for intensive production of camel milk have been implemented worldwide only in recent years. The establishment of these farming systems was synchronized with the increased consumer's interest in unprocessed raw non-bovine milk consumption. While cow milk represents 82% of the total quantity of milk produced in the world, non-bovine dairy species provided 133 million tons in 2016 [1]. Camel is considered one of the most important dairy animals contributing to about 0.3% of the milk produced in the world [1] (**Figure 2**). Raw camel milk has

Figure 1.
Dromedary camel.

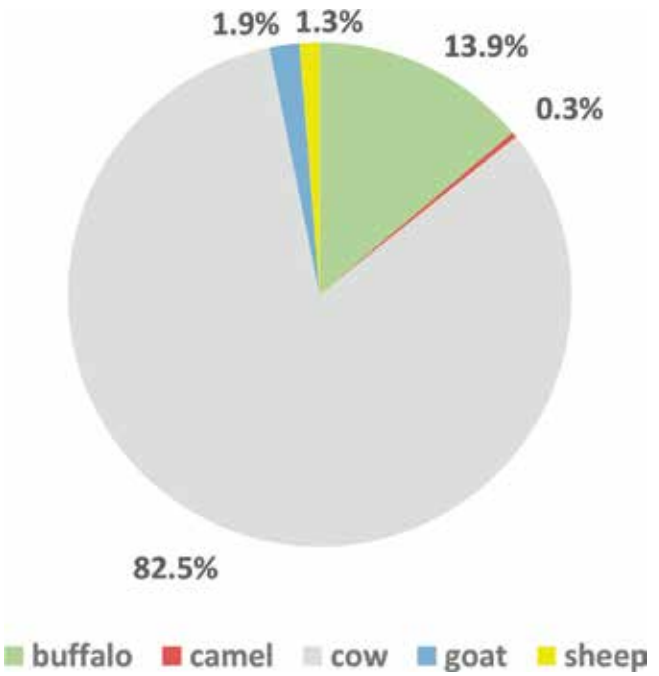

Figure 2.
Pie chart showing the percentage of the total quantity of milk produced by different species in the world.

been reported to possess several technological and medicinal advantages: (i) it can be produced in significant amounts from poor feed than any other dairy species in geographical areas with climatic constraints; (ii) it contributes to the national incomes and the international market integration; and (iii) it satisfies the growing consumer demand for functional foods that, in addition to their nutritional values, have health benefits [6]. Moreover, camel milk is a highly nutritious medium permissive for the growth of many diverse bacterial species. These bacterial populations are mainly grouped into pathogenic, spoilage, and technologically relevant bacteria (health promoter, starter cultures, and preservative agents) [7]. The microbial composition can significantly influence the safety and technological properties of this type of milk. Consequently, the consumption of raw camel milk or of dairy products made with milk that was poorly handled or not properly pasteurized can lead to serious food infections and foodborne diseases. Despite the importance of the information regarding the safety level of raw camel milk, studies investigating its microbiota are limited. Therefore, further studies in this field are required. This chapter reviews the existing knowledge on the composition, nutritional value, health beneficial properties, and economic value of camel milk and its by-products. Furthermore, the relevant studies describing the microbiota of camel milk and the approaches used for their detection are also included.

2. Past and present of camel milk

During the last 50 years, a fundamental shift has occurred in the way of housing and managing the camels. Today, in some countries, the extensive or nomadic production system for camels has become semi-intensive or intensive modern well-organized farms and industries [8]. Camels were known as "ships of the desert" commonly used as a mean of transport for thousands of years, carrying up to 600 lbs. on their backs. Currently, they are mainly considered for their milk and meat production, in particular, their milk. Camel milk contributed to the non-bovine milk production with a total amount of 2.7 million tons in 2016 [1]. Since the quantity and quality of milk production are highly dependent on the housing and management of animals, intensive production systems are currently being set up. All the world over, camel milk has proved to be suitable for producing various derived products with significant nutritional value [9].

3. Chemical composition and nutritive value of camel milk

Concurrently with the growing interest in foods that, in addition to their nutritional values, have physiological benefits, the attention toward camel milk is notably increasing [10]. The specific composition of camel milk makes it a promising alternative to bovine milk. Compared with the milk produced by other ruminants, camel milk is mainly valued for its better digestibility in the human gastrointestinal system due to the smallest milk-fat globules and its hypoallergenic properties [11]. As in human milk, dromedary camel and Bactrian camel milk do not contain β-lactoglobulins. There are no reports concerning allergy indicators possessed by these milks. Therefore, α-lactalbumin is the main whey protein in camel milk whereas this protein constitutes only 25% of the total whey proteins in cow milk [12]. Also, the fat globules in camel milk are the smallest among all ruminants, and they do not naturally aggregate due to the absence of agglutinin [9]. Consequently, camel milk can easily be digested and safely consumed by people with weak immune systems or lactose intolerance and can be considered a valid substitute to

bovine milk for children more than 2 years old [13, 14]. Moreover, camel milk has lower amounts of fat, protein, and carbohydrates compared to bovine milk [15, 16]. Milk is one of the major contributors to saturated fatty acid (SFA) and unsaturated fatty acid (USFA) intake. The SFA content of milk has received much attention due to its association with the increased cholesterol level in the plasma and subsequently the cardiovascular disease risk. However, the monounsaturated fatty acids (MUFA) are known to be a healthy cardioprotective type of fat [17]. Interestingly, the SFA content of Bactrian camel milk (average 50/100 g total FA) and dromedary camel milk (average 60/100 g total FA) is lower and slightly lower than that of cow milk, respectively. The MUFA content in dromedary camel milk (56–80/100 g total FAs) is higher than in cow milk (26/100 g total FAs) [18]. This fact may have beneficial effects on consumers with cardiovascular disease risk. Camel milk has higher amounts of certain vitamins and minerals. Its richness in vitamins, especially vitamin C (24–52 mg/kg) which is 3–5 times and 1.5 times higher than bovine and human milk, respectively, gives this milk a great significance in arid areas where green foods are not easily available [19]. Bactrian camel milk is a source of vitamin A (approximately twice that in cow milk) and is high in vitamin D and riboflavin. Two cups of camel milk supply 160% of the recommended nutrient intake of vitamin D (5 μg/day) and 0.5 mg/day of riboflavin [20]. In addition, this milk could provide the most of nutritional mineral requirements of humans due to its mineral content being almost similar to that of human milk [9]. Compared to goat and cow, it contains 55% more zinc, an essential trace element considered as the limiting dietary growth nutrient in populations suffering from deficiencies in micronutrients [21].

4. Health beneficial properties of camel milk

Camel milk is not only valued for its physicochemical composition, but traditionally, camel milk and its fermented products have also been consumed for many years due to consumer's recognition of its important health-promoting properties. Camel milk has been known for its ability to promote bone formation in infants, and its curative properties against many internal diseases [22]. Nowadays, these medicinal virtues have been scientifically supported, in particular, the association of camel milk with a decreased prevalence of diabetes type I and II by reducing the demand for insulin in patients and improving residual β-cell function in the pancreas, due to its immunomodulatory influence [23]. This hypoglycemic potential is either due to the presence of insulin-like small molecules that are easily absorbed into circulation compared to insulin from other sources or to the existence of insulin in camel milk in indigestible form, i.e., encapsulated in nanoparticles (lipid vesicles) [24]. In addition to its anti-diabetic properties, camel milk and its derived products have a therapeutic role in gastrointestinal ulcers, liver disorders, diarrhea causing viruses, and tuberculosis [25]. Moreover, this milk has received great attention for its ability to cure several diseases including jaundice, lung- and spleen-related illnesses, asthma, anemia, autism, edema, milk allergies, and dermatological autoimmune diseases [15, 16, 26, 27]. Additionally, camel milk is considered a good source of many antimicrobial substances and bioactive compounds, such as, lactoferrins and immunoglobulins, in addition to a greater quantity of lysozyme [28]. The level of lysosomal enzyme (N-acetyl-β-d-glucosaminidase) that provides antimicrobial activity is high in camel milk as compared to other ruminants. This may confer a strong innate immunity providing higher natural resistance toward infections [29]. As camel milk is a highly nutritious medium supporting the growth of various bacterial species, in particular, lactic acid bacteria, it is considered to be

Geographical area	Milk sample type	Detected genus	Bacteriocin	Reference
Kuwait	Raw camel milk	*Enterococcus faecium*	Enterocins A, B, and P	[58]
Algeria	Raw camel milk	*Enterococcus faecium*	Enterocins L50A and L50B	[43]
China	Fermented camel milk (Shubat)	*Lactobacillus casei*	Caseicin TN-2	[22]
Jordan	Raw and fermented camel milk	*Lactobacillus plantarum* *Lactobacillus rhamnosus* *Lactobacillus brevis* *Lactobacillus paracasei* *Lactobacillus fermentum*	Bacteriocin-like substances	[59]
Saudi Arabia	Raw camel milk	*Lactobacillus acidophilus*	Acidophilucin AA105	[60]
Algeria	Raw camel milk	*Leuconostoc mesenteroides*	Leucocin B	[3]
Algeria	Butter made from camel milk	*Lactobacillus plantarum*	Bacteriocin-like substance	[61]

Table 1.
Published studies on isolation of bacteriocin-producing lactic acid bacteria from camel milk and its by-products.

a potential source of probiotics and novel bioactive compounds. The few published studies on camel milk have shown that this milk is considered a niche of lactic acid bacteria producing antimicrobial peptides (bacteriocins) (**Table 1**).

5. Economic value of camel milk and its by-products

Although most of the camel milk is consumed raw or fermented by the local community, this milk and its by-products are now being promoted in the market and have considerable economic importance internationally due to the following properties: (i) It has specific nutritional features and health benefits; (ii) camels possess exceptional adaptation to poor quality and quantity of feed, and they can produce a significant amount of milk during the whole year, even during the dry season [30, 31]. Milk yield in camels is approximately 3.5 kg/day in hot summer [32], and it could reach a maximum of 6000 L/lactation in selected breeds in Saudi Arabia [33]; (iii) it has a long lactation period of about 12 months but it may last up to 24 months depending on the farming and breeding systems [21]; (iv) it can significantly contribute to the national revenues as the total production of camel milk was estimated to be 1.3 million tons [34], with the global trade of $10 billion per year; (v) machine milking of dromedaries is now under design to improve the camel milk production [35].

Shortly, a higher amount of camel milk is expected to be available to the dairy industry. There are indications of the potential for growth of this market in the future and that camel milk manufacturers will dramatically expand [36]. Many manufacturers of camel milk-based products are spread out worldwide; for example, Desert Farms (US), Camel Milk Victoria (Australia), Vital Camel Milk (Kenya), Camel Milk UK (UK), Wang Yuan Camel Milk (China), and Camel Milk

Figure 3.
Camel milk based products in Australia.

Australia (Australia). Large camel farms are being established, and increased funding for camel research is noticed. Moreover, a wide variety of camel milk-derived products are now available in the market, including various new dairy products, such as pasteurized milk, fermented milk, flavored milk, butter, cheese, and milk tea [37, 38]. As a specific example, Bactrian camel milk is used for making cheese, butter, and yogurt in Mongolia [13]. Traditional fermented camel milk is produced and consumed in several countries, such as, Shubat (Turkey, Kazakhstan, and Turkmenistan), Suusac (Eastern Africa, Kenya, and Somalia), and Garis (Sudan and Somalia). Despite the effort made by researchers to produce yogurt from camel milk, the manufacturing of this by-product needs more study due to the fact that camel milk does not easily coagulate [39]. In Dubai, a coffee shop business specializing in different hot and cold drinks and pastries made with fresh camel milk called "Cafe2go" was set up recently. Since 2011, it has expanded successfully to 10 locations in Dubai and Pakistan and is soon to be opened in Oman and Saudi Arabia. Furthermore, camel milk utilization is not limited anymore to the producer region only, but it is now marketed overseas also. It has recently been exported from the United Arab Emirates to the European Union [40]. Furthermore, recent studies have investigated the optimization of fermentation processes [41] and cheese-production from camel milk [42].

Based on the belief of many consumers that camel milk products address various allergies and skin problems, this milk reached the international markets due to the manufacturing of a broad range of healthcare products, including soap, lip balm, hand cream, and lotions. Other cosmetic product lines based on camel milk have also been developed (**Figure 3**).

6. Microbiota of camel milk

In light of the industrial interest generated in camel milk, its by-products and its medicinal features, the investigation of the safety and quality of camel milk became mandatory. In the past, the majority of the scientific studies focused on the anatomic characteristics, a physiological adaptation of camels to adverse climates, and the biomolecules present in camel milk [3]. However, information on the microbiology of camel milk is very limited. While many studies focused on the microbiology of cow, sheep, and goats milk, only a few studies have focused on camel milk despite

the fact that the difference in the composition of camel milk compared to milk from other animals, its biological features, and its production in a desert environment could underlie significant differences in its microbial ecosystem and its biological characteristics [7, 43].

Nevertheless, the available information on camel milk microbiota originates from studies based mostly on a culture-dependent approach, which is culturing the microbes using general or selective media followed by subsequent analysis.

Milk in general and camel milk, in particular, is a highly nutritious product providing an ideal environment for the growth of a diverse and complex microbial population. The nature and abundance of the microbial load are highly influenced by many parameters, such as, the surrounding environment (conditions of milk collection) and camel health status, in particular, because the mastitis disease has a great influence on the milk bacterial composition [44]. These bacterial populations are mainly grouped into two major categories: (i) beneficial and technologically relevant bacteria and (ii) pathogenic and spoilage bacteria. So far, only a few studies relying on the culture-dependent approach have been conducted on the identification of the bacterial populations of camel milk [5, 45, 46]. These studies are summarized in **Table 2**. It is well known that the specific bacterial composition of milk has a direct impact on the development of texture and flavor of the finished dairy product [47]. Several lactic acid bacteria present in raw milk have been proven to be technologically relevant in dairy products. Among these bacteria, *Lactococcus* spp., in particular, *Lactococcus lactis*, are primarily known for their role as starter cultures for the cheese industry and are also recognized for the production of flavor compounds [48]. *Lactobacillus* spp. are another example of bacteria found in raw milk, which has been used for many industrial dairy applications. They can contribute to the quality and nutritional value of dairy products through their proteolytic activity and ability to produce aroma compounds [49]. These genera are one of the dominant bacterial populations isolated from camel milk. Beside the technological properties of these microorganisms, it is also known that raw camel milk microorganisms have a health-promoting effect through aiding digestion or reducing the frequency of allergies, including asthma and atopic diseases [50–52]. In addition, camel milk is dominated by biologically active bacteria that produce many antimicrobials, including bacteriocins, antifungal agents, organic acids, and hydrogen peroxide in camel milk, which probably confer to this milk its extended shelf life, evidently resulting in safer consumption even during storage for several days in the absence of refrigeration [53]. The antimicrobial agents produced by these microbial species might be regarded as biopreservative agents that could be used to extend the shelf life and safety of camel milk products and could be of interest to the dairy industry.

On the other hand, microorganisms can also have a negative impact on the quality of camel milk and its shelf life, resulting in milk spoilage. The consumption of raw camel milk contaminated with pathogens can lead to, in some cases, severe illness [46]. Also, the presence of potentially pathogenic bacteria in raw camel milk can have implications for the animal and human health and are, therefore, relevant issues to be considered. Like other dairy animals, camels can also be affected by mastitis, which is defined as the inflammation of one or more of the teats (mammary glands) and is considered as one of the most important diseases in the dairy industry worldwide [54]. It can cause economic loss by reducing the milk production, lower probability of conception, higher treatment cost, and transmission of the disease to other species of animals [55]. It causes suffering for camels and poses a public health risk too. Recent studies on camels have described the effect of mastitis on milk hygiene and yield as well as on the immune system at the level of the mammary gland [40, 56]. Bacterial infections are considered to be the primary

Geographical area	Type of camel	Milk sample type/source	Aim of the study	Media used	Detected genus	Reference
Morocco (four zones) (n = 12)	Dromedary camel	Raw camel milk Composite samples Farm	• Assessment of the hygienic quality of camel milk • Detection of LAB and pathogens	MRS; Baird-Parker; Litsky medium	***Enterococcus*** **(58.8%)** ***Pediococcus*** **(28.2%)** ***Streptococcus*** **(4%)** ***Lactococcus*** **(8%)** ***Leuconostoc*** **(1%)**	[62]
Morocco Different regions in the south of Morocco (n = 30)	Dromedary camel	Raw camel milk Individual samples Farm	• Detection of LAB	MRS; M17; MSE	***Lactobacillus*** **(37.5%)** ***Lactococcus*** **(25.8%)** ***Leuconostoc*** **(11.7%)** ***Enterococcus*** **(10.8%)** ***Streptococcus*** **(9.2%)** ***Pediococcus*** **(5%)**	[45]
Iran Golestan (n = 10)	Dromedary camel	Raw camel milk Individual samples Farm	• Detection of LAB • Study of antimicrobial activity and probiotic traits	MRS; M17; KAA	***Enterococci*** **(51%)** ***Lactobacillus*** **(11%)** ***Leuconostoc*** **(5%)** ***Weissella*** **(2%)** ***Pediococcus*** **(2%)**	[5]
Kazakhstan (Four regions) Almaty, South Kazakhstan, Kyzylorda, and Atyrau (n = 26)	Dromedary camel	Raw camel milk Fermented camel milk Individual samples Farm	• Detection of LAB and yeast	MRS; M17	***Enterococcus*** ***(51.3%)*** ***Lactobacillus*** **(29.8%)** ***Lactococcus*** **(10.9%)** ***Leuconostoc*** **(8%)**	[63]

Geographical area	Type of camel	Milk sample type/source	Aim of the study	Media used	Detected genus	Reference
Kenya Central division of Isiolo district of Kenya (n = 15)	Dromedary camel	Fermented camel milk Individual samples Farm	• Detection of LAB	MRS	***Leuconostoc*** **(24%)** ***Lactobacillus*** **(16%)** ***Lactococcus***	[64]
Abu Dhabi (n = 50)	Dromedary camel	Raw camel milk Individual samples Farm	• Study of probiotic traits	MRS	***Lactococcus*** ***Lactobacillus***	[65]
Sudan Nine regions Central, western, northern, and eastern geographical areas of Sudan	Dromedary camel	Fermented camel milk 'Garis'	• Identification of the microbial populations present in Garis • Detection of LAB and yeast	PCA; MRS; M17; PDA	***Streptococcus*** **(68%)** ***Lactobacillus*** ***Enterococcus*** ***Lactobacillus***	[66]
Sudan Transhumance and nomadic herds (n = 28)	Dromedary camel	Fermented camel milk 'Garis'	• Assessment of the microbial contents	PCA; MRS; M17	***Streptococcus*** **(50%)** ***Lactobacillus*** **(50%)**	[67]
Sudan Dongola, Kasala, El Gadarif, El Obied, and Omdurman (n = 12)	Dromedary camel	Fermented camel milk 'Garis'	• Detection of LAB	MRS; M17	***Lactobacillus*** **(66.6%)** ***Lactococcus*** **(33.3%)**	[68]
Middle Saudi Arabia Qassim region (n = 33)	Dromedary camel	Raw camel milk	• Assessment of the microbial quality • Detection of pathogens	PCA; PDA; Baird-Parker agar	***S. aureus*** **(70%)** ***Salmonella*** **(24%)**	[69]

Geographical area	Type of camel	Milk sample type/source	Aim of the study	Media used	Detected genus	Reference
Somalia Fafen zone, Ethiopian Somali regional state (Gursum, Babile) (n = 126)	Dromedary Camel	Raw camel milk	• Assessment of the microbial quality • Detection of pathogens	Edwards Medium; MSA; MAC; EMB agar; XLD; Brilliant Green Agar; Salmonella and; Shigella Agar; TSI	***Staphylococcus*** **(89.8 %)** ***Streptococcus*** **(53.7 %)** ***E. coli*** **(31.5 %)** ***Salmonella*** **(17.6 %)** ***Klebsiella.*** **(5.6 %)** ***Enterobacter*** **(5.6 %)**	[46]
Egypt Sinai, Aswan, and Sharqia governorates (n = 185)	Dromedary camel	Raw camel milk	• Monitoring the possibility of transmission of milk borne pathogens • Detection of pathogens		***Salmonella*** ***E. coli*** ***Listeria***	[70]

Table 2.
Published studies on the bacterial populations detected in raw camel milk and its fermented products using culturing methods and subsequent analysis.

cause of mastitis in domestic animals. The major causes of camel mastitis have been investigated using a classical bacterial culturing approach [32, 54, 57]. Thus, the proper control of this disease will not only improve the quality and quantity of camel milk produced but would also reduce the public health risk to the camel milk consuming population.

7. Conclusions

Camel milk is known for its nutritional quality, being rich in vitamins C and A and low in SFA, its smaller fat globules, and being easy to digest and rich in many minerals and bioactive compounds. The medicinal virtues of camel milk and its association with a decreased prevalence of diabetes type I and II have attracted many studies, but more research is needed to dissect the mechanism of action of the insulin-like small molecules, which may be responsible for the anti-diabetic properties present in camel milk. Further studies are also required to examine the role of camel milk in treating several diseases such as gastrointestinal ulcers, liver disorders, and dermatological autoimmune diseases. In addition to that, camel milk has been proven as a highly important source of natural bioactive compounds and antimicrobial substances that could be targeted to develop functional and health promoting products for the well-being of human population in the coming years.

Conflict of interest

Authors have no conflicts of interest to declare.

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Chapter 4

In vitro Evaluation of the Phagocytosis Activity of Neutrophils and Characterization of *Staphylococcus aureus* Mastitis in Dairy Cows of Small Family Farms

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Abstract

A total of 269 cows in small family herds in the central region of México from different municipalities of México State were studied. Composed milk samples were obtained to detect subclinical mastitis and *S. aureus* infection and for characterization of phenotypes as follows: biotypes, capsular exopolysaccharide 5 and 8, ORSA/MRSA and MRSA strains; and *in vitro* phagocytosis neutrophil activity and apoptosis by *S. aureus* serotype 5. Results were evaluated by estimating proportions and chi-square test ($p < 0.05$). The microbial isolation rate was 46%; *S. aureus* isolation rate was 23.4–21.0% among cow herds; 39% of microbial isolates were in 1500–2500 cells/mL, with Wisconsin test. The phenotypes of *S. aureus* were: biotypes A and C are identified frequently that produce alpha and beta-hemolysin toxins, and a smaller proportion other hemolysins types. *S. aureus* isolates capsular serotypes 5 and 8 show differences in the *in vitro* neutrophil phagocytosis activity and apoptosis. The ORSA/MRSA isolates show that MRSA strains' mec A gene was confirmed by PCR. The *S. aureus* infection level in the dairy cow herds shows a wide municipal distribution, identifying different *S. aureus* pathotypes enclosed to virulence factors and MRSA to establish a potential health risk in small dairy cow herds in México.

Keywords: bovine mastitis, *Staphylococcus aureus*, neutrophil phagocytosis, milk quality, small dairy farms

1. Introduction

Bovine mastitis is a limiting disease of the production in dairy herd, commonly caused by *Staphylococcus aureus*. The inflammation of the glandular tissue constitutes a mechanism of natural resistance of the mammary gland infection in dairy cattle [1]. The appearance of inflammation in the mammary gland to increase the number of somatic cells (CCS), with an increased proportion of the leukocytes in milk is related to the agents present in the infection and the severity of the inflammatory response in the mammary gland [2, 3]. In addition, the inflammation provokes physical and chemical changes in the milk affecting the quality and its nutritional composition [4]. The significant increase in CCS in milk may be influenced by *S. aureus* herd infection level which stands out as one of the main contagious pathogens that are capable of producing a persistent infection and chronic inflammation in the mammary gland [5, 6]. The pathogenicity of *S. aureus* strains are related to the different virulence factors of the agent, considered primary in the development of mammary gland infection. The production of the alpha toxin shows a cytotoxic and cytolytic activity responsible for cases of gangrenous mastitis due to *S. aureus* [7]; the capsular exopolysaccharide is responsible for interfering with phagocytosis and complement activation [8]. In addition, colonization of udder skin in cows before parturition increases the risk of developing an intramammary postpartum infection, when the *S. aureus* infection level in the herd is high. The environment and body sites of animals are a frequent source of infection from cows with persistent *S. aureus* infection [9]. The potential risk for an epidemic colonization by *S. aureus* in the dairy herd occurs from the skin contamination of the nipple, and the udder lesions present favor the development of intraglandular infection. The first phase of lactation in cows is considered the most risky to new infections by *S. aureus* in cattle herds [10, 11]. Other environmental pathogens such as *Escherichia coli* and *Streptococcus agalactiae* increase the infection risk in fresh cows during postparturition period. The design of the milking parlors has a determining influence on the infection rate in the dairy herd and the elevated milk pipe lines producing large vacuum fluctuations that increase intramammary infection risk in dairy cows [12]. In comparison, the low pipe lines in the milking parlors reduce the proportion of new infections in the mammary glands. The risk of a cross infection increases at the milking time, when milking techniques are inappropriate or the milking equipment malfunctions. These factors increase the bacterial contamination of the nipple affecting the resistance mechanisms of the mammary gland. The reduction of the risk of bacterial colonization in the skin is achieved with the application of the nipple disinfectants, decreasing the skin contamination by *S. aureus* and other pathogens, without appreciably affecting the physical characteristics of the nipple skin. Keratin in the nipple duct is a natural barrier that reduces bacterial colonization and limits the development of the mammary gland infection [13, 14]. In the presence of mastitis, the chemical composition of keratin changes, and there is an increase in the proportion of polyunsaturated fatty acids and a decrease in the content of short-chain fatty acids [15, 16]. On the other hand, when the keratin is removed experimentally from the teat canal, the rate of infection with *Str. agalactiae* increases; consequently, there is a high excretion of bacteria in milk and an elevated somatic cell count in milk. This shows the importance of keratin as a natural barrier that contributes to reduce mammary glandular infection [17]. On the other hand, in infection of *S. aureus* in the mammary gland, significant changes of glandular tissue structure occur in which hyperplasia, stratification, and keratinization of the milk cistern and reduction of the glandular lumen are observed [18]. These changes are caused by the inflammatory

reaction and local leukocyte infiltration, with the formation of plaques of cellular debris and keratin present in the amorphous material. *S. aureus* experimental infection in dairy cows is affected by the lactation stage and the number of milk somatic cells [6]. The *S. aureus* strain type and the infective dose influence the development and the infection persistence in the mammary gland. In dairy cows with less than 4 months of lactation, when the number of somatic cells is less than 500,000 cells/mL, resistance to infection occurs. In turn, other studies indicate that the pathogenicity of *S. aureus* strains contributes in a decisive way to the development of mammary gland infection and its potential spread in the herd [19]. The persistence of infection by *S. aureus* in the udder of the cows increases the levels of antibodies present in the milk; this correlates positively with the number of lactations and the number of somatic cells in the cows [20]. The diagnosis and timely treatment decrease the evolution of clinical signs and the severity of damage to the mammary gland caused by *S. aureus, Str. agalactiae,* and *Str. uberi*s, resulting in a reduction in the incidence of bovine mastitis in the dairy herd [21]. The diagnostic tests allow early identification of physical-chemical milk changes caused by mastitis. The detection of agents, antigens, and antibodies favors the identification of an intramammary infection and its clinical evolution in the herd [22]. The diagnosis of the situation of subclinical mastitis in the herd, under a causal model based on clinical diagnosis, establishes an important association between the environmental factors and the causal conditions in the transmission of infectious agents in the population. In this case, the *S. aureus* infection is able to affect seriously the udder health and milk production. To evaluate herd infection level and to characterize *Staphylococcus aureus* phenotypes, dairy cows from small family farms in the Central Mexico region were studied.

2. Materials and methods

The diagnosis of the situation of subclinical mastitis in small dairy herds' family production was made under the causal model based on the clinical diagnosis of bovine mastitis and its association with the main infectious agents present in the herds and the *S. aureus* level infection and virulence factors in the different regions of the State of Mexico located in the Mexico central region. The determination of the *S. aureus* infection was associated to subclinical mastitis in dairy herds during the period of 2015–2017.

2.1 Determination of *Staphylococcus aureus* and frequency of subclinical mastitis

Milk samples obtained from 2749 cows of 182 dairy herds of family production in different municipal regions of the State of Mexico (Almoloya de Juarez, Zinacantepec, Chapultepec, Temoaya, Toluca, Tenango del Valle, and Lerma y Atlacomulco) were studied, to detect the mastitis frequency, the *S. aureus* herd level infection distribution, and dairy cow density. The isolation and identification of *S. aureus* in the milk samples was carried out using the protocol established by the National Mastitis Council [23]. The municipal regions of the entity were grouped taking into account the livestock inventory and the territorial extension of the municipalities studied to determine the population density expressed as the average number of cows per km^2. The population density of the region was classified as: low (BA), mean (ME), and high (AA). The results were evaluated using the proportion estimation test corresponding to the design ($p < 0.05$).

The level of association between the reaction of the Wisconsin Test and the isolation frequency of *S. aureus* in dairy herds was determined. A total of 243 milk pooled samples obtained from the four glandular quarters of the cows from different ages and lactation stages were obtained at random from small dairy herds of the Toluca Valley, Mexico. The inclusion criterion of cows in the study was a positive reaction to the mastitis Wisconsin Test [23]. The milk samples, were inoculated 0.01 mL, on blood agar plates, MacConkey and Vogel Jhonson agar plates (0.001 g/L potassium tellurite), blood agar plates CAMP-esculine, the plates were incubated at 37°C for 18–24 hours [24]. The identification of the isolates was carried out by routine bacteriological procedures Gram staining, coagulase test tube, catalase, Voges-Proskauer, and the standardized commercial systems API Staph and API 20E. The estimated number of somatic cells in milk was determined from tubes of the reaction level of the mastitis Wisconsin Test. The results were evaluated using the proportions estimation test corresponding to the design ($p < 0.05$).

2.2 *Staphylococcus aureus* isolation and phenotypic characterization

The isolation of *S. aureus* and its phenotypic characterization was carried out in a transversal longitudinal study during the fall-spring period of 2016, by randomly sampling 87 dairy family herds in the dairy system with an average herd size of 14.3 cows from different municipalities in the Valley of Toluca, State of Mexico. We obtained 1256 composed milk samples from the four glandular quarters to carry out the bacteriological study. The small-scale production system was characterized in the type of small dairy herd family production of rustic rural types. The racial phenotypes in dairy cow herds were Holstein, Creole and hybrid Holstein, European Swiss and *Bos indicus* crosses. A traditional productive management, hand dairy milking predominantly, feeding with diurnal grazing in native pastures and use of agricultural corn husks (*Zea mays*), a minimum supplementation diet with feedstuffs. Isolation of *S. aureus* was carried out by routine microbiological protocols [24]. About 0.01 mL was inoculated on Vogel Jhonson agar plates (0.001 g potassium tellurite/L). The agar plates were incubated at 37°C for 24 hours; colony forming units were identified by Gram stain, catalase, coagulase test tubes and aerobic fermentation of maltose, trehalose, and anaerobic mannitol tests. The final identification of the *S. aureus* was confirmed by API Staph system (Biomerieux Vitek, Durham, NC, USA).

The biotypes of *S. aureus* were identified from 90n isolates, those were grown on crystal violet media (brain heart infusion agar plates, added with 1:10000 violet crystal). The agar plates were incubated at 37°C for 24 hours, biotypes were identify when biotypes were identify when observing the colony forming units, associated with the biotypes A, B, C and D. The positive crystal violet reaction was considered with violet coloration and a slightly yellow halo formation. Biotype A was characterized as positive violet crystal, biotype B showed a whitish coloration, biotype C showed a yellowish color, and the absence of growth on the medium was related to biotype D.

The identification of the different types of hemolysins α, β, γ, and δ of *S. aureus* was made from the observation of hemolysis in blood agar plates supplemented with 7% erythrocytes obtained from: bovine, rabbit, equine, and human type O^+ [25]. The agar plates were incubated at 37°C for 24 hours under aerobic conditions and under a reduced atmosphere of CO_2, the type of hemolysis was observed in the different blood agar plates; they were compared with the control strains and the type was determined.

The *S. aureus* antibiotypes were characterized by *in vitro* sensitivity test performed by the agar diffusion method of National Committee for Clinical Laboratory Standards [26]. On the Mueller Hinton agar plates inoculated with the isolates of *S. aureus*, antibiotic discs were placed on the agar: penicillin 10 IU, 10 μg ampicillin, 1 μg dicloxacillin, 10 μg streptomycin, 30 μg cefotaxime, 30 μg cephalosporin, 2 μg lincomycin, 15 μg erythromycin, 30 μg novobiocin, and 100 μg spiramycin. Agar plates were incubated at 37°C for 18–24 hours. The bacterial growth inhibition halos on the agar plates were expressed in mm, compared to the bacterial growth inhibition halos of the control strain of *S. aureus* ATCC25923. The results were evaluated using the proportion estimation test corresponding to the design ($p < 0.05$).

2.2.1 Staphylococcus aureus *capsular polysaccharides characterization*

The capsular exopolysaccharide phenotypes were characterized from 90n *S. aureus* isolates obtained previously from small dairy family herds. They were studied for the expression of the capsule that was performed on Columbia agar added with 2% NaCl incubated at 37°C for 24 hours; the capsular expression was confirmed by capsule staining. The capsular type was observed in 4% whey soft agar tubes (brain and heart agar added with 4% v/v milk whey). The capsular serotype was determined with a rabbit polyclonal antiserum against the capsular serotypes 5 and 8 of *S. aureus*. The results obtained were evaluated by the Chi-square test ($p < 0.05$), based on the observed frequencies of bacterial isolation and the level of infection. The comparison between the growth inhibition halos was carried out by means of the hypothesis test of two proportions of the same group with mutually exclusive characteristics.

2.2.2 Staphylococcus aureus *capsular genes*

The identification of the cap5 and cap8 genes related to *S. aureus* capsular types was performed from isolates of *S. aureus*, using the polymerase chain reaction (PCR) test, were performed using the oligonucleotides Cap 5 k1 (5-GTCAAAGATT ATGTGATGCTACTGAG-3) and Cap 5 k2 (5-ACTTCGAATATAAACTTG AATCAATGTTATACAG-3) for the detection of the capsular typ. 5 and for the capsular typ. 8 the following primers 8 k1 (5GCCTTATGTTAGGTGATAAACC-3) 8 k2 (5-GGAAAAACACTATCATAGCAGG-3) were used, obtaining the PCR products amplified of 361 bp for cap 5 and 173 bp cap 8 [27].

2.3 *In vitro* induction of apoptosis in bovine neutrophils

The effect of the capsular serotyp. 5 of *S. aureus* on the induction of *in vitro* apoptosis of neutrophils from dairy cattle was evaluated by means of light field microscopy and smear staining method, May-Grünwald-Giemsa stain. The *in vitro* induction of apoptosis was used as a phagocytosis substrate of heat-inactivated *S. aureus* (120°C for 20 minutes) suspension (2×10^8 CFU/mL) at 4°C. The *in vitro* assay was performed in 1:10 neutrophil: bacteria ratio incubated during 1 hour, and then smears using May-Grünwald-Giemsa stain were prepared [28]. The microscopic observation of neutrophil apoptosis was confirmed under the epifluorescence microscope preparing ethidium bromide solution (100 μg/mL) and acridine orange (100 μg/mL). A number of apoptotic neutrophils were determined from the reddish coloration of the chromatin and nuclear condensation or fragmentation against viable neutrophils that showed a green coloration of the chromatin [29].

2.4 *Staphylococcus aureus* methicillin-resistant strains identification

With the identified resistant oxacillin and methicillin antibiotypes (ORSA/MRSA), the *in vitro* susceptibility test on antimicrobials with 90n *S. aureus* isolates was carried out by the agar diffusion method of National Committee for Clinical Laboratory Standards [30], *S. aureus* isolates were incubated in Mueller Hilton broth for 4 hours at 37°C, compared to McFarland standard 0.5; Mueller Hinton agar plates were inoculated by applying on the plates the antibiotic unidisks (BBL, Lawrence, KS, USA): 10 U penicillin, 30 μg ampicillin, 1 μg oxacillin, and 10 μg cephalothin. The *S. aureus* strains of ATCC25923 and ATCC 29213 and the *S. epidermidis* strain ATCC 12228 were used as controls. The strain of *S. aureus* ATCC 43300 was used as a control of methicillin resistance. To confirm the MRSA strains, Mueller Hinton agar plates containing 4% NaCl were inoculated and incubated at 35 and 42°C for 24 hours. The bacterial inhibition halos of the bacterial growth on the agar plates were expressed in mm and compared to the established values for the test with a difference >4 mm in diameter between the halos of inhibition containing unidisks with 1, 2, 4, and 6 μg of oxacicline [31]. The *in vitro* production of β-lactamase was determined by the modified iodometric method [32].

2.4.1 Identification of the mec A gene in Staphylococcus aureus *by polymerase chain reaction (PCR) test*

Isolation of chromosomal DNA was obtained from *S. aureus* 90n isolates, from the study groups of work strains 1, 56, 305, and 123, with the control strains of *S. epidermidis* ATCC 12228 and *S. aureus* ATCC 25923 as negative controls, and *S. aureus* ATCC 43300 as a positive control. The washed bacterial pellet was suspended in a buffered phosphate solution of pH 7.2 to proceed to bacterial lysis. The bacterial DNA solution was standardized at 0.5 ng per 1 μL and stored at −20° C. The PCR reactions were carried out with a commercial PCR kit (BioTecnologías Universitarias, México), using a thermal cycler (Genius Techne, Duckford, UK). The PCR for the identity of the *S. aureus* isolates was carried out by the amplification of nuc gene, using the oligonucleotides (Gibco BRL, Rockville, MD), Sa1 (5′GCGATTGATGGTGATACGGTT-3′), and Sa2 (5′AGCCAAGCCTTGACGAA CTAAAGC-3′) [33]; the amplification product of PCR obtained was 270 bp related to the nuc gene. The amplification of the mec A gene was performed to identify the MRSA strains of *S. aureus*, using the primers (Gibco BRL, Rockville, MD), mec1 (5′-TGGCTATCGTGTCACAATCG3′), and mec2 (5′-CTGGAACTTGTTGAGCA-GAG-3′) to obtain an amplification product of 310 bp; initial denaturation was performed at 92°C for 3 minutes and 30 cycles under the amplification conditions: 92°C for 1 minute, 56°C for 1 minute, 72°C for 1 minute, and a final extension of 2 minutes at 72°C. The amplified product was kept at 4°C, before being visualized on an agarose gel. The amplification conditions of the gene were similar with an alignment temperature of 56°C. The nuclear DNA amplification products were separated by electrophoresis in a horizontal chamber (Horizon 5B, Gibco BRL, Goithersburg, MD, USA), with a power supply of 70 Volts for 90 minutes applied to 2% agarose gel (Gibco BRL, Rockville, MD. USA), in TBE in a run with a 100 bp molecular weight marker (Gene Ruler, Fermentas, Burlington, Ontario, CA). The strains of *S. aureus* ATCC 29213, ATCC 43300, and *S. epidermidis* ATCC 12228 were used as a control. The gels were stained with ethidium bromide. The gels were photographed in a UV transluminator (UVP, Upland, CA. USA), visualized with a DC 120 digital camera (Kodak Eastman, Rochester, NY, USA) and image analysis program (ID image analysis software Windows; and 3.0, Kodak digital science, Rochester, NY, USA). The analysis of results was made from the frequency of

isolation and the expression of virulence factors of *S. aureus* related to the biotype, by estimating the absolute and relative frequency; The statistical evaluation was performed with the Pearson's Chi-square test ($\chi 2$), with an α level of 0.05 and a 95% confidence interval using Epi-Info 6.0 software (CDS, Atlanta, GA, USA). The *in vitro* sensitivity was estimated from the mean ± standard deviation of the inhibition halos (in mm) of the antibiotics.

