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Technological Approaches for Novel Applications in Dairy Processing

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Meet the editor

Nurcan Koca is currently an Associate Professor at Food Engineering Department, Ege University, Turkey, and the head of Dairy Process and Engineering Section. She received her PhD degree from the Ege University in 2002. She worked as a nurse in the hospital of Medical Faculty from 1988 to 1994 and a research assistant at Food Engineering Department, Ege University, from 1994 to 2007. She also crowned her career by doing postdoctoral research at Food Science and Technology Department, Ohio State University, in the USA from 2004 to 2006. Her main research interests include the application of nonthermal processing in dairy products, the quality effects of processing in dairy products, and the improvement of rapid methods for dairy products.

Contents

Preface

Dealing with dairy processing one needs to understand many complicated technologies and have knowledge in physics, chemistry, biochemistry, microbiology, rheology, etc., depending on the diversity of dairy products. The fact that the dairy product range is so wide provides not only both difficulties but also advantages in terms of implementation and innovation. Changes in customer expectations, technology, and economies have been forcing dairy plants to be adaptive and innovative.

In this book, some new approaches to dairy processing are presented in three sections. In the first section, several applications for the use of novel technologies for various dairy products are provided. The potential and current applications of UV light on the disinfection of air, water, food contact surfaces, packaging materials, and dairy products are introduced as well as the limitations and challenges of using UV light for future adaptations by the dairy industry. The process of using ultrasound crystallization known as sonocrystallization for the production of lactose using whey is explained. The potential use of high-pressure homogenization to develop dairy products is also presented, and subsequently, membrane separation technology and its applications in the dairy industry are comprehensively described.

In the second section, the potential improvements on functionality and quality systems for dairy products are provided. The use of plants for cheese-making and pharmaceutical applications is introduced. A detailed explanation on the recent fractionation and analytical techniques for the analysis of carbohydrates in dairy foods is introduced. The process effects on bioactive peptides are also discussed. In addition, the current challenges, guidelines, and tools used in production chains of dairy products are described by presenting a case study during primary production through the application of predictive microbiology. In the final section, the approaches for dairy waste treatment are given in terms of physical treatments.

Many authors from various countries have shared their knowledge in this book. This book will be useful for both practicing professionals and researchers in the dairy field. I would like to send my sincere thanks to all the authors for their hard work and contributions and specifically Marina Dusevic, who is the technical editor, for providing her professional guidance.

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Section 1

Novel Technologies in Dairy Processing

Chapter 1

Ultraviolet Light Applications in Dairy Processing

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Additional information is available at the end of the chapter

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Abstract

The main objective of this chapter is to discuss the potential of ultraviolet (UV) light applications in dairy industry. The principles, inactivation mechanisms, sources and devices of UV light are reviewed as well as its advantages and disadvantages. The factors affecting the efficacy of UV light are also discussed. The potential and present applications of UV light on disinfection of air, water, food contact surfaces and packaging materials are introduced. The efficacy and quality effects of UV light treatment for liquid dairy products are presented. In addition, as a promising application to prevent post-contamination after heat treatment, surface processing by UV light is emphasized. Besides its use for microbial inactivation by UV light, its possible uses such as creating novel dairy products are also introduced. The legal aspects on UV light for production, processing and handling of food briefly are given. Benefits, limitations and challenges of UV light for the future adaption in dairy industry are discussed.

Keywords: UV light, dairy products, processing, microbial inactivation, quality changes

1. Introduction

Dairy products create good growth conditions for a variety of microorganisms because they are rich in many kinds of nutrients including carbohydrates (especially lactose), lipids, proteins, essential amino acids, enzymes, vitamins and minerals. Therefore, producing safe dairy products are more challenging compared to producing many other foods.

Thermal processing is the most common decontamination method to ensure food safety and to prolong shelf life by eliminating the spoilage and pathogenic microorganisms and enzymes. In recent years, use of non-thermal technologies is increasing as an alternative to the thermal

processing in food industry. Ultraviolet (UV) light, which is a non-thermal technology, has recently attracted a lot attention to improvement of food safety. Compared to thermal processing, this promising technology can provide consumers with minimally processed, microbiologically safe and fresh-like products with minor effects on the nutritional and sensory properties of the product. On the other hand, this technology must not replace hygiene, good manufacturing or agricultural practice.

UV light application can also be introduced as an alternative to the use of chemicals in food industry. Besides, the use of UV light does not generate chemical residues. Additionally, it offers some technological advantages especially in developing countries in a small-scale production due to its low maintenance cost, low installation cost and low operational cost with minimal energy use. The operation and cleaning of the treatment is quite easy. In spite of its many advantages, its low penetration power restricts the area of use in food industry. Furthermore, its inactivation efficiency may be reduced or prevented because of physical features of food. At high doses, it can create negative effects on quality and some vitamins. In order to obtain effective results, applications should be made considering these situations.

UV irradiation of milk was first used in the mid-1900s for the purpose of vitamin D enrichment [1]. Efficacy of UV light treatment has been studied in recent years and more and more research has also been carried out to evaluate the potential applications of UV light as a non-thermal alternative to thermal processing of milk. On the other hand, due to the confirmed success and convenience of thermal processing, potential processing alternatives for milk are still limited. The use of UV light must not only be considered for microbial inactivation but also for the development of novel dairy products. The UV-treated pasteurized cow's milk was authorized as a novel food in market by European Commission. It is reported that the treatment of the pasteurized milk with UV radiation results in an increase in the vitamin D3 (cholecalciferol) concentrations by conversion of 7-dehydrocholesterol to vitamin D3 [2].

Contamination of dairy products with microorganisms may occur at several stages of production, originating from a variety of sources during production. Although heat treatment is applied for inactivation of foodborne pathogens, dairy products especially cheese can be contaminated with undesirable microorganisms. After pasteurization process, handling of the curd, equipment, processing lines, packaging or storage rooms can result in cross-contamination with a variety of microorganisms. Even if good manufacturing practices are applied, surface applications of antimicrobial agents before packaging are commonly used to prevent spoilage and extend storage life for some dairy products. Instead of chemical preservatives, additional solution is needed to control the growth of microorganisms just before or after packaging of dairy products. Surface application of UV light after production can offer an attractive alternative method to eliminate or control the growth of post-processing contamination. Other promising uses of UV light are the disinfection of air and water used in dairy plant, and surface decontamination of food contact surfaces and packaging materials.

A lot of research is mainly focused on the application of UV light to reduce microorganisms in milk, and relatively little research focuses on the decontamination of the surfaces of solid dairy products. There is lack of information about the relation of quality and safety of dairy products. Thus, the application of UV light for various dairy products needs to be investigated in

terms of both quality and safety in order to increase the use and reliability of UV light in industry. There is also need for research on various applications of UV light on dairy plant.

In this chapter, UV technology is explained in terms of its principles, inactivation mechanisms, and available UV light sources and reactors are reviewed. Then, the effects of UV light on the inactivation of microorganisms and changes in the chemical and nutritional aspects of various dairy products are discussed.

2. UV light technology

2.1. Principles of UV light technology

UV light includes the wavelengths from 100 to 400 nm on the electromagnetic spectrum. UV light can be subdivided into four regions according to their wavelength: UV-A (315–400 nm), UV-B (280–315 nm), UV-C (200–280 nm) and vacuum UV (100–200 nm). UV-C light has the most effective germicidal effect on microorganisms, such as bacteria, viruses, protozoa, fungi and algae [3, 4]. UV-C radiation in the range of 250–260 nm has the highest germicidal effect and ultraviolet energy at a wavelength of 253.7 nm shows the maximum effect, at which the absorption of DNA is stronger [3].

In principle, the photochemical reactions of biomolecules of microorganism primarily result in germicidal effect leading to inhibition of microbial growth or to inactivation of the cell. Germicidal effect of UV light on microorganisms occurs because of cross-linking between the bases of adjacent pyrimidine dimers in the same DNA strand [5]. This situation leads to inhibition of transcription and replication of nucleic acids, which is called clonogenic death [6, 7]. In some conditions, the metabolism can repair the DNA damage by photoreactivation or darkreactivation depending on the microorganism. Nevertheless, at high UV doses, the repair cannot be possible because of the wider damage [8].

2.2. Factor affecting the efficacy of UV light in food industry

The UV light efficacy depends on several factors related to UV equipment, UV sources, operating and measuring conditions, target microorganisms and material or food to be exposed in food industry, which are summarized as:

- UV light source and UV dose
- UV sensitivity of microorganisms
- The composition of target
- Physical properties of target (turbidity, opaque, color, etc.)
- Surface properties of target (roughness, dirt, etc.)

The germicidal effects of UV radiation primarily depend on the UV dose (J/m^2) which refers to the UV irradiance or UV intensity flux and is defined as the function of the intensity and time

of exposure. The UV intensity (W/m^2) is the total radiation from the specified area. In most cases, as the exposure time and intensity of UV light increase and the distance from light source to target decreases, inhibition rate of cells increases. In addition, whether the sample is located directly under lamp or not affects the inhibition ratio of microorganisms for a group of samples.

The UV light sensitivity of the target microorganism is an important parameter for the selection of the UV light dose. Microorganisms have different structures due to their many characteristics. The necessary energy can vary for a certain species of microorganism according to strain, growth medium and stage of the culture. Therefore, different doses are needed for inactivation of various microorganisms. UV doses as D values required for reducing populations of various microbial groups are reported by Koutchma [9] in **Table 1**. Besides the sensitivity of microorganism its contamination level also affects the decontamination degree. In fact, in our research on decontamination of mold on the yoghurt surface, the population of mold affected the decontamination level of mold. This can be attributed to overlapping of microorganisms which prevents UV light from reaching the population at the lower layer.

In dairy industry, one of the most important problems for dairy industry is biofilm formation which occurs with colonization of microorganisms on the surface. These biofilms block the light transmission, act as a protective barrier for microorganisms against the light and reduce the efficacy of UV treatment [10, 11].

Physical, compositional and surface properties such as thickness, viscosity, density, optical properties, color differences, dirtiness, roughness etc. can change the process efficiency. UV light has a restricted penetrability. Transparent fluids such as water are effectively disinfected by UV light, whereas opaque fluids such as milk are affected less due to poor penetration depth of light, and microorganisms cannot be affected directly [11, 12]. The composition of target is also important for the efficacy of UV light. Dissolved solids, suspended particles, organic solutes, macromolecules especially proteins and fat globules in food have shadowing effect on target microorganisms and limit the penetration and efficacy of light [11–13] Treatment efficacy also depends on the characteristics of surface exposed to light and application to

Microbial group	***D* Value (mJ/cm^2)***
Enteral bacteria	2–8
Cocci and micrococci	1.5–20
Spore formers	4–30
Enteric viruses	5–30
Yeast	2.3–8
Fungi	30–300
Protozoa	60–120
Algae	300–600

*The D value is a measure of the resistance of a microorganism. It is given as the dose needed for an exponential decay of the target microorganism.

Table 1. UV inactivation doses measured at 253.7 nm for various microbial groups [9].

smooth surfaces is more effective than rough surfaces. The dirtiness and roughness can cause to form shadows and prevents direct access of light to the microorganism. Viscosity and density determine the effectiveness of the transfer and flow model of the liquid in the system, while optical properties affect the UV light transmittance [9]. Light transmission of food and packaging material in UV application to the surface of unpacked and packaged food is a critical factor for decontamination. Higher absorbsion of light is obtained in dark foods, causing decrease of available energy for microbial inactivation [11].

2.3. UV light sources

Choosing the right UV source can increase the efficiency of microbial inactivation by increasing UV light penetration. The first and natural source of UV light is the Sun. The Sun emits radiation across a wide range of wavelengths. Other UV light sources are lamps. Many alternative UV light sources have been developed, such as low pressure mercury (LPM), medium pressure mercury (MPM), low pressure high output mercury lamp-amalgam type, mercury free amalgam lamps, pulsed-light (PL) and excimer lamps. LPM lamps are commonly used in food applications [14].

Mercury lamps have been the sources of radiation in most UV-based disinfection systems. The low and medium pressure mercury UV lamp sources are reliable sources for disinfection applications which are beneficial for their performance, and low cost. They are based on the vapor pressure of mercury while the lamps are operating. LPM lamps are designed to deliver a continuous monochromatic light at 254 nm. MPM lamps emit germicidal polychromatic light between 200 and 300 nm [9]. A breakthrough for economic UVC generation is the discovery of low pressure amalgam lamps [15]. This technology has recently been developed and incorporated into disinfection applications. The mercury emissions from lamps to the environment have encouraged the investigation of mercury-free lamps [9]. Xenon lamps are used in the Pulsed light UV technology. These lamps emit flashes in a short period of time. They have a broad spectrum of radiation between 180 and 1100 nm. Another UV light source is excimer lamps, which can emit pulsed light at 248 nm. It is possible to emit light in desired wavelength by using various gases such as He, Ne, Ar, Kr, Xe in the excimer lamps. The excimer lamps can be operated even at very low surface temperatures [7].

2.4. UV light devices

UV light applications are carried out with different equipment for solids or liquids: UV reactor designs for liquids according to flow types and UV cabinet designs for solids. It is necessary to increase the absorbed energy to the maximum level by developing the design of UV light device with appropriate lamp and size in order to achieve the desired effect.

2.4.1. Reactors

Reactors are devices used for UV light application to liquids. UV reactor contains UV lamps inside. Each UV lamp is in a separate protective quartz tube to prevent the direct contact with liquid. The liquid flowing through the UV reactor is exposed to UV rays emitted from lamps.

Thus, the microorganisms in the liquid become ineffective. In the selection of UV reactors, the physical, chemical and microbiological properties of the liquid to be disinfected and the amount of the liquid passing through are the most important parameters. In this context, the UV light dose should be determined according to the nature of the fluid and the target microorganism. In addition, to increase the efficiency of disinfection, parameters such as sediment and turbidity in liquid should be removed with sensitive filters.

The flow pattern of liquid in the UV reactor has also significant effect on total UV dose due to the differences in the position and residence times of the microorganisms in certain regions of the irradiated field [9]. The inactivation of microorganisms increases using turbulent flow in continuous flow UV reactors [16, 17].

The first reactor design is a thin film UV reactor. Thin-film reactors are characterized by laminar flow with a parabolic velocity profile [16]. Another reactor having laminar flow is laminar Taylor-Couette UV reactor. In both reactors, the two cylinders in the system are intertwined. While the system is running, the gap between the cylinders is filled with liquid product. In the thin film reactor, the UV lamps are placed in the inner cylinder, whereas in laminar Taylor-Couette UV reactor the lamps are placed on the outer cylinder and the inner cylinder turns around by creating whirlpools [18, 19]. The second design approach is turbulent flow reactors. They increase the turbulence within the reactor in order to make the liquid close to UV light source. In another approach, the UV reactor called Dean flow reactor includes a coiled Teflon tube with UV lamps and reflectors placed both inside and outside the tube, which are used to promote additional turbulence and to create a secondary swirling flow, also known as "Dean effect" [9].

2.4.2. UV cabinets and tunnels

UV cabinets are devices developed for UV light applications on the surface of solids. The number and position of lamps in the UV cabinet are the most critical factors for the disinfection of entire food surfaces. The UV processing units for solid food was well schematized by Manzocco and Nicoli [11]. If one side of the solid food is exposed to UV light, the food is placed on a support. For the exposure to the top and bottom sides, the food can be placed on a film or turned upside down during treatment. If all the surfaces of the solid food are exposed to the UV light at the same time, it is needed to increase the number of lamps and place the food on a film. For example, in dairy industry, only upper surface of the yoghurt in package is enough to be treated by UV light while all surfaces are exposed for many cheeses. If there is no food support, the product flows near the lamps coated with waterproof quartz tubes in a vessel containing water.

It is also possible to design a tunnel with a dynamic system moving with the food. In this type of cabinet system or tunnel, the food material is conveyed through UV tunnel and taken from the other end. The width and height of the tunnel are designed according to expectations of user. UV application time is adjusted by conveyor speed. Such tunnels provide convenience for industrial use. They are added to the desired point of production line and their use in the system is practical.

2.4.3. Pulsed UV light

Pulsed UV light is a modified and improved version of the UV-C light. Pulsed UV light is an application using devices containing ultraviolet lamps that emit ultraviolet light at high power at regular intervals. It is applied in a very short time (1 μs–0.1 s) in the range of 200–1100 nm [7]. In this technology, combined effect of photochemical, photothermal and photophysical conditions occurs and microorganisms become ineffective [20].

3. The applications of UV light in dairy industry

3.1. Disinfection of air in the production area

Clean and fresh air is necessary for food processing area. UV technology can be used for preventing the spread of airborne diseases by inhibition of airborne pathogenic microorganisms in the field of production, packaging, cooling, storage and ripening. For this purpose, low pressure mercury vapor lamps are successfully used as UV light sources. The efficiency of this process depends on the volume of the area and the power of the UV lamp.

3.2. Disinfection of water used during processing

UV-C light has been used to disinfect water for several years and has become a successful process that eliminates several types of microorganisms. UV-C technology is a good alternative to chlorine disinfection. In dairy industry, it is possible to use the UV systems for the disinfection of drinking water, process water, waste water and brine.

3.3. Surface applications of packaging materials and equipment

3.3.1. Packaging materials

In food industry, the use of UV light for decontamination of packaging material is becoming widespread. The number of microorganisms on the surfaces of packaging materials such as boxes, cartons, foils, films, wrappings, containers, bottles, caps, closures and lids can be reduced or eliminated by applying the appropriate UV light doses. The packages can be treated with UV light before filling or closing the lid or the packaged food can be exposed to UV-C light. The effectiveness of UV treatment is better on smooth surfaces. On the other hand, the UV light cannot reach every spot because of shadowing on irregular surfaces.

Plastic materials such as polyethylene terephthalate (PET), polyvinylchloride (PVC), polypropylene (PP) and polyethylene (PE) are increasingly being used as packaging materials for dairy products. These materials have many advantages such as availability, low cost, transparency, thermal adhesiveness and being a good barrier against oxygen, carbon dioxide, anhydrite and aromatic compounds [21]. Due to different constructions, thicknesses and various properties of these packages, their UV-C permeability is different. When the packaged food is UV treated, this permeability becomes more important. The UV permeability of PP/PP (50 μm), bone

guard bags (BG) (25 μm), polyamide/polyethylene (PA/PP) (40 μm) and oriented polypropylene (OPP) (40 μm) were reported as 64, 67, 8 and 83%, respectively, by Manzocco and Nicoli [11]. However, there was no UV-C permeability of OPP/PE, PET/PE, Polyester and oriented polypropylene/cast polypropylene (OPP/CPP).

3.3.2. Food contact surfaces

The cross-contamination of microorganisms from equipment to the products is an important issue in dairy technology. UV light can be used to provide disinfection of surfaces of conveyor and other equipment used in preparation, production and, storage areas. For an effective inhibition, microorganisms must be exposed to UV light directly. There should be no obstruction between the UV source and the surface to be sterilized. The success of this application also depends on the cleanliness of the material surfaces because dirt would absorb the radiation and hence protect the bacteria. Therefore, it is possible to say that UV light must be applied after cleaning processes of the dairy equipment.

4. Efficacy of UV light on dairy products

4.1. Liquid dairy products

Raw milk from healthy cows contains relatively few bacteria, but can be contaminated easily during handling and/or storage from a variety of sources (persons, containers, machines, pipelines etc.). Milk is also suitable for the growth of many pathogenic microorganisms carrying potential risk of transferring diseases from animals to humans. The storage conditions of milk before further processing influence the microbial population. To limit the bacterial population in the raw milk, applying effective cooling and good hygiene practices are essential. Heat application is traditionally used to kill the pathogenic bacteria and reduce the others, and extend the shelf life of milk. The success and convenience of heat treatment is proved for milk. Thus, the alternative technologies to heat treatment cannot be integrated into dairy industry easily despite studies in this field.

In literature, the results of the application of UV light technology as an alternative to thermal processing are contradictory. Some authors reported that UV light can be used effectively for the reduction of certain bacterial pathogens in milk. Cilliers et al. [22] showed the similar level of microbial efficacy obtained in milk processed with pasteurization (high temperature short time), UV light and their combination. Similarly, Crook et al. [23] investigated the effect of UV-C light on the inactivation of seven milkborne pathogens such as *Listeria monocytogenes, Serratia marcescens, Salmonella senftenberg, Yersinia enterocolitica, Aeromonas hydrophila, Escherichia coli* and *Staphylococcus aureus*. Of the seven milkborne pathogens tested, *L. monocytogenes* was the most UV resistant, requiring 2000 J/L of UV-C exposure to reach a 5-log reduction, and the most sensitive bacteria was *S. aureus*, requiring only 1450 J/L to reach a 5-log reduction. Matak et al. [24] reported that UV-C treatment could be used for the reduction of *L. monocytogenes* in goat's milk and application of a cumulative UV dose of 15.8 ± 1.6 mJ/cm^2 to goat milk led to more than

5 log reduction in *L. monocytogenes*. Engin and Karagul Yuceer [25] reported the UV irradiation was as effective against certain microorganisms as heat treatment. The authors applied the UV light as an alternative to heat treatment to bovine milk using a custom-made UV system and the growth of coliform bacteria, *E. coli* and *Staphylococcus* spp. was completely reduced by UV treatment. Similar results were found for inactivation of *S. aureus* in milk using pulsed UV light treatment by Krishnamurthy et al. [26]. It was shown that the pulsed UV light can be used as an alternative method to inactivate *S. aureus* in milk. Choudhary et al. [27] showed that *E. coli* W1485 was reduced by 7.8 log in skimmed milk, but 4.1 log in full-fat raw milk with UV light treatment by using coiled tube reactor. They also reported that *Bacillus cereus* endospores were more resistant than *E. coli* W1485 and that these endospores were reduced by only 2.72 and 2.65 log in skimmed milk and full fat milk, respectively. In another study, inactivation of *E. coli* O157: H7 in bovine milk exposed at 254 nm was higher than at 222 and 282 nm at the same UV doses. The reductions in *E. coli* O157:H7 at 254 nm using the doses of 5, 10 and 20 mJ/cm^2 were 1.81, 2.38 and 2.95 log respectively [28].

UV light efficacy on the reduction of total number of microorganisms is also proved in different studies [29–31]. Reinemann et al. [29] reported that UV treatment to raw cow's milk achieved more than 3 log reduction in total numbers of bacteria. The highest reduction was found for coliform bacteria followed by pyschrotrophs, thermodurics and spore formers. Microbial counts of UV treated milk (continuous turbulent flow system, 880 and 1760 J/L doses) were lower compared to those of control milk [30]. UV-C treatment of raw cow milk was capable of reducing total viable count by 2.3 log [31]. UV light treatment in milk can be used as a method to reduce the number of psychrotrophic bacteria to prolong the storage period of cooled raw milk [9, 22, 26, 32]. In contrast, Altic et al. [33] and Donaghy et al. [34] concluded that the UV light technology cannot be an alternative to current pasteurization process for milk. The authors found less than 1 log reduction in *Mycobacterium avium* ssp. *paratuberculosis* in milk by UV treatment. In both studies, the use of UV light was not very effective in reducing the number of *Mycobacterium avium* ssp. *paratuberculosis*.

UV radiation may be used for an alternative to pasteurization of cheese whey, valuable liquid dairy product, if the lamp fouling problem is solved [35]. In their study, for destruction of microbial population of 5.95×10^6 cells/ml in cheese whey, more than 3.3, 2.1 and 0.8 h residence times were needed in the first, second and third UV reactors, respectively. However, fouling was seen as a major problem when the temperature of cheese whey increased. As a solution to the fouling problem, coil reactor series were recommended instead of conventional reactor by Singh and Ghaly [36].

Table 2 summarizes the microbial inactivation and technical characteristics of UV light system used for milk that were reported in the studies cited above.

4.2. Surface applications of dairy products

Surface of dairy products such as cheese, yoghurt, etc. is the primary location for microbial access and quality depletion during processing and storage period. Most of the chemical, oxidative, microbial and enzymatic reactions take place on the surface of the dairy product

	Type of UV reactor	UV treatment	Test microorganisms	Results/achieved inactivation	Studies
Bovine milk-full cream	Surepure40 turbulent flow commercial system	**Dose:** 430 mJ/cm^2	Aerobic plate count Coliform bacteria *E. coli* Aerobic mesophilic spores Anaerobic mesophilic spores Aerobic thermophilic spores Anaerobic thermophilic spores	Similar level of microbial efficacy with high temperature short time heat treatment	[22]
Milk	Thin-film turbulent flow-through pilot system	**Dose:** 0–5000 J/L **Flow rate:** 4300 L/h	*Listeria monocytogenes* *Serratia marcescens* *Salmonella senftenberg* *Yersinia enterocolitica* *Aeromonas hydrophila*	Potential as a non-thermal method to reduce microorganisms	[23]
Goat milk	CiderSure 3500 apparatus	**Doses:** 0–20 mJ/cm^2	*Listeria monocytogenes*	Suggested for the reduction of *L. monocytogenes* in goat's milk	[24]
Bovine milk	Custom-made	**Intensity:** 13.87 J/mL (per single pass) **Flow rate:** 1090 mL/min	Mesophilic aerobics Coliform bacteria *E. coli* *Staphylococcus* spp. Yeasts/Molds	A major effect on total coliforms, *E. coli* and *Staphylococcus* spp.	[25]
Raw milk	Pulsed light sterilization system	**Flow rates:** 20, 30, 40 ml/min *Polychromatic 100–1100 nm*	*Staphylococcus aureus*	A potential method for inactivation of *Staphylococcus aureus* in milk	[26]
Full fat raw milk and skimmed milk	Coiled tube	**Dose:** 11.187 mJ/cm^2 **Intensity:** 1.375 mW/cm^2	*Escherichia coli* W1485, *Bacillus cereus* endospores	Higher resistance of *B. cereus* endospores to UV than *E. coli* W1485 cells, Higher inactivation efficiencies of both bacteria in skimmed milk than full fat raw milk	[27]
Bovine milk	—	**Dose:** 5–20 mJ/cm^2 **Wavelength:** *222, 254, 282 nm*	*E. coli* O157:H7	Higher inactivation efficiency and lower reactivation ratio at 254 nm than 222 and 282 nm	[28]
Raw cow milk	Pure UV system	**Doses:** 0.23, 0.46, 0.93, 1.9, 3.7, 7.4 and 1.5 kJ/L **Flow rate:** 1.1 L/s	Total viable count, Psychrotrophics Coliform bacteria Thermodurics	Suggested for reducing of bacteria not susceptible to thermal treatment and psychrotrophic in refrigerated milk stored for prolonged periods	[29]
Cow's Milk	Continuous turbulent flow	**Doses:** 880 and 1760 J/L	Aerobic plate count Aerobic sporeformers Coliform bacteria	Lower counts in UV-treated milk	[30]
Raw cow milk	Continuous flow coiled tube	**Dose:** 16.822 mJ/cm^2 **Intensity:** 1.375 mW/cm^2	Total viable count	2.3 log reduction	[31]

	Type of UV reactor	UV treatment	Test microorganisms	Results/achieved inactivation	Studies
Whole and semiskim milk	Laboratory-scale	**Dose:** 1000 mJ/ml **Flow rate:** 168 ml/min	*Mycobacterium avium* subsp. *paratuberculosis*	Not an alternative to current pasteurization	[33]
UHT milk	Pilot-scale	**Doses:** 0–1836 mJ/ml **Flow rate:** 4000 l/h *30 W UV C output*	*Mycobacterium avium* ssp. *paratuberculosis*	Not an alternative to current pasteurization	[34]
Cheese whey	Tubular-type	**Gap sizes:** 18, 13, and 6 mm	Total viable count	May be used on-line sterilization of whey if the proper reactor gap size and the appropriate residence time are used	[35]
Sterile whole milk	Pilot-scale UV light continuous flow-through unit	**Dose:** 45 J/cm^2 **Flow rate:** 65 l/min	*Listeria innocua* *Mycobacterium smegmatis,* *Salmonella serovar typhimurium* *E. coli* *S. aureus* *Streptococcus agalactiae* *Acinetobacter baumannii*	Significant reduction for all bacterial species tested except *M. smegmatis.*	[37]

Table 2. Efficacy of UV light application for liquid dairy products.

and cause undesirable changes that may reduce shelf life of the product. To prolong shelf life and reduce microbial growth and oxidative degradation of dairy products, some types of preservatives are used according to legislation limits. However, a negative public reaction is growing over the addition of chemical preservatives to foods. Although UV light application is limited for liquid dairy products because of the confirmed success of heat treatment, it is very promising for the surface applications of dairy products instead of using chemicals.

Light exposure of solid foods affects only a thin surface layer of the product, while a minimum light dose can reach its internal part [11]. Due to low penetration depth, UV light is suitable for inactivation of surface microorganisms to ensure product safety and extend shelf life with minor effects on chemical and nutritional values in dairy products. However, limited data are available on the effects of UV light on the surface decontamination, quality and organoleptic properties of dairy products.

In the surface applications of UV light, all targeted surfaces of the food must be exposed to UV light. For this purpose, flat products can be turned to allow exposure of both sides or placed on a supporting net or a film. Additional lamps can also be placed on the product sides [11].

One of the most common problems in cheese technology is molding on the surface. Application of UV light on cheese surface just before packaging can be a good solution to prevent mold growth. Lacivita et al. [38] reported 1–2 log reduction on *Pseudomonas* spp. and *Enterobacteriaceae* by applying UV light on the surface of cheese without changes in color, texture and surface

appearance. Authors concluded that this treatment showed an interesting surface microbial decontamination and prolonged cheese shelf-life with minimum transmittance inside the product. Similarly, Sık et al. [39] used different UV doses on the surface of Kashar cheese and application of UV-C (≥1.926 kJ/m^2) was able to achieve approximately 2–3 log reduction in mold population. Can et al. [40] investigated the efficacy of pulsed UV light for inactivation of inoculated *Penicillium roqueforti* and *Listeria monocytogenes* of hard cheeses packaged and unpackaged. The reduction of *P. roqueforti* was 1.32 log and 1.24 log in packaged and unpackaged cheeses, respectively. *L. monocytogenes* was reduced by over 2.8 log for packaged and unpackaged cheeses. They reported that pulsed UV light has potential to inactivate *P. roqueforti* and *L. monocytogenes* on the surface of hard cheeses. Proulx et al. [41] examined the effectiveness of pulsed-light (PL) treatment on the inactivation of the spoilage microorganisms on cheese surface in order to determine the effects of inoculum level and cheese surface topography and the presence of clear polyethylene packaging. Inoculated cheese samples were exposed to PL doses of 1.02–12.29 J/cm^2. *Listeria innocua* was the least sensitive with a maximum inactivation level of 3.37 log, followed by *P. fluorescens* with a maximum inactivation of 3.74 log and *Escherichia coli* ATCC 25922 with a maximum reduction of 5.41 log. The inactivation reached a plateau after three pulses (3.07 J/cm^2). The authors concluded that PL treatments through UV-transparent packaging and without packaging consistently resulted in similar inactivation levels.

After packaging of cheese, application of UV-C would be a good safety method to inactivate hazardous microorganisms on cheese surfaces. For this application, the transmission of UV light

Dairy product	UV treatment	Test microorganisms	Results/achieved inactivation	Studies
Sliced cheddar cheese	5 UV-C lamps **Intensity:** 3.04 mW/cm^2 **Treatment time:** 1 min	*Escherichia coli* O157: H7, *Salmonella Typhimurium*, *Listeria monocytogenes*	Suggested use of PP or PE films in conjunction with UV-C radiation for controlling foodborne pathogens	[21]
Fiordilatte cheese	**Intensity:** 20 W/m^2 **Treatment time:** up to 750 s	*Pseudomonas* spp., Enterobacteriaceae	About 1–2 log reduction without changes in color, texture and surface appearance	[38]
Kashar cheese	**Intensity:** 32.1 W/m^2 **Treatment time:** up to 300 s	Molds	Promising for surface mold reduction of pasta-filata cheese, but off-flavor at high doses	[39]
White American cheese	Pulsed Light Sterilization System **Distances:** 5, 8, and 13 cm **Treatment time:** up to 60 s	*Penicillium roqueforti*, *Listeria monocytogenes*	Suggested use of pulsed UV light for inactivation of *P. roqueforti* and *L. monocytogenes* on the surface of hard cheeses	[40]
Cheddar, process cheese	Bench top pulsed light unit **Doses:** 1.02 to 12.29 J/cm2	*Pseudomonas fluorescens*, *Escherichia coli ATCC 25922*, *Listeria innocua*	Suggested application for PL for decontamination of the cheese surface through UV-transparent packaging and without packaging	[41]
Set-type yoghurt	Batch UV light cabinet **Intensity:** 32.1 W/m^2 **Treatment time:** up to 600 s	Molds	Promising for surface mold reduction of yoghurt, but increased oxidation levels and off-flavor at high doses	[42]

Table 3. Effects of surface application of UV light on different dairy products.

through plastic film packaging and the thickness of packaging film are important parameters for eliminating or controlling growth of foodborne pathogens on the surfaces. Ha et al. [21] applied UV-C light for inactivation of food-borne pathogens on sliced cheese packaged with different types and thicknesses of plastic films. The authors' results showed that adjusted 0.07 mm thick PP or PE film packaging in conjunction with UV-C radiation can be effectively used for controlling foodborne pathogens including *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes*.

There has been really limited research carried on the surface decontamination of other dairy products with UV-light. Similar to cheese, post-processing contamination of the mold on set type yoghurt shortens its shelf life. That is why, the surface of set-type yoghurt samples contaminated naturally were exposed to UV light at different doses in a batch UV light cabinet to inactivate the mold at Ege University by chapter authors Koca and Saatli [42]. They indicated that UV light can be promising for mold inactivation of surface set-type of yoghurt and that higher doses of UV light increased oxidation levels slightly in yoghurt. Studies about the surface application of UV light to dairy products are summarized in **Table 3**.

5. Quality effects of UV light on dairy products

Milk is rich in protein, unsaturated fatty acids, metal ions, oxidases and other pro-oxidants that induce oxidative changes for lipids or protein in raw milk [43]. Dairy products are known as light sensitive products and light may decrease the nutritional value, the content of unsaturated fatty acids and vitamins especially riboflavin and α-tocopherol of the product [44, 45]. Figuring out the suitable UV doses which reduce the microbial growth enough without causing any sensorial defects is challenging. Consumer acceptance of UV treated dairy product will ultimately determine the acceptability of UV technology as an alternative or adjunct to commercial thermal treatment.

Limited research has been carried out on the effects of UV treatment on a biochemical and chemical perspective of dairy products. Some authors concluded that chemical composition of milk is not significantly affected by UV light application [43, 46]. Similarly, Cilliers et al. [22] concluded that UV light application to bovine milk did not affect most of the macro and micro-components, but reduced the cholesterol level compared to pasteurized milk. UV light application produced no change in raw milk with regard to the composition, free fatty acid profile, oxidation, or protein profile [46]. Another study showed that UV treatment to raw milk increased pH and reduced lightness, but did not change soluble solids content [43].

Lipid oxidation is known to be dependent on light exposure. In general, as the UV light dose increases, the oxidation degree and accordingly off-flavor increase in dairy products. In relation to oxidative changes of milk with UV light, increase in UV dose resulted in an increase in TBARs and acid degree values of the goat milk samples [47]. Similarly, higher values of malondialdehyde and other reactive substances in UV-treated raw cow milk were reported as an indication of oxidative degradation by Bandla et al. [31]. In contrast, Hu et al. [43] found no change in the values of TBARs of UV-C treated raw milk (11.8 W/m^2 dose), but an increase in its protein oxidation.

The nutritional value and sensory attributes of dairy products may change with the light exposure depending on the oxidation of lipids and protein and light sensitivity. Jung et al. [48] reported 'sunlight' flavor, which is characterized by a burnt and oxidized odor in milk after 2 or 3 days of UV application. Oxidized flavor in milk perceived as off-flavor results from oxidation of unsaturated fatty acid residues in milk lipids and phospholipids. The photodegradation of proteins also results in off-flavors and organoleptic changes in milk [14].

UV-C treatment has the potential to accelerate the formation of the volatile compounds in milk. In fact, Hu et al. [49] found an increase in the variety and content of volatile compounds of cow milk by the application of UV light (at 254 nm, 11.8 W/m^2). Nevertheless, no major differences were observed in terms of aroma-active compounds of milk following the UV treatment, but some new volatiles were generated [25]. In another study, no difference was found between the odor of untreated and UV treated cow milk but after 1 day of storage the UV-C treated sample had a significantly different smell from that of untreated milk [31]. The flavor defects in cow milk were clearly differentiated by panelists [30]. Cilliers et al. [22] noted the 'tallowy' flavor descriptor for the UV treated milk. In another study, odor of UV treated milk was described by panelists as manure, stinky, barnyard, and goaty [47].

Vitamin A, carotenes, vitamin B12, vitamin D, folic acid, vitamin K, riboflavin (vitamin B2) tocopherols (vitamin E), tryptophan, and unsaturated fatty acid residues in oils, solid fats, and phospholipids are well known as light sensitive nutrients [50]. The first research was carried out the increase in Vitamin D in milk. European Food Safety Authority (EFSA) concluded that the treatment of the pasteurized milk with UV radiation results in an increase in Vitamin D. The effects of UV light on vitamins A, B2, C, and E in cow and goat milk were assessed by Guneser and Karagul Yüceer [51]. UV light sensitivities of vitamins for the milk samples were found as C > E > A > B2. Authors concluded that UV light application reduces the vitamin content and their reduction levels depend on the initial amount of vitamins and the number of passes through the system. In contrast to most research, Cappozzo et al. [46] found that UV light, HTST and UHT processing of raw milk caused to decrease in vitamin D content to undetectable levels. UV light treatment reduced the content of vitamin A from 24.5 at 1045 J/L to 14.9% at 2090 J/L, but HTST and UHT processes resulted in a large reduction (96.8 and 100%, respectively). In bovine milk, vitamin B12 and riboflavin were not reduced by UV application in contrast to thermal treatment [22].

Protein oxidation in dairy systems has an important effect on protein properties and functionalities. UV light can cause the degradation or modification of proteins that lead to changes in solubility, sensitivity to heat, mechanical properties, and digestion by proteases [14]. In fact, Semagoto et al. [52] found changes in the solubility and color of milk protein concentrate. UV induced photo-oxidation decreased the solubility and contributed to the discoloration of milk protein concentrate during storage. Furthermore, exposure to UV irradiation resulted in denaturation of whey proteins but this denaturation degree is low when compared to UHT or HTST [53].

Application of UV light to raw milk used in the production of dairy products may also influence the quality of product. Some changes in rheological properties of yoghurt from UV treated milk were generated by UV treatment [25]. In this research, higher viscosity and lower syneresis were found in the sample made from UV-treated milk compared to that of heat

treated milk due to the effects of UV light on the molecular properties of proteins in milk sample. It is noted that UV treatment to raw milk limits the inactivation of native enzymes and the denaturation of whey proteins and the defects in products related to high initial bacterial counts, and shortens the ripening period of cheese. In contrast, Cilliers et al. [22] found no significant differences in the enzyme activity, α-amino acid contents and protein profiles of UV treated and pasteurized milk.

There are few data on the quality changes for surface application of UV light on dairy products such as cheese and yoghurt. Cheese treated with pulsed light at moderate (30 s at 8 cm) and extreme (40 s at 5 cm) conditions had higher values of TBARs compared to mild (5 s at 13 cm) treated and untreated samples, and the changes in color and chemical quality of cheeses were not significantly different after mild treatments. Additionally, when compared with packaged samples, unpackaged samples had slightly higher malondialdehyde values [40]. The application of UV light to surface of Kashar cheese slightly increased redness and yellowness values as the dosage of UV light increased, but these slight changes were not perceptible by the panelists [39]. However, they found that exposure of higher doses (9.630 kJ/m^2) of UV-C light led to photo-oxidation and accordingly caused flavor defects. In the other study, UV light application in batch UV cabinet to set-type yoghurt surface did not cause any significant changes with respect to hardness and color parameters [42]. On the other hand, the off-flavor was detected by panelists for the yoghurt samples treated at high dosages of UV light.

6. Legislations on UV light application in the production, processing and handling of food

The Food and Drug Administration, Department of Health and Human Services (US FDA) approved the use of UV radiation for controlling surface microorganisms of food or food product, sterilization of water used in production and reduction of human pathogens and other microorganisms in juice products under specific conditions defined by Code 21CFR179.39 [54]. These conditions are limited to the use of low pressure mercury lamps emitting 90% of the emission at a wavelength of 253.7 nm. If the pulsed UV is considered, in code 21CFR179.41, US FDA [55] approves the use of pulsed UV light for the surface microorganism control at doses not exceeding 12 J/cm^2 using xenon flashlamps, which are designed to emit broadband radiation consisting of wavelengths covering the range of 200–1100 nm, and operated no longer than 2 milliseconds for pulse duration. In addition, the minimum treatment required to obtain intended technical effect is used for food.

In European Union, UV light is accepted as irradiation [14]. The use of irradiation is limited but authorized in many European countries. According to European Commission, treating food with ionizing radiation may be authorized if there is reasonable technological need, it poses no health hazard and benefits consumers, and if it does not replace hygiene, health or good manufacturing or agricultural practice. Irradiated food or ingredients must be labeled. The UV-treated of pasteurized cow's milk was authorized as novel food in market by EC

because of the increase in vitamin D. It needs to be designated as "UV- treated milk" and also "contains vitamin D produced by UV-treatment" [2].

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References

[1] Steenbock H. Antirachitic product and process. US Patent. 1928. Patent No: 1680818

[2] Regulation (EC) No 258/97 of the European Parliament and of the Council of 19 July 2016 authorising the placing on the market of UV-treated milk as a novel food. Official Journal of the European Union: Legislation. 2016

[3] Bintsis T, Litopoulou-Tzanetaki E, Robinson RK. Existing and potential applications of ultraviolet light in the food industry – A critical review. Journal of the Science of Food and Agriculture. 2000;**80**:637-645. DOI: 10.1002/(SICI)1097-0010(20000501)80:6<637::AID-JSFA603>3.0.CO;2-1

[4] Shin JY, Kim SJ, Kim DK, Kanga DH. Fundamental characteristics of deep-UV light-emitting diodes and their application to control foodborne pathogens. Applied and Environmental Microbiology. 2016;**82**:2-10. DOI: 10.1128/AEM.01186-15

[5] Miller RV, Jeffrey W, Mitchell D, Elasri M. Bacterial responses to ultraviolet light. ASM News-American Society for Microbiology. 1999;**65**:535-541

[6] Bolton JR, Linden KG. Standardization of methods for fluence (UV dose) determination in bench-scale UV experiments. Journal of Enviromental Engineering. 2003;**129**:209-215. DOI: 10.1061/(ASCE)0733-9372(2003)129:3(209)

[7] Gomez-Lopez VM, Ragaert P, Debevere J, Devlieghere F. Pulsed light for food decontamination: A review. Trends in Food Science and Technology. 2007;**18**:464-473. DOI: 10.1016/j.tifs.2007.03.010

[8] Özkütük N. Ultraviyole lambalarının kullanımı (The use of ultraviolet lamps). In: Proceedings of 5th National Sterilization and Disenfection Congress. 4–8 April 2007; Antalya, Turkey. 2007. p. 490-496

[9] Koutchma T. Advances in ultraviolet light technology for non-thermal processing of liquid foods. Food and Bioprocess Technology. 2009;**2**:138-155. DOI: 10.1007/s11947-008-0178-3

[10] Elasri MO, Miller RV. Study of the response of a biofilm bacterial community to UV radiation. Applied and Environmental Microbiology. 1999;**65**:2025-2031

[11] Manzocco L, Nicoli MC. Surface processing: Existing and potential applications of ultraviolet light. Critical Reviews in Food Science and Nutrition. 2015;**55**:469-484. DOI: 10.1080/10408398.2012.658460

[12] Milly PJ, Toledo RT, Chen J, Kazem B. Hydrodynamic cavitation to improve bulk fluid to surface mass transfer in a nonimmersed ultraviolet system for minimal processing of opaque and transparent fluid foods. Journal of Food Science. 2007;**72**:407-413. DOI: 10.1111/j.1750-3841.2007.00518.x

[13] Guerrero-Beltrán JA, Barbosa-Cánovas GV. Review advantages and limitations on processing foods by UV light. Food Science and Technology International. 2004;**10**:137-147. DOI: 10.1177/1082013204044359

[14] Koutchma T, Forney LJ, Moraru CI. Ultraviolet Light in Food Technology: Principles and Applications. Boca Raton, FL, USA: Taylor & Francis Group; 2009. 278 p. DOI: 10.1201/9781420059519.ch5

[15] Schalk S, Adam V, Arnold E, Brieden K, Voronov A, Witzke HD. UV-lamps for disinfection and advanced oxidation – lamp types, technologies and applications. In: IUVA News. 2006;**8**(1):32-37

[16] Koutchma T, Keller S, Chirtel S, Parisi B. Ultraviolet disinfection of juice products in laminar and turbulent flow reactors. Innovative Food Science and Emerging Technologies. 2004;**5**:179-189. DOI: 10.1016/j.ifset.2004.01.004

[17] Franz CMAP, Specht I, Cho GS, Graef V, Stahl MR. UV-C-inactivation of microorganisms in naturally cloudy apple juice using novel inactivation equipment based on Dean vortex technology. Food Control. 2009;**20**:1103-1107. DOI: 10.1016/j.foodcont.2009.02.010

[18] Forney LJ, Ye Z, Koutchma T. UV disinfection of *E. coli* between concentric cylinders: Effects of the boundary layer and a wavy wall. Ozone: Science and Engineering. 2008;**30**: 405-412. DOI: 10.1080/01919510802473872

[19] Uysal Pala Ç, Kırca Toklucu A. Ultraviyole ışın (UV) teknolojisinin meyve sularına uygulanması. Akademik Gıda. 2010;**8**:17-22

[20] Gomez Lopez VM, Koutchma T, Linden K. Ultraviolet and pulsed light processing of fluid foods. In: Cullen PJ, Tiwari B, Valdramidis V, editors. Novel Thermal and Non-thermal Technologies for Fluid Foods. San Diego: Academic Press; 2012. pp. 185-223. DOI: 10.1016/C2009-0-61145-4

[21] Ha JW, Back KH, Kim YH, Kang DH. Efficacy of UV-C irradiation for inactivation of food-borne pathogens on sliced cheese packaged with different types and thicknesses of plastic films. Food Microbiology. 2016;**57**:172-177. DOI: 10.1016/j.fm.2016.02.007

[22] Cilliers FP, Gouws PA, Koutchma T, Engelbrecht Y, Adriaanse C, Swart P. A microbiological, biochemical and sensory characterization of bovine milk treated by heat and

ultraviolet (UV) light for manufacturing cheddar cheese. Innovative Food Science and Emerging Technologies. 2014;**23**:94-106. DOI: 10.1016/j.ifset.2014.03.005

[23] Crook JA, Rossito PV, Parko J, Koutchma T, Cullor JS. Efficacy of ultraviolet (UV-C) light in a thin-film turbulent flow for the reduction of milk borne pathogens. Foodborne Pathogens and Disease. 2015;**12**:506-513. DOI: 10.1089/fpd.2014.1843

[24] Matak KE, Churey JJ, Worobo RW, Sumner SS, Hovingh E, Hackney CR, Pierson MD. Efficacy of UV light for the reduction of listeria monocytogenes in goat's milk. Journal of Food Protection. 2005;**68**:2212-2216. DOI: 10.4315/0362-028X-68.10.2212

[25] Engin B, Karagul Yuceer Y. Effects of ultraviolet light and ultrasound on microbial quality and aroma-active components of milk. Journal of the Science of Food and Agriculture. 2012;**92**:1245-1252. DOI: 10.1002/jsfa.4689

[26] Krishnamurthy K, Demirci A, Irudayaraj JM. Inactivation of *Staphylococcus aureus* in milk using flow-through pulsed UV-light treatment system. Journal of Food Science. 2007;**72**: 233-239. DOI: 10.1111/j.1750-3841.2007.00438.x

[27] Choudhary R, Bandla S, Watson DG, Haddock J, Abughazaleh A, Bhattacharya B. Performance of coiled tube ultraviolet reactors to inactivate *Escherichia coli* W1485 and *Bacillus cereus* endospores in raw cow milk and commercially processed skimmed cow milk. Journal of Food Engineering. 2011;**107**:14-20. DOI: 10.1016/j.jfoodeng.2011.06.009

[28] Yin F, Zhu Y, Koutchma T, Gong J. Inactivation and potential reactivation of pathogenic *Escherichia coli* O157:H7 in bovine milk exposed to three monochromatic ultraviolet UVC lights. Food Microbiology. 2015;**49**:74-81. DOI: 10.1016/j.fm.2015.01.014

[29] Reinemann DJ, Gouws P, Cilliers T, Houck K, Bishop J. New methods for UV treatment of milk for improved food safety and product quality. American Society of Agricultural and Biological Engineers, Paper number 066088, 2006 ASABE Annual Meeting. DOI: 10.13031/2013.21493

[30] Rossito PV, Cullor JS, Crook J, Parko J, Sechi P, Cenci-Goga BT. Effects of UV irradiation in a continuous turbulent flow UV reactor on microbiological and sensory characteristics of cow's milk. Journal of Food Protection. 2012;**75**:2197-2207. DOI: 10.4315/0362-028X.JFP-12-036

[31] Bandla S, Choudhary R, Watson DG, Haddock J. UV-C treatment of soymilk in coiled tube UV reactors for inactivation of *Escherichia coli* W1485 and *Bacillus cereus* endospores. LWT – Food Science and Technology. 2012;**46**:71-76. DOI: 10.1016/j.lwt.2011.10.024

[32] Krishnamurthy K, Demirci A, Irudayaraj JM. Inactivation of *Staphylococcus aureus* by pulsed UV-light sterilization. Journal of Food Protection. 2004;**67**:1027-1030

[33] Altic LC, Rowe MT, Grant IR. UV light inactivation of *Mycobacterium avium* subsp. paratuberculosis in milk as assessed by FASTPlaque TB phage assay and culture. Applied and Environmental Microbiology. 2007;**73**:3728-3733. DOI: 10.1128/AEM.00057-07

[34] Donaghy J, Keyser M, Johnston J, Cilliers FP, Gouws PA, Rowe MT. Inactivation of *Mycobacterium avium* ssp. paratuberculosis in milk by UV treatment. Letters in Applied Microbiology. 2009;**49**:217-221. DOI: 10.1111/j.1472-765X.2009.02644.x

[35] Mahmoud NS, Ghaly AE. On-line sterilization of cheese whey using ultraviolet radiation. Biotechnology Progress. 2004;**20**:550-560. DOI: 10.1021/bp030050i

[36] Singh JP, Ghaly AE. Reduced fouling and enhanced microbial inactivation during online sterilization of cheese whey using UV coil reactors in series. Bioprocess and Biosystems Engineering. 2006;**29**:269-281. DOI: 10.1007/s00449-006-0076-x

[37] Pereira RV, Bicalho ML, Machado VS, Lima S, Teixeira AG, Warnick LD, Bicalho RC. Evaluation of the effects of ultraviolet light on bacterial contaminants inoculated into whole milk and colostrum, and on colostrum immunoglobulin G. Journal of Dairy Science. 2014;**97**:2866-2877. DOI: 10.3168/jds.2013-7601

[38] Lacivita V, Conte A, Manzocco L, Plazzotta S, Zambrini VA, Nobile MA, Nicoli MC. Surface UV-C light treatments to prolong the shelf-life of Fiordilatte cheese. Innovative Food Science and Emerging Technologies. 2016;**36**:150-155. DOI: 10.1016/j.ifset.2016.06.010

[39] Sık S, Urgu M, Koca N. The effect of UV light on the mould inactivation and the quality of fresh kashar cheese. In: Innovations in Food Science and Technology; Munich, Germany; May 10–12, 2017

[40] Can FO, Demirci A, Puri VM, Gourama H. Decontamination of hard cheeses by pulsed UV light. Journal of Food Protection. 2014;**77**:1723-1731. DOI: 10.4315/0362-028X. JFP-13-559

[41] Proulx J, Hsu LC, Miller BM, Sullivan G, Paradis K, Moraru CI. Pulsed-UV light inactivation of pathogenic and aoilage bacteria on cheese surface. Journal of Dairy Science. 2015; **98**:5890-5898

[42] Koca N, Saatli TE. The effects of ultraviolet light application on the mold growth and lipid oxidation of set-type yoghurt in batch system. Ege University Scientific Research Project. Project Number: 18-MUH-012. Izmir, Turkey

[43] Hu G, Zheng Y, Wang D, Zha B, Liu Z, Deng Y. Comparison of microbiological loads and physicochemical properties of raw milk treated with single-/multiple-cycle high hydrostatic pressure and ultraviolet-C light. High Pressure Research. 2015;**35**:330-338. DOI: 10.1080/08957959.2015.1063626

[44] Westermann S, Brüggemann DA, Olsen K, Skibsted LH. Light-induced formation of free radicals in cream cheese. Food Chemistry. 2009;**116**:974-981. DOI: 10.1016/j.foodchem. 2009.03.059

[45] Mortensen G, Bertelsen G, Mortensen BK, Stapelfeldt H. Light-induced changes in packaged cheeses – A review. International Dairy Journal. 2004;**14**:85-102. DOI: 10.1016/S0958-6946(03)00169-9

[46] Cappozzo JC, Koutchma T, Barnes G. Chemical characterization of milk after treatment with thermal (HTST and UHT) and nonthermal (turbulent flow ultraviolet) processing technologies. Journal of Dairy Science. 2015;**98**:5068-5079. DOI: 10.3168/jds.2014-9190

[47] Matak KE, Sumner SS, Duncan SE, Hovingh E, Worobo RW, Hackney CR, Pierson MD. Effects of ultraviolet irradiation on chemical and sensory properties of goat milk. Journal of Dairy Science. 2007;**90**:3178-3186. DOI: 10.3168/jds.2006-642

[48] Jung MY, Yoon SH, Lee HO, Min DB. Singlet oxygen and ascorbic acid effects on dimethyl disulfide and off-flavor in skim milk exposure to light. Journal of Food Science. 1998;**63**:408-412. DOI: 10.1111/j.1365-2621.1998.tb15753.x

[49] Hu G, Zheng Y, Liu Z, Deng Y. Effects of UV-C and single- and multiple-cycle high hydrostatic pressure treatments on flavor evolution of cow milk: Gas chromatography-mass spectrometry, electronic nose, and electronic tongue analyses. International Journal of Food Properties. 2017;**20**:1677-1688. DOI: 10.1080/10942912.2016.1217876

[50] Spikes J. Photodegradation of foods and beverages. Photochemical and Photobiological Reviews. 1981;**6**:39-81. DOI: 10.1007/978-1-4684-7003-1

[51] Guneser O, Karagul Yuceer Y. Effect of ultraviolet light on water- and fat-soluble vitamins in cow and goat milk. Journal of Dairy Science. 2012;**95**:6230-6241. DOI: 10.3168/jds.2011-5300

[52] Semagoto HM, Liu D, Koboyatau K, Hu J, Lu N, Liu X, Regenstein JM, Zhoua P. Effects of UV induced photo-oxidation on the physicochemical properties of milk protein concentrate. Food Research International. 2014;**62**:580-588. DOI: 10.1016/j.foodres.2014.04.012

[53] Kristo E, Hazizaj A, Correding M. Structural changes imposed on whey proteins by UV irradiation in a continuous UV light reactor. Journal of Agricultural and Food Chemistry. 2012;**60**:6204-6209. DOI: 10.1021/jf300278k

[54] US Food and Drug Administration. Ultraviolet Radiation for the Processing and Treatment of Food. Code of Federal Regulations 21 (Part 179.39). Washington, DC: FDA; 2016

[55] US Food and Drug Administration. Ultraviolet Radiation for the Treatment of Food. Code of Federal Regulations, Title 21, Vol. 3. (Part 179.41). Washington, DC: FDA; 2016

Chapter 2

The Application of Membrane Separation Technology in the Dairy Industry

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Additional information is available at the end of the chapter

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Abstract

Dairy industry is considered as an important food industry that provides various kinds of nutritionally rich dairy products for all age groups. Beside these nutritive values, dairy industry is contemplated as a good source of raw materials for other industries. Most importantly, dairy industry employs environment-friendly and energy-saving technologies. Membrane separation technology being one of those also focused on a cost-effective and environment-friendly manner, which can be widely applied in dairy industry for many useful purposes. In this chapter, we first define and classify the membrane separation technology and then comprehensively describe its applications, for instance, component separation, filtration, removal of bacteria, and wastewater treatment in dairy industry.

Keywords: membrane separation, milk concentration and component separation, milk sterilization, dairy wastewater treatment

1. Introduction

Milk is a complete food, and its products are a good source of essential nutrients for human health and raw materials for other industries. Dairy industry is a predominant part of food industry that has rapid growth and stability in emerging markets. With the increase of dairy industries, to control cost and make it sustainable, it is necessary to adopt new energy-efficient and eco-friendly technologies. In new technologies, membrane separation is an emerging technology with suitable properties for dairy products in which solution is passed through a membrane of microscopic pores and pressure applied to separate the components [1].

Membrane separation technique	Principle	Driving force (kPa)	Intercept component	Application
Microfiltration	Sieving	20–100	0.1–20 um	Clarification, separation, removal of bacteria, and filtration [2, 3]
Ultrafiltration	Sieving	100–1000	5–100 nm	Concentration, grading, and purification of macromolecular solution [4]
Nanofiltration	Dissolving diffusion, Donnan effect	500–1500	>1 nm	Separation, purification, and enrichment process of food, medicine, and biochemical industries [5]
Reverse osmosis	Dissolving diffusion	1000–10,000	0.1–1 nm	Concentration of low-molecular-weight components and removal of dissolved salts in aqueous solutions [6]
Electrodialysis	Ion exchange	Electrochemical potential—penetration	Large ions and water	Removal of salt and deacidifcation of solutions containing neutral components [7]
Pervaporation	Dissolving diffusion	Concentration difference	Insoluble or nonvolatile components	It is mainly used for volatile organic pollutants in the product separation and enrichment [8]

Table 1. Classification and characteristics of membrane separation techniques.

Most commonly used membrane separation techniques are microfiltration (MF), ultrafiltration (UF), nanofiltration, reverse osmosis, and electrodialysis. Their characteristics and applications are shown in **Table 1**. As these techniques have a good economic performance and are eco-friendly and uncomplicated to use, they are widely used in dairy industry for removal of bacteria, concentration, component separation, and wastewater treatment.

2. Applications of membrane separation technology in the dairy industry

2.1. Milk concentration and component separation

The removal of water from milk is known as milk concentration that reduces the cost of the packaging, storage, and transportation of milk and its products. To concentrate the milk, on the principle of heat exchange, flash [9] and falling film evaporation [10] methods are developed. However, these methods may change the composition, rheological characteristics, and heat stability and are energy consuming. As a result, the properties of final products are influenced [11]. Membrane separations are not phase separation technologies. They have advantages

of having lower cost, being environment friendly, and having a simple operation [12]. Kelly [13] and Jevons [14] applied ultrafiltration and reverse osmosis in preconcentration of quarg, soft cheese, and yogurt, respectively. The results showed that output of cheese significantly improved.

Besides milk concentration, milk components such as casein, whey proteins, mineral substance, lactose, and saccharides can also be isolated by membrane separation techniques. Milk proteins are whey protein (average diameter < 20 nm) and casein micelles (average diameter of 200 nm) that can be isolated by using membranes of 0.05 to 0.2 nm diameter [15]. Whey protein consists of lactoferrin, β-lactoglobulin, α-lactalbumin and immunoglobulins that demonstrate a range of immune-enhancing properties [16]. These components can be separated by permeation and phage retention by using one filtration process at the same time that gives approximately 60% α-lactalbumin and 40% β-lactoglobulin [17]. Al-akoum et al. [18] reported that slightly higher transmission rates, 65% for α-lactalbumin and 25–30% for β-lactoglobulin, were obtained by using vibratory shear enhanced processing. Rotating ceramic membranes are more suitable because they offered a better compromise between flux and whey protein transmission [19].

Casein is a major protein found in mammalian milk (80% of cow's milk proteins and 20–45% of human milk proteins) [20] that provides amino acids, carbohydrates, calcium, and phosphorus. Membrane separation processes do not affect the micellar structure of casein as compared to traditional methods such as acidification and rennet coagulation [21]. The temperature in membrane filtration is 45 to 50°C that is beneficial for high flux and growth control of mesophilic bacteria [22]. β-Casein exists in the serum phase that requires low temperature [23, 24]. Therefore, new separation techniques are developed such as polyethersulfone (PES) and polyvinylidene fluoride (PVDF) membrane that applied in β-casein enrichment at refrigeration temperatures (<20°C). The final casein has same composition, physicochemical properties, and protein profile. Moreover, PES membrane has a higher flux and a lower fouling [25]. Chai et al. [26] applied the transverse vibrating membrane filtration system of 0.04 μm PVDF membrane at 10°C to separate and concentrate the milk protein, and the structure of obtained protein was preserved better.