3. Results

The *S aureus* subclinical mastitis frequency determination in dairy cow population density expressed as cows number/km^2 shows the municipalities that were identified as the regions: Low (1.12) Toluca and Metepec; and Median (2.7): Atlacomulco, Chapultepec, Lerma, Tenango del Valle, Temoaya, and Zinacantepec. The high municipal livestock density (5.6) was observed only at the municipality of Almoloya de Juárez (**Table 1**).The overall *S. aureus* rate of the isolates was 21%. The *S. aureus* infection level in the herds was higher in the municipal region of Almoloya de Juárez. The *S. aureus* infection rate showed a higher tendency when the density/km^2 of cows increased.

The bacterial isolation rate of reaction level in the Wisconsin Test obtained from 243 milk samples was 46%, the main agent isolated was *S. aureus* with an overall rate of 22.4% in the studied milk samples, and the coagulase-negative Staphylococcus frequency was 12.9% (**Table 2**). Low frequencies of other environmental pathogens and minor pathogens were identified in the bacterial isolates.

When evaluating the level of somatic cells in milk by the Wisconsin Test, a significant proportion of 39% of isolates were observed in the range 1700–2500 × 103 cells/mL of the Wisconsin Test reaction distribution and the proportion of the bacterial isolates in the population sample studied (**Table 3**).

The isolation frequency of *S. aureus* in the dairy cow herds studied was 22.8%, compared to 12.29% of coagulase negative *Staphylococcus* (SCN), observed in the study ($p < 0.001$). The identification of the types of hemolysin and their relationship with the biotypes is observed (**Table 4**). The expression of hemolysins in isolates of *S. aureus* alpha-toxin was higher than other types of the identified hemolysins.

The relationship among the *S. aureus* biotypes and hemolysin type was observed; the predominant hemolysin type was α-toxin mostly related to the biotypes C and A, and β hemolysin was observed with biotype A mainly and to a less proportion

Density (cows/km^2)	Municipalities	Number of cows	Isolations *Staphylococcus aureus*	
				%
High [5.6]	Almoloya de Juárez	1021	268	46.2
Median [2.7]	Atlacomulco, Chapultepec, Lerma, Tenango del Valle, Temoaya, and y Zinacantepec	1237	211	36.3
Low [1.12]	Toluca, Metepec	491	101	17.4
Total		2749	580	21.0

$p < 0.01$.

Table 1.
Dairy cows density and frequency of staphylococcus aureus *isolates in municipalities of the State of Mexico.*

Agents	Isolation number	%
Staphylococcus aureus	82[a]	22.4
Staphylococcus coagulase negative	45[a]	12.29
Streptococcus agalactiae	14	3.82
Escherichia coli	6	1.63
Bacillus spp.	3	0.81
Micrococcus spp.	5	1.36
Klebsiella spp.	4	1.09
Enterobacter spp.	1	0.2
Streptococcus dysgalactiae	4	1.09
Streptococcus uberis	2	0.54
Negative isolations	197	53.82
Total	366	100

[a]*Significant differences ($p < 0.05$).*

Table 2.
Bacterial isolation frequency in dairy cows with subclinical mastitis.

Wisconsin test estimated somatic cells $\times 10^3$ mL	Bacterial isolates (%)
<100	10.0
100–500	8.0
500–1000	20.0
1000–1700	14.0
1700–2500	39.0
>2500	8.5
Total	100

[a]*Significant differences ($p < 0.05$).*

Table 3.
Distribution of mastitis Wisconsin Test reactions and bacterial isolates.

with C. The biotype C often expressed all hemolysin types: α-toxin, β, γ, and δ. The simultaneous expression among other hemolysin types such as α β and α β δ was observed in the biotype C.

The *S. aureus* antibiotypes resistant was frequent in β-lactam antibiotics with the highest observed proportion of antibiotics to the β-lactamases resistant was observed in the dicloxacillin (**Table 5**).

The distribution of *in vitro* sensitivity to antibiotics of the *S. aureus* isolates in the resistance pattern observed was: 65.7% and 90.3% for penicillin and ampicillin, 8.2% for dicloxacillin, 5.5% for cefotaxime, 6.0% for erythromycin, and 25.4% for lincomycin ($p < 0.05$). The evidence of the antibiotic resistance suggests a potential risk to health by antimicrobial resistance mainly to antibiotics β-lactam antibiotics and the possibility of identifying resistant strains ORSA/MRSA *S. aureus* methicillin and oxacillin resistant in the cow in the small family dairy herds studied [34].

The *in vitro* sensitivity to β-lactam antibiotics and the β-lactamase production was observed in a high percentage of the isolates evaluated (**Table 6**).

		Hemolysin types								
Biotype	**Total**	**α**	**β**	**γ**	**δ**	**α β**	**α δ**	**γ**	**α**	**α β δ**
A	48	9	11	0	2	19	2	0	3	1
C	36	23	4	1	1	5	3	1	2	0
B	6	0	0	3	0	0	0	0	0	0
Total	90	32	15	4	3	24	5	1	5	1
%	100	35.5	17.0	4.4	3.3	26.6	5.5	1.1	5.5	1.1

$p < 0.01$; total isolates of S. aureus *evaluated (90 n)*

Table 4.
Hemolysin types associated with biotypes of Staphylococcus aureus *in dairy herds of family production in Toluca Valley.*

Antimicrobial	**Isolations of *Staphylococcus aureus***		
	Sensitive (%)	**Intermediate (%)**	**Resistant (%)**
Penicillin	8.2	26.1	65.7
Ampicillin	8.2	1.5	90.3
Novobiocin	69.4 91.8	5.2 0.0	25.4 8.2
Dicloxacillin	90.3	1.5	8.2
Cephalosporin	8.2	40.3	5.5
Cefotaxime	91.8	2.2	6.0
Erythromycin	94.8	1.5	3.7
Spiramycin	69.4	5.2	25.4
Lincomycin	65.0	8.9	26.1

$p < 0.05$.

Table 5.
In vitro *sensitivity to* Staphylococcus aureus *antimicrobials in dairy herds of the Toluca Valley.*

The β-lactamase production was observed in relationship with β-lactam antibiotic in *S. aureus* resistant isolates mostly observed with penicillin and ampicillin. The oxacillin resistant isolates produce β-lactamase in a proportion of 20%.

The *S. aureus* capsular characterization results showed that total isolates of *S. aureus* expressed capsular exopolysaccharide phenotypes, expressed diffuse capsule, with the absence of the compact type in the milk serum soft agar in tube 63.33% (57/90) of the capsular strains were positive for serotyp. 5, 22.22% (20/90) for serotyp. 8 and 14.44% (13/90) were nontypable (NT) ($p < 0.05$). The municipal distribution of capsular serotypes 5 and 8 was similar ($p > 0.05$). In the Almoloya de Juárez region, a higher prevalence of capsular serotypes 5 and 8, 31.11 and 4.4%, respectively, was observed. In the municipality of Toluca, 1.75% *S. aureus* of the capsular serotyp. 5 was observed, indicating the absence of serotyp. 8; the results of the isolates were confirmed in the polymerase chain reaction (PCR) test (**Figure 1**).

The *S. aureus* capsular genes cap 5 and cap 8 were PCR confirmed, corresponding to the genes amplicons observed in the isolates obtained from dairy cows in small dairy family farms corresponding to the serotypes 5 and 8. In other hand, the serotypes non typiables NT, was not corresponded with the PCR evaluated amplicons.

Antimicrobial	Halo inhibition (mm) Halo growth inhibition				Strains resistant (%)	Production strains, β-lactamase (%)
	Maximum	Minimum	Average	SD±		
Penicillin	30	8	18.4	6.7	93.0	93.0
Ampicillin	29	10	17.3	5.7	93.0	93.0
Cefotaxime	30	0	19.1	10.2	7.0	59.0
Oxacicline	16	0	9.7	6.9	21.0	20.0

Table 6.
β-lactam In vitro *sensitivity of* Staphylococcus aureus *of cows with subclinical mastitis in small dairy family herds.*

The *in vitro* apoptosis of bovine neutrophils was evaluated by light field optical microscopy having a positive and negative control. The apoptosis values were 95.47 ± 3.07, compared with control groups. It was appreciated that *S. aureus* of capsular typ. 5 induced a greater proportion of neutrophils with apoptosis *in vitro*. The neutrophils apoptosis was confirmed in the May-Grunwald-Giemsa stained smears showing neutrophils with chromatin condensation and fragmentation.

On the other hand, the *in vitro* induction of apoptosis by *S. aureus* in bovine neutrophils was evaluated using light field and epifluorescence microscopy (**Figure 2**). The mean and standard deviation of the treatments are observed by the techniques of light field microscopy (CM) and fluorescence microscopy (MF); CC (+) positive control of neutrophils incubated with cyclophosphamide (400 μG/100 μL); negative control CC (−) only neutrophils; CC compact *S. aureus* strain; and CP capsular *S. aureus* strain serotyp. 5. The results showed differences between treatments ($p < 0.05$). The increased apoptosis induced by CP due to capsular serotyp. 5 was compared with the control groups. CC strain showed less *in vitro* neutrophil apoptosis induction.

The *S. aureus* mec A and nuc genes identified by PCR, results obtained from the characterization of the *S. aureus* phenotype isolates evaluated detecting the nuc gene (**Figure 3**), when confirming the MRSA strains mec A are shown with the PCR reaction products (**Figure 4**), that confirm presence of the amplicons in the *S.*

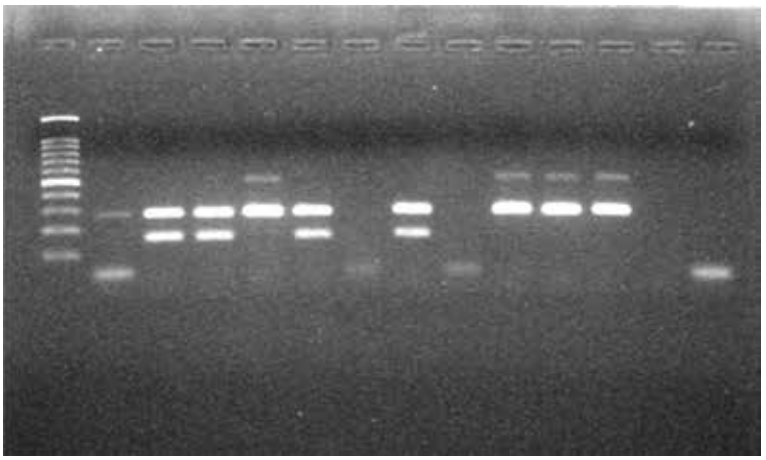

Figure 1.
Agarose gel showing the 173 bp amplicons obtained by PCR related to the cap 8 gene. Lanes 1: Molecular weight marker, 2: positive control Staphylococcus aureus *gene Nuc, 3 and 4: positive controls* Staphylococcus aureus *Cap8, 5: Positive control* Staphylococcus aureus *Cap5, 6 and 8: Positive samples of* Staphylococcus aureus *Cap8, 10–12: Positive samples for* Staphylococcus aureus *Cap5, 7, 9, 13, 14: Negative samples to* Staphylococcus aureus.

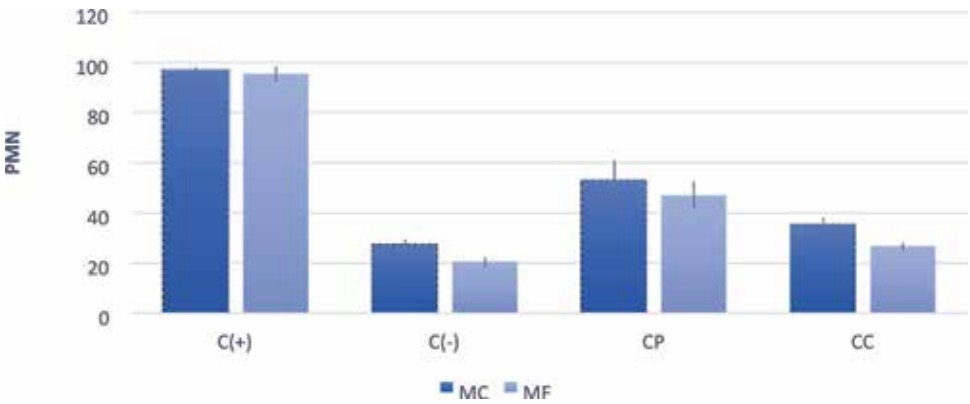

Figure 2.
Evaluation of the induction of apoptosis in vitro *in bovine neutrophils under light microscopy and epifluorescence.*

aureus isolates strains evaluated identified as *S. aureus* phenotypically as ORSA/MRSA, the nuc and mec A genes appreciated in the strains were identified is a such as 305, 123, 18, 25, 38, 44 and A53 previously identified as MRSA.

The MRSA strains were confirmed from ORSA/MRSA phenotypes detected previously; in the polymerase chain reaction (PCR) reactions, the *S. aureus* isolates were characterized by nuc gene. The MRSA isolates were identified as *S. aureus* methicillin resistant strains MRSA, identifying the mec A gene by PCR. In the ORSA/MRSA, isolates were considered to show phenotypical resistance in the different concentrations of oxacillin related to the production of β-lactamase. Those that showed resistant to 4 and 6 μg of oxacicline concentrations were confirmed by showing *in vitro* differences in the bacterial inhibition halos at 37 and 42°C, and they were considered presumptive MRSA strains. The results confirm that 90 *S. aureus* isolates were previously evaluated to detect β-lactam antibiotic-resistant phenotypes in the small dairy family farms studied, 93% the isolates produce β-lactamases and 20% of the isolates were considered ORSA/MRSA antibiotypes in which mec A gen of the MRSA strains confirmed by PCR were found.

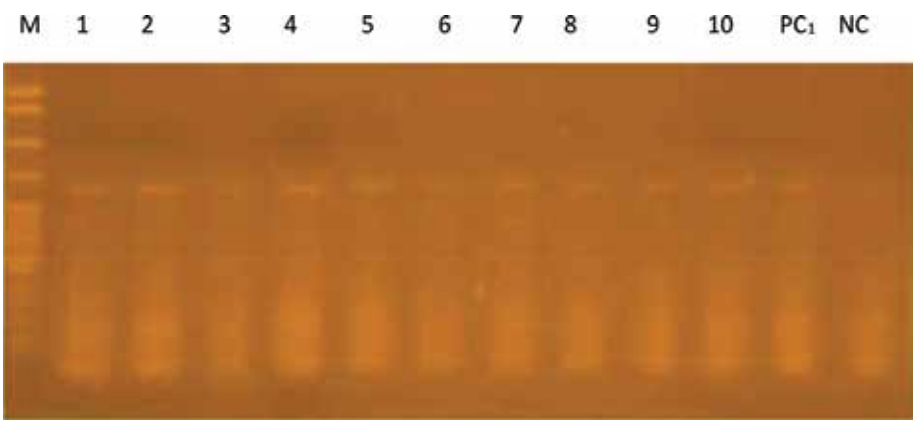

Figure 3.
PCR agarose gel electrophoresis, amplification products of the nuc gene of S. aureus *chromosomal DNA control strains ATCC 25293, ATCC 29213* S. aureus *strains as positive controls and ATCC 12228.* S. epidermidis *strain as negative control. Lanes: M, DNA manufacturer of molecular mass (100-bp ladder). Field strains; 1-8, 9-10, 305 and 113. PC1 (*S. aureus *ATCC 25293), NC (ATCC 12228* S. epidermidis*), PC2 (*S. aureus *ATCC 29213). All have an identical 270-Pb band pattern, corresponding to the* S. aureus *nuc gene.*

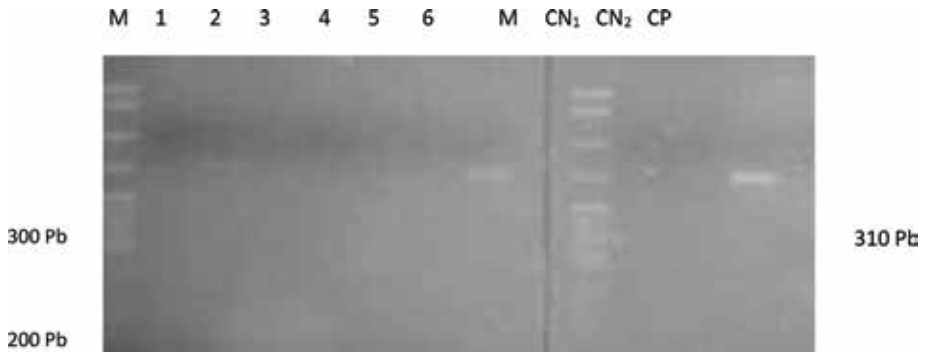

Figure 4.
Agarose gel electrophoresis shows the amplification products of the chromosomal DNA of the working strains of S. aureus*, the positive and negative controls line: M, molecular weight marker, field strains lines 1–6, the last lines correspond to SA 305, CN1 ATCC 12228* S. epidermidis *CN2 ATCC 25923 are negative controls, CP ATCC 43300* S. aureus*. SA 305 and ATCC 43300 showed a 310-bp amplicon corresponding to the mec A gene of* S. aureus*.*

4. Discussion

The wide municipal distribution of *S. aureus* mastitis and the dairy herd infection level by *S. aureus* in the studied cows were considered high as 22.4%, and *S. aureus* was identified as the main agent in the subclinical mastitis dairy cows family herds of Toluca Valley in the municipalities of central region of Mexico [35]. The herd infection level represents an important risk to the health herd and public health due to the contamination of the milk and fresh unpasteurized dairy products in the small dairy family farms in the municipal regions studied. The findings indicate the importance of *S. aureus* in the development and persistence of intramammary infection in subclinical mastitis in dairy cows [36, 37]. In it considered that dairy cows herds in family production in Mexico are widely distributed throughout the national territory, with an important contribution to regional socioeconomic development [38, 39]. In Toluca Valley, small-scale family-type production units are predominant with traditional production model, employment of family labor [40]. The danger of *S. aureus* mammary gland infection in the dairy herds occurred by poor hygiene at milking time, by increasing the bacterial contamination of the nipple and the challenge mammary gland resistance mechanisms [6, 41, 42]. In other cases, the possibility that the season of the year and the stress conditions that the cow undergoes are also indirectly affected mammary gland resistance mechanisms [43, 44]. Animal hygiene and udder health contributes to milk quality and safety food for the consumers, in opposite to subclinical mastitis, in which inflammatory reaction affects quality and milk safety [45].

Actually the importance of support of the sustainability of small cattle herds has an effect that can moderate the methane production and the adverse effects in the phenomenon of global climate change [46] because it will have a greater impact in agricultural production in geographic regions with less socioeconomic development affecting quality of life by increasing demand for food, deterioration of natural resources, water sources, and biodiversity [47]. One of the main expected effects of climate change is associated with changes in temperature and extreme weather disturbances that seriously affect the ecosystem, biodiversity, and agro-food production [48, 49]; there is a direct ecological and socioeconomic impact on human activities, the health of the human and animal population [50], and animal acclimation response in their adaptation processes [51]. Adaptive process in dairy cattle develops metabolic and behavioral physiological compensatory mechanisms to

reduce the adverse effects of climate related to the region's racial genotype [52, 53]. The risk of suffering thermal stress is increased in the animal population in certain regions with negative effects on livestock production and animal welfare [54, 55]. In extreme weather, events with a high ambient temperature, solar radiation, relative humidity and air velocity increase. Under these climatic conditions, cattle are susceptible to developing heat stress [56–59]. Thermotolerant animals expressed certain genes related to cellular stress induced by a high environmental temperature, increasing the secretion of growth hormone (b-GH), milk proteins β-casein (CSN3), and lactalbumin (LAA) [60–64].

Neutrophils phagocytosis activity in bovine mammary gland is the first line of cellular defense; its phagocytosis activity is reduced affecting its microbicidal capacity, by the presence of fat, casein [65, 66]. The bovine neutrophils are different in their capacity of phagocytosis on *S. aureus* in the mammary gland, the nipple canal and its permeability [67]. Leukocytic infiltration of the teat canal and glandular tissue occurs in response to *S. aureus*, infection at the time of mastitis [68]. Phagocytosis of leukocytes in the mammary gland shows differences in phagocytosis *in vitro*; bactericidal activity shown by neutrophils in the presence of milk whey stimulated is higher. The opsonization and intracellular killing of the *S. aureus* is affected by alpha toxin. It is possible that this effect increases intracellular survival causing failures in antibiotic therapy and mammary gland persistence infection, and the severity of the infection and the evolution of mastitis seriously affect the activity of phagocytosis with increased apoptosis and necrosis of neutrophils.

Other physiological factors of the cow modify the activity of phagocytosis in the mammary gland; during the first week of the dry period, the activity of phagocytosis increases, decreasing at the end of the dry period [69]. In other studies, one reveals a difference in the phagocytosis activity of neutrophils obtained from the glandular secretion of nulliparous and multiparous cows, when evaluating chemilumincence and peroxidase activity [70]. In lactating cows and heifers, peroxidase activation was associated with fat globules, casein, similar to that shown by zymosan phagosomes in the control group explained by the low activity of leukocyte xanthine oxidase. Other studies show differences in alkaline phosphatase of neutrophils from cows with mastitis and healthy cows. In the same way, another condition that can influence the resistance of the mammary gland is the ontogeny of the myeloid cells and their differentiation, by identifying the absence of the transferrin receptor and the expression of the antigens [43, 67, 68], BOCD11 A and BOWC5 [71]. The different surface receptors in the cell membrane of neutrophils are involved in chemotaxis, phagocytosis, and the activation of the respiratory explosion in neutrophils, evidencing the polymorphism of the functional sites of phagocytes and their modulation in phagocytosis [72]. Low neutrophils functional activity is shown at parturition, assuming an increase in susceptibility to infection at the beginning of lactation [73]. When evaluating the parameters of phagocytosis activity and milk production, a negative correlation was obtained. However, at present, there is a tendency to genetic selection of dairy cows to look for natural resistance to bovine mastitis, when choosing the progeny for the estimated data of the somatic cell count and the heritability index, evaluating the neutrophils phagocytosis activity and capacity [74].

During the *S. aureus* mammary gland infection, the somatic cells in milk are increased; these are composed of a cellular proportion of the mammary gland epithelium and another cellular portion of the leukocytes [75]. The leukocytes proportion present represent the severity inflammatory reaction affected by bovine mastitis, which causes physical-chemical and cellular alterations in milk that compromise the quality and safety of milk to increase the somatic cell count [76]. The changes that occur in milk are detected in the field and laboratory by diagnostic

techniques for detection of subclinical mastitis [77]. Other diagnostic methods for mastitis include determination of ions, leukocyte enzymes, proteins of the acute phase of inflammation, serum amyloid, and haptogloblin whose set of tests may determine the clinical course of the disease [78].

The mammary gland infection and their relationship between the average somatic cell in some cases to reflect observing persistent somatic cell counts in milk >1000,000 cells an infection with minor pathogens. The risk of intramammary infection increases when the somatic cell count of milk is >1500,000 cells/mL. When evaluating the dairy herds with high somatic cell counts in milk, the generated information is a useful collection by evolution to prevent the herd infection level [79]. Infection with *Escherichia coli* produces a pronounced increase in the somatic cell count in cows suffering from acute mastitis, in order to rapidly decline the somatic cell count after infection occurs [80, 81]. Unlike infection caused by contagious pathogens, cows have high somatic cell counts with a significantly high proportion of neutrophils [82]. The individual somatic cell count of milk and in the milk collection tank is a basic indicator of the level of mastitis in the dairy herd and health of the mammary gland of the cow [83].

The phagocytosis activity intervenes in cellular resistance and the modulation of glandular inflammation limiting the development of intraglandular mammary infection in the different stages of production of dairy cows [84]. *S. aureus* mammary gland infection in dairy cattle is important in the development of mastitis and epidemiological health risk to the population in animal and human. The phenotypic variation and genetic variability of strains of *S. aureu*s allows the expression of a greater pathogenicity potential of bacteria for the host, depending on the conditions of resistance and immunity of the gland mammary [85]. The infection by *S. aureus* is determined by the conditions of herd management and hygiene, due to the absence of measures of prevention and control of glandular disease. Intramammary infection by *S. aureus* causes a drastic reduction in production and deterioration of milk quality [86].

The infection by *S. aureus* in dairy herds develops from the contamination of the udder skin and the subsequent bacterial colonization of the nipple, which are determinants in the development of intramammary infection when proliferating the *S. aureus* in the glandular alveolus [87]. The colonization of the udder skin in cows before calving increases the risk of postpartum intramammary infection, when the level of infection in the herd by *S. aureus* is high. The production environment and the body sites of the animals are a source of infection of the agent from chronically infected cows.

The occurrence of *S. aureus* mastitis in cows increases the possibility of infection to the human population [88] through milk and unpasteurized dairy products. The pathogenicity of strains of *S. aureus* related to virulence factors are considered primary in the development of mammary infection. The production of the toxins shows a cytotoxic activity responsible for the cases of *S. aureus* mastitis. The biotypes A and C in the strains evaluated of *S. aureus* indicate their possible human and bovine origin, in which it suggests the possibility of cross infection of cow-man, extending the range of interspecies infection [89]. In isolates of *S. aureus*, the association of α and β toxins increases the cytotoxic and leukocidal capacity on neutrophils of the mammary gland, favoring the development and persistence of glandular infection [90]. The association of α hemolysin in the biotypes A and C manifests a potential risk to human health due to exotic strains of *S. aureus* of bovine origin. The capsular exopolysaccharide of *S. aureus* is responsible for interfering with phagocytosis and complement activation [67]. The capsular types of *S. aureus* predominant in dairy herds were of capsular serotypes 5 and 8. They show substantial differences in surface proteins and their ability to bind lactoferrin,

fibrinogen, fibronectin, and IgG in isolates of *S. aureus*. Different studies confirm the importance of serotypes 5 and 8 of *S. aureus* in the epidemiology of mastitis in dairy cattle [91]. The increasing occurrence of *S. aureus* strains carrying the R^+ factor to antibiotics modifies the response to antibiotic therapy. Antibiotic resistance and multidrug resistance frequently occur in isolates of *S. aureus* in livestock farms when antibiotics are used indiscriminately in drug therapy. Isolates of *S. aureus* showed resistance to β-lactam antibiotics associated with the production of β-lactamase and, to a lesser extent, related to ORSA/MRSA antibiotypes [92]. Since there is intraglandular infection due to *S. aureus*, dissemination may occur in cows and human. The mec A gene in the ORSA/MRSA phenotypes confirms a low proportion of MRSA strains in dairy cattle in family production herds. The results are similar with the low frequency reported in studies conducted in dairy herds from other countries. It is possible that human infection can occur through the consumption of dairy products contaminated with strains of animal origin and by the management of animals carrying ORSA/MRSA strains. The phenotypic expression of the virulence factors of *S. aureus* establishes additional risk conditions in the population for MRSA strains of an epidemic nature, related to their geographical distribution and genetic variability.

The MRSA strains identified in the study may be of the epidemic type (EMRSA), when related to the production of the α-toxin, which is considered a predictive marker of the virulence of *S. aureus* in EMRSA strains. The PCR amplification products demonstrated the nuc gene of *S. aureus*-specific thermonuclease in the strains evaluated. The detection of the genetic determinant of the mec A gene in the ORSA/MRSA phenotype allowed the identification of MRSA strains. The study confirms that the ORSA/MRSA phenotype carries the mec A gene that characterizes MRSA strains. The ORSA/MRSA phenotype also included the ORSA strains, which are considered sensitive methicillin (MSSA), which may include strains of β-lactamase producers called Border line that suggest a heterogeneous resistance. The use of the PCR allowed distinguishing the ORSA/MRSA isolates, the presence of MRSA strains. The procedure can be useful for the diagnosing and monitoring MRSA infection in dairy cattle [35, 93].

The case of bovine mastitis is a multifactorial disease in which several predisposing factors are identified, such as the stage of lactation, the number of births, the time of year, milking hygiene, the size, and technological level of the herd [94]. In the production environment, the presentation of subclinical mastitis is accentuated in the larger dairy herds compared with those of smaller size [95–97]. The monitory of the somatic cells in milk is an indicator of the inflammatory response of the mammary gland, and under stress, it suggests a condition of immunosuppression in cows [98, 99]. In the presence of mastitis, milk production and quality are affected by the disease presenting physicochemical and cellular alterations. When the somatic cells of total bacteria in milk increases and at the same time as it deteriorates the sanitary quality of the milk [100, 101], the poor milk quality contributes to the deterioration of the dairy products in the industrialization and increases the risk to the health of the consumers [102]. The physical-chemical alterations of milk are associated with the inflammatory reaction of the mammary gland, due to the increase in the number of leukocytes and the presence of enzymes and bacterial inhibitors that are incorporated into milk, as well as some components of blood plasma [103, 104]. The components of blood plasma contain proteases and lipases, which accelerate the decomposition of milk fat and proteins [105–107]. The increase in the number of somatic cells is related to the increase of proteins and nitrogenous substances in milk [108, 109]. The concentration of α-lactalbumin and β-lactoglobulin in milk serum decreases substantially [110]. The concentration of lactose decreases to maintain the ionic balance and the osmotic pressure of the milk,

thereby producing a variation of the mineral profile of the milk [111]. When this changes occurred, the thermotolerance of milk is reduced [112]. The glandular inflammation decreases the synthesis of casein, consequently decreasing the content of Zn, Ca, and P bound to the casein; the presence of blood serum in milk provokes an increase of the Cu, Fe, and Mn being united to serum albumin and ceruloplasmin, lactoferrin and transferrin [113, 114].

The milk pH increases from 6.6 to 7.0, due to the presence of bicarbonate, without affecting the titratable acidity and its buffer capacity and electrical conductivity, and increasing the freezing point of milk [110]. When the riboflavin and ascorbic acid concentration decreases in milk, it affects the fermentation and acidification capacity in dairy production [115]. There are many intrinsic and extrinsic factors that affect the quality of milk in the presence of mastitis, which is why a health problem is currently considered.

5. Conclusion

The *S. aureus* infection is prevalent in dairies of family production and associated to the expression of virulence factors that characterize the predominant *S. aureus* pathotypes in dairy cows in the Toluca Valley in México central region. Phenotypes and genotypes of *S. aureus* associated with the production of toxins, capsular exopolysaccharide, and MRSA strains, to establish a potential risk to health, are highlighted. The *S. aureus* infection level in the cow herds can be compromised with food safety and establishing animal and human health risk.

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Chapter 5

Quality and Safety of Bovine Raw Milk: Present Challenges and Technological Solutions

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Abstract

The dairy, as an essential agricultural activity, plays a vital role in global food production systems. Irrespective of the regions or countries, milk producers are confronted with several and sometimes contradictory challenges: achieving food security, reaching economic profitability while responding to sustainable development goals. The quality and safety of raw milk are essential for the manufacture of dairy products. For the preservation of raw milk, two options are proposed by the FAO: cold storage or the activated lactoperoxidase system. Worldwide, mostly farmers attempt to "produce" raw milk for which the bacterial content does not exceed 100,000 (10^5) cfu/ml: due to the particularities of milk production and handling and numerous contamination risks, it is difficult to always reach this goal. Our group has evaluated an N_2 gas flushing-based technology, as a supplementary or alternative hurdle to prevent bacterial development, such as to preserve the quality and safety of raw milk during its storage and transportation from farms to processing sites. We discuss here its potential compared to other options.

Keywords: food security and quality, raw milk, spoilage, bacteria, psychrotrophs, antibacterial, N_2 gas flushing

1. Introduction

Like other agricultural sectors presently challenged by environmental constraints, the dairy sector is also pushed to move towards environmental sustainability and is urged to change practices.

Despite alarming predictions, the OECD-FAO outlook for the period 2018–2027 projects an increase by 22% of the world milk production; India and Pakistan are expected to jointly account for 32% of the global milk production; for Europe, the estimations of global exports of dairy commodities are in favour of 27–29% increase for the same time period [1]. In Africa, the consumption of milk and dairy products is also expected to increase due to the population and urbanisation increase and due to economic development [2].

If processed products, like cheese or butter, still dominate the consumption of dairy products in the developed world, fresh dairy products are mostly preferred in developing regions [1].

Dairy farms around the world still show a highly contrasted picture: milk is either produced in small holder farms, where it is mainly served for family

consumption, or in large modern dairy farms equipped with rotary milking parlour or milking robots designed for up to thousands of cows.

However, irrespective of the type of farming or the local environmental constraints, raw milk as a particularly rich media is highly perishable. The quality of raw milk largely determines the quality of products manufactured at the dairy; but, milk also constitutes a health issue if consumed raw, especially.

2. Composition, physico-chemical and antimicrobial properties of bovine raw milk

Milk is synthetised in the secretory cells of the alveolar epithelium (also called alveolar cells) and further secreted into the lumen, the core of the alveolus. Alveoli, the functional units of milk synthesis and secretion, are spherical bodies found only in the mammary gland, the unique organ of the mammals. The four mammary glands of the cow (female bovine) form the single anatomic unit called udder.

Milk is the first and essential food for the newborn of the mammal, and accordingly, milk needs to fulfil all its *nutritional* needs. These needs are varying among mammalian species and consequently, the composition of milk varies considerably in carbohydrate (mainly milk sugar called lactose, as energy source), protein (source of amino acids for protein synthesis), lipid (energy source and membrane components) and mineral contents (**Table 1**). For example, bovine (cow) milk has lower lactose and higher protein contents than human (mother's) milk. In addition, the milk composition varies during the lactation period and especially, during the first days that follow calving.

The nutritional properties are not only the important characteristics of milk, but additional *protective* and *regulatory* functions are of importance, too. The protective functions are related to the survival of the newborn in the presence of various environmental microbes. Bovine milk contains antimicrobial elements like leucocytes (somatic cells), immunoglobulins, lactoperoxidase, antiadhesive glycoconjugates (oligosaccharides linked to lipids and proteins) of milk fat globule membrane (MFGM) and sialic acid residues of oligosaccharides (**Table 1**). The protective functions also may include particular prebiotics like amino sugars of the oligosaccharides that contribute to establish the optimal microbiome in the gastro-intestinal track of the newborn. The milk components having regulatory functions include hormones (e.g. insulin, somatotropin, and growth hormone), regulatory proteins (e.g. cytokines), particular bioactive lipids, and membrane-enclosed extracellular vesicles (EV) like exosomes containing bioactive miRNAs and proteins [3, 4].

All these intrinsic components of milk are of crucial importance for the growth and development of the newborn. The balance and the spectrum of the milk components are unique for each mammalian species. Because the cow (*Bos taurus*) is globally and economically the most important dairy husbandry animal, the knowledge on the bovine raw milk has the most significant impact on the dairy industry.

2.1 Sources of microbial contaminations in fresh raw milk

Raw milk is widely considered as sterile in the lumen of the alveolus in the case of a healthy cow; bacteria may be however transmitted to milk via the cow's blood in case of systemic infection. The intrinsic features of raw milk and its handling favour the presence and growth of many microbes; consequently, various viruses, moulds, yeasts, and especially bacteria take advantage of raw milk production conditions to either persist or proliferate. Bacteria by exhibiting contrasted roles in raw milk can be truly categorised as "good, bad or ugly": for ages, some are key

Principal components	Relative amounts
Water	87% (79% free, 8% bound in casein micelles)
Carbohydrates	**4.6%**
Lactose	4.6
Oligosaccharides	Traces
Lipids	**4.1%**
Neutral lipids	4.0
Polar lipids	0.05
Proteins	**3.3%**
Caseins	2.6
Whey proteins	0.6
MFGM-associated proteins	0.07
Minerals	**0.7%**
K	0.15
Ca	0.13
Cl	0.11
Phosphates	0.09
Na	0.04
Other organic compounds	**0.32%**
Citrate/citric acid	160 mg per 100 g
NPN (non-protein nitrogen)	60 mg per 100 g
Gases	
CO_2/bicarbonate	10 mg per 100 g
N_2	1.6 mg per 100 g
O_2 (fresh-cold stored)	0.15–0.6 mg per 100 g
Major redox systems	
Dehydro/ascorbic acid (C vitamin)	2.0 mg per 100 g (E_0 + 0.07 V)
Riboflavin (B2 vitamin)	0.2 mg per 100 g (E_0–0.20 V)
Milk redox potential:	
Fresh raw milk (air free)	E_h + 0.05 V
Cold stored raw milk (air dissolved)	E_h + 0.20 – +0.30 V
Major antimicrobial elements	
Leucocytes (somatic cells)	Average 150,000 cells per ml
Immunoglobulins	0.08% (77% IgG, 17% IgA, 6% IgM)
Lactoperoxidase (with SCN^- and H_2O_2)	
MFGM-associated glycoconjugates[b, c]	
Sialic acid residues of oligosaccharides[c]	

[a]*Most presented data is collected from [5].*
[b]*Ref. [6].*
[c]*Ref. [7].*

Table 1.
Some characteristics of bovine raw milk[a].

agents in the manufacture of numerous milk-based products reflecting traditions and cultures around the world; others are involved in the spoilage of raw milk and dairy products; finally, some are authentic pathogens causing severe illnesses, which have largely contributed to build the reputation that raw milk is a vehicle for spreading diseases.

Depending on the ambient conditions, the farming practices or the health of the animals, various contamination sources raise the bacterial load in raw milk. The sources of bacterial contaminations can be categorised as such:

i. The cow's udder: the teat surface can present a quite highly diverse bacterial population. Milk ducts of the udder carry epithelium-adhering commensals (like streptococci, staphylococci and micrococci), and possibly pathogens from with and within the udder. Three categories of bacterial pathogens can be distinguished: human pathogens, such as Mycobacteria or *Brucella*, which originate from within or outside the udder; others like *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Escherichia coli*, causing bovine mastitis, can also enter raw milk; finally, direct or indirect faecal contaminations lead to the addition of *Salmonella* or *Campylobacter* to raw milk.

ii. The air and soil: these factors impact differently on whether the animals are fed in- or out-doors.

iii. The farmer or the milk handler: the absence of good hygiene practices favours the distribution of contaminants and their entrance in milk. The level of contaminations promoted by a farmer or a milk handler depends on how close is the contact with raw milk: this aspect is seriously considered that as some carriers of *Salmonella* are prevented to handle milk in dairies.

iv. Water and beddings: when employed for milk production, water should be of high microbiological quality as either pathogenic or saprophytic bacteria present in water can contaminate raw milk. Several reports highlighted the crucial role of water as a major source of pseudomonal raw milk contamination [8–10]. Beddings materials may, especially at winter, carry high bacterial loads (10^8–10^{10} cfu/g), which may contaminate teats [11].

v. The dairy equipment: in the case of insufficient cleaning and disinfection, milk cans, tanks, milking machines, and pipelines constitute major contamination sources [11]. The design of certain components presenting "dead ends" constitutes ideal shelters for the settlement of biofilms: milk residues aggregate with bacteria in difficult to clean areas, detach time after time from the surface and hence raise the bacterial load in raw milk.

Due to numerous contamination sources, a rather diverse microbiota is present in raw milk; many Gram (+) or Gram (−) bacterial representatives can be present in significant numbers; their relative importance is variable and greatly depends on the elapsed time since milking. Gram (+) dominates in fresh raw milk, whereas Gram (−) takes over after cold storage [11]. If it is possible to relate high levels of coliforms to faecal contaminations, it may be more difficult to trace the source of the ubiquitous pseudomonads.

Many reports often highlight multiple, sometimes similar, contamination sources.

For example, in Brazil, the difficulties for farmers to fulfil the goals set by the Ministry of Agriculture were attributed to several causes: at first, most dairy farms

had their water contaminated with coliforms [12]. A water of poor quality combined to poor hygienic conditions (for example, in the case of insufficient cleaning of tanks) raise the contamination of raw milk; the authors also mentioned insufficient training of the farmers, which resulted in a not regular cleaning of the udders before and after milking; moreover, a majority of farmers did not systematically control mastitis in their animals [12].

In Tanzania, which belongs to the East African Community Countries (EACC) organisation, two bacteriological criteria were defined for total bacterial counts (TBCs) and for total coliform counts (TCCs) [13]: grade I, II and III raw milk is characterised by total bacterial counts below 2.10^5, between 2.10^5 and 10^6, and between 10^6 and 2.10^6 cfu/ml, respectively; raw milk of very good and good quality is characterised by coliform counts below 10^3, and between 10^3 and 5.10^4 cfu/ml, respectively. Three major factors, that impacted milk quality and caused milk-borne diseases, were identified: the doubtful health status of animals, the lack of good milking and handling practices, and the distribution, which occurs out of relevant regulations [2].

In Western African countries (Burkina Fasso, Mali, and Senegal), a campaign entitled "My milk is local" aims to replace the large consumption of imported milk powders and urges local farmers to respond to increasing demands. Producers, mainly organised as small holders, suffer to meet the challenge of producing raw milk of sufficient microbiological quality. A study reported that over 75% of raw milk samples exhibited excessive bacterial counts, with an average of 4.5×10^7 cfu/g of raw milk; the poor microbiological quality of raw milk at the farm level was due to contaminations resulting from a lack of adequate equipment and facilities, of good hygiene practices along the collection and processing steps [14].

Raw milk is frequently identified as a source of food-borne disease outbreaks. However, the consumption of raw milk continues in low-income countries because of traditions and lack of processing facilities; in high-income countries, the consumption of raw milk is encouraged by certain lobbies and life style groups for health benefit claims that vary from superior nutritional properties, lower allergenicity, reduced lactose intolerance or more efficient antimicrobial systems. In practice, the use of raw milk remains marginal, and fortunately, the consumption of milk mostly relies on processed dairy products.

3. Bacteriological quality criteria for industrial processes

The importance of bacteria in raw milk, as in other food products, is reflected by the fact that the microbiological quality criterion is a "bacteriological criteria".

Preserving and controlling the quality of raw milk is a worldwide concern and is reflected by a "common" criteria of total bacterial counts (TBCs) around 100,000 (10^5) cfu/ml (**Figure 1**).

In the European Union, the directive No 853/2004 defines "Raw milk" as the secretion of the mammary gland of farmed animals that has not been subjected to temperature above 40°C, or undergone "any treatment that has an equivalent effect" [15]. The directive also indicates that the milk, must be "cooled immediately" to not more than 8°C in case of daily collection, or not more than 6°C, if the collection does not occur daily. For cow milk, the bacteriological standard should be lower or equal to 100,000 cfu/ml (determined from the rolling geometric average over a 2-month period with at least two samples per month); for food business operators, the bacteriological level should be below or at 300,000 cfu/ml (**Figure 1**).

In Finland, for example, regarding the criteria for raw milk quality, three classes are defined depending on the bacterial load at farm level: E (excellent), I (first

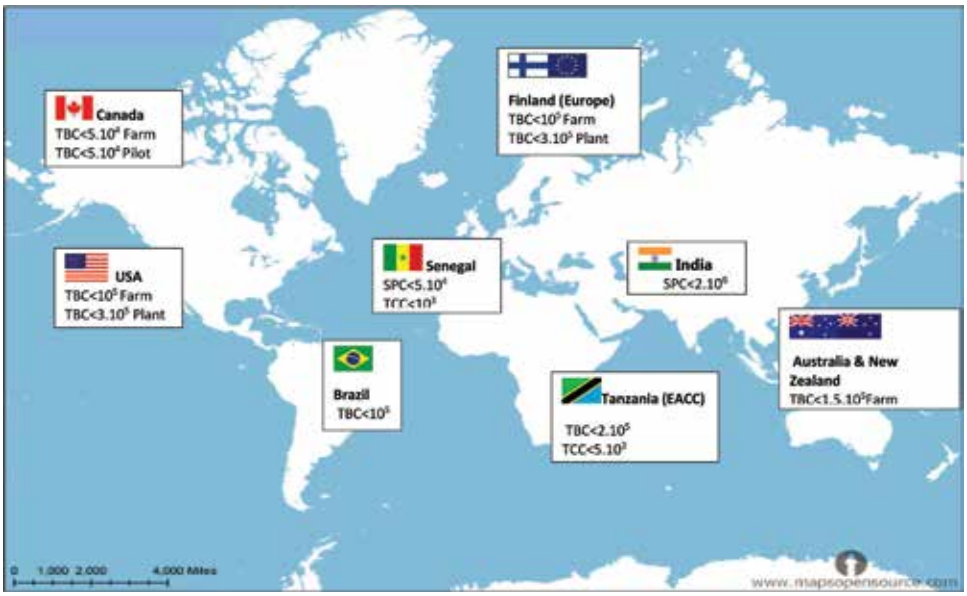

Figure 1.
The about 10^5 cfu/ml "Total bacterial counts in raw milk" world challenge [12–15, 26, 27]. Note: TBC, total bacteria counts; TCC, total coliform counts; SPC, Standard Plate Count.

class) and II (non-acceptable for dairy processes), for bacterial counts <50,000, ranging between 50 and 100,000, and exceeding 100,000 cfu/ml, respectively. In 2017, the bacterial content was on average of 5200 cfu/ml (equivalent to 3.71 $\log_{10}$ units) at farm level [16].

The microbiological criteria rely on a culture-based reference method, SPC, which accounts for Standard Plate Count agar method. Total colony forming units of bacteria, eventual yeast and moulds are determined per ml or g of milk, after 32°C for 48 h or 30°C for 72 h incubation: the so-called pour-method is recommended for the analyses [17]. Concerning bacteria, particularly viable mesophiles, aerobes and facultative anaerobes can be enumerated.

As for any method of analyses, drawbacks exist concerning the SPC method:

- The method is time-consuming as the bacterial load is only revealed after 2–3 days incubation (to overcome the time obstacle, the dairy industry uses a particle counting-based method).

- The results are method-dependent: as observed with raw milk samples, the spread-method (another common method employed for microbiological analyses) yields a slightly higher amount of colonies despite the fact that the pour-method supports growth on and within the agar; based on some analyses of raw milk samples, the spread-method yielded an average of 0.17 log unit more bacterial colonies than the pour-method ([18] and unpublished data): for example, if the pour-method would yield 1000 bacteria/ml, the spread-method would enable to enumerate 1500 bacteria/ml, for the same raw milk sample.

- The analyses may lead to underestimated bacterial contents as a large fraction of bacteria are not cultivable [19], and common bacteria can enter a viable but not cultivable (VBNC) stage [20].

- The plating result does not inform about the presence of pathogens.

- The SPC method also ignores important bacterial groups, when considering further treatments to which raw milk is subjected: the level of Gram (+) bacterial types, such as *Bacillus*, *Paenibacillus*, and *Clostridium* present as spores in raw milk, can only be revealed after a preliminary heat treatment of the raw milk samples. Hence, key spoilage bacteria that limit the shelf life of HTST milk [21, 22] are not considered by SPC.

Recently, high-throughput DNA sequencing or molecular barcoding approaches, as non-culture-based methods, were also applied to raw milk, and allowed a more accurate estimation of microbial/bacterial diversity in samples [23, 24].

However, the difficulties and costs for sampling and testing food materials also apply to raw milk testing: in USA, it was already estimated that one analysis of a cost of 5 $ (US dollars) would result in an annual cost of 150 million $, if performed daily at the farm level; similarly, if every milk tanker would be tested at the processing plant for one microbial agent, this would lead to a cost of 21 millions $ [25]. With such costs, it seems impossible to identify all risks for raw milk.

4. Effects of the storage time and temperature on bacterial growth in raw milk

4.1 Importance of low temperature

Irrespective the ecosystem, the temperature is a key determining factor of bacterial growth. Low temperature was implemented to preserve the quality of raw milk until the processing stage. When the number of dairy plants was reduced, the raw milk had to be transported over longer distances from farms to dairies. Longer distances result in increased time that elapses between milking and processing stage.

Milk, which leaves the udder, is at a temperature of about 35°C: a study that evaluated the impact of the storage temperature on bacterial growth in raw milk showed that high temperatures promoted rapid and intense bacterial growth. Importantly, the study also illustrated the "time limited effect" of cold storage at 5°C, as the bacterial growth was only inhibited for 36 h, after which a moderate increase was noticed [28]. Some reports mention "the critical age" (the time after which bacterial growth is observed) to be slightly above 48 h [29]: these variations may reflect differences in initial bacterial levels, in bacterial diversity, or in variable levels of the natural antimicrobial systems present in raw milk.

The temperature value, even at low temperature range, is of crucial importance: an illustration can be seen with the 12 raw milk samples considered in experiments I and II, listed in Table 1 [30]; for I and II, the initial average counts in log-units were 3.9 and 4.03, respectively; it can be observed that 4 days cold storage at 4 and 6°C, respectively, yielded bacterial counts of 6.4 and 7.8 log-units, respectively; the 2° shift showed an about 1.4 log-units (equivalent to a factor of 25) higher bacterial level at 6 compared to 4°C. A 2 log-units (a factor of 100) difference in psychrotrophic *Pseudomonas* levels was also reported by another study that compared optimal (4°C) and suboptimal (6°C) storage temperatures [31].

4.2 Consequences of cold storage

At bacterial population level, cold storage results in the replacement of Gram (+) by Gram (−) bacteria [11]. DGGE-based studies first highlighted that bacterial diversity in raw milk decreased during cold storage [32–34]. That cold storage-impacted bacterial diversity in raw milk was also evidenced by the determination of the amount of

Operational Taxonomic Units (OTUs) in initial and cold-stored raw milk samples: for example, after 3–4 days at 6°C, only 33% of the initial OTUs were recovered in our studies and some bacterial types did not survive the low-temperature storage condition [24].

5. Spoilage features of psychrotrophs

The reputation of psychrotrophs as key spoiling bacteria in cold-stored raw milk is extensively documented, due to their production of various enzymes, which can degrade the major milk constituents. The heat treatments, such as pasteurisation or UHT, which target bacterial populations do not affect much the hydrolytic enzymes, characterised by remarkable heat stability. Recently, the heat stability of proteases, lipases and phospholipases from selected raw milk isolates was determined in one study: after 142°C for 4 s, *Acinetobacter* frequently showed remaining lipase and phospholipase activities, whereas *Pseudomonas* exhibited highest protease activities [35].

Consequently, the spoilage is not limited to raw milk but occurs also at the level of milk-derived products: various defects or technological failures were linked to enzymatic activities [36]. Psychrotrophs and their enzymes significantly impact the quality of dairy products, which implies economic consequences.

If proteases and lipases received major attention, less studies describe other enzymatic types; however, the production of phospholipase C (PLC) was evidenced for key spoiling genera such as *Pseudomonas* and *Bacillus*. PLC causes the disruption of the milk fat globule membrane (MFGM) and is also described as a heat stable enzyme [35–37]. In our recent investigations concerning phospholipids, by a lipidomics-based approach, we also observed that PLC production was a common feature of psychrotrophic bacteria; but, consequent to bacterial growth in raw milk during its cold storage, we also evidenced the presence of various types of bacterial phospholipases that promoted hydrolysis of phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine species, together with an increase of phosphatidic acid; the changes imply the implication of phospholipases C, A, D and sphingomyelinase C activities, and show that phospholipolysis in raw milk involves many enzymatic types [38]. The analyses also revealed the presence of various lysophospholipids (LPLs) (resulting from PLA activity) in cold-stored raw milk: the fact that numerous reports described multiple physiological or pathophysiological roles of LPLs [39, 40] calls for further investigations on the significance of LPLs in cold-stored raw milk.

Like for the producing bacteria, only considered as problematic when associated to technological failures, the enzymes synthesised by these bacteria are also mainly considered under a technological point of view and to a lesser extent on their eventual impact on human health.

6. Antibiotic resistance of psychrotrophs

Earlier observations showed that bacterial isolates, retrieved from raw milk samples that apparently spent a longer time in cold storage, also exhibited higher antibiotic resistance (AR) or multi AR features [41]. We also observed that psychrotrophic bacterial populations are more risky in terms of AR compared to their corresponding mesophiles [30].

Recently, a study that evaluated the efficiency of the activated lactoperoxidase system (LPS) and N_2 gas flushing to hinder bacterial growth in raw milk showed that N_2 seemed to favour a more diverse bacterial community at 6°C, less heavily loaded with antibiotic multi-resistance features, compared to LPS [42].

Numerous reports pointed *Pseudomonas* and *Acinetobacter* as key genera associated with raw milk and spoilage of dairy products [11, 24, 35, 43–45]. The WHO, which considers that AR is nowadays one of the highest threats to global health, to food security and development, has ranked the bacterial species *Pseudomonas aeruginosa* and *Acinetobacter baumannii* as critical priorities regarding AR [46].

7. Methods to inhibit or inactivate raw milk-associated bacteria

Chemical (addition of CO_2, considered as safe), biochemical (the activated lactoperoxidase system, LPS) or physical (HHP, UHPH and LTP)-based treatments are presently in use or still under evaluation (**Table 2**).

LPS is recommended by the FAO, where economic or technical constraints prevent the use of cooling facilities: following the addition of SCN^- and H_2O_2, the shelf life of raw milk can be extended for 7–8 h under tropical conditions [47, 48].

Method/some features and applications		Advantages	Disadvantages	References
Activated LPS system	Addition of SCN^- and H_2O_2	• Raw milk is kept safe where no cold chain facilities exist	• Requests the addition of chemicals • Time-limited effect	[47, 48]
CO_2	Treatment of raw milk	• Inhibition of bacterial growth for 7 d at 4°C; no pathogen enriched		[49]
	Treatment of raw milk: impact on UHT milk	• Physicochemical and microbiological quality preserved • Proteolysis in UHT milk is delayed	• pH decrease (0.55 pH-unit difference between control and treatment)	[50]
High hydrostatic pressure (HHP) technology	• 100–1200 MPa	• Effective to inactivate microorganisms and food-borne pathogens	• Does not inactivate enzymes and spore	[51]
	• 300 MPa	• Coagulation properties of milk enhanced	• Cheese making properties altered	[52]
Ultra-high pressure homogenisation (UHPH)	• 100–300 MPa	• Equivalent to high pasteurisation 90°C/15 s • Shelf life of 14/18 d	• Physico-chemical changes	[53]
	• 400–600 MPa /800 MPa	• Inactivation of microorganisms and pathogens • Inactivation of enzymes	• Bacteria can recover • Increased level of FFA • Decrease of casein micelle size • Denaturation of whey proteins	[54]
Low temperature plasma (LTP) system	• Whole, semi and skimmed milk spiked with *E. coli*	• Reduction of bacteria by over 4 log-units (cfu/ml) within 20 min		[55]
	• 15 ml raw milk/ treatment for 20 min	• No change for the lipid composition	• Some changes with volatile compounds and increase of total aldehydes	[56]

Table 2.
Methods to tackle bacteria in raw milk.

8. A novel approach for raw milk storage: N_2 gas flushing technology

Two major observations were at the basis of the search and testing of a novel approach to better preserve the quality and safety of raw milk. In low-income countries, considerable amounts of milk are adulterated by the use of various chemicals including antibiotics to inhibit bacterial growth. On the other hand, in high-income countries, it is well known that psychrotrophs mainly considered as benign in their majority are causing significant spoilage of raw milk; our group also observed that these bacteria are heavily loaded with AR determinants.

N_2 gas, a non-finite resource and considered as chemically inert, was therefore tested at laboratory and pilot plant scales [57–59]. The so far established benefits, recorded from raw and pasteurised milk samples and from the treatment of some pure bacterial strains, are summarised in **Figure 2**.

For low- or high-income countries, the N_2 gas flushing technology presents indisputably multiple advantages considering bacteriological, biochemical, technological and nutritional aspects for the preservation of the quality and safety of raw milk.

The N_2 gas flushing technology has been recently granted a patent by the European Patent Office [63]. Future studies should consider the optimisation of the treatments and the completion of the further steps to render N_2 gas flushing technology as fully sustainable at large scale.

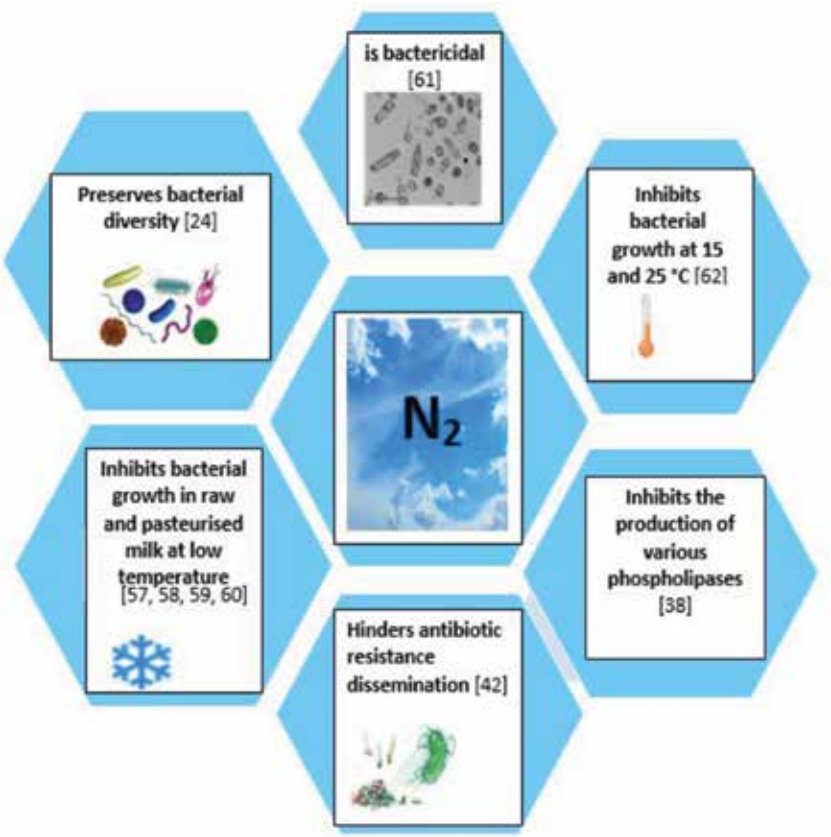

Figure 2.
Impact of N_2 flushing on raw and pasteurised milk-associated bacterial populations, and on some pure strains [24, 38, 42, 57–62].

9. Conclusions

More ancient and also recently conceived strategies that aim to reduce/eliminate bacterial populations in food materials including raw milk, are based on treatments, which are applied when the bacterial level reaches a certain threshold value: around 100,000 (10^5) cfu/ml for raw milk, which was first applied by countries having cold chain facilities; but nowadays, this threshold value is also targeted by many other countries. Depending on the production site, this goal may be difficult to reach.

The N_2 gas flushing of raw milk was initially conceived to be applied at earliest possible in farms until the processing site as an additional hurdle to cold storage, such as to preserve the initial microbiological, biochemical and nutritional features of raw milk along the cold chain of raw milk storage and transportation, before its transformation. The recent observation that N_2 gas flushing was about equivalently inhibitory of bacterial growth in raw milk at milder temperatures (15 and 25°C) compared to LPS [62], offers further perspectives for the method and especially as a replacement of numerous adulterating substances, including antibiotics, added to raw milk.

Strategies that aim to limit or control the bacterial load in raw milk should be designed to simultaneously dispel technological risks and consider human health risks: in a world that struggles with superbugs, it is reasonable to constantly evaluate practices on whether they respond at best to global challenges and interests.

N_2 gas that constitutes 78% of our atmosphere is an unlimited resource. The N_2 gas flushing technology, designed for an "open system" and successfully tested at pilot plant scale, when finalised, would simply "borrow" the gas from the atmosphere. By considering the *Sustainable Development Goals* set up by the United Nations (Agenda 2030) [64], the N_2-based treatment contributes to the achievement of several objectives: by tackling food spoilage, there are perspectives to reduce poverty, improve food security and nutrition, ensure better health, while promoting sustainable economic growth.

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Chapter 6

Analysis of Additives in Milk Powders with SPE-HPLC or 2D-HPLC Method

Xiaofang Hou, Jing Ma, Liang Chen, Xiaoshuang He and Sicen Wang

Abstract

Dairy products are beneficial to human health, especially for formula-fed newborns. According to the regulation of FDA and China national food safety standard, food additives such as benzoic acid, sorbic acid, natamycin, lysozyme, saccharin sodium, and aspartame are not permitted to be added to milk powder. So, the establishment of accurate and convenient methods for the analysis of these food additives in milk powder is critical to people's health. For the reason of the complex matrix of infant milk powders, we compared six sample pretreatment methods (liquid-liquid extraction, organic precipitation, heavy precipitation, and three different solid-phase extraction (SPE) methods (C18, HLB, MAX)) from recovery, easy operation, time cost, and organic solvent usage aspects. Finally, Poly-Sery HLB cartridge was confirmed as the most appropriate material for its high recovery and time cost merits. We are also introducing two-dimensional liquid chromatography (2DLC) method for the simultaneous determination of five major proteins and seven food additives in milk powders. Optimization of switching mode, choice of columns, mobile phase, and flow speed was discussed. We also compared limit of detection (LOD), recovery, and sample treatment with the results of high-performance liquid chromatography (HPLC). Results show that 2DLC is simpler, faster, and more accurate than the HPLC method.

Keywords: additives, milk powders, sample treatment, 2DLC, proteins

1. Introduction

Dairy products are beneficial to human health, especially for formula-fed newborns. India and the European Union both produced around 160 Mt milk in 2016 [1]. People are very concerned about the nutritional ingredients and illegal additives of the milk products. As we all know, bovine milk includes 80% caseins (CN) and 20% whey proteins. Caseins consist of α_{s1}-CN, β-CN, α_{s2}-CN, and κ-CN in an approximate 4:4:1:1 weight ratio. Whey proteins mainly consist of β-lactoglobulin (β-LgA, β-LgB) and α-lactalbumin (α-Lac) in a 3:1 weight ratio [2]. The quantity of milk fraction proteins is related to the development of the baby. As many literatures reported, preservatives and artificial sweeteners may be harmful to people's health [3–5].

According to the regulation of FDA and China national food safety standard, food additives such as benzoic acid, sorbic acid, natamycin, lysozyme, saccharin sodium, and aspartame are not permitted to be added to milk powder. So, establishment of accurate and convenient methods for the analysis of these food additives in milk powder is critical to people's health.

Here, I'll introduce two works of our groups to readers: the comparison of six sample preparation methods for the analysis of four preservatives and two artificial sweeteners in milk powders [6] and two-dimensional liquid chromatography (2DLC) for determination of five major proteins and seven additives in milk powders.

2. Comparison of six sample preparation methods for analysis of four preservatives and two artificial sweeteners in milk powders

It is not very easy to determine the trace residues or contaminants in infant milk powder for its complex matrix [7]. So, the sample pretreatment is the key step in the whole analytical procedures. According to the literatures, there are two kinds of sample preparation methods for analysis of contaminants in dairy products. One is to extract the targeted analytes, and the other is to remove the interferents. Usually, solid-phase extraction had been widely used as a milk sample preparation method. Sometimes, liquid-liquid extraction followed by a SPE cleanup step was served to remove the macromolecular protein prior to determination of the target analytes [8]. Removal of the protein in milk could be done by precipitating them with heavy metallic salt [9] or sodium tungstate [10].

Our group developed six sample preparation methods based on the literatures, that is, liquid-liquid extraction, organic precipitation, heavy precipitation, and three different solid-phase extraction methods (C18, HLB, MAX). In order to obtain the higher recovery and reduce the time cost and organic solvent dosage, the six different sample preparation methods were compared.

2.1 Sample preparation and extraction

2.1.1 Method A (liquid-liquid extraction)

This method is based on the study of preservatives in cheeses [11]. Around 2.0 g milk powder was mixed with 4.0 mL of deionized water (60°C). After 10 min of ultrasonication, 5.0 mL of ethyl acetate and 1.0 mL 10 mmol L^{-1} of formic acid were added. The samples were extracted for 40 min on a rotary mixer at 400 rpm. After that, they were centrifuged for 5 min at 3200 rpm. The supernatant was transferred to another tube, and the sediment was extracted with 5.0 mL of ethyl acetate once again. The second supernatant obtained was combined with the one from the first extraction. Then they were filtered and evaporated to dryness at ambient temperature. The residues were dissolved in 500 μL mixture solution (0.1 M acetate buffer: methanol = 2:1, v/v) and vortexed for 20 s.

2.1.2 Method B (precipitation based on sodium tungstate)

This method is based on the study of five macrolide antibiotics in milk [10]. The sample dissolving steps were the same as method A (liquid-liquid extraction). After 10 min of ultrasonication, this solution was centrifuged at 6000 rpm for 15 min. The defatted milk was transferred to a new centrifuge tube, and then 1.0 mL 10% sulfuric acid and 5.0 mL 10% sodium tungstate solutions were added. The resulting

solution was vigorously shaken for 2 min and diluted to 10 mL with water and centrifuged at 4000 rpm for 10 min. The supernatant was filtered and evaporated to dryness at ambient temperature. The residues were dissolved in 500 μL mixture solution (0.1 M acetate buffer:methanol = 2:1, v/v) and vortexed for 20 s.

2.1.3 Method C (precipitation based on potassium ferrocyanide)

This method is based on the detection of adulteration of milk with soy milk [9]. The protocols of method C were the same as method B, only except the precipitants of 3.0 mL 0.085 mol L^{-1} $K_4[Fe(CN)_6]$ and 3.0 mL 0.25 mol L^{-1} $ZnSO_4$ solutions employed in this method.

2.1.4 Method D: F (solid-phase extraction)

This method is based on the study of fluoroquinolones in milk [12] and determination of 20 pharmacologically active substances in various milk samples [13]. After 10 min of ultrasonication, 7.0 mL 1% trichloroacetic acid (TCA) and 3.0 mL acetonitrile were added. The resulting solution was vigorously shaken for 2 min and centrifuged at 4000 rpm for 10 min. The supernatant obtained extracted by SPE. Three kinds of cartridges (CNWBOND LC-C18 SPE, Poly-Sery HLB SPE, and Poly-Sery MAX SPE) were examined. The SPE protocol on three types of cartridges was consisted of the following steps: (1) activation of the cartridges with 3 mL methanol first and then conditioned with 3 mL deionized water, (2) sample loading, and (3) sample elution with 3 mL 20 mmol L^{-1} ammonium sulfate and 3 mL 80% acetonitrile. After the eluates were filtered, the steps of evaporation and residues dissolving were the same as method A (liquid-liquid extraction).

2.2 Comparison of sample preparation methods

For method A (liquid-liquid extraction), the extraction time of 10, 15, 20, 25, 30, 35, 40, 45, and 50 min was investigated. The results are illustrated in **Figure 1**, indicating 40 min was the best choice. The different extraction temperatures from 25 to 40°C were tested, and the results showed that there were no obvious differences in recoveries of each preservative. For method B (precipitation based on sodium tungstate) and method C (precipitation based on potassium ferrocyanide), the results showed that there were no significant differences between these two methods. The recoveries of benzoic acid, sorbic acid, and saccharin sodium were more than 80%. However, the recovery of lysozyme (<30%) indicated that both of the precipitate-based methods were not suitable for enzyme [14] and it might be coprecipitated with the proteins in milk powder. For method D-F (SPE), three kinds of cartridges (CNWBOND LC-C18 SPE, Poly-Sery HLB SPE, and Poly-Sery MAX SPE) and their elution solvents applied were investigated. Porous silica particles surface bonded with C18 was the most commonly used sorbents. Poly-Sery HLB is the kind of polymeric sorbents that was reported as being superior to the silica-based C18. The major difference between the HLB and MAX sorbents is the presence of the anion-exchange groups that provide high selectivity for acidic compounds. So, the three kinds of cartridges were tested to evaluate their applicability. Four elution solvents with different polarity were tested: 70% acetonitrile, 80% acetonitrile, 90% acetonitrile, and 100% acetonitrile. The recoveries of each food additive showed that Poly-Sery HLB with 80% acetonitrile provided the best results.

Figure 2 shows the recovery results of each food additive obtained by the six different abovementioned methods, respectively. Each sample was analyzed in triplicate. Both method A (liquid-liquid extraction) and method D (Poly-Sery HLB SPE)

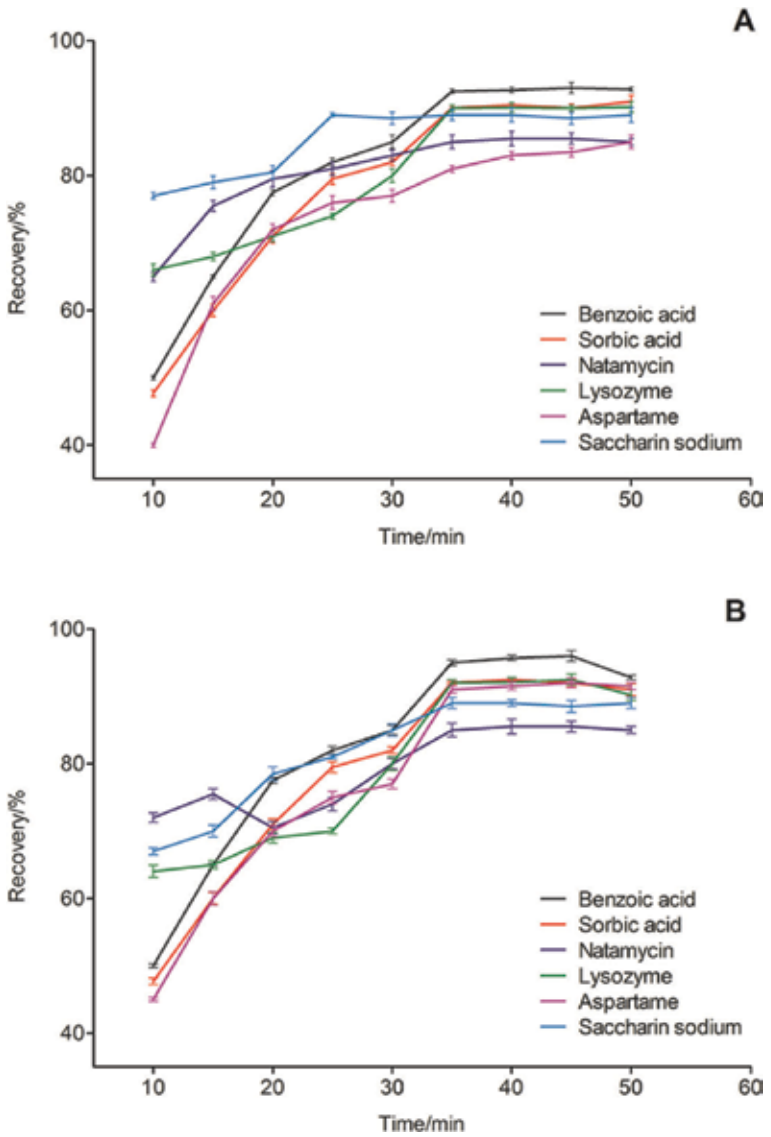


Figure 1.
Effects of extraction time on the six food additives recovery obtained with method A in the two milk samples (n = 3). (A) Whole milk powder and (B) skimmed milk powder (SMP). The six food additives: benzoic acid, sorbic acid, natamycin, lysozyme, aspartame, and saccharin sodium.

have good recoveries (>80%) for all six analytes, but results of our study showed that the average RSD% for method A of the six analytes were all between 3.7 and 5.4%, which is more than that of HLB SPE method (2.3–3.8%). Considering the environmental and economic costs, method D (Poly-Sery HLB SPE) was employed in our further study.

2.3 SPE (Poly-Sery HLB)-HPLC-DAD method validation

The proposed SPE (Poly-Sery HLB)-HPLC-DAD method was validated in terms of linearity, limit of detection, limits of quantity (LOQ), within- and between-day precision, and accuracy. **Figure 3** shows the chromatograms of the mixed standard solutions. We can see that the six preservatives and sweeteners were

baseline separated and had a good resolution (R ≥ 2.6) under the chromatographic condition.

The linearity of the method was evaluated under 210 nm with the mixed standard solutions, pooled whole milk powder matrices,and pooled skimmed milk powder matrices. The LODs and LOQs in each case were estimated based on S/N = 3 and 10, respectively. The results including the regression equations, the linear ranges, and regression coefficients are summarized in **Table 1**.

The precision and accuracy were tested in two milk powder matrices, that is, the whole milk powder and the skimmed milk powder. **Figures 4** and **5** showed the

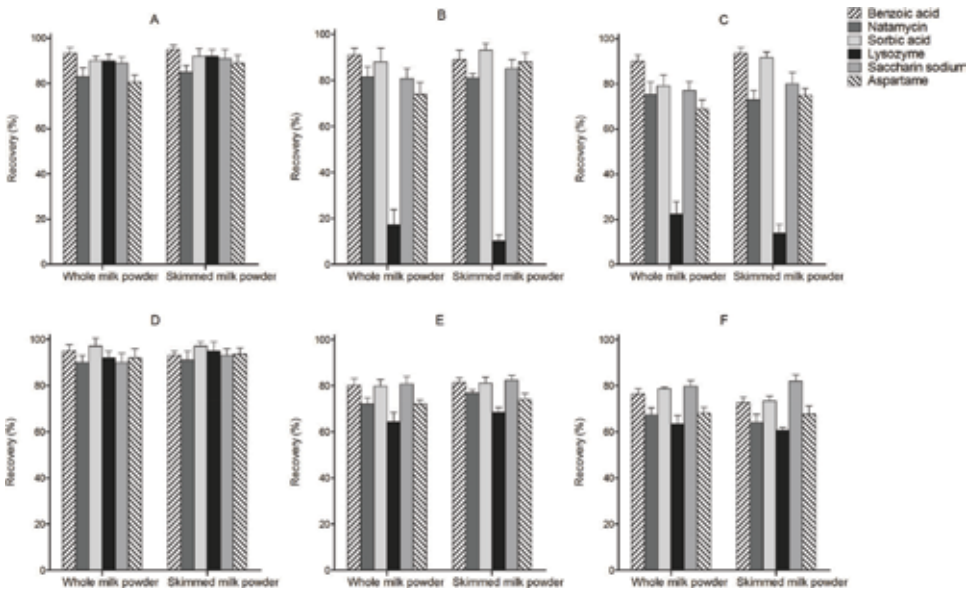


Figure 2.
Recovery results of the six different sample preparation methods. The left group in A–F represents the whole milk powder matrix, and the right group in A–F represents the skimmed milk powder matrix. The six food additives: benzoic acid, sorbic acid, natamycin, lysozyme, aspartame, and saccharin sodium. (A) Method A (liquid-liquid extraction). (B) Method B (precipitation based on sodium tungstate). (C) Method C (precipitation based on potassium ferrocyanide). (D–F) Method D-F (solid phase extraction): (D) Poly-Sery HLB SPE, (E) CNWBOND LC-C18 SPE, and (F) Poly-Sery MAX SPE.

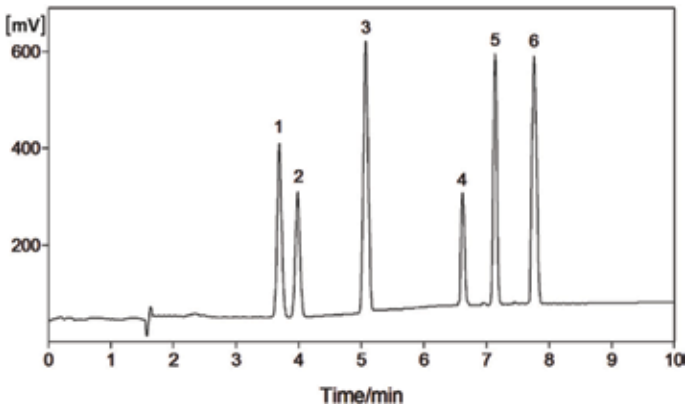


Figure 3.
Chromatogram of mixed standard solutions. (1) Benzoic acid, (2) sorbic acid, (3) saccharin sodium, (4) natamycin, (5) aspartame, and (6) lysozyme.

representative chromatograms. All chromatographic peaks were separated completely and had a good resolution ($R \geq 1.5$). Recovery studies of benzoic acid, sorbic acid, natamycin, lysozyme, saccharin sodium, and aspartame were evaluated by analysis of blank pooled samples spiked with 10 μg g^{-1} (lower level), 50 μg g^{-1} (middle level), and 100 μg g^{-1} (upper level) of each analyte. And the data were calculated based on the matrix-matched regression curves and summarized in **Table 2**. The intraday precision was studied at lower, middle, and upper concentration levels ($n = 5$). The interday precision was analyzed with spiked samples at 50 μg g^{-1} for six-day determinations (**Table 3**).

	Amounts of chemical reagents used	Time cost[e]
Method A[a]	Ethyl acetate: 5.0 + 5.0 mL Formic acid solution (10 mmol L^{-1}): 1.0 mL	>60 min
Method B[b]	Sulfuric acid solution (10%): 1.0 mL Sodium tungstate solution (10%): 5.0 mL	≈50 min
Method C[c]	$K_4[Fe(CN)_6]$ solution (0.085 mol L^{-1}): 3.0 mL $ZnSO_4$ solution (0.25 mol L^{-1}): 3.0 mL	≈50 min
Method D–F[d]	TCA solution: 7.0 mL Acetonitrile: 3.0 mL Methanol: 3.0 mL Ammonium sulfate solution: 3.0 mL Acetonitrile: 3.0 mL	≈25 min

[a]*Method A (LLE).*
[b]*Method B (precipitation based on sodium tungstate).*
[c]*Method C (precipitation based on potassium ferrocyanide).*
[d]*Method D–F (SPE).*
[e]*Time cost is the average value of the three experiments.*

Table 1.
Comparison of the cost-effectiveness of six methods.