It is notable that membrane fouling is a serious problem and becomes more severe when protein concentrated and viscosity increased during protein separation. Therefore, effective methods for fouling removal are developed. High cross-flow velocities can effectively increase the shear force that controls the membrane fouling and maintains the productivity [27]. Dynamic membrane systems such as vibratory shear enhancing process are also helpful to control the fouling problem. The sugar present in milk is lactose that is a functional ingredient used in food and pharmaceutical products. It is used in bakery goods to reduce sweetness and enhance browning and as a protective carrier for sensitive proteins and peptides. But high amounts of lactose content lead to undesirable grainy texture and cause dyspepsia [28]. The wastewater of dairy industry contains high amount of lactose that increases the level of chemical and biochemical oxygen and causes pollution. To control this pollution, it is necessary to adopt membrane separation techniques to remove lactose before draining the wastewater [29–31].

The combination of microfiltration and ultrafiltration was applied to produce protein-enriched yogurt from fractionated skim milk. Results showed that the lactose content of final product also decreased up to 50% [32]. Morr and Brandon [33] evaluated that when MF in combination with UF membrane was applied to fractionate lactose and sodium from skim milk, 90–95% of lactose and sodium fractionated without affecting the consumer acceptance, product appearance, and flavor. When the lactose is separated from goat's milk by ultrafiltration membrane, some particular components such as serum proteins, casein, and fat globules are retained. The optimization of parameters usually involve transmembrane pressure and cross-flow velocity [34].

Lactose recovery from wastewater with ultrafiltration, nanofiltration, and reverse osmosis was also reported in many previous studies [35–37]. In general, nanofiltration and reverse osmosis are more efficient in terms of lactose recovery, but they require a higher operating pressure as compared to ultrafiltration [38–41]. Chollangi and Hossain [42] found that membranes with molar weight cutoff 3, 5, and 10 kDa provided 70–80%, 90–95%, and 100% recovery rate of lactose in permeate, respectively. In addition, lactose hydrolysis was applied in a continuous stirred tank-ultrafiltration (CSTR-UF) with β-galactosidase enzyme to produce galactose and glucose [43].

Human milk oligosaccharides (HMOs) play an important role in the growth and development of infants [44]. Animal milk also contains oligosaccharides with similar structure and function as human milk oligosaccharides that can be a functional food ingredient [45]. Sialyllactose is N-acetylneuraminic acid (sialic acid) bound to β-lactose, and Luo et al. [46] showed that high permeation of 3′-sialyllactose is obtained by using an integrated UF/NF membrane system for the valorization of dairy by-products with engineered sialidase. Continuous production of sialyllactose, as a typical sialylsaccharide, was also examined with a membrane reactor by Masuda et al. [47].

2.2. Removal of bacteria in milk

Milk contains particles with different sizes such as somatic cells (15–6 um), fat globules (15–0.2 um), bacteria (6–0.2 um), and casein micelles (0.3–0.03 um) [48]. Microbial and somatic cells of milk affect quality, flavor, and shelf life of final dairy products. Milk is treated with heat to remove microbial cells [49]. However, the heat treatment change the nutritional and flavor profile of the products [50, 51]. Membrane separation techniques are operated at low temperature, which remove bacteria effectively without affecting the nutritional and flavor profile. It also reduces the processing and transportation cost; that's why microfiltration is widely used for the removal of bacteria [52–55]. Cross-flow microfiltration (CFMF) has emerged as an industrial separation technique in the dairy industry [56–58].

Sterilization with inorganic ceramic membrane not only keeps the flavor of milk but also prolongs the shelf life of product. This processing technology is combined with slight heat treatment and applied in cold pasteurization. The products are called as extended shelf-life (ESL) milk [59]. ESL milk has a shelf life of 3 weeks, longer than HTST-pasteurized milk (10 days, typically), and sensory profile analysis shows that ESL milks have no appreciable difference from the pasteurized milk during storage. It fills the gap between high-temperature short-time (HTST) pasteurized milk and ultrahigh temperature (UHT) milk [60].

2.3. Wastewater treatment

Dairy industry is the major source of wastewater in the food processing [61] that contains large amount of organic matter and nutrients [62]. Common treatments include primary treatment and secondary biological treatment. Membrane separation usually plays an important role in secondary biological treatment as it is simple and energy saving and has wastewater zero emissions [63, 64]. In this processing, protein and sugar are also recycled from the wastewater. Membrane with different molecular weight cut off plays different roles in wastewater treatment. **Table 2** shows the applications of different membrane separation technologies for wastewater treatment.

2.4. Application of electrodialysis and pervaporation in dairy industry

Apart from above mentioned membrane separation technologies, electrodialysis and pervaporation are also used in dairy industry. Electrodialysis is a unit operation applied for the separation or concentration of ions in a solution, based on their selective electromigration through semipermeable membranes under the influence of a potential gradient. Nowadays, this operation has been widely used for demineralization in the dairy industry [66] and has successfully applied electrodialysis for desalination of skimmed milk and showed that the technique is useful in demineralization of dairy products. Demineralization is helpful for better use of milk protein such as application in infant formula. Laurent et al. [67] used electrodialysis for demineralization of skim milk with rate up to 75%. This is much better than their previous study (30–40% demineralization rate) [68]. Chen et al. [69] also successfully applied electrodialysis to remove the lactate ions from acid whey in order to solve operational problems in downstream spray drying operations. Alternatively, electrodialysis has been successfully demonstrated to recover lactic acid from fermentation broths [70, 71], as well as to demineralize sweet whey prior to whey powder production [72, 73]. However, ED applications are still in their infancy, and its potentialities have not been completely exploited probably because of the high specific electromembrane costs or their short lifetime [74].

Pervaporation is a selective membrane separation process in which some feed components are concentrated to a greater degree than others with the selectivity controlled by the membrane type [75]. It can be used to concentrate certain compounds in a mixture. In hydrophobic

Membrane separation technique	MWCO	Application
Microfiltration	100–500 kDa	Remove almost all pathogenic bacterial species and mold as well as a certain amount of halogenated salt [48]
Ultrafiltration	2–150 kDa	Remove almost all of the protein, fat, and some insoluble compounds and minerals in dairy wastewater, and only lactose, soluble salts, and ash content will be allowed to pass [65]
Nanofiltration	0.2–2 kDa	Intercept the lactose in the dairy wastewater, and recover more than 90% of the acid and alkali wastewater from clean in place (CIP)
Reverse osmosis	<0.2 kDa	Intercept almost all pollutants in dairy wastewater.

Table 2. Application of different membrane separation technologies in dairy wastewater treatment.

pervaporation, volatile hydrophobic compounds such as flavors pass through the polymeric membrane more readily than water and are thereby concentrated in the permeate [76]. In previous reports, it was used to concentrate acids, esters, and ketones in model flavor mixtures, and the characteristics of the feed mixture (pH and presence of dairy ingredients) were found to alter the pervaporation behavior of the flavor compounds [77].

3. Conclusion

The applications of membrane separation technology in dairy industry are wide. These are used in milk concentration, component separation such as protein and lactose, filtration, and bacteria reduction as well as play an important role in dairy industry wastewater treatments. All these applications fully demonstrate the advantages of membrane separation: simple operation, environment-friendly, and energy saving. However, there are still some problems such as membrane fouling that limit its further application. Therefore, more attention should be paid on the mechanism and control methods.

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References

[1] Ortega-Rivas E. Membrane separations. In: Non-thermal Food Engineering Operations. Boston: Springer; 2012. pp. 199-214. DOI: 10.1007

[2] Chhaya SC, Mondal S, Majumdar GC, De S. Clarification of Stevia extract by ultrafiltration: Selection criteria of the membrane and effects of operating conditions. Food and Bioproducts Processing. 2012;**90**(C3):525-532. DOI: 10.1016/j.fbp.2011.10.002

[3] Pinto MS, Pires ACS, Sant'Ana HMP, Soares NFF, Carvalho AF. Influence of multilayer packaging and microfiltration process on milk shelf life. Food Packaging and Shelf Life. 2014;**1**(2):151-159. DOI: 10.1016/j.fpsl.2014.01.006

[4] Loginov M, Boussetta N, Lebovka N, Vorobiev E. Separation of polyphenols and proteins from flaxseed hull extracts by coagulation and ultrafiltration. Journal of Membrane Science. 2013;**442**:177-186. DOI: 10.1016/j.memsci.2013.04.036

[5] Wang KY, Xiao Y, Chung T-S. Chemically modified polybenzimidazole nanofiltration membrane for the separation of electrolytes and cephalexin. Chemical Engineering Science. 2006;**61**(17):5807-5817. DOI: 10.1016/j.ces.2006.04.031

[6] Skelton R. Membrane filtration applications in the food industry. Filtration & Separation. 2000;**37**(3):28-30. DOI: 10.1016/S0015-1882(00)88494-3

[7] Strathmann H. Electrodialysis, a mature technology with a multitude of new applications. Desalination. 2010;**264**(3):268-288. DOI: 10.1016/j.desal.2010.04.069

[8] Buvet R, Idier L, Inventors; MORGANE, assignee. Process for the concentration by pervaporation of an aqueous fluid containing volatile or carrier water vapour-distillable organic compounds; 1990. EP0380424

[9] Kiesner C, Eggers R. Concept of a sterile concentration process for milk by multistage flash evaporation. Chemical Engineering and Technology. 1994;**17**:374-381. DOI: 10.1002/ceat.270170603

[10] Liu D. Design considerations of single-effect falling film evaporator in liquid milk. Journal of Dairy Science & Technology. 2010;**4**:167-168. DOI: 10.15922/j.cnki.jdst.2010.04.008

[11] Liu D, Li J, Zhang J, Liu X, Wang M, Hemar Y, et al. Effect of partial acidification on the ultrafiltration and diafiltration of skim milk: Physico-chemical properties of the resulting milk protein concentrates. Journal of Food Engineering. 2017;**212**:55-64. DOI: 10.1016/j.jfoodeng.2017.05.019

[12] Kolfschoten RC, Janssen AEM, Boom RM. Mass diffusion-based separation of sugars in a microfluidic contactor with nanofiltration membranes. Journal of Separation Science. 2011;**34**(11):1338-1346. DOI: 10.1002/jssc.201100018

[13] Kelly PM. Application of membranes to whole milk separations: A review. Irish Journal of Food Science and Technology. 1987;**11**(2):153-161. DOI: 10.2307/25558165

[14] Jevons M. Separate and concentrate. Dairy Industries International. 1997;**8**(62):19-21, 62

[15] Heino AT, Uusi-Rauva JO, Rantamaki PR, Tossavainen O. Functional properties of native and cheese whey protein concentrate powders. International Journal of Dairy Technology. 2007;**60**(4):277-285. DOI: 10.1111/j.1471-0307.2007.00350.x

[16] Marshall K. Therapeutic applications of whey protein. Alternative Medicine Review: A Journal of Clinical Therapeutic. 2004;**9**(2):136-156

[17] Samtlebe M, Wagner N, Neve H, Heller KJ, Hinrichs J, Atamer Z. Application of a membrane technology to remove bacteriophages from whey. International Dairy Journal. 2015;**48**:38-45. DOI: 10.1016/j.idairyj.2014.12.004

[18] Al-Akoum O, Ding L, Chotard-Ghodsnia R, Jaffrin MY, Gésan-Guiziou G. Casein micelles separation from skimmed milk using a VSEP dynamic filtration module. Desalination. 2002;**144**(1):325-330. DOI: 10.1016/S0011-9164(02)00337-5

[19] Espina VS, Jaffrin MY, Ding LH. Comparison of rotating ceramic membranes and polymeric membranes in fractionation of milk proteins by microfiltration. Desalination. 2009;**245**(1):714-722. DOI: doi.org/10.1016/j.desal.2009.02.042

[20] Kunz C, Lonnerdal B. Human-milk proteins: Analysis of casein and casein subunits by anion-exchange chromatography, gel electrophoresis, and specific staining methods. The American Journal of Clinical Nutrition. 1990;**51**(1):37-46. DOI: 10.1093/ajcn/51.1.37

[21] Lawrence ND, Kentish SE, O'Connor AJ, Barber AR, Stevens GW. Microfiltration of skim milk using polymeric membranes for casein concentrate manufacture. Separation and Purification Technology. 2008;**60**(3):237-244. DOI: 10.1016/j.seppur.2007.08.016

[22] Hurt E, Zulewska J, Newbold M, Barbano DM. Micellar casein concentrate production with a 3X, 3-stage, uniform transmembrane pressure ceramic membrane process at 50 degrees C. Journal of Dairy Science. 2010;**93**(12):5588-5600. DOI: 10.3168/jds.2010-3169

[23] Payens TAJ, Van Markwijk BW. Some features of the association of β-casein. Biochimica et Biophysica Acta. 1963;**71**(Supplement C):517-530. DOI: 10.1016/0006-3002(63)91124-7

[24] Rose D. Relation between micellar and serum casein in bovine milk. Journal of Dairy Science. 1968;**51**(12):1897-1902. DOI: 10.3168/jds.S0022-0302(68)87308-4

[25] Crowley SV, Caldeo V, McCarthy NA, Fenelon MA, Kelly AL, O'Mahony JA. Processing and protein-fractionation characteristics of different polymeric membranes during filtration of skim milk at refrigeration temperatures. International Dairy Journal. 2015;**48**:23-30. DOI: 0.1016/j.idairyj.2015.01.005

[26] Chai M, Ye Y, Chen V. Separation and concentration of milk proteins with a submerged membrane vibrational system. Journal of Membrane Science. 2017;**524**:305-314. DOI: 10.1016/j.memsci.2016.11.043

[27] Punidadas P, Rizvi SSH. Separation of milk proteins into fractions rich in casein or whey proteins by cross flow filtration. Food Research International. 1998;**31**(4):265-272. DOI: 10.1016/S0963-9969(98)00088-X

[28] Somkuti GA, Holsinger VH. Microbial technologies in the production of low-lactose dairy foods. Food Science and Technology International. 1997;**3**(3):163-169. DOI: 10.1177/108201329700300302

[29] Cuartas-Uribe B, Alcaina-Miranda MI, Soriano-Costa E, Mendoza-Roca JA, Iborra-Clar MI, Lora-Garcia J. A study of the separation of lactose from whey ultrafiltration permeate using nanofiltration. Desalination. 2009;**241**(1):244-255. DOI: 10.1016/j.desal.2007.11.086

[30] Marwaha SS, Kennedy JF. Review: Whey–pollution problem and potential utilization. International Journal of Food Science and Technology. 1988;**23**(4):323-336. DOI: 10.1111/j.1365-2621.1988.tb00586.x

[31] Trägårdh G. Membrane applications in the food industry. Polymer Journal. 1991;**23**(5):521-529. DOI: 10.1295/polymj.23.521

[32] Rinaldoni AN, Campderros M, Menendez CJ, Padilla AP. Fractionation of skim milk by an integrated membrane process for yoghurt elaboration and lactose recuperation. International Journal of Food Engineering. 2009;**5**(3). DOI: 10.2202/1556-3758.1531

[33] Morr CV, Brandon SC. Membrane fractionation processes for removing 90 to 95% of the lactose and sodium from skim milk and for preparing lactose and sodium-reduced skim milk. Journal of Food Science. 2008;**73**(9):C639-CC47. DOI: 10.1111/j.1750-3841.2008.00961.x

[34] Abidin NSZ, Hussain SA, Kamal SMM. Removal of lactose from highly Goat's milk concentration through ultrafiltration membrane. In: Ahmed I, editor. Process and Advanced Materials Engineering, Applied Mechanics and Materials. Vol. 6252014. pp. 596-599. DOI: 10.4028/www.scientific.net/AMM.625.596

[35] Barba D, Beolchini F, VegliÓ F. Minimizing water use in Diafiltration of whey protein concentrates. Separation Science and Technology. 2000;**35**(7):951-965. DOI: 10.1081/SS-100100204

[36] Barba D, Beolchini F, Cifoni D, Veglio F. Whey protein concentrate production in a pilot scale two-stage diafiltration process. Separation Science and Technology. 2001;**36**(4):587-603. DOI: 10.1081/SS-100102948

[37] Pouliot Y, Wijers MC, Gauthier SF, Nadeau L. Fractionation of whey protein hydrolysates using charged UF NF membranes. Journal of Membrane Science. 1999;**1;158**(1-2):105-114. DOI: 10.1016/S0376-7388(99)00006-X

[38] Cheang BL, Zydney AL. A two-stage ultrafiltration process for fractionation of whey protein isolate. Journal of Membrane Science. 2004;**231**(1-2):159-167. DOI: 10.1016/j.memsci.2003.11.014

[39] Atra R, Vatai G, Bekassy-Molnar E, Balint A. Investigation of ultra- and nanofiltration for utilization of whey protein and lactose. Journal of Food Engineering. 2005;**67**(3):325-332. DOI: 10.1016/j.jfoodeng.2004.04.035

[40] Barba D, Beolchini F, Veglio F. Water saving in a two stage diafiltration for the production of whey protein concentrates. Desalination. 1998;**119**(1):187-188. DOI: 10.1016/S0011-9164(98)00146-5

[41] Barba D, Beolchini F, Cifoni D, Veglio F. Whey ultrafiltration in a tubular membrane: Effect of selected operating parameters. Separation Science and Technology. 2002;**37**(8):1771-1788. DOI: doi.org/10.1081/SS-120003043

[42] Chollangi A, Hossain MM. Separation of proteins and lactose from dairy wastewater. Chemical Engineering and Processing. 2007;**46**(5):398-404. DOI: 10.1016/j.cep.2006.05.022

[43] Namvar-Mahboub M, Pakizeh M. Experimental study of lactose hydrolysis and separation in CSTR-UF membrane reactor. Brazilian Journal of Chemical Engineering. 2012;**29**(3):613-618. DOI: 10.1590/S0104-66322012000300018

[44] Hickey RM. The role of oligosaccharides from human milk and other sources in prevention of pathogen adhesion. International Dairy Journal. 2012;**22**(2):141-146. DOI: 10.1016/j.idairyj.2011.09.012

[45] Oliveira DL, Wilbey RA, Grandison AS, Roseiro LB. Milk oligosaccharides: A review. International Journal of Dairy Technology. 2015;**68**(3):305-321. DOI: 10.1111/1471-0307.12209

[46] Luo J, Nordvang RT, Morthensen ST, Zeuner B, Meyer AS, Mikkelsen JD, et al. An integrated membrane system for the biocatalytic production of 3'-sialyllactose from dairy by-products. Bioresource Technology. 2014;**166**:9-16. DOI: 10.1016/j.biortech.2014.05.003

[47] Masuda M, Kawase Y, Kawase M. Continuous production of sialyllactose from colominic acid using a membrane reactor. Journal of Bioscience and Bioengineering. 2000;**89**(2):119-125. DOI: 10.1016/S1389-1723(00)88724-1

[48] Saboya LV, Maubois JL. Current developments of microfiltration technology in the dairy industry. Le Lait. 2000;**80**(6):541-553. DOI: 10.1051/lait:2000144

[49] Hinrichs J, Atamer Z. Heat Treatment of Milk | Sterilization of Milk and Other Products A2 - Fuquay. In: John W, editor. Encyclopedia of Dairy Sciences. 2nd ed. San Diego: Academic Press; 2011. pp. 714-724. DOI: 10.1016/B978-0-12-374407-4.00218-1

[50] Kilshaw PJ, Heppell LM, Ford JE. Effects of heat treatment of cow's milk and whey on the nutritional quality and antigenic properties. Archives of Disease in Childhood. 1982;**57**(11):842-847. DOI: 10.1136/adc.57.11.842

[51] Greenbank GR, Wright PA. The deaeration of raw whole milk before heat treatment as a factor in retarding the development of the tallowy flavor in its dried product. Journal of Dairy Science. 1951;**34**(8):815-818. DOI: 10.3168/jds.S0022-0302(51)91786-9

[52] Solanki G, Rizvi SSH. Physico-chemical properties of skim milk retentates from microfiltration. Journal of Dairy Science. 2001;**84**(11):2381-2391. DOI: 10.3168/jds.S0022-0302(01)74687-5

[53] Pafylias I, Cheryan M, Mehaia MA, Saglam N. Microfiltration of milk with ceramic membranes. Food Research International. 1996;**29**(2):141-146. DOI: 10.3168/jds.S0022-0302(01)74687-5

[54] Fritsch J, Moraru CI. Development and optimization of a carbon dioxide-aided cold microfiltration process for the physical removal of microorganisms and somatic cells from skim milk. Journal of Dairy Science. 2008;**91**(10):3744-3760. DOI: 10.3168/jds.2007-0899

[55] Madaeni SS, Tavakolian HR, Rahimpour F. Cleaning optimization of microfiltration membrane employed for milk sterilization. Separation Science and Technology. 2011;**46**(4):571-580. DOI: 10.1080/01496395.2010.534118

[56] Tomasula PM, Mukhopadhyay S, Datta N, Porto-Fett A, Call JE, Luchansky JB, et al. Pilot-scale crossflow-microfiltration and pasteurization to remove spores of *Bacillus anthracis* (Sterne) from milk. Journal of Dairy Science. 2011;**94**(9):4277-4291. DOI: 10.3168/jds.2010-3879

[57] Fauquant J, Beaucher E, Sinet C, Robert B, Lopez C. Combination of homogenization and cross-flow microfiltration to remove microorganisms from industrial buttermilks with an efficient permeation of proteins and lipids. Innovative Food Science & Emerging Technologies. 2014;**21**:131-141. DOI: 10.1016/j.ifset.2013.10.004

[58] Schmidt VSJ, Kaufmann V, Kulozik U, Scherer S, Wenning M. Microbial biodiversity, quality and shelf life of microfiltered and pasteurized extended shelf life (ESL) milk from Germany, Austria and Switzerland. International Journal of Food Microbiology. 2012;**154**(1-2):1-9. DOI: 10.1016/j.ijfoodmicro.2011.12.002

[59] Hoffmann W, Kiesner C, Clawin-RÄDecker I, Martin D, Einhoff K, Lorenzen PC, et al. Processing of extended shelf life milk using microfiltration. International Journal of Dairy Technology. 2006;**59**(4):229-235. DOI: 10.1111/j.1471-0307.2006.00275.x

[60] Lorenzen PC, Clawin-Raedecker I, Einhoff K, Hammer P, Hartmann R, Hoffmann W, et al. A survey of the quality of extended shelf life (ESL) milk in relation to HTST and UHT milk. International Journal of Dairy Technology. 2011;**64**(2):166-178. DOI: 10.1111/j.1471-0307.2010.00656.x

[61] Rahimi Z, Zinatizadeh AA, Zinadini S. Milk processing wastewater treatment in a bioreactor followed by an antifouling O-carboxymethyl chitosan modified Fe_3O_4/PVDF ultrafiltration membrane. Journal of Industrial and Engineering Chemistry. 2016;**38**:103-112. DOI: 10.1016/j.jiec.2016.04.011

[62] Perle M, Kimchie S, Shelef G. Some biochemical aspects of the anaerobic degradation of dairy wastewater. Water Research. 1995;**29**(6):1549-1554. DOI: 10.1016/0043-1354(94)00248-6

[63] Luo J, Ding L, Wan Y, Paullier P, Jaffrin MY. Application of NF-RDM (nanofiltration rotating disk membrane) module under extreme hydraulic conditions for the treatment of dairy wastewater. Chemical Engineering Journal. 2010;**163**(3):307-316. DOI: 10.1016/j.cej.2010.08.007

[64] Andrade LH, Mendes FDS, Espindola JC, Amaral MCS. Nanofiltration as tertiary treatment for the reuse of dairy wastewater treated by membrane bioreactor. Separation and Purification Technology. 2014;**126**:21-29. DOI: 10.1016/j.seppur.2014.01.056

[65] Luo J, Ding L, Qi B, Jaffrin MY, Wan Y. A two-stage ultrafiltration and nanofiltration process for recycling dairy wastewater. Bioresource Technology. 2011;**102**(16):7437-7442. DOI: 10.1016/j.biortech.2011.05.012

[66] Andrés LJ, Riera FA, Alvarez R. Skimmed milk demineralization by electrodialysis: Conventional versus selective membranes. Journal of Food Engineering. 1995;**26**(1):57-66. DOI: 10.1016/0260-8774(94)00042-8

[67] Bazinet L, Ippersiel D, Gendron C, René-Paradis J, Tétrault C, Beaudry J. Bipolar membrane electroacidification of demineralized skim milk. Journal of Agricultural and Food Chemistry. 2001;**49**(6):2812-2818. DOI: 10.1021/jf000982r

[68] Bazinet L, Lamarche F, Ippersiel D, Amiot J. Bipolar membrane electroacidification to produce bovine milk casein isolate. Journal of Agricultural and Food Chemistry. 1999;**47**(12):5291-5296. DOI: 10.1021/jf990524m

[69] Chen GQ, Eschbach FII, Weeks M, Gras SL, Kentish SE. Removal of lactic acid from acid whey using electrodialysis. Separation & Purification Technology. 2016;**158**:230-237. DOI: 10.1016/j.seppur.2015.12.016

[70] Lee EG, Moon SH, Yong KC, Yoo IK, Chang HN. Lactic acid recovery using two-stage electrodialysis and its modelling. Journal of Membrane Science. 1998;**145**:53-66. DOI: 10.1016/S0376-7388(98)00065-9

[71] Wee YJ, Yun JS, Lee YY, Zeng AP, Ryu HW. Recovery of lactic acid by repeated batch electrodialysis and lactic acid production using electrodialysis wastewater. Journal of Bioscience and Bioengineering. 2005;**99**:104-108. DOI: 10.1263/jbb.99.104

[72] Pérez A, Andrés LJ, Álvarez R, Coca JCGH Jr. Electrodialysis of whey permeates and retentates obtained by ultrafiltration. Journal of Food Process Engineering. 1994;**17**:177-190. DOI: 10.1111/j.1745-4530.1994.tb00334.x

[73] Greiter M, Novalin S, Wendland M, Kulbe KD, Fischer J. Desalination of whey by electrodialysis and ion exchange resins: analysis of both processes with regard to sustainability by calculating their cumulative energy demand. Journal of Membrane Science. 2002;**210**:91-102. DOI: 10.1016/S0376-7388(02)00378-2

[74] Fidaleo M, Moresi M. Electrodialysis Applications in the Food Industry. Advances in Food & Nutrition Research. 2006;**51**(51):265-360. DOI: 10.1016/S1043-4526(06)51005-8

[75] Karlsson HOE, Trägårdh G. Pervaporation of dilute organic-waters mixtures. A literature review on modelling studies and applications to aroma compound recovery. Journal of Membrane Science. 1993;**76**(2-3):121-146. DOI: 10.1016/0376-7388(93)85211-E

[76] Lipnizki F, Hausmanns S, Ten PK, Field RW, Laufenberg G. Organophilic pervaporation: Prospects and performance. Chemical Engineering Journal. **73**:113-129. DOI: 10.1016/S1385-8947(99)00024-8

[77] Overington AR, Wong M, Harrison JA. Effect of feed pH and non-volatile dairy components on flavour concentration by pervaporation. Journal of Food Engineering. 2011;**107**(1):60-70. DOI: 10.1016/j.jfoodeng.2011.05.045

Chapter 3

Potential of High Pressure Homogenization and Functional Strains for the Development of Novel Functional Dairy Foods

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Additional information is available at the end of the chapter

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Abstract

Functional foods are one of the fastest increasing fields in the global food industry since they are positively perceived by the consumers as dietary strategies to reduce the incidence of illness in the humankind. Actually, the use of biotechnological strategies, based on the use of functional and specific strains and sustainable technologies, such as high-pressure homogenization, can be a great chance to create innovation in the dairy field. Critical discussion on the actual scenario is the main topic of this chapter.

Keywords: high pressure homogenization, functional strains, dairy applications, novel product application, innovation

1. Introduction

Functional foods are one of the fastest increasing fields in the global food industry since they are positively perceived by the consumers as dietary strategies to reduce the incidence of illness in the humankind as established by the European Commission's Concerted Action on Functional Food Science in Europe (FuFoSE) (**Figure 1**) [1, 2].

Currently, the most important and interesting applications have been studied and applied for the dairy ingredients and products due to their great potential as functional and nutraceuticals. Although the health-promoting dairy products can be represented by several food types such as products with intrinsic functionality and products fortified with natural ingredients

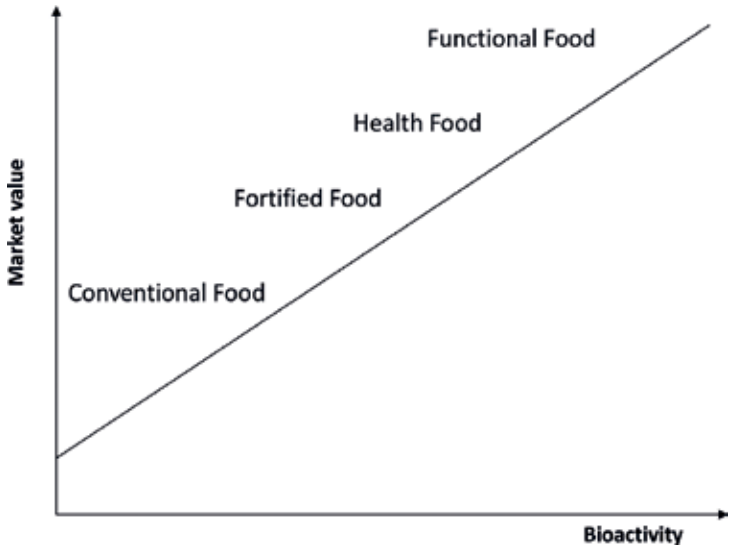

Figure 1. Functional food market. Source: Adapted from koronen, 2002.

to obtain a desired functionality, the probiotic products have received the highest attention due to their importance as a suitable vehicle for probiotic microorganisms, defined as "*live microorganisms which when consumed in adequate numbers confer a health benefit on the host*" [3]. In fact, it is scientifically proved that the intake of probiotics, in relation to the species and strain, can positively affect the host at gastrointestinal level and in the respiratory and urogenital tracts. Also lactose intolerance, diarrhea, gastroenteritis, irritable bowel syndrome, inflammatory bowel disease, depressed immune function, cancer and genitourinary tract infections can take benefit with probiotic-based diet. Thus, the development of dairy products containing probiotic bacteria has become an important challenge in the food industry and several attempts have been made to identify the most suitable carriers, strains and process conditions for probiotic products [3]. The increasing demand for high-value health-promoting dairy foods has encouraged the industries and consequently, the research field, to investigate new emerging food processes and biotechnological strategies to obtain functional but innovative products without detrimental effect on safety, quality and nutritional value. In particular, the literature has pointed out the potential of non-thermal technologies, such as high-pressure processing treatments, in the development of new functional dairy foods. Also the use of selected microbial strains used as fermentation starters, co-starters, adjuncts or bio-control agents, imparting specific functionalities but maintaining high product quality and safe standards, can contribute to designing new bio-functional dairy foods. Moreover, the literature has underlined the potential of tailor-made lactic acid bacteria to create gender foods with defined functionalities in relation to the strains used [4]. Thus, the use of an integrated biotechnological approach, based on new technologies and functional microbial cultures, could be the new challenge and an alternative to the engineered strains that are always lacking robustness and characterized by high production costs. Among the non-thermal technologies, the high-pressure homogenization (HPH) represents a potential technology to develop

new functional applications, also easily implementable at industrial level. Several literature reports have highlighted its use in dairy field to increase product functionality by both treating the milk designated to cheese making or fermented product preparation and treating the bacteria cells to increase their probiotic features [5–8]. Moreover, high pressure homogenization was used also to develop suitable microcapsules to carry the probiotic cultures in order to increase their viability during dairy product storage under refrigeration conditions [9, 10].

The innovation of dairy products can be also achieved using specific microorganisms able to generate specific functionalities. In fact, functional foods could differently affect human health in relation to the gender, and this research aspect is particularly interesting for women since, in recent decades, research on the female gender has been neglected, and the results obtained in men have been directly translated to women both in medicine and in nutrition fields. In this sense, a recent contribution of Siroli et al. [4] has highlighted the challenge to study the technological properties of some *Lactobacillus* strains isolated from the vagina of healthy women and endowed with anti-Candida and anti-Chlamydia activities for potential use in dairy food to contribute to women's well-being by a dietary strategy. The exploitation of a *Lactobacillus salivarius* strain, with a strong anti-*Helicobacter pylori* activity, and a nisin producer *Lactococcus lactis* strain were used as co-starters in cheese making in order to contribute to product innovation and to increase the overall welfare [11].

Thus, this chapter will outline the most important findings on the attempts made in dairy product innovation, exploiting both an emerging technology such as high-pressure homogenization and the use of functional strains endowed with specific functionalities.

2. Biotechnological strategies for dairy innovation

2.1. Innovation in dairy field throughout technological approach

Innovation is the major driving force of the economic growth worldwide and it is directly connected with the needs of the consumers or final users. In 2008, Grunert et al. [12] created the term "user-oriented innovation" defining it "*as a process towards the development of a new product or service in which an integrated analysis and understanding of the users' wants, needs and preference formation play a key role*". In this last decade, the consumer interests and needs have been directed towards food able to confer specific functionalities to human well-being. For this reason, the scenario has notably changed in many food sectors, pointing out the fast development of new products being able to satisfy the consumer needs. In particular, the dual request for guaranteeing the safety and meeting the consumer demands for more fresh and high-quality functional foods necessitated the development of new strategies such as non-thermal technologies able to inactivate microorganisms at room or near-room temperatures but preserving flavor, color and nutritional value. Much attention has been focused on new food processing trends to offer interesting solutions to these challenges. Currently, the most important and interesting applications have been studied and applied for the dairy ingredients or products due to their innate great potential as functional and nutraceuticals. The first product innovation in this sector was represented by the use of different types of milk, to cow

milk, recognized for their intrinsic functionalities, to produce new functional dairy products. For example, sheep dairy products have gained market size due to the product's quality, high yield and nutritional value due to the high concentrations of proteins, fats, vitamins and minerals [13]. However, these types of productions have grown over the years reaching a plateau in the market. For this reason, new dairy products have been developed and, among these, probiotic ones have gained major attention due to their driving force on the market [1, 2]. The literature data show that many attempts have been made to increase both functionality and product differentiation throughout biotechnological approaches. Among technologies, pulsed electric field (PEF), high hydrostatic pressure (HHP), high-pressure homogenization (HPH), ultrasounds, oscillating magnetic fields and high-intensity light pulses have been exploited in dairy product application with the purpose not to decontaminate milk but to increase the final product functionalities [14]. Among these technologies, most of them investigated and tested at least at laboratory level, the sector of high-pressure processing (HHP and HPH) [15], together with the PEF field [16], is probably one the most scientifically developed and with already-established applications at industrial level for the production of probiotic/prebiotic ingredients and foods.

2.2. Principles of high-pressure homogenization and its application

Although HHP and HPH share some action mechanisms, the latter induces major changes to macromolecules of the system with respect to HHP, throughout cavitation, turbulence and viscous shears, which seem the most probable mechanisms of action. Moreover, HHP can be applied both on liquid and on solid matrix while HPH can be used only for liquid foods. However, both offer interesting possibilities to restructure food proteins, affecting protein conformation, leading to protein denaturation, gelation or aggregation and, consequently, creating new products with new/improved texture [17, 18]. They are also used on dairy proteins for providing low-temperature enzyme activity modification and stabilization of fermented dairy products and also to improve coagulation of milk and to prepare dairy gels and emulsions characterized by novel textures. Moreover, according to the literature, they are involved in functional dairy formulation, and for HHP the most evidences are on bioactive milk proteins [19]. Although HHP is more consolidated at the industrial level in many fields, the potentialities of HPH in the dairy sector are multiple. In addition, this process can be applied, contrary to HHP, in a continuous manner, offering a great advantage from an industrial point of view and increasing the competition among the enterprises in process innovation and products. The word "homogenization" is referred to the ability to produce a homogeneous size distribution of particles suspended in a liquid, by forcing the liquid under the effect of pressure through a specifically designed homogenization valve (**Figure 2**) [17, 18].

Nowadays, homogenizers able to treat fluid matrices for pressure ranging between 10 and 40 MPa are well implemented in different sectors, that is, dairy, beverage, pharmaceutical and cosmetic industries, with the principal aim to reduce particle size and increase stability. However, the first application of HPH dealt with the cell disruption and recovery of intracellular bioproducts [17] reaching pressures of 100 MPa. The successful results obtained on cell rupture of microbial cultures motivated researches on the application of HPH for food safety and shelf life extension. In the food industry, the interest in mild non-thermal processes, able

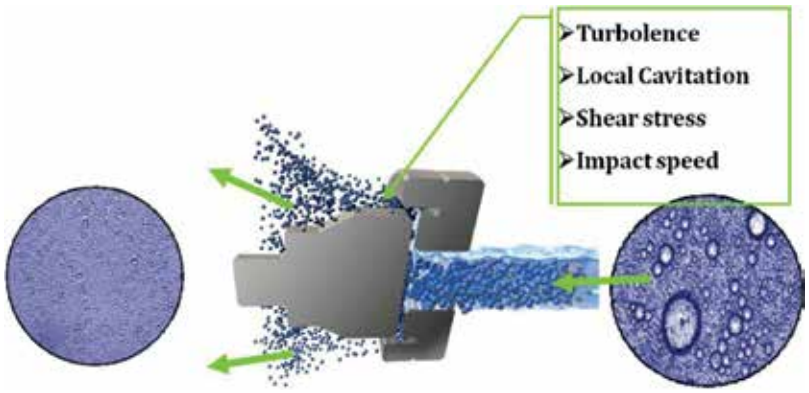

Figure 2. Principal action mechanisms of high-pressure homogenization.

to achieve efficient microbial reduction with a maximal retention of physicochemical product properties, as well as nutritional and sensory feature retention, is very high. Among the non-thermal treatments, HPH is regarded as one of the most encouraging alternatives to traditional heat treatments for food preservation and product diversification for dairy and beverages. Its efficacy against spoilage microorganisms in model and real systems has been well proven since 1994 [17].

A homogenizer is composed above all of a pump and a homogenizing valve. The pump is used to force the fluid into the valve where the homogenization takes place. In the homogenizing valve, the fluid is forced under pressure through a small orifice between the valve and the valve seat. The operating pressure is controlled by adjusting the distance between the valve and seat. Pressure, temperature and flow rate are the main parameters influencing the success of homogenization for microbial inactivation. As in HHP, the level of microbial inactivation by the application of HPH increases with the pressure level. Temperature effects have to be necessarily taken into account in HPH, since during homogenization, there is an increase of temperature (about 2.5°C per 10 MPa) related to the fluid food treated. This increase is due to the viscous stress caused by the high speed of the fluid flow and due to the loss of a significant fraction of the mechanical energy which is lost as heat in the fluid [17]. According to the literature, the HPH has been used instead of the conventional homogenization for the modification of the microstructure and rheology of food emulsions [20, 21], the improvement of the body and texture of yogurts and cheeses [22, 23], the increase of cheese yield [5] and the reduction of cheese ripening time due to the enhanced susceptibility of proteins and triglycerides to proteolysis and lipolysis, respectively [24]. Some papers report also the exploitation of HPH for the activation or inactivation of enzymes [25, 26] and to reduce the biogenic amine content of ripened cheeses.

2.3. Potential of high-pressure homogenization in dairy sector for the development of functional dairy products

HPH has demonstrated great potential in the dairy sector also for the development of new products, differentiated from traditional ones by sensory and structural characteristics or

functional properties [27]. Moreover, Iordache and Jelen [28] showed HPH as a suitable approach for producing soluble whey protein concentrates/isolates for the production of several dairy products, as well as meat or egg substitutes. Bury et al. [29], comparing sonification, bead milling and HPH, showed that HPH was suitable for the large-scale disruption of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 for the recovery of β-galactosidase for the production of lactose-hydrolyzed dairy products. In addition to the production of yeast autolisate for food aroma improvement, HPH has been used for the recovery of (1–6)-β-D-glucan from Saccharomyces cerevisiae. This molecule is a well-known immune modulator with a positive influence on the human and animal immune system. Because HPH has been extensively used to emulsify, mix [30] and reduce the mean droplet size, simultaneously narrowing the width of the size distribution by reducing the number of microparticles and the polydispersity index, one of the most recent applications regards the use of HPH processes to produce nanodispersions and nanoparticles containing bioactive compounds, including functional lipids, with substantial health benefits [31].

In the functional dairy sector, HPH has been proposed to produce probiotic fermented milk, bio-yogurt and probiotic cheeses with improved sensorial or functional properties [3, 5, 6, 22]. In general, fermented dairy products are considered by consumers as healthy foods since they are good sources of vitamins and minerals and have low lipid content. The use of probiotic (health-promoting) microorganisms in different fermented milk or yogurt-like products can also amplify their acclaimed healthful properties. In fact, *Streptococcus thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus*, the traditional starter cultures of yogurt, improve themselves the nutritional content and digestibility of yogurt, although they are not usually part of the native microbiota of the mammalian intestine and have limited survival after oral intake. In order to beneficially affect the host, improve the balance of intestinal bacterial ecosystem and influence positively human immune responses, the probiotic microorganisms need to be viable, active and sufficiently abundant (i.e. in concentrations of at least 10^7 cfu/g in the product) throughout the specified shelf life [24]. Also other properties of fermented milk, such as acidity, lipid content, the presence of diacetyl, acetaldehyde and acetoin and the nutritional value are important and affect acceptability by consumers. Thus, the ability to impart good sensory properties to the final product is included in the criteria of selection for probiotic strains [24], because fermented milk obtained from the direct and sole use of probiotic strains are often characterized by the lack of desirable sensory features [7].

Several options have been proposed in order to increase the textural properties of fermented milk. Among these, the exploitation of exopolysaccharide-producing strains has been suggested as an option to additives such as xanthan gum, gelatin, pectin, and carrageenan [24], which can adversely influence the product flavor, aroma and mouth feel [6]. Also, co-inocula of probiotic strains with *Lb. delbrueckii* ssp. *bulgaricus* and *Strep. thermophilus* has been reported to improve the sensorial properties of fermented milk [6]. Moreover, *Strep. thermophilus* is applied to sustain the mild acid fermentation of yogurt containing probiotic lactobacilli. Also the modulation of some physicochemical and technological variables permitted to increase the technological strain features and sensory properties of fermented milk. Mainly, some papers indicate response surface methodology as a good approach to evaluate the simultaneous effects of some important technological, compositional and microbiological variables on

the acidification rate of starter bacteria (including probiotic strains), on their viability losses during product storage and on product sensory traits [6].

Among the technological variables that are potentially useful, the HPH of milk has been regarded: (1) to increase or modulate the sensorial features of probiotic fermented milk and cheese without detrimental effects on shelf life and safety [5, 6]; (2) to improve the technological performances of probiotic strains used alone or in combination with yogurt starter cultures [24]; and (3) to modify the functional features of lactic acid bacteria used as starters [32] and of probiotic bacteria [7, 8]. Regarding the use of homogenizing pressure to milk, the use of HPH tested between 20 and 100 MPa showed ability to improve the sensorial features of fermented milk using a probiotic strain, *Lactobacillus paracasei* BFE 5264, as starter culture in combination with compositional variables such as milk fat content and non-fat milk solids content as reported by Patrignani et al. [33]. These authors highlighted that the rheological parameters, such as firmness, viscosity index and consistency of probiotic fermented milk, increased with the increase in pressure level for added non-fat milk solid concentrations lower than 3%. When non-fat milk solids were higher than 3%, different rheological behaviors were observed. The improvement of textural properties of fermented milk can be explained with the progressive increase in the extent and strength of protein hydrophobic associations which the pressure can promote. Also, the content of characteristic molecules such as diacetyl and acetaldehyde, able to affect the flavor and taste of fermented milk, increased with the use of homogenizing pressure. Moreover, the HPH treatment affected significantly also some technological performances of the employed probiotic strain such as fermentation rate and its viability loss during fermented milk storage at refrigeration temperature. In fact, Patrignani et al. [33] found that *Lb. paracasei* BFE 5264 coagulation times were significantly affected, in addition to the added milk fat, by the increase of pressure. However, the technological performances of the probiotic strain were affected by the addition phase of milk fat (before or after the pressure treatment). When the addition of milk fat was performed before HPH treatment, the strain fermentation rate decreased and its viability during the refrigerated storage was reduced. The addition of UHT cream before high pressure homogenization treatment decreased the nutrient diffusion, the microbial growth and the acidification rates since it generated compartmentalization of the aqueous phase in the lipid-protein gel matrix. HPH showed good potentialities also when used to produce fermented milk containing the traditional yogurt starters (i.e. *Strep. thermophilus* and *Lb.s delbrueckii* subsp. *bulgaricus*) and probiotic strains of *Lactobacillus acidophilus* 08 and *Lb. paracasei* A13. In particular, Patrignani et al. [6] studied four types of fermented milk, obtained from HPH-treated and heat-treated (HT) milk with and without added probiotics. The results showed that HPH treatment favored the viability of starter cultures, particularly *Strep. thermophilus*, even at the end of the storage period without detrimental effect on the viability of probiotic bacteria. Higher levels of viable lactic acid bacteria LAB at the end of the shelf life is, in any case, an interesting feature for this type of product due to the now recognized probiotic characteristics of yogurt cultures. In addition, the probiotic strains of *Lb. acidophilus* and *Lb. paracasei*, employed by Patrignani et al. [6], were found at levels of 5 and 7 log orders, respectively, at the end of the shelf life of the fermented milk from HPH-treated milk. Moreover, the fermented milk obtained from HPH milk was characterized by significant higher values of firmness ($p < 0.05$) with respect to

those from HT milk. Similar results were observed for consistency, cohesiveness and viscosity indexes. All the samples obtained from HPH milk received high sensory analysis scores for each descriptor considered. Also, significant higher amounts of acetaldehyde and 2-propanone were detected in fermented milk obtained from HPH milk than those from HT milk.

According to the literature available, HPH technology has shown good potential for the manufacturing of probiotic cheeses. In fact, soft cheeses have a number of advantages over yogurt and fermented milk as a delivery system for viable probiotic microorganisms because they generally have higher pH and buffering capacity, more solid consistency and relatively higher fat content [27]. On the other hand, in order to give protection to probiotic bacteria during storage and passage through the gastrointestinal tract, some cheese varieties such as Gouda [34], Argentinean Fresco cheese [35], white cheese [36], Arzua-Ulloa [37], Minas fresh cheese [38], Cheddar [39] and cottage cheese [40] have also been studied as vehicles of probiotic microorganisms. In addition, Burns et al. [5] studied the potential of HPH treatment of milk for the production of Crescenza cheese carrying probiotic bacteria. More specifically, these authors studied the viability of commercial probiotic cultures of *Lb. acidophilus* and *Lb. paracasei*, added as adjunct cultures during production, and the implications of their addition in the physicochemical and sensory characteristics of the product obtained. In fact, a previous work performed by Gobbetti et al. [41] showed that Crescenza cheese is suitable to serve as a carrier for probiotic bacteria because no prolonged periods of ripening are necessary, and storage occurs at refrigeration temperatures. However, the inclusion of probiotic bacteria affected negatively the organoleptic properties of this traditional Italian cheese. To evaluate the potential of milk treated by HPH for the production of probiotic Crescenza, Burns et al. [5] compared four types of cheeses made from HPH and pasteurized milk with and without probiotics, respectively. A strain of *Strep. thermophilus* was used as starter culture for all the cheese types. The results of compositional analyses carried out during the refrigerated storage (4°C) showed no significant differences for gross composition (protein, fat, moisture) and pH. Differently, the HPH treatment to milk induced an increase in cheese yield of about 1%. Also the viability of the used probiotic strain maintained cell loads of 8 log cfu/g after 12 days of refrigerated storage. *Lb. acidophilus* 05 showed, in probiotic Crescenza cheese from pasteurized milk, a cell load decrease of about 1 log cfu/g with respect to the cell loads reached in Crescenza-obtained HPH milk. Moreover, the hyperbaric treatment had a significant positive effect on release of free fatty acids, cheese proteolysis and organoleptic properties of cheeses tested by a sensorial analysis.

The modification of the rheological and sensorial properties of fermented milk and cheeses induced by HPH can be explained mainly with the modification induced by HPH treatment on casein-casein or casein-fat interactions. The ability of HPH treatment to increase the exposure of the hydrophobic regions of proteins and extent and strength of hydrophobic associations between proteins is well documented. Moreover, HPH of milk is reported to improve the coagulation characteristics of milk due to the modification of the balance between insoluble and soluble forms of calcium, phosphorus and nitrogen [27]. Also, the modification of sensorial profile in terms of volatile molecules and the different retention of flavor compounds can be dependent on the different gel networks of proteins. The release of flavor compounds and their perception during consumption, which are key quality parameters for foodstuff, are

undoubtedly affected also by the food matrix and microstructure [27]. Moreover the different volatile profiles of fermented milk and cheeses obtained from HPH-treated milk could be due to the combination of events associated with homogenization. In fact, HPH is reported to increase the nitrogen fraction soluble at pH 4.6, the susceptibility to proteolysis of whey proteins and caseins, and, consequently, the availability of free amino acids regarded as several aroma precursors including acetaldehyde. The increase of viability for yogurt starters and probiotic cultures observed by Patrignani et al. [6] and Burns et al. [5], respectively, can be attributed to the increased precocious availability in the products obtained from HPH-treated milk of low molecular weight peptides and/or free fatty acids such as oleic acid, essential for the growth of many LAB. Moreover, Patrignani et al. [33] reported levels ranging between 60 and 80 MPa as optimal both for the viability of probiotic and for the sensorial features of fermented milk obtained with the sole use of probiotic strains. Moreover, because some literature papers proposed HPH to control and enhance the proteolytic and fermentative activities of some *Lactobacillus* species [32], Tabanelli et al. [8] have adopted this technology to modify/enhance some functional properties of already-known probiotic strains, considering that probiotic properties are related to the cell wall, also the principal target of HPH. Thus, viability and cell-surface hydrophobicity of *Lb. paracasei*. A13, *Lb. acidophilus* DRU, *Lb. delbrueccki* subsp. *lactis* 200 and their bile-resistant derivative DRU+ and 200+, inoculated in phosphate buffer solution and buttermilk, were evaluated. Moreover, at the same conditions, bacterial aggregation characteristics, response to simulated stomach duodenum passage and resistance to simulated gastric acid conditions were evaluated. The strains were treated by HPH at 50 MPa and the results were compared with control data obtained under the same conditions but without the pressure application. Among the tested strains, only *Lb. paracasei* A13 increased its hydrophobicity when subjected to HPH, while bacterial aggregation characteristics, viability and resistance were strain dependent and affected by the media employed. In particular, among the treated strains, the more resistant ones to gastric acid conditions were those inoculated in phosphate buffer solution. This study documented that HPH treatment could increase some important probiotic characteristics such as hydrophobicity and resistance to simulated gastric juice but the response varied according to the species and the characteristics of the individual strains as well as the time of refrigerated storage and the media containing probiotic strains.

2.4. Use of high-pressure homogenization to develop new carriers for probiotic strains

From the technological point of view, probiotic strains have to maintain not only a good viability but also a good functionality during manufacture, storage and even during consumption. Since probiotic cultures run across acidic conditions already in food products and during gastric transit, their tolerance to low pH is a critical factor that has an influence on probiotic functionality. The use of an appropriate technology for the preservation of probiotic viability is a key step for the industrial production of functional foods, since probiotic microorganisms are subjected to lose their viability during the fermentation process or during the product storage. In general, this is affected by several factors such as from 'strain sensitivity to process factors (low pH, oxygen and fermentation temperature), food matrix composition (water activity, pH, presence of natural antimicrobials and nutrient availability) and

packaging and storage conditions (i.e. refrigeration temperature). Also, the gastrointestinal tract conditions can influence the viability of the probiotic bacteria during the passage. Many attempts have been performed by many researchers to maintain high viability of probiotic strains in food products. Recently, the literature data pointed out the use of polymers such as pectin, alginate, carrageenan, chitosan, whey, gelatin and lipids for microencapsulation of bacteria with positive effects in protection of probiotic cells during storage condition and gastric intestinal environment. Patrignani et al. [10] investigated the microencapsulation of two probiotic bacteria, *Lb. paracasei* A13 and *Lb. salivarius* CET 4063, performed by HPH at lab scale for the manufacture of functional fermented milk endowed with high functional strain viability. Specifically, the two probiotic bacteria were encapsulated by HPH at 50 MPa using 5 cycles, using sodium alginate vegetable oil emulsion. The microencapsulated bacteria were used as adjuncts for the production of functional fermented milks. The viability of the strains in the product was monitored over 2 months of refrigerated storage. The survival of lactic acid bacteria following the simulation of the gastric duodenal passage were evaluated. Textural parameters of fermented milk and the presence of exo-polysaccharides were also determined over storage. In addition, the profiles of volatile compounds of the products were evaluated by the GC/MS/SPMR (solid phase micro extraction) technique. The obtained microcapsules resulted in homogeneous and having a size <100 μM without detrimental effect on the sensory properties of the fermented milk (**Figure 3**).

The main effect of the encapsulation resulted in the decrease of the hyperacidity phenomena generally connected to the addition of probiotic bacteria in fermented milk. This result was fundamental for the improvement of the viability of the starter culture and the sensorial features of the products. Moreover, the microencapsulation conditions preserved the viability of the two used probiotic bacteria, even if the strain *Lb. paracasei* A13 showed a higher resistance

Figure 3. Micro-beads containing *Lb. salivarius* CET 4063 obtained by high-pressure homogenization.

to the gastric barrier with respect to *Lb. salivarius* CECT 4063. In contrast, the data obtained showed a reduction of exo-polysaccharides production in presence of microencapsulated bacteria. The results of this study underlined the applicative potential of HPH for microencapsulation of probiotic microorganisms to produce fermented milk with improved functionality and with enhanced sensory properties.

2.5. Future trends: gender foods based on biotechnological approach

Another great challenge to create new functional foods and develop product innovation is the exploitation of microbial strains, with great technological potential, able to provide specific functionalities in relation to the gender. In fact, today, foods are not intended to only satisfy hunger and to provide necessary nutrients for humans but also to prevent nutrition-related diseases and improve physical and mental well-being of the consumers [2]. The literature has suggested that functional foods could differently influence the male and female health. Until the last decade, research on women has been neglected and the results obtained in men were directly translated to women in medicine and nutrition fields [42]. Reproductive-aged women are often subjected to gynecological disturbances due to abnormalities in vaginal or gut microbiota and the occurrence of vaginal infections, including vulvovaginal candidiasis, bacterial vaginosis and aerobic vaginitis. Vulvovaginal candidiasis (30–35% due to Candida albicans) is a yeast infection compromising the life quality of many women. Bacterial vaginosis is an imbalance in the ecology of the normal vaginal microbiota, characterized by a decrease in *Lactobacillus* species, which predominate in the healthy vagina, and an increase of several pathogenic bacteria, mainly anaerobes. Aerobic vaginitis is another major abnormality of the vaginal microbiota where lactobacilli are replaced with aerobic organisms, that is, streptococci, enterococci, Escherichia coli and Staphylococcus aureus. Bacterial vaginosis and aerobic vaginitis interfere with female reproductive health, abortion, preterm delivery, premature rupture of membranes and chorioamnionitis. Conventional therapies failed in the treatments of these disorders. The administration of probiotics has been shown to be effective in restoring a normal vaginal microbiota. Recent studies highlighted the anti-Candida and anti-Chlamydia activities of strains, belonging to *Lactobacillus crispatus*, *Lactobacillus gasseri* and *Lactobacillus vaginalis*, isolated from the vagina of healthy women. Those strains were also characterized for some functional and technological properties by Siroli et al. [4] in order to evaluate their potential inclusion in a dairy products. Several strains of *Lb. crispatus* showed a significant antagonistic activity against spoilage and pathogenic microorganisms of food interest, as well as against the principal urogenital pathogens. Moreover, their fermentation kinetics in milk and their ability to survive at 4°C indicated the potential application of the selected strains as adjunct cultures for the production of female gender foods. In this view, their use for the preparation of a functional gender food could be a great scientific challenge. In particular, it is known that soft cheese could be an optimal carrier and dietary strategy to transfer of probiotic strains able to control the healthy status of the human vaginal microbiota to protect the woman from vaginal dysbiosis and infections. In fact, cheese is characterized by a protective protein and fat matrix, neutral or sub-alkaline pH and higher buffering capacity [27]. These properties allow for a great protection towards probiotic bacteria during their gastrointestinal transit. Indeed, cheeses represent a good source of calcium and vitamins. In this

regard, dairy products produced with functional lactic acid bacteria strains used as co-starters have been recognized as functional foods able to provide potential benefits in preventing some diseases. Thus, in this scenario the formulation of a soft cheese containing *Lb. crispatus* BC4, isolated from the vagina of healthy women and selected for its antimicrobial activity and its ability to survive during the refrigerated storage, has been assessed in order to develop a new functional soft cheese able to promote the woman's well-being. The results obtained showed that the strain could survive very well in cheese during its refrigerated storage and also survive the simulated stomach duodenum passage maintaining cell loads higher than 6 log cfu/g over the storage. The ability of this strain to interact with the gut microbial population was also studied by using a dynamic model (called SHIME) of the gastrointestinal tract to study physicochemical, enzymatic and microbial parameters, in a controlled in vitro setting, able to affect the viability of *Lb. crispatus* BC4.

2.6. Conclusions

The results presented in this chapter have highlighted that the innovation in dairy field is achievable using different strategies. The field of high-pressure homogenization certainly represents one of the most important technological tools to reach this aim due to the tangible effects of this non-thermal approach. Also the use of safe and well-characterized probiotic/health-promoting strains can contribute to the development of products able to increase the general human well-being. Also the interaction between these two strategies could represent a challenge in the future for the sector's innovation.