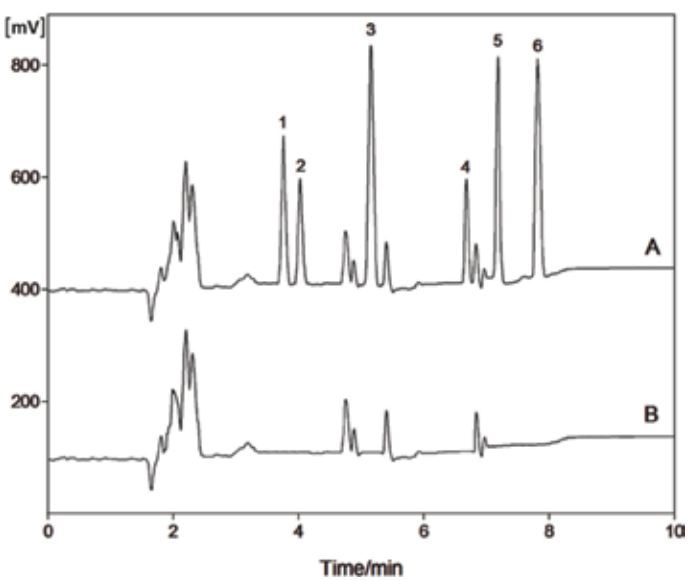


Figure 4.
Chromatograms of the blank whole milk powder sample and spiked whole milk powder sample. (A) The spiked whole milk powder sample: (1) benzoic acid, (2) sorbic acid, (3) saccharin sodium, (4) natamycin, (5) aspartame, and (6) lysozyme (50 μg g^{-1} of each food additives). (B) The blank whole milk powder sample.

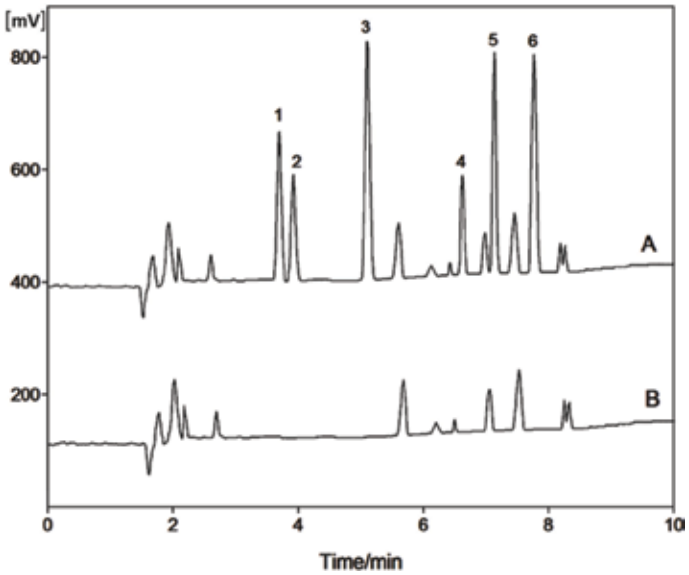


Figure 5.
Chromatograms of the blank skimmed milk powder sample and spiked skimmed milk powder sample. (A) The spiked skimmed milk powder sample: (1) benzoic acid, (2) sorbic acid, (3) saccharin sodium, (4) natamycin, (5) aspartame, and (6) lysozyme (50 μg g^{-1} of each food additives). (B) The blank skimmed milk powder sample.

Analytes	Sample matrix	Regression equation[b] y = ax ± b	Regression coefficient	Linear range (μg L^{-1})	LOD (μg L^{-1})	LOQ (μg L^{-1})
Benzoic acid	A[a]	y = 0.582x − 0.035	0.9999	100–20,000	45	95
	B[a]	y = 0.497x − 0.028	0.9998	250–20,000	70	200
	C[a]	y = 0.501x + 0.037	0.9998	250–20,000	60	200
Sorbic acid	A[a]	y = 0.362x + 0.017	0.9998	150–25,000	60	130
	B[a]	y = 0.297x + 0.028	0.9994	300–25,000	90	255
	C[a]	y = 0.500x − 0.054	0.9995	300–25,000	90	240
Natamycin	A[a]	y = 0.313x + 0.039	0.9999	200–30,000	60	135
	B[a]	y = 0.364x + 0.023	0.9997	500–30,000	80	215
	C[a]	y = 0.288x + 0.060	0.9997	500–30,000	90	230
Saccharin sodium	A[a]	y = 0.405x + 0.097	0.9999	100–25,000	30	75
	B[a]	y = 0.329x − 0.010	0.9992	200–25,000	50	120
	C[a]	y = 0.525x − 0.091	0.9995	200–25,000	70	150
Aspartame	A[a]	y = 0.617x − 0.043	0.9999	100–30,000	50	95
	B[a]	y = 0.577x + 0.033	0.9996	200–30,000	60	165
	C[a]	y = 0.505x − 0.023	0.9997	200–30,000	70	180
Lysozyme	A[a]	y = 0.405x + 0.097	0.9997	100–30,000	45	95
	B[a]	y = 0.381x + 0.059	0.9995	250–30,000	60	170
	C[a]	y = 0.370x + 0.068	0.9996	250–30,000	60	170

[a] *(A) Aqueous, (B) pooled whole milk powder, and (C) pooled skimmed milk powder.*
[b] *y is the average peak area of each analyte (n = 3), and x is the mass concentration of the analyte in μg L^{-1}.*

Table 2.
Linearity and LOD of the developed method (n = 3).

Analytes	Added ($\mu g\ g^{-1}$)	Whole milk powder		Skimmed milk powder	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Benzoic acid	10	93	4.8	95	3.3
	50	95	3.5	93	2.9
	100	93	1.4	95	2.5
Sorbic acid	10	94	3.7	94	4.1
	50	97	1.5	97	3.0
	100	92	1.2	99	2.2
Natamycin	10	89	4.9	90	3.9
	50	90	2.0	91	3.5
	100	96	1.0	99	2.7
Lysozyme	10	91	4.5	92	4.4
	50	92	5.0	95	1.9
	100	101	2.8	103	2.3
Saccharin sodium	10	95	3.3	89	4.6
	50	90	4.5	93	4.1
	100	97	2.0	98	2.4
Aspartame	10	90	2.9	93	3.6
	50	92	4.5	94	1.1
	100	94	2.4	94	1.2

Table 3.
Precision and accuracy of the assay for whole milk powder and skimmed milk powder analysis (n = 3).

2.4 Conclusion

Among the six different sample extraction methods, two precipitate-based methods (method B and method C) were not suitable for the low recovery of lysozyme. Both method A and method D obtained good recoveries of six food additives simultaneously, but the major problem of method A is the lower reproducibility and much more time cost than method D. SPE was a simple and rapid method for the extraction of six food additives. From the results of method D–F, Poly-Sery HLB cartridge was confirmed as the most appropriate material for its high recovery.

3. 2DLC for determination of five major proteins and seven additives in milk powders

Two-dimensional liquid chromatography has been used in many aspects. Herein, a 2DLC method was introduced for the simultaneous determination of five major proteins and seven additives in milk powders. Considering the macromolecular proteins, C4 column was placed in the first dimension (1D), and C18 column was placed in the second dimension (1D) for analysis of the seven additives. Finally, the five proteins in milk powders were separated completely on the 1D column, and the seven additives can be simultaneously analyzed on the 2D column. In the middle of 1D and 2D, a trapping column and a ten-port switching valve was served. This method was compared with the conventional one-dimensional liquid

chromatography (1DLC) in terms of sample preparation, limit of detection (LOD), and recovery.

3.1 1DLC separation of additives

3.1.1 Sample preparation for 1DLC

0.5 g milk powder samples were diluted in 2.0 mL ultrapure water. After 10 min of ultrasonication, 0.5 mL of Carrez I solution (500 mM aqueous potassium ferrocyanide), 0.5 mL of Carrez II solution (500 mM aqueous zinc acetate), and 1 mL of ACN were added in order to precipitate the proteins.

3.1.2 1DLC analysis

Maltol, ethyl maltol, vanillin, ethyl vanillin, benzoic acid, sorbic acid, and saccharin sodium were separated on a 2010 AT chromatographic instrument from Shimadzu Corporation (Kyoto, Japan). A C18 analytical column (4.6 × 150 mm, 5 μm) was used. The mobile phases were ammonium acetate buffer (25 mM, pH 6.6) (solvent A) and ACN/water = 90/10 (v/v) (solvent B); the following gradient program was used: 0–2.8 min, 0% ACN; 2.8–5 min, progressing linearly to 45% ACN; and 5–15 min, maintaining at 45% ACN. The flow rate was 1.0 mL/min, and the column temperature was maintained at 40°C. The injection volume was 5 μL, and the detection wavelength was 214 nm. The peak area was calculated for quantification, and each sample or standard was injected in triplicate.

3.2 2DLC separation of proteins and additives

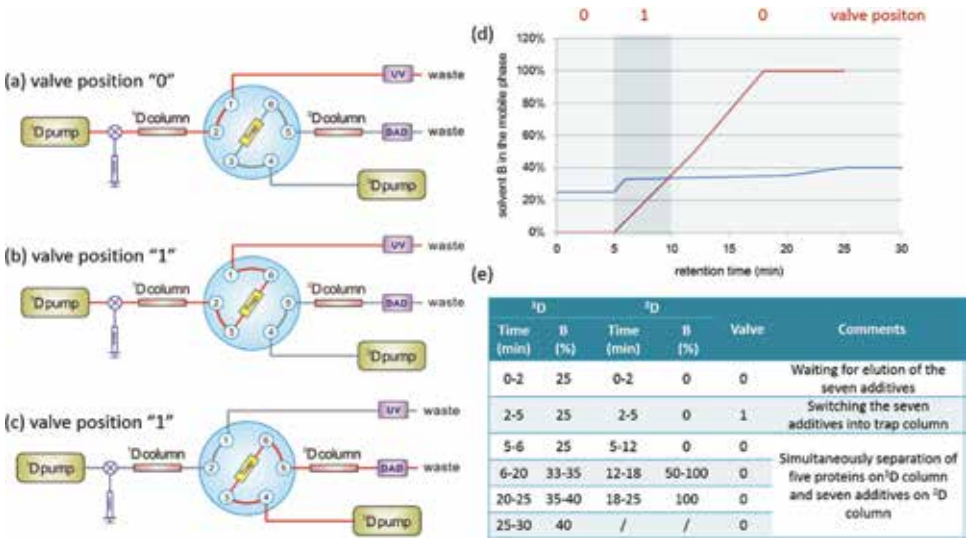
3.2.1 Sample preparation for 2DLC

Around 0.2 g of the milk powder samples were dissolved for 10 min in 5 mL of buffer (6 M urea, 0.5% OG). The samples were then filtered through a 0.45 μm nylon membrane before injected into the 2DLC system for analysis.

3.2.2 2DLC analysis

The 2DLC system consisted of two LC-20AB binary gradient pumps (Shimadzu Technologies), one six-port two-position switching valve (VICI Valco Instruments, Houston, TX, USA), a SIL-20A autosampler, two DGU-20A3 degassers, a CTO-20A column oven, and two SPD-M20A diode array detectors.

A scheme of the 2DLC system is shown in **Figure 6**. For the first dimension (1D), a Venusil XBP-C4 analytical column (4.6 mm × 100 mm, 5 μm) coupled with a C4 guard column was used. One aspect is for separation of proteins and additives, and the other is for five proteins analysis. The target fractions (polar substances) from 1D were enriched by a trapping column (ODS C18, 4.6 mm × 50 mm, 5 μm) and switched into the 2D through a six-port valve. A Hypersil ODS-2 C18 column (4.6 × 150 mm, 5 μm) was used to completely separate the seven food additives in 2D. The mobile phase consisted of ACN/water/TFA (10/90/0.1, v/v/v) (solvent A) and ACN/water/TFA (90/10/0.1, v/v/v) (solvent B) for 1D and ammonium acetate (25 mM, pH 6.6) (solvent A) and ACN/water (50/50, v/v, pH 7.2) with 25 mM ammonium acetate (solvent B) for 2D. The column temperature was 40°C. The detection wavelengths for 1D was 214 nm, for 2D were 254 nm and 278 nm. The eluted program is shown in **Figure 6**. The injection volume was 5 μL, and each sample or standard was injected in triplicate.



1D Time (min)	1D B (%)	2D Time (min)	2D B (%)	Valve	Comments
0-2	25	0-2	0	0	Waiting for elution of the seven additives
2-5	25	2-5	0	1	Switching the seven additives into trap column
5-6	25	5-12	0	0	Simultaneously separation of five proteins on 1D column and seven additives on 2D column
6-20	33-35	12-18	50-100	0	
20-25	35-40	18-25	100	0	
25-30	40	/	/	0	

Figure 6.
Schematic representation (a–c) and gradient, flow rates, and switching times (d and e) of the stop-flow heart-cutting 2DLC system.

3.3 Optimization of chromatographic conditions

In order to accurately quantify the five proteins and seven additives in milk powder samples using the 2DLC method, some important parameters were optimized, including the stationary phase, mobile phase, and switching time.

According to the literature, a shorter switching time in the 2DLC system means better shape of the peaks in the 2D chromatogram [15]. Because the milk powder matrix is so complex, the ideal 1D column would be able to separate the proteins and additives into two groups. The additives with higher polarity were concentrated within a short period of time and eluted rapidly, while the proteins were separated completely after the elution of additives by adjusting the mobile phase. In order to achieve this goal, two columns were tested: a Venusil XBP-C4 column (4.6 mm × 100 mm, 5 μm) and a Venusil XBP-C8 column (4.6 mm × 100 mm, 5 μm). These two types of columns could separate the seven additives and five proteins as two groups. The seven additives were concentrated at 2.0–5.0 min on the C4 column and 2.0–6.0 min on the C8 column. Therefore, the Venusil XBP-C4 column was chosen as the 1D column because of the shorter switching time. **Figure 7A** shows the chromatogram of the seven additives and five major proteins. The seven additives were focused at the first minutes, and the five major proteins could be separated later. For 2D separation, Hypersil ODS-2 C18 column showed better separation performance for the seven additives. A trapping column was used as the interface between 1D and 2D, which should result in better enrichment of the targets [16]. For online 2DLC, the choice of the mobile phase is very important. Because of protein separation, ACN was chosen as the organic mobile phase. 0.1% v/v TFA was added to all mobile phases to improve the protein separation effect.

The mobile phases for 1D were A1, ACN/water (10/90 v/v, 0.1% TFA), and B1, ACN/water (90/10 v/v, 0.1% TFA). Solvent B1 was set at 25% from 0 to 5 min in order to elute the additives quickly. Due to the little polarity difference of proteins, a gentle gradient of 0.14% B min^{-1} was used to achieve good separation of the five proteins, which was consistent with the literature [17–19]. As shown in **Figure 7**, the proteins were eluted in the following order: α_{s2}-CN, α_{s1}-CN, α-Lac, β-CN, β-LgB, and β-LgA. It should be noted that there were no standards for α_{s1}-CN and α_{s2}-CN

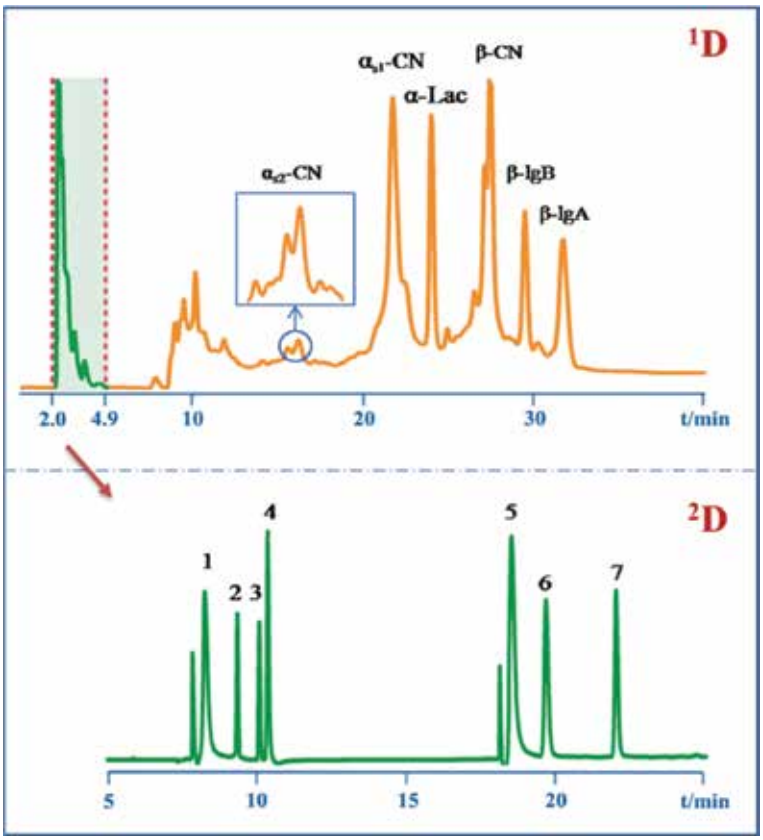


Figure 7.
The 2DLC chromatogram of the 12 mixed standards substances. The 1D chromatogram of seven additives (2.0–5.0 min) and five proteins on the 1D C4 column (4.6 mm × 100 mm, 5 μm) (up) and the 2D chromatogram of the seven additives on the C18 analytical column (down). (1) Maltol, (2) saccharin sodium, (3) benzoic acid, (4) sorbic acid, (5) ethyl maltol, (6) ethyl vanillin, and (7) vanillin.

proteins, only for their mixture [10]. The chromatographic profiles showed no carryover effects of these proteins. A shoulder for the α_{s1}-CN standard can be seen due to the presence of its two variants (α_{s1}-CN and α_{s2}-CN), which are very difficult to separate completely. From the different findings from previous reports, the monomorphic α-Lac was eluted firstly than β-CN [17–19]. The three shoulders of β-CN corresponded to its variants. As previously reported, γ-CN is the proteolytic product of β-CN, so they could be eluted together [18]. For β-Lg, variant B eluted before variant A, which is consistent with the literature [18]. During the process of quantitative analysis, α_{s1}-CN and α_{s2}-CN were quantified together, as for the three variants of β-CN.

Acetic ammonia is often used as the modifier in liquid chromatography separation. To obtain better separation of the seven additives in 2D, a series of acetic ammonia concentrations (15, 20, 25, 30 mM) were tested. When 25 mM acetic ammonia was added, the baseline was much more stable, and the peak shape was greatly improved. Therefore, the 2D mobile phase were as follows: A2, 25 mM acetic ammonia, and B2 ACN/water (50/50 v/v) with 25 mM acetic ammonia. The gradient program is shown in **Figure 6**. The initial mobile phase of 1D was optimized and set at 25% B1. If lower than 25% B1, elution of the seven additives would be taken too long in the 1D column, which could lead to sample loss in the trapping column before switching; if higher than 25% B1, maltol and saccharin sodium could be separated incompletely in 2D because of ACN in the trapping column.

The switching time is a key parameter in this method. Three switching time (2.0–4.5, 2.0–5.0, and 2.0–5.5 min) were tested. When the switching time was between 2.0 and 4.5 min, maltol and saccharin sodium were separated incompletely, and the sorbic acid peak was less sharp than that for 2.0–5.0 min; when between 2.0 and 5.5 min, some analytes were lost in the trapping column. Therefore, 2.0–5.0 min was chosen as the final switching time for the experiment. **Figure 7** shows the chromatogram of the 12 mixed standard substances using the optimized 2DLC method. In **Figure 7A**, the seven additives were eluted between 2.0 and 5.0 min due to their higher polarity, and the proteins were separated on the 1D column (8.0–30.0 min); **Figure 7B** shows the 2D chromatogram of the seven additives that were switched from the 1D column at 2.0–5.0 min. The whole analysis process was less than 30 min, which provide a highly efficient analysis method.

3.4 Comparison of analysis parameters for 1DLC and 2DLC

The matrix effect, linearity, LOD, intra- and interday precision, and accuracy were validated under the optimized conditions for 1DLC and 2DLC.

The method validation parameters of 1DLC and 2DLC were shown in **Table 4**. The correlation coefficient values (R^2) for both methods are higher than 0.9988 for all the additives. And R^2 of ethyl maltol in 1DLC is lower than that in 2DLC.

Considering the complexity of milk powder, the possibility of a matrix effect was investigated by comparing the slope ratio of the calibration curves for the seven additives obtained in the presence and absence of blank milk powder [20]. For example, the slope ratio is closer to 1.0, which means a lower matrix effect in the method. The results in **Table 4** show that 2DLC (slope ratio: 0.94–1.09) had a lower matrix effect than 1DLC (slope ratio: 0.84–1.21). The sample matrix effect for the determination of the seven additives for both 1DLC and 2DLC can be seen in **Figure 8**. The milk powder sample matrix chromatogram of 2DLC (b′) is much clean and flat than that in 1DLC (a′), and there has no interference peak for the analytes. Although the matrix effect of 2DLC is low, we still chose the matrix-matched standard curve for the sample analysis [19]. The LOD values of the 2DLC method were higher than that of the 1DLC method, as the peak width obtained with the new method is broader than that with the conventional method. Those are the advantages and disadvantages of these two methods.

Table 5 shows the precision and recovery results of 1DLC and 2DLC. The intra-day and interday data showed that the precision of the two methods is satisfactory. However, the recovery of the 2DLC method (89.6–103.5%) was much better than that for the 1DLC method (65.5–99.2%), which is mainly benefit from the "one-step" sample preparation method. Analytes may be lost during the processes of traditional sample pretreatment (such as solid-phase extraction, liquid–liquid extraction, and precipitation). In this method, the whole analysis time was less than 1 h. So, 2DLC is much more efficient than 1DLC. Overall considering the environmental protection and time saving, the automation offered by 2DLC possesses more advantages.

3.5 Commercial sample analysis

Four different commercial milk and milk powder samples purchased from local supermarkets were analyzed using the developed 2DLC method. The chromatograms are shown in **Figure 9**. **Figure 9A** and **B** were infant formula milk powder (IFMP), **Figure 9C** was skimmed milk powder, and **Figure 9D** was fresh bovine milk. Benzoic acid and ethyl vanillin were detected only in the IFMP 1 sample. α-CN, β-CN, and α-Lac were detected in the four milk products. β-LgB and β-LgA were detected in the IFMP 2 and SMP samples.

Analytes	Methods	Sample matrix	Regression equation[a] y = ax ± b	R^2	Slope ratio (matrix/blank)	Linear range (μg mL^{-1})	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
MAL	1DLC	Blank	y = 34636x − 34306	0.9996	1.21	0.28–28	0.051	0.21
		Matrix	y = 41933x − 77788	0.9995	/	1.0–100	0.41	1.02
	2DLC	Blank	y = 33548x + 367540	0.9991	1.09	0.28–28	0.065	0.187
		Matrix	y = 36410x + 354210	0.9990	/	0.28–28	0.105	0.25
Saccharin sodium	1DLC	Blank	y = 24706x + 43056	0.9998	0.97	0.22–22	0.026	0.10
		Matrix	y = 23945x + 96114	0.9998	/	0.5–50	0.067	0.21
	2DLC	Blank	y = 4328x + 7786	0.9991	0.94	0.22–22	0.044	0.11
		Matrix	y = 4086x + 10206	0.9991	/	0.22–22	0.074	0.19
Benzoic acid	1DLC	Blank	y = 31513x − 244	1.0000	1.08	0.25–25	0.018	0.051
		Matrix	y = 34007x − 3087	1.0000	/	0.5–50	0.18	0.42
	2DLC	Blank	y = 4497x + 318	0.9999	1.06	0.25–25	0.09	0.25
		Matrix	y = 4757x − 485	0.9999	/	0.3–30	0.10	0.28
Sorbic acid	1DLC	Blank	y = 147925x − 1384	1.0000	0.89	0.2–20	0.01	0.025
		Matrix	y = 131050x − 3021	1.0000	/	0.5–50	0.056	0.136
	2DLC	Blank	y = 57114x + 186559	0.9992	0.96	0.2–15	0.054	0.133
		Matrix	y = 54623x + 228303	0.9990	/	0.25–18	0.075	0.20
EMA	1DLC	Blank	y = 34036x − 197820	0.9977	1.04	2.5–50	0.14	0.42
		Matrix	y = 35360x − 87395	0.9985	/	5–100	1.14	3.33
	2DLC	Blank	y = 82117x + 167643	0.9996	1.07	0.5–50	0.165	0.50
		Matrix	y = 87714x + 47480	0.9995	/	1–100	0.18	0.56

Analytes	Methods	Sample matrix	Regression equation[a] y = ax ± b	R^2	Slope ratio (matrix/blank)	Linear range (μg mL^{-1})	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
EVA	1DLC	Blank	y = 45172x + 459	1.0000	0.90	0.2–20	0.017	0.055
		Matrix	y = 40683x + 521	1.0000	/	0.5–50	0.043	0.15
	2DLC	Blank	y = 42319x + 4981	0.9996	1.01	0.2–20	0.016	0.051
		Matrix	y = 42898x – 1620	0.9995	/	0.1–10	0.019	0.056
VAN	1DLC	Blank	y = 65925x + 54232	0.9999	0.84	0.2–20	0.015	0.048
		Matrix	y = 55386x + 47261	0.9998	/	0.5–50	0.039	0.12
	2DLC	Blank	y = 45492x + 4220	0.9994	1.06	0.2–20	0.018	0.049
		Matrix	y = 48056x – 1552	0.9997	/	0.1–10	0.018	0.054
α-CN	1DLC	Blank	y = 1596833x + 58163	0.9984	/	100–5000	50	92.7
α-Lac	1DLC	Blank	y = 4334341x – 13906	0.9997	/	10–500	3.0	9.9
β-CN	1DLC	Blank	y = 2982340x + 12008	1.0000	/	16–780	4.0	10.2
β-LgB	1DLC	Blank	y = 707669x + 1006	0.9997	/	15–750	5.1	18.4
β-LgA	1DLC	Blank	y = 2393184x + 4246	1.0000	/	15–750	3.8	12.4

[a]*y is the average peak area of each additive (n = 3), and x is the mass concentration of the additive in mg mL^{-1}.*

Table 4.
Method validation parameters of 1DLC and 2DLC (n = 3).

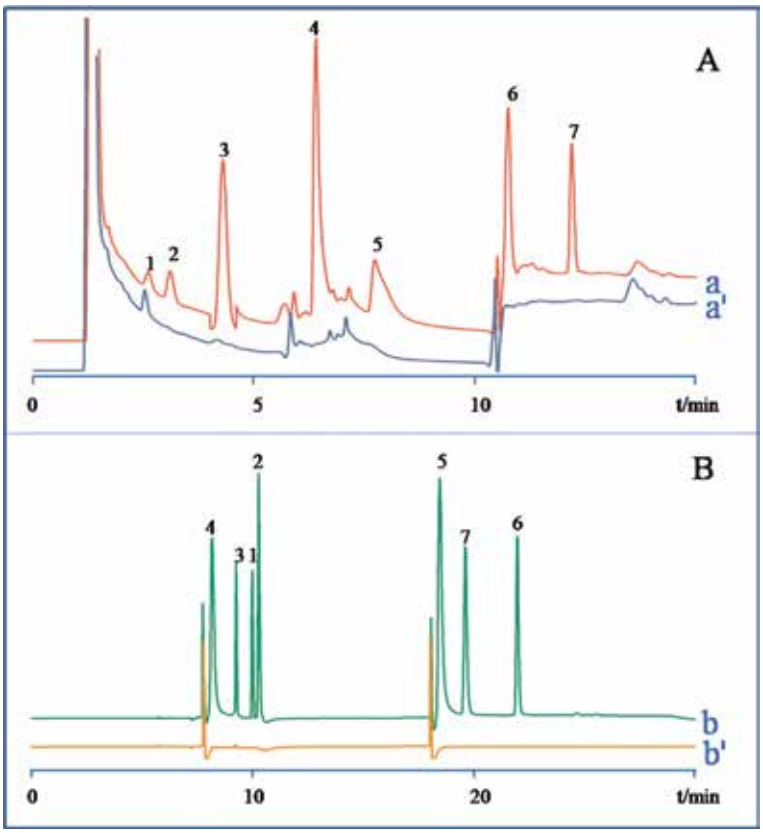


Figure 8.
1DLC (A) and 2DLC (B) chromatograms for testing sample matrix effect. (a' and b') Sample matrix without standard substances. (a and b) Sample matrix with standard substances. Chromatographic peaks: (1) benzoic acid, (2) sorbic acid, (3) saccharin sodium, (4) maltol, (5) ethyl maltol, (6) vanillin, and (7) ethyl vanillin.

	Concentration ($\mu g\,mL^{-1}$)		Precision				Spiked (ng)		Recovery			
			Intraday RSD		Interday RSD				Recovery (%)		RSD	
	1D	2D	1D	2D	1D	2D	1D	2D	1D	2D	1D	2D
Maltol	1.25	0.5	2.93	2.86	3.85	2.93	3	1.4	73.6	92.1	0.55	3.66
	3	6.25	0.85	0.19	0.43	2.17	9	7	72.6	97.5	2.07	1.68
	18.75	18.75	0.72	2.70	1.19	2.73	45	21	72.1	103.5	1.14	3.92
Saccharin sodium	1	0.4	0.12	1.44	1.13	1.43	0.7	1.1	67.5	93.4	2.75	2.36
	2.4	5	0.77	0.58	2.04	3.28	5	5.5	70.1	93.9	0.33	2.84
	15	15	0.42	1.93	0.77	2.11	22.5	16.5	78.4	91.2	0.80	1.78
Benzoic acid	1	0.5	1.03	1.99	1.18	3.29	0.7	1.5	65.5	90.6	3.42	3.09
	2.4	6.25	0.11	1.88	0.46	4.96	4	7.5	71.0	95.2	0.42	0.34
	15	18.75	0.13	2.53	0.40	1.87	20	22.5	79.1	91.1	0.36	0.34
Sorbic acid	1	0.4	0.06	2.25	1.54	5.44	1.25	1.25	71.2	100.6	0.08	1.09
	2.4	5	0.19	0.90	0.25	3.74	5	6.25	72.2	102.8	0.16	0.79
	15	15	0.21	2.22	0.20	4.82	22.5	18.75	75.7	94.5	0.18	2.65

	Concentration ($\mu g\ mL^{-1}$)		Precision				Spiked (ng)		Recovery			
			Intraday RSD		Interday RSD				Recovery (%)		RSD	
	1D	**2D**	**1D**	**2D**	**1D**	**2D**	**1D**	**2D**	**1D**	**2D**	**1D**	**2D**
Ethyl maltol	2.5	2	3.72	0.90	3.48	0.73	9	5	71.6	105.4	4.00	0.58
	6	25	0.76	0.98	4.80	2.90	22.5	25	70.1	98.2	0.85	2.26
	37.5	75	0.16	1.28	1.89	2.98	45	75	80.2	96.5	0.87	1.25
Ethyl vanillin	1	0.4	0.41	0.38	2.65	1.52	2	0.51	82.8	92.1	0.05	3.36
	2.4	5	0.12	0.83	1.05	4.31	4	2.55	84.5	92.6	0.17	3.92
	15	15	0.04	1.00	0.29	1.84	20	7.65	79.3	98.0	0.09	0.55
Vanillin	1	0.4	1.81	2.24	1.82	1.68	2.5	0.49	81.7	92.6	1.36	1.14
	2.4	5	0.70	0.33	1.21	0.84	7	2.45	86.5	93.7	0.69	2.66
	15	15	0.69	0.11	1.29	0.47	22.5	7.35	85.5	98.4	1.46	3.00
α-CN	200	/	4.55	/	4.28	/	506	/	86.6	/	3.07	/
	1200	/	0.46	/	4.14	/	1210	/	99.2	/	1.12	/
	5000	/	0.15	/	6.45	/	4050	/	94.7	/	3.73	/
α-Lac	20	/	1.99	/	2.48	/	49	/	87.7	/	3.07	/
	120	/	0.51	/	3.39	/	120	/	86.4	/	1.49	/
	500	/	0.33	/	4.11	/	395	/	88.3	/	2.75	/
β-CN	30	/	3.70	/	3.71	/	78	/	105.2	/	1.66	/
	180	/	0.42	/	2.68	/	188	/	94.0	/	2.28	/
	780	/	0.41	/	1.12	/	392	/	101.7	/	1.93	/
β-LgB	30	/	2.57	/	4.60	/	73.5	/	101.1	/	2.22	/
	180	/	1.55	/	3.24	/	176.4	/	99.2	/	3.24	/
	750	/	0.67	/	3.08	/	588	/	83.5	/	2.78	/
β-LgA	30	/	2.93	/	3.10	/	52	/	99.4	/	3.39	/
	180	/	1.06	/	1.14	/	125	/	98.2	/	1.99	/
	750	/	0.03	/	0.86	/	600		91.7	/	4.84	/

Table 5.
Accuracy of the two methods (n = 6).

Table 6 showed the contents of the five major proteins and the seven additives. The contents of α-CN and β-CN were much higher than that of α-Lac, β-LgB, and β-LgA in all the milk products. The contents of α-Lac, β-LgB, and β-LgA were lower in the infant formula milk powder than that in the skimmed milk powder. The results are consistent with those from the literature [17], which is probably due to the denaturation of the thermosensitive whey proteins [21] or intentional removal of β-LgB to prevent allergic reactions [22].

In order to evaluate the accuracy of protein determination using the proposed method in this work, four brands of commercial milk products were analyzed using both the 2DLC and Kjeldahl methods. **Table 7** shows the total major protein contents in the various milk matrices determined by these methods, 2DLC, the Kjeldahl method, and TPC, as given by the manufacturers. The RSD of the three groups were less than 3%, which means that the milk protein contents were similar for our method and the Kjeldahl method as well as that given by the manufacturers.

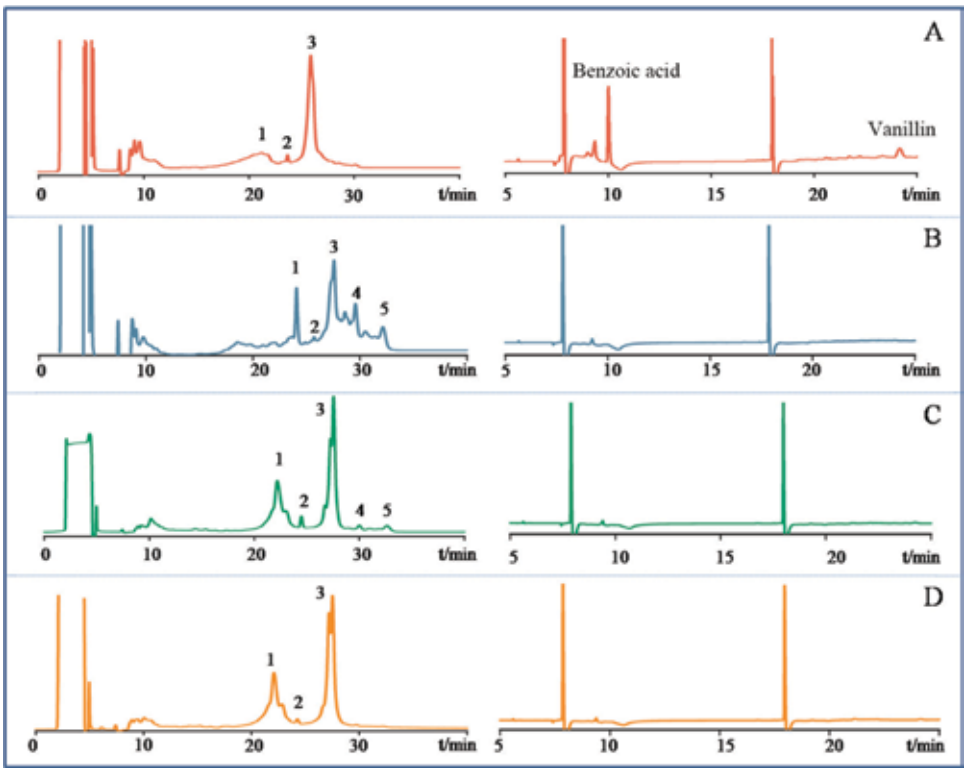


Figure 9.
Chromatograms of four brands of commercial milk and milk powders. (A) Infant formula milk powder 1, (B) infant formula milk powder 2, (C) skimmed milk powder, and (D) bovine milk. (1) α-Casein (α-CN), (2) α-lactalbumin (α-Lac), (3) β-casein (β-CN), (4) β-lactoglobulin B (β-LgB), and (5) β-lactoglobulin A (β-LgA).

Sample (μg g^{-1})	IFPM[a]1	IFPM2	SMP[a]	BM[a]
α-CN	43.11 ± 0.45[c]	67.59 ± 0.60	225.41 ± 3.00	22.65 ± 0.30
α-Lac	0.87 ± 0.02	2.48 ± 0.05	4.47 ± 0.14	0.28 ± 0.01
β-CN	56.38 ± 0.52	27.91 ± 0.32	98.59 ± 1.89	9.00 ± 0.11
β-LgB	ND[b]	4.97 ± 0.28	5.52 ± 0.19	ND
β-LgA	ND	3.75 ± 0.12	6.19 ± 0.16	ND
MAL	ND	ND	ND	ND
Saccharin sodium	ND	ND	ND	ND
Benzoic acid	1553.00 ± 0.04	ND	ND	ND
Sorbic acid	ND	ND	ND	ND
EMA	ND	ND	ND	ND
EVA	ND	ND	ND	ND
VAN	20.51 ± 0.24	ND	ND	ND

[a]*IFPM, infant formula powder milk; SMP, skimmed milk powder; BM, bovine milk.*
[b]*ND, not detected.*
[c]*The values of the concentration are means ± SD (n = 3).*

Table 6.
Contents of food additives determined in milk powder samples by 2DLC (n = 3).

	TMPC[a] with 2DLC	Kjeldahl method	TPC[b] indicated by manufacturers	RSD[d]
IFMP 1	10.04 ± 0.10[c e]	9.77 ± 0.09	10.4	0.03
IFMP 2	10.67 ± 0.14	10.83 ± 0.07	11.4	0.03
SMP	32.85 ± 0.54	34.19 ± 0.35	33.0	0.02
Milk	3.19 ± 0.04	2.96 ± 0.08	3.1	0.03

[a]*TMPC, the total major protein concentrations.*
[b]*TPC, the total protein concentration.*
[c]*Powder milks in g/100 g and liquid milks in g/100 ml.*
[d]*RSD among the data determined by the two methods and indicated by manufacturers.*
[e]*The values of the concentration are means ± SD (n = 3).*

Table 7.
Comparison between the total major protein concentrations (TMPC) in the various milks determined with 2DLC method and the total protein concentration (TPC) determined with Kjeldahl method and TPC given by the manufacturers.

4. Conclusions

In this chapter, two kinds of analysis methods for common additives are introduced. One is HPLC, and the other is 2DLC. Poly-Sery HLB cartridge was confirmed as the most appropriate material for HPLC because of the higher recovery. As to 2DLC, the sample preparation method is much easier, time-saving, and efficient, and this method possesses much higher recovery of food additives by avoiding the sample loss; and the analysis process was performed on an automated instrument within 30 mins. Therefore, it is simpler, faster, and more accurate than current standard methods.

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Conflict of interest

Sicen Wang on behalf of other authors declares that all authors of this article have no conflict of interest. This article does not contain any studies with human or animal subjects.

Abbreviations

HPLC	high-performance liquid chromatography
RPLC	reversed-performance liquid chromatography
2DLC	two-dimensional liquid chromatography

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Chapter 7

Dulce de Leche—Chemistry and Processing Technology

Rodrigo Stephani, Júlia Francisquini, Ítalo Tuler Perrone, Antônio Fernandes de Carvalho and Luiz Fernando Cappa de Oliveira

Abstract

Originally hailing from Latin America, dulce de leche (DL) is one of the most widely manufactured dairy products in South America, where it is marketed as a paste or bar. Due to DL's low moisture content, the product can be safely stored at room temperature, which facilitates storage and transportation logistics. The primary ingredients used to manufacture DL are milk, sucrose, and an acidity reducer. Needless to say, the raw materials must be of good quality from reliable suppliers in order for the final product to have the desired characteristics. The milk used to make DL must be microbiologically safe, remain stable during thermal processing, and preferably exhibit a high solid content. Dulce de leche is defined as a product made with or without the addition of other food substances that is obtained from milk or reconstituted milk and added sucrose (either partially substituted or not by monosaccharides and/or other disaccharides) via concentration and heat action at normal or reduced pressure. This chapter aims to explore the chemistry, processing technology, and most common industrial practices for manufacturing DL in South America.

Keywords: dairy products, Maillard reaction, milk, evaporation, manufacturing technology

1. Introduction

Dulce de leche (DL) is a dairy product that has widespread success and acceptance due to its pleasant sensorial characteristics. Nutritionally, it has a high energetic value and boasts high concentration of proteins, minerals, and carbohydrates.

During the manufacturing process, DL is submitted to high temperatures for long periods. This, along with the types of ingredients used in its formulation, stimulates an acceleration of the Maillard reaction rate and consequently an increase of 5-hydroxymethylfurfural index. The latter corresponds to one of the indicators used to verify the intensity of the heat treatment.

DL is formulated in plants that range from small, artisanal plants to small, medium, and large industrial dairy factories. The quality of the raw material, the type of formulation used, and the technology hurdles related to a lack of standardization of time and temperature during the manufacturing process have made it difficult for brands on the Brazilian market to be standardized.

It is therefore imperative to analyze, study, and prioritize how the different ingredients, manufacturing technologies, and heat treatment intensities influence the quality and uniformity of the product. Moreover, the lack of technical and scientific literature on this product underscores the need for further studies in the domain.

2. Dulce de leche

DL is a dairy product that is manufactured via heat action concentration. It is produced in small- and medium-sized dairy industries on a large scale throughout South America, particularly in Brazil and Argentina [1].

According to the most recent data from the Annual Industrial Survey (PIA) released by the Brazilian Institute of Geography and Statistics (IBGE), DL production in Brazil represents 345.19 million reais, a sum equivalent to 0.82% of dairy sector production in the country.

In 2009, 50% of Brazil's milk production was concentrated in the state of Minas Gerais, according to the Integrated Development Institute of Minas Gerais [2, 3]. Despite the importance of dulce de leche on the Brazilian market since 2007, the country has maintained a trade balance deficit for the dairy product. Argentina and Uruguay have remained the primary suppliers to Brazil [4, 5].

In 2015, the Foreign Trade Information Analysis System via Internet (ALICEWeb) determined that 95.46% of Brazil's total DL imports came from Argentina, while 4.54% came from rom Uruguay. As to destination countries for DL in 2015, the United States of America (USA), Bolivia, Paraguay, Costa Rica, United Kingdom, Chile, Uruguay, and the Netherlands accounted for 56.37, 15, 13.92, 9.67, 2.62, 0.99, 0.81, and 0.61%, respectively.

Dulce de leche is defined as a product made from milk or reconstituted milk and added sucrose (either partially substituted or not by monosaccharides and/or other disaccharides), with or without the addition of other food substances, obtained via concentration and heat action at normal or reduced pressure [6].

Regulations require DL to contain milk and/or reconstituted milk and indicate minimum and maximum values for moisture, fat, ash, and protein.

DL may not contain nondairy fat or protein. It is produced by concentration of milk to which sugar, usually sucrose, has been added. In some cases, it is possible to replace part of the sucrose with glucose, which improves the texture and color while simultaneously reducing lactose crystal growth and formation and increasing product viscosity [7].

The heat and the concentration processes during DL processing can lead to a series of product defects, such as protein destabilization, which results in protein precipitation. Acidity reducers can be used to reduce or avoid precipitation, as well as enhance product color. Sodium bicarbonate is one of the most common acidity reducers used in DL production [7].

The DL sensorial characteristics such as flavor and coloring are fundamentally due to the nonenzymatic browning reaction (the Maillard reaction) that occurs during the intense thermal treatment to which the milk and sugar syrup is submitted during manufacturing. Despite the sensory benefits it imparts, this reaction can have adverse effects on the nutritional characteristics of the food, such as reducing the concentration of essential amino acids like lysine [7].

The Technical Regulation of DL classifies products according to fat content and additional ingredients as shown in **Table 1**.

The term DL (dulce de leche) is reserved for products that do not contain fat and/or protein of nondairy origin.

Parameter	Classification
Fat content	DL (6.0–9.0 g.100 g^{-1})
	DL with cream (higher than 9.0 g.100 g^{-1})
Additional ingredients	DL or DL without additional ingredients
	DL with additional ingredients

Table 1.
Dulce de leche (DL) classification for fat content and addition of other food substances.

The sales terms for dulce de leche established by the Technical Regulation are as follows:

a. DL

b. Confectionery DL

c. DL with _____ filling (in the blank with the name(s) of the product(s) added. It may optionally be called "Mixed DL.")

d. Ice Cream DL or DL for Ice Cream with _____ as appropriate, when the product is intended for making ice cream

The Technical Regulation further defines the sensory requirements for paste DL consistencies, for DL without apparent crystals or in semisolid or solid form, and partially crystallized DL where the moisture does not surpass 20 g.100 g^{-1} of product.

DL can be produced as a paste or bar. The production processes for both differ in the amount of sucrose used and in end product determination.

2.1 Paste dulce de leche

DL produced for the consumer is classified as paste DL and bar DL. Paste DL, as the name would indicate, is produced as a creamy paste, with uniform texture, no crystals, a distinctive brownish color, and a characteristic taste. It is generally made to be eaten as a dessert and used in pie fillings, roll cakes, and bars, among other sweets.

2.2 Bar dulce de leche

Bar DL is crystallized into blocks, which have a uniform coloration and texture. The bars are typically eaten in pieces.

The difference in sweetness between paste and bar DL is determined by the total solid and sucrose content and from the manufacturing process. For paste DL, production involves a slow, manual process that induces lactose and sucrose crystallization in the product.

Sucrose and lactose content is higher in the syrup used to make bar DL than it is in that used to paste DL and consequently increases crystallization. The increased crystallization is necessary for the product to become solid and firm enough to be cut and served in pieces. To this end, the original solution is subjected to a controlled-crystallization process or batching. In controlled crystallization or churning, the product undergoes intense stirring as it is slowly cooled. This process induces

the formation of numerous sucrose and lactose crystals, which ultimately alter the texture of the product.

Due to the greater crystal concentration and manual stirring of bar DL, the processing time is increased, which also implies in an increase in costs (steam, electric power, ice water, etc.) and payroll. However, the higher sucrose content results in a higher yield, which can offset rising costs as long as operational losses are controlled.

Figure 1 presents the compositional differences between bar and paste DL made from the same milk, with a dry matter (DM) concentration of 12.21 g.100 mL^{-1}. The addition of sucrose to the bar DL is greater (300 kg) than that for the paste DL (200 kg), in this example.

2.3 Dulce de leche with cream

According to the Technical Regulation, the term "with cream" must be included when the physicochemical fat content of a product is greater than 9.0 g.100 g^{-1} of DL.

Although consumers enjoy the texture of DL with cream, the product is not generally manufactured on a large scale, because of its higher caloric content. Only a small number of consumers are willing to indulge and enjoy this delicacy.

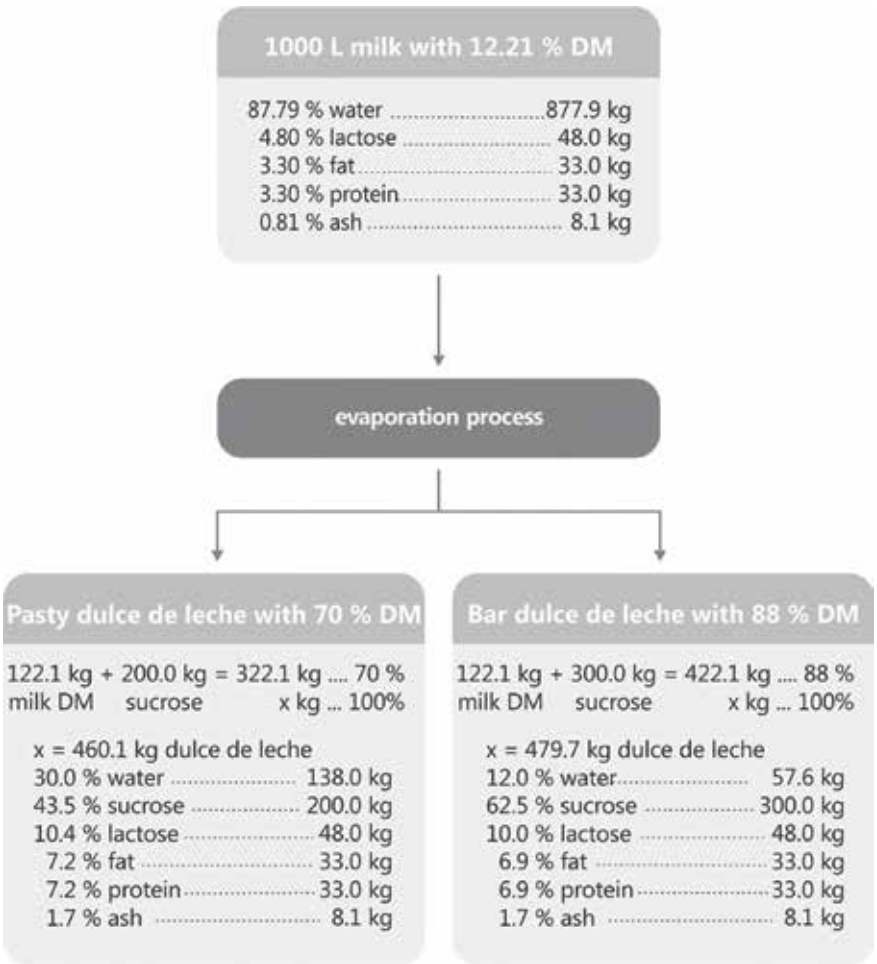

Figure 1.
Differentiation between bar and paste DL compositions.

Few large manufacturers in Brazil produce this type of dulce de leche. Because the cost of manufacturing is higher, the companies that do make DL with cream need to develop specific marketing strategies because not many consumers know how to identify the difference between DL with cream and conventional paste DL.

2.4 Dulce de leche with additional ingredients

DL with cocoa, chocolate, almonds, peanuts, dried fruits, cereals, and/or other ingredients (apart or mixed in), which are not thickening/stabilizing/wetting additives authorized in the Technical Regulation, must be labeled "DL with _____" with the name (s) of the product (s) added entered into the blank space. It may optionally be labeled "Mixed DL."

DL with additional ingredients has attracted consumer interest because of the new flavor opportunities it provides that can be extremely pleasing to the palate. Manufacturers have also shown great interest in DL with additional ingredients as a means to add value to specific products.

There are two primary methods for adding ingredients to dulce de leche.

The first is the direct addition of the product to the mass of the dulce de leche, which results in a uniform end product. These additional ingredients must be added at the end of the manufacturing process in order to be adequately heat-treated during production. The most common DL flavors found on the market are plum, peanut, coffee, chocolate, coconut, strawberry, and walnuts. These DLs are generally used to make desserts.

The second type of DL with additional ingredients involves packaging a fruit jelly or compote with the DL product to allow consumers to choose flavors and mix combinations according to their own tastes. Dessert DLs with additional ingredients are very common in the southern region of the State of Minas Gerais. A large market for these products can also be found in the northeastern region of Brazil where it is considered a specialty of the region. For these DL products, seeing the product itself (and not just the packaging) is very important to consumer acceptance. Pineapple, açaí, plum, blackberry, cashew fruit, cherry, cupuassu, cherimoya, guava, kiwi, passion fruit, strawberry, grape, and peach jellies are the most common DL add-ins.

It is important to note that optional additives are allowed according to Principle of Transfer of Food Additives (Codex Alimentarius Vol. 1A, 1995 Section 5.3), but their concentration in the final product must not exceed the corresponding maximum proportions permitted and, in regard to the additives indicated in the Technical Regulation, should not exceed the maximum limits authorized.

2.5 Dulce de leche for ice cream and dulce de leche for ice cream with additional ingredients

DL may be labeled "DL for Ice Cream" or "DL for Ice Cream with _____" when its intended use is in the preparation of ice creams. This sales description is required when the product is added as caramel (INS 150 a, b, c, d).

Production of this type of dulce de leche has also grown due to the increase in the demand for new frozen desserts.

Because it is one of many ingredients used in ice cream and frozen desserts, dulce de leche manufactured for ice cream use needs to have a pronounced flavor and intense color. Moreover, characteristics that inhibit freezing need to be verified during production. Lactose crystallization plays the most important role in determining these characteristics. Manufacturing technologies that use the enzymatic hydrolysis of lactose during DL production for ice cream are recommended to inhibit crystallization.

2.6 Confectionery dulce de leche

DL that has been enhanced with authorized thickening/stabilizing additives and/or humectants allowed in the Technical Regulation are referred to as "Confectionery DL."

This market probably is probably the most competitive among DL manufacturers. It is characterized by 10-pound can packaging and large-scale manufacturing processes. Confectionery DL must meet certain requirements such as pronounced flavor (to enhance filling taste), high viscosity (for easy molding), and low microbial counts (to account for the additional manipulations involved). The technology used must address these needs, which is why thickeners and higher sucrose contents are used. The Technical Regulation allows for the addition of several thickeners; however, starch remains the most commonly used thickener (maximum of 0.5 g.100 mL^{-1} on milk volume). Sucrose can be used up to 30 g.100 mL^{-1} per milk volume.

2.7 Dulce de leche for industrial use only

When DL is produced exclusively as a raw material for industrial preparation of other foods and contains a concentration of sorbic acid and/or its Na, K, or Ca salts higher than 600 mg.1000 g^{-1} up to 1000 mg.1000 g^{-1} (both expressed in sorbic acid), the label must include the term "Industrial Use Only."

2.8 Special types of dulce de leche

2.8.1 Dulce de leche base paste and similar products

Because there are no specific technical regulations for products found on the Brazilian Market such as DL Base Paste, DL Paste, and DL Candy Flavor, at time of printing, we will not include a discussion of their legislation.

The use of starch and glucose is almost a requirement when manufacturing these products. It is currently possible to develop different product profiles for a variety of applications by the precise selection and inclusion of a starch in the product during manufacturing. The starches are modified in order to give the final product the plasticity, brightness, and consistency that baking and candy-making professionals expect for their fillings.

In sum, modified starches with improved gelling ability have been used for this purpose, which, in addition to lowering the cost, are easy to implement in the manufacturing process. Gelling occurs during the cooling and storage phases of product production. The gelation is due to intermolecular interactions of the polymers that form a three-dimensional network and confer the viscoelastic properties on it. Gelling time depends on the formulation type, package size, and storage temperature. Average gelling in this type of DL varies from 4 to 14 days after the end of the concentration/evaporation phase.

2.8.2 Diet dulce de leche

The already strong market for diet products continues to grow each year at rates that have driven some companies to specialize in the production of diet DL. The production technology for diet dulce de leche is advanced and very complex due to the limits placed on sucrose addition; these have a direct impact on the characteristics of the product both during manufacturing and after packaging.

Sucrose in dulce de leche influences taste, yield, consistency, brightness of the final product, and protein adherence to equipment surfaces.

Thus, unlike other dairy products where sucrose is substituted for other high power sweeteners and substantially minimizes sugar withdrawal, the technology needed to make diet DL (without the addition of saccharose) calls for different ingredients that answer them. Sorbitol, pectin, modified starches, dairy proteins, and sweeteners are the most common additives used to make diet DL.

2.8.3 Milk drop (Pingo de Leite)

Marketed in small drop-like pieces, milk drops have a firm coating with a soft, creamy interior. The dual texture characteristics are achieved by rapidly cooling and partially dehydrating the DL as it is poured in thin layers onto special trays. Generally, these trays have designs (company name or logo) that are embossed into the product.

3. Dulce de leche manufacturing technology

Originally hailing from Latin America, dulce de leche (DL) is one of the most widely manufactured dairy products in Brazil, where it is marketed as a paste or bar. Due to DL's low moisture content, the product can be safely stored at room temperature [8]. The primary ingredients used to manufacture DL are milk, sucrose, and an acidity reducer. Needless to say, the raw materials must be of good quality from reliable suppliers in order for the final product to have the desired characteristics. The milk used to make DL must be microbiologically safe (total bacteria count <300,000 cfu mL^{-1} in raw milk), remain stable during thermal processing, and preferably exhibit a high solid content (>11.4 g.100 g^{-1} of dry matter) [1].

Total processing time can vary from 40 minutes to 4 hours. Processing time depends on the type of equipment used and the amount of steam injected. Processing time plays an important role in a product's viscosity, color, and flavor and ultimately determines the characteristics of the final product [7].

Processing end time for dulce de leche can be verified in two different ways: by determining the soluble solids content (above 66°Brix) or by observing how a small droplet of DL behaves when submerged in water. Once the desired solid content/consistency is reached, the final product should be cooled to 75–80°C and then packaged while still warm in cans or glass containers that have been filled to the top to eliminate any air and prevent contamination. In general, the yield is 2.5 L/kg of DL. The product is then stored at room temperature for a period ranging from 160 to 180 days [8].

DL can either be handmade in batches or continuously produced in pans and vacuum evaporators or evaporators attached to pans. The pan used is generally a dual-ply container with a stainless steel interior and an outer wall, which lets steam pass between the two layers while also conducting heat [7].

3.1 Open top pan

The technology used to manufacture dulce de leche is determined by the equipment used. Often, quality improvement is limited not by cost or lack of skills, but by the very equipment that is used to make the product.

It is possible to make DL in 30 minutes or up to 6 hours using the same volume of milk. This shows how important equipment choice can be, since time factor can directly impact the competitiveness of a specific dairy plant.

In the traditional manufacturing process (most common in Brazil), the milk and sugar mixture (syrup) is concentrated directly in double-walled stainless steel pans, through which steam flows. After the syrup has been prepared, gradual heating is applied to the liquid, which is continuously stirred. This stirring prevents the liquid in direct contact with the walls from scorching. Stirring also reduces foam formation, which allows water to evaporate more quickly from the syrup. Once the syrup reaches the desired consistency, the cooling process begins until the dulce de leche temperature has dropped to 75°C. The cooling step helps avoid high temperatures within the DL mass, which would otherwise affect the color and texture uniformity (**Figure 2**).

Primary components of an open-top dulce de leche pan:

1. Manometer: Pressure gauge inside the steam jacket. Controls the processing time and regulates safety during processing.

2. Purge: Eliminates the condensate from the steam jacket, increasing energy exchange (heat) efficiency.

3. Stirrer: Promotes syrup movement during the concentration phase, homogenizes temperature, facilitates water evaporation, and prevents scorching.

4. Reducing box: Reduces engine rotation and transfers motion to the shaker.

5. Steam jacket: Provides indirect heat via steam circulation. It covers approximately one-third of the area of the tank.

6. Security valve: Opens and expels steam when the pressure in the pan is above the working pressure of the equipment in order to prevent excess pressure.

7. Reducing pressure valve: Keeps the pressure in the steam jacket constant to avoid levels above safe working pressure.

8. Exhaust system: Accelerates the concentration process by removing excess steam from interior of the pan to prevent it from condensing.

9. Lid: The pan lid usually provided at the time of purchase by the pan manufacturer. However, this device is not often used in many plants, especially those where is no exhaust installed in the equipment (though this is not recommended). Using the open pan without a lid can lead to foreign objects and debris falling into the product during processing, mainly due to operator carelessness. Operating the system without a lid can also lead to unprotected bulb breakage and lining, top, accessory, and other equipment (stirrer locks and set screws) deterioration.

3.2 Vacuum evaporation

Vacuum evaporation consists of withdrawing water by applying energy in the form of heat to milk in a pressurized chamber that is lower than atmospheric pressure. This process promotes evaporation at temperatures between 40 and 75°C, which minimizes the changes caused to milk constituents due to heating. Downstream tubular evaporators are the most commonly used vacuum evaporators in dairy production. These can be coupled to systems as a finisher and flash cooler that finalize the concentration phase and rapidly cool the concentrated product [9].

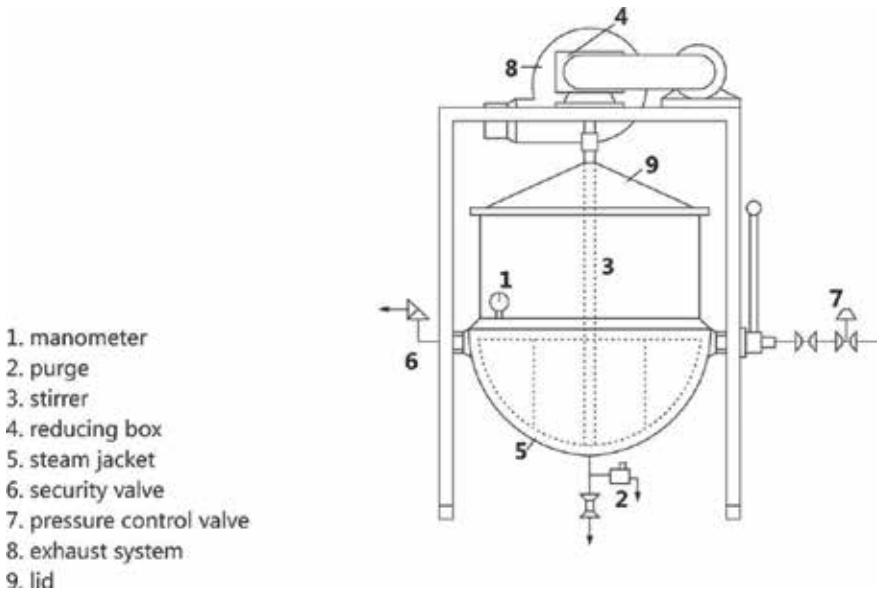

Figure 2.
Open-top dulce de leche pan.

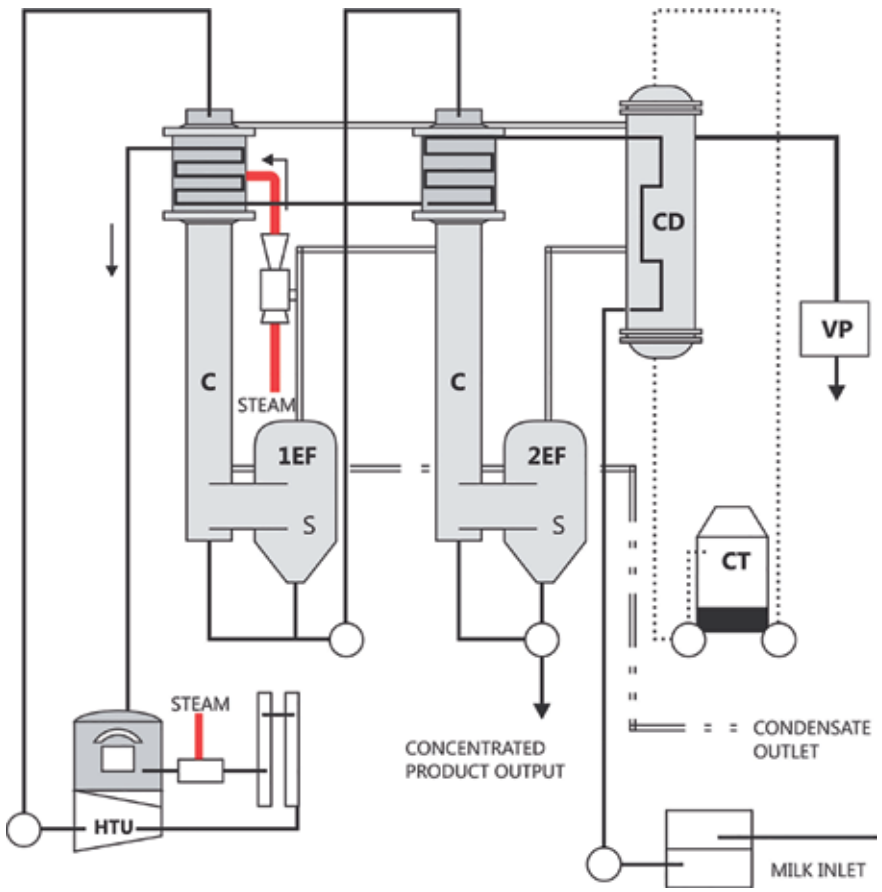

Figure 3.
Diagram of a vacuum evaporator. C = evaporator calandria; S = liquid vapor separator; EF = effect; HTU = heat treatment unit; CT = cooling tower; CD = condenser; VP = vacuum pump.

The four main components of a vacuum evaporator are the evaporator calandria, the liquid vapor separator, the condenser, and the vacuum pump. The evaporator calandria and separator set is called the evaporation effect, so a machine that features five evaporator calandrias and five liquid vapor separators is a five-effect evaporator. **Figure 3** shows a diagram of a two-effect vacuum evaporator.

The evaporator calandria consists of a heat exchanger made up of tubes and an outer casing surrounding these tubes. The product to be concentrated is passed through the tubes while the heating medium is propelled (by steam or evaporation) inside the casing. An evaporator calandria's inner tubes transfer heat energy from the heating medium to the boiling product with the sole aim of promoting the passage of water from the liquid state to the gaseous state. The liquid vapor separators are directly connected to the evaporator calandrias and separate the concentrated product from the evaporated mass produced. The condenser, along with the vacuum pump, decreases pressure throughout the equipment.

4. Dulce de leche manufacturing processes

DL can be produced in four ways:

a. Manually

b. Open-top pan

c. Split addition of the mixture to the open-top pan

d. Preconcentration in vacuum evaporator and termination in open-top pan

We will address the latter three processes because they represent the bulk of DL production in Brazil. DL-processing technology consists of the evaporation of water by indirect application of heat in equipment known as evaporators, or, more commonly, pans. The manufacturing process is shown in **Figure 4**.

The mixture of milk, sucrose, and other ingredients and additives is called the syrup. The DL syrup is subjected to continuous water evaporation by means of transferring heat energy indirectly using steam (pressure range: 100–600 kPa) from boilers. The vapor used becomes vapor condensate, water at 85–90°C, and the

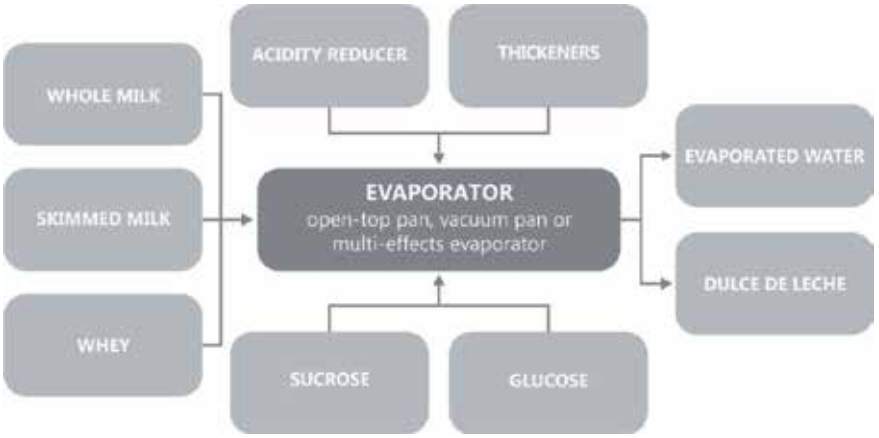

Figure 4.
Dulce de leche (DL) manufacturing process diagram.

quantification of this makes it possible to determine the total mass of steam used in the manufacturing process.

The water withdrawn in the gaseous state is called evaporated water and must be removed from the equipment rapidly to avoid condensation and reincorporation into the syrup. Hence, an exhaust fan system is required to remove the evaporate.

During evaporation, constant stirring is performed by mechanical agitators at a speed of 72 and 80 rpm. This stirring is necessary to minimize deposit formation on the heat exchanger equipment walls. Processing time may vary from 30 minutes to 4 hours, depending on heat, exchange area, milk volume, and steam pressure used.

Color, flavor, and viscosity of the dulce de leche are directly related to its processing time, which ultimately determines the characteristics of the final product. DLs produced in a very short time have a pale color, a less pronounced flavor, and a low viscosity.

The most common heat exchanger used to produce dulce de leche is a pan made of an inner stainless steel wall and an outer wall that allows steam to enter and circulate between the two. The working capacity of pans varies from 25 to 1500 L of syrup (**Figure 5**).

In most cases, the evaporation phase is started when all milk and sugar are added at the beginning of the process, though in some factories, only some of the milk and sugar are used at the beginning of the process and the rest is gradually added throughout (split addition of the mixture to the open-top pan). This second method allows a higher quantity of syrup to be processed than that which volume capacity of the pan would ordinarily allow, as shown in **Figure 6**. An additional advantage of this type of technology is related to reduction on process time as a consequence of a higher pressure in the heating medium during evaporation. The higher solid content in the syrup during evaporation process leads to boiling over reduction (**Figure 7**).

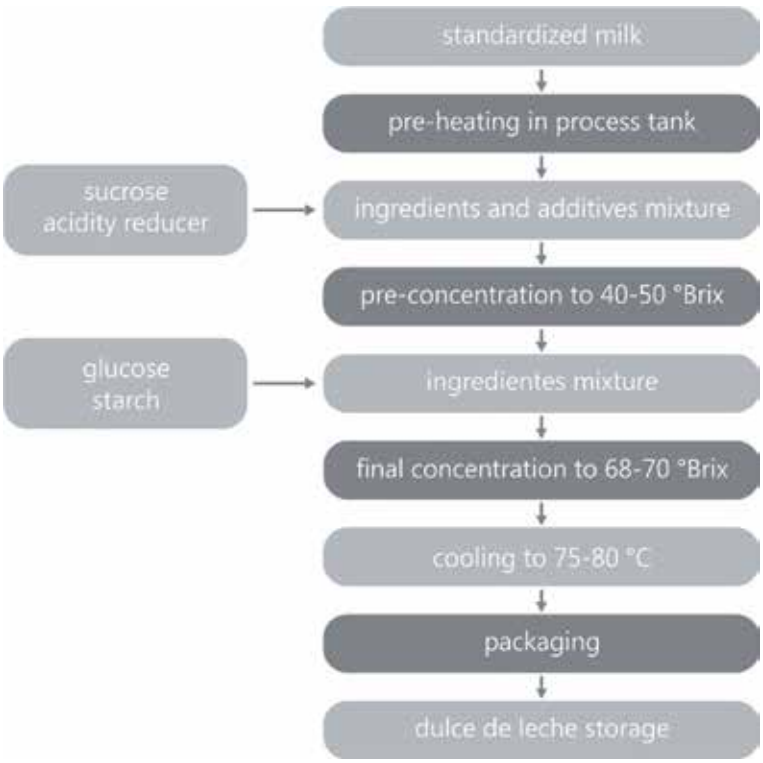

Figure 5.
Open-top pan manufacturing process.

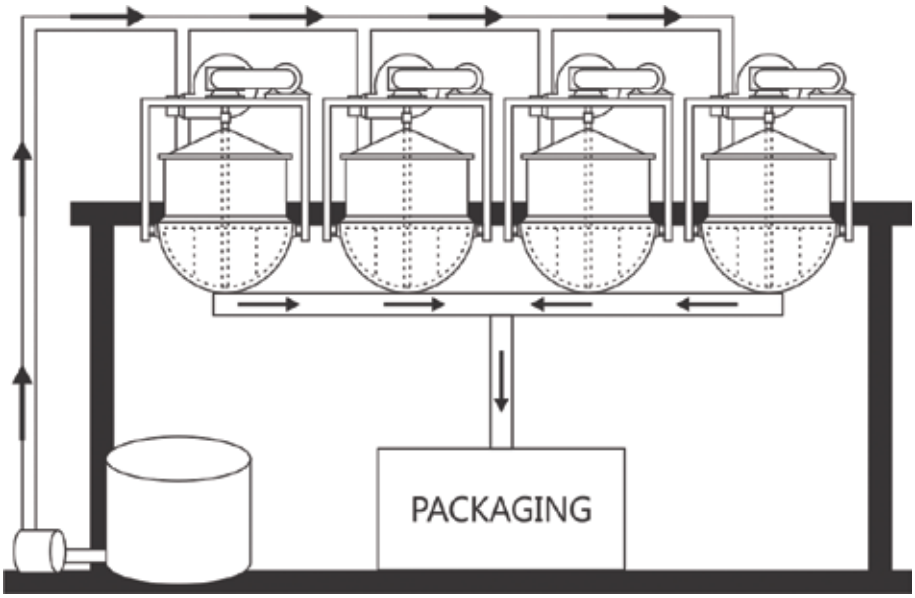

Figure 6.
Split mixture addition to an open-top pan.

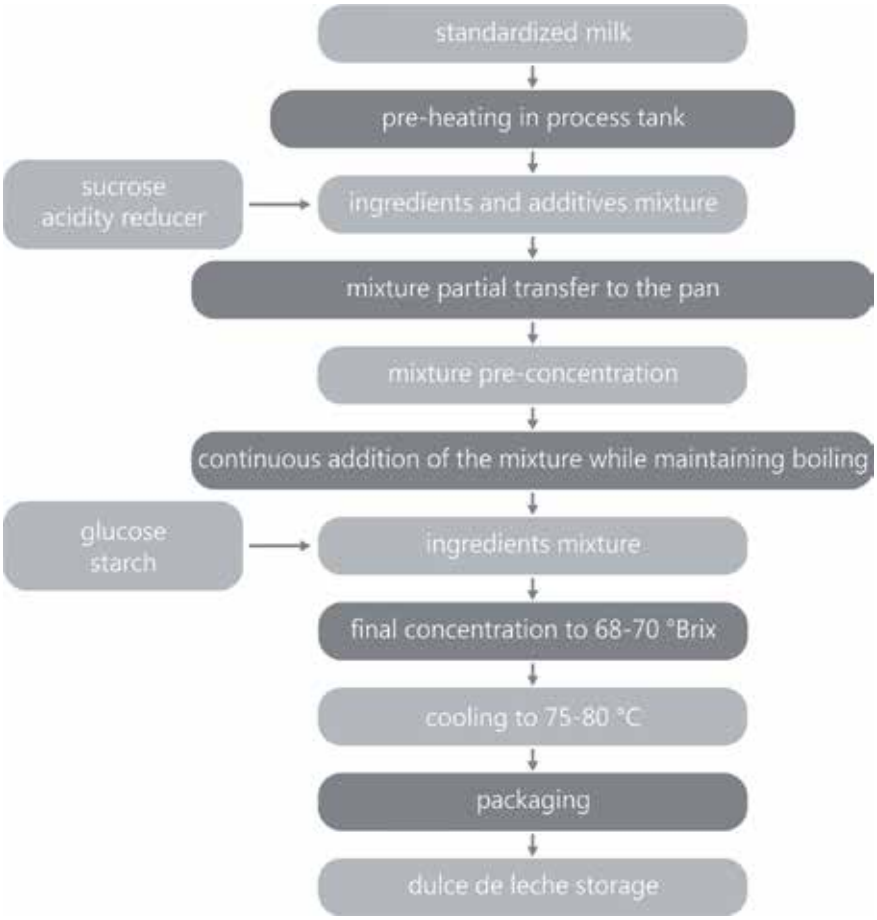

Figure 7.
Split addition of the mixture processing in an open-top pan.

In large-scale productions, the volume of milk to be evaporated exceeds 30,000 kg of milk per day; it is common to use vacuum evaporators in the preconcentration stage of the fluid milk, with the objective of lower energy waste (preconcentration in vacuum evaporator and termination in open-top pan). As shown in **Figure 8**, DL can be obtained using vacuum evaporators combined with open-top pan.

The vacuum evaporator considerably reduces steam consumption in the industrial manufacturing settings and increases production capacity. While an open pan uses approximately 1.1–1.3 kg of steam to evaporate 1.0 kg of milk water, an evaporator requires only 0.1–0.3 kg of steam to evaporate the same amount of water. The disadvantage of the vacuum evaporator is that it cannot be used as the sole equipment to produce dulce de leche because it does not allow the necessary physicochemical modifications in the syrup. Thus, the process calls for concentrating the milk and sugar syrup under vacuum conditions of 62–66°Brix, and then the syrup is concentrated in an open-top pan for another 20–40 minutes so that it can achieve the viscosity and color required to meet desired standards (**Figure 9**).

Concentration in multistage evaporators is common in dairy plants that process milk powder, sweetened condensed milk, and evaporated milk. However, due to the high initial cost of equipment, these evaporators are only feasible in companies that process large volumes of milk. An evaporator is a device that works with a partial vacuum in order to lower the boiling temperature. Thus, the effects of heat on the milk protein structure and lactose caramelization are minimized. Each stage is actually an evaporator, but all stages subsequent to the first use the steam released from the precedent stage as a thermal source. In the multieffects system, the steam generated in the first stage and the partially concentrated liquid flow into the vapor separator where they are separated by centrifugal force; the pressurized product is then forced into the vaporization chamber of the next stage, while the vapor is also pushed into the evaporator calandria where it is condensed by yielding energy in the form of heat to the liquid to concentrate.