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References

[1] Linares DM, Gómez C, Renes E, Fresno JM, Tornadijo ME, Ross RP, Stanton C. Lactic Acid Bacteria and Bifidobacteria with potential to design natural biofunctional health-promoting dairy foods. Frontiers in Microbiology. 2017;**8**:846. DOI: 0.3389/fmicb.2017.00846

[2] Siro I, Kápolna E, Kápolna B, Lugasi A. Functional food product development, marketing and consumer acceptance: A review. Appetite. 2008;**51**:456-467. DOI: 10.1016/j.appet.2008.0

[3] Patrignani F, Lanciotti R, Guerzoni ME. Emerging technologies for probiotic and prebiotic food. In: Probiotic and Prebiotic Food 2011. ISBN 978-1-61668-842-4

[4] Siroli L, Patrignani F, Serrazanetti DI, Parolin C, Palomino RAN, Vitali B, Lanciotti R. Determination of antibacterial and technological properties of vaginal Lactobacilli for their potential application in dairy products. Frontiers in Microbiology. 2017;**8**:166. DOI: 10.3389/fmicb.2017.00166

[5] Burns P, Patrignani F, Serrazanetti D, Vinderola G, Reinheimer J, Lanciotti R, Guerzoni ME. Probiotic Crescenza cheese containing *Lactobacillus paracasei* and *Lactobacillus acidophilus* manufactured with high pressure-homogenised milk. Journal of Dairy Science. 2008;**91**:500-512. DOI: 10.3168/jds.2007-0516

[6] Patrignani F, Burns P, Serrazanetti D, Vinderola G, Reinheimer JA, Lanciotti R, Guerzoni ME. Suitability of high pressure-homogenized milk for the production of probiotic fermented milk containing *Lactobacillus paracasei* and *Lactobacillus acidophilus*. Journal of Dairy Research. 2009;**76**:74-82. DOI: 10.1017/S0022029908003828

[7] Muramalla T, Aryana KJ. Some low homogenization pressures improve certain probiotic characteristics of yogurt culture bacteria and Lactobacillus acidophilus LA-K. Journal of Dairy Science. 2010;**94**:3725-3738. DOI: 10.3168/jds.2010-3737

[8] Tabanelli G, Patrignani F, Vinderola G, Reinheimer JA, Gardini F, Lanciotti R. Effect of sub-lethal high pressure homogenization treatments on the in vitro functional and biological properties of lactic acid bacteria. LWT—Food Science and Technology. 2013;**53**:580-586. DOI: 10.1016/j.lwt.2013.03.013

[9] Ding WK, Shah NP. An improved method of microencapsulation of probiotic bacteria for their stability in acidic and bile conditions during storage. Journal of Food Science. 2009;**74**:M53-M61

[10] Patrignani F, Siroli L, Serrazanetti D, Braschi G, Betoret E, Reinheimer J, Lanciotti R. Microencapsulation of functional strains by high pressure homogenization for a potential use in fermented milk. Food Research International. 2017;**97**:250, 257. DOI: 10.1016/j.foodres.2017.04.020

[11] Serrazanetti DI, Siroli L, Patrignani F, Braschi G, Castellone V, Vinderola GC, Vitali B, Reinheimer JA, Lanciotti R. Set up of biotechnological strategies for the production of fresh cheeses having different functionalities. In: dalla Rosa M, Maupoey PF, editors. FoodInnova 2017; 31 January–3 February; Cesena. Cesena: Knowledge Management for Food Innovation—KM4FI; 2017. p. 208. ISBN: 978-2-12-345680-3

[12] Grunert KG, Boutrup Jensen B, Sonne AM, Brunsø KV, Byrne D, Clausen C, Friis A, Holm L, Hyldig G, Kristensen NH, Lettl C, Scholderer J. User-oriented innovation in the food sector: Relevant streams of research and agenda for future work. Trends in Food Science & Technology. 2008;**19**:590-602. DOI: 10.1016/j.tifs.2008.03.008

[13] Balthazar CF, Pimentel LL, Ferrao CN, Almada A, Santillo M, Albenzio N, Mollakhalili N, Mortazavian AM, Nascimento JS, Silva MC, Freitas MQ, Sant'Ana AS, Granato D, Cruz AG. Physicochemical characteristics and relevance for functional food development. Comprehensive Reviews in Food Science and Food Safety. 2017;**16**:247-262. DOI: 10.1111/1541-4337.12250

[14] Senorans FJ, Ibanez E, Cifuentes A. New trends in food processing. Critical Reviews in Food Science and Nutrition. 2003;**43**:507-256

[15] Wan J, Mawson R, Ashokkumar M, Ronacher K, Coventry MJ, Roginski H, Versteeg C. Emerging processing technologies for functional foods. The Australian Journal of Dairy Technology. 2005;**60**:167-169

[16] Wan J, Conventry J, Sanguansri P, Versteeg C. Advances in innovative technologies for microbial inactivation and enhancement of food safety—Pulsed electric field and low-temperature plasma. Trends in Food Science and Technology. 2009;**20**:414-424

[17] Patrignani F, Lanciotti R. Applications of high and ultra high pressure homogenization for food safety. Frontiers in Microbiology. 2016;**7**:article 1132. DOI: 10.3389/fmicb.2016.01132

[18] Zamora A, Guamis B. Opportunities for ultra-high-pressure homogenisation (UHPH) for the food industry. Food Engineering Reviews. 2015;**7**:130-142. DOI: 10.1007/s12393-014-9097-4

[19] Kananen A, Savolainen J, Mäkinen J, Perttilä U, Myllykoski L, Pihlanto-Leppälä A. Influence of chemical modification of whey protein conformation on hydrolysis with pepsin and trypsin. International Dairy Journal. 2000;**10**:691-697

[20] Floury J, Bellettre J, Legrand J, Desrumaux A. Analysis of a new type of homogenizer. A study of the flown pattern. Chemical Engineering & Science. 2004;**59**:843-853

[21] Guerzoni ME, Lanciotti R, Westall F, Pittia P. Interrelation between chemicophysical variables, microstructure and growth of listeria monocytogenes and Yarrowia lipolytica in food model systems. Science des les Aliments. 1997;**17**:507-522

[22] Lanciotti R, Chaves-Lopez C, Patrignani F, Papparella A, Guerzoni ME, Serio A, et al. Effects of milk treatment with dynamic high pressure on microbial populations, and lipolytic and proteolytic profiles of Crescenza cheese. International Journal of Dairy Technology. 2004;**57**:19-25

[23] Lanciotti R, Vannini L, Pittia P, Guerzoni ME. Suitability of high dynamic-pressure-treated milk for the production of yogurt. Food Microbiology. 2004;**21**:753-760

[24] Patrignani F, Serrazanetti DI, Mathara JM, Siroli L, Gardini F, Holzapfel WH, et al. Use of homogenisation pressure to improve quality and functionality of probiotic fermented milks containing Lactobacillus rhamnosus BFE 5264. International Journal of Dairy Technology. 2016;**69**:262-271

[25] Hayes MG, Kelly AL. High pressure homogenisation of raw whole bovine milk (a) effects on fat globules size and other properties. Journal of Dairy Research. 2003;**70**:297-305

[26] Vannini L, Lanciotti R, Baldi D, Guerzoni ME. Interactions between high pressure homogenization and antimicrobial activity of lysozyme and lactoperoxidase. Journal of Food Microbiology. 2004;**94**:123-135

[27] Burns P, Patrignani F, Tabanelli G, Vinderola G, Siroli L, Reinheimer J, et al. Potential of high pressure homogenisation on probiotic Caciotta cheese quality and functionality. Journal of Functional Foods. 2015;**13**:126-136. DOI: 10.1016/j.jff.2014.12.037

[28] Iordache M, Jelen P. High pressure microfluidization treatment of heat denatured whey proteins for improved functionality. Innovative Food Science and Emerging Technologies. 2003;**4**:367-376

[29] Bury D, Jelen P, Kaláb M. Disruption of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 cells for lactose hydrolysis in dairy products: A comparison of sonication, high-pressure homogenization and bead milling. Innovative Food Science and Emerging Technologies. 2001;**2**:23-29

[30] Roesch R, Corredig M. Texture and microstructure of emulsions prepared with soy protein concentrate by high-pressure homogenization. LWT—Food Science and Technology. 2003;**36**:13-124

[31] Saito M, Lin Y, Kobayashi I, Nakajima M. Preparation characteristics of oil-in-water emulsions stabilized by proteins in straight-through microchannel emulsification. Food Hydrocolloids. 2005;**19**:745-751

[32] Lanciotti R, Patrignani F, Iucci L, Saracino P, Guerzoni ME. Potential of high pressure homogenization in the control and enhancement of proteolytic and fermentative activities of some Lactobacillus species. Food Chemistry. 2007;**102**:542-550. DOI: 10.1016/j.foodchem.2006.06.043

[33] Patrignani F, Iucci L, Lanciotti R, Vallicelli M, Maina Mathara J, Holzapfel WH, et al. Effect of high pressure homogenization, not fat milk solids and milkfat on the technological performances of a functional strain for the production of probiotic fermented milks. Journal of Dairy Science. 2007;**90**:4513-4523

[34] Gomes AMP, Malcata FX, FAM K, Grande HJ. Incorporation and survival of Bifidobacterium sp. strain Boand lactobacillus acidophilus strain Ki in a cheese product. Netherlands Milk and Dairy Journal. 1995;**49**:71-95

[35] Vinderola CG, Bailo N, Reinheimer JA. Survival of probiotic microflora in Argentinean yogurts during refrigerated storage. Food Research International. 2000;**33**:97-102

[36] Kasımoglu A, Goncuoglu M, Akgu S. Probiotic white cheese with lactobacillus acidophilus. International Dairy Journal. 2004;**14**:1067-1073

[37] Menendez S, Centeno JA, Godınez R, Rodrıguez-Otero JL. Effect of Lactobacillus strains on the ripening and organoleptic characteristics of Arzua´–Ulloa cheese. International Journal of Food Microbiology. 2000;**59**:37-46

[38] Buriti FCA, da Rocha JS, Saad SMI. Incorporation of Lactobacillus acidophilus in minas fresh cheese and its implications for textural and sensorial properties during storage. International Dairy Journal. 2005;**15**:1279-1288

[39] Ong L, Henriksson A, Shah NP. Chemical analysis and sensory evaluation of Cheddar cheese produced with Lactobacillus acidophilus, Lb. casei, Lb. paracasei or Bifidobacterium sp. International Dairy Journal. 2007;**17**:937-945

[40] Blanchette L, Roy D, Belanger G, Gauthier SF. Production of cottage cheese using dressing fermented by bifidobacteria. Journal of Dairy Science. 1996;**79**:8-15

[41] Gobbetti M, Corsetti A, Smacchi E, Zocchetti A, De Angelis M. Production of Crescenza cheese by incorporation of bifidobacteria. Journal of Dairy Science. 1998;**81**:37-47

[42] Marino M, Masella R, Bulzomi P, Campesi I, Malorni W, Franconi F. Nutrition and human health from a sex-gender perspective. Molecular Aspects of Medicine. 2011;**32**: 1-70. DOI: 10.1016/j.mam.2011.02.001

Chapter 4

Sonocrystallization of Lactose from Whey

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Additional information is available at the end of the chapter

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Abstract

Whey is a by-product obtained from the cheese-making industry. This by-product is the primary source of high-value products such as whey protein concentrates and lactose. The partial removal of water from the whey is the first step in the recovery of lactose. Then, lactose in the concentrated whey is forced to crystallize through a cooling stage. This conventional process of crystallization is very slow up to 72 h accompanied by the generation of a mixture of lactose types (α, β, and amorphous) and low yield of lactose. These issues have been addressed through the seeding of lactose, the antisolvent crystallization, and more recently, by the crystallization of lactose assisted with low-frequency power ultrasound. Sonocrystallization is known to have a number of specific features that include the enhancement of the primary and secondary nucleation, as well as the development of smaller crystals with more uniform sizes and higher purity. Nowadays, there are a number of studies that provide relevant information on the effects of ultrasound on lactose crystallization, although some of these effects are still not fully understood. This book chapter discusses the current knowledge on lactose sonocrystallization and describes the basic principles of lactose crystallization and sonocrystallization.

Keywords: lactose, whey, crystallization, sonocrystallization

1. Introduction

The composition of whey varies according to the cheese process, but in general, this contains 0.8–1.0% soluble proteins, 0.05–0.5% fat, >1% salts, 5–6% lactose, and up to 93% of water [1–4]. The volume of whey recovered from a cheese process makes up 70–90% of the original

volume of milk [3]. Therefore, it is estimated that more than 80 million tons of whey are produced annually all over the world [4, 5].

Most of the small-scale dairy companies dispose of the whey into the municipal sewage, rivers, lakes, or use this by-product as fertilizer and animal feed [4, 5]. The disposal of cheese whey into water bodies and lands should be strongly discouraged because it produces serious environmental problems. The bacterial degradation of whey causes a depletion of oxygen in the water and soil killing aerobic organisms, such as fish, insects, plants, and microorganisms. The high biological and chemical oxygen demand (BOD: 30–50 g L^{-1}; COD: 60–80 g L^{-1}) of the whey arise from its large content of carbohydrates, chiefly lactose (5–6%) [2, 6–8]. In consequence, the removal of lactose reduces more than 80% of the BOD and COD of whey, which minimizes the negative environmental impact of this by-product [9, 10].

Besides the ecological benefits of lactose removal from whey, this by-product also has a great relevance for the food and pharmaceutical industries [11]. It is estimated that 400,000 tons of crystalline lactose are worldwide produced each year. In comparison with other carbohydrates, lactose has a low caloric value, low glycemic index, good plasticity, compressibility, and low level of sweetness. This sugar is used in the food industries in a wide variety of products such as instant coffee, infant formula, and baked foods. Meanwhile, lactose is used as an excipient for tablets and dry powder inhalers in the pharmaceutical industry [11, 12]. The general steps in the recovery of lactose from the whey involve a step for the partial removal of water followed by a crystallization step. Some of the challenges to overcome in the recovery of lactose from the whey are the long crystallization times, low yields, and low quality of lactose crystals. These problems on lactose crystallization have been approached through the seeding of lactose, the use of antisolvent, and more recently, by the sonocrystallization of lactose [1, 3, 5]. In the last years, the number of research studies of the crystallization of lactose assisted with ultrasound has increased considerably. Hitherto, it has been established that sonocrystallization decreases the size of crystals and improves the crystal size distribution but also might speed up the crystallization process or enhance the purity of lactose crystals. However, the effect that ultrasound has on lactose crystallization is by far not fully understood. This chapter discusses the current knowledge on lactose sonocrystallization (fifth section) but also addresses the basic principles of lactose crystallization (second section) and sonocrystallization (fourth section). Furthermore, the conventional process of lactose recovery from whey is described in the third section of this chapter.

2. Crystallization of lactose

Lactose is the principal carbohydrate in the milk of mammalians, which is a reducing disaccharide made up of galactose and glucose joint by a glycosidic bond (β 1–4). Lactose comprises of two stereoisomers α- and β-anomers. In solution, lactose opens and reforms the ring structure interchanging between α and β anomers (mutarotation). The mutarotation equilibrium of lactose at 20°C is attained, when the ratio of β/α isomers is 1.70 (63:37), although this proportion is highly dependent on temperature. In equilibrium, the isomer β form is more abundant and more soluble (500 g L^{-1}) than α-lactose isomer (70 g L^{-1}) [12, 14]. Therefore, the

α isomer will crystallize first in a supersaturated solution of lactose, like a whey concentrate. In this section, the three main phases of lactose crystallization are described: supersaturation, nucleation (appearance of crystals), and crystal growth [15].

2.1. Supersaturation

Supersaturation of lactose solutions is the first step in the crystallization process, since a non-equilibrium condition is required for the spontaneous birth of nuclei [16]. At any given temperature, a maximum amount of solute can be dissolved in a solvent. When a solution is saturated with a solute, this is considered being in a thermodynamic equilibrium. Any further increase in the concentration above the saturation (solubility) point disturbs the equilibrium and induces a pseudo-equilibrium state or supersaturation. The nucleation and hence crystallization won't occur at the supersaturation point (at least not spontaneously), since the energy available is insufficient to induce the nuclei formation. However, beyond the pseudo-equilibrium state (labile zone), nucleation takes place spontaneously. The region between solubility and supersolubility (supersaturation) is known as the metastable zone (MZ). The width of this region (MZW) is obtained by plotting the solubility and supersolubility of the solute as a function of temperature. From these curves, it is possible to establish the temperature and solute concentration required in a crystallization process [5, 17]. The conventional process of lactose crystallization has a wide MZW, which means that a very high supersaturation is necessary to induce nucleation [18, 19].

2.2. Nucleation

Nucleation has a major influence on crystallization and consequently on the quality properties of lactose crystals like its structure and size distribution [21]. The formation of a new solid phase from a supersaturated solution is called nucleation, and the nucleation rate is the change in the number of particles in solution with time [22]. There are two kinds of nucleations: the primary and secondary; the former occurs when a crystal is nucleated without an interphase in the solution. Nucleation in the absence of solid surfaces is called homogeneous nucleation, and if there is a foreign interphase in the solution, the process is referred as heterogeneous nucleation. In contrast, the secondary nucleation is induced by pre-existing crystals [13]. Two theories try to explain the nucleation mechanism, the Classical Nucleation Theory (CNT) and the Two-Step Nucleation Theory (TSNT). The basics of the CNT are that from a supersaturated solution, a number of ordered subcritical clusters of solute molecules are formed under certain temperature and concentration conditions. When the number of molecules in the cluster increases (until reaching a critical cluster size n^*) (~100 to 1000 atoms), the total free energy (ΔG) in the system rises. Above this n^*, the total free energy decreases continuously and the formation of a crystal nuclei becomes favorable. However, a cluster of size n^* has equal possibilities to form a crystal nucleus or to disaggregate. Therefore, the height of the free energy barrier for nucleation (ΔG^*) and the nucleation rate are determined largely by the n^* [16, 21]. The CNT gives some insights about the n^* and nucleation rate but does not provide information on the structure of aggregates or pathways leading to the formation of solid crystal from the solution [16]. On the other hand, the major difference between the CNT and the TSNT is

that the latter considers the formation of disordered (liquid-like) clusters instead of ordered subcritical clusters. Besides, the TSNT suggests the formation of a crystalline nucleus inside the liquid-like clusters beyond the n^* [16]. Although the theories of the nucleation process have advanced considerably in recent years, the particular ordering within the solid state via the nucleation process remains ambiguous. Moreover, some of the parameters described by these nucleation theories are difficult to verify experimentally, like the critical cluster size (n^*). The number of particles can be measured by methods such as light scattering, direct particle counting (microscopy), and turbidity measurements [22]. The problem arises from the fact that n^* typically falls in a range of 100–1000 atoms, which is hardly accessible to most of the current experimental methods [16].

2.3. Crystal growth

The growth of lactose crystals is controlled by several factors but the key variable determining the rate of nucleation is the supersaturation [23]. If nucleation is fast, many crystals form simultaneously and they will grow to approximately identical sizes. In contrast, if the nucleation is slow and fewer crystals nucleate at a time, the supersaturation in the solution drops slowly, the nucleation of new crystals continues, and the solution presents a wider crystal size distribution (CSD) [21]. Other variables that affect the crystal growth are the temperature, viscosity, pH, presence of salts, and whey proteins, which modify the levels of supersaturation and consequently the nucleation and crystal growth [24–26]. Speaking of impurities like salts and proteins, these can either accelerate or inhibit the crystal growth. The impurities induce a heterogeneous nucleation and are incorporated frequently into the crystal lattice. In addition, the presence of impurities affects the solubility and supersolubility of the substance being crystalized, modifying the nucleation and crystal growth. It is well established that salts may either increase or decrease the growth rate of lactose crystals. The presence of calcium chloride, calcium lactate, magnesium sulfate, and lithium chloride increases the crystallization velocity, at the difference of potassium phosphate [24]. In the same way, the whey proteins promote nucleation but slow down the growth of lactose crystals. This effect is attributed to its high water-binding capacity that creates areas of lactose supersaturation, which are favorable for nucleation [25].

3. Conventional recovering of lactose from whey

The process of recovery of lactose from cheese whey is described in **Figure 1**. Before whey processing, curd fines and fat are separated from the whey by centrifugation [27]. This clarified and defatted (0.07%) whey must be deproteinized in advance to the concentration step. The presence of whey proteins decreases the solubility of lactose [24], promotes nucleation, accelerates the lactose crystallization [25], and reduces the purity of lactose crystals [5]. Besides, proteins increase significantly the viscosity of the concentrated whey, hindering the recovery of lactose crystals [5]. The heat-acid precipitation of whey proteins is the easiest and cheapest method for whey deproteinization, although this method leaves between 0.1 and 0.2% of the residual protein in the whey [28]. Proteins can also be removed by ultrafiltration (UF) using membranes with a molecular weight cut-off (MWCO) ranging from 3 to 10 kDa. When UF is

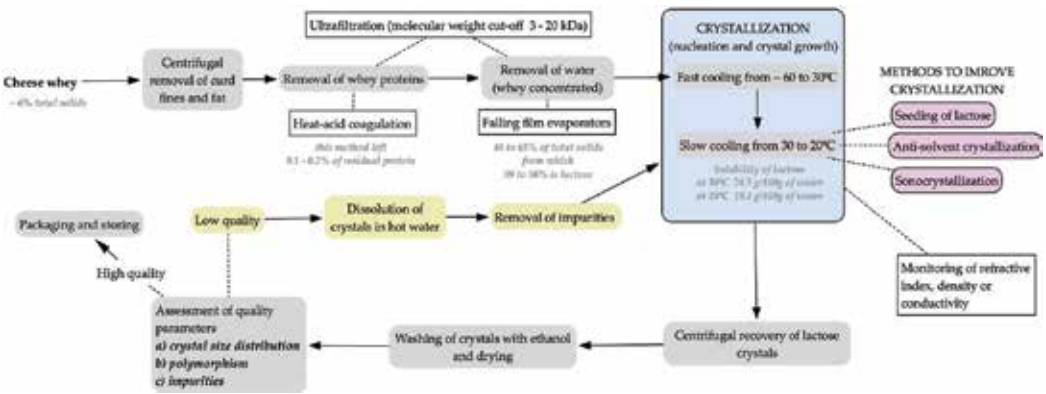

Figure 1. Schematic procedure for the recovery of lactose from whey.

carried out with this MWCO, the fat and protein fraction are retained in retentate; meanwhile, permeate keeps the lactose, vitamins, and minerals. The major drawback of this technology is the high cost of UF equipment and membranes because most of the small and medium-scale dairy processors cannot afford it [4]. Finally, if the deproteinized whey is not evaporated immediately, this must be pasteurized to avoid the fermentation of lactose by microorganisms.

The clarified, defatted, and deproteinized whey is sent to the evaporators for concentration. Evaporation is performed under reduced pressure in falling film (single and multiple effect) evaporators. These evaporation units allow the concentration of total solids in the whey by nearly ten folds (concentration factor Q = 9.5). The content of dry matter in the whey is measured during the whole evaporation process through the refractive index *n*. When the whey reaches 40 to 65% of dry matter, the evaporation process is stopped. The temperature of the final concentrate is ~60°C, and the lactose content ranges from 39 to 56%. At this point, lactose is supersaturated in the whey concentrate but won't crystallize as the temperature is high [1, 4, 28, 29]. The whey concentrate (still being hot) is then transferred into a large stirred tank where it is cooled fast enough to induce crystallization of lactose. Once in the crystallizer, the whey is first cooled rapidly from 60 to 30°C and then slowly from 30 to 20–25°C (1–3°C h^{-1}) [17, 26, 29]. The nucleation and crystallization of lactose will occur spontaneously just beyond the metastable zone (MZ), that is a region between the supersaturation point where nucleation occurs and the saturation equilibrium of lactose [14, 30]. This MZ is attained mostly during the second cooling stage when the temperature drops below 30°C, and the lactose supersaturation rises considerably [26]. The progress of crystallization can be followed measuring the changes of lactose concentration in the liquor either by refractive index, density, or conductivity [31]. The complete process of crystallization is prolonged and may take up to 48 h. Crude lactose crystals are separated from the liquor by centrifugation, filtration, or both and then washed with a nonsolvent compound (such as ethanol) to remove impurities and water. The resulting crystals are air dried and further characterized by its size distribution and purity. The yield of crystallization depends upon many variables, but typically 65% of lactose is recovered from this process [12, 17, 29]. Crude lactose is further recrystallized, if some quality parameters are not achieved such as the crystal size distribution (CSD), form,

and purity. For this lactose refining, the crystals are re-dissolved, treated with charcoal to remove impurities (salts and proteins), and recrystallized as previously described [4, 31].

The process of lactose crystallization is very slow (up to 72 h), the quality of lactose is usually poor, and the yields of crystallization are very low. One of the oldest methods used to improve the process of crystallization is the seeding of lactose. This approach consists in the addition of small lactose crystals into whey concentrate (seeding of nuclei) just before the second cooling step. The addition of lactose crystals may induce a secondary nucleation that accelerates the crystallization process and reduces the CSD [5]. However, this method has low reproducibility because its success depends on the addition of crystals in the appropriate timing [13]. More recently, alternative methods such as the use of antisolvent or sonocrystallization have been explored to assist the crystallization of lactose. The addition of nonsolvent compounds into whey concentrate (antisolvent crystallization) decreases the solubility of lactose, narrows the metastable zone, and reduces the induction times of nucleation. In general, the antisolvent crystallization improves the yield of crystallization and reduces the size of lactose crystals [20, 32]. The main drawbacks of antisolvent crystallization are the large amounts of solvent used, and the expensive separation and purification steps required to remove the antisolvent from the product [5, 9]. The crystallization of lactose assisted with low-frequency power ultrasound (sonocrystallization) is discussed later in the chapter.

4. General principles of ultrasound and sonocrystallization

Sonochemistry and sonoprocessing have a wide range of applications in food technology, medicine, nanotechnology, chemical synthesis, materials extraction, polymerization, phase separation, surface and water cleaning, catalysis, enhancing the enzyme activity, and so on. Sonication of a liquid generates acoustic cavitation depending upon the experimental conditions used. Strong physical effects and highly reactive radicals are generated during acoustic cavitation [33]. An overview of the general principles of ultrasound is discussed in this section.

4.1. Ultrasound

Ultrasound refers to sound waves of a frequency that cannot be detected by human ear. The ultrasonic frequency ranges from 20 kHz to >10 MHz within which they are further divided into low frequency (20–100 kHz), intermediate frequency (100 kHz–1 MHz), and high frequency (1–10 MHz) regions. The interaction of ultrasound gas bubbles in liquids can lead to the generation of chemical reactions and physical forces. The driving force behind such forces is acoustic cavitation [34].

4.2. Acoustic cavitation

Acoustic cavitation is the phenomenon of formation, growth, and violent collapse of microbubbles in a liquid medium under the influence of acoustic field (**Figure 2**). Bubbles which are inherently present as small nuclei will grow to a critical size under the applied ultrasonic energy. The growth of the acoustic bubbles is due to the phenomenon called "rectified

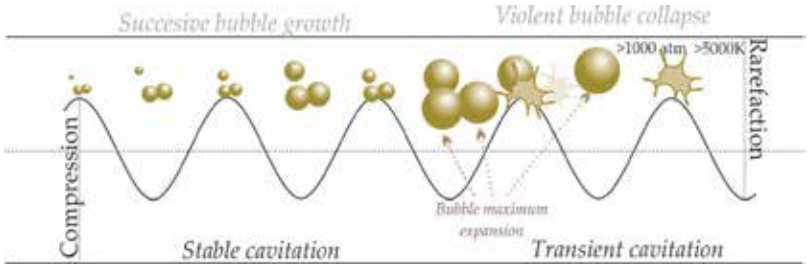

Figure 2. Schematic representation of acoustic cavitation.

diffusion" which is defined as slow growth of the acoustic bubble as a function of time due to unequal mass transfer across the air/water interface [35]. There are two types of cavitation bubbles that exist depending upon the ultrasonic intensity, i.e., transient cavitation bubbles and stable cavitation bubbles. When the ultrasonic intensity is very high, transient cavitation bubbles last for a few acoustic cycles. On the other hand, stable cavitation bubbles can oscillate for many acoustic cycles. The size of stable cavitation bubbles grows over time due to coalescence and also by rectified diffusion until the size is reached, where the coupling of bubble's resonance frequency and driving frequency of the ultrasound occurs. In a multibubble cavitation field, bubbles with a range of size are generated and grown toward the critical size. Miinaert's equation (Eq. 1) provides a relationship between linear resonance radius (critical size) of the bubble with frequency [36].

$$R_{res} = \frac{3}{f} \tag{1}$$

where R_{res} is the linear resonance radius (m) and f is the ultrasonic frequency (Hz).

Transient cavitation bubbles dominate at the lower frequency where they can grow rapidly above a threshold size during the rarefaction cycle. The nature of cavitation bubble is controlled by numerous parameters, such as acoustic pressure, frequency, type of reactor, and bubble size.

4.3. Chemical and physical effects of ultrasound

The collapse of cavitation bubble leads to the generation of a very high temperature of >5000 K and pressure (>1000 atm) within the bubble (**Figure 2**) [34, 36, 39]. The collapse of the bubble takes place in a very short period of time, and thermodynamically, the work done leads to a near adiabatic heating of the bubble contents, which lead to extreme conditions [36–38]. The maximum temperature and pressure generated within the cavitation bubble can be theoretically calculated using Eqs. (2) and (3), respectively, to a near adiabatic heating of the bubble contents which lead to extreme conditions [36–38].

$$T_{max} = T_0 \left\{ \frac{P_m(\gamma - 1)}{P_v} \right\} \tag{2}$$

where T_0 is the temperature of the solution, P_m is the pressure inside the liquid, γ is the ratio of specific heat of gas-vapor mixture, and P_v is the pressure of the bubble when it has maximum size.

$$P_{max} = P_v \left\{ \frac{P_m(\gamma - 1)}{P_v} \right\}^{\frac{\gamma}{(\gamma-1)}} \tag{3}$$

The extreme conditions generated on bubble collapse results in: (1) light emission—sonoluminescence, (2) radical generation, and (3) shock waves, microjetting, microstreaming, shear forces, and microturbulence [40]. The violent collapse of cavitation bubbles can sometimes lead to the emission of light called sonoluminescence [41]. The intensity of light emitted and the sonochemical yield depends on different factors, such as amount and type of dissolved gases in a liquid, ultrasonic frequency and power, hydrostatic pressure, and addition of some solutes. When ultrasound is passed through a liquid medium, the formation of standing waves takes place. Mostly, active bubble formation occurs at the pressure antinodes; therefore, the number of bubbles increases with an increase in the antinodes formed in a liquid. The increase in ultrasonic frequency leads to (i) an increase in number of antinodes and active cavitation bubbles and (ii) a decrease in size and collapse intensity of cavitation bubbles [40].

In air-saturated water, different radicals and molecular products such as H_2O_2, HO_2, H, and OH radicals are generated. These radical are formed through the following reactions: (i) $H_2O \rightarrow H^{\cdot} + OH^{\cdot}$, (ii) $OH^{\cdot} + OH^{\cdot} \rightarrow H_2O_2$, (iii) $H^{\cdot} + O_2 \rightarrow HO_2^{\cdot}$. Primary radicals can be used to initiate a number of chemical reactions such as polymerization, synthesis, and degradation. The detailed information on the fundamentals and applications of the ultrasound could be found in literature [42–44]. The physical and chemical effects of ultrasound can be utilized and applied in a number of fields such as material synthesis, water treatment, and crystallization. In the following section, various reported investigations dealing with crystallization processes under ultrasound and possible mechanisms involved are summarized.

4.4. Sonocrystallization (ultrasound-assisted crystallization)

Ultrasound is found to influence crystallization process, which is referred to as sonocrystallization. Ultrasound has been used from a long time to initiate nucleation and control growth during cooling and antisolvent crystallization process, but the mechanism behind sonocrystallization is still debatable and unclear. It is generally accepted that the physical effects of acoustic cavitation are responsible for the effects observed during sonocrystallization [13]. Sonocrystallization is known to have a number of specific features. For most materials, such features include enhancing the primary nucleation, due to uniform mixing throughout the liquid medium; relatively easier nucleation in some systems which are otherwise hard to nucleate under conventional procedures; ultrasound also has the tendency to initiate secondary nucleation and formation crystals with uniform and small size with high purity. Supersaturation is the driving force behind crystallization process, which is accompanied by nucleation and growth of the crystals, and ultrasound can affect both processes. Active pharmaceutical ingredients are found in a variety of crystalline solid forms, which include polymorphs, hydrates, salts, co-crystals, and amorphous solids. Such different solid forms exhibit different and unique

physical as well as chemical properties that can affect the bioavailability, solubility, stability, and other characteristics of the drug. Reduction in particle size can significantly enhance the bioavailability and solubility in most of the pharmaceutical drugs. Therefore, production of smaller size particles with uniform size distribution and desired properties is very important in the development of pharmaceutical drugs. Applying ultrasound during crystallization also results in a number of other benefits, such as nucleation at lower level of supersaturation, narrowing of metastable zone width, highly repeatable and predictable crystallization, reduction in the induction time, improved morphology, and polymorphs selectivity [45–47].

Ultrasound has also shown the tendency to significantly influence the agglomeration of the particles. There are different physical effects generated by ultrasound that can contribute to reduce the agglomeration. This includes shock waves generated due to acoustic cavitation, which can decrease the time of contact between particles that can hinder the interaction of particles together. Also, sometimes agglomeration occurs at the stage of nucleation. Nuclei usually have high surface area to volume ratio, which can lead to high surface tension and nuclei tend to lower the surface tension by interacting to one another. Then, the surface tension tends to drop during the crystal growth when the particles become more stable, which can prohibit agglomeration [48]. Lastly, the uniform mixing of the sonication mixture due to physical forces of the ultrasound can help to reduce agglomeration by locally controlling the nucleus population [48].

Hunt and Jackson [49] demonstrated that nucleation occurs during the collapse of a cavitation bubble rather than when it expands. They have demonstrated this by slowing down the formation and collapse of cavitation bubble in pure liquid sealed in U-tube during isolated cavitation events. The pressure variations and very high pressures generated when the cavity collapses tend to lower the crystallization temperature of the liquid, which results in nucleation [50]. Another reason may be the rapid cooling that occurs after the bubble collapse around the collapsing bubble, thus creating a region of high supersaturation. According to another report [36], nucleation occurs due to the negative pressures generated during the collapse of cavitation bubbles. A possible nucleation mechanism, proposed during ice crystallization, is that the concentration and agglomeration of ice clusters can occur near the bubble surface because of the diffusion of species from low- to high-pressure zones. Louisnard et al. [52] have also suggested that high-pressure gradients are required, for pressure diffusion to be effective and that can only be attained during the collapse of the bubbles, and thus, stable cavitation can also act as a potential nucleation initiator. It has been provided different physical mechanisms that can possibly influence crystallization process with sonication. It was suggested that high pressures due to cavitation, agitation intensive mixing of the liquid medium by ultrasound, supercooling at the bubble surface, and enhanced heterogeneous nucleation are the possible factors responsible for the observed benefits of the sonocrystallization [52]. Author [53] has suggested that high pressure generated is strong enough to initiate the nucleation, as it increases the melting point of the liquid; therefore, cavitation bubble is important to initiate the crystallization. The enhancement of heterogeneous nucleation occurred as the ultrasound can lead to production of different nuclei from the single seed [53]. On the other hand, Virone et al. [54] determined the physical mechanism of ultrasound-induced crystallization based on the bubble dynamics for the first time. The authors correlated the nucleation

rate to the maximum pressure reached inside the cavitation bubble. To correlate such factors, they used numerical simulations on bubble dynamics.

Since sonocrystallization has many benefits over the conventional crystallization, numerous studies have been reported on the impact of various ultrasonic parameters on crystallization process for a variety of solutes such as acetylsalicylic acid, sodium acetate, sucrose, glycine, lactose, adipic acid, carbamazepine, NaCl, KCl, benzoic acid, and paracetamol. Various parameters investigated include sonication time, frequency and power, horn diameter, and supersaturation ratio. Besides affecting the MZW, crystal size distribution (CSD), and yield, ultrasound also provides control over polymorph forms of some solutes. It was shown that sonication can influence the primary nucleation and crystal growth of roxithromycin during antisolvent crystallization. With intensive amount of shear generated, ultrasound helped to reduce agglomeration and change the roxithromycin crystal morphology from a hexagonal to rhombus shape [55]. Further study by Hatkar et al. [51] on salicylic acid clearly established that ultrasound can be effectively used to control the antisolvent crystallization process in terms of the mean size of obtained crystals and size distribution. During sonocrystallization experiments, ultrasound-related variables like irradiation time and power of ultrasound were found to affect the crystal size distribution, whereas frequency did not have much effect over the range of frequencies investigated. It was found that irradiation time and power of ultrasound decreased the average particle size, as well as a reduction in the agglomeration was observed [36].

5. Sonocrystallization of lactose

Sonocrystallization of lactose has aroused great interest in the last decade. Consequently, there are a number of studies that provide relevant information on the effect of ultrasound on lactose crystallization, although such effect is not completely understood yet. The current information concerning the sonocrystallization of lactose is condensed in **Tables 1** and **2**.

5.1. Effects on lactose supersaturation and nucleation

It has already been discussed that sonication favors the formation of supersaturation and modifies the metastable zone [13, 14, 30]. The effect of ultrasound on lactose supersaturation has been scarcely documented chiefly because almost all the studies on lactose sonocrystallization have been done in combination with nonsolvents that greatly modify the solubility of lactose (**Table 1**) [4, 9, 18, 20, 28, 32]. The antisolvents used for these studies include ethanol, propanol, glycerol, and acetone, all of which decrease the solubility of lactose sharply and speed up the attainment of supersaturation [5]. On the other hand, there are a couple of studies that have been conducted in the absence of nonsolvent compounds (**Table 2**) [15, 22, 55]. From these works, it is reported that ultrasound energy densities of up to 0.15 W g^{-1} (at 20 kHz) narrow the MZW of lactose [22].

On the other hand, the effect of ultrasound on nucleation is not entirely clear, but it is generally accepted that sonication increases the rate of nucleation [15]. The long time of crystallization

	Effect on	Antisolvent	Experimental setup	Key outcomes	Ref.
Model solution system	Crystal growth rate	85% n-propanol	12–18% lactose, 20 kHz, 120 W	Growth rates from 0.007 to 0.027 μm s^{-1}	[32]
	Size and morphology	80% Acetone	12–16% lactose, 120 W	The crystal diameter decreased from 4 to 2.48 μm. Appearance of rod shaped crystals	[20]
		Ethanol	20–30% lactose, 20 kHz, 10–30 W	There was no correlation between ultrasound and particle size. Appearance of rods, needles and tomahawks shape.	[19]
		85% n-propanol	12–18% lactose, 20 kHz, 120 W	Mean diameter from 12 to 15 μm. Appearance of elongated and rod/needle-shaped lactose crystals in sonicated samples and tomahawk shape for stirring samples.	[32]
		85% Ethanol	11.5–17.5% lactose, 22 kHz, 12.3 W	Smaller crystals with more uniform shape than those obtained in the absence of sonication (stirring). Tomahawk and needle-shaped crystals were observed.	[28]
	Yield	85% Ethanol	11.5–17.5%lactose, 22 kHz, 12.3 W	Lactose recovery after sonication treatment (91.48%) was much higher that nonsonicated samples (14.63%)	[28]
	Purity	Ethanol	20–30% lactose, 20 kHz, 10–30 W	Decreases β-lactose incorporation.	[19]
Concentrated cheese whey	Size and morphology	65–85% Acetone	5–15% of lactose content, 120 W	Narrow crystal size distribution (2.5–6.5 μm) at pH 6.5, 15% of lactose concentration and 75% acetone concentration. Appearance of needle shaped crystals.	[58]
		85% Ethanol	22–33 kHz, 40–120 W	An increase in power from 40 to 120 W and frequency from 22 to 33 kHz had a marked reduction in particle size.	[6]
	Yield	85% Ethanol	22–33 kHz, 40–120 W	The yield without sonication was 74.7% and increased to 97.6% with 40 W of ultrasonic power. In a range between 60 and 120 W the yield decreased from 98.6 to 75.6%. An increase in frequency from 22 to 33 kHz did not change the lactose recovery (94 to 92%)	[6]
	Purity	85% Ethanol	22–33 kHz, 40–120 W	An increase in dissipation power (40 to 120 W) decreases the lactose purity from 96.9 to 88.7%. An increase in frequency from 22 to 33 kHz increased the purity from 79.5 to 91.5%.	[6]

Table 1. Reported effects of ultrasound in combination with antisolvents on lactose crystallization.

	Effect on	Experimental setup		Ref.
Model solution system	Supersaturation	60% lactose, 0.46 W g^{-1}	Ultrasound affected the heterogeneous nucleation.	[22]
			More prominent effect of ultrasound at low supersaturation between 1.6 and 2.1	
	Nucleation	30–50% lactose, 20 kHz, 750 W	Sonication showed a very rapid nuclei induction.	[18]
	Induction time	60% lactose, 0.46 W g^{-1}	Induction time was faster than stirring but this decrease with an increasing power from 0.15 to 1.15 W g^{-1}. Sonication resulted in a significantly faster nucleation rates than stirring, 5.3×10^5 and 1.6×10^4 mL^{-1} min^{-1}	[22]
	Crystal growth rate	60% lactose, 0.46 W g^{-1}	Did not change growth rate between ultrasound and stirring (0.14 μm min^{-1})	[22]
	Size and morphology	30–50% lactose, 20 kHz, 750 W	Particles from 15 to 30 μm. Production of rod-shaped lactose crystals with high elongation ratio. Appearance of rod-shaped crystals with high elongated ratio.	[18]
		60% lactose, 0.46 W g^{-1}	Number of crystals mL^{-1} was 2.8×10^6 and 4.6×10^5 for sonicated and nonsonicated samples.	[22]
		33% lactose, 20 kHz, 10-70 W, (oscillatory)	Batch produces bigger crystals than continuous treatment. An increase in power produces smaller crystals. Tomahawk crystals were observed.	[55]
	Yield	30–50% lactose, 20 kHz, 750 W	Yield of 84% with 5 min of sonication	[18]
		33% lactose, 20 kHz, 10-70 W, (oscillatory)	Sonication power from 0.10 to 0.15 W g^{-1} increased the yield from 17.4 to 25.1% (2.5 h of crystallization; and 19.7 to 28.3% after 4 h).	[56]
Concentrated cheese whey	Crystal growth rate	3 to 16 J mL^{-1}, 20 kHz	A faster rate of crystallization were obtained for whey sonicated at a flow rate of 11 L min^{-1} and applied energy density of 3.3 J mL^{-1}	[15]
	Size and morphology	3 to 16 J mL^{-1}, 20 kHz	Narrow distribution of crystal sizes (38 ± 10 μm) than stirred solutions (57 ± 17 μm).	[15]
	Yield	3 to 16 J mL^{-1}, 20 kHz	Yield obtained from 75 to 85% after 24 h.	[15]

Table 2. Reported effects of ultrasound on lactose crystallization (without antisolvents).

is one of the major issues to deal with during the recovering of lactose. Therefore, ultrasound has been used in lactose crystallization chiefly to accelerate nucleation and consequently to reduce the crystallization time [5]. The theoretical effects of ultrasound on nucleation can be summarized as follows:

- during the stable cavitation, the bubbles remain without collapsing for a number of ultrasonic cycles. The movement of these bubbles or flow streams enhances the mass and heat

transfers, as well as the aeration, so does the nucleation rate [5, 14]. The stable bubbles may act as nucleation centers, since the rapid growth of bubbles during the acoustic cycles drops the temperature locally and increases the supersaturation nearby the bubbles [5]. Besides, the pressure gradient around the cavitation bubbles induces a controlled diffusion of particles or embryos (segregation effect) that also favor the nucleation process [57].

- during the transient cavitation, the vigorous collapse of bubbles releases shockwaves and creates local zones of high pressure and temperature. These release of energy promotes mass transfer, molecular collisions, and supply the driving force for instantaneous nucleation (ΔG^*) [5, 13, 30]. Moreover, cavitation bubbles tend to locate themselves near the boundaries of the earlier formed crystals. When these bubbles collapse, the crystals are disrupted in many small fragments promoting the secondary nucleation [14].

Only a few works have addressed the effect of ultrasound on lactose nucleation, like in that reported by Dincer et al. [22]. In such study, it was observed that sonication of lactose solutions (60%) with an ultrasonic power density of 0.46 W g^{-1} (at 20 kHz) enhances the nucleation rate (5.3×10^5 crystals mL^{-1} min^{-1}) as compared to simple stirring at 300 rpm (0.16×10^5 crystals mL^{-1} min^{-1}). The ultrasound affected primarily the heterogeneous nucleation, and this effect was improved at low levels of lactose supersaturation (1.6–2.1). This study also reported that sonication decreased nearly ten-fold the induction time of nucleation. Induction time has been used to determine the nucleation rate, and this is defined as the time elapsed from the creation of supersaturated solution and the appearance of the first crystals [13]. The conventional crystallization of lactose exhibits a long induction time, which makes the process uneconomical [32]. Therefore, the reduction of induction time by lactose sonocrystallization becomes relevant.

5.2. Effects on the crystal size distribution (CSD) and yield

In contrast to nucleation, there is no consensus to the effect of ultrasound on crystal growth. Although, it is theorized that ultrasound might promote the growth of crystals through the mass, and heat transfers enhancement [14]. Patel and Murthy [32] reported crystal growth rates between 0.007 and 0.027 μm s^{-1} for a crystallization process of lactose assisted with ultrasound (120 W) and antisolvents (n-propanol 85%). Meanwhile, Dincer et al. (2014) reported a growth rate of 0.14 μm min^{-1} for a lactose solution (60%) sonicated at 0.46 W g^{-1}. Nevertheless, these authors did not observe a difference between the growth rates of sonication and stirring.

The size of lactose crystals commonly falls between 2 and 50 μm. The desired lactose crystal size varies depending on the specific use. For instance, when lactose is employed as an excipient in dry powder inhalers, its size must range between 2 and 6 μm for an optimum drug delivery to the lung. When lactose is destined to the food industry, the size of crystals is regularly bigger than 20 μm. No matter the intended use, a narrow crystal size distribution (CSD) is always preferable [5, 19]. The principal factors that affect the crystal size of lactose are (a) initial levels of saturation, (b) presence of salts and proteins, (c) rate of nucleation/crystallization, and (d) extent of secondary nucleation [5, 24, 32, 58]. Similarly, the addition of nonsolvents, as well as the seeding of lactose, decreases the size of lactose crystals and narrows the CSD [13, 18].

The shape and size of lactose crystals are also modified by sonication. The tomahawk shape (characteristic of α-lactose monohydrate) is the most reported in sonocrystallized lactose [5], although elongated shapes are described in some reports [9]. According to Dhumal et al. [18], applying ultrasound causes some faces of the lactose crystals grow faster than other producing elongated rod-shaped crystals. Besides, ultrasound increases the surface roughness of lactose crystals by reducing the incorporation of β-lactose into the crystal lattice [9, 18].

Regarding the crystal size, all the works agree that ultrasound increases the number of lactose crystals and produces smaller crystals with more homogeneous sizes [6, 15, 18, 20, 22, 32, 56]. Only one study has reported that there is no correlation between ultrasound (10–30 W, 20 kHz) and the size of lactose crystals [9]. The effects on the number and size of lactose crystal are primarily attributed to the increase in the number of nuclei that promotes the ultrasound, whether during the primary or secondary nucleation [56]. The extent of size reduction that is attained through sonocrystallization depends upon the ultrasound energy density (or power), time of sonication, and frequency applied (**Tables 1** and **2**). For example, we have observed that ultrasound energy densities of 9 J mL^{-1} were enough to decrease the size of lactose crystals by half and to significantly narrow the CSD Nevertheless, a higher energy density (50 J mL^{-1}) did not produce a further change in the size of crystals or the CSD (**Figure 3A**). On the other hand, the effect of different ultrasonic frequencies on the size of lactose crystals has been hardly reported, because nearly all the studies have used the similar frequencies (20–22 kHz). So far, only the work of Gajendragadkar and Gogate [6] has explored an ultrasonic frequency of 33 kHz. According to these authors, a frequency increase from 22 to 33 kHz reduced the crystal size and improved the lactose purity but decreased the yield of crystallization.

The process of lactose crystallization is conventionally carried out in presence of residual whey proteins (0.1–0.2%), which also decrease significantly the crystals size. The water-binding capacity of whey proteins creates supersaturation spots that favor nucleation [24, 25]. A few studies have addressed the effect of whey proteins on lactose sonocrystallization. Bund and Pandit [28] reported an increase in the crystal size of lactose sonocrystallized with ethanol (85%) in the presence of 0.4% of bovine serum albumin (BSA). Patel and Murthy [32] described that 0.2 to 0.8% of BSA widened the CSD of lactose sonocrystallized (120 W, 20 kHz) with n-propanol (85%). In contrast, we have noted that 0.64% of whey proteins decreased the

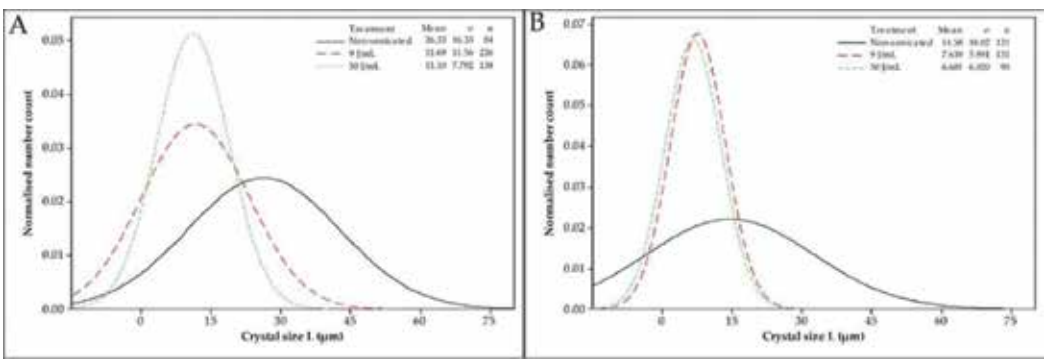

Figure 3. Effect of different ultrasound energy densities on the crystal size distribution (CSD) of lactose: (A) solutions saturated with 25% (w/v) of lactose; (B) solutions with 25% (w/v) of lactose and 0.64% (w/v) of whey proteins.

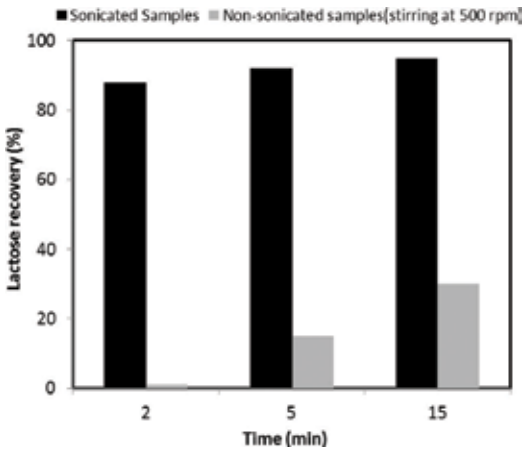

Figure 4. Effect of time on lactose recovery in sonicated and nonsonicated samples (reproduced from Bund and Pandit [28]).

size of crystal size significantly (9 J mL^{-1}) in an aqueous system without nonsolvents. Besides, the CDS was narrowed by the presence of whey proteins (**Figure 3B**).

Speaking of lactose recovering, there is a consensus that ultrasound improves the yield of lactose crystallization. **Tables 1** and **2** summarize the published data on crystallization yield obtained from lactose sonocrystallization with or without nonsolvents. Just to mention some examples, Bund and Pandit [28] showed that sonicated samples had higher lactose recoveries both in absence and presence of protein compared to the nonsonicated samples at different pH values with an antisolvent crystallization method. They recovered ~88% of lactose with 2 min of sonication as compared to 55–60% in 12 to 72 h with conventional lactose recovery (**Figure 4**). Similarly, the recovery of lactose was reported from the paneer whey with the use of ethanol as an antisolvent. The ultrasonic frequency and power utilized were 22 kHz and 120 W, respectively. Almost 90% of lactose was recovered in just 20 min with ultrasound.

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References

[1] Walstra P, Wouters J, Geurts T. Dairy Science and Technology. 2nd Ed. CRC press; 2005, 782 p

[2] Das B, Sarkar S, Sarkar A, Bhattacharjee S, Bhattacharjee, C. Recovery of whey proteins and lactose from dairy waste: A step towards green waste management. Process Safety and Environmental Protection. 2015;**110**:27-33. DOI: 10.1016/j.psep.2015.05.006

[3] De Souza R, Bergamasco R, da Costa S, Feng X, Faria S, Gimenes M. Recovery and purification of lactose from whey. Chemical Engineering and Processing: Process Intensification. 2010;**49**:1137-1143. DOI: 10.1016/j.cep.2010.08.015

[4] Patel S, Murthy Z. Lactose recovery processes from whey: A comparative study based on sonocrystallization. Separation & Purification Reviews. 2012;**41**:251-266. DOI: 10.1080/15422119.2011.594142

[5] Zamanipoor M, Mancera R. The emerging application of ultrasound in lactose crystallization. Trends in Food Science & Technology. 2014;**38**:47-59. DOI: 10.1016/j.tifs.2014.04.005

[6] Gajendragadkar C, Gogate P. Ultrasound assisted intensified recovery of lactose from whey based on anti-solvent crystallization. Ultrasonics Sonochemistry. 2016;**38**:754-765. DOI: 10.1016/j.ultsonch.2016.08.011

[7] Jelen P. Industrial whey processing technology: An overview. Journal of Agricultural and Food Chemistry. 1979;**27**:658-661. DOI: 10.1021/jf60224a037

[8] Prazeres A, Carvalho F, Rivas J. Cheese whey management: A review. Journal of Environmental Management. 2012;**110**:48-68. DOI: 10.1016/j.jenvman.2012.05.018

[9] Kougoulos E, Marziano I, Miller P. Lactose particle engineering: Influence of ultrasound and anti-solvent on crystal habit and particle size. Journal of Crystal Growth. 2010; **312**:3509. DOI: 10.1016/j.jcrysgro.2010.09.022

[10] Patel S, Murthy Z. Optimization of process parameters by Taguchi method in the recovery of lactose from whey using sonocrystallization. Crystal Research and Technology. 2010;**45**:747-752. DOI: 10.1002/crat.201000139

[11] Listiohadi Y, Hourigan J, Sleigh R, Steele R. Properties of lactose and its caking behaviour. Australian Journal of Dairy Technology. 2005;**60**:33-52

[12] Fox P. Lactose: Chemistry and properties. In: McSweeney P, Fox P, editors. Advanced Dairy Chemistry: volume 3: Lactose, water, salts and minor constituents. 3rd ed. Springer Science & Business Media. 2009; p. 1-15

[13] De Castro M, Priego-Capote F. Ultrasound-assisted crystallization (sonocrystallization). Ultrasonics Sonochemistry. 2007;**14**:717-724. DOI: 10.1016/j.ultsonch.2006.12.004

[14] Zhang Z, Sun D, Zhu Z, Cheng L. Enhancement of crystallization processes by power ultrasound: current state-of-the-art and research advances. Comprehensive Reviews in Food Science and Food Safety. 2015;**14**:303-316. DOI: 10.1111/1541-4337.12132/pdf

[15] Zisu B, Sciberras M, Jayasena V, Weeks M, Palmer M, Dincer T. Sonocrystallisation of lactose in concentrated whey. Sonocrystallization of lactose in concentrated whey. 2014; **21**:2117-2121. DOI: 10.1016/j.ultsonch.2014.03.031

[16] Erdemir D, Lee A, Myerson A. Nucleation of crystals from solution: Classical and two-step models. Accounts of Chemical research. 2009;**42**:621-629. DOI:10.1021/ar800217x

[17] Wong S, Hartel R. Crystallization in lactose refining-A review. Journal of Food Science. 2014;**79**:257-272. DOI: 10.1111/1750-3841.12349

[18] Dhumal R, Biradar S, Paradkar A, York P. Ultrasound assisted engineering of lactose crystals. Pharmaceutical Research. 2008;**25**:2835-2844. DOI: 10.1007/s11095-008-9653-9

[19] Raghavan S, Ristic R, Sheen D, Sherwood J. Morphology of crystals of r-lactose hydrate grown from aqueous solution. The Journal of Physical Chemestry. 2000;**104**:12256-12262. DOI: 10.1021/jp002051o

[20] Patel S, Murthy Z. Ultrasound assisted crystallization for the recovery of lactose in an anti-solvent acetone. Crystal Research and Technology. 2009;**44**:889-896. DOI: 10.1002/crat.200900227

[21] Vekilov P. Nucleation. Crystal Growth & Design. 2010;**10**:5007-5019. DOI: 10.1021/cg1011633

[22] Dincer T, Zisu B, Vallet C, Jayasena V, Palmer M, Weeks M. Sonocrystallization of lactose in an aqueous system. International Dairy Journal. 2014;**35**:43-48. DOI: 10.1016/j.idairyj.2013.10.001

[23] McLeod J, Paterson A, Jones J, Bronlund J. Primary nucleation of alpha-lactose monohydrate: The effect of supersaturation and temperature. International Dairy Journal. 2011;**21**:455-461. DOI: 10.1016/j.idairyj.2011.01.006

[24] Bhargava A, Jelen P. Lactose solubility and crystal growth as affected by mineral impurities. Journal of Food Science. 1996;**61**:180-184. DOI: 10.1111/j.1365-2621.1996.tb14754.x

[25] Huppertz T, Gazi I. Lactose in dairy ingredients: Effect on processing and storage stability. Journal of Dairy Science. 2015;**98**:1-10. DOI: 10.3168/jds.2015-10033

[26] Pisponen A, Pajumägi S, Mootse H, Sats A, Poikalainen V, Karus A. Effect of cooling rates and low crystallization temperatures on morphology of lactose crystals obtained from Ricotta cheese whey. Agronomy Research. 2014;**12**:787-792

[27] McSweeney P. Cheese Problems Solved. 2nd ed. Elselvier. 2007

[28] Bund R, Pandit A. Sonocrystallization: effect on lactose recovery and crystal habit. Ultrasonics sonochemistry. 2007;**14**:143-152. DOI: 10.1016/j.ultsonch.2006.06.003

[29] Wong Y, Bund R, Connelly R, Hartel R. Designing a lactose crystallization process based on dynamic metastable limit. Journal of Food Engineering. 2012;**111**:642-654. DOI: 10.1016/j.jfoodeng.2012.03.003

[30] Sander J, Zeiger B, Suslick K. Sonocrystallization and sonofragmentation. Ultrasonics Sonochemistry. 2014;**21**:1908-1915. DOI: 10.1016/j.ultsonch.2014.02.005 1350-4177/

[31] Hartel R. Advances in food crystallization. Annual Review of Food Science and Technology.2013;**4**:277-292

[32] Patel S, Murthy Z. Anti-solvent sonocrystallization of lactose. Chemical and Process Engineering. 2011;**32**:379-389

[33] Mason T. Sonochemistry and sonoprocessing: The link, the trends and (probably) the future. Ultrasonics Sonochemistry. 2003;**10**:175-179

[34] Ashokkumar M, Mason T. Sonochemistry. Kirk-Othmer Encyclopedia of Chemical Technology. 2007

[35] Crum L. Acoustic cavitation series: Part five rectified diffusion. Ultrasonics. 1984;**22**:215-223. DOI: 10.1016/0041-624X(84)90016-7

[36] Brotchie A, Grieser F, Ashokkumar M. Effect of power and frequency on bubble-size distributions in acoustic cavitation. Physical Review Letters. 2009;**102**:084302. DOI: 10.1103/PhysRevLett.102.084302

[37] Mason T. Ultrasound in synthetic organic chemistry. Chemical Society Reviews. 1997;**26**. DOI: 10.1039/CS9972600443

[38] Mason T, Tiehm A. Advances in Sonochemistry: Ultrasound in Environmental Protection. 6th ed. Elsevier. 2001

[39] Young. Cavitation. World Scientific. 1999

[40] Ashokkumar M. Ultrasonic synthesis of functional materials ultrasonic synthesis of functional materials. Spring. 2016

[41] Suslick K, Crum L. Sonochemistry and Sonoluminescence. New York: Wiley-Interscience; 1998

[42] Leighton T. The Acoustic Bubble. England: Academic Press; 2012

[43] Leong T, Wu S, Kentish S, Ashokkumar M. Growth of bubbles by rectified diffusion in aqueous surfactant solutions. The Journal of Physical Chemistry. 2010;**114**:20141-20145. DOI: 10.1021/jp107731j

[44] Lorimer J, Mason T. Sonochemistry: Part 1-The physical aspects. Chemical Society Reviews. 1987;**16**:239-274. DOI: 10.1039/CS9871600239

[45] Guo Z, Zhang M, Li H, Wang J, Kougoulos E. Effect of ultrasound on anti-solvent crystallization process. Journal of Crystal Growth. 2005;**272**:555-563. DOI: 10.1016/j.jcrysgro.2004.09.049

[46] Lyczko N, Espitalier F, Louisnard O, Schwartzentruber J. Effect of ultrasound on the induction time and the metastable zone widths of potassium sulphate. Chemical Engineering Journal. 2002;**86**:233-241. DOI: 10.1016/S1385-8947(01)00164-4

[47] Young S. Mechanical stimulus to crystallization in super-cooled liquids. Journal of the American Chemical Society. 1911;**32**:148-162. DOI:10.1021/ja02215a003

[48] Beckmann W. Crystallization: Basic Concepts and Industrial Applications. Germany: John Wiley & Sons; 2013

[49] Hunt J, Jackson K. Nucleation of solid in an undercooled liquid by cavitation. Journal of Applied Physics. 1966;**27**:254-257. DOI: 10.1063/1.1707821

[50] Saclier M, Peczalski R, Andrieu J. A theoretical model for ice primary nucleation induced by acoustic cavitation. Ultrasonics Sonochemistry. 2010;**17**:98-105. DOI: 10.1016/j.ultsonch.2009.04.008

[51] Hatkar U, Gogate P. Process intensification of anti-solvent crystallization of salicylic acid using ultrasonic irradiations. Chemical Engineering and Processing: Process Intensification. 2012;**57**:16-24. DOI: 10.1016/j.cep.2012.04.005

[52] Hem S. The effect of ultrasonic vibrations on crystallization processes. Ultrasonics. 1967; **5**:202-207. DOI: 10.1016/0041-624X(67)90061-3

[53] Schmid G, Roll A. The effects of ultrasound on the kinetics of crystallization. Consultants Bureau, New York: Zeitschrift fur Elektrochemie; 1939

[54] Virone C, Kramer H, Van Rosmalen G, Stoop A, Bakker T. Primary nucleation induced by ultrasonic cavitation. Journal of Crystal Growth. 2006;**294**:9-15. DOI: 10.1016/j.jcrysgro.2006.05.025

[55] Park S, Yeo S. Liquid antisolvent recrystallization of phenylbutazone and the effect of process parameters. Separation Science and Technology. 2011;**46**:1273-1279. DOI: 10.1080/01496395.2010.551167

[56] Siddique H, Brown C, Houson I, Florence A. Establishment of a continuous sonocrystallization process for lactose in an oscillatory Baffled crystallizer. Organic Process Research & Development. 2015;**19**:1871-1881. DOI: 10.1021/acs.oprd.5b0012

[57] Kiani H, Zhang Z, Delgado A, Sun D. Ultrasound assisted nucleation of some liquid and solid model foods during. Food Research International. 2011;**44**:2915-2921. DOI: 10.1016/j.foodres.2011.06.051

[58] Lifran E, Vu T, Durham R, Hourigan J, Sleigh R. Crystallisation kinetics of lactose in the presence of lactose phosphate. Powder Technology. 2007;**179**:43-54. DOI: 10.1016/j.powtec.2006.11.010

Section 2

Approaches on Improving Functionalities and Quality Systems of Dairy Products

Chapter 5

Cynara cardunculus: Use in Cheesemaking and Pharmaceutical Applications

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Additional information is available at the end of the chapter

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Abstract

Cynara cardunculus L. is the most widespread species of *Cynara* genus (f. *Asteraceae*). This herbaceous perennial plant is native to the Mediterranean region and invasive in other parts of the world, growing naturally in harsh habitat conditions. There are three subspecies: globe artichoke; cultivated cardoon and the progenitor of the two, the wild cardoon. The culture of *Cynara cardunculus* L. follows an annual growth cycle, emerging in autumn and harvesting in summer. *Cynara cardunculus* has been considered as a multi-purpose crop due to its relevant biochemical profiles. Inflorescences have been used as food, whereas leaves are a rich source of bioactive compounds. Consequently, larger plants without spines have been selected for technological purposes. Due to its high cellulose and hemicellulose content, the lignocellulosic fraction has been used as solid biofuel, biogas and bioethanol. Both pulp fibers production and seeds oil are suitable for biodiesel production. Over the centuries, the inflorescence pistils of *Cynara cardunculus* L. have been widely used for cheesemaking. The present chapter gives an overview of the *Cynara cardunculus* L. emphasizing recent knowledge regarding the use, conservation, preparation and application of *Cynara cardunculus* in ovine milk cheesemaking, as well as other biotechnological applications.