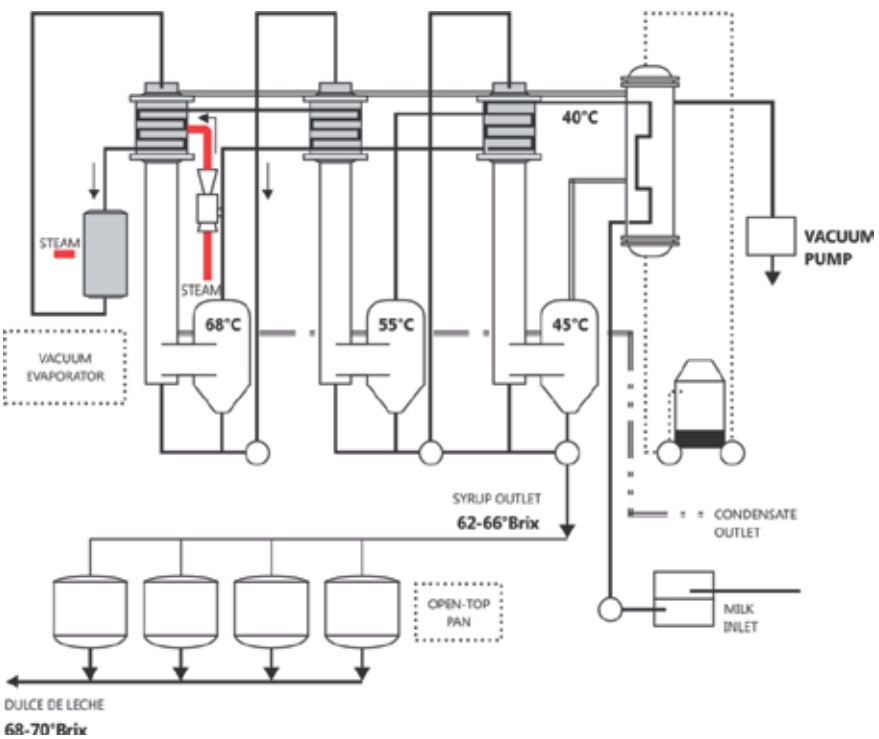

Figure 8.
DL processing with preconcentration in a vacuum evaporator and final phase in an open-top pan.

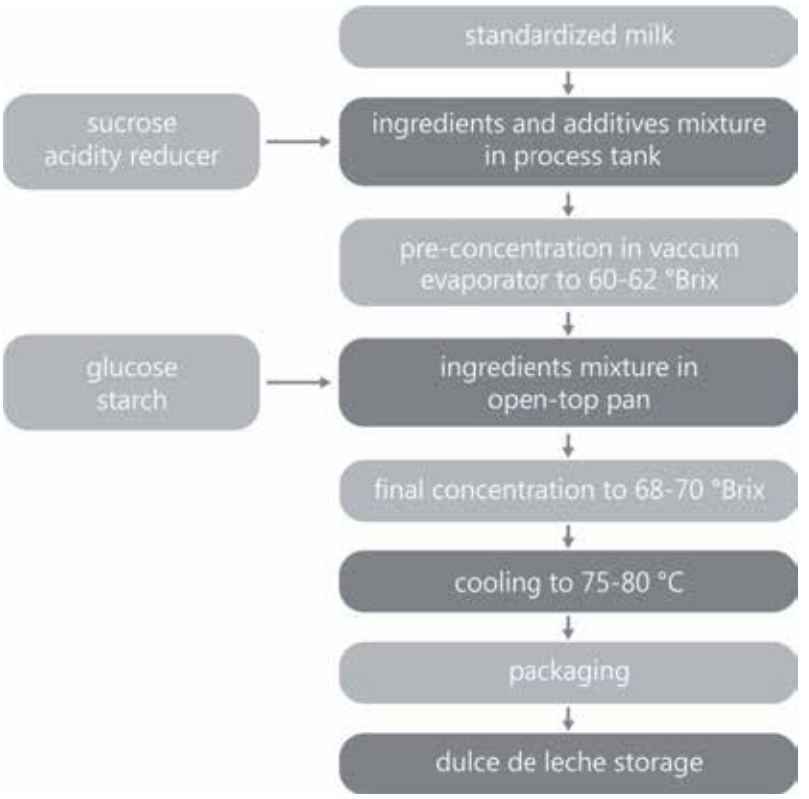

Figure 9.
Processing with preconcentration in vacuum evaporator and termination in open-top pan.

Falling film evaporators fitted with a top feed that use gravity to draw the liquid down before it enters centrifuge stage in heated tubes are the most common evaporators used to make dulce de leche. Plate evaporators have also been used; these concentrate the milk quickly, which saves saving time, steam, and labor during processing.

Multistage evaporator concentration occurs at low temperatures (compared to the conventional open-top process) of 70–48°C. Therefore, neither the Maillard reaction nor a substantial increase in product viscosity (fundamental characteristics in DL have a chance to develop). This makes it necessary to finish processing in a conventional open-top pan to give the dulce de leche the desired organoleptic characteristics.

5. The Maillard reaction

Different types of food darkening can occur in both home and industrial kitchens. Enzymatic darkening and discoloration originates from reactions catalyzed by an enzyme known as polyphenol oxidase (PPO). This enzyme acts mainly on fruits and vegetables, generating negative consequences such as economic losses, decreased nutritional value, and undesirable changes in flavor and appearance of foods such as such as tea, coffee, cocoa, and prunes [10].

There is also a nonenzymatic browning that can occur. It is slower than enzymatic browning because it does not have the reaction catalyzing enzyme. This nonenzymatic browning is characterized by caramelization, the Maillard reaction,

and ascorbic acid (vitamin C) oxidation. Each food has its own specific darkening profile since reaction speed is dependent on the nature of the reactive components of the food [11].

Among the nonenzymatic darkening reactions, the Maillard reaction will be highlighted here. The reaction was discovered in 1912 by Louis-Camille Maillard while attempting peptide synthesis under physiological conditions. It is of great interest at present time as it has been shown to be related to chemical, organoleptic, nutritional, toxicological, and in vivo manifestations [12].

The Maillard reaction is in fact a complex cascade of reactions (**Figure 10**), which take place primarily during heating and prolonged storage of food products. These reactions can result in positive or negative changes in food quality because it favors the formation of compounds responsible for aroma, flavor, and color of heat-treated foods. The Maillard reaction is divided into three stages: the initial stage, intermediate stage, and final stage [10].

The initial stage involves the condensation of the carbonyl group of the reducing sugar with the free amino group of amino acids, peptides, or proteins. It occurs through the nucleophilic attack of the nitrogen's electron pair of the amino group, leading to the beginning of the reaction. As a result of the condensation, an unstable Schiff base is formed, which in turn releases water and then forms a glycosylamine. The Schiff base undergoes these sequential rearrangements to produce a reasonably stable aminoketose known as the Amadori product (aldose sugar) or Heyns product (ketose sugar). These initial stage products are stable and do not have color, fluorescence, or ultraviolet visible absorption. As a result, there is a huge variety of products in different proportions [12].

The second phase of the Maillard reaction takes place upon prolonged heating prolongation or storage. The Amadori products or Heyns products become fragmented and give rise to a series of reactions including dehydration, enolization, and

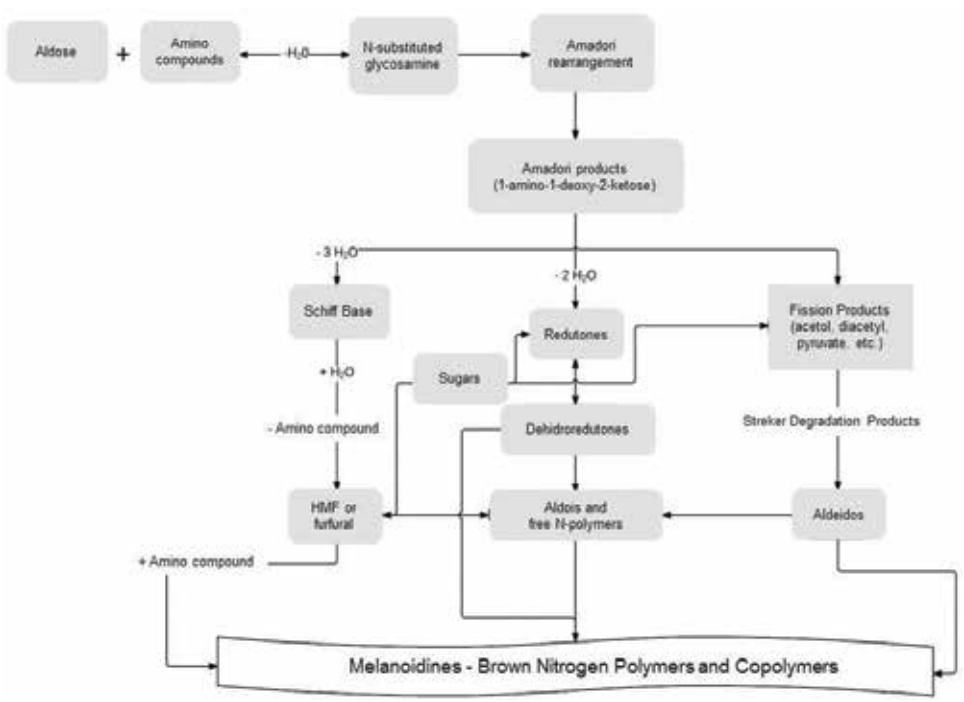

Figure 10.
Three stages of the Maillard reaction in food, adapted by the authors from Refs. [13–15].

retro-aldolization. In this intermediate stage, dicarbonilic compounds, redutones, furfural derivatives, and Strecker degradation products appear and induce the appearance of a furan derivative that becomes the origin of a hexose commonly known as 5-hydroxymethylfurfural.

The compounds that originate during the intermediate phase are fluorescent and absorb ultraviolet radiation. They are cyclic and highly reactive, polymerizing with lysine or arginine residues in proteins to create stable compounds that culminate in the formation of dark pigments known as melanoidins. These pigments lead to the desirable or undesirable coloring of foods that make up part of the final stage of the Maillard reaction [12].

The Maillard reaction can be affected by temperature and pH, among other factors. The Maillard reaction rate is slower at lower temperatures and practically doubles at every 10°C increase between 40 and 70°C. pH also exerts an effect on the intensity of the reaction, with maximum discoloration occurring in an alkaline range between pH 9 and 10 [12].

The amine type directly influences onset of the Maillard reaction. Highly reactive amino acids such as lysine, glycine, tryptophan, and tyrosine facilitate the reaction, whereas proline, leucine, isoleucine, hydroxyproline, and methionine show medium reactivity and histidine, threonine, aspartic acid, glutamic acid, and cysteine demonstrate low reactivity. Lysine, because it has the free epsilon amino group, demonstrates high reactivity because it is more susceptible to the reaction (carbonylamino). It may therefore reduce the nutritional value of the food [10].

A reducing sugar is essential for the Maillard reaction to occur; pentoses are more reactive than hexoses, which in turn are more reactive than disaccharides. This type of browning occurs more frequently with intermediate values of water activity (0.5 and 0.8) and a relative humidity between 30 and 70%. At low water activity (0.2–0.25), the velocity tends to zero due to a decrease in solvent. At high water values (0.9), the reactants are extremely diluted, which decreases the darkening rate [10].

In addition to these factors, metal ions (iron and copper), sulfite, storage conditions, light, type, time, and temperature of the heat treatment and cooking methods may interfere with the reaction [12].

The extent to which the Maillard reaction occurs can be monitored by the appearance of certain compounds, including furosine, hydroxymethylfurfural and carboxymethyllysine. The appearance of these compounds offers an indication of the intensity of the thermal processing and nutritional changes related to the reaction as well [16].

Thus, the color of the dulce de leche is basically due to the Maillard reaction. The use of different ingredients, with modification of, for example, the type of sugar and the amount of acidity reducer during processing may influence the development of the Maillard reaction. Among the ingredients used to make DL, glucose and acidity reducers exert the most influence over the development of this nonenzymatic darkening reaction.

Glucose, an optional ingredient in dulce de leche production, is a monosaccharide made up of monomeric units that form sugars of greater size when united. It is less sweet than sucrose, which enables it to reduce the development and growth of lactose crystals while increasing the viscosity and brightness of the final product. The maximum glucose/sucrose substitution is 40% according to the Technical Regulation of Identity and Quality of DL. The authors suggest adding 2 g.100 g^{-1} of glucose by weight of milk in order to improve the final product's texture and brightness. The color of dulce de leche made with glucose tends to be darker because glucose is a reducing sugar, which promotes an increase of the nonenzymatic darkening reaction.

Baking soda is a fundamental element in DL technology because it plays the role of acidity reducer. Adding bicarbonate at the beginning of the manufacturing

process helps reduce initial acidity of the syrup (milk + sugar) and maintains the pH of the milk during the concentration stage. Thus, sodium bicarbonate acts as an extra source of alkalinity. It prevents the destabilization of casein micelles caused by the decrease of the pH that occurs during evaporation. This decrease could be due to the concentration of calcium phosphate and the formation of organic acids that occur during lactose degradation and phosphoric casein ester hydrolysis [1].

6. Conclusions

It has been shown that dulce de leche is typically produced, marketed, and consumed by small, medium, and large dairy manufacturers in South America. Dulce de leche is made via concentration and heat processing of liquid milk plus sucrose. The color and taste of dulce de leche are primarily determined by the Maillard reaction, which is enhanced by the heating processes used. Dulce de leche may be consumed as a paste or bar. Both may be manufactured on small and large scales, in open-top pans or in evaporators. The DL Technical Regulation classifies dulce de leche according to fat content and additional ingredients. It has been noted that certain production variables can influence dulce de leche's physical-chemical, compositional, and sensorial characteristics. Therefore, understanding the product's chemistry, the technology used to manufacture it is relevant in order to develop more extensive standardization practices of the products available to consumers.

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Conflict of interest

There is no conflict of interest.

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Chapter 8

Production, Processing, Commercialization and Analysis of Costumer Preferences of Sheep Cheese in Chile

María C. Barrón Rivas, Carlos Palacios Riocerezo, Ignacio A. Dominguez Vara, Manuel Gonzalez Ronquillo and Sergio Radic Schilling

Abstract

The constant increase in the demand for dairy products and their derivatives has generated a higher consumption of dairy products (113.5 t/year). Chile has not been the exception; in 2017, it presented an average per capita consumption of 11.1 kg of cheese, making it the largest consumer in Latin America, followed by Argentina (7.5 kg) and Uruguay (5 kg). Although Chile does not have a strong tradition of consuming sheep's milk cheese compared with other (European) countries, in recent years, there have been changes in the demands and expectations of cheese consumers, who demand higher quality and a wider range of varieties, thus establishing the potential for further products to enter the market. The global cheese industry has taken charge of these trends through the phenomenon of granting a premium value to certain products, adding new ingredients and flavors, producing limited editions of certain products, favoring traditional recipes and craft methods, and highlighting specific localities of origin. Given a greater appreciation for and popularity of locally produced cheeses and value-added products, particularly those with the highly valued "Designation of Origin" label, this would offer a potential for expansion in the Chilean market.

Keywords: sheep, cheese production, marketing, Chile

1. Milk production

1.1 Global production of sheep's milk

The constant population growth and the increase in the demand for dairy products and their derivatives have generated a continuous increase in the world per capita consumption of milk, which reached 111.3 kg in 2015, and an increase of 12.5% is estimated by 2025 [1]. However, there are significant regional disparities among developing countries, where fresh dairy products remain, by a large margin, the most consumed, unlike in developed countries where consumer preferences

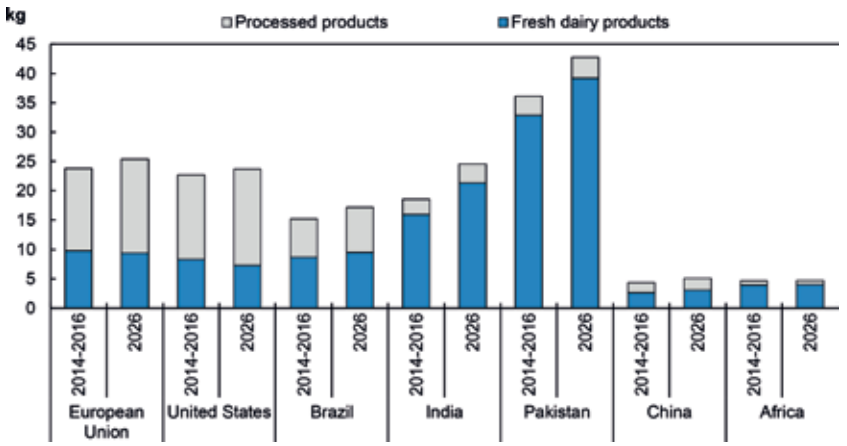

Figure 1.
Per capita consumption of processed and fresh dairy products. OECD-FAO, 2016 (adapted from [2]).

incline toward processed products (butter, cheese, skimmed milk powder and whole milk powder) (**Figure 1**) [2].

The world production of milk of all species has followed an upward trend in recent decades, reaching in 2015 approximately 818 million t [1]. The highest percentage of dairy production is derived from the dairy industry (82.6%), followed by buffaloes (13.9%), goats (1.9%), sheep (1.3%), and finally, camels, which contribute only 0.3% of the total [2] (**Figure 2**).

The dairy industry is markedly regionalized and is associated with a long tradition of production and consumption of dairy products, mainly cheeses. The world production of fresh sheep's milk registered in 2016 by FAO estimates 10,366,980 t. This is mainly produced in the Asian region (44.6%), which houses the two main producing countries: China (1,361,360 t) and Turkey (929,432 t). In the European continent (32.7%), Greece (711,577 t) and Romania (631,419 t) stand out, ranking as the third and fifth largest producers of sheep's milk, respectively. The African

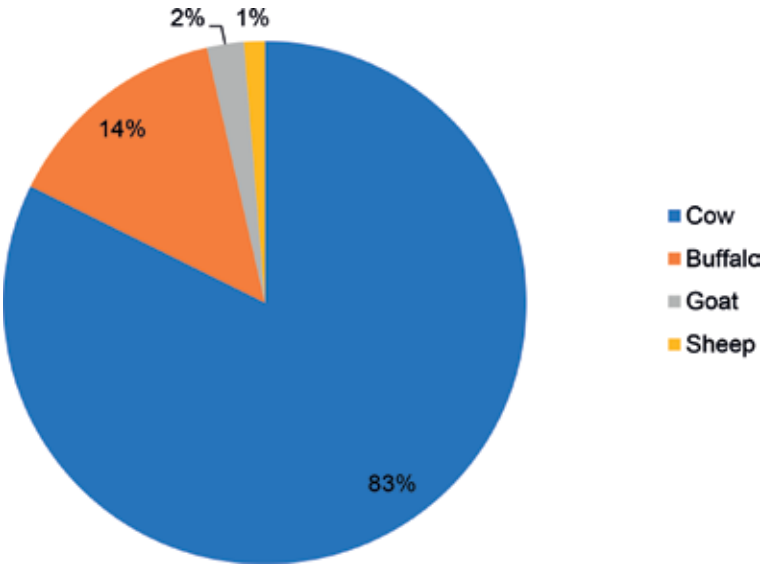

Figure 2.
World milk production by species. FAO 2016 [2].

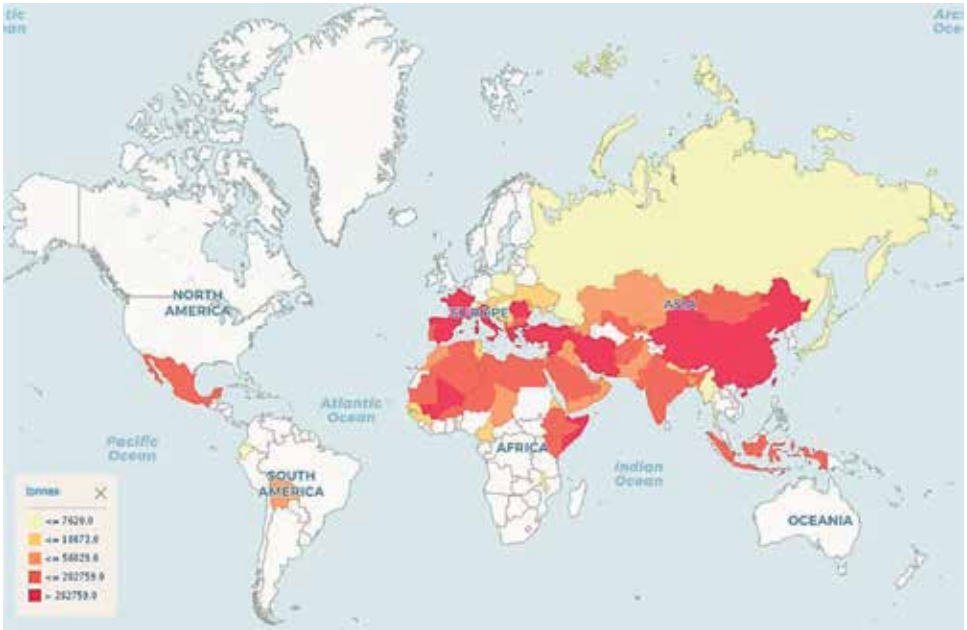

Figure 3.
Geographical distribution of sheep's milk production. FAO 2016 [3].

continent produces almost a quarter of the international sheep milk inventory (21.8%), with subsistence production systems; countries such as Syria (651,867 t), Mali (529,373 t) and Sudan (403,008 t) are among the top 10 producing countries worldwide. The American continent has a relatively minimal participation in the world production of sheep's milk (0.9%); only the following countries provide official productivity data: Mexico (57,589 t), Bolivia (29,617 t) and Ecuador (3617 t) (**Figure 3**) [3].

Sheep's milk production in the North American countries is not significant; it is estimated that Mexico, the United States and Canada have approximately 200 production units dedicated exclusively to the production of milk, with less than 10,000 sheep each [3, 4]. Data from associations of producers estimate that in 2010, there were 20 production units with the potential to milk sheep with an approximate inventory of 6000 animals distributed in the states of Coahuila, Guanajuato, State of Mexico, Puebla, Querétaro and Veracruz (**Table 1**). In 2009, Querétaro was the state that presented the highest production, with 30,000 liters. Likewise, in this state, the first association of dairy sheep called "Producers of Milk and Derivatives of Sheep S.A. of C.V." united 13 producers and integrated the collective brand "Del Rebaño" [5].

In Chile, the sheep cheese market, unlike that for goat cheeses, is very underdeveloped, given the low national consumption tradition and the low consumer culture regarding this product. However, in the country, there are foreign colonies of immigrants from Mediterranean countries that have favored the expansion of these products. In 1995, the first exploratory imports of sheep's milk cheese were carried out, with a volume of 1.25 t, and a positive evolution was observed, which was manifested the following year with an increase to 3.88 t. In 1998, the total national supply in the sheep cheese market reached 7.2 t, of which two were of national origin and the rest imported mainly from Spain and France. Currently, the national demand is less than 6 t per year, a figure that represents a volume of milk of less than 40,000 liters. The national production of sheep's milk associated with its industrialization and cheese making is estimated between 20,500

Local name	Localization
Rancho Santa Marina	Queretaro
Rancho San Josemaría	Queretaro
Quesos del Rebaño	Queretaro
Rancho San Francisco de Mariana	Queretaro
Escuela de Pastores	State of Mexico
Rancho Tierra Nueva	Puebla

Table 1.
Some units of sheep milk production in Mexico [8, 16].

and 22,000 liters per year, equivalent to between 3 and 4 t of cheese [6]. In 2014, the *El PASO* location assessed five regions that produce most of the country's cheeses: Los Ríos (49,394 t), Los Lagos (30,939 t), La Araucanía (6202 t), Biobío (906) and Metropolitana de Santiago (273 t) [7].

In Chile, two sheep dairy breeds have been introduced: Milchschaf and Latxa, which have been fostered through the crossing of dairy males with sheep from Chilean cattle ranchers, with the aim of reducing the initial investment costs. The dairy breed of the male or the genetic improvement program that is chosen will basically depend on three aspects: forage potential, adaptation of the dairy breed that is being introduced to the area, and the reproductive and productive characteristics of each one [8]. Within the dairy production and ovine cheese industry, a highlight is the initiative developed by the University of Magallanes in the city of Puerto Natales (Chilean Patagonia) that produces the southernmost cheese of Chile [9].

1.2 Sheep's milk: physicochemical characteristics and nutritional properties

Recent studies have determined that the chemical composition of milk varies depending on the feeding of the sheep, modifying the percentages of fat, protein, lactose and the fatty acid profile of the milk depending on the ratio of forage to concentrate in the diet or of the supplementation with protected fat. The results show that diets with the highest forage content (>40% DM) have a significantly lower milk yield (0.8 g/kg), but with a higher fat content (0.32 g) and concentration of conjugated linoleic acid (CLA) (2.28 mg/kg). The addition of protected fat in the rumen in the diets has a positive effect on the concentration of fat (0.22 g/100 g) and CLA (3.98 mg/g) in milk, but the protein concentration is reduced ($P < 0.001$). On the other hand, diets with a higher proportion of concentrate (>40% DM) affect the biodegradation processes and the synthesis of CLA (**Table 2**) [10].

The particular characteristics in the chemical composition of sheep's milk, mainly in relation to its high levels of total solids and protein, make it especially suitable for transformation into yogurt and cheese. About 95% of sheep's milk is converted into dairy products, which also acquire a regional connotation of origin and quality [11, 12]. For this reason, when we refer to the quality of sheep's milk, we must concentrate mainly on its ability to be transformed into high-quality dairy products, generate high yields of these products per liter of milk and ensure the safety of these foods [11]. In this way, three types of "quality" can be distinguished when analyzing products of animal origin: hygienic and sanitary quality, dietetic and nutritional quality and gustatory and gastronomic quality [12].

The unique characteristics of sheep's milk have been discussed in extensive reviews of literature [11–15], in which the quality of sheep's milk is evaluated in terms of its technological and coagulation properties. High concentrations of

Variable		Mean	Median	SD	Min	Max	Skewness	Kurtosis
Forage: concentrate ratio (F:C)								
Milk yield (L/d)	F	1.29	1.01	0.72	0.58	2.58	0.66	−1.26
	C	1.43	1.18	0.86	0.63	2.85	0.7	−1.27
Fat content (g/100 g)	F	6.17	5.95	0.75	5.1	7.31	0.16	−1.49
	C	5.87	5.97	0.76	4.5	6.88	−0.49	−1.13
Protein content (g/100 g)	F	5.48	5.34	0.7	4.9	6.85	1.12	−0.4
	C	5.56	5.45	0.79	4.8	7.04	0.76	−0.89
Lactose content (g/100 g)	F	4.72	4.69	0.12	4.6	4.98	0.96	−0.43
	C	4.79	4.77	0.15	4.55	5.05	0.23	−0.76
CLA content[1]	F	7.57	8.8	3.88	0.79	13.7	−0.41	−1.08
	C	5.4	6.4	2.48	1.33	8.6	−0.35	−1.55
Protected fat supplementation (S) or un supplemented (U)								
Milk yield (L)	S	1.58	1.56	0.75	0.18	3.17	0.3	−0.94
	U	1.56	1.52	0.79	0.2	3.06	0.39	−1.03
Fat content (g/100g)	S	7.45	6.47	2.21	4.35	12.1	0.46	−1.21
	U	7.03	6.48	1.7	4.8	9.91	0.34	−1.28
Protein content (g/100g)	S	5.2	4.93	0.87	3.74	7.49	0.77	0
	U	5.44	5.2	0.86	4.38	7.52	0.77	−0.3
CLA content[1]	S	1.89	1.59	1.2	0.33	5.6	0.93	0.46
	U	1.18	0.68	0.84	0.35	3.0	0.85	−0.81

[1]*mg/g of methylated fatty acids; F, forage based rations; C, concentrate based ration; S, protected fat supplemented rations; U, unsupplemented fat protected rations.*

Table 2.
Descriptive statistics of data used in forage:concentrate ratio and protected fat supplementation meta-analysis [10].

protein, fat and total solids in milk are associated with high yields in the production of dairy products; therefore, the aforementioned studies conclude that sheep's milk has higher yields compared to goat's milk and cow's milk because of its superior chemical composition (**Table 3**) [13].

Milk proteins include caseins and whey proteins; caseins are a family of phosphoproteins synthesized in the mammary gland in response to lactogenic hormones [17], which represent the highest protein share of sheep's milk (76–83%) [18]. The heterogeneity of the caseins is determined mainly by the presence of genetic variants; four genetic variants of caseins are recognized: αs1-CN, αs2-CN, β-CN and κ-CN. Sheep's milk has higher concentrations of the four casein variants compared to sheep and goat milk [17]. Whey proteins represent 17–22% of the total protein content; 75% of whey proteins are albumins (α-lactoalbumin and β-lactoglobulin), with a high content of the AA phenotype of β-lactoglobulin, which has been shown to provide greater efficiency in the manufacture of sheep cheese [19, 20].

In recent decades, there has been an increase in interest in foods with specific nutritional properties. The nutritional advantages of sheep's milk over other species does not derive from its content of protein, minerals or vitamins; the superiority as a functional food lies in its lipid content, more specifically in its fatty acid profile; ovine products have received direct attention due to the possibility of being enriched with fatty acids' potential benefits to health, especially vaccenic acid (VA, C18: 1 t11), c9, t11CLA, also

Component	Sheep	Goat	Dairy cattle	Human
Fat (%)	7.9	3.8	3.6	4.0
Non solid fat (%)	12.0	8.9	9.0	8.9
Lactose (%)	4.9	4.1	4.7	6.9
Protein (%)	6.2	3.4	3.2	1.2
Casein (%)	4.2	2.4	2.6	0.4
Albumin-globulin (%)	1.0	0.6	0.6	0.7
NNP (%)	0.8	0.4	0.2	0.5
Ash (%)	0.9	0.8	0.7	0.4
Calories/ml	105	70	69	68
Ca (mg)	1950–2000	1260	1200	320
P (mg)	1240–1580	970	920	150
Cl (mg)	1100–1120	1600	1100	450
Na (mg)	440–580	380	450	200
K (mg)	1360–1400	1900	1500	550
Mg (mg)	180–210	130	110	40
Zn (μg)	5200–7470	3400	3800	3000
Fe (μg)	720–1222	550	460	600
Cu (μg)	400–680	300	220	360
Mn (μg)	53–90	80	60	30
Iodine (μg)	104	80	70	80
Se (μg)	31	20	30	20
Vitamin A(mg)[1]	0.8	0.04	0.04	0.06
Vitamin D (μg)	0.18	0.06	0.08	0.06
Vitamin E(mg)[2]	0.11	0.04	0.11	0.23
Vitamin B1 (mg)[3]	0.08	0.05	0.04	0.02
Vitamin B2 (mg)[4]	0.35	0.14	0.17	0.03
Vitamin B3 (mg)[5]	0.42	0.20	0.09	0.16
Vitamin B5 (mg)[6]	0.41	0.31	0.34	0.18
Vitamin B6 (mg)[7]	0.08	0.05	0.04	0.01
Vitamin B8 (μg)[8]	nd	2.0	2.0	0.70
Vitamin B9 (μg)[9]	5.0	1.0	5.3	5.2
Vitamin B12 (μg)[10]	0.71	0.06	0.35	0.04

NNP, non-protein nitrogen. [1]Retinol. [2]Tocopherol. [3]Thiamine. [4]Riboflavin. [5]Niacin. [6]Pantothenic acid. [7]Pyridoxine. [8]Biotin. [9]Folic acid. [10]Cobalamin.

Table 3.
Comparison of the chemical composition of different species (adapted from [13, 18, 22]).

called rumenic acid, and α-linolenic acid (ALA, C18: 3, n3). Sheep's milk, compared to cow's milk, contains three to four times the amount of VA and CLA c9T11 [15].

The milk of small ruminants and their derivatives are the largest source of CLA in human food [21]. The content of CLA in ruminant milk decreases in the following order: sheep > cow > goat, with contents 1.08, 1.01 and 0.65%, respectively [22]. However, the particular management conditions of sheep herds determine seasonal oscillations in CLA concentrations due to the variability in the availability and quality of forage [23].

1.3 Processing of milk

Milk is a food that has a reduced shelf life and is highly perishable: it is an excellent medium for the growth of microorganisms, especially bacterial pathogens that can cause the deterioration of the product and diseases in consumers. The processing of milk allows it to be kept for days, weeks or months and helps to reduce the diseases transmitted by this food [24].

The production of dairy products offers small dairy producers greater cash income than the sale of raw milk and greater opportunities to reach regional and urban markets. In addition, it helps to cope with the fluctuations caused by the seasonality of the milk supply that limits the elaboration and commercialization of dairy products in many developing countries, causing important variations in the supply. The transformation of milk, as in the case of cheese making, contributes to the generation of jobs both in production unit to obtain the raw material and outside the production unit, involved in the collection, processing and marketing of the product [24].

2. Cheese production

2.1 World production of sheep's cheese

Within the top 10 countries that produce sheep's cheese are mainly European countries: Greece (125,000 t) is the top producer of sheep's cheeses worldwide, followed by Spain (65,544 t) and Italy (57,595 t) at third and fifth place, respectively. There are also France (26,448 t) and Romania (24,000 t) in the eighth and ninth places, respectively (**Figure 4**). The Asian continent is the second highest producer of sheep's cheese; countries such as China (108,000 t), Syria (60,500 t), Turkey (39,600 t) and Iran (18,750 t) are positioned in the second, fourth, sixth and tenth place, respectively. In Africa, Niger is the seventh highest producer of sheep's cheese globally, with 27,927 t. The American continent has a minimal participation in the production of cheeses; the countries of interest for this chapter lack official records on the production of cheeses, so only the total production of the continent is considered (7843 t) [25].

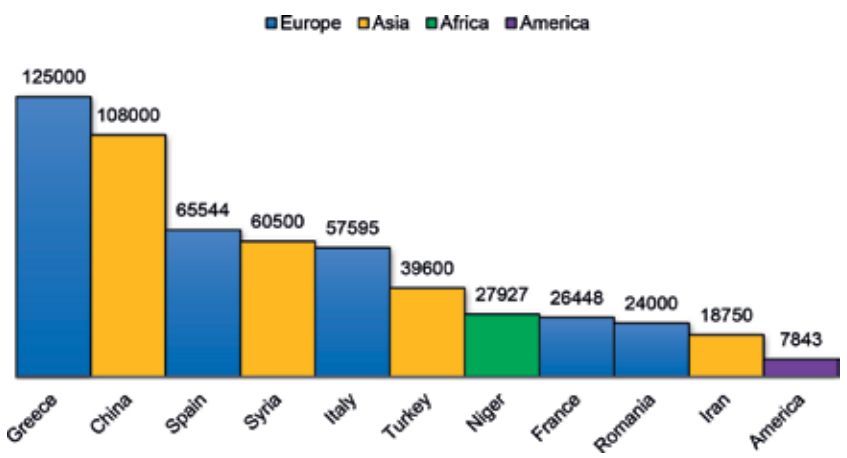

Figure 4.
World production of sheep cheeses in tons. FAO 2016 (adapted from [25]).

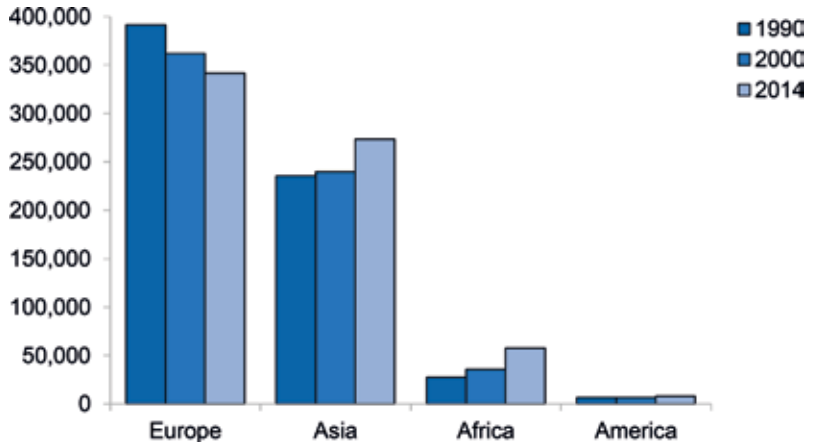

Figure 5.
Production trend in tons of sheep cheeses per continent in the years 1990, 2000 and 2014. FAO 2014 (adapted from [25]).

In 1990, Asia, Africa and the Americas had lower production than the most recent data released by the FAO in 2014, evincing a tendency toward an increase in the production of sheep's cheeses on these continents. In Europe, in contrast, production has been declining since 1990 (**Figure 5**). From 1990 to 2014, it fell 12.74% and is the only continent showing this trend. Asia, on the other hand, has increased its production by 15.99%, and Africa has considerably expanded its production of sheep's cheese, doubling the amount produced in the last century, increasing production by more than 110%. America has increased its local production of sheep's cheese more modestly, from 1990 to 2014 by 21.92% [25].

2.2 National production

PASO registered that during the January–May 2017 period, production in Chile was 33,300 t, with an increase of 5.4% compared to the same period of the previous year [26]. Despite this increase in national production, it continues to focus mainly on basic and mass-produced varieties, such as Gouda and Chanco, which in 2012 accounted for 90% of the total national production [27]. The remaining 10% corresponds to industrial Mozzarella cheeses (which are booming due to the growth of fast food chains that offer pizzas), melted cheeses, spreadable cheeses, Edam-type cheeses and, in smaller volumes, gourmet profile cheeses as well as craft companies engaged in the production of so-called "field cheeses" [26].

2.3 Sheep cheese: physicochemical characteristics

Cheese is a product derived from milk that is obtained by the coagulation of the milk protein (casein) that is partially separated from the whey. Cheeses can be hard, semi-hard, soft ripened or unripened [28]. The differences in composition between sheep's milk and that produced by cattle and goats determine their milk coagulation properties (**Table 2**). Milk coagulation is the most important factor in cheese making, which is affected by the following properties of milk—pH, casein concentration, amount of calcium (Ca) per casein and concentration of other minerals—that define the differences in coagulation time, coagulation rate, firmness of the curd and amount of rennet used [29]. The high concentrations of protein, fat and total solids present in sheep's milk result in high yields in the production of dairy products; this species has the best cheese yield compared to goat and cow milk due to

its superior chemical composition [16]. Approximately 5.5 liters of milk are needed to make 1 kg of sheep's cheese, while twice as much milk is required to make 1 kg of cow's cheese [30]. Sheep's milk produces a hard curd, the result of higher levels of casein. In general, cheese products have a particular appearance and flavor: the pasta is white and the appearance of bitter flavors is difficult [31].

The nutritional and organoleptic characteristics of the cheeses depend on the properties and nutritional qualities of the milk with which they were made, as well as the procedure in their preparation [32]. The cheese of sheep is a cheese of intense aroma, given by the volatile fatty acids present in the milk; its color is determined by the masking of the fat globules, responsible for the yellow pigmentation present in the milk of other species. For this reason, sheep cheeses are whiter and have slight grayish tones compared with cheeses made with milk from other species [30].