Keywords: cardoon, *Cynara cardunculus* L., pistilis, vegetable coagulant, cardosins, bioactive compounds, ovine milk cheese

1. Introduction

Cynara cardunculus L. is a herbaceous perennial diploid plant. It is the most widespread species of the genus *Cynara* and belongs to the Asteraceae family. Recent revisions about *Cynara* genus classification have sparked discussion over whether wild cardoon (*C. cardunculus* var. *sylvestris*), cultivated cardoon (*C. cardunculus* var. *altilis*) and globe artichoke (*C. cardunculus* var. *scolymus* L.) should be classified as different species, or as subspecies [1]. Studies about morphology and phytogeography of the *Cynara* genus supports that the referred plants belongs to a single species and should consequently be classified as subspecies [2–8]. Regardless the subspecies, cardoon is part of Mediterranean flora, distributed throughout the Mediterranean basin, Macaronesia (Madeira and Canary Islands), North Africa, Cyprus and Turkey [2, 9–11], is also a colonizer in Mexico, California, Argentina, Chile, Peru, Australia, China and West Africa [11–13]. Regarding artichoke, its production is worldwide disseminated, with great economic impact, especially in Italy, Spain, France and Turkey [12]. The production of cultivated cardoon seems to be more restricted to South Europe, namely in Spain, Italy, France, Greece and Portugal [13–17].

Wild cardoon grows spontaneously in marginal areas of field crops, pastures and along paths in dry areas and in soils of various characteristics. The plant, either cultivated or wild, can persist for a number of years, over 10 years, re-sprouting annually from its large perennial taproot [18, 19]. New seedlings usually germinate after the autumn rains, then first cotyledons emerge, growing slowly through in a rosette arrangement. Cardoon plants hold in the rosette stage during winter and early spring, when stalks start to elongate. As the flower stems develop, the lower rosette leaves begin to die off. Plants usually flower in the early summer, followed by the dieback of their aerial growth. Seedlings do not generally flower in their first year, as their energy is absorbed on the development of its deep taproot. New growth occurs with the autumn rains, and the cycle starts over [16].

Both plant agronomic characteristics and human selection of certain phenotypes, over the years, can possibly explain the use specificity, of each subspecies, for different purposes. *C. cardunculus* L. species lignocellulosic fraction shows great potential as solid biofuel [14], as well as for biogas production [20, 21] and bioethanol [22, 23]. *C. cardunculus* L. biomass productions can reach 14–20 t DW/ha per year, while according the studies of Pesce et al. [24], the wild cardoon accession was less productive in terms of biomass accumulation (11.8 t DW/ha per year) compared to cultivated forms (*Altilis* 41′ and 'Bianco avorio') producing, respectively, 19.1 t and 16.8 t DW/ha per year. Stalks and capitula of cultivated cardoon may also be used to produce pulp fibers [14, 25, 26]. In Southern Portugal, a region characterized by very hot and dry summers, a large scale cultivation of the cardoon *C. cardunculus* L. for biomass production was installed in a total of 77.4 ha. The field biomass yield was estimated at 7.5 t/ha, and the seed yield was 603 kg/ha after the second growing season [16].

C. cardunculus seed's oil fatty acid composition has also revealed a great potential for biodiesel production [14, 27]. *C. cardunculus* seed yield, and see oil composition is quite similar to sunflower oil. *Cynara* crop seed yield has been estimated at 1.36 t/ha per year [28], and a maximum seed oil content of 32.47% has been described by Curt et al. [29], while oil composition is on average 11% palmitic, 4% stearic, 25% oleic and 60% linoleic fatty acids.

Artichoke extracts make part of commercial dietary juices and capsules for digestion dysfunction treatment, being related with the bioactive extractives composition [30]. Nevertheless, cultivated cardoon leaves can be a good substitute for green forage during wintertime [14], and a great biomass source of the sesquiterpenic lactone cynaropicrin [31]. Moreover *C. cardunculus* L. presents diverse nutraceutical, pharmacologic and/or therapeutic properties [32–38].

In Portugal, *C. cardunculus* is known as "cardo de coalho" or "cardo hortense" [39, 40]. The richness of inflorescence pistils in aspartic proteases, named cardosins, has potentiated its wide use for cheesemaking for centuries [41]. Aqueous extracts of *C. cardunculus* pistils have been successfully employed for centuries in the manufacture of French, Italian, Spanish and Portuguese cheeses, and legally required to manufacture a number of protected designation of origin (PDO) cheeses in Portugal and Spain. The coagulant enzymes extracted from *C. cardunculus L.* flowers, cardosins, are aspartic proteinases, which have been assigned specific and technologic consequences, namely in sheep cheeses [42]. In cheesemaking, these enzymes show a similar action to other aspartic proteinases used in cheesemaking, such as chymosin. Cleaving the Phe105-Met106 binding of ovine and bovine κ-caseins [43–45] allowing the casein micelles aggregation by the milk ionic calcium. Cardosins reveal a more intense secondary proteolytic action on cheese α_s- and β-casein than other coagulants, with impact on the cheeses biochemical and sensory properties.

The most recent knowledge regarding the use, conservation, preparation and application of *Cynara cardunculus* plants in cheesemaking and others biotechnological applications are reviewed in the present chapter.

2. The species *Cynara cardunculus* L.

2.1. Origin and population structure

The taxonomy on the gender *Cynara* has evolved over time, and it seems to exist some confusion about the type of plants that fall under the designation of *C. cardunculus* L. species; even for the use in cheese manufacture, the descriptions of plants have been a little variable. In fact, the definition of the species that comprises the genus *Cynara* is somewhat complex, because some of them take different names, appearing eventually placed in other genera, with which there are affinities. For example, the number of species considered in the genus review of Wiklund [2] is eight, whereas the previous treatment of the same genus, in 1838, considered only three.

Illustrating the designations complexity or classification, Bailey and Bailey [46] refer to 10 Mediterranean species, 7 of which are referred to by Tutin et al. [47] as European: *C. scolymus*, *C. tournefortii*, *C. humilis*, *C. cardunculus*, *C. cornigera*, *C. alba* and *C. algarbiensis*. However, the species *C. humilis* is often identified as belonging to the genus *Bourgea* (*Bourgea humilis* L.) [2, 48] and *C. alba* corresponds, according to Valdes et al. [48], to the species *C. baetica*, also referred as belonging to the genus *Cirsium*. From this, one species, *Cirsium vulgare*, was used by Wiklund [2] as a test for its study of the genus *Cynara*. *C. scolymus* is referred to as spontaneously unknown by Tutin et al. [47] and Franco [49]. Moreover, Foury [3] distinguish only three species: (i) *C. cardunculus*, whose distribution coincides with that found by Wiklund [2]

for *C. cardunculus* spp. *flavescens* (the NW Mediterranean region, whereas *C. cardunculus* spp. *Cardunculus* generally occurs in regions with coastal influence, Macaronesia, Portugal, Central and NE Mediterranean distribution); (ii) *C. sibthorpiana* (Greece, Cyprus and Crete), designated by Tutin et al. [47] by *C. cornigera* and (iii) *C. syriaca*, in Palestine. Valdés et al. [48] distinguish *C. cardunculus* L., *C. humilis* L., *C. algarbiensis* and *C. baetica* in Andaluzia. Franco [49], in Portugal flora, refers to *C. scolymus*, *C. cardunculus*, *C. humilis*, *C. algarbiensis* and *C. tournefortii*, the last one being excluded of *Cynara* genus by Wiklund [2].

The recent revisions on *Cynara* genus classification have sparked discussion over whether the wild cardoon (*C. cardunculus* var. *sylvestris*), cultivated cardoon (*C. cardunculus* var. *altilis*) and globe artichoke (*C. cardunculus* var. *scolymus* L.) should be classified as different species, or as subspecies [50]. According to the results of Gatto et al. [12], the origin of these forms may have new scenarios: (i) the globe artichoke was domesticated a long time ago from wild material in Sicily/northern Africa; (ii) the leafy cardoon was derived from western Mediterranean (Portugal, Spain) wild cardoon and (iii) the eastern wild cardoon from Italy, Greece, Tunisia and Malta represents the only original wild form, that gave origin to both globe artichoke and cultivated cardoon. Furthermore, the leafy cardoon might have returned to wild forms, giving rise to the so-called western wild cardoon (Spain, Portugal). So, plant types considered before as different species or varieties, as *C. scolymus* L., cultivated for artichoke, and *C. cardunculus* L. var. *altilis* DC, known for its juicy young leaves [4], or *C. cardunculus* L. subsp. *cardunculus* and *C. cardunculus* L. subsp. *flavescens* [2], with differences on size, leaves and flowers of plants, and on spinyness of bracts, are considered now as coming from the perennial wild, *C. cardunculus* L. var. *sylvestris* (Lam.).

All these different forms of plants belong actually to a single species, *C. cardunculus* L. [4]. Studies about morphology and phytogeography of the *Cynara* genus support that the referred plants belong to a single species and should consequently be classified as subspecies [2–8]. The variability of characteristics within the species is a conclusion drawn by different authors [2, 3] and could be observed before in Portugal by Morbey [51], who collected plants of *C. cardunculus* prior to settle of experimental fields. However, despite the richness of wild cardoon germplasm, the identification and characterization of its genetic resources are scarcely investigated. In Portugal, *C. cardunculus* L. has an increasingly limited distribution, becoming restricted to certain areas where it has benefited of some care, even being sometimes cultivated [52].

Molecular data provided evidence that the western wild cardoon, the *C. cardunculus* L. subsp. *flavescens*, distributed in Spain and Portugal and characterized by more robust plants, is genetically closer related to cultivated cardoon, while the eastern wild cardoon, the *C. cardunculus L.* subsp. *cardunculus*, might be the progenitor of the globe artichoke [1, 7, 50], confirming the conclusions of recent revisions of *Cynara* genus. The two crops have possibly been derived from human pressure selection for either large non-spiny heads on one side, or non-spiny large stalked tender leaves on the other side [1, 5]. In the last decades, several molecular markers (RAPD, AFLP, SSR and ISSR) have been used for *C. cardunculus* population characterization. Among genetic markers available, the simple sequence repeats (SSR) are highly informative since they are codominant and generally highly polymorphic [53]. Recent studies have been conducted in Tunisia by Ben Ammar et al. [54] and Khaldi et al. [55], which revealed a large variability among wild cardoon populations.

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The agronomic plant characteristics, combined with human selection over the years, possibly explain the specificity of the use of each subspecies for each different purpose. Different parts of the plant, such as leaves and inflorescences, with high relevant biochemical profiles, are used as food providing the selection for larger, tender and non-spiny plants [12, 56].

2.2. Historical and etymological archives

Throughout history, cardoon use had curious applications including torment weapon, confectionery, medicine, besides its role as a coagulant in cheesemaking. According to Barreira [57], the reference of cardoon in the Bible is associated with "torment" or "suffering", as, for example, (i) "*But Jehoash king of Israel replied to Amaziah king of Judah: A thistle in Lebanon sent a message to a cedar in Lebanon, 'Give your daughter to my son in marriage.' Then a wild beast in Lebanon came along and trampled the thistle underfoot"* (2 Chronicles 25:18); (ii) "*It will produce thorns and thistles for you, and you will eat the plants of the field"* (Genesis 3:18); (iii) "*But land that produces thorns and thistles is worthless and is in danger of being cursed. In the end it will be burned"* (Hebrews 6:8).

Columella [58], in the treatise "*De Re rustica"* (1st century BC), mentioned some interesting references about the use of cardoon as a milk coagulant, for example, "*It will be necessary too not to neglect the task of cheese-making, especially in distant parts of the country, where it is not convenient to take milk to the market in pails. Further, if the cheese is made of a thin consistency, it must be sold as quickly as possible while it is still fresh and retains its moisture; if, however, it is of a rich and thick consistency, it bears being kept for a longer period. Cheese should be made of pure milk which is as fresh as possible, for if it is left to stand or mixed with water, it quickly turns sour. It should usually be curdled with rennet obtained from a lamb or a kid, though it can also be coagulated with the flower of the wild thistle or the seeds of the safflower, and equally well with the liquid which flows from a fig-tree if you make an incision in the bark while it is still green*.".

The only treatise on the ancient gastronomy that is known nowadays is "*Artis magiricoe libri X*", also known as "*De Re coquinaria*" written by Apicio in the first century AD [4]. In this treatise some recipes using cardoon can be found in Chapter XIX of Book III [5], as, *for example, "CARDUI ("cardoons"): thistles are eaten with a salty dressing, olive oil, and hard-boiled eggs"* (adapted)*; "ALITER CARDUI ("other cardoons"): rind, mint, coriander, finely chopped fennel, pepper, levis and salt water and olive oil"* (adapted)*; "ALITER CARDUOS ELIXOS ("other cooked cardoons"): served with pepper, cumin, sauce and olive oil"* (adapted). In addition, in Spanish manuscripts of fifteenth century [59], it is possible to find a recipe of candied *C. cardunculus* describing that the ideal months for *C. cardunculus* picking are April/May, when the stalks are more tender and sweet. The process of confection begins with stalk cutting, then rinsed in cold clear water for 1 or 2 days, followed by cooking in clear water and ending in a process of soaking in a syrup of sugar and water. Later, in the seventeenth century, the use of *C. cardunculus* is also mentioned in the elaboration of dishes along with butter, onion, pepper, nutmeg, ginger, eggs, among other ingredients, like milk clot (e.g., "*Almojavanas de quajada*"). The first cookbook printed in Portugal, known as *"Art of Cooking divided in two parts"* (from the original *"Arte de Cozinha dividida em duas partes"*) brought up some references of recipes of the banquets to be served during April, with dishes using cardoon such as "*Cardoon curdled with eggs*", "*Bundle of cardoon garnished with the same cardoon*" and "*Italian style cardoon garnished with cream*". Also in

medicine, several references on the use of cardoon can be found in treatises, such as "*Pedacio Dioscorides anazarbeo, Acerca de la materia medicinal y de los venenos mortiferos traduzido de lengua griega en la vulgar castellana & illustrado con claras y substantiales annotationes, y con las figuras de innumeras plantas exquisitas y raras*". This work consists of the translation from Greek to Castilian of the treatise *De Materia Medica*, by Pedanius Dioscorides (first century BC) with the inclusion of personal annotations. In Chapter XIII is included the description of the benefits of cardoon on the stomach, liver, bladder and kidneys, but also in the prevention of the bad odors from the human body.

As in a previous treatise [58], several ways of preparing and consuming cardoon are reported, especially using young and tender plants, like the preparation using wine, salt and pepper. Andres Laguna [60] differentiates the "cardoon" from the "artichoke" referring to the latter on as "lush" for which reason should be placed in the list of foods recommended to the bride and groom, however can be used as milk coagulant. A manuscript about the cheesemaking process in the Alentejo region, reports different ways of preparing *C. cardunculus* aqueous extracts [61]. The author also points out that the consumption of cardoon can lead to food craving, and mentions a method for growing cardoon: "*The truth is that planting them* [the thistles], *covering all them with earth, makes them more white, more tender and tastier, like they were from a different species*" [60]. This description is in line where Priest Isidoro de Barreira [57], who, referring to the biblical meaning of the cardoon, describes: "(…) *that* [torment] *which he suffers* [thistle] *before being suitable for eating*: (…) *when they bind him, and cover him with earth, in which will be mortified to lose its bitterness*." Cardoon crops are present in paintings from the beginning of the seventeenth century by Caravaggio and Juan Sanchez Cótan [50]. Later during the seventeenth century, de Cabreira [62] included the use of "holy thistle" in the treatment of sores in his compendium of medicines for surgery.

2.3. The natural growth cycle of *Cynara cardunculus* L.

C. cardunculus, as other Mediterranean species, is a plant fully adapted to the local climate conditions, where raining is low, irregular and mainly concentrated in autumn/winter. On the other hand, the hot dry summers are not favorable for plant growth, especially if no irrigation is used. The first stage of *C. cardunculus* growing cycle initiates after seeds germinate, usually in the first weeks of autumn. In this stage, two fresh cotyledons arise from the ground, followed by several leaves and, later, a leaf rosette. This rosette usually grows in a slow but steady manner and, the *Cynara* spp. may take all wintertime to early spring at this rosette stage. By late spring, *Cynara* spp. develops a leaf-branched floral scape including several heads. After full blossom and flower fertilization, the fruits ripen and finally the aerial biomass dries up in the summer. When the weather conditions become milder the perennating buds on the basal plant part sprout and a new development cycle starts. This succession of annual growth cycles may last several years [14].

The cultivation as an industrial crop for industrial application of *Cynara* spp. resembles most of the stages of the natural growth reported before, that is, as a perennial field crop in dry farming. As a perennial crop, and with a very deep plant root system, a basal dressing before sowing is recommended. After subsoiling and plowing are recommended, a thorough harrowing should

be followed. The seed germination occurs when the soils water content and the environmental temperature are favorable, normally in the autumn or spring of Mediterranean climates. After this initial growth cycle, the aerial biomass production is usually lower due to the larger development of the root system, but can increase in the next growth cycles, depending on the environmental conditions. In the case of a cold autumn (early frosts), spring sowing may constitute a better option. It is advisable to accomplish the spring sowing as soon as the period of frosts is over. Usually, plants will reach summertime in the rosette stage; after, and due to the high temperatures, some leaves dry up. Later, when the environmental conditions are milder, *Cynara* spp. resumes its vegetative growth, and the size of the leaf rosette becomes bigger.

After, the growing process of the plant can be considered finished in the next summer and *Cynara* spp. can be harvested [14, 26]. Harvest process should be performed after the conclusion of the plants growing cycle,but before seed dispersal. The aerial biomass of *Cynara* spp. should be dry (less than 15% moisture) and seeds must be ripe. The research and development of proper machinery for harvesting *Cynara* spp. plantations is under process aiming for harvesting the whole biomass in one operation.

2.4. Harvest and conservation of *Cynara cardunculus* L. flower

C. cardunculus adult plant can reach a height of 3 m, spread over an area of 1.5 m in diameter [16], and can contain 15 inflorescences, 7 in average [16]. These inflorescences do not open all at the same time because they have different maturation states.

The flower harvest is performed through a cut, and should be done when the inflorescences are mature and open. To obtain high-quality flower with a minimum of impurities, like straw, the pistils should be collected as high as possible on the plant. It is empirically and generally accepted that the more blue-purple the collected material is, the more value it has for traditional cheesemaking [16], concerning at least the milk clotting activity. The harvest of the flower is usually done between the end of June and the beginning of July [51, 63], depending on the year and on the varieties; it is usually performed manually, with a bucket with two hooks where the inflorescence fits. The scissors used by the pickers are long and very sharp, being able to collect all of the flower at once. There are some recent developments regarding mechanical harvesting attempts, but so far, there is no specific device commercially available.

The traditional preservation process for cardoon flower is at room temperature (25–30°C) with air dehydration for about 30–60 days [67]. The purple parts of the cardoon flower (styles and stigmas) are collected along the flowering season and placed to dry at room temperature, protected from sunlight, and with regular turnings of the material to prevent unwanted fermentations and fungi growth [51, 64]. The drying process can decrease the coagulant activity [65]. In spite the fact that average flower milk clotting activity (MCA)/g of dried, and non-dried flower was similar, the authors refer to losses of milk clotting activity varying from 20 to 50% when expressed on dry basis or nitrogen (N) total basis. The traditional drying process, used to preserve the flower throughout the cheese production season, tends to standardize the flower composition and coagulant activity and although carried out at low temperature leads to high losses of flower enzymatic activity as measured by coagulant activity. The evaluation of the amount of these losses, together with the decline in clotting activity during conservation,

was estimated to be about 75% of the potential enzymatic coagulant available in the flower expressed per unit of dry matter [66]. Reducing exposure time to traditional drying conditions may limit such losses, and it is possible to use dehydration at higher temperatures while shortening the drying time, with MCA losses still lower than those with traditional drying.

Martins [66] also studied the effect of cardoon flower drying under different conditions (25–30°C for 7 days, 50°C for 5 days, and 100°C for 5 h). The author concluded that when compared to traditional drying process (25–30°C for 30 days), MCA average was significantly higher drying only for 7 days (MCA in dry matter about 35% higher). MCA of dried flower at 55°C for 5 days in a dry basis, was about 17% higher, while drying at 100°C for 5 h caused a loss for flower MCA of about 5% in a dry basis. The moisture content of the dried flower at 25–30°C for 7 days, about 6% (w/w), was similar to that of traditionally dried flower, showing average water activity (a_w) of 0.585. This means that by controlling the moisture content throughout, the drying period can be decreased, and thus the MCA can be significantly preserved if adequate storage conditions are initially respected. Although there are always losses related to flower storage, the dried flower remains well preserved until next harvest period under conditions of reduced relative humidity and at room temperature. After 300 days of dry flower storage at 25°C, Martins [66] found MCA losses of about 35% of the original MCA, near the MCA losses after storage at 4°C for 150 days.

3. *Cynara cardunculus* L. cheesemaking applications

In most Mediterranean countries, Asia and Africa the milk from small ruminants (goat and sheep) is widely used for human consumption, or mainly processed into typical cheeses by traditional manufacture methods, in contrast to most Anglo-Saxon and Northern European countries, where small ruminants meat production is the only purpose [67]. Although, the amount of milk produced per ewe or goat is extremely variable, depending on the geographical location and prevailing weather conditions, and the farming system, a marginal farm where ewes are milking after weaning the young, in contrast to a dairy farm, where milking occur during whole lactation period [68, 69].

Milk and cheese production from small ruminants in Mediterranean countries has a great socio-economic relevance, mainly in rural areas. In Portugal, according to the latest published statistics [70], sheep milk production reached 68.6×10^6 L/year, and goat milk 25.6×10^6 L/year, and almost all is used for cheesemaking, recording 11,400 and 2400 tons of sheep and goat cheese, respectively.

Specific sensory characteristics of ewe and goat cheeses are related with the chemical composition of raw ewe and goat milk, the coagulant enzymes, raw milk microbiota or milk inoculation with autochthonous strains, and some distinctive manufacturing cheese practices [71]. Traditionally, pistils of wild and cultivated cardoons are used to produce several traditional ewe's and goat 's cheeses, namely Serra da Estrela, Serpa, Nisa, Azeitão and Évora in Portugal [72]; La Serena, Los Pedroches, Torta del Casar, Los Ibores and Flor de Guía in Spain [42, 65, 71, 73–80] and Caciofiore, Fiore sardo, Cacio Fiore and Cacioricotta cheeses in Italy [81]. Some

of these cheeses benefit from protected designation of origin (PDO) status in which *C. cardunculus* flower aqueous extracts have been successfully employed and legally required (**Table 1**).

3.1. *Cynara cardunculus* L. flower aqueous extracts

With the exception of some standardized formulas recently commercially available, the general preparation of cardoon flower extracts for cheesemaking use, remains generally as described in very old references. Coagulating enzymes are extracted from dry flower (styles and stigmas) on a day-to-day basis [83]. The required amount of cardoon flower is placed in bottled water infusion during a variable period of time, being carefully macerated and ground with water, usually in a mortar. The mixture is then filtered, obtaining a purplish or brown liquid which is added to the milk [71, 80].

This traditional use is based on the observation of previous clotting times, and when appropriate, compensation for any loss of enzyme activity coagulant is made by correcting the amount of flower applied per liter of milk as a correction given the empirical use of the coagulant. Control of extracts coagulant activity is performed by controlling the amount of flower necessary to the milk batch volume, frequently equivalent to 0.2–0.6 g flower/L milk [84].

So, the cardoon extract is an aqueous extract of edible parts of flowers from plants, prepared with a variable proportion of bottled water, sometimes with some salt addition during maceration for flower proteinases extraction, therefore usually not standardized. With the maceration in a mortar, with a 5% sodium chloride solution and sand added as an abrasive

Cheese	Country	Type of milk	Coagulant (reference in law)
Mestiço de Tololosa	Portugal	Ewe and goat	Animal Rennet or *Cynara cardunculus*
de Nisa	Portugal	Ewe	*Cynara cardunculus*
de Castelo Branco	Portugal	Ewe	*Cynara cardunculus*
de Évora	Portugal	Ewe	*Cynara cardunculus*
Serpa	Portugal	Ewe	*Cynara cardunculus*
de Azeitão	Portugal	Ewe	*Cynara cardunculus*
Serra da Estrela	Portugal	Ewe	*Cynara cardunculus*
La Serena	Spain	Ewe	*Cynara cardunculus*
Torta del Casar	Spain	Ewe	*Cynara cardunculus*
Flor de Guía	Spain	Ewe, goat and cow	*Cynara cardunculus* or *Cynara scolymus*
Media Flor de Guía	Spain/Gran Canaria Island	Ewe, goat and cow	≥50% *Cynara cardunculus* or *Cynara scolymus* ≤50% animal rennet
Guía	Spain	Ewe, goat and cow	*Cynara cardunculus* or *Cynara scolymus* or animal rennet

Table 1. PDO cheeses made with aqueous extract of ***Cynara cardunculus*** L. in Portugal and Spain [82].

agent, followed by infusion over 2 days under agitation, Christen and Virasoro [85] managed to obtain almost the maximum flower coagulant activity. With a similar extraction process, Tsouli [86] concluded that the sodium chloride concentration of the extracting solution does not influence the enzyme extraction; however, it is necessary to ensure a minimum ionic strength since less active solutions are obtained with water. Perhaps the ionic strength is not so important in this type of extraction, since extraction follow the destruction of the vegetal tissues, although not complete. On the other hand, the results of Tsouli [86] support the use of warm water for extraction. The author obtained more effective extractions at temperatures of 23°C than at 4°C, although very long extractions at higher temperatures may lead to the development of molds; high salt concentrations may have a favorable effect, acting as inhibitors of microbial growth in the extracts.

Regarding more intrusive destructive processes, Sousa and Malcata [87] obtained effective extractions with less than 1 min flower mill time, an extremely destructive process; the optimum pH for extraction was 5.9 (similar to water), being salt concentration and homogenization time not relevant parameters for extraction efficiency. Martins [66] found that traditional extraction using mortar maceration produced extracts with MCA 3 times greater than with ultrasonic extraction, and about 1.5 times higher than with an high speed blender, using the same extracting solution (5% NaCl solution) and same temperature extraction. However, the latter process is more practical for the preparation of large quantities of coagulant and is widely used in dairies, both in Portugal and in Spain.

The purple-brown liquid used for milk coagulation is frequently contaminated with other flower components not relevant for cheesemaking, for example, phenolic compounds, which may affect the enzymatic activity and even hinder any purification and concentration processes [88, 89]. This liquid can be preserved to further use at 4°C, although some losses on MCA where noticed from different authors. Tavaria [9, 90] obtained a decrease in extract coagulant activity (65%), after 4 weeks stored at 4°C, lower than for the same lyophilized extracts (34 and 38%, respectively, as extraction was prepared with water, or citrate buffer at pH 5.4). Although, after 1 week the activity losses were reversed, 23% for fresh extracts and 44 and 61% for the lyophilizates, attributed to a spontaneous loss of the catalytically active conformation over time. The proteolytic activity of lyophilized extracts tended to decrease with shelf life and with lyophilization, mainly due to the lower degradation of alpha-casein and also beta-casein, which led to an increase in the coagulant activity/proteolytic activity ratio. Martins [66] also obtained MCA losses of aqueous extracts, from about 27 to 40%, over a 90 day storage period at 4°C. The results revealed similar losses to those obtained for flower conservation, which indicates the possibility to consider extracts preservation at 4°C, allowing the availability of liquid standard solutions for use in cheesemaking. As with other cheesemaking coagulants, according to the same author, finding suitable formulations for better preservation will enhance the exploitation of the cardoon coagulant and its proteolytic potential.

Obviously, there are small variations which are adaptations designed to meet the latest hygienic requirements, or to solve difficulties in cheese manufacture originated by the evolution of production systems. In fact, some different ways to prepare cardoon aqueous extracts are described [71].

The traditional method of preparing coagulant extracts from *C. cardunculus L.* dried flower is so far mandatory for the manufacture of some Portuguese cheeses with PDO [84], the same occurring for some spanish PDO cheeses [42], where it is also possible to use powder vegetable coagulant [91]. Standardized solutions of these vegetable proteinases can be obtained in the market for the production of fresh and ripened sheep's milk cheeses in Portugal and Spain, whereas other preparations of proteases from *C. cardunculus* have been recently approved in Canada and accepted in different countries for use in cheesemaking [92].

3.2. *Cynara cardunculus* L. proteases as milk clotting enzymes in cheesemaking

The cardoon extract used in traditional cheesemaking is an aqueous extract of edible parts from *C. cardunculus* L. flower, especially the styles and stigmas. The final crude extract contains a mixture of acid or aspartic proteinases (endopeptidases), the same type of enzymes used for cheesemaking, like chymosin, pepsin and some other proteases from microbial origin. These proteinase combinations allow the milk casein micelles destabilization, and their subsequent coagulation by micellar aggregation using the milk ionic calcium, the most common milk coagulation process for cheesemaking.

Aspartic proteinases are widely distributed in nature, and have been extensively detected and isolated in seeds, leaves and flowers in different plants [93–95] . These enzymes are assigned important functions in animal biological systems, namely protein degradation (pepsin, chymosin and cathepsin D) or blood pressure regulation (renin), among many others, but their biological functions in plants are not yet clear, remaining still as hypotheses [94, 95]. In general, they have been involved in protein processing or degradation in different plant organs, pollen-pistil interaction, as well as in plant senescence, stress responses, programmed cell death and reproduction [94–96].

Several aspartic proteinases have been identified in *C. cardunculus* L. flower extracts, which have been assigned different names, cardosins A–H [41, 96, 97], cyprosins 1–3 [98], cynarase [93, 99], which are generally designated at MEROPS database by Phytepsin, ID A01.020 (Clan AA, Family A1), belonging to the IUBMB group EC 3.4.23, namely EC 3.4.23.40 with the chemical name of phytepsins, including cardosins and cyprosins.

C. cardunculus pistils are known to express several aspartic proteinases; the latest studies bring the total number of aspartic proteinases isolated from pistils of *C. cardunculus* L. to nine, one of the highest numbers of aspartic proteinases isolated from a single organism, suggesting important and specific biological functions within *C. cardunculus* [97]. This multiplicity in pistils is unusual since most plant aspartic proteinases described were mainly isolated from seeds or leaves, at lower concentrations [100]. In this species, the presence of these enzymes is restricted to the pistil, namely in the violet fraction, corresponding to the stigma and stylet, or to the upper part of it [95, 96, 98]. The continuous accumulation of aspartic proteinases increases during flower development, and maturation [41, 98, 99]; Veríssimo et al. [94] reports that the enzymatic complex concentrates mostly within stigmas, and can constitute more than 60% of the total protein of mature stigmas, which, according to the authors, constitutes the first example of extremely high levels of aspartic proteases in higher plants.

These enzymes are characterized, in mature form, by having a tertiary structure with two heterodimeric lobes or domains, two glycosylated subunits with different molecular weight (30 and 15 kDa, variable, depending on the different enzymes which have been identified in the enzyme complex), between which is located a large cleft where the catalytic aspartic centers are located [93–95]. The proteases are inhibited by pepstatin, and shown to be active at acidic pH, with increased proteolytic activity at pH values of about 4.5–5.5 [94, 97, 101–103]. A characteristic feature, of the majority of plant aspartic proteinase precursors, is the presence of an extra segment of about 100 amino acids, known as the plant-specific insert, which is usually removed during processing and is absent from the mature form of the enzyme [95], bearing no sequence similarity with aspartic proteinases of mammalian or microbial origins [94]. As the majority of coagulating enzymes used in cheese manufacture, aspartic proteinases from cardoon flower crude extract reveal primary affinity for breaking the link Phe105-Met106 of κ-casein, an action that triggers enzymatic milk coagulation process, having a subsequent action on the α_s- and β-casein, with preferential affinity for peptide bonds involving hydrophobic amino acids [41, 44, 97].

Aside from the primary role of cardoon flower extract in cheese manufacturing, as milk clotting agent, there are other important actions during draining and pressing steps, and, in particular, throughout the ripening phase, which have impact in the final product characteristics, depending on the technologies and therefore on the cheese type. After milk preparation, cardoon flower extract is added and dispersed homogeneously by stirring, as all other enzyme coagulant type, after which the milk is left to stand for a gel formation. The extract aspartic proteinases have as primary action the cleavage of the κ-casein Phe105-Met106 link, which triggers the destabilization of the milk micellar casein structure [104, 105]. This reaction allows the calcium sensitive α_s- and β-casein to gradually aggregate, forming a progressively structured protein mesh in which the fat, and other components of milk are retained, the curd.

The cheese production proceeds acting differently on the curd to produce different types of cheese. After milk coagulation, the next phase is whey draining, through various operations, which vary with the cheese type to be manufactured. Although most of the added coagulation enzymes are lost through whey draining, as with other aspartic proteases used in cheese production, residual proteinases, added via cardoon flower extract, remain in cheese. This residual fraction plays, however, an important role in defining cheese properties, and typical characteristics through proteolytic action on casein fraction after coagulation. The residual proteinases influence the progress of proteolysis along cheese ripening, which takes place at lower temperatures (8–16°C). Thus, cardoon flower extract plays a complementary role, which is essential for cheese properties being assigned to specific and technologically essential consequences, such as particular textures in milk sheep cheeses, which will be considered on Section 3.4.

With a good milk quality, the endogenous factors do not cause any inactivation of clotting action of the cardoon crude extract proteases. The ideal temperature of the clotting enzymes depends on the technological temperature profile, and not on enzymes temperature sensitivity, since they reveal proteolytic activity within the temperature range normally used in coagulation phase of cheese manufacture (28–36°C). For cardoon flower extracts, the cheesemaking temperature is limited by lower minimum temperature necessary for the micellar aggregation (about 20°C), being the upper limit dependent on the protease inactivation

temperature (60–70°C), substantially higher than the chymosin inactivation temperature [66, 80], allowing higher cheesemaking temperatures as those used in the manufacture of some traditional fresh white Portuguese cheeses.

Vieira de Sá and Barbosa [80, 106], at pH 6.6, report a significant increase in coagulant activity to about 50°C, increasing them slower up to 70°C, where the activity is maximal, followed by a sharp decrease above this temperature and disappearing at 75°C. Christen and Virasoso [85, 107] referred before a maximum of activity to 68°C, well above 41°C for animal rennet, while Campos et al. [108] observed coagulation difficulties at 20°C and a rapid increase in coagulant activity up to a maximum in the range of 40–60°C, from which activity is lost due to protein denaturation. For *C. humilis*, Martinez and Esteban [109] found similar results, with the coagulant activity remaining around 75°C, decreasing quickly thereafter; for the different enzymes studied, this profile was only surpassed by papain, which remained active until the highest temperature used (80°C).

The temperature effect is more crucial during micellar aggregation than in the primary (enzymatic) coagulation phase. While at low temperatures the enzyme phase becomes slower, the micellar aggregation phase hardly occurs at temperatures below 20°C; at a lower temperature, we can perform the primary coagulation phase without causing the milk to coagulate [110, 111]. Using the Optigraph to study the effect of different technological factors, Alves et al. [112] concluded that milk flocculation time, after cardoon flower extract addition, seemed to be more influenced by temperature than rennet, especially near the limits of the temperature range used in milk clotting for cheesemaking (30–36°C) made from enzymatic coagulation, although these differences were smaller in sheep's milk compared with cow's milk coagulation. However, the micellar aggregation rate increased more with increasing temperature with the cardoon extract than with rennet; from 26 to 34°C, even though the impact on the flocculation time is lower. This means that the use of lower coagulation temperatures, below 30°C, does not benefit the manufacturing technology, and may even create difficulties in draining and subsequent problems during ripening by the instability created in the inner cheese ripening conditions [111].

The milk pH, an important milk property depending on its composition and preservation, is one of the factors that most influence the coagulation in its different phases, primary or enzymatic phase, micellar aggregation and gel firming and syneresis [113]. Modification of milk pH before coagulation is often used as a standardization process in the cheese industry but in traditional technologies the milk pH variability, which originates from the milk composition or preservation, can explain much of the milk behavior within coagulation, which usually leads to problems in the syneresis and draining and, later, in cheese ripening; the aforementioned heterogeneity in traditional cheeses may even have its origin in this fact [111].

The lowering of pH tends to decrease coagulation time by approaching the optimum pH for the proteolytic activity of coagulating agents such as aspartic proteinases of rennet, or *C. cardunculus* flower extracts, whereas above pH 7 there is no coagulation due to the rapid inactivation of rennet enzymes [110, 114]. Likewise, the gel is firmer at a lower pH, favoring the micelle aggregation reaction by decreasing the micelle stability, coupled to the negative charge neutralization and the release of calcium ions from the dissolved complexes and the colloidal phase [110, 114, 115].

The decrease in pH accelerates the enzymatic action and the micellar aggregation [111, 112, 116]; the milk with a pH higher than 6.7 is slow to coagulate, and the gel firmness is affected while milk of pH lower than 6.6 show rapid coagulations and the gel firmness is higher and reached faster [111, 112, 117]. Milk that naturally provide weaker curds suffer more with lowering the pH; in goat's milk with very low pH (6.3–6.4) the curd spontaneously breaks and it becomes very difficult to control the characteristics of curd and fresh cheese [111, 118]. When comparing the effect of pH on the rennet and the cardoon flower extract coagulant activity, the main difference in cow's milk is that the former is more effective mainly at lower pH, although the differences between coagulants are less evident for pH values as low as 5.8 [80, 106]. However, with sheep's milk, the cardoon flower extract is more effective than the rennet for all the pH levels studied by these authors, with differences between coagulants much more pronounced than with cow's milk. Similar results were obtained by Martinez and Estebán [109] for extracts of *C. humilis*, which showed that, for the common pH range for sheep's milk, the coagulant action of vegetable coagulant extracts is less affected than the rennet activity. The authors concluded that the coagulants of animal origin are more sensitive to the milk pH variability, and may reveal some difficulties in the coagulation of sheep milk with high pH (6.7–6.8), as it frequently happens.

The micellar aggregation phase of milk enzyme coagulation is a set of reactions dependent on milk composition in terms of protein and mineral elements, in particular calcium in ionic form, that is, a set of reactions not directly dependent on the coagulating enzymes. However, the type of coagulant influences it indirectly through the proteolytic action exerted on the protein destabilized by the coagulant primary action, interfering with the speed and the firmness of the gel. In fact, the coagulants play an important role in the definition of the characteristics of the curds [115], in conjugation with the more intense proteolytic action that is recognized for the enzymes of the *C. cardunculus* flower extracts. In fact, several authors pointed out a significantly higher proteolytic activity/coagulant activity ratio (P/C ratio) for cardoon extract enzymes compared to chymosin, or even rennet, which includes a percentage of pepsin, although this effect is less evident in cow's milk when compared to what happens in ewe's milk [119]. Alves et al. [112] observed that micellar aggregation rate and gel firmness were lower for milk clotted with cardoon flower extract when compared to rennet coagulation and attributed some of this behavior to the characteristic higher non-specific proteolytic activity of proteases of *C. cardunculus* L. flower, since the micellar aggregation phase is not directly influenced by the coagulant enzymes.

The effect of calcium in the enzymatic coagulation for cheesemaking is well known and it is considered an important technological factor; it is essential for micellar aggregation, especially its Ca^{2+} ionic form, whose proportion depends on milk pH [110, 114, 120]. For this reason, calcium chloride addition to cow's milk, is a common practice in the cheesemaking industry, as an attempt to optimize both the cheese yield, and the curd properties [115, 121], assuming that the milk does not provide the required amount of calcium. It is generally considered that this need does not apply to milk from small ruminants [122], but this may not be true nowadays considering the intensification of sheep and goat milk production. In cow's milk coagulation with cardoon flower extract, Alves [116] and Alves et al. [112], using Optigraph, concluded that gel firmness tends to increase with the addition of calcium, albeit on a lower scale than that for the rennet, which can be attributed to the conjugate effect of calcium chloride addition and the higher non-specific proteolytic activity of the cardoon flower enzymes. In accordance with Vieira De Sá and Barbosa [80], the same authors also concluded that calcium chloride

addition decreased milk coagulation time with cardoon extract and rennet, but from a calcium chloride addition of 0.06%, the difference in the coagulation time from both coagulants almost disappeared, as pointed out by Martinez and Estebán [109] for *C. humilis* and pepsin. In fact, since calcium does not participate directly in the primary (enzymatic) coagulation phase, the decrease in coagulation time is due to a decrease in milk pH, which as mentioned, influences coagulation time [115, 123, 124].

Finally, concerning the traditional cheesemaking technologies, the addition of salt to milk must also be considered as an additional technological factor, since it is a practice present in some cases, as in the Portuguese cheeses of Azeitão, Serpa and Serra da Estrela [84], all of them made with cardoon flower extract as the coagulant agent. The addition of sodium chloride causes a decrease in milk pH, resulting in calcium and phosphorus solubilization. As a consequence, the curd tends to form slowly, and the syneresis is considerably inhibited, contributing to a retention of whey in the curd [125–127]. Although favored by a slight increase in ionic strength, the enzymatic reaction is affected by its excessive increase, despite the pH decrease [114], with animal rennet being less affected than some coagulants of microbial origin [128]. In the manufacture of Azeitão cheese the salting in milk is performed by the addition of 15–25 g salt/L, which can decrease the milk pH in 0.2–0.4 units [129]. Alves et al. [112] found that rennet was clearly inhibited by the addition of salt to milk, whereas for cardoon extract coagulation time and gel firmness remained almost unaffected. Cow and goat milks are very sensitive to salting in milk, but sheep milk shows a greater resistance to this effect, starting from a gel firmness characteristically superior, which is based on the content and type of caseins of this milk type [111].

Despite the studies already done and the knowledge available on the properties of the cardoon flower enzymes, the enzymatic content of the extracts and the effect of the flower variability/enzymatic profile is not fully understood, and the use of cardoon flower extracts still remains somewhat empirical, without any kind of standardization or evaluation of coagulant solutions [65]. However, in recent years, there has been some effort toward the availability of aspartic proteinases or extracts from *C. cardunculus* with guaranteed efficiency in the manufacture of cheese. A laboratory in Spain has been selling a coagulant extract of plant origin for a number of years, claimed to be obtained from *C. cardunculus*. Almeida et al. [130, 131] have developed and characterized a new cardosin B-derived coagulant produced in the generally regarded as safe (GRAS) yeast *K. lactis*, which they claim to be effective in the manufacture of cheese from milk of different species. In 2015, a cyprosin was claimed to be authorized for use in Portugal, Spain, France and the Netherlands, and joined the list of authorized food enzymes in Canada, after favorable decision from Health Canada's Food Directorate [92], and since 2015 a number of applications by different entities for the inclusion of cardoon enzymes or extracts in the European list of food enzymes in accordance with Regulation (EC) No 1331/2008 are waiting for official approval.

3.3. Effect of *Cynara cardunculus* L. on physicochemical, texture and sensory cheese properties

Cheese maturation is a dynamic process, in which many metabolites, resulting from primary degradation act as substrate for secondary reactions [132, 133]. Proteolysis, the main biochemical process that occurs during cheese maturation [111, 134], has a central role in cheese texture development [42], and is usually divided in primary and secondary proteolysis.

Proteolysis is translated by the hydrolysis of the Phe105-Met106 binding of k-casein, which leads to the formation of para-κ-casein and glycomacropeptide, causing micellar destabilization, which results in the existence of a more hydrophilic part of k-casein [104, 132, 135]. Most of the glycomacropeptide is eliminated to the whey, but the para-κ-casein remains in the casein micelles and is therefore incorporated into the cheese [132]. The residual coagulant agent, as well as plasmids present in the curd, will act on α- and β-caseins, giving rise to insoluble and water-soluble fractions, which are high and medium molecular weight peptides [74, 135, 136] (primary proteolysis). These are then degraded into small peptides and amino acids [136, 137] by proteases, and from starter or secondary cultures (secondary proteolysis), which will subsequently contribute to cheese flavor formation [43, 74, 136].

Therefore, it seems obvious that the residual cardoon enzyme fraction remaining in cheese after whey draining should play a specific and technologically important role, promoting particular cheese properties, like textures (softening) in milk sheep cheeses [74, 138]. Vasconcelos et al. [129] studied the Azeitão cheese, having established the basis for its definition and certification as a PDO, and found that the cardoon is the main factor of the typicality of this cheese, and based in detailed studies on different sheep microflora and the milk cheeses whose technology include cardoon as coagulant, many authors concluded by the unique properties resulting from the technological use of the vegetable extracts.

The type of coagulant agent is one of the main factors responsible for the variability in cheese characteristics, being therefore its effect on the proteolysis a subject of profound study. In general, differences in coagulant action on protein can affect the concentration of α-casein and β-casein, and influence the concentration of degradation product, γ-caseins and para-α_s-caseins, which in turn contribute for cheese properties. Thus, several studies have been performed in order to evaluate the influence of different preparations, of the amount of *Cynara* aqueous extract, in comparing it with other coagulating agents or the possibility to use *C. cardunculus* enzymes for cheese ripening acceleration.

Roa et al. [73] demonstrated that in La Serene cheeses the residual coagulant of *C. cardunculus* in cheese, and whey are, respectively, 27 and 78%, of the total amount added to milk. In this type of cheese, cardoon flower extract proteinases play an important complementary role for cheese properties; cheeses made with cardoon extract showed a more intense secondary proteolysis than those made with rennet or microbial coagulants, with hydrolysis of α_s- and β-casein up to 82 and 76% higher, respectively.

In Torta del Casar, Delgado et al. [74] indicate, in general, a weak proteolysis at the first 30 days of ripening, more intense between 30 and 60 days, but without differences between 60 and 90 days of ripening. For this type of cheese, the results show a slow degradation of α_{s1}-casein in the first 30 days in contrast with a high degradation level between day 30 and day 60 of ripening. Unlike α_{s1}-casein, β-casein showed the fastest degradation levels in the first 30 days of maturation and after this time it was slight and constant up to the end of ripening. At 90 days of ripening, the degradation of α_{s1}-case was higher than β-casein, which demonstrated a lower proteolysis level, reaching 38% of the initial level at 90 days maturation. For Serena cheeses, Roa et al. [73] reported a similar proteolysis pattern along ripening period, with a higher proteolysis level of β-casein than for α-casein during the first 30 days of ripening

(percentage of degradation of β1-, β2- and α_{s1}-casein was 41, 58 and 9%, respectively). This rate of casein matrix is associated to cheese texture variation along maturation, decreasing the hardness and consistency of cheeses and increasing their adhesiveness [73].

The proteolysis pattern in cardoon flower cheeses seems to confirm the suggestions that proteinases from *C. cardunculus* displayed a stronger preference for peptide bonds between bulky hydrophobic amino acid residues than chymosin [44, 104]. The use of raw ewe milk and plant coagulant provides these cheeses a spreadable texture, and a peculiar slight bitter taste. The slight bitter flavor of Torta del Casar cheese may be due to certain sequences in the caseins which are particularly hydrophobic and, when excised by proteinases can lead to bitterness [139]. Nevertheless, others studies show that, while α-casein decreased throughout ripening, β-casein only decreased slightly, confirming its greater resistance to hydrolysis [140].

A number of studies tried to evaluate differences between the utilization of animal and vegetable coagulants or to investigate the possibility of reciprocal substitution of rennet by cardoon extracts, concerning the effect of different proteolytic pattern of the different enzymatic complexes and the effect on cheese properties.

For Los Pedroches cheese manufactured with both animal and vegetable coagulating agents, Férnandez-Salguero and Sanjuán [141] demonstrated a decrease in the relative proportion of α_s-caseins during the maturation in cheeses. The initial proportion of α_s-caseins was higher for the vegetable coagulant (42.3%), and their decrease was also faster during the maturation period. β-Caseins showed a slight decrease in their proportion during cheeses maturation with a higher residual protein content in cheeses produced with animal rennet. The content of compounds located in the γ-casein region were similar for the two coagulants types, increasing from 14.6% (at the beginning of maturation) to 24–25% at the end. These compounds are the result of the proteolytic action of animal or vegetable coagulant agents on β-caseins.

Freitas and Malcata [79] in Picante da Beira Baixa cheese, from Portugal, concluded that coagulation with vegetable coagulant results in more extensive degradation of α-casein, comparatively to animal rennet. β-casein shows a greater resistance to the coagulant agent enzymatic activity, when compared with α-casein. The water-soluble nitrogen for cheeses coagulated with animal rennet were in general lower than those for cheeses coagulated with plant rennet, but much minor differences were identified on non-protein nitrogen. These results are in agreement with those presented by Marcos et al. [142] which reported a small degradation of β-casein in several Portuguese sheep, goat and bovine milk cheeses.

For Serpa cheese, Roseiro [71] compared the effect of replacing *C. cardunculus* by animal or microbial rennet as coagulant. The pH 4.4 soluble nitrogen (pH 4.4-SN) was significantly higher for cheeses made with *C. cardunculus*, while trichloroacetic acid soluble nitrogen (TCA-SN) and phosphotungstic acid soluble nitrogen (PTA-SN) were significantly higher for cheeses made with animal, or microbial rennet. However, at 60 days of ripening no significant differences were observed. The proteolysis of α_{s1}-casein was faster than the one of β-casein, with α_{s1}-casein being completely degraded at the end of maturation. After 90 days of maturation β-casein showed a lower proteolysis level, but a faster degradation, when compared to α_{s1}-casein during the first 30 days period; up to the end of ripening, the levels of β-casein

remained practically constant. The electropherograms of Serpa cheese caseins, obtained through capillary zone electrophoresis (CZE), showed a peak that remained throughout maturation, and was not detected in others cheeses. This peak was suggested as arising from β-casein and allows the identification of the coagulant type employed in the cheesemaking [71]. Alvarenga et al. [143] concluded that the softening of Serpa cheese occurs during the first 2–3 weeks, and it can be explained by the breakage of the protein-protein bonds, which occurred during proteolysis, and to the dissociation of sub-micelles caused by a pH decrease.

O'Mahony et al. [144] studied the effect of type and amounts of coagulant (100% *C. cardunculus*, 50% *C. cardunculus*/50% chymosin, 25% *C. cardunculus* and 75% *C. cardunculus*, Chymosin, 100%) in miniature Cheddar-type cheeses. According to the results, there were no substantial differences between the compositions of cheeses made using any of the four coagulant mixtures. Otherwise cheeses manufactured with coagulant mixtures containing *C. cardunculus* proteinases showed higher levels of pH 4.6-soluble nitrogen, and higher degradation of α_{s1}-casein, than cheese made with chymosin as coagulant.

C. cardunculus proteinases, incorporated in coagulant mixtures, has the possibility of accelerating proteolysis of Cheddar cheese. Tejada et al. [77] on Murcia al Vino (goat's cheese) observed an intense proteolytic activity during the maturation process, particularly during the first 30 days, when *C. cardunculus* aqueous extract were used. At 15 days of maturation the levels of α_s-caseins and β-caseins were significantly lower in cheeses produced with vegetable coagulant, meaning that α_s-casein degradation during maturation was more intense than in the cheeses produced with the animal origin coagulant (59.5 and 40.2%, respectively). However, the degradation of β-casein was very similar in both cases. The γ-CN concentration was significantly higher in cheeses manufactured with animal rennet as cyprosins in the vegetable rennet cheeses hydrolyse such fractions or maybe inhibit plasmin action. Similar results were observed by Pino et al. [43] in goat's cheese made with animal coagulant and with powdered vegetable coagulant. Significant differences in the remaining percentage of β-caseins from day 2 onwards were not observed, with this remaining practically constant until 120 days of maturation. However, α_s-caseins levels decreased between day 2 and day 15, for both types of coagulants, decreasing slightly thereafter and more intensely in cheese made with *C. cardunculus*.

In Évora cheese [145, 146] the proteolysis is not very pronounced in contrast to the high lipolysis, resulting mainly in secondary metabolites compounds, such as amino acids, products of amino acid catabolism and mainly free fatty acids and other volatile compounds (esters, ketones, aldehydes, alcohols, lactones, among others) with consequences on the sensorial characteristics of cheese The differences between cheeses manufactured with *C. cardunculus* or with animal rennet is more pronounced in the early stages of maturation (30 days vs. 45 and 60 days) and were explained by the different peptide profile, the greater increase of amino acids (40 vs. 8%) and higher intensity of ewe flavor and "typical flavor" supported biochemically by the free fatty acids profile (particularly short and medium-chain-fatty acids) and components such as 3-methyl propanoic, 2-methyl butanoic and 3-methy butanoic (products of amino acid catabolism). The formation of hydrophobic peptides and the ratio of hydrophobic/hydrophilic peptides throughout the ripening are higher in cheeses made with *C. cardunculus* than in those made with animal rennet.

Galán et al. [147] investigated the effect of different amounts of *C. cardunculus* coagulant (normal and the double amount) and calf rennet in sheep milk cheese. After 2 days of ripening, significantly higher levels of cheese casein hydrolysis was achieved (measured as SN-soluble nitrogen, NPN-non-protein nitrogen in cheeses produced with double amount of *Cynara* compared with those made with normal amount and animal rennet. There were significant differences between the SN values of the cheeses clotted with two types of coagulants. In the early stages of ripening, the observed high SN levels were caused by the intense proteolytic enzymes action of vegetable coagulant. The taste intensity of the cheese produced with vegetable coagulant was higher than the one produced with calf rennet except for day 180. Generally, the cheeses made with double *C. cardunculus* were more bitter and acidic than cheeses made with calf rennet but no constant significant differences were observed. Later on, in 2012, Galan et al. [138] tested three coagulants (100% *C. cardunculus*, Calf rennet and 50:50 mixture). For most of the chemical and microbiological parameters no differences were observed between the coagulants used but the proteolysis index was higher in cheeses made with vegetal rennet and mixture cheeses which acquired the typical sensory characteristics faster than animal rennet cheeses.

In the last decade, special attention has also been paid to the effect of cardoon flower enzyme composition on cheese properties, following the hypothesis that the diversity of the thistle flower enzymatic profile may influence the cheese characteristics, since it is possible to differentiate at least the intensity of the proteolysis in the cheese manufacture and ripening.

In 2013, Ordiales et al. [140] analyzed the influence of rennet from different *C. cardunculus* ecotypes plants, selected for its clotting and proteolytic activity on caseins, on the characteristics of manufactured 'Torta del Casar' cheeses. The rennet with higher clotting activity after 24 h of maceration allowed higher creaminess, viscosity, and overall acceptability of the cheese. Nevertheless, the rennet with high proteolytic activity negatively influenced the acidity, bitterness and creaminess parameters. Consequently, it was concluded that the most appropriate cardoons for making this cheese are those with higher clotting activities and moderate proteolytic activities especially on β-casein. Concerning to the relationship between the characteristics of the rennet and the sensorial analysis, the degradation of β-casein was positively correlated with the compactness of the cheese paste, while is negatively correlated with the creaminess and the bitter and acid taste (the greater degradation the less the creaminess, and less bitter taste).

Recently, Guiné et al. [148] evaluated the physicochemical and sensorial properties of the Portuguese cheese "Serra da Estrela" made with six different ecotypes of cardoon flower extract. The results confirmed that the type used rennet, and in particular the cardoon flower ecotype, greatly influenced the cheese properties. A great variability in the chemical composition was verified. Texture characteristics also diverged importantly among samples and color parameters also revealed noticeable differences. The sensorial analysis allowed to clearly identify some differences, particularly in terms of creaminess, rind thickness and uniformity. In a similar studied carried out by Correia et al. [149], cheeses were also manufactured with extracts of different cardoon flower. The results showed that cardoon ecotype had a considerable influence within clotting time, and color parameters. The ecotype that provided the lowest clotting time was also the one with the highest concentration of total cardosins.

This confirms cardosins relevance in clotting time reduction. Cheeses produced with the different cardoons ecotypes were significantly different concerning rind and paste properties, as well as for global sensorial grade.

4. *Cynara cardunculus* L. other traditional and industrial applications: the biopharmaceutical potential

Traditionally, infusions of artichoke and wild cardoon leaves have been used since the fourth century B.C. [150], based on well accepted health benefits, regarding liver protection [151] and stimulating bile flow from the gallbladder (choleretic action) [150, 152, 153]. Artichoke leaves and seed extracts are also consumed to protect toward atherosclerosis, arterial hypertension and hyperuricemia [11, 154]. Wild cardoon leaves are popular in folk medicine, given to their cardiotonic, antihemorrhodial, and antidiabetic actions [155] mainly due to the biological effects of the secondary compounds. Among the different *C. cardunculus* physiological comparts, leaves appeared to accumulate a wide range of compounds with known biological activities [31, 36, 37, 156].

In order to pull out compounds of interest from *C. cardunculus* leaves, extraction processes must be done and optimized. Divided in conventional and non-conventional, extraction methodologies applied to *C. cardunculus* are commonly conventional, as batch and Soxhlet extraction [31, 36, 37, 156, 157], while application of non-convention extraction methodologies, as ultrasound, microwave, supercritic fluid or ionic liquid solutions, are still very scarce [158].

With interesting biological activities, the study of cynaropicrin extraction, a sesquiterpene lactone, found for the first time by Ramos et al. [31] in *C. cardunculus* leaves with a Soxhlet extraction, is an important step for the recovery of this added value compound. Some studies are recently appearing, applying new and non-conventional methodologies for cynaropicrin extraction from *C. cardunculus* leaves, such as ultrasound assisted extraction, where an increase of 36% on cynaropicrin concentration was achieved as well a reduction on energetic costs [159, 160]. *C. cardunculus* biological potential is tremendous, but the challenge is to transfer this knowledge to industry, toward new value chains, being crucial the cost reduction of extraction/purification processes ensuring safety, and end products functionality.

The lignocellulosic fraction, especially of cultivated cardoon, over the years has demonstrated a great potential as solid biofuel. The first research on *C. cardunculus* potential as an energy crop was carried out in the 1980s [161]. Currently research within energy and cardoon is wide, with several reports which highlight different possibilities: solid biofuel [14, 162], liquid biofuel (seed oil [29], biodiesel [163] and bioethanol [22, 164, 165]), and biogas production [20, 24]. Clearly, within energy production, *C. cardunculus* represents a high potential as an alternative to fossil materials.

5. New perspectives for economic valorization

Portugal has applied recently for the registration of traditional *C. cardunculus* crude extract as an enzymatic extract for cheesemaking, which will enable the traditional utilization of cardoon

as coagulant for cheesemaking to legally proceed. However, deeper knowledge concerning genetic variability within the plant, and enzymatic profiles is critical to improve traditional cheese quality, allowing to find a basis for certified and guaranteed enzymatic formulas for commercial use in cheesemaking, and to reinforce and valorise all the innovative potential associated to *C. cardunculus* use. In the near future, regarding the cardoon as extractives source, the higher demand is searching for cleaner and more sustainable processes, capable of combining bioactivies extraction efficiency, with reduced costs and great biological action, potentiating new biopharma-products development. The fulfill comprehension of the genetic natural variability of *C. cardunculus* plants will be out breaking in terms of full capability to economic crop valorization.

6. Concluding remarks

In agro-industries, there is an increasing interest in promoting integrated exploitation of different biomass resources, in order to maximize crop value. Consequently, a global socio-economic and environmental impact of these industries is expected in the future.

Cynara cardunculus biochemical profile unveils a great potential for different applications within energy generation, as well as in the food and pharmaceutical industries. In parallel, the traditional applications will be maintained, such as the use of *C. cardunculus* flower aqueous extracts for cheese production. For a number of cheeses regulated by PDO in the Iberian Peninsula, the use of this vegetable coagulant is mandatory. The increasing use of vegetable proteases as a coagulating agent is related not only to the unique sensory characteristics of the resulting cheese but also with a growing consumer interest in reducing the consumption of animal-derived products.

Due to the high variability of biochemical profiles of *C. cardunculus*, the development of a basis for certified and guaranteed enzymatic formulas for use in cheese manufacture is mandatory to develop the full innovation potential in this agro-industrial sector. Overall, the assessment of the genetic, chemical and biological diversity of natural occurring populations of *C. cardunculus* will also promote a wider economic valorization, adding new biotechnological applications to the traditional activities. In this way, the consolidation of the knowledge transfer from the perspective of maximizing the exploitation of a value chain around cardoon production will be achieved.

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Conflict of interest

The authors confirm that this article content has no conflict of interest.

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References

[1] Sonnante G, Carluccio AV, Vilatersana R, Pignone D. On the origin of artichoke and cardoon from the Cynara gene pool as revealed by rDNA sequence variation. Genetic Resources and Crop Evolution. 2007;**54**:483-495. DOI: 10.1007/s10722-006-9199-9

[2] Wiklund A. The genus *Cynara* L. (Asteraceae-Cardueae). Botanical Journal of the Linnean Society. 1992;**109**:75-123. DOI: 10.1111/j.1095-8339.1992.tb00260.x

[3] Foury C. Ressources genetiques et diversification de l'arti chaut (*Cynara scolymus* L.). Acta Horticulturae. 1989;**242**:155-166

[4] Rottenberg A, Zohary D. The wild ancestry of cultivated artichoke. Genetic Resources and Crop Evolution. 1996;**43**:53-58. DOI: 10.1007/BF00126940

[5] Basnizki J, Zohary D. Breeding of seed-planted artichoke. In: Janicks J, editor. Plant Breeding Review. Vol. 12. Oxford, UK: John Wiley & Sons, Inc; 1994. pp. 253-269. DOI: 10.1002/9780470650493.ch9

[6] Pignone D, Sonnante G. Wild artichokes of south Italy: Did the story begin here? Genetic Resources and Crop Evolution. 2004;**51**:577-580. DOI: 10.1023/B:GRES.0000024786.01004.71

[7] Sonnante G, Carluccio AV, De Paolis A, Pignone D. Identification of artichoke SSR markers: Molecular variation and patterns of diversity in genetically cohesive taxa and wild allies. Genetic Resources and Crop Evolution. 2008;**55**:1029-1046. DOI: 10.1007/s10722-008-9310-5

[8] Boury S, Jacob AM, Egea-Gilabert C, Fernández JA, Sonnante G, Pignone D, Pagnotta MA. Assessment of genetic variation in an artichoke European collection by means of molecular markers. In: VII International Symposium on Artichoke, Cardoon Their Wild Relat.; June 2009; Saint Pol de Léon. p. 81-88. DOI: 10.17660/ActaHortic.2012.942.8

[9] Tavaria FK, Sousa MJ, Malcata FX. Storage and lyophilization effects of extracts of *Cynara cardunculus* on the degradation of ovine and caprine caseins. Food Chemistry. 2001;**72**:79-88. DOI: 10.1016/S0308-8146(00)00213-2

[10] Quiroz D. Invasive Species Compendium [Internet]. 2016. Available from: http://www.cabi.org/isc/datasheet/17584 [Accessed: 2017-03-31]

[11] Rottenberg A. The wild gene pool of globe artichoke. Israel Journal of Plant Sciences. 2015;**62**:33-38. DOI: 10.1080/07929978.2014.887379

[12] Gatto A, De Paola D, Bagnoli F, Vendramin GG, Sonnante G. Population structure of *Cynara cardunculus* complex and the origin of the conspecific crops artichoke and cardoon. Annals of Botany. 2013;**112**:855-865. DOI: 10.1093/aob/mct150

[13] Ierna A, Mauromicale G. *Cynara cardunculus* L. genotypes as a crop for energy purposes in a Mediterranean environment. Biomass and Bioenergy. 2010;**34**:754-760. DOI: 10.1016/j.biombioe.2010.01.018

[14] Fernández-Salguero J, Curt MD, Aguado PL. Industrial applications of *Cynara cardunculus* L. for energy and other uses. Industrial Crops and Products. 2006;**24**:222-229. DOI: 10.1016/j.indcrop.2006.06.010

[15] Pinelli P, Agostini F, Comino C, Lanteri S, Portis E, Romani A. Simultaneous quantification of caffeoyl esters and flavonoids in wild and cultivated cardoon leaves. Food Chemistry. 2007;**105**:1695-1701. DOI: 10.1016/j.foodchem.2007.05.014

[16] Gominho J, Lourenço A, Palma P, Lourenço ME, Curt MD, Fernández J, Pereira H. Large scale cultivation of *Cynara cardunculus* L. for biomass production—A case study. Industrial Crops and Products. 2011;**33**:1-6. DOI: 10.1016/j.indcrop.2010.09.011

[17] Lourenço A, Neiva DM, Gominho J, Curt MD, Fernández J, Marques AV, Pereira H. Biomass production of four *Cynara cardunculus* clones and lignin composition analysis. Biomass and Bioenergy. 2015;**76**:86-95. DOI: 10.1016/j.biombioe.2015.03.009

[18] Kelly M. Cynara cardunculus. In: Bossard CC, Randall JM, Hoshovsky MC, editors. Invasive Plants of California's Wildlands. Berkeley: University California Press; 2000. pp. 139-145

[19] Archontoulis SV, Struik PC, Vos J, Danalatos NG. Phenological growth stages of *Cynara cardunculus*: Codification and description according to the BBCH scale. The Annals of Applied Biology. 2010;**156**:253-270. DOI: 10.1111/j.1744-7348.2009.00384.x

[20] Oliveira I, Gominho J, Diberardino S, Duarte E. Characterization of *Cynara cardunculus* L. stalks and their suitability for biogas production. Industrial Crops and Products. 2012;**40**:318-323. DOI: 10.1016/j.indcrop.2012.03.029

[21] Fritsche J, Beindorff C, Dachtler M, Zhang H, Lammers J. Isolation, characterization and determination of minor artichoke (*Cynara scolymus* L.) leaf extract compounds. European Food Research and Technology. 2002;**215**:149-157. DOI: 10.1007/s00217-002-0507-0

[22] Fernandes MC, Ferro MD, Paulino AFC, Mendes JAS, Gravitis J, Evtuguin DV, Xavier AMRB. Enzymatic saccharification and bioethanol production from *Cynara cardunculus* pretreated by steam explosion. Bioresource Technology. 2015;**186**:309-315. DOI: 10.1016/j.biortech.2015.03.037

[23] Gouveia SC, Castilho PC. Phenolic composition and antioxidant capacity of cultivated artichoke, Madeira cardoon and artichoke-based dietary supplements. Food Research International. 2012;**48**:712-724. DOI: 10.1016/j.foodres.2012.05.029

[24] Pesce GR, Negri M, Bacenetti J, Mauromicale G. The biomethane, silage and biomass yield obtainable from three accessions of *Cynara cardunculus*. Industrial Crops and Products. 2017;**103**:233-239. DOI: 10.1016/j.indcrop.2017.04.003

[25] Gominho J, Lourenço A, Curt M, Fernández J, Pereira H. Characterization of hairs and pappi from *Cynara cardunculus* capitula and their suitability for paper production. Industrial Crops and Products. 2009;**29**:116-125. DOI: 10.1016/j.indcrop.2008.04.022

[26] Gominho J, Fernandez J, Pereira H. *Cynara cardunculus* L.—A new fibre crop for pulp and paper production. Industrial Crops and Products. 2001;**13**:1-10. DOI: 10.1016/S0926-6690(00)00044-3

[27] Schütz K, Kammerer D, Carle R. Identification and quantification of caffeoylquinic acids and flavonoids from artichoke (*Cynara scolymus* L.) heads, juice, and pomace by HPLC-DAD-ESI/MSn. Journal of Agricultural and Food Chemistry. 2004;**52**:4090-4096. DOI: 10.1021/jf049625x

[28] Fernández J, Curt MD. Low-cost biodiesel from Cynara oil. In: Proceedings 2nd Conference and Exhibition on Biomass for Energy, Industry and Climate Protection; 10-14 May 2004; Rome, Italy. p. 10-14

[29] Curt MD, Sanchez G, Fernandez J. The potential of *Cynara cardunculus* L. for seed oil production in a perennial cultivation system. Biomass and Bioenergy. 2002;**23**:33-46. DOI: 10.1016/s0961-9534(02)00030-2

[30] Angelini LG, Ceccarini L, Nassi o Di Nasso N, Bonari E. Long-term evaluation of biomass production and quality of two cardoon (*Cynara cardunculus* L.) cultivars for energy use. Biomass and Bioenergy. 2009;**33**:810-816. DOI: 10.1016/j.biombioe.2008.12.004

[31] Ramos PA, Guerra AR, Guerreiro O, Freire CS, Silva AM, Duarte MF, Silvestre AJ. Lipophilic extracts of *Cynara cardunculus* L. var. *altilis* (DC): A source of valuable bioactive terpenic compounds. Journal of Agricultural and Food Chemistry. 2013;**64**:8420-8429. DOI: 10.1021/jf402253a

[32] Christaki E, Bonos E, Florou-Paneri P. Nutritional and functional properties of Cynara crops (globe artichoke and cardoon) and their potential applications: A review. International Journal of Applied Science and Technology. 2012;**2**:64-70. ISSN: 2277-8691

[33] Figueiredo ACS. Cultura de *Cynara cardunculus*: Produção de Biomassa e Obtenção de Proteases [thesis]. Lisboa: Universidade de Lisboa; 1987

[34] Colak E, Ustuner MC, Tekin N, Colak E, Burukoglu D, Degirmenci I, Gunes HV. The hepatocurative effects of *Cynara scolymus* L. leaf extract on carbon tetrachloride-induced oxidative stress and hepatic injury in rats. Springerplus. 2016;**5**:2-9. DOI: 10.1186/s40064-016-1894-1

[35] Ferro AM, Ramos P, Guerreiro O, Jerónimo E, Pires I, Capel C, Lozano R, Duarte MF, Oliveira MM, Gonçalves S. Impact of novel SNPs identified in *Cynara cardunculus* genes on functionality of proteins regulating phenylpropanoid pathway and their association with biological activities. BMC Genomics. 2017;**18**:1-16. DOI: 10.1186/s12864-0173534-8

[36] Ramos PAB, Santos SAO, Guerra ÂR, Guerreiro O, Freire CSR, Rocha SM, Duarte MF, Silvestre AJD. Phenolic composition and antioxidant activity of different morphological parts of Cynara *cardunculus* L. var. *altilis* (DC). Industrial Crops and Products. 2014;**61**:460-471. DOI: 10.1016/j.indcrop.2014.07.042

[37] Brás T, Guerreiro O, Duarte MF, Neves LA. Impact of extraction parameters and concentration by nanofiltration on the recovery of phenolic compounds from *Cynara cardunculus* var. *altilis*: Assessment of antioxidant activity. Industrial Crops and Products. 2015;**67**:137-142. DOI: 10.1016/j.indcrop.2015.01.005

[38] Ramos PAB, Guerra ÂR, Guerreiro O, Santos SAO, Oliveira H, Freire CSR, Silvestre AJD, Duarte MD. Antiproliferative effects of *Cynara cardunculus* L. var. *altilis* (DC) lipophilic extracts. International Journal of Molecular Sciences. 2017;**18**:63. DOI: 10.3390/ijms18010063

[39] Vieira De Sá F. Cuajos vegetales. Rev Española Leche n° 92 1974:107-109

[40] Barbosa M. O cardo (*Cynara cardunculus*) como coagulante vegetal. In: 1º Congresso Nacional das Indústrias Agro-Alimentares; 22-25 Março 1983. LNETI-DTIA: Lisboa; 1983

[41] Faro C, Verissimo P, Lin Y, Tang J, Pires E. Cardosin A and B, aspartic proteases from the flowers of cardoon. In: Takahashi K, editor. Aspartic Proteinases. Advances in Experimental Medicine and Biology, 362. Boston, MA: Springer; 1995. pp. 373-377. DOI: 10.1007/978-1-4615-1871-6_49

[42] Nuñez M, Del Pozo BF, Rodríguez-Marin MA, Gaya P, Medina M. Effect of vegetable and animal rennet on chemical, microbiological, rheological and sensory characteristics of La Serena cheese. The Journal of Dairy Research. 1991;**58**:511-519. DOI: 10.1017/S0022029900030120

[43] Pino A, Prados F, Galán E, McSweeney PLH, Fernández-Salguero J. Proteolysis during the ripening of goats' milk cheese made with plant coagulant or calf rennet. Food Research International. 2009;**42**:324-330. DOI: 10.1016/j.foodres.2008.12.009

[44] Macedo IQ, Faro CJ, Pires EM. Caseinolytic specificity of cardosin, an aspartic protease from the cardoon *Cynara cardunculus* L.: Action on bovine αs-and β-casein and comparison with chymosin. Journal of Agricultural and Food Chemistry. 1996;**44**:42-47. DOI: 10.1021/jf9406929

[45] Faro CJ, Moir AJG, Pires EV. Specificity of a milk clotting enzyme extracted from the thistle *Cynara cardunculus* L.: Action on oxidised insulin and k-casein. Biotechnology Letters. 1992;**14**:841-846. DOI: 10.1007/BF01029150

[46] Bailey LH, Bailey EZ. Hortus Third—$ Concise Dictionary of Plants Cultivated in the United States and Canada. Gominho. 1st ed. New York: Macmillan Publishing Company; 1976. 1312 p. ISBN: 0025054708

[47] Tutin TG, Heywood VH, Burges NA, Valentine DH, Walters SM, editors. Flora Europaea. Plantaginaceae to Compositae (and Rubiaceae). Vol. IV. Cambridge: Cambridge University Press; 1976. 534 p. ISBN: 9780521153690

[48] Valdés B, Talavera S, Fernández-Galiano E, editors. Flora vascular de Andalucía Occidental. Barcelona: Ketres Editora S.A; 1978. ISBN: 84-8525526-63-8

[49] Carneiro A, Antas J, Coelho JML. Nova Flora de Portugal (Continente e Açores). Clethraceae—Compositae. Vol. II. Escolar Editora: Lisboa; 1984. 230 p. ISBN: 978111 5551113

[50] Sonnante G, Pignone D, Hammer K. The domestication of artichoke and cardoon: From roman times to the genomic age. Annals of Botany. 2007;**100**:1095-1100. DOI: 10.1093/aob/mcm127

[51] Morbey TM. Projecto "Cardo." Relatório sucinto da Act. desenvolvida entre 1980 e 1990, Oeiras, Dezembro: ENTPA, INIA.; 1990

[52] Gaião D. CARDOP—Queijos Serra da Estrela. Cardo [Internet]. 2017. Available from: http://cardop-queijoserradaestrela.blogspot.pt/p/blog-page_3.html [Accessed: 2017-07-22]

[53] Koreth J, O'Leary JJ, McGee JOD. Microsatellites and PCR genomic analysis. The Journal of Pathology. 1996;**178**:239-248. DOI: 10.1002/(SICI)1096-9896(199603)178:3<239::AID-PATH506>3.0.CO;2-5

[54] Ben Ammar I, Harzallah-Skhiri F, Al Mohandes Dridi B. Morphological variability of wild cardoon (*Cynara cardunculus* L. var. *sylvestris*) populations in north of Tunisia. ISRN Agron. 2014;**2014**:1-9. DOI: 10.1155/2014/656937

[55] Khaldi S, Sonnante G, El Gazzah M. Analysis of molecular genetic diversity of cardoon (*Cynara cardunculus* L.) in Tunisia. Comptes Rendus Biologies. 2012;**335**:389-397. DOI: 10.1016/j.crvi.2012.04.005

[56] Sonnante G, D'Amore R, Blanco E, Pierri CL, De Palma M, Luo J, Tucci M, Martin C. Novel hydroxycinnamoyl-coenzyme A quinate transferase genes from artichoke are involved in the synthesis of chlorogenic acid. Plant Physiology. 2010;**153**:1224-1238. DOI: 10.1104/pp.109.150144

[57] Barreira I. Tractado das significaçoens das plantas, flores, e fructos que se referem na Sagrada Escriptura: Tiradas de divinas, & humanas letras, cõ suas breves consideraçoes [Internet]. 1622. Available from: http://purl.pt/13995 [Accessed: 2017-09-05]

[58] Columella LJM. On Agriculture, Volume I: Books 1-4. Translated by Harrison Boyd Ash. Loeb Classical Library 361. Cambridge, MA: Harvard University Press; 1941

[59] Vergel de señores, en el cual se muestran a hacer con mucha excelencia todas las conservas, electuarios, confituras, turrones y otras cosas de azúcar y miel [Internet]. 1401. Available from: http://bdh.bne.es/bnesearch/detalle/bdh0000145867 [Accessed: 2017-09-10]

[60] Dioscórides Laguna. Pedacio Dioscorides anazarbeo, Acerca de la materia medicinal y de los venenos mortiferos traduzido de lengua griega en la vulgar castellana & illustrado con claras y substantiales annotationes, y con las figuras de innumeras plantas exquisitas y raras por Andres de Laguna … [Internet]. 1555. Available from: http://bdh.bne.es/bnesearch/detalle/bdh0000037225 [Accessed: 2017-09-01]

[61] Pinheiro CMSC. Contributo para a caracterização do queijo de ovelha produzido na região de Évora: Aspectos químicos, bioquímicos do leite obtido em diferentes sistemas de produção e físico-químicos, bioquímicos, tecnológicos e organolépticos do queijo [Phd thesis]. Évora: Universidade de Évora; 2002

[62] Cabreira GR. Compendio de muitos e vários remedios de Cirurgia, & outras coufas curiofas. Recopiladas do Thesouro de Pobres, & outros Authores. Em Lisboa: na officina de Francisco Villela; 1671. http://purl.pt/13994 [Accessed: 2017-09-05]

[63] Pinheiro C, Machado G, Bettencourt C, Matos C. Avaliação sensorial do queijo: Definição dos atributos de qualidade. Revista de Ciências Agrárias. 2007:350-357. DOI: v30n1/v30n1a37

[64] Bettencourt CMV, Pinheiro CMSC, Carrasco AMC. Queijo Serpa—Um património a preservar. Rota do Guadiana, Associação de Desenvolvimento Integrado e Confraria do Queijo de Serpa. Serpa (Portugal). 2008. 150 p. ISBN: 978-989-96023-1-1

[65] Martins APL, De Vasconcelos MMP, De Sousas RB. Thistle (*Cynara cardunculus* L) flower as a coagulant agent for cheesemaking. Short characterization. Lait. 1996;**76**:473-477. DOI: 10.1051/lait:1996536

[66] Martins APL. A flor de cardo (*Cynara cardunculus* L.) como agente coagulante no fabrico de queijo. Caracterização e influência dos processos de conservação na actividade coagulante [thesis]. Lisbon: University of Lisbon; 1999

[67] Le Jaouen JC. Production du lait et marché des produits laitiers caprins dans le bassin méditerranéen. In: Les petits ruminants et leurs productions laitières dans la région mediterranéennee; 09-09 mars 1990; Paris: Bougler J, Tisserand JL, CIHEAM (Centre International de HautesEtudes Agronomiques Méditerranéennes), CENECA (Centre National de Expositions et Concours Agricoles; 1990. pp. 73-80. ISBN: 85352-0978

[68] Flamant JC, Morand-Fehr P. Milk production in sheep and goats. In: Coop IE, editor. Sheep and Goat Production. World Animal Science Series No C1. Amsterdam, Netherlands: Elsevier Scientific Publishing Co.; 1982. pp. 275-295. DOI: 10.1016/0301-6226(83)90012-X

[69] Dubeuf JP, Morand-Fehr P, Rubino R. Situation, changes and future of goat industry around the world. Small Ruminant Research. 2004;**51**:165-173. DOI: 10.1016/j.smallrumres.2003.08.007

[70] INE. Anuário Estatistico de Portugal. In: Lisboa. 2016

[71] Roseiro LB, Barbosa M, Ames JM, Wilbey RA. Cheesemaking with vegetable coagulants—The use of *Cynara* L. for the production of ovine milk cheeses. International Journal of Dairy Technology. 2003;**56**:76-85. DOI: 10.1046/j.1471-0307.2003.00080.x

[72] Macedo IQ, Faro CJ, Pires EM. Specificity and kinetics of the milk-clotting enzyme from cardoon (*Cynara cardunculus* L.) toward bovine κ-casein. Journal of Agricultural and Food Chemistry. 1993;**41**:1537-1540. DOI: 10.1021/jf00034a001

[73] Roa I, Belén López M, Javier Mendiola F. Residual clotting activity and ripening properties of vegetable rennet from *Cynara cardunculus* in La Serena cheese. Food Research International. 1999;**32**:413-419. DOI: 10.1016/S0963-9969(99)00098-8

[74] Delgado FJ, Rodríguez-Pinilla J, González-Crespo J, Ramírez R, Roa I. Proteolysis and texture changes of a Spanish soft cheese ('Torta del Casar') manufactured with raw ewe milk and vegetable rennet during ripening. International Journal of Food Science and Technology. 2010;**45**:512-519. DOI: 10.1111/j.1365-2621.2009.02157.x

[75] Rincón AA, Pino V, Fresno MR, Jiménez-Abizanda AI, Álvarez S, Ayala JH, Afonso AM. Influence of vegetable coagulant and ripening time on the lipolytic and sensory profile of cheeses made with raw goat milk from canary breeds. Food Science and Technology International. 2017;**23**:254-264. DOI: 10.1177/1082013216685202

[76] Sousa M, Malcata FX. Advances in the role of a plant coagulant (*Cynara cardunculus*) in vitro and during ripening of cheeses from several milk species. Le Lait. 2002;**82**:151-170. DOI: 10.1051/lait

[77] Tejada L, Abellán A, Cayuela JM, Martínez-Cacha A, Fernández-Salguero J. Proteolysis in goats' milk cheese made with calf rennet and plant coagulant. International Dairy Journal. 2008;**18**:139-146. DOI: 10.1016/j.idairyj.2007.08.010

[78] Barbosa M, Valles E, Vassal L, Mocquot G, Nicolas M, Pitel G, Vachot JC. L'utilisation d'extrait de *Cynara cardunculus* L. comme agent coagulant en fabrication de fromages à pâte molle et à pâte cuite. Le Lait. 1976;**56**:1-17. DOI: 10.1051/lait:1976551-5521

[79] Freitas AC, Malcata FX. Influence of milk type, coagulant, salting procedure and ripening time on the final characteristics of picante cheese. International Dairy Journal. 1996;**6**:1099-1116. DOI: 10.1016/S0958-6946(96)00035-0

[80] Vieira De Sá F, Barbosa M. Cheesemaking with a vegetable rennet from cardo (*Cynara cardunculus* L.). The Journal of Dairy Research. 1972;**39**:335-343. DOI: 10.1017/S0022029900014163

[81] Aquilanti L, Babini V, Santarelli S, Osimani A, Petruzzelli A, Clementi F. Bacterial dynamics in a raw cow's milk Caciotta cheese manufactured with aqueous extract of *Cynara cardunculus* dried flowers. Letters in Applied Microbiology. 2011;**52**:651-659. DOI: 10.1111/j.1472-765X.2011.03053.x

[82] European Commission—Agriculture and Rural Development – DOOR n.d. Available from: http://ec.europa.eu/agriculture/quality/door/list.html [Accessed: 2017-08-12]

[83] Jacob M, Jaros D, Rohm H. Recent advances in milk clotting enzymes. International Journal of Dairy Technology. 2011;**64**:14-33. DOI: 10.1111/j.1471-0307.2010.00633.x

[84] Vasconcelos MP. Estudo do queijo de Azeitão, melhoramento da tecnologia tradicional e sua influência nas características do queijo [thesis]. Oeiras: INIAV; 1990

[85] Christen C, Virasoro E. Présures végétales. Extraction et propriétés (fin). Le Lait. 1935;**15**:496-505

[86] Tsouli J. Étude comparée de l'activité enzymatique de 3 variétés d'artichauls du genre *Cynara Cardunculus* L. sur la coagulation du lait. Le Lait. 1974;**537**:415-421. DOI: 10.1051/lait:197453721

[87] Sousa MJ, Malcata FX. Effects of processing conditions on the caseinolytic activity of crude extracts of *Cynara cardunculus* L. Food science and technology international.1996;**2**:255-263. DOI: 10.1177/108201329600200408

[88] Scopes RK. Protein Purification. Principles and Practice. 2nd ed. New York: Springer-Verlag; 1987. 380 p. DOI: 10.1007/978-1-4757-2333-5

[89] Jervis L, Pierpoint WS. Purification technologies for plant proteins. Journal of Biotechnology. 1989;**11**:161-198. DOI: 10.1016/0168-1656(89)90117-X

[90] Tavaria FK, Sousa MJ, Malcata FX. Effect of storage and lyophilization on ovine and caprine casein degradation by extracts of *Cynara cardunculus* L. In: 3º Encontro de Química de Alimentos—Alimentação Mediterrânica; Faro; 1997. pp. 346-348

[91] Tejada L, Fernández-Salguero J. Chemical and microbiological characteristics of ewe milk cheese (Los Pedroches) made with a powdered vegetable coagulant or calf rennet. Italian Journal of Food Science. 2003;**15**:125-132. ISSN: 1120-1770

[92] Health Canada. Notice of Modification to the List of Permitted Food Enzymes to Enable the Use of Cyprosin Obtained from the Flower of *Cynara cardunculus* L. var. altilis DC. as a Food Enzyme in Various Standardized Cheeses. 2015. Available from: http://www.hc-sc.gc.ca/fn-an/consult/nom-adm-0049/index-eng.php [Accessed: 2017-09-13]

[93] Kervinen J, Törmäkangas K, Runeberg-Roos P, Guruprasad K, Blundell T, Teeri TH. Structure and possible function of aspartic proteinases in barley and other plants. In: Takahashi K, editor. Aspartic Proteinases. Advances in Experimental Medicine and Biology. Vol. 362. Boston, MA: Springer; 1995. pp. 241-254. DOI: 10.1007/978-1-4615-1871-6_28

[94] Veríssimo P, Faro C, Moir AJ, Lin Y, Tang J, Pires E. Purification, characterization and partial amino acid sequencing of two new aspartic proteinases from fresh flowers of *Cynara cardunculus* L. The FEBS Journal. 1996;**235**:762-768. DOI: 10.1111/j.1432-1033.1996.00762.x

[95] Simões I, Faro C. Structure and function of plant aspartic proteinases. The FEBS Journal. 2004;**271**:2067-2075. DOI: 10.1111/j.1432-1033.2004.04136.x

[96] Ramalho-Santos M, Pissarra J, Veríssimo P, Pereira S, Salema R, Pires E, Faro CJ. Cardosin A, an abundant aspartic proteinase, accumulates in protein storage vacuoles in the stigmatic papillae of *Cynara cardunculus* L. Planta. 1997;**203**:204-212. DOI: 10.1007/s004250050183

[97] Sarmento AC, Lopes H, Oliveira CS, Vitorino R, Samyn B, Sergeant K, Debyser G, Beeumen JV, Domingues P, Amado F, Pires E, Domingues MRM, Barros MT. Multiplicity of aspartic proteinases from *Cynara cardunculus* L. Planta. 2009;**230**:429-439. DOI: 10.1007/s00425-009-0948-9

[98] Brodelius PE, Cordeiro MC, Pais MS. Aspartic proteinases (Cyprosins) from *Cynara cardunculus* spp. *Flavescens* Cv. cardoon; purification, characterization, and tissue-specific expression. In: Takahashi K, editor. Aspartic Proteinases. Advances in Experimental Medicine and Biology. Vol. 362. Boston, MA: Springer; 1995. pp. 255-266. DOI: 10.1007/978-1-4615-1871-6_29

[99] Heimgartner U, Pietrzak M, Geertsen R, Brodelius P, da Silva Figueiredo AC, Pais MSS. Purification and partial characterization of milk clotting proteases from flowers of *Cynara cardunculus*. Phytochemistry 1990;**29**:1405-1410. DOI:10.1016/0031-9422(90)80090-4

[100] Mutlu A, Gal S. Plant aspartic proteinases: Enzymes on the way to a function. Physiologia Plantarum. 1999;**105**:569-576. DOI: 10.1034/j.1399-3054.1999.105324.x

[101] Dubeuf JP, De F, Castel Genis JM. Initiatives and projects to promote the Mediterranean local cheeses and their relations to the development of livestock systems and activities. Small Ruminant Research. 2010;**93**:67-75. DOI: 10.1016/j.smallrumres.2010.03.001

[102] Vairo Cavalli S, Lufrano D, Colombo ML, Priolo N. Properties and applications of phytepsins from thistle flowers. Phytochemistry. 2013;**92**:16-32. DOI: 10.1016/j.phytochem.2013.04.013

[103] Llorente BE, Obregón WD, Avilés FX, Caffini NO, Vairo-Cavalli S. Use of artichoke (*Cynara scolymus*) flower extract as a substitute for bovine rennet in the manufacture of Gouda-type cheese: Characterization of aspartic proteases. Food Chemistry. 2014;**159**:55-63. DOI: 10.1016/j.foodchem.2014.03.007

[104] Pires E, Faro C, Macedo I, Esteves C, Morgado J, Veríssimo P, Dia-Pereira GD. Flor do cardo versus quimosina no fabrico de queijos artesanais. Boletim da Sociedade Portuguesa de Química. 1994;**54**:66-68 http://www.spq.pt/periodicos/browsing_new.aspx

[105] Ben Amira A, Besbes S, Attia H, Blecker C. Milk-clotting properties of plant rennets and their enzymatic, rheological, and sensory role in cheese making: A review. International Journal of Food Properties. 2017;**20**:1-18. DOI: 10.1080/10942912.2017.1289959

[106] Vieira De Sá F, Barbosa M. Activité coagulante comparée d'une presure vegetale extraite du chardon (*Cynara cardunculus*) et de Ia presure animale. In: Proceesing XVIII International Dairy Congress. Vol. 1. Sydney. 1970. p. 292

[107] Christen C, Virasoro E. Présures végétales. Extraction et propriétés. Le Lait. 1935;**15**:354-563. DOI: 10.1051/lait:193514414

[108] Campos R, Guerra R, Aguilar M, Ventura O, Camacho L. Chemical characterization of proteases extracted from wild thistle (*Cynara cardunculus*). Food Chemistry. 1990;**35**:89-97. DOI: 10.1016/0308-8146(90)90023-W

[109] Martinez E, Esteban MA. Actividad coagulante del extracto del cardo *Cynara humilis* L. Archivos de Zootecnia. 1980;**29**:107-116. Available from: http://helvia.uco.es/xmlui/bitstream/handle/10396/3085/01_21_03_114_1.pdf Accessed: 2017-09-01

[110] Lenoir J, Schneider N. L'aptitude du lait à la coagulation par la présure. In: Eck A, Gillis JC, editors. Le Fromage. 3rd ed. Lavoisier, Paris: Technique et Documentation; 1997. pp. 229-256. ISBN: 9782743008918

[111] Moreira CPM. Desenvolvimento de metodologias analíticas para queijos. Estudo de caso: queijos da Beira Interior. [PhD.thesis]. ISA/UTL: Lisboa; 2011

[112] Alves SMP, Martins APL, Mourato MP, Vasconcelos MM, Fontes AML. Effect of technological parameters on coagulation properties of sheep milk. In: Symposium Internacional "El Futuro de los Sectores Lecheros ovino y caprino" (FIL/IDF, CIHEAM); 28-30 October 2004; Zaragoza; 2004. pp. 4-22

[113] Delacroix-Buchet A, Barillet F, Lagriffoul G. Caractérisation de l'aptitude fromagère des laits de brebis Lacaune à l'aide d'un Formagraph. Le Lait. 1994;**74**:173-186. DOI: 10.1051/lait:1994315

[114] Dalgleish DG. The enzymatic coagulation of milk. In: Fox PF, editor. Cheese: Chemistry, Physics and Microbiology. Boston, MA: Springer US; 1993. pp. 69-100. DOI: 10.1007/978-1-4615-2650-6_3

[115] Ramet JP, Weber F. Contribution à l'étude de l'influence des facteurs de milieu sur la coagulation enzymatique du lait reconstitué. Le Lait. 1980;**60**:1-13. DOI: 10.1051/lait:1980591-5921

[116] Alves S. Estudo do efeito de alguns factores tecnologicos na coagulacao do leite com extractos de cardo (*Cynara cardunculus* L.). [Relatório de Fim de Curso]: Lisboa, UTL; 2003

[117] Esteves CLC, Lucey JA, Hyslop DB, Pires EMV. Effect of gelation temperature on the properties of skim milk gels made from plant coagulants and chymosin. International Dairy Journal. 2003;**13**:877-885. DOI: 10.1016/S0958-6946(03)00114-6

[118] Martins APL, Dias JCL, Sanchez M, Vasconcelos MM, Leal CCS, Palmilha MILC, Fontes AML. Effect of heating and salt and calcium addition on coagulation of goat milk and curd properties. Int Dairy Fed Spec Issue Issue 0501/Part 4 2005:280-281. ISBN: 92 9098 039-7

[119] Cordeiro M, Jakob E, Puhan Z, Pais MS, Brodelius PE. Milk clotting and proteolytic activities of purified cynarases from Cynara cardunculus—A comparison to chymosin. Milchwissenschaft. 1992;**47**:683-687. Available from: http://agris.fao.org/agris-search/search.do?recordID=DE94V0073

[120] Lucey JA, Fox PF. Importance of calcium and phosphate in cheese manufacture—A review. Journal of Dairy Science. 1993;**76**:1714-1724. DOI: 10.3168/jds.S0022-0302(93)77504-9

[121] Van Hooydonk ACM, Hagedoorn HG, Boerrigter IJ. The effect of various cations on the renneting of milk. Netherlands Milk and Dairy Journal. 1986;**40**:369-390 Available from: https://eurekamag.com/research/001/707/001707163.php

[122] Anifantakis EM. Manufacture of sheep's milk products. In: Proceedings of the XXIII International Dairy Congress. Vol. 1. 1990. pp. 420-432

[123] Van Hooydonk ACM, Berg G. Control and determination of the curd-setting during cheesemaking. IDF Bulletin, n° 225. Brussels: International Dairy Federations; 1988. pp. 1-10

[124] Balcones E, Olano A, Calvo MM. Factors affecting the rennet clotting properties of Ewe's milk. Journal of Agricultural and Food Chemistry. 1996;**44**:1993-1996. DOI: 10.1021/jf960138u

[125] Baumy JJ, Brulé G. Etude comparée de la solubilité et de la viscosité des solutions de caséinate et paracaséinate de sodium en présence de calcium. Le Lait. 1968;**66**:65-77. DOI: 10.1051/lait:198614

[126] Green ML, Grandison AS. Secondary (non-enzymatic) phase of rennet coagulation and post-coagulation phenomena. In: Fox PF, editor. Cheese: Chemistry, Physics and Microbiology. 2nd ed. Boston, MA: Springer; 1999. pp. 101-140. DOI: 10.1007/978-1-4615-2650-6_4

[127] Walstra P. The syneresis of curd. In: Fox PF, editor. Cheese: Chemistry, Physics and Microbiology. 2nd ed. Boston, MA: Springer; 1993. pp. 141-190. DOI: 10.1007/978-1-4615-2650-6

[128] Abd El-Salam MH. Domiati and feta-type cheeses major cheese groups. In: Fox PF, editor. Cheese: Chemistry, Physics and Microbiology. 2nd ed. Boston, MA: Springer; 1999. pp. 301-335. DOI: 10.1007/978-1-4615-2800-5_11

[129] Vasconcelos MM, Martins APL, Carreira DFC. Improvement of Azeitão cheese traditional technology. In: II Cong. Int. Quim. la ANQUE—Cienc. y Tecnol. los Aliment.; Burgos, Espanha; 1992. pp. 539-544

[130] Almeida CM, Gomes D, Faro C, Simões I. Engineering a cardosin B-derived rennet for sheep and goat cheese manufacture. Applied Microbiology and Biotechnology. 2015;**99**:269-281. DOI: 10.1007/s00253-014-5902-5

[131] Almeida CM, Manso JA, Figueiredo AC, Antunes L, Cruz R, Manadas B, Bur D, Pereira JBP, Faro C, Simões I. Functional and structural characterization of synthetic cardosin B-derived rennet. Applied Microbiology and Biotechnology. 2017;**101**:6951-6968. DOI: 10.1007/s00253-017-8445-8

[132] Veiros CBR. Contributo para a caracterização do queijo terrincho: estudo da proteólise e avaliação da autenticidade por HPLC/UV. [MSc thesis]. Porto: Faculdade de Farmácia da Universidade do Porto; 2005

[133] Medina M, Nuñez MM. Cheeses from ewe and goat milk. In: McSweeney PLH, Fox PF, Cotter P, David W, editors. Cheese. Chemistry, Physics and Microbiology. 4th ed. San Diego; 2017. pp. 1069-1091. DOI: 10.1016/B978-0-12-417012-4.00041-7

[134] Irigoyen A, Izco JM, Ibáñez FC, Torre P. Influence of calf or lamb rennet on the physicochemical, proteolytic, and sensory characteristics of an ewe's-milk cheese. International Dairy Journal. 2002;**12**:27-34. DOI: 10.1016/S0958-6946(01)00147-9

[135] Veloso ACA. Análise das caseínas de leite e queijos por HPLC/UV e por Ureia-PAGE. [MSc Thesis]. Porto: Faculdade de Farmácia da Universidade do Porto; 2001

[136] Sousa MJ, Ardö Y, McSweeney PLH. Advances in the study of proteolysis during cheese ripening. International Dairy Journal. 2001;**11**:327-345. DOI: 10.1016/S0958-6946(01)00062-0

[137] Fox PF, McSweeney PLH. Proteolysis in cheese during ripening. Food Reviews International. 1996;**12**:457-509. DOI: 10.1080/87559129609541091

[138] Galán E, Cabezas L, Fernández-Salguero J. Proteolysis, microbiology and sensory properties of ewes' milk cheese produced with plant coagulant from cardoon *Cynara cardunculus*, calf rennet or a mixture thereof. International Dairy Journal. 2012;**25**:92-96. DOI: 10.1016/j.idairyj.2012.02.001

[139] McSweeney PLH, Fox PF. Chemical methods for the characterization of proteolysis in cheese during ripening. Le Lait. 1997;**77**:41-76. DOI: 10.1051/lait:199713

[140] Ordiales E, Benito MJ, Martin A, Fernández M, Hernández A, de Guia Córdoba M. Proteolytic effect of *Cynara cardunculus* rennet for use in the elaboration of "Torta del Casar" cheese. The Journal of Dairy Research 2013;**80**:429-438. DOI:10.1017/S0022029913000411

[141] Fernández-Salguero J, Sanjuán E. Influence of vegetable and animal rennet on proteolysis during ripening in ewes' milk cheese. Food Chemistry. 1999;**64**:177-183. DOI: 10.1016/S0308-8146(98)00149-6

[142] Marcos A, Esteban MA, León F, Fernández-Salguero J. Electrophoretic patterns of European cheeses: Comparison and quantitation. Journal of Dairy Science. 1979;**62**:892-900. DOI: 10.3168/jds.S0022-0302(79)83345-7

[143] Alvarenga N, Silva P, Rodriguez-Garcia J, Sousa I. Estimation of Serpa cheese ripening time using multiple linear regression (MLR) considering rheological, physical and chemical data. The Journal of Dairy Research. 2008;**75**:233-239. DOI: 10.1017/S0022029908003191

[144] O'Mahony JA, Sousa MJ, McSweeney PLH. Proteolysis in miniature cheddar-type cheeses made using blends of chymosin and *Cynara cardunculus* proteinases as coagulant. International Journal of Dairy Technology. 2003;**56**:52-58. DOI: 10.1046/j.1471-0307.2003.00078.x

[145] Pinheiro C, Banks J, Brechany E. Volatile components in Évora cheese manufactured with calf rennet or an extract from Cynara. In: Symposium: Quality and Microbiology of Traditional Raw Milk Cheese. France: Dijon. p. 1998

[146] Pinheiro C, Banks J, Brechany E. Amino acid profiles in the ripening of Evora cheese manufactured with calf rennet or an extract from Cynara. In: Symposium: Quality and Microbiology of Traditional Raw Milk Cheese. France: Dijon. p. 1998

[147] Galán E, Prados F, Pino A, Tejada L, Fernández-Salguero J. Influence of different amounts of vegetable coagulant from cardoon *Cynara cardunculus* and calf rennet on the proteolysis and sensory characteristics of cheeses made with sheep milk. International Dairy Journal. 2008;**18**:93-98. DOI: 10.1016/j.idairyj.2007.06.003

[148] Guiné RPF, Correia PMR, Correia AC. Avaliação Comparativa de Queijos Portugueses de Cabra e Ovelha. Millenium. 2015;**49**:111-130. DOI: 10400.19/3992

[149] Correia P, Vítor A, Tenreiro M, Guiné R. Effect of different thistle flower ecotypes as milk-clotting in Serra da Estrela cheese. Nutrition & Food Science. 2016;**46**:458-475. DOI: 10.1108/NFS-12-2015-0157

[150] Kraft K. Artichoke leaf extract—Recent findings reflecting effects on lipid metabolism, liver and gastrointestinal tracts. Phytomedicine. 1997;**4**:369-378. DOI: 10.1016/S0944-7113(97)80049-9

[151] Adzet T, Camarasa J, Laguna JC. Hepatoprotective activity of polyphenolic compounds from *Cynara scolymus* against CCl 4 toxicity in isolated rat hepatocytes. Journal of Natural Products. 1987;**50**:612-617. DOI: 10.1021/np50052a004

[152] Kirchhoff R, Beckers C, Kirchhoff GM, Trinczek-Gärtner H, Petrowicz O, Reimann HJ. Increase in choleresis by means of artichoke extract. Phytomedicine. 1994;**1**:107-115. DOI: 10.1016/S0944-7113(11)80027-9

[153] Gebhardt R. Choleretic and anticholestatic activities of flavonoids of artichoke (*Cynara cardunculus* L. subsp. *scolymus* L. Hayek). Acta Horticulturae. 2005;**681**:429-436. DOI: 10.17660/ActaHortic.2005.681.60

[154] Proença da Cunha A, da Silva AP, Roque OR. Plantas e Produtos Vegetais em Fitoterapia, 4ª Edição. Fundação Calouste Gulbenkian: Lisbon; 2012. 729 p. ISBN: 978-972-31-1435-5

[155] Koubaa I, Damak M, McKillop A, Simmonds M. Constituents of *Cynara cardunculus*. Fitoterapia. 1999;**70**:212-213. DOI: 10.1016/s0367-326x(99)00007-6

[156] Velez Z, Campinho M, Guerra Â, García L, Ramos P, Guerreiro O, Felício L, Schmitt F, Duarte M. Biological characterization of *Cynara cardunculus* L. methanolic extracts: Antioxidant, anti-proliferative, anti-migratory and anti-angiogenic activities. Agriculture. 2012;**2**:472-492. DOI: 10.3390/agriculture2040472

[157] Falleh H, Ksouri R, Chaieb K, Karray-Bouraoui N, Trabelsi N, Boulaaba M, Abdelly C. Phenolic composition of *Cynara cardunculus* L. organs, and their biological activities. Comptes Rendus Biologies. 2008;**331**:372-379. DOI: 10.1016/j.crvi.2008.02.008

[158] de Faria EL, Gomes MV, Cláudio AFM, Freire CS, Silvestre AJ, Freire MG. Extraction and recovery processes for cynaropicrin from *Cynara cardunculus* L. using aqueous solutions of surface-active ionic liquids. Biophysical Reviews. 2018 Jan;**2**:1-11. DOI: 10.1007/ s12551-017-0387-y

[159] Teresa Brás LAN, Crespo JG, Duarte MF. Extraction methodologies of cynaropicrin from *Cynara cardunculus* var. *altilis*. In: 6th International Congress of Aromatic Medicinal Plants (CIPAM 2016); 29 May–1 June 2016; Coimbra. 2016

[160] Bras T, Neves LA, Crespo JG, Duarte MF. Processos de extração da cinaropicrina presente nas folhas de *Cynara cardunculus* L. In: PCT/IB2017/053152. May 2017

[161] Fernández J, Manzanares P. Lignocellulosic biomass production from annual energy crops. Report EUR 12631 EN-. Comission of European Cummunities, Luxembourg. 1990. 54 p

[162] Grammelis P, Malliopoulou A, Basinas P, Danalatos NG. Cultivation and characterization of *Cynara cardunculus* for solid biofuels production in the mediterranean region. International Journal of Molecular Sciences. 2008;**9**:1241-1258. DOI: 10.3390/ijms9071241

[163] Sengo I, Gominho J, D'Orey L, Martins M, D'Almeida-Duarte E, Pereira H, Ferreira-Dias S. Response surface modeling and optimization of biodiesel production from *Cynara cardunculus* oil. European Journal of Lipid Science and Technology. 2010;**112**:310-320. DOI: 10.1002/ejlt.200900135

[164] Fernandes DLA, Pereira SR, Serafim LS, Evtuguin D, Xavier AMRB. Second Generation Bioethanol from Lignocellulosics: Processing of Hardwood Sulphite Spent Liquor. In: Prof. Marco Aurelio Pinheiro Lima, editor. Bioethanol. InTech; 2012. pp. 123-52. DOI: 10.5772/23825. Available from: http://www.intechopen.com/books/bioethanol/second-generation-bioethanol-from-lignocellulosics-processingof-hardwood-sulphite-spent-liquor

[165] Ballesteros I, Ballesteros M, Manzanares P, Negro MJ, Oliva JM, Sáez F. Dilute sulfuric acid pretreatment of cardoon for ethanol production. Biochemical Engineering Journal. 2008;**42**:84-91. DOI: 10.1016/j.bej.2008.06.001

Chapter 6

Adjusting Bioactive Functions of Dairy Products via Processing

Katrin A. Kopf-Bolanz

Additional information is available at the end of the chapter

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Abstract

Milk is known for its high nutrient content that helps to maintain important body functions. In this regard, bioactive peptides that are encrypted in milk proteins and get released during processing and/or digestion might play a role. These peptides are able to inhibit enzymes, influence cell growth, or target specific receptors. The peptide profile that arises after protein digestion in the jejunum before the absorption into the blood takes place includes these bioactive peptides. The composition of the peptide profile is influenced strongly via processing and a modification in processing might target specific functionalities. Thermal, chemical, biochemical, and physical treatments affect protein digestion mainly by changing the protein structure for example via denaturation or protease actions. Parameters influencing this are external ones, like the matrix of the product, and internal ones, like specific enzyme deficiencies. However, considering all the important aspects that are involved, there might be the possibility in the future to adjust a bioactive function via processing.

Keywords: bioactive peptides, processing, bioavailability, bioactive function, dairy products

1. Introduction

Dairy products are appreciated for their high nutritional value [1]. Not only the high contents of protein, vitamins, and minerals determine the positive health effect of dairy products. Hidden components called bioactive peptides, encrypted in parent milk proteins, exhibit special functions that might influence our well-being. So far peptides with antihypertensive, anti-oxidative, anti-thrombotic, anticancer, immune-modulatory, antimicrobial, cholesterol-lowering, antidiabetic, mineral-binding, opioid and satiety properties were identified. These peptides occur directly in the dairy products after processing and are resistant to digestion enzymes or they are encrypted in dairy proteins and get released during digestion.

Interestingly, the processing method of the dairy products can influence the number and sequence of the resulting peptides after digestion and therefore also the content of bioactive peptides. Heat treatment, chemical and biochemical, and physical treatment can influence the bioactive functionality transmitted by the selected dairy product. In the future, it might be possible to target a wished bioactive function via processing. This book chapter just deals with the effects exhibited by bioactive peptides. However, also other milk components are affected by processing and can exhibit bioactive functions, e.g. effects on the lipids, minerals, and vitamins. Furthermore, the addition of certain bioactive ingredients to dairy products is also not discussed in this chapter. The focus is given to the possible effects transmitted via bioactive peptides and the effect of processing on the peptide profile.

2. Bioactive peptides and their functionalities

The health potential of dairy protein is not only originating from the unique amino acid composition and their great bioavailability (**Figure 1**). Especially the high content of essential amino acids and their fast release as free amino acids during digestion [2], plus the high content of certain vitamins and minerals is important for the high nutritional value of bovine milk. However, there is a more hidden health potential of dairy products that is displayed by bioactive peptides. Bioactive peptides contain usually 3–20 amino acid residues and their composition and sequence determine their activity. They are encrypted in the primary sequence of proteins and get released via three different ways [3]:

1. Hydrolysis by digestive enzymes
2. Hydrolysis by proteolytic microorganisms
3. Action of proteolytic enzymes derived from microorganisms or plants

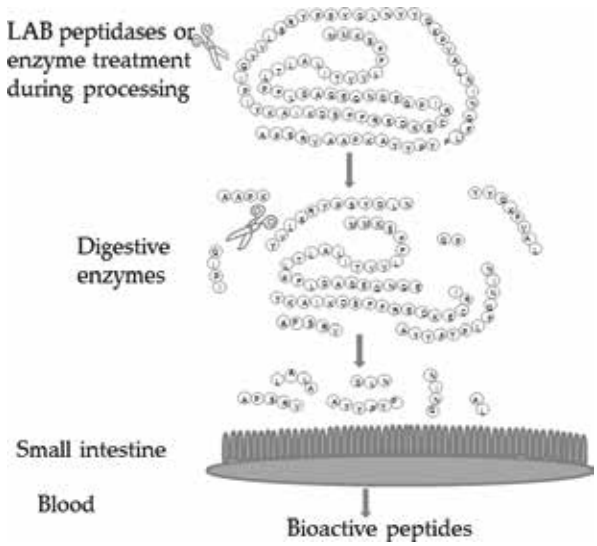

Figure 1. Illustration of milk protein degradation by lactic acid bacteria (LAB) and during digestion via digestive enzyme action resulting in bioactive peptides.

These are the ways to produce bioactive peptides that can be afterwards purified and used as ingredients for manufacturers of functional foods. However, also the more natural way of processing via hydrolysis by proteolytic microorganisms can be an approach to enrich a specific bioactive function in a product. Bioactive peptides have been discovered not only in dairy products, but also in meat, eggs, fish, and other marine organisms and also in plant sources like certain grains, legumes, pulses, and oilseeds [4–6].

The production of bioactive peptides for use as additives can be done by enzymatic hydrolysis or microbial fermentation [7]. Enzymatic hydrolysis applies digestion enzymes. Mostly trypsin, a pancreatic proteinase is used, but also chymotrypsin, pepsin thermolysin, pancreatin, elastase, carboxypeptidase or a proline-specific endopeptidase can deliver bioactive peptides. Additionally, proteases from bacteria, fungi, and plants also showed interesting properties [7]. Microbial fermentation uses bacteria or yeast that exhibit proteolytic activity to generate peptides. They are grown and added in their exponential phase to the protein of interest. The degree of hydrolysis is then dependent on the strain and its proteolytic activity. In both ways, a purification of the peptides is necessary. This can be for example reached by centrifugation methods, freeze drying, desalting, and membrane filtration techniques [8]. Examples are the production of caseinophosphopeptides from α-s-casein with an immobilized trypsin in a fluidized bed bioreactor [9] and a combination of diafiltration and anion-exchange chromatography [10]. The peptide additives can be added to a product of interest to generate a functional food. For this purpose, also the stability of the peptides with regard to pH, temperature, and food matrix has to be considered. Furthermore, the more natural way to enhance dairy products with bioactive peptides is to directly add a bacterial culture to the dairy product and generate a fermented product containing bioactive peptides. This is the general processing method applied already for each fermented dairy product. If protein is not taken out, all dairy products result in a high quantity of bioactive peptides that might be absorbed in the small intestine. For the functionality of these peptides, the selection of bacteria strains is important to aim for a specific bioactive function via processing (see Section 3).

The possible, so far detected, functionalities of bioactive peptides are summarized in **Figure 2**.

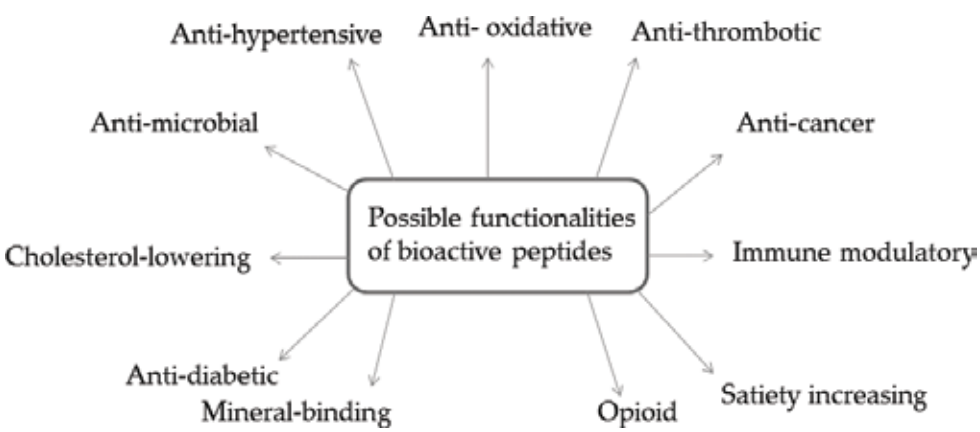

Figure 2. Possible functionalities of bioactive peptides.

2.1. Antihypertensive peptides

Antihypertensive peptides can inhibit the angiotensin I-converting enzyme (ACE) (EC 3.4.15.1;ACE) that is involved in blood pressure regulation. ACE increases the blood pressure by converting angiotensin I into the vasoconstrictor angiotensin II and additionally degrades vasodilative bradykinin. ACE-inhibitory peptides were detected in different food proteins like bovine casein and human casein, whey protein, zein, gelatin, yeast, and corn [11]. The most ACE-inhibitory peptide in studies of [28], had an IC_{50} value of 77 μM and was originating from α-lactalbumin with the peptide sequence α-lactalbumin f(104–108). Different studies showed the bioavailability of the ACE-inhibitory peptides Ile-Pro-Pro and Val-Pro-Pro in humans [12, 13]. These two tripeptides are the ones that are studied the most and show the highest evidence for their bioefficacy.

2.2. Anti-oxidative peptides

Anti-oxidative peptides help against the oxidative damage caused by reactive oxygen species. The amino acids cysteine, lysine, histidine, methionine, tryptophan, and tyrosine can act as radical scavengers [14]. Therefore, they act as potential antioxidants.

2.3. Antithrombotic peptides

The formation of blood clots can be reduced by antithrombotic peptides. Especially known is the glycomacropeptide (GMP) originating from kappa-casein after enzymatic milk coagulation. GMP can inhibit the aggregation of blood platelets and binding of the human fibrinogen gamma-chain to platelet surface fibrinogen receptors [15]. Also, the absorption into the plasma could be observed in humans for 2 anti-thrombotic peptides [16].

2.4. Anticancer peptides

Anticancer peptides can inhibit cancer cell growth. In vitro experiments with HL-60 human leukemia cells showed for example, that after skimmed milk digestion with a proteolytic enzyme from yeast *Saccharomyces cerevisiae* apoptosis can be induced [17].

2.5. Immune-modulatory peptides

Immune-modulatory peptides are mostly found in dairy products. Enzymatic hydrolysis resulted in a number of biologically active peptides that can influence immune cells and release specific signals [18]. Some peptides can stimulate or inhibit immune responses and their positive health effects have been investigated mostly in vitro. These assays are performed with immune cells and target for example proliferation, phagocytosis, differentiation, and cytokine production. A survey of these assays can be found in the review of Maestri et al. [6]. The immunomodulatory potential of peptides originating from whey protein is discussed in the study of Gauthier et al. [19]. Interestingly, there are some in vivo studies that have

demonstrated promising results. Otani et al. showed for example that feeding mice a dietary casein phosphopeptide influenced the level of serum IgA and intestinal antigen-specific IgA [20]. The exact mechanism of the action exhibited by immune-modulatory peptides still has to be determined.

2.6. Satiety peptides

Peptides that inhibit dipeptidyl peptidase 4 (DPP-IV, EC 3.4.14.5) are known as satiety increasing peptides. DPP-IV degrades the satiety regulating glucagon-like-peptide 1 [21]. Kopf-Bolanz et al, monitored the fates of specific peptides with known satiety increasing action. They compared different dairy products and found the relative abundance of three potent DPP-IV inhibitory peptides deriving from β-casein. The best source for these peptides was Gruyere cheese. Two other peptides deriving from α-S1-casein were not detectable anymore after the pancreatic phase of the digestion [22]. Tryptophan seems to be an important amino acid in peptides that exhibit a DPP-IV inhibitory potential [23]. Three dipeptides containing tryptophan Trp-Arg, Trp-Lys, and Trp-Leu with half maximum inhibitory concentrations (IC_{50}) < 45 μM could be detected in a study of Nongonierma and Fitzgerald that are potent inhibitors of DPP-IV [24].

Another interesting peptide that has an influence on satiety is the glycomacropeptide (GMP) resulting from cheese production. It has demonstrated in several animals and human studies that it can stimulate the release of cholecystokinin and promote satiety. However, further studies would be necessary to demonstrate a clear bioefficacy [25, 26]. Peptides that increase the satiety are also known as anti-obesity peptides [27].

2.7. Opioid peptides

Peptides that have an affinity for the opioid receptor are categorized in this group. There are receptors that are responsible for specific physiological effects like emotional behavior and food intake. Opioid peptides have the same N-terminal sequence Tyr-Gly-Gly-Phe. There are also atypical opioid peptides with the ending of Tyr-X-Phe or Tyr-X1-X2-Phe. A tyrosine residue at the N-terminal and another aromatic amino acid at the third or fourth position are specific binding motifs of the opioid receptor. The first food-derived opioid peptides were β-casomorphins. Also, casoxins, lactorphins, and exorphins can bind to opioid receptors [28]. So far, a weak opioid activity for α-lactorphin (α-lactalbumin f(50–53)) and β-lactorphin (β-lactoglobulin f(102–105)) was detected in guinea pigs [11], but human data are still missing. Concentrations released from in vivo digestion of milk are quite low. The total amount of α-lactorphin and β-lactorphin in 1 L of bovine milk would be 32 mg (64 μmol), respectively 90 mg (162 μmol), but it might be difficult to obtain a full release of the possible peptide during in vivo digestion. It is so far not clear whether they can get liberated by in vivo digestion at all, but it was demonstrated that casomorphins are liberated in vivo [11].

2.8. Antidiabetic peptides

Diabetes is treated by synthetic antidiabetic drugs that can result in side effects like hypoglycemia or weight gain [8]. To overcome this issue, the application of antidiabetic peptides originating from food sources might be a solution. Antidiabetic peptides could be for example detected in sheep milk [29].

2.9. Mineral-binding peptides

Mineral-binding phosphopeptides can carry different minerals by forming soluble organophosphate salts [30]. Caseinophosphopeptides (CPP) can increase calcium absorption by limiting calcium precipitation in the ileum. Caseins are phosphorylated in the mammary gland at primary sequences rich in serine and glutamic acid forming triplet regions SerP-SerP-SerP-Glu-Glu that occur in α-S1-casein (66–70), α-S2-casein (8–12), (56–60), (129–133) and β-casein (15–19). The presence of CPPs has been shown in vivo. Several animal studies have demonstrated the effect of CPP to enhance calcium bioavailability. In contrast, convincing results from human are still missing [31]. A human study with CPP-enriched preparations (containing candidate functional food ingredients) on calcium absorption from a calcium lactate drink showed no significant results [32]. Another interesting peptide is lactoferricin consisting of 25 amino acid residues. The molecule is folded into two globular units, each capable of binding one ferric (Fe^{3+}) ion [33].

2.10. Cholesterol-lowering peptides

So far mainly peptides derived from soy proteins have been shown to suppress cholesterol in the blood. Some can for example target the cholesterol receptor or suppress the presence of LDL. Important for the functions are mainly the hydrophobic residues [34]. A novel peptide (Ile-Ile-Ala-Glu-Lys) from a trypsin-treated hydrolysate of β-lactoglobulin showed a hypocholesterolemic effect in an animal study [35].