3. Foreign cheese trade in Chile

3.1 Imports

According to data from the "Office of Agricultural Studies and Policies" ODEPA 2011 [33], Chile began a significant increase in import of cheese, which reached its annual maximum in the 2012 season, when 18,400 t were imported. The partial figure up to May 2013 shows an increase of more than 40% with respect to the previous year's period [6]. In 2017, the following imports were made: mainly from the United States (8955 t), followed by the Netherlands (8637 t), Germany (7692 t), New Zealand (6830 t), Argentina (5584 t), Mexico (1367 t), Uruguay (1257 t), Spain (1076 t), France (1040 t), Brazil (0.872 t) and other countries (1105 t), registering a total of 44,439 imported tons (**Figure 6**) [33].

3.2 Exports

Worldwide, about 82% of exports of dairy products come from developed countries and it is expected that this rate will increase to 83% by 2026 [2]. In Chile, of the

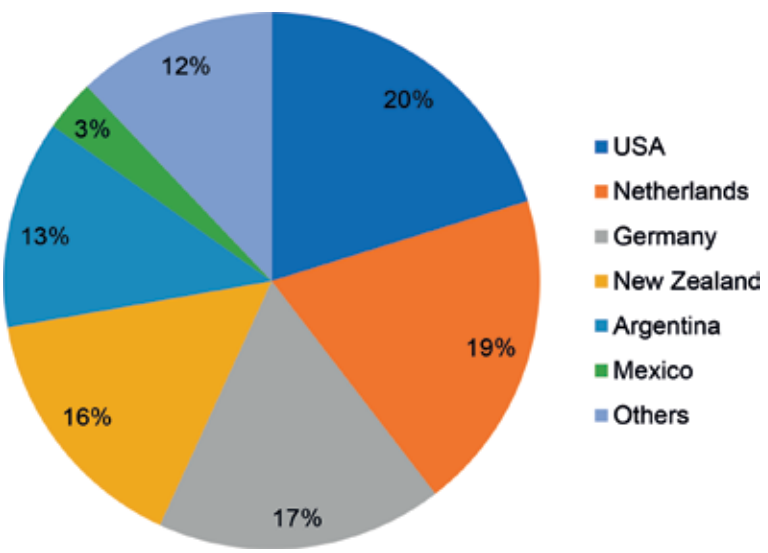

Figure 6.
Imports of cheese by country of origin. ODEPA 2017 [33].

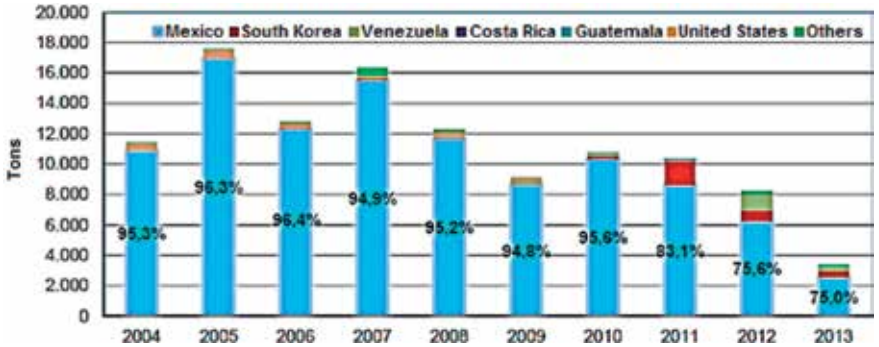

Figure 7.
Chilean export of cheeses by destination country from 2004 to 2013 [27].

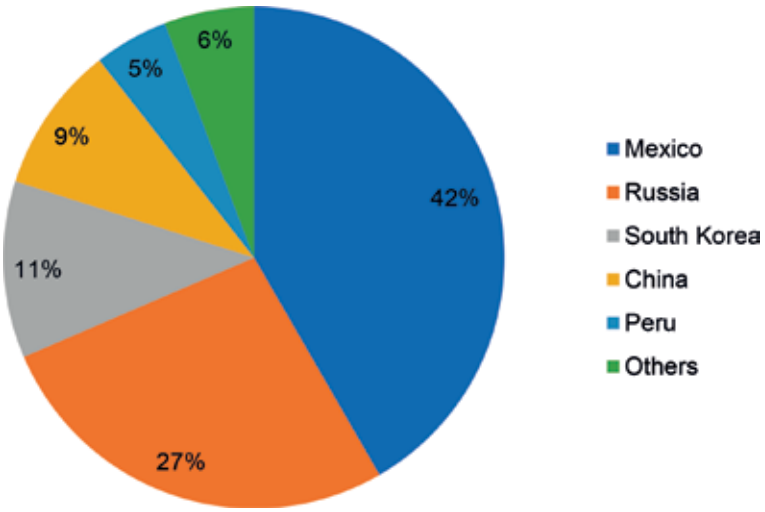

Figure 8.
Chilean export of cheeses by destination country [33].

total of dairy products exported between 2004 and 2005, Mexico bought over 60%. Between 2008 and 2010, exports to Mexico declined, and as of 2011 and 2012, exports and sales of dairy products were diversified. The importance of cheese in exports is even greater: between 2004 and 2010, 95% of the total exported cheese went to Mexico. In 2011, this share fell to 83.1%, as well as in 2012, and in 2013, fell to 75% of the total volume. At the same time, the volumes exported to Korea, Venezuela, Costa Rica, Guatemala and the United States increased. However, it must be recognized that Chilean cheese exports are still Mexico-dependent (**Figure 7**) [27].

Cheese exports by variety, according to PASO 2018 records during the January–August period, are as follows: Gouda cheese and Gouda type (3038 t), followed by Parmesan and Parmesan type (1013.1 t), Mozzarella (271.8 t), Edam and Edam type (110.6 t), and finally, other varieties or types, including sheep and goat cheeses (65.4 t) [33].

Cheese exports in 2017 were as follows: Mexico (3898 t), Russia (2512 t), South Korea (1061 t), China (886 t) and Peru (447 t) (**Figure 8**) [33].

4. Cheese consumption

In recent years, according to a study conducted in 2016 by the consultancy Euromonitor, the cheese market in Chile has experienced a sustained increase,

reaching a total consumption of 201,000 t in 2016, which represents 34.4% growth in the last 5 years. During this same period, sales in value increased from US $ 1449.6 million to US $ 2378.9 million, which represents an increase of 64.1% [26].

Currently, Chile is the largest per capita consumer of cheeses in Latin America, with an average of 11.1 kg per capita, a figure that represents an increase of 27.6% compared to 2011, during which consumption was 8.7 kg. Two countries with a strong dairy tradition follow: Argentina (7.5 kg) and Uruguay (5 kg). The same study indicates that Chile also leads the average expenditure per capita in this category, reaching US $ 131.2 in 2016, a figure that represents a growth of 55.6% in relation to the US $ 84.3 expense for the year 2011. At the second and third places are Argentina (US $ 73.8) and Uruguay ($ 65.9), respectively [26].

During the last 5 years in Chile, there have been two important changes with respect to the cheese market. The first refers to consumer behavior patterns, which are demanding higher quality cheeses and a wider range of varieties. The second is related to the development of dairy agroindustries specialized in the production of goat cheese and, to a lesser extent, sheep cheese [6].

Chilean consumers prefer fresh and soft cheeses, and sheep and goat cheeses are generally considered to be gourmet products, with a higher price compared to cow's milk cheeses [33]. The FIA indicates that the consumer segment of sheep cheeses is very small but stable. The consumption and commercialization of sheep's cheese traditionally corresponds to consumers of foreign origin and niches determined as gourmet stores, hotels or restaurants, with a high influx from among the foreign public. Of these places, restaurants are where there is the highest consumption of sheep cheeses, by including this product in their dishes [30]. Another type of consumer is one with some type of intolerance to cow's milk; ovine products, being more digestible, represent an option for this niche of the population [6].

4.1 Expansion of the market and its relationship with the consumer

The Chilean population is mostly urban (87%), with around 40% residing in the metropolitan region around the capital, Santiago de Chile. This is the great center of Chilean consumption, from which the vast majority of importers and distributors from all over the country operate. It is also where most of the transforming industries are concentrated. It is a market in which a large number of international companies compete, so the buyer is used to comparing a wide range of products and assessing the technical information provided by the supplier. The Chilean consumer is also one of the most demanding exercisers of purchasing power in Latin America, particularly appreciative of the quality-price ratio of products [35].

Along with the increase in cheese consumption in Chile, there have also been changes in consumer expectations and the setting of a premium value for a certain product. The Chile gourmet study carried out by the consultants iCuadrado, Contexto and Whizzy, seeking to recognize the preferences and trends of the market, points out the appearance of a phenomenon of consumerization among the consumers of medium and high groups; among consumers of socioeconomic groups C1 and C2, aged 25 and over, 45% describe themselves as gourmet. Cheeses rank second in the list of products most perceived as gourmet by this consumer profile. Gourmet foods are characterized by high quality, craftsmanship and limited availability. Among the attributes that consumers value most are the outstanding flavor, the mixture of flavors, the quality of the ingredients and a higher price. The panel also included experts in gastronomy who pointed out that this consumer will opt in the medium term for authentic and natural foods, few ingredients and sustainable production [26].

For its part, the global cheese industry has taken over these trends through the phenomenon of granting a premium value to products, adding new ingredients and flavors, producing limited editions of certain products, preferring recipes and

methods of traditional craftsmanship and highlighting specific localities of origin. A higher value and popularity of locally produced cheeses have been observed, with the cheeses that have "Designation of Origin" being very valued and value-added products [36]. Therefore, the implementation of a denomination of origin in sheep cheeses would offer potential for expansion in the Chilean market.

Other studies have focused on measuring consumer preferences through surveys in order to expand the market to the consumption of sheep's milk cheese. Within an analysis of potentials and limitations to evaluate the weak and strong points of the product in the Punta Arenas region, surveys and analyses of the cheese supply data were carried out by questioning potential purchasers of cheese such as hotels, restaurants and supermarkets. A test of consumers' preference according to the type of milk used in cheese preparation revealed that 55% preferred cow, 26% cow with goat, 3% prefer sheep's cheese, another 3% cow's cheese with sheep and the rest (13%) did not differentiate between one product and the other [30].

With regard to cheese from cows, 76% of respondents prefer it because it is the most demanded by customers and more used in preparations, 6% prefer it for being cheaper in comparison to cheeses of other species and the rest (18%) named both options in choosing the product. Among the brands most consumed by respondents, including Quillayes and Colun, are brands that offer more varieties of cheese (15 and 7, respectively); respondents agree that less-consumed brands are those that offer fewer varieties of product [30].

The population of consumers was classified as 56% local and national population as the main consumers of cheese, while 11% indicate that it is foreign tourists who most want the product and 33% said it makes no difference. In terms of knowledge of the product, 16% indicate that they do not know about sheep's cheese, the remaining 84% are familiar with sheep's cheese, with the main reason for familiarization being current or past consumption (44%), because they use or sell the product (41%) or because they have heard it mentioned (15%). Regarding the brand, 34% of consumers are not aware of brands of sheep's cheese, while 66% do know about one, chiefly the brand Universidad de Magallanes (UMAG) (50%) followed by the brand Pére André, with 7%. The regional elaboration of such cheese represents for 90% of the respondents an added value to the product, indicating that they would prefer this cheese over others if it is elaborated in the region. The remaining 10% do not consider the origin of the cheese as an important reason when choosing. According to the prices collected in the market of gourmet cheeses similar to sheep's cheese, 57% of the sources state that they would buy the product at the suggested price. On the contrary, 33% said they would not buy it and the remaining 10% believe it unlikely [30].

Although there is no refusal on the part of the respondents in the Region of Magallanes and Chilean Antarctica to commercialize sheep's cheese in their respective establishments, the characteristics of that possibility differ between groups. For hotels and pubs, the possibility of purchase is associated with the market behavior of supply and demand of the product, where if there is demand, standardized processing characteristics and adequate sales prices, they would include the product in their assortment. Shops, supermarkets and restaurants would incorporate it immediately as they believe that there is a potential demand for sheep's cheese. However, when informed of the sale price at which it is marketed, the purchase percentages decrease, and the number of respondents that believe that a possible purchase is unlikely or null increases [30].

In another study conducted in the Province of Santiago in Chile, the behavioral response of the respondents was identified in which the processes of acquisition, use and possession of the product were analyzed. By means of questionnaires carried out in supermarkets, the data showed that the highest proportion of people

Potentialities	Limitations
P1. Demand/Intent to purchase and test	L1. Price
P2. Market offer low	L2. Competition (origin and by species)
P3. Brand recognition/regional product	L3. Low popularity
P4. Degree of differentiation with other cheeses	L4. Resistance to change
P5. Positive perception	
P6. New geographic market	
P7. Denomination of origin	

Table 4.
Potentialities and limitations of sheep's cheese and its insertion in the Punta Arenas market [29].

surveyed turned out to be women (62%) between 20 and 25 years of age (34%). The purchases are made by 52% women and the most important aspects to consider the purchase are: brand and origin (70%) and price (30%). In terms of species, cheese of cow origin is the most consumed (77%), followed by goat cheese (6%), cow and goat cheese (15%) and those who consumed cow, goat and sheep cheese (2%). The main use of cheese is to make sandwiches (81%), indicating the need to find a more appropriate way to enter the cheese market of small ruminants—a more practical and habitual use [34].

4.2 Analysis of potentialities and limitations of sheep's cheese

The aforementioned data establish an analysis of sheep cheese potentialities and limitations that indicate a broader perception of the product and its insertion in the Chilean market (**Table 4**). It is worth mentioning that the mere insertion of the product in the city is not enough; it should be known and generate interest in the consumer to bring the population closer to the demand side [30].

5. Marketing

5.1 Distribution channels in Chile

5.1.1 Traditional channel

The traditional channel of consumption is mainly grocery stores, with little specialization, which offer a limited variety of products and brands. In addition, one has to consider the butchers, fishmongers and greengrocers and the itinerant markets of small traders that have a great importance in the retail distribution of fresh products and, in particular, of fruit and fish [35].

5.1.2 Modern channel

In Chile, there is a huge concentration of retail distribution in a few large business groups, among which DyS and CENCOSUD stand out, with a combined market share of close to 60%. DyS is part of the Walmart group, which currently operates in Chile through the LIDER, Ekono and Super Bodega Acuenta brands. CENCOSUD is one of the largest Latin American distribution groups, with operations in Chile and other countries in the region. In Chile, there are the Jumbo Hypermarkets, the Paris Johnson department stores and the Santa Isabel supermarkets. Other relevant retail distribution groups are the SMU Group, with activity in wholesale and retail distribution and brands such as Construmart, OKMarket or Unimarc Supermarkets, and other relevant retail distribution groups [35].

5.1.3 Institutional channel

Hotel and restaurant supply is very concentrated in a few companies, such as Aramark Central de Restaurantes or Compass Group-Eurest. It is estimated that around 15% of the products consumed in restaurants and hotels are imported. In Chile, this distribution channel is where the sale of sheep's cheese and its consumption are mainly located, being mainly the point of sale of gourmet product stores, hotels or restaurants with a high affluence of the foreign public [35]. The highest consumption is in restaurants that include sheep cheeses in their dishes [30].

5.1.4 Internet

The Internet has expanded rapidly and plays a very important role as a means of advertising and retail. Virtually all the major chains mentioned have very developed websites through which individuals can acquire a wide variety of products, from cosmetics to food. It is estimated that almost 70% of retail outlets allow the online purchase of their products. Chains such as Telemarkets, Jumbo or Lider are very active in the online distribution of their products [35].

5.2 Advertising media in Chile

The main advertising media are, in order of popularity, television, newspapers, radio, specialized magazines and billboards. Direct marketing is not usually very successful in Chile. The enormous importance that the Internet has received as a means of advertising and marketing all types of products in Chile, principally food and consumer goods, should be noted. With regard to industrial inputs, the introduction of new products must be carried out through specialized means and provide ample technical information [35].

6. Cheese products

Some of the brands of sheep cheese found in Chile are the Chilozábal cheese made in Chiloé, a mature cheese made with sheep's milk that simulates the process involved in the making of Idiázabal cheese, a cheese of Spanish origin with designation of origin. It has a stock of 140 Latxa animals and a production of 700 kg/year. A second example of sheep's cheese in Chile is the Boladero cheese manufactured in Aysén, marketed in the local market and in the metropolitan region. This company offers the market cheese made with pasteurized sheep's milk. A third example is the one promoted by Quillayes Peteroa in conjunction with the Universidad Austral de Chile. This facility is located in Futrono XIV region and has approximately 400 sheep of the Latxa breed for cheese production [30].

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Chapter 9

Goat and Sheep Milk as Raw Material for Yogurt

António Monteiro, Soraia Loureiro, Susana Matos and Paula Correia

Abstract

Yogurts are prepared by bacterial fermentation of milk using bacterial cultures composed of a mixture of *Streptococcus ssp. thermophilus* and *Lactobacillus delbrueckii ssp. bulgaricus*. In the regions where small ruminants are important to the economy, the development of new products may and the diversification of the offer might represent good strategies to attract new consumers since it allows producers to go beyond the usual cheesemaking. Those were the reasons that led to the production of a yogurt that would include different proportions of sheep and goats milk, a final product with the right physicochemical quality properties and sensory attributes. The addition of sheep milk is meant to attract more and more potential consumers and to additionally improve the nutritional value of the product, mainly with respect to the amount of fatty acid and mineral contents. Consumers tend to prefer yogurts made of cow milk, but this work shows that people enjoy and accept yogurts produced with goat and sheep milk as well. Therefore, it seems evident that the milk produced by these small ruminants can be an alternative and has the potential to become a good food product.

Keywords: sheep, goat, milk, yogurt, physicochemical properties, sensory analysis

1. Introduction

Goats and sheep were the first animals to be domesticated by humans for livestock husbandry, about 10,000 years ago [1, 2]. These animals were raised around the world in hundreds of different breeds. There are currently more than 750 million goats and 1000 million sheep [3].

Portugal is one of the largest producers of sheep milk worldwide. However, a substantial part of the goat and sheep milk production comes from family-scale farms and is normally intended for the owners' own consumption.

Even so, a significant part of the national milk production is already fully industrialised and has already generated some products that have been awarded with a protected designation of origin (PDO). Such designation grants them a high economic impact.

This is the case of the famous Serra da Estrela cheese and of the Transmontano goat cheese made of the milk obtained from Serra da Estrela sheep and Serrana goats, respectively.

Milk has a high nutritional value; however, small differences in the composition of the different types of milk may generate large nutritional and technological differences in milk processing industry [4]. Goat milk is quite important since it has high biological value and important nutritional qualities. Its higher digestibility, alkalinity and dietary characteristics make it highly recommended for infant feeding and for adults who are sensitive or allergic to cow milk [5, 6]. These benefits can be attributed to the micellar structure of casein protein in goat milk and to the fact that it contains a large quantity of fatty acids with higher digestibility [5, 7]. On the other hand, sheep milk is characterised not only by its higher total solids, fat, protein and caseins but also by its larger amount of minerals and vitamins [5, 8].

Yogurt has been known to mankind for over 6,000 years. The word "yogurt" seems to be derived from the Turkish word "jugurt" which first appeared in the eighth century [9]. The same author mentions that yogurt comes from the Middle East, where milk was scarce due to the desert environment.

Moreover, in milk technology yogurt and its derivatives are called fermented milk products. This process results from the development of certain microorganisms that modify the normal components of milk. Lactose is partially transformed into lactic acid. In certain milks, it also produces ethyl alcohol. Furthermore, proteins may suffer peptonisation, which improves digestibility [8].

The Food and Agriculture Organisation (1984) defined yogurt as "the coagulated milk product obtained by lactic acid fermentation through the action of *Lactobacillus delbrueckii ssp. bulgaricus* (*Lb. bulgaricus*) and *Streptococcus thermophilus* from milk and milk products. The microorganisms in the final product must be viable and abundant". The *Codex Alimentarius* (Codex STAN 243-2003) specifies that yogurt should contain a minimum of 2.7% (m/m) milk proteins, a maximum of 15% milk fate, a minimum of 0.6% titratable acidity (expressed as % of lactic acid) and a minimum of 107 CFU/g of microorganisms (total microorganisms in the starter culture).

The objective of this work was to address the issue of producing yogurts from goat and/or sheep milk with high consumer acceptability and high nutritional value so customers could be offered a new and alternative product in the competitive market of fermented milk and in a context where cow milk yogurt has the largest market share. In order to support the findings observed in this study, a complementary characterisation of the goat, sheep and cow milks was also carried out.

2. Materials and methods

To support this work, a well-documented research on goat and ewe milks and on their suitability for yogurt making was conducted. The main sources of information were scientific papers and books. A research previously carried out by the authors and that included information about the milk obtained through mechanical milking of Serrana Jarmelista goats and Serra da Estrela sheep in the centre region of Portugal was also considered. Milk was pasteurised at 65°C during 30 minutes and then it was cooled to 4°C.

Yogurt was produced using goat and sheep milk in accordance with experimental group obeying to the following proportions of sheep and goat milk: 100% of sheep milk (O100C0), 80% of sheep milk and 20% of goat milk (O80C20), 50% of sheep milk and 50% of goat milk (O50C50), 40% of sheep milk and 60% of goat milk (O40C60), 20% of sheep milk and 80% of goat milk (O20C80) and 100% of goat milk (O0C100). The lactic bacteria used in the yogurt production were lyophilized *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. Commercial cow milk powder (12%) was added to all the yogurts that were produced.

The yogurt samples were placed in hermetically sealed bottles for fermentation for 12 hours at 45°C. Then, they were refrigerated at 5°C. The total nitrogen content was measured by the micro-Kjeldahl method [10]. Protein was calculated as N × 5.38. The fat content was determined by the Geber's method [11]. Ash content was determined by incinerating the samples for 24 hours at 550°C. Moisture content was determined by drying samples overnight at 105°C [10]. Total solids content was determined using the gravimetric method as the samples were dried in an oven at 105°C for 24 hours [10]. Phosphorus was determined by spectrophotometric UV/VIS, 720 nm [12], and calcium, magnesium, potassium and sodium were estimated using flame atomic spectrophotometry at 750 nm [13]. The determination of fatty acids was done using a gas-chromatographic method (GLC), total titratable acidity was determined by reference method [10] and the pH using the potentiometric method.

For the sensorial analysis of samples, an acceptance test with untrained panellists was used. The panel consisted of 25 tasters who analysed the samples of goat and ewe yogurt. A commercial yogurt produced with cow milk and bought at a local market was analysed as well. The grades awarded by each panellist ranged from 1 to 9, where 1 is "extremely unpleasant" and 9 is "extremely pleasant" for attributes such as sweetness, colour, aroma, flavour, texture and overall assessment. To assess the tasters' overall preference, a last question was asked: "Which sample did you prefer?"

Statistical analysis was performed using the Statistica 12 programme [14] where mean, standard deviation and mode, median, minimum and maximum values were determined.

The findings were analysed using one-way analysis of variance. Means were compared at a 5% level of significance using LSD test to check significant difference. The Kruskal-Wallis test was used to verify the panellists' preferences regarding the different yogurt samples.

3. Goat and sheep milk

Goat milk has a white-matte colour, does not contain β-carotene and has a sweet and pleasant distinctive "freshly milked taste"; however, it can sometimes, at the end of lactation or after a period of storage in a cold environment, acquire a certain flavour one can describe as "animalic". Sheep milk, on the other hand, shows a more marked white opacity and has a distinctive odour originally called "suarda" or "sheepy". This feature is relatively less evident in milk that is stored in good hygienic condition. The intense flavour of goat milk may be due to the release of short-chain fatty acids during the handling of milk [5], and it has a density that ranges between 1.026 and 1.042 with a pH ranging from 6.3 to 6.7 [15]. It is naturally alkaline, unlike cow's milk which is slightly acidic.

The major components of any mammalian milk are water, fat, protein, lactose and minerals [16, 17], as shown in **Table 1** for goat, sheep and cow milk. The water content found in goat milk is similar to cow milk and is approximately 87% [18]. Goat milk also has a higher content of nonprotein nitrogenous substances and contains fewer types of casein than sheep and cow milk [6]. This specific characteristic leads to a weaker structure in goat milk yogurt, unlike sheep milk that has good coagulation capacity [6]. Moreover, the differences in sheep milk caseins are the main factors for curd fitness time to be shorter and rennet coagulation time to be firmer [19]. Goat and sheep milk also contains higher amounts of minerals and vitamins than cow milk [6].

Milk	Fat (%)	Nonfat dry extract (%)	Lactose (%)	Protein (%)	Casein (%)	Ash (%)
Goat	4.25–3.80	8.68–8.90	4.08–4.27	2.90–3.52	2.40–2.47	0.79–0.86
Sheep	7.62–7.90	10.33–12.00	3.70–4.90	5.23–6.21	4.20–5.16	0.90
Cow	3.60–3.70	9.00–9.10	4.70–4.81	3.20–3.50	2.60–2.63	0.70–0.73

Adapted from [5, 6, 39].

Table 1.
Comparison of the physicochemical characteristics of goat, sheep and cow milk.

The micellar structures of sheep and goat milk differ from cow milk in the diameter, hydration and mineralisation. Goat casein micelles contain more calcium and inorganic phosphorus, are less solvated and less stable to heat and lose casein more easily than bovine casein micelles [20]. Lipids appear in the form of smaller-sized globules in goat and sheep milk, contributing to a better digestibility [3]. An intensive research on sheep and goat milk has revealed that lipid components might have a great deal of benefits. Studies focused on trans-acid and conjugated isomers of linoleic acid, since the latter are believed to have beneficial effects on human health while the former seem to have certain negative effects. As for minerals, the differences between the three types of milks are shown in **Table 2**.

Sheep milk is the type of milk that has the highest amount of calcium, phosphorus and magnesium, while goat milk has higher amounts of potassium, chlorine and manganese. Minerals have great importance in the composition of the milk of any species. The most important minerals in the constitution of milk are calcium, sodium, potassium and magnesium [21]. Thus, milk mineral fraction is characterised mainly by its high calcium content linked to casein phosphoserine, and it is the calcium-protein binding that gives milk its irreplaceable character [17].

Calcium and phosphorus are two fundamental elements of the micelle structure of caseins and will condition the stability of the colloidal phase, particularly calcium which is also very important biologically [22].

Mineral constituents	Goat	Sheep	Cow
Calcium (mg)	126–135	193–197.5	120–122
Phosphorous (mg)	97–130	141–158	92–119
Magnesium (mg)	13–16	18–19.5	11–12
Potassium (mg)	181–190	136–138	150–152
Sodium (mg)	38–41	44–51	45–58
Chlorine (mg)	150–160	111–160	100–110
Sulphur (mg)	28	29	32
Iron (mg)	0.07	0.08	0.08
Copper (mg)	0.03–0.05	0.04–0.05	0.02–0.06
Manganese (mg)	0.008–0.032	0.007	0.006–0.02
Zinc (mg)	0.34–0.56	0.57–0.63	0.38–0.53
Iodine (mg)	0.008–0.022	0.020–0.097	0.007–0.021
Selenium (ug)	1.33–2	1.00–3.1	0.96–3
Aluminium (mg)	—	0.05–0.18	—

Adapted from [6, 8, 21].

Table 2.
Mineral constituents of three milks.

4. Microbial cultures used in the yogurt manufacture

Bacterial populations which are traditionally used in the manufacture of yogurt include species such as *Streptococcus thermophilus* (**Figure 1**) and *Lactobacillus bulgaricus* (**Figure 2**). The young cells of *S. thermophilus* are spherical in shape and occur in chains. In the dairy industry, they are often called "cocos". These cultures usually have weak milk clotting because of low acid production. Strains of *S. thermophilus* are commonly used in association with *Lactobacillus delbrueckii* ssp. *bulgaricus*. The latter is commonly referred as "rod" in the dairy industry, and the combination of the two bacterial populations is called the "coconut stick" [23].

When a single population of *Lactobacillus delbrueckii* ssp. *bulgaricus* or *Streptococcus thermophilus* is used, the production of lactic acid and acetaldehyde was greatly reduced when compared to that of combined commercial cultures [24, 25].

In the first fermentation stages of the yogurt, in which the action of those bacteria are evident, *S. thermophilus* grow much faster because of their greater aerotolerance, whereas at this stage, the populations of *L. bulgaricus* grow more slowly; however, and due to their greater proteolytic activity, these species provides enough peptides to stimulate and guarantee the growth of *S. thermophilus* [23]. The slow growth of *Lactobacillus* populations may be due to the fact that they are microaerophilic [26]. Thus, at the end of the first phase, the growth of *S. thermophilus* slows down because of the high concentration of lactic acid produced. Besides, in this phase the production of formic acid is high enough to stimulate the growth

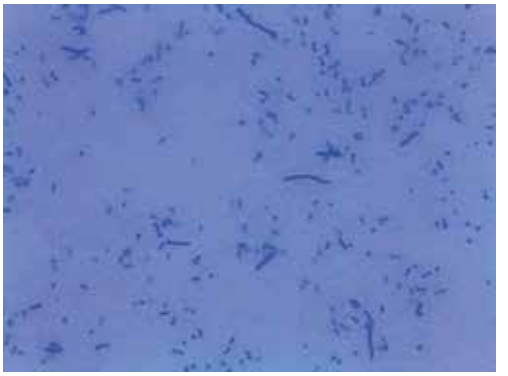

Figure 1.
Streptococcus thermophilus.

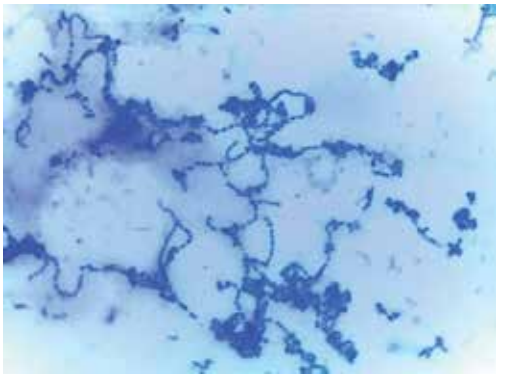

Figure 2.
Lactobacillus bulgaricus.

of *Lactobacillus* [25]. Furthermore, due to this complementary action of the two species, the desirable acidity of the yogurt can be achieved. Sá and Barbosa (1990) [27] report that these two species of microorganisms develop in cooperation at an optimum temperature of 45°C; this temperature can, however, decrease to 42°C.

The optimum ratio between the two species that will enable the existence of the flavour and aroma that are characteristic of the product depends on the properties of the strains used. Nonetheless the most common ratio is approximately 1:1 [28]. The characteristic flavour of yogurt is also related to bacteria, as they produce acetaldehyde, acetone, acetoin and a small amount of diacetyl. Among all these, the best flavour compound of yogurt is acetaldehyde, and *L. bulgaricus* is the bacteria that produce most of that compound. However, and in smaller quantities, *S. thermophilus* also produce acetaldehyde and support its conversation into threonine [29].

It should also be noted that the predominance of any of these species may lead to defects in the final product. The main factors that can affect the proper balance between the two microorganisms are time, incubation temperature and the percentage of inoculum. For example, a shorter incubation time results in a product with lower coccus content and a poorer flavour; on the other hand, a longer incubation time would result in a product with bitter flavour [30].

5. Sheep and goat yogurt

In yogurt made from sheep milk as with those produced with cow milk, homogenisation increases product firmness and reduces product serum separation. Sheep yogurt is characterised by higher values of hardness, adhesiveness and extrusion, such factors are explained [31] by its high solids content.

A research related with the analyses of the microstructure of goat, cow and sheep yogurts observed that in sheep yogurt the protein matrix of milk consists mainly of chains of large individual casein micelles. There were also small and regular voids. As a result, sheep milk produces a stronger gel that is more resistant to deformation [32]. Another study reports that sheep milk has a high viscosity that will influence the firmness of the product and may be caused by an increase in water binding capacity provided by its milk proteins [33]. Moreover, an investigation carried out by [34] shows that sheep milk is used in the production of mixed yogurts—goat milk was also used in the cases depicted—due to the higher amount of protein found in sheep milk that will improve the consistency of goat yogurt. On the other hand, some research [35] found out that goat yogurt has a poorer consistency, hardness and stability when compared, for example, with sheep and cow yogurt. In the case of the protein matrix, casein micelles of small size are bound in thick chains presenting large agglomerates as well as large empty spaces filled with serum or occupied by yogurt bacteria. This yogurt reveals a less compact gel and is therefore more delicate, brittle and less resistant to deformation. These properties affect the results of the instrumental analysis conducted on yogurt texture. This yogurt will then present low texture values and high syneresis values [30]. Several studies have been carried out on the low consistency caused by the presence of goat milk and [36], in a study based on samples of cow and goat milk yogurt and a mixture of the two, found out that as goat milk was added the firmness and consistency of the gel decreased.

The higher porosity of the protein network observed through the analysis of the microstructure and the subsequent lower degree of micellar aggregation also contribute to the mechanics observed, since a gel with low porosity is characterised by a compact matrix, which would contribute to increase the firmness and consistency

of the gel. The poor firmness of the gel can still be influenced by the size of the fat globules and their mechanical properties as explained by [37] who reports a positive correlation between poor firmness and the smaller size of the globules.

Goat milk yogurt is less commercially produced, although it has high digestibility and good nutritional and organoleptic properties [6].

6. Effect of the mixture of sheep and goat's milk on yogurt characteristics

The results shown in **Table 3** reveal that the moisture value of goat yogurt is higher than the one observed in sheep yogurt, since the addition of milk powder during yogurt production causes a decrease in available water which consequently generates lower water content in the product [18]. On the other hand, sheep milk has higher solids content, and its addition causes a decrease in the moisture value.

The composition of the yogurt is identical to the composition of the milk that served as its raw material, although there are some differences arising from the bacterial lactose fermentation and the addition of milk powder usually used to increase milk solids and that consequently generates an increase in protein content [38]. Yogurts with goat milk have lower protein values than those produced using sheep milk. This value decreases as the proportion of goat milk in the product increases. [3, 5, 13] also found out that the protein values for goat milk presented values that are between 2.90 and 3.52% lower than those found for sheep milk whose values range between 5.23 and 6.20%. Evidence also showed that the 9.59% protein content obtained for sheep yogurt was lower than that presented in similar studies carried out by other researchers [39–41]. Those studies presented values of 5.05, 6.34 and 4.55% of protein content for sheep yogurt, respectively.

The highest percentage of fat content in sheep's yogurt can reach 80%. Lower percentages of fat content are observed and could be related with several factors such as the animals' diet, the climate, their breed and their lactation stage. Curiously the goat milk yogurt presents contents of approximately 7% of fat, when the expected values are around 5% [6]. This difference may be explained by the breed of the animals.

As it was the case with the fat and the protein values, the highest value of dry extract is found in sheep milk yogurt (24.95%), while 21.17% is the value found in goat milk yogurt. These findings are in accordance with the content of the raw material, since the dry extract content found in sheep milk can reach 19.06%, while in goat milk dry extract may be up to 12.73%. Besides, the ash content is indicative of the amount of minerals present. The results found for goat and sheep yogurt are higher than the values reported by other authors [34]. The addition of powdered milk may be the source of these particularly high values. The maximum acidity value was found in sheep milk yogurt (18.9 ml/100 g) and tends to decrease as the quantity of goat milk increases. These acidity values are not in accordance with the Portuguese standard NP-694. This standard requires a maximum acidity of 13 cm^3/100 g, although this value is reported to cow milk yogurt.

Table 4 shows an increase in the values of mineral contents probably due to the addition of milk powder. The addition of sheep milk caused an increase in phosphorus, calcium and magnesium contents, since this type of milk contains a higher amount of these minerals [5]. These results are in accordance with those obtained by other authors [34].

Centesimal composition	O100C0	O80C20	O60C40	O50C50	O40C60	O20C80	O0C100
Humidity (%)	73.65 ± 0.19	76.20 ± 0.86	76.02 ± 0.17	76.81 ± 0.97	77.89 ± 0.06	77.05 ± 0.43	79.08 ± 0.06
Protein (%)	9.59	8.62	8.68	8.72	8.24	7.32	7.98
Fat (%)	9.33 ± 0.23	8.60 ± 0.20	8.27 ± 0.23	8.20 ± 0.20	8.40 ± 0.20	7.87 ± 0.42	6.93 ± 0.61
Ashes (%)	1.80 ± 0.05	1.68 ± 0.03	1.70 0 ± 0.09	1.71 ± 0.06	1.69 ± 0.14	1.68 ± 0.06	1.77 ± 0.14
Dry extract (%)	24.95 ± 0.77	22.35 ± 0.04	24.67 ± 0.65	23.03 ± 1.24	23.84 ± 2.63	21.00 ± 1.72	21.17 ± 0.24
Nonfat dry extract (%)	20.29 ± 5.82	18.05 ± 6.04	20.53 ± 6.50	18.93 ± 4.56	15.44 ± 2.63	13.13 ± 1.72	14.24 ± 0.24
Acidity (ml/100 g)	18.90	17.90	17.30	16.80	18.33	18.13	17.37

Table 3.
Physical-chemical characteristics.

Minerals	O100C0	O80C20	O60C40	O50C50	O40C60	O20C80	O0C100
Calcium	295.65	316.98	322.403	332.91	180.48	179.8	160.85
Sodium	65.58	144.49	154.91	131.27	56.49	58.65	60.11
Magnesium	22.16	18.19	17.65	16.83	16.85	16.32	15.54
Potassium	223.21	279.09	310.27	331.44	170.78	157.63	162.45
Phosphorous	268	300.29	278.75	191.37	165.85	152.43	140.12

Table 4.
Mineral contents.

7. Fatty acids

Table 5 presents the differences between the saturated and unsaturated fatty acids. The yogurt produced with sheep milk alone presents the highest content of unsaturated fatty acids (30.64%), while yogurts whose composition contains 50% of goat milk and 50% of sheep milk have the highest saturated fatty acid value (71.67%). These values have to do with differences found in the composition of milks and are in accordance with those of [42] who mentions that the proportion of saturated fatty acids in the sheep milk ranges between 68 and 78% and the unsaturated fatty acids proportion is about 31%. However, total unsaturated fatty acids were usually higher in sheep milk. Therefore, in yogurts the percentage of unsaturated fatty acids may increase with the addition of sheep milk, a fact that is in accordance with another of the studies [22] already conducted.

In terms of yogurts' fatty acid content and of the type of milk used to produce them (**Table 6**), evidence showed that saturated pentadecanoic fatty acid (C15: 0) that according to [6] is contained in all samples is lower than would be expected for goat and sheep milk with values of 0.71 and 0.99, respectively.

Milk taken from goats and sheep has a higher amount of short- and medium-chain fatty acids that are responsible for their characteristic flavours.

The quantity of caprylic acid (C8: 0) found in sheep and goat milk is 2.6 and 2.7%, respectively; the quantity of capric acid (C10: 0) is 7.8 and 10%, and the lauric acid (C12: 0) found in both those milks is 4 and 5.5%, respectively. It is also worth mentioning that goat milk has a higher amount of these fatty acids than sheep milk [6]. These authors have observed that as the goat milk concentration increases, the percentage of this capric acid (C10: 0) increases as well. This phenomenon was expected since this was the milk's main component and will play a major role in giving it its distinctive flavour and aroma.