2.11. Antimicrobial peptides

Peptides that induce for example the lysis of bacterial membranes are antimicrobial peptides. They could be detected in α-lactalbumin, β-lactoglobulin, all casein fractions, and lactoferrin [36].

2.12. Safety issues

The safety and toxicity of bioactive peptides has to be considered. Different studies demonstrated that casein hydrolysates and Val-Pro-Pro from powdered fermented milk did not show any toxicological potential [37–39]. Processing can lead to Maillard reaction and result in the production of allergenic compounds [40]. Processing changes the protein structure and might influence the protein degradation and therefore also the response of the immune system. Therefore, it is important to determine the allergic potential that can arise from bioactive peptides. If fermentation takes place, for example, de novo peptides might originate and their allergenic potential has to be determined. First, a comparison with already known allergenic sequences can be done, followed by laboratory tests. The problem is that allergenic sequences

can occur over the whole dairy protein sequences, and there can be rare cases that people are allergic to a new peptide sequence arising from fermentation. However so far, mostly positive reports about the effect of fermentation are published [41, 42]. It is also important to mention that these functionalities were observed to a great extent with in vitro methods. Only very few human studies have demonstrated an effect of bioactive peptides in vivo.

2.13. Physiology of digestion

To exhibit really a bioactive function in vivo, the peptides must be released during digestion from their originating protein or if they are already in the product as such, they have to be resistant to digestion enzymes. During digestion, the proteins get denatured by gastric acidification and subsequently degraded by pepsin and pancreatic peptidases like trypsin into peptides and amino acids. Furthermore, the final actions of the enzymes at the brush-border membrane in the small intestine have to be taken into account. There are peptidases that cleave amino acids or dipeptides from the N- or C-terminal of the interior bond of the oligopeptides. The mean size of the peptides in the jejunum considering the action of aminopeptidases and dipeptidases from the enterocytes is 3–6 amino acids. Di- and tripeptides can be transported actively by the peptide transporter PEPT1 [43]. Longer peptides can probably get absorbed ether via paracellular or transcellular pathways. The possible transport of a heptapeptide was shown using a cell culture model [44]. In the blood, the peptides must be able to reach their target site in the peripheral organs. In a human study of van Platerink et al., 17 ACE-inhibiting peptides with 5–6 amino acids length could be detected in the blood after consumption of drinks enriched with those peptides [13]. The first proof that the tripeptide Ile-Pro-Pro does not undergo intestinal degradation and can reach the circulation intact was shown from Foltz et al. [12]. Another human study showed the presence of a longer peptide after soybean consumption in the blood [45]. At the target cells, it is assumed that peptides can internalize via endocytosis and get digested in the lysosome. Peptides that do not enter target cells can accumulate in the liver and kidney and can be detected in urine or bile [6]. There is still the need to demonstrate a clear bioefficacy of the peptides and confirm the positive health effects in human studies. In the future possibly health claims for certain bioactive peptides could be developed. So far Japan declared certain antihypertensive peptides such as Val-Pro-Pro, Ile-Pro-Pro, Val-Tyr, and Cys-Pro-Pro as Food of Specific Health Use (FOSHU). In contrast, the European Food and Drug Association (EFSA) did not authorize any claims regarding the effect of bioactive peptides in foods yet [46].

2.14. Detection of bioactive peptides

Experiments concerning bioactive peptides are mainly done in vitro. Most of the time, a dairy product is inserted into an in vitro digestion model that mimics human digestion.

There are numerous in vitro digestion models that can be applied. It is important that a model close to human physiology and validated is applied. Recently, a harmonized digestion model was established during the COST digestion action. This model is very physiological and might be used for mimicking digestion [47]. The resulting peptides generated during the digestion process can be detected by peptidomic methods. Analytics of bioactive peptides aim toward three main directions [48]:

1. Tracing the pathways of formation of bioactive peptides from the parent proteins
2. Identifying the biological properties
3. Improving the "positive" properties discovered in natural peptides by design of synthetic structural analogues or peptide mimetics

Peptidomics is the comprehensive qualitative and quantitative analysis of all peptides in a biological sample. In earlier days, protein digestion could be followed by HPLC or Edman sequencing [49]. Nowadays, MS-based techniques such as Liquid chromatography coupled to mass spectrometry (LC-MS) can be applied [22, 50]. Peptidomics of food hydrolysates, for example, led to the discovery of the exact sites of rennet cleavage on kappa-casein or the cleavage sites produced by bacteria during cheese ripening [49]. The detailed human study of Boutrou et al. was identified in the jejuna effluents of healthy adults, after consumption of 30 g milk casein and whey proteins, 356 and 146 peptides [50]. The in vitro model developed by Minekus et al., almost resulted in similar peptides [47]. The different analytical approaches that can be applied are summarized in the review of Dallas et al. [49]. Technology allows the prediction of the peptide sequence and can generate a peptide fingerprint. The peptides can be then compared to the known bioactive peptides from the literature in various databases. An example is the milk bioactive peptide database by Nielsen et al. [51]. This database comprises information on bioactive peptides from across hundreds of original research articles and is available to the public. Furthermore, whole in silico strategies for bioactive function generation including computational modeling might be applied, that still have limitations, but might be used in the future for the design of new products.

3. Influence of processing on protein digestion and peptide profile

Dairy products are processed by the application of different physical and chemical methods. These methods change the protein structure irreversible or reversible depending on the impact of the treatment. The protein can be mainly denatured, hydrolysed, or glycosylated. This structural change can influence the access of the digestion enzymes to the protein and therefore changes the action of the digestion enzymes. An impact on the peptide profile that is generated before absorption into the blood takes place is the result. It is necessary to determine which processing methods and which processing variables are necessary to be able to reach or maintain a specific bioactivity.

3.1. Thermal treatment

Thermal processing is an important step to improve the microbial quality of milk. Additionally, enzyme activities are inactivated and some physicochemical changes can occur that might support processing. The nutritional value is greatly affected by thermal processing. Denaturation, β-elimination, racemization, or iso-peptide bond formation can occur that influence the nutritional value [52]. Denaturation is influenced by pH, protein concentration, ionic environment, genetic variant, and presence of ligands [53]. Heating might even particularly destroy tryptophan, can convert Arginine into citrulline and ornithine, can deamidate glutamine and

asparagine, and desulphur cysteine and cysteine. Resulting end products might be lanthionine, lysine-alanine, iso-peptides and ornitho-alanine [52]. The digestibility of whey proteins increases after thermal treatment because the sites for enzymatic hydrolysis are easier to reach for the digestive enzymes. However, strong denaturation reduces digestibility [54]. Kopf-Bolanz et al. showed that heat treatment of dairy products led to an increased number of β-lactoglobulin peptides after in vitro digestion [22]. There is a greater susceptibility to hydrolysis following heat treatment [55]. Regarding the antidiabetic action of casein, there was a significant reduction observed after boiling compared to the raw casein [29]. The denaturation of whey protein via thermal processing led to an increase in the antibacterial activity of α-lactalbumin [56] and lysozyme [57]. The antioxidant action of whey proteins can be maintained by low-temperature processing. This results in high levels of specific dipeptides that can promote the synthesis of the antioxidant glutathione [58]. Extrusion cooking might also affect protein digestibility shown for example in a study of Onwulata et al. [59]. Data on the effect of ohmic heating are rare. Depending on the used temperatures, similar effects like with application of other heating methods might be expected [52]. It was also shown that spray drying or freeze drying did not exhibit negative effects on the immunomodulatory activity of a whey protein hydrolysate. The study also used whey protein concentrate (WPC) and sodium alginate as carriers for encapsulation to reduce bitter taste and resistance to hygroscopicity. They showed that spray drying of whey protein concentrate hydrolysate with the proper carriers did not affect the immunomodulatory activity and might therefore widen its application in food systems [60].

3.2. Chemical treatment

Hydrolysis by acid is applied which is known to improve their protein digestibility. It is used for example for enteral and hypoallergenic infant nutrition. For Mozzarella, the type of acid used is important for the protein yield obtained in the pre-cheeses [61] and might therefore also affect the profile of bioactive peptides. Treatment with alkali for hydrolysis is rarely applied in the food industry. It would result in racemization and loss of protein digestibility [62].

3.3. Biochemical treatment

Fermented dairy products like yoghurt and cheese result in a high number of bioactive peptides produced by the lactic acid bacteria. Especially the type of the starter culture, type of probiotic bacteria, and the fermentation parameters play an important role for the bioactive effect that the product might have. Furthermore, only via this way de novo peptides can be generated that do not occur after digestion of milk as such. *Streptococcus thermophilus* and *Lactobacillus bulgaricus* possess bacterial activity against Streptococci in vivo that probably derives from the antimicrobial peptides that they produce during fermentation [63]. It is very promising to test different lactic acid bacteria strains for their effect on a bioactive function. One study of Gobbetti et al. showed that a fermentation with *L. delbrueckii* ssp. *Bulgaricus SS1* versus a fermentation with *Lactobacillus lactis* subspecies *cremoris FT4* resulted in a higher ACE-inhibitory activity [64]. The most investigated ACE-inhibitory peptides were obtained after fermentations with *L. helveticus* and *L. helveticus CP790*. Also, the Finnish milk product Evolus contained *L. helveticus LBK-16H* strain as a starter and

they all contained the tripeptide IPP and exerted a hypertensive effect [65–67]. Another study demonstrated the effect of the time of cheese ripening on the ACE-inhibitory activity. Cheese was produced with a mixture of 12 different strains and showed an increase of the inhibitory effect during ripening as long as a certain level of proteolysis was not exceeded [68]. In 10 Swiss cheese types, the ACE-inhibiting peptides V-P-P and I-P-P were quantified. They detected contents of 19.1 mg/kg to 182.2 mg/kg depending on the cheese type that shows the huge effect of different processing ways probably via different lactic acid bacteria [69]. Also, the application of new techniques like next-generation sequencing that reveals the whole genome of bacteria strains might help to select promising strains with specific protease expressions. It was also demonstrated that fermentation reduced the allergenic potential of α-lactalbumin and β-lactoglobulin [41, 42]. The peptides that result after fermentation and enzyme hydrolysis might remain susceptible to further hydrolysis as long as the process goes on. This might lead to a decrease of bioactive function of these peptides. More important is also the stability of the generated peptides. They might be degraded by the digestive enzymes and result in zero activity in the body. The stability versus the action of gastric and pancreatic enzymes has to be tested beforehand. Another problematic point is that the microbial fermentations have to be reproducible [8]. Fermentation with known and established lactic acid bacteria cultures is a great strategy to enrich certain bioactive peptides with a special functionality. This would be a possibility to enhance a bioactive function in a natural way with a minimal processing approach that meets the interests of the consumer. The functionality and bioavailability of bioactive peptides generated via fermentation has to be more clarified.

The use of milk-clotting enzymes and digestive enzymes to produce bioactive peptides is another processing approach. However, most of the resulting peptides had a bitter taste [7]. Membrane-separation technique is applied to enrich peptides with a specific molecular weight [3]. It was also shown that hydrolysed infant formulas show a different peptide profile compared to the standard formulas assuming that infants fed hydrolysed formulas might obtain bioactive peptides that promote other bioactive functions than the ones provided by the standard formulas [70].

3.4. Physical treatment

Homogenization applies pressure (14–18 MPa) and shear stress that alter the protein structure and improve the digestibility [52]. Use of ultra-high pressure homogenization with pressure around 400 MPa results in more severe protein denaturation [71]. Application of high hydrostatic pressure processing increased digestibility of β-lactoglobulin with pepsin with increasing pressures (400–800 MPa) [72]. Penas et al. also combined high hydrostatic pressure processing with selected food-grade proteases and demonstrated a reduction in antigenicity of the whey protein hydrolysates that can be used as ingredients of hypoallergenic infant formulae [73]. Ultrasound treatment is a non-conventional processing technique that can denature α-lactalbumin and β-lactoglobulin. In whole milk compared to skim milk, the denaturation was stronger and heat addition even increased this effect [74]. A very soft technology is membrane filtration that enables to separate proteins in their native state. This technology only enables a fractionation of different milk components and does not alter the protein structure as such, and it only influences the milk composition.

4. Influence of other factors on bioavailability of bioactive peptides

Not only processing can influence the profile of bioactive peptides. Also, other external factors can influence protein digestion and therefore the bioavailability and generation of bioactive peptides. It is important to consider the effect of the food matrix and meal composition on digestion. For example, the addition of inulin to the dairy product can influence digestion and peptide bioavailability [75]. Also, proteins can form complexes with polyphenols, etc. that could lower protein bioavailability [76]. Furthermore, internal factors can influence the peptide profile. Children and the elderly have different enzyme activities and therefore the digestion enzymes will act slightly different and change the peptide profile [77, 78]. Genetic variations in people for example enzyme deficiencies or changes in the composition of the digestion juices due to different transporter expression can have an impact. The action of digestion enzymes depends on daytime, age and on *Helicobacter pylori* infection [79]. Also for a lot of other special physiological states, certain diseases and so on, the enzyme activity is affected and might therefore result in a different bioactive peptide bioavailability. It is very important to consider all the factors that can affect peptide bioavailability in the target group of the product.

5. Possible approach for design of a dairy product with a satiety increasing effect

An enrichment of peptides that can inhibit DPP-IV could result in increasing satiety after consumption of a dairy product. The most promising approach to steer the peptide profile of the

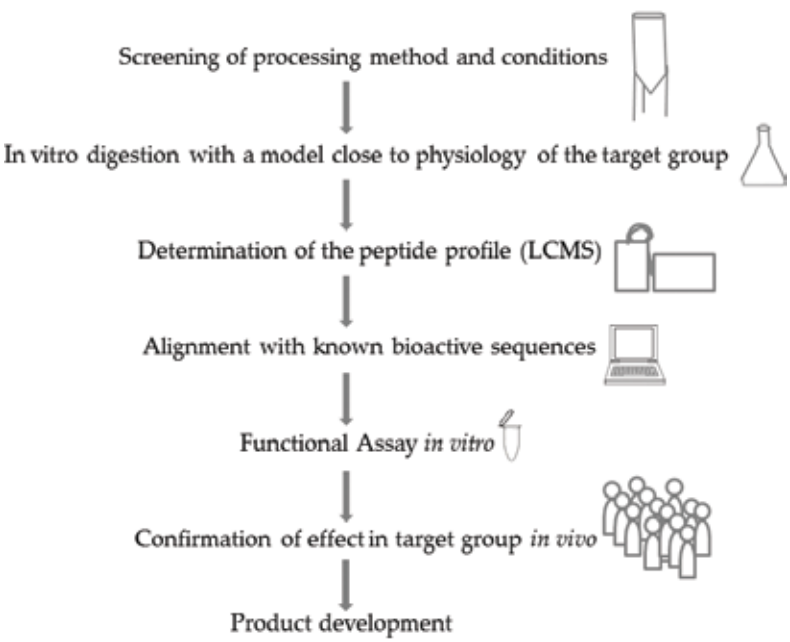

Figure 3. Targeting bioactive function in product development.

product is via fermentation with lactic acid bacteria. It would be promising to test different starter bacteria with different protease activities. Differences in their proteolytic action result in different peptide profiles. Then, an in vitro digestion model might be applied that is mimicking the digestion physiology of the target group. Next step would be peptide profiling and alignment of the results with already known sequences of DPP-IV inhibitory peptides. Furthermore, the inhibitory effect could be also tested using directly an enzyme assay. It is important to consider also all the other processing parameters that can affect protein digestion. Finally, the bioactive effect has to be confirmed in humans that represent the target group of the product (**Figure 3**).

6. Conclusion

After ingestion of food containing protein, a peptide pattern is generated that probably contains bioactive sequences and is present in the jejunum before absorption takes place. Especially for dairy products, many different bioactive peptides could be identified that might be very interesting for the development of products with a specific functionality. The peptide patterns are strongly influenced by processing. Thermal treatments are used in general to ensure the microbial quality of dairy proteins. They exhibit a great influence on the protein structure for example by protein unfolding. Easier access is then given to the digestion enzymes and the resulting peptide profile is changed. Highly interesting is the fermentation of dairy products with different lactic acid bacteria strains. Certain strains have different protease activities and increase for example the number of antihypertensive peptides resulting after digestion. The angiotensin-converting enzyme inhibition is the most studied functionality and there are reports that could detect bioavailable peptides in the blood. The peptide concentrations reached for example in cheeses are promising to exhibit a bioactive function. Chemical and physical approaches can also influence the protein structure and therefore the protein digestion. The impact of new processing techniques on protein digestion should be always monitored. For the adjustment of a specific bioactive function in a product, an example approach is mentioned. However, it will always also depend on other factors whether the wished functionality is really reached or not. External factors like the meal composition and internal factors like age or genetic preconditions can also have an impact and have to be considered. Furthermore, for safety reasons, there is the small chance that generated de-novo peptides might act as epitopes for rare cases of cow milk allergy. However so far, fermentation with established lactic acid bacteria strains seems to reduce the allergenic potential of dairy products in general. In the future, it is necessary to perform well designed human studies that ensure a bioactive effect and allow the admission of health claims.

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References

[1] Haug A, Høstmark AT, Harstad OM. Bovine milk in human nutrition—A review. Lipids in Health and Disease. 2007;**6**:25. DOI: 10.1186/1476-511X-6-25

[2] Kopf-Bolanz KA, Schwander F, Gijs M, Vergères G, Portmann R, Egger L. Validation of an in vitro digestive system for studying macronutrient decomposition in humans. The Journal of Nutrition. 2012;**142**:245-250. DOI: 10.3945/jn.111.148635

[3] Korhonen H, Pihlanto A. Bioactive peptides: Production and functionality. International Dairy Journal. 2006;**16**:945-960. DOI: 10.1016/j.idairyj.2005.10.012

[4] Hartmann RMH. Food-derived peptides with biological activity: From research to food applications. Current Opinion in Biotechnology. 2007;**18**:163-169. DOI: 10.1016/j.copbio.2007.01.013

[5] Kim S-K, Wijesekara I. Development and biological activities of marine-derived bioactive peptides: A review. Journal of Functional Foods. 2010;**2**:1-9. DOI: 10.1016/j.jff.2010.01.003

[6] Maestri E, Marmiroli M, Marmiroli N. Bioactive peptides in plant-derived foodstuffs. Journal of Proteomics. 2016;**147**:140-155. DOI: 10.1016/j.jprot.2016.03.048

[7] Choi J, Sabikhi L, Hassan A, Anand S. Bioactive peptides in dairy products. International Journal of Dairy Technology. 2012;**65**:1-12. DOI: 10.1111/j.1471-0307.2011.00725.x

[8] Daliri EB-M, Oh DH, Lee BH. Bioactive peptides. Foods. 2017;**6**(5):32. DOI: 10.3390/foods6050032

[9] Park O, Allen JC. Preparation of phosphopeptides derived from αs-casein and β-casein using immobilized glutamic acid-specific endopeptidase and characterization of their calcium binding. Journal of Dairy Science. 1998;**81**:2858-2865. DOI: 10.3168/jds.S0022-0302(98)75845-X

[10] Ellegård KH, Gammelgård-Larsen C, Sørensen ES, Fedosov S. Process scale chromatographic isolation, characterization and identification of tryptic bioactive casein phosphopeptides. International Dairy Journal. 1999;**9**:639-652. DOI: 10.1016/S0958-6946(99)00135-1

[11] Pihlanto-Leppälä A, Paakkari I, Rinta-Koski M, Antila P. Bioactive peptide derived from in vitro proteolysis of bovine β-lactoglobulin and its effect on smooth muscle. The Journal of Dairy Research. 1997;**64**:149-155. DOI: 10.1017/S0022029996001926

[12] Foltz M, Meynen EE, Bianco V, van Platerink C, Koning TM, Kloek J. Angiotensin converting enzyme inhibitory peptides from a lactotripeptide-enriched milk beverage are absorbed intact into the circulation. The Journal of Nutrition. April. 2007;**137**:953-958

[13] van Platerink CJ, Janssen H-GM, Horsten R, Haverkamp J. Quantification of ACE inhibiting peptides in human plasma using high performance liquid chromatography-mass spectrometry. Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences. 2006;**830**:151-157. DOI: 10.1016/j.jchromb.2005.10.036

[14] Udenigwe CC, Aluko RE. Food protein-derived bioactive peptides: Production, processing, and potential health benefits. Journal of Food Science. 2012;**77**:R11-R24. DOI: 10.1111/j.1750-3841.2011.02455.x

[15] Fiat A-M, Migliore-Samour D, Jollès P, Drouet L, Sollier CBD, Caen J. Biologically active peptides from milk proteins with emphasis on two examples concerning antithrombotic and Immunomodulating activities. Journal of Dairy Science. 1993;**76**:301-310. DOI: 10.3168/jds.S0022-0302(93)77351-8

[16] Chabance B, Marteau P, Rambaud JC, Migliore-Samour D, Boynard M, Perrotin P, Guillet R, Jollès P, Fiat AM. Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. Biochimie. 1998;**80**:155-165

[17] Roy MK, Watanabe Y, Tamai Y. Induction of apoptosis in HL-60 cells by skimmed milk digested with a proteolytic enzyme from the yeast *Saccharomyces cerevisiae*. Journal of Bioscience and Bioengineering. 1999;**88**:426-432. DOI: 10.1016/S1389-1723(99)80221-7

[18] Gill HS, Doull F, Rutherfurd KJ, Cross ML. Immunoregulatory peptides in bovine milk. British Journal of Nutrition. 2000;(Suppl 1):S111-S117

[19] Gauthier SF, Pouliot Y, Saint-Sauveur D. Immunomodulatory peptides obtained by the enzymatic hydrolysis of whey proteins. International Dairy Journal. 2006;**16**:1315-1323. DOI: 10.1016/j.idairyj.2006.06.014

[20] Otani H, Kihara Y, Park M. The Immunoenhancing property of a dietary casein phosphopeptide preparation in mice. Food and Agricultural Immunology. 2010;**12**:165-173. DOI: 10.1080/095401000404102

[21] Darmoul D, Voisin T, Couvineau A, Rouyerfessard C, Salomon R, Wang YX, et al. Regional expression of epithelial dipeptidyl peptidase IV in the human intestines. Biochemical and Biophysical Research Communications. 1994;**203**:1224-1229. DOI: 10.1006/bbrc.1994.2313

[22] Kopf-Bolanz KA, Schwander F, Gijs M, Vergères G, Portmann R, Egger L. Impact of milk processing on the generation of peptides during digestion. International Dairy Journal. 2014;**35**:130-138. DOI: 10.1016/j.idairyj.2013.10.012

[23] Sila A, Martinez-Alvarez O, Haddar A, Gómez-Guillén MC, Nasri M, Montero MP, Bougatef A. Recovery, viscoelastic and functional properties of Barbel skin gelatine: Investigation of anti-DPP-IV and anti-prolyl endopeptidase activities of generated gelatine polypeptides. Food Chemistry. 2015;**168**:478-486. DOI: 10.1016/j.foodchem.2014.07.086

[24] Nongonierma AB, Fitzgerald RJ. Inhibition of dipeptidyl peptidase IV (DPP-IV) by tryptophan containing dipeptides. Food & Function. 2013;**4**:1843-1849. DOI: 10.1039/c3fo60262a

[25] Beucher S, Levenez F, Yvon M, Corring T. Effects of gastric digestive products from casein on CCK release by intestinal cells in rat. The Journal of Nutritional Biochemistry. 1994;**5**:578-584. DOI: 10.1016/0955-2863(94)90012-4

[26] Yvon M, Beucher S, Guilloteau P, Le Huerou-Luron I, Corring T. Effects of caseinomacropeptide (CMP) on digestion regulation. Reproduction, Nutrition, Development. 1994;**34**:527-537. DOI: 10.1051/rnd:19940602

[27] Ricci-Cabello I, Herrera MO, Artacho R. Possible role of milk-derived bioactive peptides in the treatment and prevention of metabolic syndrome. Nutrition Reviews. 2012;**70**:241-255. DOI: 10.1111/j.1753-4887.2011.00448.x

[28] Pihlanto-Leppälä A. Bioactive peptides derived from bovine whey proteins. Trends in Food Science & Technology. 2000;**11**:347-356. DOI: 10.1016/S0924-2244(01)00003-6

[29] Jan F, Kumar S, Jha R. Effect of boiling on the antidiabetic property of enzyme treated sheep milk casein. Veterinary World. 2016;**9**:1152-1156. DOI: 10.14202/vetworld.2016.1152-1156

[30] Park YW, Nam MS. Bioactive peptides in milk and dairy products: A review. Korean Journal for Food Science of Animal Resources. 2015;**35**:831-840. DOI: 10.5851/kosfa.2015.35.6.831

[31] Meisel H, FitzGerald J. Biofunctional peptides from milk proteins: Mineral binding and cytomodulatory effects. CPD. 2003;**9**:1289-1295. DOI: 10.2174/1381612033454847

[32] Teucher B, Majsak-Newman G, Dainty JR, McDonagh D, FitzGerald RJ, Fairweather-Tait SJ. Calcium absorption is not increased by caseinophosphopeptides. The American Journal of Clinical Nutrition. 2006;**84**:162-166

[33] Shah NP. Effects of milk-derived bioactives: An overview. BJN. 2000;**84**:58. DOI: 10.1017/S000711450000218X

[34] Howard A, Udenigwe CC. Mechanisms and prospects of food protein hydrolysates and peptide-induced hypolipidaemia. Food & Function. 2013;**4**:40-51. DOI: 10.1039/c2fo30216k

[35] Nagaoka S, Futamura Y, Miwa K, Awano T, Yamauchi K, Kanamaru Y, et al. Identification of novel hypocholesterolemic peptides derived from bovine milk beta-lactoglobulin. Biochemical and Biophysical Research Communications. 2001;**281**:11-17. DOI: 10.1006/bbrc.2001.4298

[36] Clare D, Catignani G, Swaisgood H. Biodefense properties of milk: The role of antimicrobial proteins and peptides. CPD. 2003;**9**:1239-1255. DOI: 10.2174/1381612033454874

[37] Maeno M, Mizuno S, Mennear JH, Bernard BK. Studies of the toxicological potential of tripeptides (L-valyl-L-prolyl-L-proline and L-isoleucyl-L-prolyl-L-proline): VIII. Assessment of cytotoxicity and clastogenicity of tripeptides-containing casein hydrolysate and Lactobacillus helveticus-fermented milk powders in Chinese hamster lung cells. International Journal of Toxicology. 2005;**24**(Suppl 4):97-105. DOI: 10.1080/10915810500259663

[38] Nakamura Y, Bando I, Mennear JH, Bernard BK. Studies of the toxicological potential of tripeptides (L-valyl-L-prolyl-L-proline and L-isoleucyl-L-prolyl-L-proline): IV. Assessment of the repeated-dose toxicological potential of synthesized L-valyl-L-prolyl-L-proline in male and female rats and dogs. International Journal of Toxicology. 2005;**24**(Suppl 4):25-39. DOI: 10.1080/10915810500259580

[39] Bernard BK, Nakamura Y, Bando I, Mennear JH. Studies of the toxicological potential of tripeptides (L-valyl-L-prolyl-L-proline and L-isoleucyl-L-prolyI-L-proline): II. Introduction. International Journal of Toxicology. 2005;**24**(Suppl 4):5-11. DOI: 10.1080/10915810500259531

[40] Davis PJ, Smales CM, James DC. How can thermal processing modify the antigenicity of proteins? Allergy. 2001;**56**:56-60. DOI: 10.1034/j.1398-9995.2001.00918.x

[41] Bu G, Luo Y, Zhang Y, Chen F. Effects of fermentation by lactic acid bacteria on the antigenicity of bovine whey proteins. Journal of the Science of Food and Agriculture. 2010;**90**:2015-2020. DOI: 10.1002/jsfa.4046

[42] Ahmadova A, El-Ghaish S, Choiset Y, Rabesona H, Drouet M, Chobert J-M, Kuliev AA, Haertle T. Modification of IgE binding to β- and α S1-caseins by proteolytic activity of Lactobacillus helveticus A75. Journal of Food Biochemistry. 2013;**37**:491-500. DOI: 10.1111/j.1745-4514.2012.00664.x

[43] Daniel H. Molecular and integrative physiology of intestinal peptide transport. Annual Review of Physiology. 2004;**66**:361-384. DOI: 10.1146/annurev.physiol.66.032102.144149

[44] Vermeirssen V, Deplancke B, Tappenden KA, van Camp J, Gaskins HR, Verstraete W. Intestinal transport of the lactokinin Ala-Leu-Pro-Met-is-Ile-Arg through a Caco-2 Bbe monolayer. Journal of Peptide Science. 2002;**8**:95-100. DOI: 10.1002/psc.371

[45] Dia VP, Torres S, de Lumen BO, Erdman JW, de Mejia EG. Presence of lunasin in plasma of men after soy protein consumption. Journal of Agricultural and Food Chemistry. 2009;**57**:1260-1266. DOI: 10.1021/jf803303k

[46] Walther B, Sieber R. Bioactive proteins and peptides in foods. International Journal for Vitamin and Nutrition Research. 2011;**81**:181-192. DOI: 10.1024/0300-9831/a000054

[47] Minekus M, Alminger M, Alvito P, Ballance S, Bohn T, Bourlieu C, et al. A standardised static in vitro digestion method suitable for food - an international consensus. Food & Function. 2014;**5**:1113-1124. DOI: 10.1039/c3fo60702j

[48] Mamone G, Picariello G, Caira S, Addeo F, Ferranti P. Analysis of food proteins and peptides by mass spectrometry-based techniques. Journal of Chromatography. A. 2009;**1216**:7130-7142. DOI: 10.1016/j.chroma.2009.07.052

[49] Dallas DC, Guerrero A, Parker EA, Robinson RC, Gan J, German JB, et al. Current peptidomics: Applications, purification, identification, quantification, and functional analysis. Proteomics. 2015;**15**:1026-1038. DOI: 10.1002/pmic.201400310

[50] Boutrou R, Gaudichon C, Dupont D, Jardin J, Airinei G, Marsset-Baglieri A, et al. Sequential release of milk protein-derived bioactive peptides in the jejunum in healthy humans. The American Journal of Clinical Nutrition. 2013;**97**:1314-1323. DOI: 10.3945/ajcn.112.055202

[51] Nielsen SD, Beverly RL, Qu Y, Dallas DC. Milk bioactive peptide database: A comprehensive database of milk protein-derived bioactive peptides and novel visualization. Food Chemistry. 2017;**232**:673-682. DOI: 10.1016/j.foodchem.2017.04.056

[52] Borad SG, Kumar A, Singh AK. Effect of processing on nutritive values of milk protein. Critical Reviews in Food Science and Nutrition. 2017;**57**:3690-3702. DOI: 10.1080/10408398.2016.1160361

[53] Relkin P, Eynard L, Launay B. Thermodynamic parameters of β-lactoglobulin and α-lactalbumin. A DSC study of denaturation by heating. Thermochimica Acta. 1992;**204**:111-121. DOI: 10.1016/0040-6031(92)80320-V

[54] alKanhal HA, Al-Othman AA, Hewedi FM. Changes in protein nutritional quality in fresh and recombined ultra high temperature treated milk during storage. International Journal of Food Sciences and Nutrition. 2001;**52**:509-514

[55] Barbé F, Ménard O, Le Gouar Y, Buffière C, Famelart M-H, Laroche B, et al. The heat treatment and the gelation are strong determinants of the kinetics of milk proteins digestion and of the peripheral availability of amino acids. Food Chemistry. 2013;**136**:1203-1212. DOI: 10.1016/j.foodchem.2012.09.022

[56] Agyei D, Ongkudon CM, Wei CY, Chan AS, Danquah MK. Bioprocess challenges to the isolation and purification of bioactive peptides. Food and Bioproducts Processing. 2016;**98**:244-256. DOI: 10.1016/j.fbp.2016.02.003

[57] Takahashi H, Tsuchiya T, Takahashi M, Nakazawa M, Watanabe T, Takeuchi A, et al. Viability of murine norovirus in salads and dressings and its inactivation using heat-denatured lysozyme. International Journal of Food Microbiology. 2016;**233**:29-33. DOI: 10.1016/j.ijfoodmicro.2016.06.006

[58] Bounous GGP. The biological activity of undenatured dietary whey proteins: Role of glutathione. Clinical and Investigative Medicine. 1991;**14**:296-309

[59] Onwulata CI, Konstance RP, Cooke PH, Farrell HM. Functionality of extrusion—Texturized whey proteins. Journal of Dairy Science. 2003;**86**:3775-3782. DOI: 10.3168/jds.S0022-0302(03)73984-8

[60] Ma J-J, Mao X-Y, Wang Q, Yang S, Zhang D, Chen S-W, Li Y-H. Effect of spray drying and freeze drying on the immunomodulatory activity, bitter taste and hygroscopicity of hydrolysate derived from whey protein concentrate. LWT—Food Science and Technology. 2014;**56**:296-302. DOI: 10.1016/j.lwt.2013.12.019

[61] Seth K, Bajwa U. Effect of acidulants on the recovery of milk constituents and quality of Mozzarella processed cheese. Journal of Food Science and Technology. 2015;**52**:1561-1569. DOI: 10.1007/s13197-013-1176-7

[62] Hayashi RKI. Decreased proteolysis of alkali-treated protein: Consequences of racemization in food processing. Journal of Food Science. 1980;**45**:1430-1431. DOI: 10.1111/j.1365-2621.1980.tb06572.x

[63] Petti S, Tarsitani G, Simonetti D'Arca A. Antibacterial activity of yoghurt against viridans streptococci in vitro. Archives of Oral Biology. 2008;**53**:985-990. DOI: 10.1016/j.archoralbio.2008.04.009

[64] Gobbetti M, Ferranti P, Smacchi E, Goffredi F, Addeo F. Production of angiotensin-I-converting-enzyme-inhibitory peptides in fermented milks started by Lactobacillus delbrueckii subsp. bulgaricus SS1 and Lactococcus lactis subsp. cremoris FT4. Applied and Environmental Microbiology. 2000 Sep;**66**:3898-3904

[65] Jauhiainen T, Vapaatalo H, Poussa T, Kyrönpalo S, Rasmussen M, Korpela R. Lactobacillus helveticus fermented milk lowers blood pressure in hypertensive subjects in 24-h ambulatory blood pressure measurement. American Journal of Hypertension. 2005;**18**:1600-1605. DOI: 10.1016/j.amjhyper.2005.06.006

[66] Yamamoto N, Akino A, Takano T. Antihypertensive effects of different kinds of fermented milk in spontaneously hypertensive rats. Bioscience, Biotechnology, and Biochemistry. 2014;**58**:776-778. DOI: 10.1271/bbb.58.776

[67] Yamamoto N, Akino A, Takano T. Antihypertensive effect of the peptides derived from casein by an extracellular proteinase from lactobacillus helveticus CP790. Journal of Dairy Science. 1994;**77**:917-922. DOI: 10.3168/jds.S0022-0302(94)77026-0

[68] Ryhänen E-L, Pihlanto-Leppälä A, Pahkala E. A new type of ripened, low-fat cheese with bioactive properties. International Dairy Journal. 2001;**11**:441-447. DOI: 10.1016/S0958-6946(01)00079-6

[69] Bütikofer U, Meyer J, Sieber R, Walther B, Wechsler D. Occurrence of the angiotensin-converting enzyme inhibiting tripeptides Val-Pro-Pro and Ile-Pro-Pro in different cheese varieties of Swiss origin. Journal of Dairy Science. 2008;**91**:29-38. DOI: 10.3168/jds.2007-0413

[70] Wada Y, Lönnerdal B. Bioactive peptides released by in vitro digestion of standard and hydrolyzed infant formulas. Peptides. 2015;**73**:101-105. DOI: 10.1016/j.peptides.2015.09.005

[71] Hayes MG, Fox PF, Kelly AL. Potential applications of high pressure homogenisation in processing of liquid milk. Journal of Dairy Research. 1999;**72**:25-33. DOI: 10.1017/S0022029904000524

[72] Zeece M, Huppertz T, Kelly A. Effect of high-pressure treatment on in-vitro digestibility of β-lactoglobulin. Innovative Food Science & Emerging Technologies. 2008;**9**:62-69. DOI: 10.1016/j.ifset.2007.05.004

[73] Penas E, Restani P, Ballabio C, Préstamo G, Fiocci A, Gomez R. Evaluation of the residual antigenicity of dairy whey hydrolysates obtained by combination of enzymatic hydrolysis and high-pressure treatment. Journal of Food Protection. 2006;**69**:1707-1712. DOI: 10.4315/0362-028X-69.7.1707

[74] Villamiel M, de Jong P. Influence of high-intensity ultrasound and heat treatment in continuous flow on fat, proteins, and native enzymes of milk. Journal of Agricultural and Food Chemistry. 2000;**48**:3068. DOI: 10.1021/jf0006224

[75] Simsek S, Sánchez-Rivera L, El SN, Karakaya S, Recio I. Characterisation of in vitro gastrointestinal digests from low fat caprine kefir enriched with inulin. International Dairy Journal. 2017;**75**:68-74. DOI: 10.1016/j.idairyj.2017.07.004

[76] Ozdal T, Capanoglu E, Altay F. A review on protein–phenolic interactions and associated changes. Food Research International. 2013;**51**:954-970. DOI: 10.1016/j.foodres.2013.02.009

[77] Rémond D, Shahar DR, Gille D, Pinto P, Kachal J, Peyron M-A, et al. Understanding the gastrointestinal tract of the elderly to develop dietary solutions that prevent malnutrition. Oncotarget. 2015;**6**:13858-13898. DOI: 10.18632/oncotarget.4030

[78] Dallas DC, Underwood MA, Zivkovic AM, German JB. Digestion of protein in premature and term infants. Journal of Nutritional Disorders & Therapy. 2012;**2**:112. DOI: 10.4172/2161-0509.1000112

[79] Newton JL, James OF, Williams GV, Allen A. The diurnal profile of gastric pepsin activity is reduced with *Helicobacter pylori* infection. Digestive Diseases and Sciences. 2004 Aug;**49**:1103-1108

Chapter 7

Advances in Fractionation and Analysis of Milk Carbohydrates

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Additional information is available at the end of the chapter

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Abstract

Lactose is a principal component of the milk obtained from the mammals. Milk also contains several other sugars such as monosaccharides and oligosaccharides in low concentrations. Lactose has reactive functional groups and can be converted to valuable food-grade derivatives for commercial applications through chemical and/or enzymatic reactions. Physical and chemical properties of carbohydrates determine the methods that can be used for their fractionation and purification. In this chapter, the advanced extraction techniques for fractionation and analytical methodologies applied for the determination of different carbohydrates of milk (lactose, lactulose, and oligosaccharides) are summarized. The main aim of this contribution is to provide the reader with a broad view on the recent fractionation and analytical techniques employed for the analysis of carbohydrates in dairy foods and their applications in food and pharmaceutical industry.

Keywords: lactose, fractionation, purification, analytical techniques, applications

1. Introduction

Lactose is the principal carbohydrate present in milk obtained from all mammalian species, such as cow, buffalo, goat, and sheep. The lactose content in milk is relatively constant, although it varies among different dairy products. Lactose is a disaccharide composed of glucose and galactose molecules linked through β(1–4) glycosidic bond and is synthesized in the mammary gland. Small amounts of free glucose and galactose may also be present [1]. Other minor carbohydrates such as oligosaccharides, glycopeptides, glycoproteins, and nucleotide sugars are also found in milk in very small amounts [2]. The core structures of milk

oligosaccharides have a lactose unit at their reducing end to which specific neutral monosaccharides/oligosaccharides (*N*-acetylglucosamine or *N*-acetylgalactosamine, galactose or glucose, and fucose or 6-deoxyhexose) or acidic oligosaccharides [*N*-acetylneuraminic acid (NANA)] can be attached [3]. However, β-galactooligosaccharides (GOSs) are oligosaccharides composed primarily of galactose and often terminate with a glucose residue at the reducing end, and they occur naturally in the milk of many animals including humans and cows. But, GOSs are normally produced industrially by transgalactosylation of lactose using β-galactosidase.

Substances such as lactulose, lactitol, and lactobionic acid are derived from lactose and are not present in natural sources. When lactose in milk is subjected to moderate heating, its isomerization can occur with the lactulose (4-*O*-β-D-galactopyranosyl-D-fructofuranose) formation through Lobry de Bruyn-Alberda van Ekenstein reaction, through the intermediate compound 1,2-enediol [4]. Consequently, the quantity of lactulose is directly proportional to the intensity of the heat treatment applied and could be useful as indicators of the quality of processing of milk [5]. Lactobionic acid (4-*O*-β-D-galactopyranosyl-D-gluconic acid) is an aldonic acid, comprising of galactose and gluconic acid, obtained through lactose oxidation using a metal catalyst or by enzymatic/microbiological oxidation, while lactitol (4-*O*-β-D-galactopyranosyl-D-glucitol) is a sugar alcohol derived from lactose by catalytic hydrogenation. In this chapter, we describe the different techniques employed for the fractionation of milk carbohydrates, the methods of analysis of fractionated carbohydrates, and applications of fractionated carbohydrates in food and pharmaceutical industry.

2. Need of fractionation of carbohydrates

Intensive biochemical characterization of the carbohydrate molecules and their various bioactivities may facilitate an understanding of their importance in human nutrition and may suggest individual carbohydrate structures to target for industrial production [6]. Recently, many studies reveal the biological significance of some carbohydrates and their potential role as nutraceuticals. However, detailed analysis is beneficial for the identification of the specific oligosaccharides responsible for such activities, as the activity may be attributed to one oligosaccharide or even a fraction of oligosaccharides within the entire milk oligosaccharide pool. Extraction and fractionation techniques represent useful tool in the analysis of such carbohydrates from food samples. Thus, it is important to investigate the structure of oligosaccharides to understand the relationship between their structure and biological function.

3. Techniques employed for fractionation of carbohydrates

The fractionation of carbohydrates from dairy foods is carried out by using some kind of cleanup or extraction methods prior to their analysis. The general scheme for obtaining lactose and other sugars from dairy foods is to first precipitate fat and protein by different reagents (Carrez solution, Biggs-Szijarto solution, and 60% methanol), giving a clear serum adequate for carbohydrate analysis [7]. After precipitation, filtration or centrifugation step yields a clear solution. The ulterior analysis may require an additional step. In this section, the techniques employed for extraction and fractionation of carbohydrates are described.

3.1. Pressurized liquid extraction

Pressurized liquid extraction (PLE) is based on the use of solvents at high temperatures (50–200°C) and pressures (1450–2175 psi) to ensure the rapid extraction rate of compounds [8]. The high temperature enables higher solubility and higher rate of solute diffusion in the solvent, while the application of high pressure maintains the solvent below its boiling point, thereby allowing a high penetration of the solvent into the sample [9]. Recently, the extraction and purification of lactulose from a mixture with lactose have been carried out by using PLE (at 1500 psi) with ethanol/water (70:30, w/w) mixture at 40°C for 30 min, and the recovery of lactulose reached up to 84.4% with a purity of over 90% [10]. Despite the advantages over conventional extraction methods, this method is not found to be suitable for thermo-labile compounds as high temperature can have deleterious effects on their structural and functional activities [11].

3.2. Supercritical fluid extraction

The use of supercritical fluid extraction consists of the separation of the analyte from the matrix using supercritical fluids as the extracting solvent. Carbon dioxide (probably the most used supercritical fluid) is nontoxic, nonflammable, can act at low temperatures, and is relatively cheap; unfortunately, the solubility of carbohydrates in the supercritical phase of this fluid is low [12]. Some of the advantages of supercritical fluid extraction are solvating powers similar to liquid organic solvents, high solute diffusivities, lower viscosity, lower surface tension, and the possibility of adjusting the solvating power by changing pressure or temperature [9]. Carbon dioxide is sometimes modified by co-solvents such as ethanol that change its polarity. This technique is useful for the separation of lactulose and tagatose from their isomeric aldoses (i.e., lactose and galactose, respectively) [13] and GOS from monosaccharides in a commercial sample using CO_2 with ethanol/water as co-solvent (at 150 bar and 80°C) [14].

3.3. Solid phase extraction

Solid phase extraction (SPE) is the very popular technique currently available for rapid and selective sample preparation. The versatility of SPE allows the use of this technique for several purposes, such as purification, trace enrichment, desalting, derivatization, and class fractionation. The selection of an appropriate SPE extraction sorbent depends on understanding the mechanism of interaction between sorbent and analyte of interest [15]. Reverse-phase (RP) cartridges are commonly used for the purification of carbohydrates. Octyl (C_8) and octadecyl (C_{18}) silica phases are the most common RP cartridges used for carbohydrate cleanup. These sorbents show high affinity for hydrophobic compounds but less affinity for hydrophilic solutes such as oligosaccharides [16]. Moreover, C_{18} cartridges are useful for the fractionation of (1–4)-α-glucans depending on their degree of polymerization. Ion-exchange SPEs are used for desalting oligosaccharides mixtures, but care should be taken to avoid the loss of charged sugars during their purification [12]. Solid-phase extraction on graphitized carbon material upon enzymatic amyloglucosidase pre-treatment enabled a good recovery and a selective purification of the different GOS structures from the exceeding amounts of particularly lactose- and maltodextrin-rich preparations [17]. These cartridges are also used effectively to remove salts and residual contaminants (traces of protein and lipids) from whey permeate

samples obtained by ultrafiltration [18] and purified oligosaccharide-rich solutions from bovine colostrums [19], thus allowing proper oligosaccharide identification by mass spectrometry without the need of any further purification.

3.4. Chromatography-based methods

Chromatographic techniques, usually set up in open columns with stationary phases based on anion exchange, adsorption, or gel-filtration/permeation mechanisms, are commonly used for the fractionation of carbohydrates. Brand-Miller et al. [20] used charcoal column chromatography for the separation of human milk oligosaccharides (HMOs) from the other constituents in milk. In this method, milk fat was first removed using centrifugation, and protein precipitated with organic solvents followed by enzymatically converting lactose to glucose and galactose to facilitate separation. The extract was filtered through a column packed with granular charcoal to separate the sugars. Glucose and galactose were eluted from the column initially with water and then with 2% v/v ethanol. The HMOs were then eluted from the column with 50% ethanol. In order to improve detection and characterization of less abundant oligosaccharides from bovine colostrums, fractionation of 2-aminobenzamide-labeled sample into neutral and acidic oligosaccharide fractions was performed by weak anionic exchange chromatography, and its separation ability is based on the combination of the charge and size of molecules [21]. Carbohydrates can be readily fractionated by gel-filtration chromatography on the basis of their relative sizes. To separate the HMO from lactose and salts, gel-filtration chromatography (G25 Sephadex column) has often been used [22–25]. When gel permeation chromatography is used for further separation of the different oligosaccharide fractions, lactose can be obtained separately in one of the fractions [Fractogel TSK HW 40 (S)] [26]. Various problems, however, have limited the development of gel-filtration methods for oligosaccharides. First, many of the commercially available gel-filtration matrices are themselves carbohydrates (e.g., Sephadex, Sepharose, etc.), shedding milligram quantities of heterodisperse carbohydrate polymers into the mobile phase. Second, nonspecific interactions with matrix materials are common because sugars are essentially amphipathic with a hydrophobic ring structure and hydrophilic functional groups [27]. Despite these problems, however, gel-filtration chromatography still remains an important option for the purification of complex oligosaccharides.

3.5. Membrane filtration

Ultrafiltration (UF) and nanofiltration (NF) are increasingly used for the removal of lactose and other soluble components from milk, desalting and separation of interfering compounds; the resulting permeate has numerous applications including the production of lactose. The choice for selecting the UF and NF membrane is mainly based on the value of the molecular weight cutoff (MWCO), which is the molecular mass of the smallest compound retained to an extent larger than 90% [12]. Mehra et al. [28] employed the membrane filtration technology to produce powders enriched in bovine milk oligosaccharides (BMOs) using mother liquor (the liquid remaining after the separation of lactose crystals from whey UF permeate) as a starting raw material. The microfiltrate of mother liquor from the microfiltration step was utilized as the feed to the ultrafiltration (spirally wound membranes with a porosity of 1 kDa MWCO) for fractionation and enrichment of milk oligosaccharides from lactose and mineral salts. NF

is an attractive method for HMO isolation due to the speed with which separations can be performed, and it does not require the use of organic solvents. An easily scalable approach to the recovery of HMO from milk has been developed by Sarney et al. [23], which relay on the combination of enzymatic treatment of defatted and deproteinated milk using β-galactosidase and NF and compared the resulting HMO produced with gel filtration. The authors obtained a yield of 6.7 g of HMO from 1 L of milk in just four NF cycles, yet residual lactose appeared in the oligosaccharide fraction produced with NF but not in that prepared using gel filtration.

4. Methods of analysis of fractionated carbohydrates

4.1. Enzymatic methods

An enzymatic method has been adopted by the IDF [29] for the determination of lactulose content of milk in the presence of much higher concentrations of lactose. Lactulose is often determined by the enzymatic methods using commercially available kits supplied by companies such as Boehringer-Mannheim and Merck. An enzymatic electrochemical method for the detection of lactulose content in milk samples was developed by Moscone et al. [30]. This method uses the enzyme β-galactosidase in solution to hydrolyze lactulose to galactose and fructose, and then the latter is oxidized by a fructose dehydrogenase enzyme reactor using potassium ferricyanide as mediator and platinum-based electrochemical transducer. The sensitivity of the procedure allowed pasteurized, UHT, and in-container sterilized milk can be distinguished. Lactulose content can also be determined by enzymatic method based on amperometric detection [31, 32].

4.2. Spectrophotometric methods

High sensitivity, sufficient accuracy, simplicity, speed, and the necessity of less expensive apparatus make spectrophotometric method as an attractive method for the determination of lactose and lactulose in different dairy products. For the analysis of lactulose preparation, spectrophotometric-enzymatic methods were applied to sugar mixtures produced during isomerization of lactose [33]. A simple spectrophotometric method for lactulose detection was based on hydrolysis of lactulose under acidic conditions followed by reaction with resorcinol, giving absorption peaks at 398 and 480 nm [34]. There are several enzymatic methods based on spectrophotometric detection that have been reported for the determination of lactose or lactulose in milk based on hydrolysis of lactose or lactulose by β-galactosidase [35–37]. A rapid and nondestructive front-face fluorescence spectroscopic method to quantify furosine and lactulose in heat-treated milk has been reported by Kulmyrzaev and Dufour [38]. Zhang et al. [39] developed a sensitive and simple spectrophotometric method for the quantification of lactulose without interference from aldoses. The method was based on hydrolysis of lactulose under acidic conditions. The hydrolyzed product reacted with cysteine hydrochloride-tryptophan reagent, giving an absorption peak at 518 nm.

4.3. Capillary electrophoresis

Capillary electrophoresis (CE) is the technique of choice for the analysis of hydrophilic mono- and oligosaccharides, with an impressive number of different separation approaches and different

detection modes, due to their simplicity, short analysis time, efficiency, and low sample consumption [40, 41]. Bao et al. [42] developed a method for the quantification of sialyl oligosaccharides by CE with detection at 205 nm. However, carbohydrates lack a light-absorbing chromophore, which makes direct UV detection impossible unless a derivatization procedure is involved prior to analysis. Baross et al. [43] determined the lactose in milk and milk products by CE with indirect UV detection. More recently, HMOs have been detected by single CE with laser-induced fluorescence (LIF) [42, 44] or additionally coupled online with MS [45–47]. Labeling of HMO with 8-aminopyrene-1,3,6-trisulfonic acid introduces a fluorophore for the LIF detection, simultaneously adding the negative charge needed for the separation. Albrecht et al. [17] also developed a method for qualitative and quantitative analysis of GOS in different food matrices using CE-LIF. A recent study by Kottler et al. [48] describes the use of multiplexed capillary gel electrophoresis with LIF as a high-throughput method for glycol-analysis, demonstrating the ability to identify and quantify approximately 17 oligosaccharide structures based on "fingerprint" electropherograms.

4.4. Infrared spectrometry methods

Infrared (IR) spectroscopy works by expressing vibrational modes of covalent bonds in molecules and assists in the quantification of any component present in a sample that absorbs IR radiation. The spectral ranges from 750 to 2500 nm and 2500 to 25,000 nm, respectively, are the near infra-red (NIR) and mid-infra-red (MIR) regions. NIR spectra are a result of combination of complex overtones and high frequency, while MIR is due to fundamental stretching, bending, and rotating vibrations in the sample [49]. The use of short-wave NIR wavelengths from 700 to 1100 nm has a key role in the analysis of protein, moisture, fat, and lactose contents in a wide range of dairy products including liquid milk, milk powder, cream, and processed cheese. NIR also offers the possibility of on-line analysis, which avoids the need for batch sampling and minimizes sampling error by averaging of virtually instantaneous, continuous measurements [50]. More recent instrument based on Fourier transform infrared (FTIR) spectroscopy is a rapid, accurate, and nondestructive method that can detect a range of functional groups and is sensitive to change in molecular structure. FTIR provides information on the basis of chemical composition and physical state of the whole sample [51]. Lactulose content in freeze-dried heat-treated milk was determined using diffuse reflectance FTIR spectroscopy in the MIR spectral region of 1286–754 cm^{-1} without any chemical treatment of milk. The authors suggested a broader range of 1300–750 cm^{-1} to be an important region for the study of carbohydrates [52]. FTIR spectroscopy was also used to monitor the lactose and protein concentration, during the UF and NF of whey. A range of 1220–800 cm^{-1} provides details about the lactose content of the sample [53].

4.5. Liquid chromatography

The HPLC is one of the most extensively used techniques employed for the separation of a large variety of carbohydrates in foods [54], as it is particularly advantageous in terms of speed, simplicity of sample preparation (without a prior derivatization), and obtaining a high-resolved chromatogram in a short period of time. A considerable research has been carried out on the quantitative determination of lactose and lactulose by using HPLC. Over all

detectors coupled to HPLC, the refractive index detector (RID) is the most widely used for sugars because no fluorophore (fluorescence detector) or chromophore (UV detector) is necessary; in other words, no derivatization is required. The HPLC-RID was commonly used for the determination of lactose, lactulose, and some other monosaccharides in variety of dairy foods, for instance, milk [55–58], whey permeate [59], cheese [60], and also in some of the milk-based infant formulae [61–63].

The evaporating light scattering detector (ELSD) is a universal, nonspecific detector, which detects all nonvolatile solutes after evaporation of the solvent of the column effluent by light dispersion on the solid analyte particles formed. Recently, Schuster-Wolff-Bühring et al. [64] developed a sensitive and reliable method for simultaneous detection and quantification of lactose and lactulose in milk-based products using HPLC with ELSD and also compared four analytical methods using different HPLC columns. A HPLC column with an amino-bonded polymeric matrix yielded better results compared to amino-bonded silica-phase resin or cation-exchange resin. The detection of nonchromophoric and nonvolatile compounds makes ELSD a suitable technique for the detection of sugars.

High performance (high-pH) anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) takes advantage of the affinity between the ionized group of sugars at alkaline pH (pH 12) and a pellicular quaternary amine stationary phase. Therefore, high resolution and highly selective separation of nonderivatized mono-, di- and oligosaccharides can be achieved [65]. This very good match between liquid chromatography and electrochemical detection has allowed the determination of carbohydrates in various complex matrices including dairy foods. Cataldi et al. [66] gave a comprehensive overview of analytical applications in food for carbohydrate analysis by HPAEC-PAD. The sensitive and accurate quantification of lactulose in sterilized milk by HPAEC-PAD has been demonstrated, and it makes the differentiation between sterilized and pasteurized milks possible [67]. The separation of monosaccharides (glucose, galactose, and fructose) and disaccharides (lactose, lactulose, epilactose, sucrose, and maltose) in dairy products [68, 69] and infant formulas [70, 71] by HPAEC-PAD has been described. The quantitative determination of NANA present at the terminal position of many glycoprotein and glycolipid-oligosaccharides by HPAEC-PAD has been reported by Rohrer [72]. Underivatized neutral or acidic oligosaccharides from human milk have been resolved by HPAEC-PAD [73–79]. Sialyllactose isomers (3′- and 6′-sialyllactose) from whey streams were also quantified by HPAEC-PAD [28].

However, since most sugars have no significant UV absorbing and/or fluorophore groups, derivatization prior to separation usually results in higher sensitivity. For detection by UV, a derivatization reagent is required. Reagents such as 1-phenyl-3-methyl-5-pyrazolone (PMP) and p-aminobenzoic ethyl ester are the most popular labels that react with reducing carbohydrates under mild conditions [80, 81]. Common fluorescent tags used for labeling the monosaccharides prior to HPLC analysis are anthranilic acid, 2-aminobenzamide, 2-aminopyridine, phenyl isothiocyanate, 9-fluorenylmethoxycarbonylhydrazine, 7-amino-4-methylcoumarin, and 7-amino-1, 3-naphthalene-disulphonate [82]. Normal-phase [83] and reversed-phase HPLCs [83–92] have been used efficiently for the analysis of derivatized milk oligosaccharides. Retention and separation of the HMO on reversed-phase liquid chromatography depend thus mainly on the method of derivatization; some isomer separation was

obtained so far, but no method has emerged that provides comprehensive isomer separation [93]. Underivatized acidic oligosaccharides or derivatized neutral oligosaccharides have also been resolved by ion-exchange HPLC with detection at 200 nm absorbance [94]. However, the lack of structural discrimination by UV detection can make identification of peaks ambiguous.

Labeling with chromophoric active tags such as 1-phenyl-3-methyl-5-pyrazolone, 2-aminopyridine, and 2-aminobenzoicacid as well as perbenzoylation has been applied for the analysis of oligosaccharides [93]. Recently, hydrophilic interaction liquid chromatography (HILIC)-HPLC has been used for separation and characterization of 2-aminobenzamide-labeled milk oligosaccharides (N- and O-glycans) from bovine colostrum and has proven efficient and robust method for oligosaccharide analysis [21]. Using exoglycosidases, the structures of 37 bovine milk oligosaccharides could be confirmed, which revealed the separation of several structural isomers.

Ultra performance liquid chromatography (UPLC) has also shown improved resolution of *N*-glycans released from glycoproteins [95], with reduced run times and solvent consumption, and it could provide a further improvement in the detection and separation of larger isomeric oligosaccharides.

All the analytical techniques described so far are based on separation alone; however, structural confirmation can in such cases only be obtained based on standards. These standards are expensive and not available for all types of oligosaccharides. For better identification, coupling of the separation with mass spectrometry has proven to be effective. Several applications are reported where sugars were analyzed as their sodium adduct, acetate adduct, or chloride adduct. Indeed, the combination of liquid chromatography and time of flight (TOF) mass spectrometry by the HPLC-chip technology has been extensively and successfully used for oligosaccharide profiling in human milk [96, 97] and bovine milk [19, 98]. Nano-liquid chromatography porous graphitic carbon TOF MS in the positive mode has also been applied in the analysis of HMO [99–101]. Both neutral and sialylated compounds may be separated in one run, and using a library containing retention time, mass, and fragmentation information, immediate identification is possible [100, 101]. Using this method, >200 HMO structures can be separated. More recently, oligosaccharides in bovine colostrum were investigated by Aldredge et al. [102], who employing nano-liquid chromatography tandem mass spectrometry, identified five fucosylated oligosaccharides that were found to be in common with human milk. Nano-LC chip/TOF MS offers an orthogonal dimension of retention time and accurate mass, making it possible to separate isomeric HMOs, with and without sialic acids, in a single-chromatographic separation. Ultra high performance chromatography coupled to mass spectrometry or tandem mass spectrometer (UHPLC-MS or MS/MS) is highly specific and sensitive if single- or multi-reaction monitoring is used. Trani et al. [58] carried out a very interesting comparison of HPLC-RI, LC/MS-MS, and enzymatic assays for the analysis of residual lactose in lactose-free milk. The enzymatic methods as well as HPLC coupled to RI detector are not suitable for quantitative determination of residual lactose in lactose-free milk. But, LC-MS/MS method based on the use of lactose formate adduct was proved to be very sensitive and offered highly reproducible results even at the lowest lactose concentrations.

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Porous graphitized carbon (PGC) has also been recognized as a valuable stationary phase for the analysis of native oligosaccharides including neutral [100] and sialylated oligosaccharides [101], mostly because of the extensive separation of isomers that may be obtained. PGC-LC-MS was recently described as the stationary phase of choice for N-glycan analysis [103]. Bao et al. [104] developed a quantitative method for the analysis of HMO using PGC as the stationary phase and single-quadrupole MS detector. This analytical technique provides sensitive, precise, accurate quantification for each of 11 milk oligosaccharides and allows measurement of differences in milk oligosaccharide patterns between individuals and at different stages of lactation.

4.6. Gas chromatography (GC)

Beside LC, GC has also been extensively applied for the determination of carbohydrates in dairy foods. Compared to LC, the main advantage of GC is much higher separation power. However, derivatization is crucial for nonvolatile carbohydrates converted to volatile derivatives amenable to GC analysis. Most of the GC methods are based on the previous trimethylsilylation of sugars. Mainly hexamethyldisilazine, trimethylchlorosilane, *N*,*O*-bis(trimethylsilyl) trifluoroacetamide, trimethylsilylimidazole, and *N*,*O*-bis(trimethylsilyl)acetamide have been used as silylation reagents, and the good volatility and stability characteristics of the derivatives formed make trimethylsilyl (TMS) ethers the most popular derivatives applied to GC analysis of saccharides. Due to the relatively low volatility of carbohydrates, GC analysis is limited to derivatized sugars of low molecular weight, mainly mono-, di-, and trisaccharides [105]. In general, GC methods with flame ionization detection (FID) or MS detection provide a good separation of sugars and a good sensitivity. Recently, the use of MS as detector has increased over the FIDs for the identification of carbohydrates and the determination of molecular mass. Analysis of free carbohydrates in milk and milk products by GC-MS has been described by Reineccius et al. [106]. In this study, free carbohydrates (galactose, glucose, and lactose) in milk products, i.e., milk, cream, and cheeses, were isolated by dialysis, converted to trimethylsilyl ethers, and then analyzed by GC-MS. Troyano et al. [107, 108] developed a GC method for the analysis of free monosaccharides in raw and market milk samples. Valero et al. [109] determined the amount of lactulose formed in pasteurized milk by GC of the TMS derivatives of the free sugar, besides monosaccharides were also determined. Lactulose content was also determined by GC analysis of their corresponding TMS ethers separated on a 50% diphenyl/50% dimethylsiloxane capillary column [110]. Using this method, it was possible to quantify lactulose on different treated milks (UHT, sterilized, pasteurized, condensed, and powder milk). Although GC is a sensitive method for sugar analysis, the sample preparation is laborious and not very practical in routine analysis. Besides in the GC procedure, the anomeric composition of α- and β-anomers is obtained, which means more than one peak area for each compound.

4.7. Mass spectrometry methods

Mass spectrometry provides a sensitive technique to assist in the determination of the structure of carbohydrates, but it is essential that the samples are pure to achieve maximum ionization.

The most widely used ionization methods for oligosaccharides are matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), coupled with tandem techniques such as collision-induced dissociation (CID). Such advances have improved carbohydrate research with mass spectrometry. The tandem mass spectrometry (MS/MS) has been used for detailed structural analysis of oligosaccharide molecules in which a molecular ion is selected by a first stage of MS, which undergoes activation and fragmentation, and the product ions are analyzed to provide information about the sequence monosaccharide compositions, linkages, and locations of various modifications [111].

Milk oligosaccharides have been analyzed using tandem mass spectrometry such as collision-induced dissociation (CID) and infrared multiphoton dissociation (IRMPD) [112]. A comprehensive label-free procedure to identify and quantify milk oligosaccharides and other glycans using nano-liquid chromatography-time of flight MS was recently developed [113]. During this study, MALDI-Fourier transform ion cyclotron resonance (FTICR) was used for accurate mass measurement compositional analysis. FTICR MS provides exceptional mass accuracy at even few parts per million with extremely high mass resolution and has been extensively and successfully used in complex samples. Oligosaccharides in bovine cheese whey permeate were characterized by a combination of nanoelectrospray (nESI)-FTICR-MS and MALDI-FTICR-MS, and this method was possible to identify 15 (8 were neutral and 7 were acidic) milk oligosaccharides [18]. Recently, Mehra et al. [28] identified 25 oligosaccharides including 6 high molecular weight fucosylated oligosaccharides in a mother liquor of dairy streams using MALDI-FTICR mass spectrometer, and the researchers also confirmed the fucosylated oligosaccharides composition and putative structure by using accurate tandem mass spectrometry.

4.8. Nuclear magnetic resonance (NMR)

Many different analytical techniques have been used to elucidate the free glycan profile present in milk samples. Compared with LC-MS and GC-MS, one of the advantages of NMR spectroscopy is the direct and quantitative relationship between the molar concentration and the intensity of the NMRs. For instance, NMR has played an important role in the structural characterization of free oligosaccharides in human milk [114–117]. Monakhova et al. [118] suggest the use of NMR spectroscopy as screening tool to validate nutrition labeling of lactose-free milk, reporting a detection limit of 30 mg/L.

5. Applications of fractionated carbohydrates

Lactose is used as an agglomerating agent, a flavor enhancer, and a diluent in many foods, beverages, and bakery and confectionery products. In the pharmaceutical industry, it is employed as a diluent in tablets and a carrier in medicines [119, 120]. In the last year, the interest in lactulose has increased to a high extent due to its application in the pharmaceutical and food industry. Lactulose is considered as a prebiotic because it promotes the proliferation of *Bifidobacteria* in the human intestine. Therefore, this disaccharide is used as a food supplement in pediatric and geriatric diets [121] as well as for prevention and treatment of chronic constipation, portal systemic encephalopathy, and other intestinal or hepatical disorders [122]. It has been suggested that lactulose has anti-inflammatory properties, which make it an appropriate and useful adjunctive treatment to inflammatory bowel diseases in humans [123].

Most recently, lactulose has been proposed as a treatment for the *Salmonella* carrier state, a preventative and treatment for high cholesterol, a preventative for gall stones, and an adjunctive treatment for colorectal cancers [124]. Moreover, lactulose is used in pharmaceuticals as a mild laxative and to treat hyperammonemia.

The main characteristics of lactobionic acid include moisturizing, antioxidant, stabilizing, and acidifying capacities. Lactobionic acid appears to be a less commonly used prebiotic for both *Lactobacilli* and *Bifidobacteria*. However, lactobionic acid has been suggested to improve gut health [125]. Other applications of lactobionic acid include as a calcium supplement (calcium lactobionate) in pharmaceutical solutions for stabilizing the organs during transplant [126] and in the cosmetic industry [127]. Lactitol is widely used in chocolates, biscuits, sweets, ice cream, and confectionary manufacture as an alternative to other sugar alcohols, such as mannitol, sorbitol, and xylitol. It is used in low-energy, low-fat, and glycemic foods for diabetics and is also recognized as not causing dental caries [128].

It is widely accepted that milk oligosaccharides play several important protective, physiological, and biological roles including selective growth stimulation for beneficial gut microbiota, inhibition of pathogen adhesion, and immunoregulation [88, 129]. The use of free oligosaccharides and their conjugated derivatives in anti-adhesion therapy may provide an effective therapeutic approach to prevent diseases [21]. GOSs are widely used in infant milk formula, infant foods, follow-on formula, beverages, and fermented milks and in confectionary and bread making, of their functional properties as well as health-enhancing properties. The use of GOS in the livestock feed and a pet food industry is also increasing, especially in the poultry, pig, and aqua-culture [130].

6. Conclusion

There are several fractionations and analytical techniques currently being used for the determination of carbohydrates in different milk and milk products. A problem with studying carbohydrate bioactivities is the limited access to well-defined oligosaccharides. Purification of oligosaccharides from natural sources is laborious, and it is difficult to obtain preparation free of contaminants. In addition, the nonlinear nature of oligosaccharides creates a high structural diversity and their overall complexity makes it difficult for a single analytical method to characterize them. Numerous health benefits of milk oligosaccharides and lactose derivatives are well established, including the pharmaceutical and food applications.

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References

[1] Haenlein GFW. Goat milk in human nutrition. Small Ruminant Research. 2004;**51**(2): 155-163

[2] Park YW, Júarez M, Ramos M, Haenlein GFW. Physico-chemical characteristics of goat and sheep milk. Small Ruminant Research. 2007;**68**(1-2):88-113

[3] Kunz C, Rudloff S. Health promoting aspects of milk oligosaccharides. International Dairy Journal. 2006;**16**(11):1341-1346

[4] O'Brien J. Non-enzymatic degradation pathways of lactose and their significance in dairy products. In: McSweeney P, Fox PF, editors. Advanced Dairy Chemistry. New York: Springer; 2009. pp. 231-294

[5] Morales FJ, Romero C, Jiménez-Pérez S. Characterization of industrial processed milk by analysis of heat-induced changes. International Journal of Food Science & Technology. 2000;**35**(2):193-200

[6] Ward RE. Isolation of milk oligosaccharides using solid-phase extraction. Open Glycoscience. 2009;**2**:9-15

[7] Egge H, Dell A, Von Nicolai H. Fucose containing oligosaccharides from human milk. Archives of Biochemistry and Biophysics. 1983;**224**(1):235-253

[8] Dunford N, Irmak S, Jonnala R. Pressurised solvent extraction of policosanol from wheat straw, germ and bran. Food Chemistry. 2010;**119**(3):1246-1249

[9] Baiano A. Recovery of biomolecules from food wastes – A review. Molecules. 2014;**19**(9): 14821-14842

[10] Ruiz-Matute AI, Sanz ML, Corzo N, Martín-Álvarez PJ, Ibáñez E, Martínez-Castro I, Olano A. Pressurized liquid extraction with ethanol–water at different temperatures. Journal of Agricultural and Food Chemistry. 2007;**55**(9):3346-3350

[11] Ajila CM, Brar SK, Verma M, Tyagi RD, Godbout S, Valero JR. Extraction and analysis of polyphenols: Recent trends. Critical Reviews in Biotechnology. 2011;**31**(3):227-249

[12] Soria AC, Brok M, Sanz ML, Martínez-Castro I. Sample preparation for the determination of carbohydrates in food and beverages. In: Pawliszyn J, Mondello L, Dugo P, editors. Comprehensive Sampling and Sample Preparation. 1st ed. Oxford, UK: Elsevier, Academic Press; 2012. pp. 213-243

[13] Montañés F, Corzo N, Olano A, Reglero G, Ibáñez E, Fornari T. Selective fractionation of carbohydrate complex mixtures by supercritical fluid extraction with CO_2 and different CO-solvents. The Journal of Supercritical Fluids. 2008;**45**(2):189-194

[14] Montañés F, Olano A, Reglero G, Ibáñez E, Fornari T. Supercritical technology as an alternative to fractionate prebiotic galactooligosaccharides. Separation and Purification Technology. 2009;**66**(2):383-389

[15] Żwir-Ferenc A, Biziuk M. Solid phase extraction technique – Trends, opportunities and applications. Polish Journal of Environmental Studies. 2006;**15**(5):677-690

[16] Packer NH, Lawson MA, Jardine DR, Redmon JW. A general approach to desalting oligosaccharides reseased from glycoproteins. Glycoconjugate Journal. 1998;**15**(8):737-747

[17] Albrecht S, Schols HA, Klarenbeek B, Voragen AGJ, Gruppen H. Introducing capillary electrophoresis with laser-induced fluorescence (CE-LIF) as a potential analysis and quantification tool for galactooligosaccharides extracted from complex food matrices. Journal of Agricultural and Food Chemistry. 2010;**58**(5):2787-2794

[18] Barile D, Tao N, Lebrillab CB, Coisson JD, Arlorio M, Germana JB. Permeate from cheese whey ultrafiltration is a source of milk oligosaccharides. International Dairy Journal. 2009;**19**(9):524-530

[19] Barile D, Marotta M, Chu C, Mehra R, Grimm R, Lebrilla CB, German JB. Neutral and acidic oligosaccharides in Holstein-Friesian colostrum during the first 3 days of lactation measured by high performance liquid chromatography on a microfluidic chip and time-of-flight mass spectrometry. Journal of Dairy Science. 2010;**93**(9):3940-3949

[20] Brand-Miller JC, McVeagh P, McNeil Y, Messer M. Digestion of human milk oligosaccharides by healthy infants evaluated by the lactulose hydrogen breath test. Journal of Pediatrics. 1998;**133**(1):95-98

[21] Mariño K, Lane JA, Abrahams JL, Struwe WB, Harvey DJ, Marotta M, Hickey RM, Rudd PM. Method for milk oligosaccharide profiling by 2-Aminobenzamide Labeling and hydrophilic interaction chromatography. Glycobiology. 2011;**21**(10):1317-1330

[22] Kobata A, Ginsburg V, Tsuda M. Oligosaccharides of human milk. I. Isolation and characterization. Archives of Biochemistry and Biophysics. 1969;**130**(1):509-513

[23] Sarney DB, Hale C, Frankel G, Vulfson EN. A novel approach to the recovery of biologically active oligosaccharides from milk using a combination of enzymatic treatment and nanofiltration. Biotechnology and Bioengineering. 2000;**69**(4):461-467

[24] Jantscher-Krenn E, Zherebtsov M, Nissan C, Goth K, Guner YS, Naidu N, Choudhury B, Grishin AV, Ford HR, Bode L. The human milk oligosaccharide Disialyllacto-N-Tetraose prevents necrotising enterocolitis in neonatal rats. Gut. 2011;**61**(10):1417-1425

[25] Jantscher-Krenn E, Lauwaet T, Bliss LA, Reed SL, Gillin FD, Bode L. Human milk oligosaccharides reduce *Entamoeba histolytica* attachment and cytotoxicity in vitro. The British Journal of Nutrition. 2012;**108**(10):1839-1846

[26] Thurl S, Offermanns J, Muller-Werner B, Sawatzki G. Determination of neutral oligosaccharides fractions from human milk by gel permeation chromatography. Journal of Chromatography B. 1991;**568**(2):291-300

[27] Hodgson J. Chromatogrpahy of complex carbohydrates. Biotechnology. 1991;**9**(2):149-150

[28] Mehra R, Barile D, Marotta M, Lebrilla CB, Chu C, German JB. Novel high-molecular weight fucosylated milk oligosaccharides identified in dairy streams. PLoS One. 2014;**9**(5):e96040

[29] IDF. Provisional Standard. Milk. Determination of Lactulose Content. Enzymatic Method. Standard 175, Brussels: International Dairy Federation; 1995

[30] Moscone D, Bernardo RA, Marconi E, Amine A, Palleschi G. Rapid determination of lactulose in milk by microdialysis and biosensors. Analyst. 1999;**124**(3):325-329

[31] Mayer M, Genrich M, Kunnecke W, Bilitewski U. Automated determination of lactulose in milk using an enzyme reactor and flow analysis with integrated dialysis. AnalyticaChimicaActa. 1996;**324**(1):37-45

[32] Sekine Y, Hall EA. A lactulose sensor based on coupled enzyme reactions with a ring electrode fabricated from Tetrathiafulvalen-Tetracyanoquinodimetane. Biosensors & Bioelectronics. 1998;**13**(9):995-1005

[33] Geier H, Klostermeyer H. Formation of lactulose during heat treatment of milk. Milchwissenschaft. 1983;**38**:475-477

[34] Khan MA, Iqbal Z, Jan MR, Shah J, Ahmad W, Haq ZU. Obaidullah. A spectrophotometric method for quantitative determination of lactulose in pharmaceutical preparations. Journal of Analytical Chemistry. 2006;**61**(1):32-36

[35] Andrews GR. Distinguishing pasteurized, UHT and sterilized milks by their lactulose content. International Journal Dairy Technology. 1984;**37**(3):92-95

[36] Amine A, Moscone D, Palleschi G. Rapid determination of lactulose in milk using Seliwanoff's reaction. Analytical Letters. 2000;**33**(1):125-135

[37] Marconi E, Messia MC, Amine A, et al. Heat-treated milk differentiation by a sensitive lactulose assay. Food Chemistry. 2004;**84**(3):447-450

[38] Kulmyrzaev A, Dufour É. Determination of lactulose and furosine in milk using front-face fluorescence spectroscopy. Le Lait. 2002;**82**(6):725-735

[39] Zhang Z, Wang H, Yang RJ, Jiang XY. A novel spectrophotometric method for quantitative determination of lactulose in food industries. International Journal of Food Science & Technology. 2010;**45**(2):258-264

[40] Wang X, Chen Y. Determination of carbohydrates as their p-sulfophenylhydrazones by capillary zone electrophoresis. Carbohydrate Research. 2001;**332**(2):191-196

[41] Wang X, Wang Q, Chen Y, Han H. Determination of carbohydrates as their 3-aminophthalhydrazide derivatives by capillary zone electrophoresis with on-line chemiluminescence detection. Journal of Chromatography A. 2003;**992**(1-2):181-191

[42] Bao Y, Zhu L, Newburg DS. Simultaneous quantification of sialyloligosaccharides from human milk by capillary electrophoresis. Analytical Biochemistry. 2007;**370**(2):206-214

[43] Baross E, Bolyán D, Ganzler K. Qualitative and quantitative determination of lactose in milk and dairy products by capillary electrophoresis. Periodica Polytechnica Chemical Engineering. 1996;**40**(1-2):117-124

[44] Bao Y, Newburg DS. Capillary electrophoresis of acidic oligosaccharides from human milk. Electrophoresis. 2008;**29**(12):2508-2515

[45] Albrecht S, Schols HA, Van Den Heuvel EG, Voragen AG, Gruppen H. CE-LIF-MS n profiling of oligosaccharides in human milk and feces of breast-fed babies. Electrophoresis. 2010;**31**(7):1264-1273

[46] Albrecht S, Schols HA, Van Den Heuvel EG, Voragen AG, Gruppen H. Occurrence of oligosaccharides in feces of breast-fed babies in their first six months of life and the corresponding breast milk. Carbohydrate Research. 2011;**346**(16):2540-2550

[47] Albrecht S, Schols HA, Van Zoeren D, Van Lingen RA, Groot Jebbink LJ, Van Den Heuvel EG, Voragen AG, Gruppen H. Oligosaccharides in feces of breast- and formula-fed babies. Carbohydrate Research. 2011;**346**(14):2173-2181

[48] Kottler R, Mank M, Hennig R, Müller-Werner B, Stahl B, Reichl U, Rapp E. Development of a high-throughput Glycoanalysis method for the characterization of oligosaccharides in human milk utilizing multiplexed capillary gel electrophoresis with laser-induced fluorescence detection. Electrophoresis. 2013;**34**(16):2323-2336

[49] Upadhyay N, Jaiswal P, Jha SN. Application of attenuated total reflectance Fourier transform infrared spectroscopy (ATReFTIR) in MIR range coupled with chemometrics for detection of pig body fat in pure ghee (heat clarified milk fat). Journal of Molecular Structure. 2018;**1153**:275-281

[50] Osborne B G. Near-infrared spectroscopy in food analysis. In: Meyers RA, editor. Encyclopedia of Analytical Chemistry. USA: John Wiley& Sons, Inc.; 2006. DOI: 10.1002/9780470027318.a1018

[51] Cocchi M, Foca G, Lucisano M, Marchetti A, Paean MA, Tassi L, Ulrici A. Classification of cereal flours by chemometrie analysis of MIR spectra. Journal of Agriculture and Food Chemistry. 2004;**52**(5):1062-1067

[52] Pappas CS, Sakkas L, Moschopoulou E, Moatsou G. Direct determination of lactulose in heat-treated milk using diffuse reflectance infrared Fourier transform spectroscopy and partial least squares regression. International Journal of Dairy Technology. 2015;**68**(3):448-453

[53] Solís-Oba M, Teniza-García O, Rojas-López M, Delgado-Macuil R, Díaz-Reyes J, Ruiz R. Application of infrared spectroscopy to the monitoring of lactose and protein from whey after ultra and nano filtration process. Journal of the Mexican Chemical Society. 2011;**55**(3):190-193

[54] Corzo N, Olano A, Martínez-Castro I. Carbohyrates. In: Nollet LML, Toldra F, editors. Handbook of Dairy Foods Analysis. Boca Raton, FL, USA: Taylor & Francis; 2009. pp. 139-167

[55] Manzi P, Pizzoferrato L. HPLC determination of lactulose in heat treated milk. Food and Bioprocess Technology. 2013;**6**(3):851-857

[56] Silveira MF, Masson LMP, Martins JFP, Álvares TS, Paschoalin VMF, Torre CL, Conte-Junior CA. Simultaneous determination of lactulose and lactose in conserved milk by HPLC-RID. Journal of Chemistry. 2015;**2015**:1-6

[57] Veena N, Nath BS. HPLC determination of polydextrose in fortified milk. Indian Journal of Dairy Science. 2017;**70**(1):123-126

[58] Trani A, Gambacorta G, Loizzo P, Cassone A, Fasciano C, Zambrini AV, Faccia M. Comparison of HPLC-RI, LC/MS-MS and enzymatic assays for the analysis of residual lactose in lactose-free milk. Food Chemistry. 2017;**233**:385-390

[59] Hu M, Kurth MJ, Hsieh YL, Krochta JM. HPLC and NMR study on reduction of sweet whey permeate. Journal of Agricultural and Food Chemistry. 1996;**44**(12):3757-3762

[60] Garde S, Ávila M, Arias R, Gaya P, Nuñez M. Sugars and organic acids in raw and pasteurized milk Manchego cheeses with different degrees of late blowing defect. International Dairy Journal. 2012;**25**(2):87-91

[61] Ferreira IMPLVO, Gomes AMP, Ferreira MA. Determination of sugars and other compounds in infant formulae, follow-up milks and human milk by HPLC-UV/RI. Carbohydrate Polymers. 1998;**37**(3):225-229

[62] Chávez-Servin JL, Castellote AI, López-Sabater MC. Analysis of mono- and disaccharides in milk based formulae by high-performance liquid chromatography with refractive index detection. Journal of Chromatography A. 2004;**1043**(2):211-215

[63] Xinmin W, Ruili Z, Zhihua L, Yuanhong W, Tingfu J. Determination of glucosamine and lactose in milk-based formulae by high-performance liquid chromatography. Journal of Food Composition and Analysis. 2008;**21**:255-258

[64] Schuster-Wolff-Bühring R, Michel R, Hinrichs J. A new liquid chromatography method for the simultaneous and sensitive quantification of lactose and lactulose in milk. Dairy Science & Technology. 2011;**91**(1):27-37

[65] Davidek T, Clety N, Devaud S, Robert F, Blank I. Simultaneous quantification analysis of Maillard reaction precursors and products by high-performance anion exchange chromatography. Journal of Agricultural and Food Chemistry. 2003;**51**(25):7259-7265

[66] Cataldi TRI, Campa C, De Benedetto GE, Fresenius J. Carbohydrate analysis by high-performance anion-exchange chromatography with pulsed amperometric detection: The potential is still growing. Analytical Chemistry. 2000;**368**(8):739-758

[67] Liu Y, Avdalovic N, Pohl C, Matt R, Dhillon H, Kiser R. An on-line, high purity acid and base eluent generation system for ion chromatography. American Laboratory. 1998:48C-54C

[68] Reil JV, Olieman C. Selectivity control in the anion-exchange chromatographic determination of saccharides in dairy products using pulsed amperometric detection. Carbohydrate Research. 1991;**215**(1):39-46

[69] Cataldi TR, Angelotti M, Bufo SA. Method development for the quantitative determination of lactulose in heat-treated milks by HPAEC with pulsed amperometric detection. Analytical Chemistry. 1999;**71**(21):4919-4925

[70] Kaine LA, Wolnik KA. Detection of counterfeit and relabeled infant formulas by high-pH anion-exchange chromatography-pulsed aAmperometric detection for the determination of sugar profiles. Journal of Chromatography A. 1998;**804**(1-2):279-287

[71] Austin S, Bénet T, Michaud J, Cuany D, Rohfritsch P. Determination of β-galactooligosaccharides by liquid chromatography. International Journal of Analytical Chemistry. 2014. http://dx.doi.org/10.1155/2014/768406

[72] Rohrer JS. Analyzing sialic acids using high-performance anion-exchange chromatography with pulsed amperometric detection. Analytical Biochemistry. 2000;**283**(1):3-9

[73] Thurl S, Muller-Werner B, Sawatzki G. Quantification of individual oligosaccharide compounds from human milk using high-pH anion exchange chromatography. Analytical Biochemistry. 1996;**235**(2):202-206

[74] Nakhla T, Fu D, Zopf D, Brodsky NL, Hurt H. Neutral oligosaccharide content of preterm human milk. British Journal of Nutrition. 1999;**82**(5):361-367

[75] Coppa G V, Pierani P, Zampini L, Carloni I, Carlucci A, Gabrielli O. Oligosaccharides in human milk during different phases of lactation. Acta Paediatrica Suppliment. 1999;**88**(430):89-94

[76] Finke B, Stahl B, Pfenninger A, Karas M, Daniel H, Sawatzki G. Analysis of high-molecular-weight oligosaccharides from human milk by liquid chromatography and MALDI-MS. Analytical Chemistry. 1999;**71**(17):3755-3762

[77] Bode L, Rudloff S, Kunz C, Strobel S, Klein N. Human milk oligosaccharides reduce platelet-neutrophil complex formation leading to a decrease in neutrophil Beta 2 integrin expression. Journal of Leukocyte Biology. 2004;**76**(4):820-826

[78] Thurl S, Munzert M, Henker J, Boehm G, Muller-Werner B, Jelinek J, Stahl B. Variation of human milk oligosaccharides in relation to milk groups and Lactational periods. The British Journal of Nutrition. 2010;**104**(9):1261-1271

[79] Coppa GV, Gabrielli O, Zampini L, Galeazzi T, Ficcadenti A, Padella L, Santoro L, Soldi S, Carlucci A, Bertino E, Morelli L. Oligosaccharides in 4 different milk groups, Bifidobacteria, and Ruminococcusobeum. Journal of Pediatric Gastroenterology and Nutrition. 2011;**53**(1):80-87

[80] Gomis DB, Tamayo DM, Alonso JM. Determination of monosaccharides in cider by reversed-phase liquid chromatography. AnalyticaChimicaActa. 2001;**436**(1):172-180

[81] Lv Y, Yang X, Zhao Y, Ruan Y, Yang Y, Wang Z. Separation and quantification of component Monosaccharides of the tea polysaccharides from *Gynostemmapentaphyllum* by HPLC with indirect UV detection. Food Chemistry. 2009;**112**(3):742-746

[82] Racaityte K, Kiessig S, Kalman F. Application of capillary zone electrophoresis and reversed phase high-performance liquid chromatography in the biopharmaceutical industry for the quantitative analysis of the monosaccharides released from a highly glycosylated therapeutic protein. Journal of Chromatography A. 2005;**1079**(1-2):354-365

[83] Charlwood J, Tolson D, Dwek M, Camilleri P. A detailed analysis of neutral and acidic carbohydrates in human milk. Analytical Biochemistry. 1999;**273**(2):261-277

[84] Chaturvedi P, Warren CD, Altaye M, Morrow AL, Ruiz-Palacios G, Pickering LK, Newburg DS. Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. Glycobiology. 2001;**11**(5):365-372

[85] Warren CD, Chaturvedi P, Newburg AR, Oftedal OT, Tilde CD, Newburg DS. Comparison of oligosaccharides in milk specimens from humans and twelve other species. In: Newburg DS, editor. Bioactive Components of Human Milk. Boston, MA: Springer; 2001. pp. 325-332. DOI: 10.1007/978-1-4615-1371-1_40

[86] Martin-Sosa S, Martin MJ, Hueso P. The sialylated fraction of milk oligosaccharides is partially responsible for binding to enterotoxigenic and uropathogenic *Escherichia coli* human strains. The Journal of Nutrition. 2002;**132**(10):3067-3072

[87] Sumiyoshi W, Urashima T, Nakamura T, Arai I, Saito T, Tsumura N, Wang B, Brand-Miller J, Watanabe Y, Kimura K. Determination of each neutral oligosaccharide in the milk of Japanese women during the course of lactation. The British Journal of Nutrition. 2003;**89**(1):61-69

[88] Asakuma S, Akahori M, Kimura K, Watanabe Y, Nakamura T, Tsunemi M, Arai I, Sanai Y, Urashima T. Sialyl oligosaccharides of human colostrum: Changes in concentration during the first three days of lactation. Bioscience, Biotechnology and Biochemistry. 2007;**71**(6):1447-1451

[89] Asakuma S, Urashima T, Akahori M, Obayashi H, Nakamura T, Kimura K, Watanabe Y, Arai I, Sanai Y. Variation of major neutral oligosaccharides levels in human colostrum. European Journal of Clinical Nutrition. 2008;**62**(4):488-494

[90] Leo F, Asakuma S, Nakamura T, Fukuda K, Senda A, Urashima T. Improved determination of milk oligosaccharides using a single derivatization with anthranilic acid and separation by reversed-phase high performance liquid chromatography. Journal of Chromatography A. 2009;**1216**(9):1520-1523

[91] Leo F, Asakuma S, Fukuda K, Senda A, Urashima T. Determination of sialyl and neutral oligosaccharide levels in transition and mature milks of Samoan women, using anthranilic derivatization followed by reverse phase high performance liquid chromatography. Bioscience, Biotechnology and Biochemistry. 2010;**74**(2):298-303

[92] Erich S, Anzmann T, Fischer L. Quantification of lactose using ion-pair RP-HPLC during enzymatic lactose hydrolysis of skim milk. Food Chemistry. 2012;**135**(4):2393-2396

[93] Ruhaak LR, Lebrilla CB. Advances in analysis of human milk oligosaccharides. Advances in Nutrition. 2012;**3**(3):406S-414S

[94] Cardon P, Parente JP, Leroy Y, Montreuil J, Fournet B. Separation of sialyl-oligosaccharides by high-performance liquid chromatography. Application to the analysis of mono-, di-, tri- and tetrasialyl-oligosaccharides obtained by hydrazinolysis of alpha 1-acid glycoprotein. Journal of Chromatography. 1986;**356**(1):135-146

[95] Ahn J, Bones J, Yu YQ, Rudd PM, Gilar M. Separation of 2-aminobenzamide labeled glycans using hydrophilic interaction chromatography columns packed with 1.7 μm sorbent. Journal of Chromatography B. 2010;**878**(3-4):403-408

[96] Ninonuevo MR, Park Y, Yin H, Zhang J, Ward RE, Clowers BH, German JB, Freeman SL, Killeen K, Grimm R, Lebrilla CB. A strategy for annotating the human milk Glycome. Journal of Agricultural and Food Chemistry. 2006;**54**(20):7471-7480

[97] Ninonuevo MR, Ward RE, LoCascio RG, German JB, Freeman SL, Barboza M, Mills DA, Lebrilla CB. Methods for the quantitation of human milk oligosaccharides in bacterial fermentation by mass spectrometry. Analytical Biochemistry. 2007;**361**(1):15-23

[98] Tao N, Depeters EJ, Freeman S, German JB, Grimm R, Lebrilla CB. Bovine milk Glycome. Journal of Dairy Science. 2008;**91**(10):3768-3778

[99] Niñonuevo M, An H, Yin H, Killeen K, Grimm R, Ward R, German B, Lebrilla C. Nanoliquid chromatography-mass spectrometry of oligosaccharides employing graphitized carbon chromatography on microchip with a high-accuracy mass Analyzer. Electrophoresis. 2005;**26**(19):3641-3649

[100] Wu S, Tao N, German JB, Grimm R, Lebrilla CB. Development of anannotated library of neutral human milk oligosaccharides. Journal of Proteome Research. 2010;**9**(8):4138-4151

[101] Wu S, Grimm R, German JB, Lebrilla CB. Annotation and structural analysis of sialylated human milk oligosaccharides. Journal of Proteome Research. 2011;**10**(2):856-868

[102] Aldredge DL, Geronimo MR, Hua S, Nwosu CC, Lebrilla CB, Barile D. Annotation and structural elucidation of bovine milk oligosaccharides and determination of novel fucosylated structures. Glycobiology. 2013;**23**(6):664-676

[103] Jensen PH, Karlsson NG, Kolarich D, Packer NH. Structural analysis of N- and O-glycans released from glycoproteins. Nature Protocols. 2012;**7**(7):1299-1310

[104] Bao Y, Chen C, Newburg DS. Quantification of neutral human milk oligosaccharides by graphitic carbon high-performance liquid chromatography with tandem mass spectrometry. Analytical Biochemistry. 2013;**433**(1):28-35

[105] Ruiz-Matute A I, Hernández-Hernández O, Rodríguez-Sánchez S, Sanz M L, Martínez-Castro I. Derivatization of carbohydrates for GC and GC-MS analyses. Journal of Chromatography B. 2011;**879**(17-18):1226-1240

[106] Reineccius GA, Kavanagh TE, Keeney PG. Identification and quantitation of free neutral carbohydrates in milk products by gas-liquid chromatography and mass spectrometry. Journal of Dairy Science. 1970;**53**(8):1018-1022

[107] Troyano E, Olano A, Fernández-Díaz M, Sanz J, Martínez-Castro I. Gas chromatographic analysis of free monosaccharides in milk. Chromatographia. 1991;**32**(7):379-382

[108] Troyano E, Villamiel M, Olano A, Sanz J, Martinez-Castro I. Monosaccharides and *Myo*-inositol in commercial milks. Journal of Agricultural and Food Chemistry. 1996;**44**(3):815-817

[109] Valero E, Villamiel M, Sanz J, Martínez Castro I. Chemical and sensorial changes in milk pasteurised by microwave and conventional systems during cold storage. Food Chemistry. 2000;**70**(1):77-81

[110] Montilla A, Moreno FJ, Olano A. A reliable gas capillary chromatographic determination of lactulose in dairy samples. Chromatographia. 2005;**62**(5):311-314

[111] An HJ, Lebrilla CB. Structure elucidation of native N- and O-linked glycans by tandem mass spectrometry (tutorial). Mass Spectrometry Reviews. 2011;**30**(4):560-578

[112] Zhang H, Zhang S, Tao G, Zhang Y, Mulloy B, Zhan X, Chai W. Typing of blood-group antigens on neutral oligosaccharides by negative-ion electrospray ionization tandem mass spectrometry. Analytical Chemistry. 2013;**85**(12):5940-5949

[113] De Leoz ML, Wu S, Strum J, Ninonuevo MR, Gaerlan S, Mirmiran M, Ninonuevo M, German JB, Mills DA, Lebrilla CB, Underwood MA. A quantitative and comprehensive method to analyze human milk oligosaccharide structures in the urine and feces of infants. Analytical and Bioanalytical Chemistry. 2013;**405**(12):4089-4105

[114] Martin-Pastor M, Bush CA. Conformational studies of human milk oligosaccharides using ^{1}H-^{13}C one-bond NMR residual dipolar couplings. Biochemistry. 2000;**39**(16):4674-4683

[115] Hu F, Furihata K, Ito-Ishida M, Kaminogawa S, Tanokura M. Nondestructive observation of bovine milk by NMR spectroscopy: Analysis of existing states of compounds and detection of new compounds. Journal Agricultural and Food Chemistry. 2004;**52**(16):4969-4974

[116] Kogelberg H, Piskarev VE, Zhang Y, Lawson A, Chai W. Determination by electrospray mass spectrometry and ^{1}H-NMR spectroscopy of primary structures of variously fucosylated neutral oligosaccharides based on the iso-lacto-n-octaose core. European Journal of Biochemistry. 2004;**271**(6):1172-1186

[117] Chai W, Piskarev VE, Zhang Y, Lawson AM, Kogelberg H. Structural determination of novel lacto-N-decaose and its monofucosylated analogue from human milk by electrospray tandem mass spectrometry and ^{1}H-NMR spectroscopy. Archives of Biochemistry and Biophysics. 2005;**434**(1):116-127

[118] Monakhova YB, Kuballa T, Leitz J, Andlauer C, Lachenmeier DW. NMR spectroscopy as a screening tool to validate nutrition labeling of milk, lactose-free milk, and milk substitutes based on soy and grains. Dairy Science & Technology. 2012;**92**(2):109-120

[119] Seki N, Saito H. Lactose as a source for lactulose and other functional lactose derivatives. International Dairy Journal. 2012;**22**(2):110-135

[120] Majid M, Madadlou A. Maillard conjugation of lactulose with potentially bioactive peptides. Food Chemistry. 2016;**192**:831-836

[121] Schwartz SJ, Sieber FE. The geriatric patient. In: Anesthesia and Uncommon Diseases. 6th ed. Philadelphia: WB Saunders; 2012. pp. 573-585

[122] Corzo-Martínez M, Megías-Pérez R, Montilla A, Olano A., Moreno F J, Villamiel M. Impact of power ultrasound on formation and degradation of lactulose in basic media. EuroCarb. 2013;**17**. Available from: http://hdl.handle.net/10261/115019

[123] Manzanares W, Hardy G. The role of prebiotics and synbiotics in critically ill patients. Current Opinion in Clinical Nutrition and Metabolic Care. 2008;**11**(6):782-789

[124] Bovee-Oudenhoven IM, Termont DS, Heidt PJ, Van der Meer R. Increasing intestinal resistance of rats to the invasive pathogen *Salmonella enteritidis*: Additive effects of dietary lactulose and calcium. Gut. 1997;**40**(4):497-504

[125] Schaafsma G. Lactose and lactose derivatives as bioactive ingredients in human nutrition. International Dairy Journal. 2008;**18**(5):458-465

[126] Saarela M, Hallamaa K, Mattila-Sandholm T, Matt J. The effect of lactose derivatives lactulose, lactitol and lactobionic acid on the functional and technological properties of potentially probiotic *Lactobacillus* strains. International Dairy Journal. 2003;**13**(4):291-302

[127] Alonso S, Rendueles M, Díaz M. Feeding strategies for enhanced lactobionic acid production from whey by *Pseudomonas taetrolens*. Bioresource Technology. 2013;**134**:134-142

[128] Playne MJ, Crittenden RG. Galacto-oligosaccharides and other products derived from lactose. In: McSweeney PLH, Fox PF, editors. Advanced Dairy Chemistry: Lactose, Water, Salts and Minor Constituents. 3rd ed. New York, USA: Springer; 2009. pp. 121-188

[129] Coppa GV, Zampini L, Galeazzi T, Facinelli B, Ferrante L, Capretti R, Orazio G. Human milk oligosaccharides inhibit the adhesion to CaCO-2 cells of diarrheal pathogens: *Escherichia coli*, *Vibrio cholerae*, and *Salmonella fyris*. Pediatric Research. 2006;**59**(3):377-382

[130] Torres DPM, Gonçalves MPF, Teixeira JA, Rodrigues LR. Galacto-oligosaccharides: Production, properties, applications, and significance as prebiotics. Comprehensive Reviews in Food Science and Food Safety. 2010;**9**(5):438-454

Chapter 8

Food Quality Management Systems in the Dairy Industry: A Case Study on the Application of Predictive Microbiology in the Microbial Quality of Milk

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Additional information is available at the end of the chapter

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Abstract

Agri-food industries must guarantee the safety of the produced foods through the application of the existing regulations, by correctly implementing quality control systems. In relation to the quality of drinking milk, it is extremely important to monitor the industrial treatments to which it is subjected to avoid the multiplication of spoilage and pathogenic microorganisms. Raw milk must undergo strict quality controls at the primary production level based on the knowledge of the main factors that influence their quality and microbiological safety: hygienic practices, health status of cows, frequency and moment of collection, storage temperature and time of transportation. To improve food safety and estimate food shelf life, predictive microbiology is a widely used tool for the estimation of microbial behavior as a function of intrinsic and extrinsic by using mathematical models. Throughout this chapter, a description of the current food quality management systems (FQMS) carried out by dairy industries will be provided by reflecting the current challenges, the guidelines, and available tools. A case study based on the application of predictive microbiology considering the importance of controlling certain factors in the primary production dairy chain will be developed.

Keywords: quality management system, dairy industry, MicroHibro, HACCP, predictive microbiology

1. Introduction

Food industries are responsible of assuring the quality and food safety by means of the implementation of quality management systems (QMS). Industrial QMS allows to accomplish with the specifications between the food companies and customers, claiming the quality along

the time [1]. The food production chain connects different actors and stages. In the case of dairy production, the chain includes some steps as milking, milk collection, milk reception, industrial treatment and dairy transformation, packaging, storage, distribution, and consumption [1, 2]. Each stage should offer added value to the product with minimal cost [3] and apply the European Union legislation such as: Regulation (EC) 178/2002, laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety, Regulation (EC) 852/2004 on the hygiene of foodstuffs, Regulation (EC) 853/2004 laying down specific hygiene rules for on the hygiene of foodstuffs, and Regulation (EC) 854/2004 on the organization of official checks on products of animal origin intended for human consumption.

Milk is a nutritious food, which allows the growth of microorganisms. Its composition includes proteins, fats, carbohydrates, vitamins, minerals, and essential amino acids, which provide an adequate environment for the microorganisms' proliferation because of a neutral pH and a high-water activity on milk. Some microorganisms use these nutrients directly, and others unleash the metabolism of them. Their proliferation is determined by temperature, time, and nutrient availability [3, 4]. For this reason, good manufacturing and good hygiene practices (GMP and GHP) applied at all the stages of the chain must to be adopted.

QMSs are focused in the control of the main hazard sources that can occur in the food industry. The dairy chain implies several limitation and potential hazard that can reduce the quality and safety of milk. The intrinsic characteristic of milk, as an ideal environment for microorganisms, [4] combined with its direct contact with equipment and facilities during the different stages of milk production make the implementation of QMS in the dairy production chain a challenge [5]. Two types of hazards have been considered the most relevant: chemical hazards are more associated to feed and farm and microbiological hazards more related with farm and dairy processing [6]. Application of validated predictive models is recognized as a valuable tool able to estimate behavior of potential microbiological hazards or spoilage microorganisms on raw milk during production chain. The obtained outcome can provide an estimation of the impact of relevant factors within the milk production chain and corrective measures to be applied.

2. Quality management system of the dairy production chain

2.1. General consideration of the self-control and quality management systems (QMSs)

The self-control systems are based on the Hazard Analysis of Critical Control Point System (HACCP). This is a systematic procedure focused to identify microbiological, chemical, and physical hazards as early as food manufacturing and to eliminate these hazards by taking the suitable measures [7, 8]. Correct practices and conditions described on the Codex Alimentarius [9] and Regulation (EC) 853/2004 define prerequisites to be deemed before the development of HACCP.

The International Organization for Standardization (ISO) created the international standards which give specifications for products, services, and systems, to ensure quality, safety, and

efficiency. They comprise standards for the development of a quality system of any type of organization and any part of the world. They were directed for the assurance of the process quality and the establishing of an international normative frame for the management and quality control [2, 10]. The requirements applied by the food industry can be tougher than the compliance of legal rules. The ISO certification has become a very important strategy for the companies because it provides the confidence to customers and other stakeholders to control food safety hazards. In addition, international standards enhance transparency in the development of food quality and safety procedures, thus helping to improve and update food safety systems [11]. The implementation of these standards is fixed with the ISO 9000:2000—Quality management systems—fundamentals and vocabulary, which takes part of others onto NMX ISO 9000:2000 with guidance, technical reports, and specifications [2, 10].

2.2. QMS applied in farms and transport

Although HACCP are systems implemented in the industries, the primary production has no duty of developing them. On this case guidance and advices for the correct management are followed in relation with animal health, food, traceability, hygiene, and cleaning [12].

The milk collection by the trucks in farms must to be done in hygienic conditions to prevent microbial contamination. For the correct handling of raw milk, the truck driver must be trained to use suitable clothes and to avoid the entrance to stables. Milk samples will be collected for quality control, verifying the temperature of storage (8°C for daily collection and 6°C for each 48 h collection), and trucks must maintain the milk temperature on tanks below 10°C according to EC Regulation 853/2004 [13].

Current transportation unit designing must have an efficient cleaning and drainage system to prevent corrosion and the transference of foreign substances to milk [14]. Complete cleaning and disinfection of truck are made after use and for 48 h. Trucks must have available the Agreement on the International Carriage of Perishable Foodstuffs and on the Special Equipment to be Used for such Carriage (ATP), which is a treaty of United Nations laying down rules about international transport of perishable food like milk between states members of the treaty [15].

Finally, the round-trip sheet of the truck must include information of the address industry, of the driver, of the suppliers of milk or farms, and of the date, hour, deposit, or liters collected.

2.3. QMS applied to the dairy industry

There are seven principles on the development of the HACCP on a dairy industry [16]. They are conducting a hazard analysis, identifying the critical points in the process, establishing critical limits, critical control point monitoring requirements, corrective actions verification, as well as record-keeping procedures and documentation of the system. The principles of HACCP are mainly oriented to achieve the following objectives—identifying, assessing, and controlling health hazards—to increase the level of product quality and safety and to low product liability risks and to enhance consumers' protection and confidence [9, 17].

The process and product description lead to describe the different production stages, and it helps to detect and define the hazard and critical points in the industry. Onto the different stages of the dairy industry, there are some where milk is not heat-treated (so it is raw) and others where milk receives a heat treatment. Regarding raw milk, time and temperature conditions are some of the main factors affecting milk quality. Bacterial counts can be highly influenced by the time since the milk is collected in tanks until it is thermally treated in the industry [18, 19]. Despite that the most spoilage and pathogenic microorganisms are inactivated by the thermal treatment, the previous stages in which milk remains untreated are very important from the assurance of quality point of view, to minimize undesirable effect of microbiology in the treated and packaged product [7]. These stages are tank truck reception and storage on silos. Truck's reception involves the stage in which milk go from tank of truck to silo of industry. Silo is a big container for the storage of milk in isothermal conditions. The temperature of milk must to be below 6°C until its transformation except when milk will be transformed immediately after or over the next 4 h. The truck reception is an important quality control point where quality control technicians collect milk samples for analyses before content passes to silo. Because raw milk is stored on tanker trucks and silos under isothermal conditions, if refrigeration temperature of tanks is not working properly, initial microorganisms could grow until reaching unacceptable levels. Thus, cooling and storage temperature of milk in farms should be controlled to prevent microbial contamination.

For safety assurance, antibiotic waste, mycotoxins, somatic cells, and bacterial counts in milk of trucks before discharging from cisterns to silos should be analyzed. Besides, from a quality point of view, additional physicochemical parameters of milk such as color, odor, appearance, acidity, alcohol stability, cryoscopic point, and milk temperature at arrival to industry are usually monitored. Likewise, chemical composition (mainly fat, proteins, and dry matter) is also analyzed [20].

Hazards must be identified to be eliminated or reduced to acceptable safety levels. The hazard occurrence probability and importance (i.e., severity of foodborne illness) allow to define different tolerance levels for hazard (**Table 1**). The source of hazards can be produced by biological, chemical, or physical agents. The incidences can be determined by the presence of any of these agents or favorable conditions for the effect of them on the human health. Incidences by biological agents can be caused by the reception of milk with higher somatic cells or bacteria than legal limits accepted. Biological agents can be originated by mastitis cows, poor management practices during the milking, cooling, and storage or through damaged equipment. All these factors

Probability	**Importance**		
	Low	**Medium**	**High**
Unlikely	Tolerable	Tolerable	Medium
Temporary	Tolerable	Medium	Not admitted
Likely	Medium	Not admitted	Not admitted

Table 1. Classification of hazards according to their level of tolerance based on their occurrence probability and importance.

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involve tissue damages on udder or proliferation of pathogenic microorganisms related to mastitis process. Incidences by chemical hazard can occur because the presence of disinfectant waste associated to milk blended with water cleaning. Incidences by physical hazard can be caused by the presence of foreign material when the maintenance of tank, truck, or silo does not have been verified consistently [8].

3. Case study on the application of predictive microbiology tools to determine the effect of production chain conditions on the microbial quality of milk

The optimization of the milk production chain to reduce spoilage before the heat treatments at industry greatly relies on the knowledge of storage temperature and times at the different steps in the primary production chain [21].

The temperature of milk below 7°C for a long storage period is associated with the proliferation of certain psychrotrophic bacterial species. The glycolytic, proteolytic, and lipolytic activity of these types of bacteria can produce deterioration of milk quality after heat treatment [22]. The most common species of psychrotrophic bacteria are *Pseudomonas* spp., *Alcaligenes* spp., *Bacillus cereus*, *Lactobacillus*, *Micrococcus*, *Streptococcus*, and *Enterobacteriaceae* family [4]. *B. cereus*, *Streptococcus*, and *Pseudomonas* spp. are known as the most persistent bacteria able to survive forming biofilms on equipment in dairy industries [23]. The main concern of these psychrotrophic species is related to the survival capacity of their enzymes and spores to typical heat treatments applied to milk. Species as *Pseudomonas fluorescens* produce extracellular enzymes when bacterial population reaches or exceeds 10^6 CFU/ml in food [21]. They can contribute to casein and lipid degradation, causing product alteration during distribution and home storage [24]. In addition, an increase in the number of milking times from two milking per collection and truck (only 1-day milking) to four milking per collection and truck (2-day milking) can result in a larger hydrolysis of fat globules and higher production of oxidation and browning phenomena because of temperature rises during different milkings [25].

Predictive microbiology is a scientist branch within food microbiology aimed at predicting microbial behavior in foods at different processing and storage conditions. Predictive microbiology is gaining relevance in the establishment of HACCP systems in food industries as tool to identify microbial hazards, set control limits, and/or define corrective measures to be implemented. One of the most relevant applications is focused on the study of the bacterial behavior on foods under different environmental conditions. The kinetic parameters (i.e., maximum growth rate, lag time, inactivation rate, etc.) are estimated by means of mathematical equations. The use of predictive microbiology is very interesting in order to optimize food processes and to provide assistance in decision-making in a short time frame to food industries. To this sense, the use of mathematical models by the food industry will depend on the development of appropriate and easy-to-use software tools, which encompass predictive models and allow different users to retrieve information from them in a rapid and convenient way.

On this case study, microbial growth was assessed at different time and temperature conditions during the milk production chain, from farm to industry, by the application of predictive microbiology. Further, corrective measures and recommendations to industrials are provided on the storage and handling practices to avoid milk spoilage before application of thermal treatments.

3.1. Selection of a predictive microbiology model

Pseudomonas spp. was selected as the reference spoilage microorganism group given its relevance as psychrotrophic bacteria and its influence on milk stability [23].

The model for *Pseudomonas* spp. corresponded to the one developed by Lin et al. [21] for *P. fluorescens* in UHT milk, considering storage temperature as the prediction variable:

$$\sqrt{\mu} = c(T - T_0) \tag{1}$$

where μ is the growth rate, c is the slope of regression line, T is the storage temperature, and T_0 is the hypothetical minimum growth temperature where the extrapolation of the regression line intersects the T axis.

3.2. Definition of the stages and variables for dairy chain

The different stages considered in the present study are represented in **Figure 1**. At *Farm*, milk is stored up to its collection by tank trucks, and it can be split into four different steps: (1) the cooling process after the first milking, (2) storage at low temperature, (3) the cooling process after the second milking, and (4) storage at low temperature up to collection. The stage *Tanker* in **Figure 1** represents for milk storage during tank truck transport from farm to industry. Finally, *Silo* stands for the milk storage in the containers at industry.

Since the purpose was to determine *Pseudomonas* spp. growth along the different represented steps by applying the selected predictive model, growth factors along the different considered steps and initial bacterial concentration at farm had to be defined beforehand.

To this end, an expert knowledge elicitation was carried out using the Delphi method [26, 27] where five experts were identified in base of their experience (**Table 2**) and asked for their

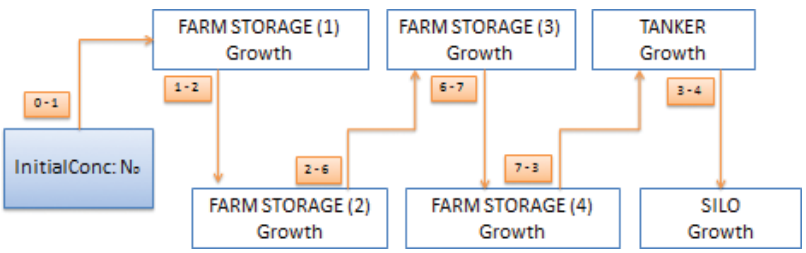

Figure 1. Flow diagram showing the stages considered for the exposure assessment model.

No.	Responsibilities, education, and specialization			
	Job position	**Qualification**	**Experience**	**Experience (years)**
1	Cistern reception control	Technician	Responsible for ensuring specifications of raw milk during reception of cistern trucks by means of analyzing milk quality and composition	4
2	Cistern reception control	Technician	Responsible for ensuring specifications of raw milk during reception of cistern trucks by means of analyzing milk quality and composition	3
3	Milk quality control chief	Graduate	Manager responsible for the quality of the milk, dairy products, and industrial processes	15
4	Cistern truck driver	Operator	Responsible for the hygienic collection of milk from tanks to cistern of truck and collection of quality control samples from farms	25
5	R + D responsible	Graduate	Responsible for the Research and Development Department	3

Table 2. Description of the profile of selected experts.

estimation for time and storage temperature of raw milk at the different stages studied: during storage at farm, in tankers and silos at industry facilities.

The values obtained from the Delphi method are presented in **Table 3** together with the initial concentration of psychrotrophic bacteria on raw milk at farm based on the study of Cempírková [28]. These values were used to define probability distributions that were then used to input the selected growth predictive models to predict *Pseudomonas* growth from farm to industry.

3.3. Case study assumptions

It should be mentioned that model by Lin et al. [21] has certain limitations concerning its application in the present case study. While the targeted product in the present study was raw milk as the study scope encompasses from farm to industry (i.e., before processing), the predictive model used was performed in UHT and low-fat milk. Therefore, predictions from the model could overestimate the actual growth in raw milk, in which competing microbiota and higher fat content are expected to reduce bacterial growth in comparison with treated milk. Nonetheless, estimates are still useful to represent for a worse-case scenario in which

Parameters	**T (°C)**			**Time (h)**			***Pseudomonas* spp. (log CFU/ml)**		
Stage	**Max**	**Min**	**Med**	**Max**	**Min**	**Med**	**Max**	**Min**	**Med**
1. Tank	3.9	3.5	3.7	18	1	12	4.81	2.84	3.66
2. Tanker	6	3.5	4.5	8	1	5	—	—	—
3. Silo	6.5	3.5	4.0	24	4	14	—	—	—

Table 3. Representative values for temperature (°C) and time (h) along the different steps from farm to industry for the milk production chain and initial concentration of *Pseudomonas* spp. at farm (log CFU/ml) [28].

bacterial growth is not influenced by the accompanying microbial population. Besides, the model domain of Lin et al. [21] was between 4 and 29°C so that temperatures below 4°C have not been considered for the exposure assessment model.

To enable to assess the suitability of the milk production chain in terms of microbial quality, a cut-off value was set determining spoilage associated with microbial protease activity after heat treatment. This reference value corresponded to 10^6 CFU/ml in food as discussed previously [21].

3.4. Exposure assessment model

An exposure assessment model was implemented in MicroHibro software v 1.7.7. (www.microhibro.com) including the stages abovementioned and based on the application of the selected predictive microbiology model. Storage time and temperature of milk at each stage were introduced by defining triangular distributions based on data presented in **Table 3**. Since distributions were used, a Monte Carlo simulation was performed in MicroHibro with 10,000 iterations. The Monte Carlo method implemented in MicroHibro enables to run the *Pseudomonas* growth model with a total of 10,000 random combinations of time and temperature for each step in the milk production chain and returns a probability distribution reflecting variability in the output which, in our case, corresponded to the concentration of *Pseudomonas* after storage in Silo.

The output from the model simulation provided the final concentration distribution for *Pseudomonas* after silo storage and just before heat treatment at industry as shown in **Figure 2**.

The simulated data in MicroHibro software yielded a mean concentration corresponded to 3.8 log CFU/ml, which means an increase in 0.2 log CFU/ml with respect to the initial concentration defined at farm second expert specifications in **Table 3**. The maximum value also resulted in a slightly higher increase of 0.4 log CFU/ml than that at farm. These results evidence that the milk production chain as defined in this study was adequate to significantly reduce milk spoilage due to psychrotrophic bacteria growth.

A scenario analysis was also performed in which different constant values for time and temperature during storage in silo were tested. According to the simulated data, the 5th and 99th

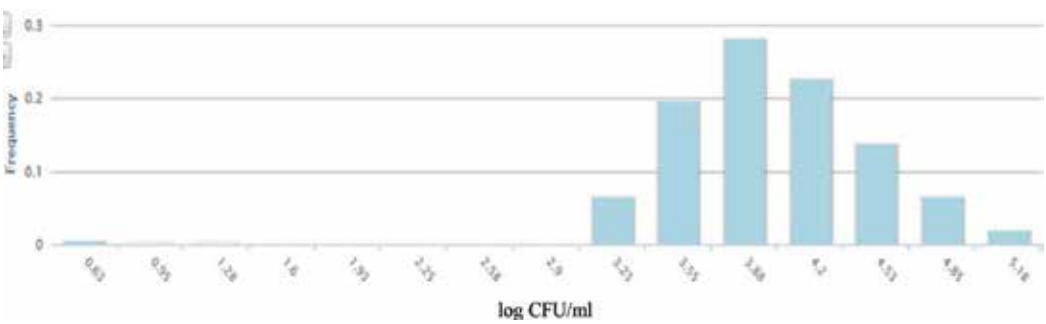

Figure 2. Simulated output distribution for final concentration of *Pseudomonas* after silo storage and before heat treatment at industry obtained using MicroHibro software.

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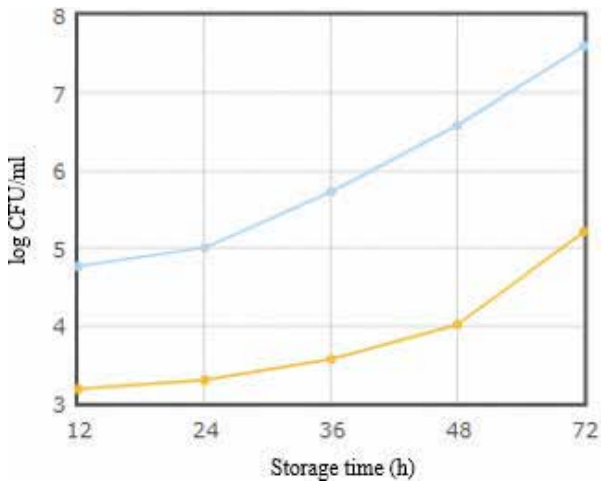

Figure 3. Simulated percentiles for the concentration of *Pseudomonas* versus storage time (h). The blue and yellow lines represent for the 99th and 5th percentiles, respectively.

percentiles (log CFU/ml) of the final concentration of *Pseudomonas* spp. were calculated. Percentile is a statistic widely used in exposure assessment studies to indicate the values below which a percentage of observations fall. In **Figure 3** the 5th and 99th percentiles are represented for obtained final levels of *Pseudomonas* for different storage time in silo. According to **Figure 3**, both percentiles showed a significant increase as time increased. For times higher than 36 h, the 99th percentile for *P. fluorescens* concentration was above 5.5 log CFU/ml (i.e., 1% of simulated values exceeded this limit). Considering a worst-case scenario (99th percentile), predicted microbial concentration was above 6 log CFU/ml (microbial quality criterion) when the storage time was around 40 h. This fact shows the importance of maintaining a short-time milk storage in silos

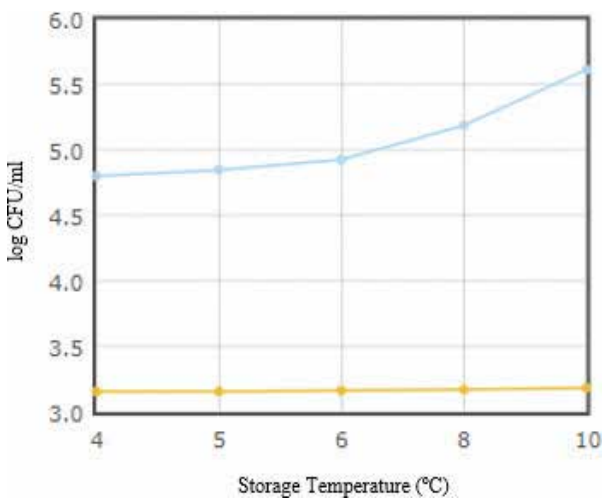

Figure 4. Simulated percentiles for the concentration of *Pseudomonas* versus storage temperature (°C). The blue and yellow lines represent for the 99th and 5th percentiles, respectively.

before processing. Besides, although considering relatively low counts of *P. fluorescens*, it is recommended that milk should not be stored in tanks for more than 24 h since unloaded milk remaining inside tanks could have a high risk of milk contamination to silos.

Figure 4 shows the effect of temperature. Results are only significant if considering extreme values (99th percentile) from the exposure assessment model. Even though, storage temperatures did not yield to increase levels of *P. fluorescens* above 6 log CFU/ml when simulated 99th percentile was considered for 10°C of storage temperature. However, extreme combinations of long storage times and high temperatures should be avoided to prevent milk from microbial spoilage. Storage temperature maintenance between 4 and 6°C seems to be enough to prevent the high proliferation of bacteria.

4. Conclusions

Quality system of food industries must apply the current regulations and guarantee the compliance of specifications indicated by customer. Industries can be tougher from the quality point of view by means the application of ISO 22000 and 9000:2000 Standards whose compliance provides confidence and positioning with respect to other companies. One of the most relevant parts when developing a HACCP consists in the identification and classification of hazards and analyzes possible incidences and correcting measures. Predictive microbiology is an efficient tool for the prediction of microbial safety and quality associated with specific steps along the milk production and distribution chain. To this respect, the case study presented herein evidenced that the current temperature and time values observed in the milk production chain are suitable to maintain milk microbial quality. In addition, specific critical limits were identified, especially for storage time at silo, where times shorter than 36 h could be a reliable measure to reduce milk spoilage due to microbial protease activity after heat treatment.