The results obtained show a great quantity of stearic and oleic acids in both milks, and as far as the presence of C18: 0 was concerned, there were no significant differences between the different yogurts. However, sheep milk had a higher amount of C18: 1. As a consequence, the addition of sheep milk as yogurts are being produced will lead to a considerable increase in the percentage of this MUFA.

% Fatty acids	O100C0	O80C20	O60C40	O50C50	O40C60	O20C80	O0C100
Unsaturated	30.64	26.56	29.06	26.75	30.94	28.32	27.55
Saturated	68.46	70.11	70.93	71.67	63.94	66.8	67.57

Table 5.
Differences between the contents of saturated fatty acids and unsaturated in the yogurts.

Fatty acids	O100C0	O80C20	O60C40	O50C50	O40C60	O20C80	O0C100
C10: 0	7.76	8.56	8.63	10.09	9.19	9.49	9.5
C12: 0	4.56	4.9	4.74	5.03	4.66	4.57	4.47
C14: 0	11.75	11.69	11.43	11.36	10.95	10.77	10.61
C15: 0	n.d.	0.62	0.71	0.73	n.d.	0.51	0.6
C15: 1	0.79	1.28	1.26	1.22	1.21	1.16	1.14
C16: 0	24.78	24.85	25.73	25.46	25.04	27.06	27.98
C16: 1	0.83	0.82	1.85	0.86	0.8	0.8	0.9
C18: 0	13.1	12.1	12.34	11.78	11.2	11.49	11.52
C18: 1 oleic	28.63	24.47	25.97	24.76	24.38	22.44	21.32
C18: 2	3.06	3.23	3.32	2.73	2.11	2.5	2.68
CLA	1.18	1.27	1.24	1.13	2.44	1.42	1.51

Table 6.
Fatty acid contents in the yogurts.

As far as linoleic acid is concerned, data are not very consistent, although sheep milk generally has a higher proportion of this acid. However, this fact is not supported by the results.

Evidence showed that conjugated linoleic acid (CLA) content, for both samples, was higher than it would be predicted. It should be noted, however, that goat milk contains a lower proportion of conjugated linoleic acid than sheep milk [11].

The AG content in dairy products presents a variable value range resulting from numerous factors, the most important being the animal's diet and the type of production system [43].

8. Sensory analysis

The sensory analysis was performed by panellists and included yogurts containing 100, 80, 60 and 50% of both sheep and goat milk. The score given by the panellists (**Table 7**) to the sweetness, colour, aroma, taste, texture and their overall appreciation for the different yogurts analysed was not significantly different: most of them were awarded a 7 (pleasant) on a scale of 1 (extremely unpleasant) to 9 (extremely pleasant), as previously mentioned.

		Sweetness	Colour	Aroma	Taste	Texture	Overall appreciation
O100C0	Mean	3.64	7.24	5.00	4.04	6.48	4.76
	Std. dev.	1.73	1.13	1.83	2.17	1.73	1.81
	Mode	4	7	5	Multiple	7	Multiple
O80C20	Mean	4.28	6.96	4.84	4.24	6.12	4.76
	Std. dev.	1.86	1.43	1.77	1.67	1.81	1.56
	Mode	5	7	5	3.	6	3
O60C40	Mean	4.36	6.84	5.20	5.00	5.96	4.92
	Std. dev.	2.29	1.70	2.18	2.08	2.09	2.10
	Mode	3	7	Multiple	6	7	3

		Sweetness	Colour	Aroma	Taste	Texture	Overall appreciation
O50C50	Mean	4.14	7.16	5.38	4.72	6.02	5.12
	Std. dev.	1.92	1.33	1.86	1.99	1.77	1.86
	Mode	Multiple	7	Multiple	4	7	3
O40C60	Mean	4.28	6.76	5.36	4.40	5.76	5.04
	Std. dev.	1.79	1.88	1.82	1.80	1.83	1.72
	Mode	6	7	6	Multiple	6	6
O20C80	Mean	4.52	6.96	5.48	4.84	6.20	5.64
	Std. dev	1.69	1.72	1.53	1.68	1.78	1.38
	Mode	4	7	6	4	Multiple	6
O0C100	Mean	4.60	6.96	5.32	4.88	5.96	5.36
	Std. dev.	1.73	1.51	1.89	1.76	1.72	1.66
	Mode	Multiple	7	7	Multiple	7	6
Cow	Mean	5.10	7.00	5.82	5.58	6.08	5.80
	Std. dev.	2.01	1.46	1.89	2.09	1.84	1.81
	Mode	Multiple	7	Multiple	5	7	Multiple

Table 7.
Mean, standard deviation and mode of sweetness, colour, aroma, taste, texture and overall appreciation.

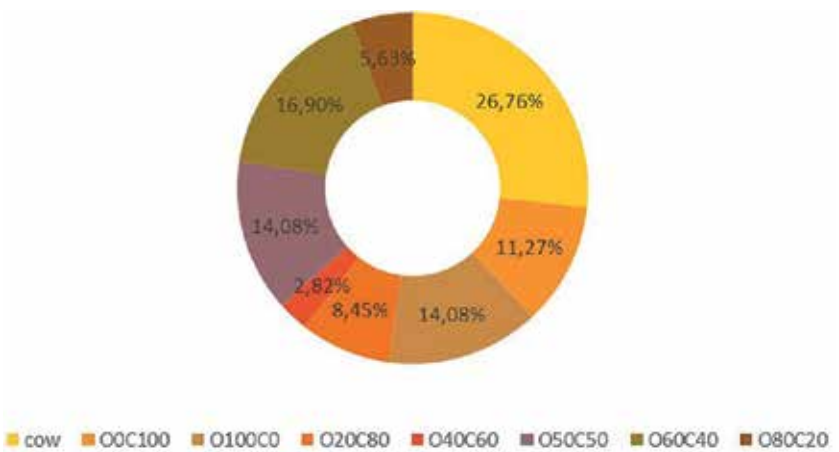

Figure 3.
Yogurt preferences identified by the panellists.

Although there were no significant differences between yogurts, **Figure 3** shows that 26.76% of panellists preferred the commercial yogurt (produced with cow milk). Nevertheless, some of the goat and sheep yogurts assessed were well appreciated.

In the group of yogurts with a greater presence of sheep milk, 16.90% of the panellists preferred the O60C40 yogurt (60% sheep milk and 40% of goat milk). The O100C0 yogurt (100% milk of sheep) and the O50C50 yogurt (50% of sheep milk and 50% goat milk) were chosen by 14.08% of the tasters. Moreover, 11.27% of the panellists chose the yogurt that contained the highest proportion of goat milk, the O0C100 (100% goat milk).

9. Conclusion

Yogurt made of goat and sheep milk represents a firmer and creamier product whose features increasingly attract consumers. These characteristics also increase the nutritional value of the product, mainly because they improve its level of fatty acids and minerals.

The increase in the proportion of sheep milk used in the production of yogurts promotes a significant increase in fat but also tends to increase the proportion of unsaturated fatty acids.

It was observed that the majority of the panellists who took part in the study were quite pleased with the yogurts produced with goat and sheep milk. So, being capable of developing strategies that will help increase the use of goat and sheep milk in yogurts and that will in turn have an important role in attracting more consumers to the product is undoubtedly a challenging endeavour.

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Chapter 10

Optimal Procedures to Valorize High-Quality Traditional Dairy Products

Catia Pasta, Rosario Petriglieri and Margherita Caccamo

Abstract

Traditional cheeses represent by themselves high-quality productions that are expressions of a production system highly linked to the territory. Biodiversity factors rising from the natural system have been extensively studied, and their effect on the quality of dairy products has been scientifically shown. CoRFiLaC is among the main contributors to this field with many studies developed on traditional productions in different environments. The main goal of the chapter is to highlight through case studies the methods applied in scientific protocols in order to define, besides the identification of biodiversity factors affecting quality, and valorize health properties and safety aspects and to understand consumer reactions and intentions to purchase depending on specific characteristics of final products. The combination of research performed in every single aspect of traditional production is the real challenge and good strategy to valorize high-quality goods.

Keywords: biodiversity, consumer expectations, traditional products, marketing

1. Introduction

Traditional productions, result of a historical evolution of a territory, provide a population with richness and dignity, besides being socially useful and environmentally sustainable. All these concepts are valid for all the productions that respect natural biological cycles, but nevertheless the agro-food sector finds its best expression: the traditional production of milk and cheese implies a perfect equilibrium among mankind, animals and environment. For example, livestock overcrowding per surface unit is not allowed as it causes lack of resources. Environmental cycles and the related processes (e.g. seasonality) cannot be altered.

Globalized processes cannot follow these nature-driven rules, by altering farming systems through intensive strategies. This implies a huge increase in investments, an exasperation of production processes and, unfortunately quite often, an alteration of the environment. Thus, creating a condition of illusory wealth undergoes downward market rules and will create only the effect of subjection and poverty.

With all of this premised, this does not mean that traditional production processes cannot be supported by advanced technology. On the contrary, research has to improve and not to alter production processes.

In order to valorize any traditional production process, it is necessary primarily to understand their origins and then the motivations at the basis of their development

and establishment, to study historical events, the human characteristics of the operators, the characteristics of bred animals, the culture of farmers ended down by generations and the technology that allowed to obtain high-quality products for that specific area. As an example, it is sufficient to think about seasonality and the availability of forage and water, technology and services.

Research needs to understand traditional production processes from inside in order to appreciate quantitative and qualitative richness of feeds, to study their nutritional and aromatic characteristics and to assess their transfer both to milk and consequently to the deriving productions.

After a preliminary phase of investigation based on the study of the production process, it is possible to evaluate eventual intervention plan for each specific sector of milk production (land cultivation, forage quality, breeding systems, farm management, milk quality) and of the dairy production process while being careful to not alter tradition and history.

However, very often the sector that needs most interventions lays in the post-production phase and in marketing, promotion and selling activities. Traditional productions are the results of a particular environment and non-replicable expression of culture and tradition. Traditional dairy products and cheeses in particular give the consumers the possibility to taste and experience a unique product deeply linked with the territory of origin, from which it draws its peculiarities. For this reason the concept of "traditional dairy product" became relevant and is conceived as a territorial brand. It represents the interaction mean between the market and consumers to promote both cheese varieties and territories.

It is important to make these products recognizable and traceable for consumers, by characterizing them scientifically and describing their properties in order to promote high-quality traditional dairy products.

2. Identification of factors influencing traditional cheese productions

CoRFiLaC is a dairy research centre that carries out research actions taking into account all the aspects of the dairy production chain, from the animal nutrition, management and animal welfare and quality of milk and dairy products to finally their consumers' acceptance and product valorization. For these purposes, it has developed several projects, together with other national and international partners, with the aim of creating a network between the research and public institutions involved in the agricultural sector, the control bodies that certify quality products and cheese producers. To strengthen this network, a common shared strategy to support the actors of the dairy chain is necessary in order to improve the quality of the productions, to reduce the geographical isolation limits and to raise consumers' awareness.

The real challenge is to demonstrate scientifically that traditional dairy products are healthy, high-quality and pleasant for consumers and carriers of cultural elements that provide an identity to a specific area of production.

Several and specific factors are responsible for the bio-organoleptic diversity of the traditional dairy products.

Every traditional cheese originates from complex production systems characterized by several "biodiversity factors", such as the environment, the macro- and microclimate, the natural pasture, the breed (often autochthonous) of the animals, the use of raw milk and its natural microflora, the use of natural coagulants, the use of natural ingredients, the use of traditional equipment and the ageing conditions [1]. The characterization of such production systems is basically

important both from a scientific point of view and for the valorization of the deriving productions.

3. Marketing cheese valorization

To understand market and consumer habits is the key point to better position goods. This rule of thumb is valid also for niche products such as traditional cheeses strongly tied to the territory of origin. It is necessary to keep in mind that not all consumers are alike and that not all areas are similar in terms of background, rules, laws, custom habits and preferences. Even if brand managers are marketing experts in logistics, price promotions and advertising, most often they are not in consumer acceptance or compliance [2]. What managers miss is the level of consumer knowledge.

The influence of consumer knowledge [3] extends beyond product choice and consumption. Beliefs, attitudes and perceptions are all aspects that strongly drive consumer act of purchase. In order to better valorize cheese on the market, consumers' knowledge is the first step to face to adopt correct marketing strategies. Besides, consumer research constitutes the base for getting hints on a potential wow effect [4] that is often personally induced when not only expectations are satisfied but consumers obtain something more.

In order to valorize niche productions strongly tied to the originating area, CoRFiLaC has always supported technical with marketing research trying to highlight important aspects under consumers' point of view to improve market share and product selling power.

3.1 Consumer research

There are many ways to approach consumers. Primary and secondary data and qualitative and quantitative research approaches are developed in order to deeply understand consumer thinking processes. Even the new area of the Marketing 4.0 studies focus on consumers by defining a new trip across five principal A's: aware, appeal, ask, act and advocate [4]. In various studies carried out by CoRFiLaC, one of the main objectives was to understand consumer awareness and knowledge to obtain relevant hints to affect the appealing process. In the aware phase, consumers know for what they have already experienced, listened, suggested and indirectly known through the word of mouth (WOM). In 2003 [1, 5], through a phone survey (933 Sicilian people), CoRFiLaC aimed to show the main criteria of consumer selection for *traditional Sicilian cheeses* and people personal definition of *traditional cheese*. The main criteria driving cheese consumers' decision-making resulted to be safety food (92.3%), natural raw ingredients in the method of production (82%), healthy properties (75%) and local product (73%) and then followed by product denomination of origin (PDO) brand of recognition (68%), artisanal products (67%) and typical flavour (66%). However, external cues that have been the focus of many marketing research and have been considered to be the most important criteria for consumer decision-making (i.e. price and brand), in this study, seemed to be less relevant according to participant claims. Through a factorial analysis, we detected two main factors according to the elbow plot which together explained 83.83% of the total information generated:

- New criterion believers (51.47%), with the only exception for "price"
- Classic criterion believers (32.38%) (**Table 1**)

Rotated factor pattern		Factor % explanation		
		VAR	%	CUM
New criterion believers		8.75	51.47	51.47
Artisanal product	0.93	5.50	32.36	83.83
Safety food	0.93			
Previous use	0.92			
Locally produced	0.92			
Natural raw ingredients	0.88			
Price	0.88			
Product denomination of origin (PDO)	0.87			
Easy to use	0.76			
Healthy properties	0.73			
Classic criterion believers				
WOM	0.91			
Plain flavour	0.86			
Industrial product	0.83			
Packaging	0.77			
Advertising	0.76			
No OGM	0.68			
Brand	0.66			

Table 1.
Principal component (PC) factor analysis criteria of selection.

Besides, participants considered that traditional cheese had a different flavour compared to industrial one. According to a PC factor analysis, two main factors explained consumer tendency for 90.69%. Among participants there were:

- Flavour oriented, 54%
- Biodiversity fellows, 36% (**Table 2**)

This information was very important in building related strategies for Sicilian cheeses. In fact communication highlighted the importance of factors such as raw milk, tools, local method of production, product safety and better flavour. Similarly, in 2013 in an international project (T-Cheesimal), CoRFiLaC carried out a study that implied different steps: (a) consumer pilot study at the beginning (56 Maltese people) and (b) face-to-face survey with Maltese consumers (1194 people) and restaurants (131 restaurants) to support marketing strategies for a local cheese called Gbejna.

Through the pilot study, CoRFiLaC tried to withdraw information concerning Maltese consumers such as consumption habits, preferences, cheese awareness, traditional cheese definitions and attitude towards both Sicilian and local Maltese cheeses. Besides, typical average portion for packaged cheeses, types of cheeses present on the shelves, prices for both local cheeses and competitors and cheese merchandising were gathered in the pilot study. The first analysis showed that Italian cheeses were highly mentioned (more than 50% of people) either for the

Rotated factor pattern		Factor % explanation		
		VAR	%	CUM
Flavour oriented		3.23	53.85	53.85
Different flavour	0.95	2.21	36.83	90.96
Better flavour	0.94			
Biodiversity fellows				
Tools	0.94			
Local usage	0.87			
Raw milk	0.86			
Healthy properties	0.85			

Table 2.
Principal component (PC) factor analysis of traditional cheese.

larger variety compared to other countries or for the territorial proximity. Maltese cheeses did not have a good recognition among dwellers: in fact people had a positive quality perception for imported rather than local cheeses. Maltese people, independently from the sex, defined a "traditional cheese" *as a fresh product with a different taste, made both with pasture locally produced goat milk and according to the tradition of the place,* and *a well-known product*. Therefore, from few data we found out that there was a general confusion. Italian cheeses, including Sicilian ones, due to the halo effect of the country of origin, Italy, [6] were considered per se good and identified as traditional. However, once we asked to describe what a traditional cheese meant for them, they overall indicated a fresh cheese, locally produced even if the perception about the local Gbejna was not so good. In comparison with other Sicilian products after a taste, the Gbejna was considered, on a nine-point Likert scale, appetizing, pleasant, satisfying, quite exciting as a product and not expensive and obtained a good overall evaluation. After the trial, the quality of the Gbejna was not considered so high and not so unique. The willingness to buy (WTB) was low compared to the others [7]. And people did not consider Gbejna "exactly for me" (**Figure 1**). The halo effect for Italian cheeses was still there. In fact, the pilot study showed that Maltese people tended to buy imported cheeses among which Italians were the preferred and often identified as traditional cheeses. The Gbejna was well known among dwellers. They used the cheese as part of their own culture but unconsciously preferred and considered imported cheeses as better products with higher quality. This implied a strategy to improve and fortify product intrinsic quality. Under a marketing point of view, it was necessary to raise product positive perception by highlighting good aspects of the product not only among dwellers but also focusing on tourists by pulling Gbejna use inside of the restaurants.

To confirm pilot study indications and deeply understand both consumer habits on a large scale, two surveys were carried out with representative samples: one on consumer population over 15 years old and another one with restaurants. A part of both studies focused on criteria affecting quality cheese perception in terms of fortifying the image of the Gbejna. In both studies sampling was withdrawn proportionally to the actual composition of the targeted population [8, 9]. For consumers, a list of nine criteria on a nine-point Likert scale (1 = very unimportant; 9 = very important) was presented [10], and a PC analysis for consistency was performed taking into account age (15–24; 25–34; 50–64; over 64), sex (female, F; male, M) and area of residence in Malta (Southern Harbour, SH; Northern Harbour, NH; Souther Eastern, SE; Western District, WE; Northern District, ND).

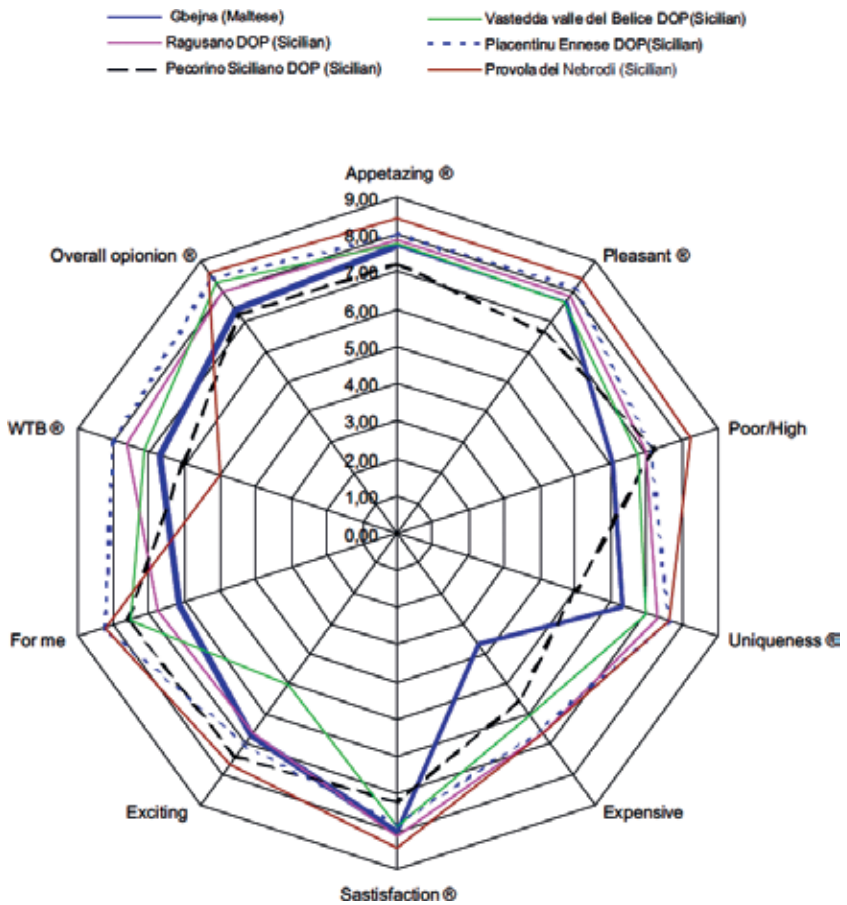

Figure 1.
Consumer attitude towards cheeses after tasting.

Two main components explained in the PCA are more than 65% of the variance. The main component was represented by intrinsic product qualities such as taste, pleasantness and high quality and, on the other side, perceived characteristics, such as superior quality, healthy, appetizing, uniqueness of the product and the product representing consumer personality. According to age, sex and area, the criteria worked in different ways affecting consumers (**Figure 2**).

In the PCA, the data showed that Gbejna was considered of a certain quality mainly from consumers between 35 and 64 years old. Women in the range 35–49 dwelling in the Western and Northern District and Southern Harbour considered the Gbejna as "A cheese for me", appetizing, unique and pleasant. Rather men in the western area focused on the flavour and on the pleasantness of the cheese, and similarly men in the Southern Harbour considered the quality a little bit superior. However, the main target for the cheese was people in the range 50–64 years old with no area distinction. In specific men of the southern area considered the Gbejna "A cheese for them" with a good flavour, pleasant, with a superior quality, and unique. In fact, from the survey these two targets of people represented the heavy Gbejna cheese consumers (35–49 daily consumers for 14%, 50–64 daily consumers 13%, over 64 daily consumers for 10%). Hence, these consumers represented the main targets to focus in order to push Gbejna quality awareness and perception. What was interesting was the range 15–24 years old that did not consider the Gbejna as "A cheese for them". Young people considered the product low in quality, with

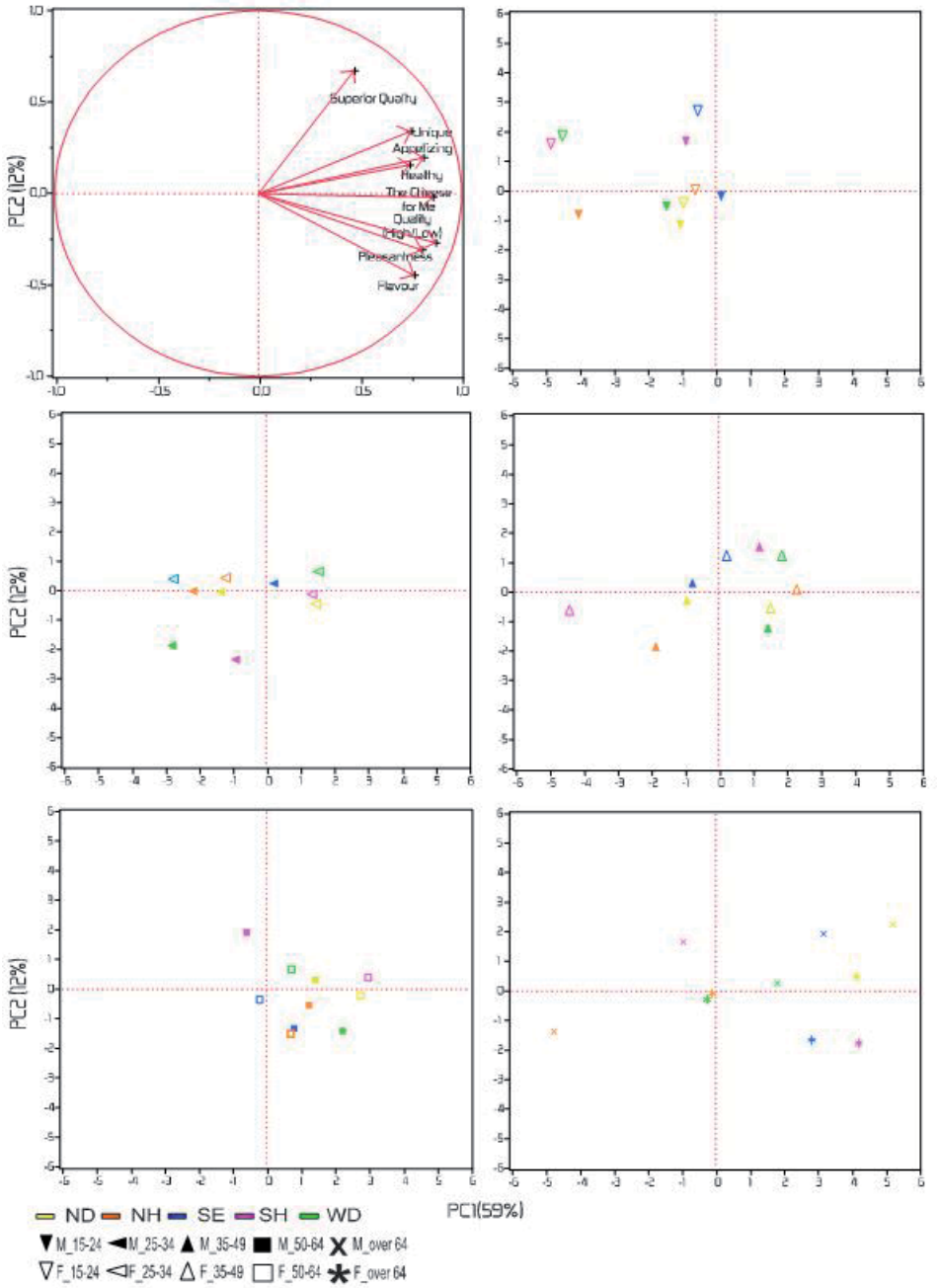

Figure 2.
Mean square PCA score per age, sex and area.

an unpleasant flavour. Actually, the same target tended to identify the traditional cheese with a product they were used to since the childhood but did not represent actually a typical or local product tied with the territory. Very often the youngest tended to mention industrial imported cheeses [11].

Similarly to consumer survey, we tried to understand quality product perception under restaurants' point of view. Managers and owners were interviewed. To get information on criteria affecting overall cheese quality perception, we proposed once again on a seven-point Likert scale (1 = very unimportant, 7 = very important)

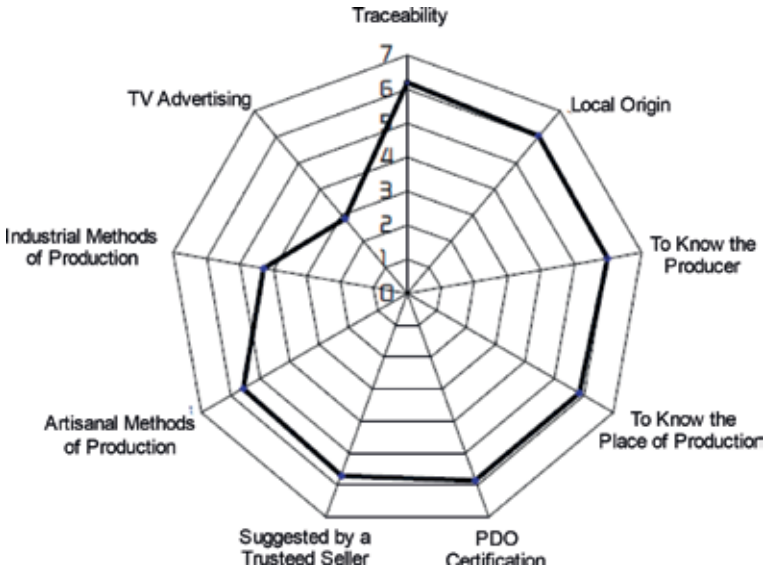

Figure 3.
Criteria affecting quality perception in restaurants.

[10] nine criteria of course considering that the target was different (**Figure 3**): the importance of (1) knowing the producer, (2) PDO certification, (3) TV advertising, (4) industrial systems, (5) awareness of the place of production, (6) recommendation by sellers, (7) local origins, (8) artisanal systems and (9) traceability of the product was withdrawn by a face-to-face questionnaire (**Figure 3**).

Data showed that restaurants are considered as main criteria for quality perception traceability, local origin, awareness of the producer, awareness of the place of production and PDO certification. TV advertisement was considered less important. Restaurants tended to look for information from producers, sellers and strongly trusted blogs and websites. A good strategy to affect their act of purchase was to propose trials and certified products (i.e. PDO). A quality cheese was identified with a traced product, a product with local origin or whose origins were easily recognizable, a PDO certified product and a well-known place of production. Besides, they required more frequent deliveries in order to have very fresh products [12]. All these aspects were relevant to generate an advantage for Gbejna cheese compared to the imported cheeses. Therefore, a suggested strategy was to cooperate and to set up activities in order to fortify the image of the product pushing on the origin, the place and the producers and setting up a process for obtaining the PDO certification, very important for restaurants as a consequence of the tourist targets. The relevance of a brand or recognition such as the PDO is attributable to the fact that in Malta region guests exceeded 1.5 million [13] and the main tourists come from France, Germany, the United Kingdom and Italy. It is easy to get the need for restaurants to present certified products to these very high demanding customers especially for cheeses.

3.2 Packaging

In many of our project, CoRFiLaC supported local producers by realizing packaging prototypes to help small realities to better sell their products. In all studies there was a frequent problem: producers did not have time to dedicate for marketing activities. For this reason, in some projects a pack prototype was defined as an indicative way to communicate through product qualities. The prototype was generally the result of a multilevel analysis. Four levels are worth to be considered.

The first level included (1) definition of the objectives for the packaging, (2) analysis of the actual selling variety in the targeted channels and (3) analysis of the correlated communicative context by evaluating packaging dimensions, materials, merchandising, exhibition on shelves and counters and formats of all direct and indirect competitors. This approach is really powerful to define packaging strategy and to follow in order to create the right suit for the product under analysis able to communicate correctly to consumers [14].

The second level included the study of the most important elements (materials, shape, dimensions, colours, graphics elements, brand, labelling and slogan). Therefore, graphic elements and in turn brand and labelling along with slogan definitions are strongly related to consumer market analysis.

The third level was the conceptualization of the analysis in prototypes, subsequently subject to qualitative analysis, depending of course on money availability and time (focus groups, laddering interview, depth interview).

The fourth level was a first selection of prototypes based on the qualitative analysis and according to people actual message perception and selecting prototypes in line with the packaging communication objective. Then these prototypes were proposed to a larger number of final consumers, by gathering quantitative data, and the final packaging was chosen according to its ability to achieve the communication object with consumers.

In the T-Cheesimal project, the goal of the packaging was to communicate to consumers that the Gbejna:

- Was a cheese locally produced
- Was obtained using milk from local breed cows fed with natural pasture
- Represented somehow Malta
- Presented a higher quality
- Was traditional

Once data were gathered from the pilot study and from the survey with consumers, a group of ten prototypes of packaging were set up with two different slogans. A focus group with experts and with students of the University of Malta was run. People attributed each prototype a vote from 1 (the most liked packaging) to 10 (the less liked packaging), after which discussion started in order to understand their motivations. On the external aspect, we realized that they appreciated red and green colours, shape and different appearances compared to what was already present on the market, and they liked very much a graphic element present on some of them: a wave. Besides, people tended to prefer the same slogan. Hence, among the 10 packagings, we selected 5 that were evaluated from 175 people. In a random way, packaging was presented in one exhibition in Malta, and we asked to choose two of them. Then, we gave two pieces of paper to put inside the corresponding bag under the package asking to write the motivation of their choice. The packaging was selected not just according to the most preferred but considering people perception from it. The one indicated in **Figure 4** was the most liked because:

- Local animals were represented in the packaging.
- The green wave both represented the natural pasture and unconsciously recalled the sea and the Maltese landscape.

Figure 4.
Prototype defined from the analysis.

- The colours were liked because they represented the Maltese flag.
- The writing was easy to get, clear and simple.

With this packaging we achieved the main goals, but not all of them. Of course for us it was a starting point to let producers raise the perception of higher-quality Gbejna cheese.

3.3 Training and word of mouth

Once a new product idea enters into a market, in order to succeed, it is important that features, characteristics and advantages reach final consumers through available information. Word of mouth is the most powerful way [15, 16]. In consumers' perception the word of mouth reduces the risks and becomes the most important referring source especially when finding information on specific products is difficult. Nowadays [4] social influence is determining the success or the decline on the market of many products. All of us strongly trust what is communicated through the network. In the social, however, the new tendency is to jump in and out the Net to verify the information collected, strongly relying on the F factors: family, friends, fan and followers [4]. For this reason we believe that a strategy we pursued in the 2009 for Sicilian cheeses would be successful for other cheeses around the world. By collecting data on supermarkets, specialty shops and big distributions' point of sales, we realized that people working at the daily counter did not have expertise on dairy product. This was very common inside the big distribution. Therefore, we decided to start a collaboration with a local chain to support traditional cheese selling. We set up training on local traditional cheeses by combing training and awareness but most importantly *emotional experience* playing on sensorial aspects and environmental comfort. We tried to combine research knowledge and selling experience in a simple way to define consumer strategy approach. To involve emotionally counters that were daily in touch with final consumers, it was the main strategy to fortify their abilities in selling and in positively affecting consumers looking for specific information. Consequently, it turned to be a strategy to increase consumers' willingness to choose and buy these niche products. A positive word of mouth was generated on

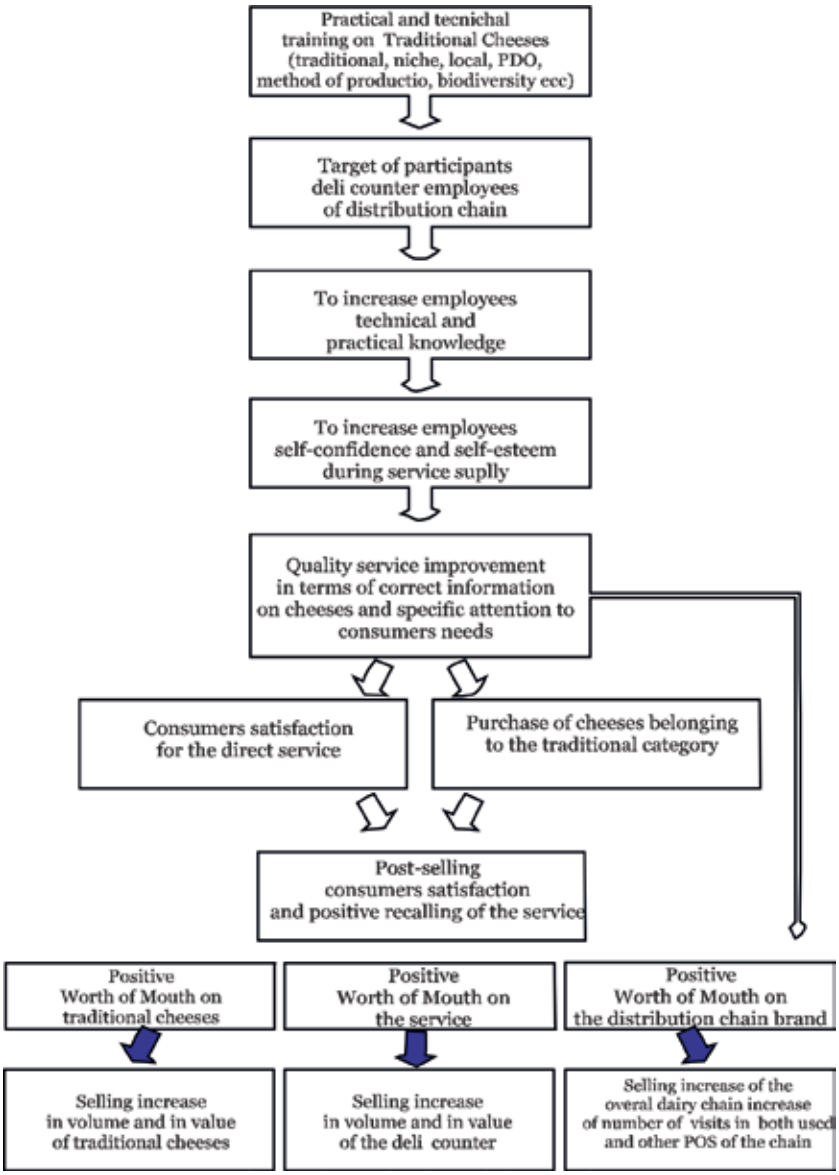

Figure 5.
Multistep flow generated by technical training.

specific products, on counter service and on the distribution chain brand [17]. A multistep flow allowed at first on Sicilian cheeses and subsequently on other projects (i.e. Gbejna) to increase product awareness, selling skills and in turn selling volume and value (**Figure 5**). The high turnover of people employed as counters requires a frequent and continuous training in order to support and improve the selling volume of niche products sector that does not have the possibility to invest a huge amount of money in advertising and publicity.

4. Conclusions

Every traditional cheese originates from complex production systems from which the specific bio-organoleptic properties are drawn. In order to valorize high-quality

traditional dairy products from any place of the planet, some specific practical steps are needed. First of all research needs to understand traditional production processes from the inside in order to deeply appreciate quantitative and qualitative richness of feeds, to study their nutritional and aromatic characteristics and to assess their transfer to milk and consequently to the deriving productions.

This preliminary phase tied to the production systems is the base of an action plan to follow in the production chain.

However, small producers, even when they are favourable to apply and correct specific aspects of their own method of production by adapting their farms to the research, face the lack of knowledge on consumers and final user needs under a marketing point of view.

The best product will remain unknown until consumers become not only *aware of* but get *involved with*. Therefore, research should be twofold oriented. On the one hand, research should be addressed to improve and determine traditional cheese internal qualities and characteristics to prevent contamination and to guarantee consumers safety, to provide high-quality products in any conditions standardized and not standardized and to safeguard the territory of origin and all the biodiversity factors determining the peculiarity of these dairy products.

On the other hand, research should get insights from consumer/final user criteria driving decision-making on traditional cheeses, by segmenting targets and elements on which to build incisive marketing strategies suitable to targets. These strategies should be able to affect consumer appeal and to maximize their trust process, resulting in consumers' repeated purchasing acts, positive word of mouth and of course positive returns to small producers. In this chapter, we attempted to explore some of the ways in which behavioural aspects should be analysed along with technical product aspects in order to set up better selling and marketing strategies for traditional dairy products.

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Milk is considered as a complete diet for an infant and contains essential nutrients for the development of young mammals. The substances in milk provide energy and antibodies that help protect against infection. Most farmers are paid for the quality and composition of their milk. Whole milk, once approved for use, is pumped into storage silos where it undergoes pasteurization, homogenization, separation, and further processing. Milk is a highly perishable commodity because it is an excellent medium for the growth of microorganisms—particularly bacterial pathogens—that can cause spoilage as well as diseases in consumers. Milk processing allows the preservation of milk for days, weeks, or months and helps to reduce food-borne illness.

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