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References

[1] Khanson Q. An introduction to HACCP. 1st ed. Toronto, Canada: Qamrul A. Khan; 2011 ISBN 978-0-9739024-4-0

[2] Russel S. ISO 9000:2000 and the EFQM excellence model: Competition or co-operation? Journal of Total Quality Management. 2000;**11**(4–6):657-665

[3] Marth EH, Steele JL, editors. Raw milk and fluid milk products. Applied Dairy Microbiology. 2nd ed. New York, USA: Marcel Dekker; 2001. 736 p

[4] Quigley L, Sullivan O, Stanton C, Beresford TP, Ross RP, Fitzgerald GF, Cotter PD. The complex microbiota on raw milk. FEMS: Microbiology Reviews. 2013;**37**(5):664-698. DOI: 10.1111/1574-6976.12030

[5] Signorini ML, Sequeira GJ, Bonazza JC, Dalla SR, Martí LE, Frizzo LS, Rosmini MR. Use of indicator microorganisms for the hygienic-sanitary conditions evaluation in the milk primary production. Revista Científica FCV-LUZ. 2008;**18**(2):207-217

[6] Valeeva NI, Meuwissen MP, Lansink AG, Huirne RB. Improving food safety within the dairy chain: An application of conjoint analysis. Journal of Dairy Science. 2005;**88**(4):1601-1612

[7] Shah NP. Psychrotrophs in milk: A review. Milchwissenschaft. 1994;**494**:32-437

[8] CAC/ RCP 1-1969, Rev. 4-2003. HACCP/General Principles of Food Hygiene. http://www.fao.org/input/download/standards/23/CXP_001e.pdf

[9] Codex Alimentarius Commission (CAC). Hazard analysis and critical control point (HACCP) system and guidelines for its application. Report of the 29th session of the Codex Committee on Food Hygiene, Alinorm. 97/13A, Appendix II. Rome: Codex Alimentarius Commission; 1997

[10] Barendsz AW. Food safety and total quality management. Food Control. 1998;**9**(2–3):163-170. DOI: 10.1016/S0956-7135(97)00074-1

[11] ISO 22000:2005. Food Safety Management Systems—Requirements for any Organization in the Food Chain [Internet]. 2005. Available from: https://www.iso.org/iso-22000-food-safety-management.html [Accessed: 2017-02-15]

[12] CAC/RCP 57-2004. Code of Hygienic Practice for Milk and Milk Products [Internet]. 2004. Available from: www.fao.org/input/download/standards/10087/CXP_057s.pdf [Accessed: 2017-02-15]

[13] Regulation (EC) 853/2004 Laying Down Specific Hygiene Rules for on the Hygiene of Foodstuffs [Internet]. 2004. Available from: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2004:139:0055:0205:en:PDF [Accessed: 2016-04-17]

[14] CAC/RCP 47-2001 Code of Hygienic Practice for the Transport of Food in Bulk and Semi-Packed Food [Internet]. 2001. Available from: http://www.ico.org/projects/Good-Hygiene-Practices/cnt/cnt_en/sec_3/docs_3.5/Codex%20bulk%20transport.pdf [Accessed: 2016-12-01]

[15] Agreement on the International Carriage of Perishable Foodstuffs and on the Special Equipment to be Used for Such Carriage [Internet]. 2017. Available from: https://www.unece.org/fileadmin/DAM/trans/main/wp11/ATP_publication/2017/ATP_E_ECE_TRANS_271_WEB.pdf [Accessed: 2017-04-04]

[16] Peristeropoulou M, Fragkaki AG, Printzos N, Laina I. Implementation of the hazard analysis critical control point (HACCP) system to a dairy industry: Evaluation of benefits and barriers. Journal of Food Nutrition and Dietetics. 2015;**1**(1):102. DOI: 10.19104/jfnd.2015.102

[17] Lizcano L, López de la Torre C, Roldán C, Ercilla A, Rodríguez F, Santero MJ. Manual de Aplicación del Sistema APPCC en Industrias Lácteas de Castilla la Mancha. 1st ed. Toledo, Spain: Cecam, 2009. ISBN: 978-84-7788-546-7

[18] De Oliveira G, Favarin L, Luchese RH, McIntosh D. Psychrotrophic bacteria in milk: How do we really know? Brazilian Journal of Microbiology. 2015;**46**(2):313-321

[19] Godič Torkar K, Golc Teger S. The microbiological quality of raw milk after introducing the two-day's milk collecting system. Acta Agriculturae Slovenica. 2008;**92**(1):61-74

[20] Regulation (EC) No. 854/2004 on the Organisation of Official Checks on Products of Animal Origin Intended for Human Consumption [Internet]. 2004. Available from: http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex:32004R0854 [Accessed: 2017-03-22]

[21] Lin H, Shavezipur M, Yousef A, Maleky F. Prediction of growth of *Pseudomonas fluorescens* in milk during storage under fluctuating temperature. Journal of Dairy Science. 2016;**99**:1822-1830. DOI: 10.3168/jds.2015-10179

[22] Samardžija D, Zamberlin Š, Pogačić T. Psychrotrophic bacteria and milk and dairy products quality. Mljekarsivo. 2012;**62**(2):77-95

[23] Ozer B. Akdemir-Evrendilek G. Dairy Microbiology and Biochemistry: Recent Developments. 1st ed. Ankara, Turkey: CRC Press, 2014. 464 p. ISBN: 978-81482235029

[24] Baur C, Krewinkel M, Kranz B, Von Neubeck M, Wenning M, Scherer S, Stoeckel M, Hinrichs J, Stressler T, Fischer L. Quantification of the proteolytic and lipolytic activity of microorganisms isolated from raw milk. International Dairy Journal. 2015;**49**:23-29

[25] Callejo A, Díaz-Barcos V. Calidad de la Leche. Lipolisis. Frisona Española. 2008;**166**:98-102. ISSN 0211-3767

[26] Okoli C, Pawlowski SD. The Delphi method as a research tool: An example, design considerations and applications. Information Management. 2004;**42**(1):15-29

[27] European Food Safety Authority. Guidance on expert knowledge elicitation in food and feed safety risk assessment. EFSA Journal. 2014;**12**(6):3734

[28] Cempírková R. Psychrotrophic vs. total bacterial counts in bulks milk samples. Veterinarni Medicina-Praha-Czech. 2002;**47**:227-233

Section 3

Approaches on Water Treatment in Dairy Processing

Chapter 9

Membrane Technology and Water Reuse in a Dairy Industry

Douglas Felipe Galvão

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76464

Abstract

The dairy industry uses substantial amount of water to achieve the production process, especially for the maintenance of hygiene and cleanliness conditions. With this, is generating considerable volume of wastewater requiring treatment to meet the discharge standards before discarded? With the shortage of water resources and charging for water use, minimizing water consumption in industries is of paramount importance to maintain its market competitiveness. In this sense, there has been increasing interest in the reuse of wastewater, in order to decrease the good water consumption by processes that do not require such high quality feature. Membrane separation processes are highlighted in generating good-quality reuse water that can meet the demand of various industrial processes. Thus, the present study aimed to evaluate the membrane separation processes for the treatment of dairy industry effluent to reuse.

Keywords: milk, membrane, water, reuse, technology

1. Introduction

The dairy industry is responsible for the consumption of high volume of water, being the natural resource most used in the productive process of the sector. The need for water mainly occurs to maintain the conditions of cleaning, sanitary, and hygiene within the production sectors. With the high consumption of water, the generation of considerable effluent flow occurs, which makes the dairy industry a potential polluter. The effluents of the dairy industry contain organic matter, whey, and other constituents of milk, cleaning products, and soaps.

It is necessary to treat this effluent in order to meet the release standards set forth in the legislation and, consequently, reduce the pollutant loads released into the water environment. In

this sense, it becomes important the study for the proposal of methods of treatment of effluents and water reuse in the dairy industry. The possibilities of reuse of the effluent after treatment for industrial processes are restricted to the dairy industry, depending on the country and the legislation applied to the segment, since it is a food production industry. However, even in these industries, there is the possibility of reuse for cleaning floors, garden watering, cooling water, and use water in boilers.

Membrane separation processes have been prominent in recent years regarding the treatment of effluents for their reuse, since it allows the use of compact treatment systems, generating good-quality reuse water, which makes it possible for industries to save. In membrane separation systems, synthetic membranes are used, which imitate the selectivity characteristics of natural membranes, in order to separate, concentrate, or purify the substances present in the water, making it of better quality. A hydraulic pressure gradient or electric field must be applied in order for the separation to occur [1]. In this way, membrane separation processes have generated interest in the dairy industry, especially for the possibility of generating good-quality reuse water that can help to minimize consumption, as well as reduce the generation of effluents by these industries.

With the objective of elucidating membrane separation processes in the treatment of dairy effluents, research was carried out in the national and international literature, using previously published studies on the subject, verifying results obtained and the importance with regard to the reuse of water and the valorization of products.

2. Water in the dairy industry

In industry, water has several types of use; the main ones are presented below [2]:

A. human consumption: Water used in sanitary environments, changing rooms, kitchens and dining rooms, drinking fountains, safety equipment, or any domestic activity with direct human contact

B. raw material: As raw material, water will be incorporated into the final product, as is the case in the beer and soft drinks industries, or water is used to obtain other products, for example, hydrogen by electrolysis of water

C. use as an auxiliary fluid: Water, as auxiliary fluid, stands out for the preparation of chemical suspensions and solutions, intermediate compounds, chemical reagents, and vehicle or for washing operations

D. use for energy generation: Transformation of kinetic, potential, or thermal energy, accumulated in water, in mechanical and electrical energy

E. use as heating and/or cooling fluid: In these cases water is used as heat transfer fluid to remove heat from reactive mixtures or other devices that require cooling

F. other uses: The use of water for firefighting, irrigation of green areas, or incorporation in various byproducts generated in industrial processes, whether in the solid, liquid, or gas phase

In the dairy industry, water consumption is significant, being the natural resource most used in this sector [3]. The authors, Saraiva et al. [4, 5], investigating the consumption of water in the dairy industry found the coefficients shown in **Table 1**, which refer to the types of products produced.

The consumption of water is quite variable, related to the size of the dairy, the standardization of activities, reuse practices, the technologies employed, and the type of product produced. The mean intakes reported for different European and Nordic countries are shown in **Table 2**.

The high consumption of water in the dairy industry is related to the need to maintain sanitary and hygiene conditions and is mainly due to cleaning operations, milk washing, cooling, and steam generation [3]. According to Vourch et al. [6], the water consumption of the dairy industries will depend on the volume of milk that is processed, the water quality required for the different industrial processes, and the water management practices in the industry.

By analyzing the data presented in **Tables 1** and **2**, it is possible to notice that the highest coefficient of consumption found by Saraiva et al. [4] occurs in the production of yogurt, presenting a consumption of 10 liters of water for each liter of processed milk. This figure was above the other production lines and was well above the figures cited by Maganha [3] for European and Nordic countries. Differently from Saraiva et al. [4, 5] found high consumption for the production of milk candy, but they mentioned the possibility of standardization of processes, reduction of waste by dairy employees, and adoption of reuse practices as ways of reducing consumption of this production line. Looking at the data, it can be observed that consumption can vary widely according to the production lines, as well as the water management practices of the dairy industries, which makes relevant the development of studies referring to better forms of use and reuse of water.

2.1. Liquid effluents from the dairy industry

Industrial effluents are liquid streams from processes, operations, and utilities in industries [7]. It is also possible to consider the currents originated from the processes and operations in which water is used, but there is no incorporation of this in the final product, besides the net

Activity/product	Water/consumption (L L^{-1} of processed milk)	
	(1)	(2)
Yogurt	10	5.15
Butter	1	—
Mozzarella cheese	1.55	—
Ricotta cheese	0.2	—
Curd	1.4	—
Creamy cheese	1.39	—
Milk candy	—	9.14

Source: (1) adapted from [4] and (2) adapted [5].

Table 1. Water consumption in the dairy industry in different production lines.

Product	Water consumption (L L^{-1} of processed milk)			
	Sweden	Denmark	Finland	Norway
Milk and yogurt	0.96–2.8	0.6–0.97	1.2–2.9	4.1
Cheese	2.0–2.5	1.2–1.7	2.0–3.1	2.5–3.8
Powdered milk and/or liquid products	1.7–4.0	0.69–1.9	1.4–4.6	4.6–6.3

Source: adapted from [3].

Table 2. Water consumption in dairy products from different Nordic and European countries.

part from the raw material that is removed in the industrial processes [8]. Industrial effluents vary according to the technologies that are used in the production processes, the values of raw materials and inputs, the age of the industry, and the specialization of the equipment operators, besides the way of operation if it is continuous or intermittent [1].

The characteristics of the effluents can be biodegradable, similar to sanitary sewage, or completely nonbiodegradable, especially those from industries of metallic products such as electroplating. The food, paper, and cellulose and sugar-alcohol industries are characterized by the generation of biodegradable effluents rich in organic matter [8].

In the case of food industries, the dairy industry is characterized by high water consumption and, consequently, high effluent production. **Table 3** lists the volumes of effluents generated in the dairy industry, according to the type of product produced.

For Tchamango et al. [9], dairy products are characterized by the high generation of liquid effluents, with high oxygen demand (COD) due to their high organic load. In many cases the generated effluent ends up being released directly into the rivers, contributing to its eutrophication by the phosphorus and nitrogen components present in the effluent. The characteristics of the generated effluents vary widely according to the standard and technologies used in the dairy industry. In general, they present high concentrations of organic matter and considerable amounts of nutrients, suspended solids, organic pollutants, and infective agents, as well as milk residues, proteins, carbohydrates, fats, and residues of cleaning agents [4, 10].

In addition, **Table 4** shows the characteristics for the different parameters of the raw dairy effluent, according to Maganha [3].

Product type	Volume of liquid effluents (L kg^{-1} of processed milk)
"White" products (milk, cream, and yogurt)	3
"Yellow" products (butter and cheese)	4
"Special" products (concentrated of milk or whey and dehydrated milk products)	5

Source: [3].

Table 3. Effluent volume generated per kilogram of milk processed in the different production lines of the dairy industry.

Parameter	Variation range	
	(1)	(2)
Suspended solids (mg L^{-1})	24–5700	100–1000
Total suspended solids (mg L^{-1})	135–8500	100–2000
COD (mg L^{-1})	500–4500	6000
BOD (mg L^{-1})	450–4790	4000
Protein (mg L^{-1})	210–560	Undefined
Grease/oils and greases (mg L^{-1})	35–500	95–550
Carbohydrate (mg L^{-1})	252–931	Undefined
Ammonia (mg L^{-1})	10–100	Undefined
Nitrogen (mg L^{-1})	15–180	116
Phosphor (mg L^{-1})	20–250	0.1–46
Sodium (mg L^{-1})	60–807	Undefined
Chlorides (mg L^{-1})	48–469	Undefined
Calcium (mg L^{-1})	57–112	Undefined
Magnesium (mg L^{-1})	22–49	Undefined
Potassium (mg L^{-1})	11–160	Undefined
pH	5.3–9.4	1–12
Temperature (°C)	12–40	20–30

(1) Environment Agency of England and Wales, 2000; European Commission—Integrated Pollution Prevention and Control Jan/2006. (2) Associação Brasileira da Indústria de Queijo (ABIQ).

Table 4. Physical and chemical characterization of effluents from the dairy industry.

The characteristics of the effluents vary considerably between different activities of milk industrialization. **Table 5** shows the generation of BOD in the effluent related to the population equivalent, which can demonstrate the high organic load released in the dairy effluent.

These wastewaters, if disposed of in water resources without adequate treatment, may cause a number of problems, such as the mortality of aquatic species, damage to public health through the consumption of contaminated water, and an increase in the costs of treatment of this resource [12].

Thus, it is important to use optimized effluent treatment systems that are integrated with the identification of the liquid waste generation points in the production process, so that sustainable production can be achieved [4].

2.2. Water reuse in industry

The use of water combined with its high consumption in industry has led this segment to seek internal reuse alternatives and to consider the purchase of treated effluents from sanitation companies at prices lower than those of drinking water [13]. In this sense, Hespanhol [14]

Industrial unit	(DBO) (mg L^{-1})	Specific load of BOD (kg BOD/m^3 of processed milk)	Population equivalent (equivalent $inhab/L^{-1}$ of processed milk)
Milk reception and cooling station	600–1200	1.2	24
Milk and butter packaging	800–1600	3.0	60
Cheese factory	3000–6000	18.0	368
Yogurt	1500–3500	5.0	100
Milk drying tower	600–1200	1.3	27

Source: [11].

Table 5. Comparison between the BOD generated in a dairy and population equivalent of the generated organic load.

states that one should choose to satisfy those demands that require water of not very high quality by less noble sources and use of sources of superior quality only for nobler uses, such as domestic supply. In response to such conditions, the development of effluent treatment technologies has increased in order to meet the quality limits for reuse. Thus, reuse waters can become an important contribution to the water supply in the industry [6, 15–17].

It is possible to define reuse as the use of the effluent directly or after some treatment steps in other processes, according to the water quality required. Not all of the effluent generated needs to be treated for reuse, but in some cases, there is a need for specific purification treatments [18]. Reuse can also be conceived as the use of treated or untreated effluents for purposes that are beneficial, such as irrigation, industrial use, and urban purposes that are not potable [1]. Mierzwa [19] asserts, however, that the practice of reuse should not be seen as the goal of any model of water resource management in an industry, not meeting the requirements of Agenda 21, which exposes the rational use of water as a goal main.

Some industrial processes, more specifically those of the food industry, do not allow the use of reuse water in their production processes. However, even in these industries, there is the possibility of reusing water in processes that demand less noble water quality, such as for cooling, boiler feed, floor cleaning, and sanitary discharges in toilets, among others.

Due to the existing treatment techniques in the industries, a treated effluent can present similar, or even better, physical, chemical, or biological characteristics than the raw water. In the same way, effluents from certain processes present adequate quality to other processes, without the need for treatment [1].

In this sense, industrial water reuse can be classified into two broad forms of application, namely, external macro reuse, which refers to the use of treated effluents from stations administered by utilities or other industries, and the internal macro reuse, referring to the internal use of effluents, treated or not, from activities carried out in the industry itself [20].

One form of reuse that has gained relevance is cascading reuse. This form of reuse is a type of internal macro use, in which the industrial effluent originated in a certain process is directly used in a subsequent process [2]. It is important to emphasize the need to verify if the water

quality of the effluent is adequate for the next process. This type of reuse is the most interesting for the industries, because, just as the amount of water used will decrease, the amount of effluent generated also decreases. However, the concentration of specific pollutants and pollutants increases with this type of reuse [1]. In addition, cascade reuse does not require treatment of the effluent, since it is in the appropriate quality to be reused in another process that does not require a high quality, which ends up decreasing the amount spent for the reuse of water.

2.2.1. Reuse of treated effluents

It is the form of reuse that has been most applied in industry and is characterized by the use of the effluents generated in the site in other industrial processes, after adequate treatment to reach the required quality to the usage patterns that were preestablished (FIRJAN, 2006).

According to the water quality required in the process and the specific reuse scope, the effluent treatment levels will be established, in which safety criteria will be adopted, as well as related capital, operating, and maintenance costs [14]. Due to the techniques that are used in the treatment of effluents in the industries, the treated effluent sometimes has better characteristics than the raw water or may have aspects suitable for its use in certain processes [1].

The reuse of effluents after treatment can be done directly and after complementary treatment techniques. Direct reuse is accomplished by routing the treatment plant effluent to the site where it will be used. If there is still a contaminant in this effluent that needs to be eliminated to meet the required quality indices, complementary techniques must be adopted for the treatment and subsequent reuse [1].

It is also worth mentioning three categories of reuse water for industry that offer great possibilities of reuse: reuse water in cooling towers and lakes, open cycle (cooling) systems and process, and boiler feed [13].

2.3. Membrane separation process

Membrane separation processes use synthetic membranes to separate substances and solids that have small diameters, as well as molecules and ionic compounds, through the application of some type of external force. The external forces used in membrane filtration may be pressure, suction, or even electric potential [21, 22]. After the passage of the liquid through the membrane, this happens to be called permeate; what is retained is called concentrated or retentate [23]. **Figure 1**, below, shows the simplified inlet and outlet diagram of the solution in the membrane.

Mainly because of the charge for water use and the need to preserve the environment, interest in water reuse has gained relevance. In this sense, membrane separation processes in the treatment of effluents are presented as one of the most promising technologies, enabling the reuse of water and, consequently, reducing the consumption of good-quality water in processes that do not demand such high quality, optimizing its use in industrial processes [16]. According to Mierzwa and Hespanhol [1], membrane separation processes differ from conventional filtration systems because the feed flow is parallel to the membranes. These processes have numerous advantages in that membranes are efficient in retaining small-sized solid particles

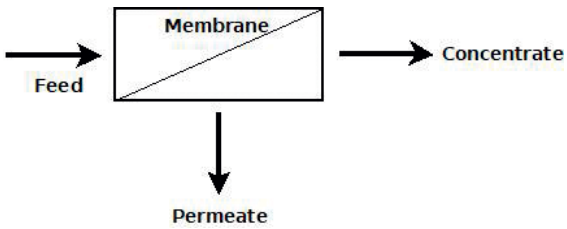

Figure 1. Scheme representing the inlet of the liquid, the outlet of the permeate, and the concentrate (retentate). Source: Adapted from Mierzwa [23].

and dissolved organic and inorganic compounds. In addition, the pressure of the membrane separation system is higher than in conventional filtration processes [1].

The materials used in the production of membranes vary. There are membranes prepared with polymeric materials—organic—and those that are produced with inorganic materials. Inorganic materials have been used in the production of membranes for more than 20 years. However, they have gained more space in the market only recently [22]. The organic materials for the production of the membranes are basically the polymers, and the inorganics are the metals and the ceramics. The most commonly used polymers in the manufacture of membranes are set out below in **Table 6**.

The processes using membranes are basically five: microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), reverse osmosis (RO), and electrodialysis (ED) [21]. The difference of each is the size of the particles and molecules that are retained and with what intensity the driving force promotes the separation, as visualized in **Figure 2** and in **Table 7**, respectively [7, 22, 24].

Membrane	Material
Microfiltration (MF)	Ceramics
	Polypropylene (PP)
Ultrafiltration (UF)	Ceramics
	Cellulose acetate (CA)
	Polysulfone (PS)
	Polyethersulfone (PES)
	Polyvinylpyrrolidone
	Polyacrylonitrile (PAN)
	Polyvinylidene fluoride (PVDF)
Nanofiltration (NF)	Polyacrylamide
Reverse osmosis (RO)	Polyamide
	Polyacrylamide

Source: [7, 24].

Table 6. Types of polymers used in the production of different membranes.

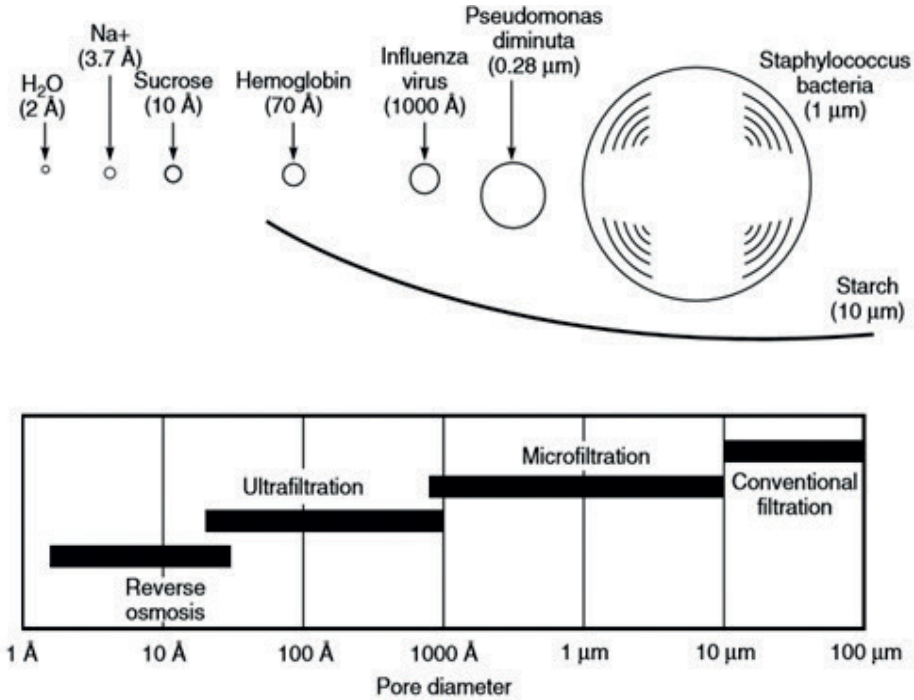

Figure 2. Particles and molecules retained according to the type of membrane separation process. Source: [25].

Membrane	Driving force
Microfiltration (MF)	<2 bar
Ultrafiltration (UF)	1–10 bar
Nanofiltration (NF)	5–35 bar
Reverse osmosis (RO)	Difference of concentration (15–150 bar)
Electrodialysis	Voltage and electric current

Source: [7, 24].

Table 7. Membrane type and driving force.

Compared with traditional physicochemical techniques to membrane treatment systems, a number of advantages can be cited, such as the need for chemical treatment only for membrane cleaning and high transfer rate occurring during the process, while maintaining the purity of the membrane product in ambient conditions [26]. The advantages highlighted are important because they reduce environmental pollution and increase the safety and efficiency of the process.

For the use of the treatment systems by membrane separation processes, it is necessary to use the so-called membrane modules. According to Schneider and Tsutyia [21], the module is a basic system that allows the use of membranes as separation unit. Its composition is

as follows: membranes, pressure support structures (vacuum or electric current), and feed channels and removal of concentrate and permeate. The main modules are modules with membrane plates, tubular modules, spiral modules, modules with hollow fibers, and modules with rotary disks [21].

2.3.1. Operation of membrane separation processes and factors influencing flow

Analyzing a membrane treatment system, Schneider and Tsutyia [21] state that, in addition to the module, these systems are formed by a pressurizing system to pressurize the channel that feeds the membrane, a valve in the concentrate channel that regulates the pressure in the feed channel, a permeate collection channel, and the surface material removal elements.

In membrane separation processes, permeate flow is time independent, depending specifically on the applied pressure potential or the diffusion by the membrane material. This independence of time indicates the mechanical stability of the membrane, the purity of the solvent, and its low interaction with the membrane fabricating material. When there is no observation of one of these conditions, the permeate flux will decrease over time, indicating that there is some change with the membrane [22].

Habert et al. [22] Indicate that the main causes of flux decline the mechanical deformation, the interaction of the solvent with the membrane material, or the presence of impurities in the solvent that causes the clogging of the pore. Schneider and Tsutyia [21] cite that the flow of liquid through the membrane is dependent on some factors, such as pore diameter, porosity of the membrane (fraction of the membrane area occupied by pores), membrane thickness, layer of concentration and polarization, filter cakes (retained material), chemical fouling (precipitated salts or gels), and biofilms.

One of the worst problems cited by the authors during the operation of membrane systems is the decay of the flow over time. This phenomenon is called “fouling” or “membrane fouling” [27–31].

This decrease in flow occurs for two essential reasons: membrane pore blockage, which increases flow resistance, and formation of a fouling layer which creates an additional layer of resistance to permeate flow. Other factors such as adsorption of the solute, deposition of particles within the pores of the membrane, and changes in the characteristics of the fouling layer can affect the fouling of the membrane or increase it or modify both essential fouling phenomena (pore clogging and formation of the scale layer). The formation of the polarization concentration layer may also add a layer of resistance [30].

According to Giacobbo et al. [29], fouling refers to the accumulation of contaminants on the surface or inside the pores of the membrane, causing the decrease of permeate flow. The phenomenon of polarization by concentration is the increase of the solute concentration at the interface membrane/solution, which ends up generating a backscatter of the solute toward the solution. That is, the solute is concentrated in the region close to the membrane/solution interface, preventing the passage of the solvent. According to the authors [27], the reduction in the permeate flux during the filtration, applying constant pressure, occurs due to the deposition of colloidal particles and macromolecules on the surface of the membrane. Thus, the authors state that membrane cleaning should be performed periodically during the operation of the system to prevent the possibility of extreme membrane fouling and even irreversible fouling.

Fouling eventually increases the complexity of operation of membrane separation systems because the system often needs to be stopped to restore flow through backwashing, resulting in increased cost savings and less use of UF and MF systems for many processes [30].

The ways of cleaning the membranes can be physical and chemical. Physics are dependent on mechanical forces to remove particles accumulated on the surface of the membrane. They include flushing and/or reverse flushing and backwashing [27]. Chemical methods depend on chemical reactions, which break down the forces that bind the adhered substances on the surface of the membrane. Chemical cleaning methods are hydrolysis, digestion, saponification, solubilization, and dispersion [27].

2.3.2. Membrane separation processes in the treatment of dairy effluents

Studies using membrane separation processes have varied greatly, mainly because of the great capacity of this process in concentrating, separating, purifying, and treating substances. In the treatment of dairy effluents, some studies will be cited using membranes of microfiltration, ultrafiltration, and nanofiltration, since they are the most used today. It should be noted that such processes are not the only ones but reverse osmosis and dialysis, for example, may also be used in the treatment.

The use of membrane separation processes, in the treatment of industrial effluents, allows high efficiency and the possibility of reuse of effluents in the industry itself. Thus, reuse of wastewater has become an environmentally and economically viable option for industries [16]. Using condensed steam from the dairy industry, Chmiel et al. [32] carried out the nanofiltration in the treatment of this wastewater, being able to maintain all the parameters required for the use as reuse water in the boilers. According to the authors, the reuse of water in the boilers is important for the industries, because in these processes, the volume of water used is considerable. Reuse minimizes waste of water.

In the study of the removal of biochemical oxygen demand (BOD) and chemical oxygen demand (COD) from dairy effluent, Khider et al. [33] used a commercial ultrafiltration membrane and a clay support ultrafiltration membrane. The crude effluent samples were filtered and were able to remove 4000 mg L^{-1} and 1800 mg L^{-1} of BOD and COD, respectively, at 90 and 62 mg L^{-1} with the commercial membrane. For the membrane with clay support, the permeate characteristics reached 60 mg L^{-1} for BOD and 42 mg L^{-1} for COD. Since BOD and COD represent the organic load of the effluent, the removal of such parameters is essential for the quality of the effluent to be released.

Baldasso [34] used membranes to separate and fractionate-specific proteins found in whey, discarded material in the treatment of dairy effluents where there is mainly cheese production and which is responsible for great pollution, especially for the large amount of organic matter. Using ultrafiltration membranes, the author found a great possibility for protein fractionation, which is extremely important for the dairy industry.

Luo [35] tested seven ultrafiltration membranes in the treatment of synthetic dairy effluent. UF membranes had cut molecular weight (MWCO) ranging from 5 to 100 kDa. For the membranes of 100, 30, 20, and 5 (kDa) obtained high efficiency for removal of turbidity, a very relevant result, because the reduced turbidity in effluent has greater acceptance by the population.

In membrane separation processes, besides the treated effluent being reused as reuse water within the processes, there is the possibility of concentration of the material retained by the membrane and its reuse in the manufacture of new products. This allows the valuation of the resources of the industry. Studies such as Brião and Tavares' [36] that used ultrafiltration as a process of treatment and reuse of dairy effluents found the possibility of reusing the permeate in the cleaning of equipment and the concentrate, after pasteurization, in the production of dairy byproducts such as milk candy.

The use of coagulation associated with membrane separation processes allows high removal of contaminants from the effluents. In this sense, Schimitt et al. [37] used effluent from the process of cleaning pasteurizers from a dairy industry. The UF membrane used was ceramic, with a pore size of 0.1 μm and molar retention of 4 kDa. They found removals of COD and turbidity above 96% for treatments using UF associated with *Moringa oleifera* coagulation.

The authors Kumar et al. [38] fabricated a low-cost ceramic microfiltration membrane and used it in the treatment of dairy effluent. They found that, by increasing permeate flow and pressure, there is a decrease in the removal of chemical oxygen demand. They attributed this fact to the greater pressure and flow force the structure of the membrane, which allows the greater passage of pollutants through it. They achieved a 91% removal efficiency of COD (135 mg L^{-1}), which was below the limit allowed for effluent discharge, concluding that the low-cost membrane was successful in treating the effluent.

Galvão [39] studied microfiltration and ultrafiltration membranes in posttreatment of dairy effluent. It found that the efficiency of microfiltration and ultrafiltration membrane separation processes proved to be quite considerable, both in the retention of organic matter and microorganisms and in the removal of nutrients such as nitrogen and phosphorus. As a complementary treatment of dairy effluent, after the treatment by the physical–chemical float, the MF membrane presented a considerable improvement in the effluent quality in both COD and BOD_5 retention, as well as in the retention of nutrients such as nitrogen and phosphorus.

3. Final considerations

Dairy industries, as potential polluters, need to seek improvements in processes and technologies, to remain competitive and to meet quality requirements in a sustainable way.

Membrane separation processes have been studied and used in the dairy industry, mainly aimed at treating effluents for reuse water generation, which ends up contributing to the preservation of water resources, generates savings for industries, and contributes to sustainability.

The possibilities of study are broadened in the case of membranes, as there is the possibility of reusing the permeate and the concentrate, allowing the reuse of water and materials for the production of byproducts. The studies presented demonstrate the relevance of membrane separation processes and their efficiency in the production of quality reuse water and the possibility of concentrating, separating, and purifying substances.

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References

[1] Mierzwa JC, Hespanhol I. Água na indústria: uso racional e reúso. 1st ed. São Paulo: Oficina de Textos; 2005

[2] Federação Das Indústrias Do Estado De São Paulo - FIESP. Conservação e reúso de Água: Manual de orientações para o setor industrial. 1st ed. São Paulo: CIRRA/IRCWR e DCT Engenharia; 2004

[3] Maganha MFB (Elab.). Guia técnico ambiental da indústria de produtos lácteos—Série P+L. 1st ed. São Paulo: CETESB; 2008

[4] Saraiva CB et al. Consumo de água e geração de efluentes em uma indústria de laticínios. Revista Inst. Latic. "Cândido Tostes". 2009;**367/368**:10-18

[5] Silva DJP. Gestão ambiental em uma indústria de produtos lácteos. Revista Leite e Derivados. 2006;**94**:52-63

[6] Vourch M et al. Treatment of dairy industry wastewater by reverse osmosis for reuse water. Dessalination. 2008;**219**:190-202

[7] Cavalcanti JEW d A. Manual de tratamento de efluentes industriais. 2nd ed. São Paulo: Engenho Editora Técnica; 2012

[8] Pires ECD, Del Nery MHRZ. Tratamento de águas residuárias industriais. In: Calijuri MdoC, Cunha DGF (coord), editors. Engenharia ambiental: conceitos, tecnologia e gestão. 1st ed. Rio de Janeiro: Elsevier; 2013

[9] Tchamango S et al. Treatment of dairy effluents by electrocoagulation using aluminium electrodes. Science of the Total Environment. 2010;**408**:947-952

[10] Lásló, et al. Effect of preozonation on the filterability of model dairy wastewater in nanofiltration. Desalination. 2009;**240**:170-177

[11] Federação Das Indústrias do Estado De Minas Gerais – FIEMG. Guia técnico ambiental da indústria de laticínios. 1st ed. Minas Gerais: FIEMG; 2014

[12] Schmitt DMF et al. Estudo da eficiência do composto ativo de Moringa Oleífera extraída com soluções salinas na tratabilidade de águas residuárias da indústria de laticínios. Revista Engevista. 2014;**16**:221-231

[13] Constanzi RN. Tratamento de efluentes domésticos por sistemas integrados de lodos ativados e membranas de ultrafiltração visando o reuso de água [thesis]. São Paulo: Escola Politécnica de São Paulo; 2007

[14] Hespanhol I. Potencial de Reuso de Água no Brasil Agricultura, Indústria, Municípios, Recarga de Aquíferos. Revista Brasileira de Recursos Hídricos. 2002;**7**:75-95

[15] Árevalo J et al. Wastewater reuse after treatment by MBR. Microfiltration or ultrafiltration? Desalination Journal. 2012:299

[16] Andrade LH et al. Nanofiltration as a post treatment to membrane bioreactor effluent for dairy wastewater reuse. Procedia Engineering. 2012;**4**:1956-1960

[17] Andrade LH, Mendes FDdeS, Cerqueira N, Espíndola JCA, Amaral MCS. Distribuição de massa molar em um biorreator com membrana para tratamento de efluente de laticínios. Revista de Engenharia Sanitária e Ambiental. 2014;19

[18] Telles DD; Costa RHPG (coord), editors. Reúso da água: conceitos, teorias e práticas. 2nd ed. São Paulo: Blucher; 2010

[19] Mierzwa JC. O uso racional e o reúso como ferramentas para o gerenciamento de águas e efluentes na indústria – estudo de caso da Kodak Brasileira [thesis]. São Paulo: Universidade de São Paulo; 2002

[20] Federação Das Indústrias Do Estado Do Rio De Janeiro – FIRJAN. Manual de conservação e reúso da água na indústria. 1st ed. Rio de Janeiro: DIM; 2006

[21] Schneider RP, Tsutyia MT. Membranas Filtrantes para o tratamento de água, esgoto e água de reúso. 1st ed. São Paulo: ABES; 2001

[22] Habert AC, Borges CP, Nobrega R. Processos de separação por membranas. 1st ed. Rio de Janeiro: E-papers; 2006

[23] Mierzwa JC. Processos de separação por membranas: conceitos para uma aplicação eficiente [Internet]. 2007 [Updated: 2007]. Available from: http://www.cepema.usp.br/wp-content/uploads/2011/06/IV-EPOA_-4.pdf> [Accessed: 15 março. 2015]

[24] Wagner J. Membrane Filtration Handbook: Practical tips and hints. 2nd ed. Osmonics; 2001

[25] Baker RW. Membrane Technology and Applications. 2nd ed. California: John Wiley & Sons, Ltda; 2004

[26] Acero JL. Membrane filtration technologies applied to municipal secondary effluents for potential reuse. Journal of Hazardous Materials. 2010;**177**:390-398

[27] Lautenschlager RS, Filho SSF, Pereira O. Modelação matemática e otimização operacional de processos de membrana de ultrafiltração. Revista de Engenharia Sanitária e Ambiental. 2009;**14**:215-222

[28] Hasan A, Peluso CR, Hull TS, Fieschko J, Chatterjee SG. A surface-renewall model of cross-flow microfiltration. Brazilian Journal of Chemical Engineering. 2013;**30**:167-186

[29] Giacobbo A, Rodrigues MAS, Bernardes AM, Ferreira JZ, Meneguzzi A. Microfiltração aplicada ao tratamento de efluentes de curtume. VII Simpósio Internacional de Qualidade Ambiental. Porto Alegre: Rio Grande do Sul; 2010

[30] Song L. Flux decline in crossflow microfiltration and ultrafiltration: Mechanisms and modeling of membrane fouling. Journal of Membrane Science. 1998;**139**:183-200

[31] Vidal CMS, Campos JR. Coagulação associada à microfiltração para o tratamento avançado de esgoto sanitário. Revista Ambiência. 2009;**5**:101-114

[32] Chmiel H, Mavrov V, Belières E. Reuse of vapour condensate from milk processing using nanofiltration. Filtration+Separation. 2000: 24-27

[33] Khider K, Akretche DE, Larbot A. Purification of water effluent from a milk factory by ultrafiltration using Algerian clay support. Dessalination. 2004;**167**:147-151

[34] Baldasso C. Concentração, purificação e fracionamento das proteínas do Soro Lácteo através da Tecnologia de Separação por Membranas [dissertation]. Porto Alegre: Programa de Pós-Graduação em Engenharia Química; 2008

[35] Luo J. Treatment of dairy effluent by shear-enhanced membrane filtration: The role of foulants. Separation and Purification Technology. 2012;**96**:194-203

[36] Brião VB, Tavares CRG. Ultrafiltração como processo de tratamento para o reúso de efluentes de laticínios. Revista de Engenharia Sanitária e Ambiental. 2007;**12**:134-138

[37] Schimitt DMF et al. Ultrafiltration combined with coagulation/flocculation/sedimentation using Moringa oleifera as coagulant to treat dairy industry wastewater. Water, Air, and Soil Pollution. 2013;**224**:1-10

[38] Kumar RV, Goswami L, Pakshirajan K, Pugazhenthi G. Dairy wastewater treatment using a novel low cost tubular ceramic membrane and membrane fouling mechanism using pore blocking models. Journal of Water Process Engineering. 2016;**13**:168-175

[39] Galvão DF. Pós-tratamento de efluentes por processos de separação por membranas e reúso de água em uma indústria de laticínios [dissertation]. Medianeira: Programa de Pós-Graduação em Tecnologias Ambientais; 2016. p. 117

Chapter 10

Physico-Chemical Treatment of Dairy Industry Wastewaters: A Review

Taner Yonar, Özge Sivrioğlu and Nihan Özengin

Additional information is available at the end of the chapter

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Abstract

Dairy industries have grown in most countries because of the demand in milk and milk products. This rise has led to the growth of dairy industries. The wastewaters discharged from this industry contain high concentrations of nutrients, chemical oxygen demand (COD), biological oxygen demand (BOD), total suspended solids (TSS) and organic and inorganic contents, which can cause serious environmental problems if not properly treated. The conventional biological treatment methods are suitable for dairy wastewaters due to its high biodegradability. However, long chain fatty acids formed during the hydrolysis of lipids show the inhibitory action during anaerobic treatment. Sequencing batch reactor (SBR) and up flow anaerobic sludge blanket (UASB) systems seem to be the most promising technology for the biological treatment of dairy wastewaters. Several research papers have been published on the application of aerobic and anaerobic treatment technologies for dairy industry wastewater, but both treatment methods still have some disadvantages. The most important challenge is to find cost-efficient and environmentally sustainable approaches to enable water reuse and waste management. Therefore, alternative treatment technologies against biological treatment methods such as coagulation, adsorption, membrane and electrolysis processes are under investigation. This chapter provides a critical review focusing on physico-chemical treatment technologies of dairy wastewater.

Keywords: dairy, wastewater treatment, physicochemical

1. Introduction

Industrialisation has a big role for development of a country which causes serious pollution problems throughout the earth [1]. With increase in demand for milk and milk products, dairy industries

have shown enormous growth in number and size in many countries all around the world [2]. The total milk production was estimated 818 million tonnes according to the İnternational Dairy Federation's World Dairy Report 2016, approximately 2% more than 2014 [3].

Dairy industry is the major source of food processing which has one of the highest consumption of water which used through every steps of dairy industry [4–6]. Therewith the amount of wastewater discharged from dairy industry has also raised [2]. For this reason, treatment of dairy wastes becomes very important before disposal [7]. Therefore, it is necessary to know how the processes take place in dairy industry.

In the dairy industry, the products are very diverse, which are mainly pasteurised and sterilised milk, yogurt, ayran, cheese, cream, butter, ice cream, and milk powder. Wastewater is produced both from production of products and from packaging units.

In the milking process, raw milk is collected from the producers, samples are taken and sent to the factory. Wastewater arises from the water coming from milk cans, storage tanks, washing places and cooling systems.

In the packaging unit, wastewater occurs during the cleaning of bottles, jars, tanks and related equipment with packaging.

In the cream production unit, butter is made with sweet cream and sour cream. Milk is centrifuged to separate the cream from the milk. While the cream-free milk is sent to the needed processes, butter is produced by churning the remaining cream. Wastewater is formed during the washing of the places and the cleaning of the tools.

In cheese making process, there are many steps. These include coagulation of the milk, cutting of the curd, cooking, when draining, placing curd in cheese moulds, and pressing the moulds. The cheese in the moulds is shaped and packaged. The most important wastewater source in the state is whey. However, whey can be re-used by mostly drying. For this reason, it is used again in ready-made food production (biscuit, chocolate, etc.) from being given as wastewater [8].

In the ice cream production unit, milk, additives, sugar and thickeners are mixed. After being pasteurised and cooled, aromas are added and packaged afterwards. Detergents and disinfectant-containing wastewaters form during cleaning and disinfection at the unit.

In condensed milk production, heated milk is evaporated and homogenised to yield sugar free milk. Sweet condensed milk is also produced using this method.

In the production of milk powder, it is obtained by applying vacuum evaporation and then spray drying.

The sources of the dairy industry wastewater are given in **Table 1**.

Operating methods, production program, type of product being processed, water management being applied and design of the processing plant are effecting the composition and concentration of dairy effluents. Processing waters, cleaning wastewaters and sanitary wastewater are the three major sources of dairy industry wastewaters [9]. Most of milk processing

Dairy processes	Sources of waste	
Preparation stages		
Milk receiving/ storage	• Poor drainage of tankers • Spills and leaks from hoses and pipes • Spills from storage silos/ tanks	• Foaming • Cleaning operations
Pasteurisation/ ultra heat treatment	• Liquid losses/ leaks • Recovery of downgraded product • Cleaning operations	• Foaming • Deposits on surfaces of pasteurisation and heating equipment
Homogenisation	• Liquid losses/ leaks	• Cleaning operations
Separation/ clarification (centrifuge, reverse osmosis)	• Foaming • Cleaning operations	• Pipe leaks
Product processing stages		
Market milk	• Foaming • Product washing • Cleaning operations • Overfilling • Poor drainage	• Sludge removal from clarifiers/ separators • Leaks • Damaged milk packages • Cleaning of filling machinery
Cheese making	• Overfilling vats • Incomplete separation of whey from curd • Using salt in cheese making	• Spills and leaks • Cleaning operations
Butter making	• Cleaning operations • Produce washing	• Vacreation (reduced pressure pasteurisation using steam) and salt use
Powder manufacture	• Spills of powder handling • Start-up and shut-down losses • Plant malfunction	• Stack losses • Cleaning of evaporators and driers • Bagging losses

Table 1. The sources of the dairy industry wastewater [15].

industries use clean in place (CIP) system which uses caustic, phosphoric/nitric, sodium hypochlorite solutions for cleaning, and these chemicals became a part of wastewater [1].

Dairy industry wastewaters contain suspended and dissolved solids, soluble and trace organics, nutrients, fats, chlorides, sulphate, lactose, and they are characterised by high chemical oxygen demand (COD) and biological oxygen demand (BOD) [2, 12–14]. The wastewater may also contain germicides, detergents and other types of chemicals [10]. These all have significant impact on wastewater. The characteristics and standards for discharge of dairy effluents are given in **Table 2**.

The characteristics of dairy wastewaters have shown variable effluent composition and differ from industry to industry. This makes it hard to use same methods for each wastewater for treatment.

Traditional approaches (aerobic and anaerobic processes) for the treatment of dairy wastewater have many disadvantages such as land cost, climatic conditions, need of sludge recycling, and so on [7]. The most preferred treatment method for dairy wastewater is a biological method including processes such as activated sludge, tricking filters, aerated lagoons, sequential batch reactor (SBR), upflow anaerobic sludge blanket (UASB), anaerobic filters, and so on. Aerobic processes are high energy intensive, but they have to be combined with anaerobic processes to achieve discharge standards [2, 16]. On the other hand, physicochemical methods are promising and effective methods for wastewater treatment.

	Milk and dairy products factory	Dairy effluent	Arab dairy factory	Dairy wastewater	World Bank report	Turkish discharge standards
pH	8.34	7.2–8.8	7.9 ± 1.2	7.2–7.5	6–9	6–9
Biochemical oxygen demand (BOD) (mg/L)	4840.6	1200–1800	1941 ± 864	1300–1600	50	—
Chemical oxygen demand (COD) (mg/L)	10251.2	1900–2700	3383 ± 1345	2500–3000	250	160
Total suspended solids (TSS) (mg/L)	5802.6	500–740	831 ± 392	72,000–80,000	50	—
Oil & grease (mg/L)	—	—	—	—	10	30
Total nitrogen (TN) (mg/L)	—	—	—	—	10	—
Total phosphorus (TP) (mg/L)	—	—	—	—	2	—
References	[17]	[18]	[19]	[20]	[21]	[22]

Table 2. Characteristics of some dairy industry wastewaters and discharge standards of dairy effluents (adapted from [2, 14]).

2. Assessment of physicochemical treatment processes on dairy wastewater

Wastewater characterisation plays an important role when the wastewater treatment system is designed. The COD concentration of dairy wastewater varies considerably [23]. Pollution load of a company wastewater producing yogurt in the sector and pollution load of a company wastewater producing cheese are very different. Since yogurt and ayran production plants have low oil-grease and COD parameters, they generally provide only physical + biological treatment and discharge standards. However, since the oil-grease and KOI parameters are high in the cheese producing plants, the physical + chemical + biological treatment units are generally preferred in the small-scale plants.

In many countries, the wastewater of dairy and dairy products is among the sources that cause significant pollution of natural aquatic environments. Numerous studies have been conducted to date to considerably reduce the adverse effects of these wastewaters [24].

Physicochemical processes are widely used for treatment of industrial wastewaters. Summarised literature of the dairy industry wastewater treated with physico-chemical processes are given in **Table 3**.

2.1. Chemical precipitation and coagulation/flocculation processes

Some physical-chemical-biological processes are usually interacting such as chemical precipitation, colloids' aggregation by coagulation-flocculation processes. In most processes, both precipitation and coagulation-flocculation happen simultaneously.

Chemical precipitation involves the addition of chemicals to separate the dissolved and suspended solids by sedimentation and used for primary settling facilities. In current practice, phosphorus and heavy metal removal can be realised. Many substances have been used as precipitants over the years such as alum, ferric sulphate, ferrous sulphate, and so on. They are used primarily for the treatment of metallic cations, anions, organic molecules, detergents and oily emulsions [44].

Coagulation/flocculation processes are used basically to separate suspended, colloidal and dissolved contents from wastewater and they applied directly to raw wastewater [45]. The process can be divided into two categories. The first one named coagulation is the process where chemicals (coagulant agents) such as iron or aluminium are used to overcome the factors which promote the stability of the system. The second process named flocculation makes destabilised particles come together and they can be separated easily through gravity settling [46]. A few studies have been studied in the literature for the coagulation of dairy wastewater. The literature studies are summarised at **Table 3**.

2.2. Adsorption process

Adsorption has been found to be attractive for the removal of organic compounds from wastewater [47]. There are many types of adsorbents including activated carbon, synthetic polymeric

Treatment process	Characterisation	Remove/removal efficiency (%)	References
Chemical precipitation	Ferrous sulphate and ferric chloride as coagulant	BOD: 64% (ferrous sulphate) and 85% (ferric chloride)	[25]
Chemical precipitation	Pre-treatment $Ca(OH)_2$ and $FeSO_4$ used	High COD removal	[26]
Coagulation	Alum and ferrous sulphate as coagulant	Alum was more effective than ferrous sulphate and it removed 5% more COD than ferrous sulphate.	[27]
Coagulation	Iron chloride, aluminium sulphate and calcium chloride as coagulant	Calcium hydroxide: organic matter: 40%, suspended solid: 94%, phosphorus: 89%	[28]
Coagulation	$FeCl_3$ as coagulant Pre-treatment	Addition of 0.10–0.15 mg $FeCl_3$-$6H_2O$/mg COD, or about 0.20 mg $Al_2(SO_4)_3.18H_2O$/mg COD, was sufficient to obtain good removal of organic matter. Maximum removal efficiencies of 67–90% total COD	[29]
Coagulation/flocculation	$FeCl_3$, $Fe_2(SO_4)_3$ and alum Pre-treatment	$FeCl_3$ ve $Fe2(SO_4)_3$: COD: >70% Alum: COD: >65%	[30]

Treatment process	Characterisation	Remove/removal efficiency (%)	References
Coagulation/flocculation	$FeCl_3$ as coagulant	$FeCl_3$	[11]
		Weak wastewater: Doses: 550,180, 180 mg/l	
		COD: 76, 88 and 82%, respectively	
		Strong wastewater: Doses: 500, 500, 500 mg/l	
		COD: 45, 28 and 29%, respectively	
Adsorption	low cost adsorbents like powdered activated carbon, bagasse, straw dust, saw dust, fly ash and coconut coir as adsorbent	TSS: activated carbon had a better removal efficiency	[31]
Adsorption	lanthaum modified benthonite as adsorbent	Phospate: 100% in the first 15 min.	[32]
Membrane process	Reverse osmosis	95% water recovery with an average flux around 10–11 L/h.m^2	[33]
		TOC: 99.8%,	
		TKN: 96%, conductivity: 97% and lactose: 99.5%	
Membrane process	Reverse osmosis	Conductivity: 98.2%, COD: 97.8%	[34]
Membrane process	Ultrafiltration + reverse osmosis (pre-treated the wastewater with coagulant and PAC before)	Dairy industry wastewater can be recycled and reused	[35]
Membrane process	Membrane bioreactor + nanofiltration	MBR: COD: 98%, nutrients: 86% (86% nitrogen and 89% phosphorus)	[36]
		NF: COD: 99.9%, TSS: 93.1%	
Electrocoagulation		COD: 98% (at optimum conditions at electrolysis time of 7 min)	[16]
Electrocoagulation	Soluble aluminium anode as used	Phosphorus: 89%, nitrogen: 81%, COD: 61%	[37]
Electroflocculation	Iron electrodes	organic matter: 97.4% (at final pH of 7,4)	[38]
Combined electrode system	Iron and aluminium electrodes	20 min electrolysis was enough for the treatment of COD.	[39]
Electrochemical oxidation	IrO_2-Pt/Ti coated anodes	After 360 min 3700 mg/L COD removal was completed at a current density of 100 mA/cm^2 by using IrO_2/Ti electrode and complete decolourisation was achieved less than 60 min	[40]
Electrochemical process	Sn/Sb/Ni-Ti coated anodes	COD: 98% at a current density of 50 mA/cm^2 at 10 min	[41]

Treatment process	Characterisation	Remove/removal efficiency (%)	References
Electrocoagulation	Aluminium electrodes were used in the presence of potassium chloride as electrolytes	98.84% COD removal, 97.95% BOD5 removal, 97.75% TSS removal, and >99.9% bacterial indicators at 60 V during 60 min	[42]
Electrocoagulation	Direct current-aluminium plates were used as sacrificial electrodes	COD: 87% (the optimum current intensity, pH and electrolysis time for 1070 mg/dm^3 and were 3A, 9, 75 min, respectively. Mean energy consumption was 112.9 kWh/kg)	[43]

Table 3. Summarised literature of the dairy industry wastewater treated with physico-chemical processes.

and silica-based adsorbents. The most useful one is activated carbon because of cost efficiency and ability to adsorb wide range of organic compounds. Adsorption can be classified as physical and chemical adsorption. Van der Waals forces are used in physical adsorption and activated carbon is the best example of physical adsorption. A chemical reaction occurs between adsorbate and adsorbent, but it does not have a wide application in wastewater treatment [48].

Adsorption onto solid surfaces has various applications and used to remove organics, chemicals, heavy metals, and so on [49]. Fly ash, rice husk ash, and bagasse fly ash and activated carbon are some of low-cost adsorbents.

2.3. Membrane processes

Membrane processes such as microfiltration, ultrafiltration, nanofiltration, dialysis, electrodialysis and reverse osmosis are very promising methods [49]. Membrane filtration can be defined as removal or separation of particulate and colloidal substances from a liquid which work as selective barrier and are typically 0.0001–1.0 μm.

Several works focused on treatment of dairy wastewater by membrane operations. The use of membrane filtration technology offers a wide range of advantages for the consumer. The membrane technology is a novel nonthermal environmental friendly technology within future possibilities that minimises the adverse effect of temperature rise such as change in phase, denaturation of proteins and change in sensory attributes of the product.

2.4. Electrochemical process

Electrolysis is the degradation of organic or inorganic substances by using electrical charge. Oxidation and reduction reactions occur in electrolytic cell which contains an anode and cathode. When you apply electric to cell, negative ions will migrate to anode and positive ions will migrate to cathode and cations will be reduced and anions will be oxidised at both electrodes [48]. Electrocoagulation, electroflotation and anodic oxidation processes are some examples used for dairy treatment.

Electrocoagulation is an effective and promising treatment method subject of numerous publications. It has been shown that this method is particularly effective for a wide range of pollutants (heavy metals, organic compounds, microorganisms and various others). For this reason, it is considered as one of the more promising water remediation techniques.

EC is a primary wastewater treatment for inducing the controlled electrogeneration of flocculants/coagulants on site, usually under the application of a constant current. It is a complex process involving several chemical and physical phenomena with the formation of iron or aluminium cations from the dissolution of the corresponding sacrificial anode(s) and the simultaneous production of OH^- anions by cathodic reduction of water. The polymeric metal hydroxides formed act as excellent coagulating agents to favour the removal of dissolved, colloidal, or suspended matter, eventually yielding great percentages of removal of colour and turbidity. Coagulation mainly occurs by destabilisation, once the metal cations combine with the negatively charged particles moving towards the anode by electrophoretic motion [49].

3. Conclusions

Milk and dairy products are among the sources of industrial wastewater that cause significant pollution of natural aquatic environments. Wastewater generally comes from the dilution of milk or dairy products. In addition, detergents, disinfectant materials, machine oils and cloth fibres used in cleaning take place in wastewater. Dairy effluent nature is slightly alkaline, high temperature, unpleasant rancid odours, bitter or medicinal taste, hard, scaly deposits, and so on when it is disposed without treatments, it may result in adverse effects in fish growth, reproduction and immunity in water bodies, harmful effect on beneficial microorganism's and plant growth due to decrease micronutrients solubility, serious problems of health and hygiene, eutrophication.

In order to treat industrial wastewater of milk and dairy products, quite different systems have been developed in different countries of the world. Factors such as the initial investment and operating costs in the selection of treatment technologies, the presence of appropriate staff for the enterprise and the need for treatment to ensure the regulations are taken into account.

The use of membrane technology in wastewater treatment by biological treatment has a short history covering the last 20–30 years. It is in a rapid development process, since it removes many disadvantages of classical systems. Membrane processes are in their process of being an effective remedy for most wastewater treatment with their unique properties. They can be used alone or together with other wastewater treatment systems. Membrane bioreactors offer effective solid-liquid separation, high yields of effluent, smaller plant sizes and low sludge production.

Treatment methods supported by chemical substances (coagulation-flocculation, oxidation-reduction, flotation, etc.) implemented for organic matter in water and wastewater treatment,

solid material, turbidity, heavy metal, colour removal purposes. The treatment efficiency is affected by such factors such as the parameter to be eliminated, the chemical substance used, the duration of the detention, the intensity of the mixture; the amount of sludge formed can be more or less than the chemical substance. Compared to biological processes, advantages such as ease of operation, removal of the non-degradable part of the organic material, removal of the treatment efficiency from changes are caused to be particularly preferred.

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References

[1] Tikariha A, Omprakash S. Study of characteristics and treatments of dairy industry waste water. Journal of Applied & Environmental Microbiology. 2014;**2**(1):16-22. DOI: 10.12691/jaem-2-1-4

[2] Kushwaha JP, Srivastana C, Mall ID. An overview of various technologies for the treatment of dairy wastewaters. Critical Reviews in Food Science and Nutrition. 2011;**51**:442-452. DOI: 10.1080/10408391003663879

[3] The World Dairy Situation. Bulletin of the International Dairy Federation 485/2016; 2016. ISSN 0250-5118

[4] Sivrioğlu Ö, Yonar T. Determination of the acute toxicities of physicochemical pretreatment and advanced oxidation processes applied to dairy effluents on activated sludge. Journal of Dairy Science. 2015;**98**(4):2337-2344. DOI: 10.3168/jds.2014-8278

[5] Singh NB, Singh R, Imam MM. Waste water management in dairy effluents: Pollution abatement and preventive attitudes. International Journal of Science, Environment and Technology. 2014;**3**(2):672-683. ISSN: 2278-3687 (O)

[6] Sarkar B, Chakrabari PP, Viyajkumar A, Kale V. Wastewater treatment in dairy industries-possibility of reuse. Desalination. 2006;**195**:141-152. DOI: 10.1016/j.desal.2005.11.015

[7] Deshmukh DS. Wastewater generation and its treatment in dairy industries. International Journal of Application of Engineering and Technology. 2017;**2**(3):25-35. ISSN: 2321-8134

[8] Kiliç A. Süt endüstrisi atıksularının arıtımında ardışık kesikli reaktörde (SBR) hareketli biofilm uygulaması [thesis]. Selçuk Üniversity; 2006

[9] Britz TJ, Van Sckalkwyk C, Hung YT. Treatment of Dairy Processing Wastewater: Handbook of Industrial and Hazardous Waste Treatment. 2nd ed. New York: Marcel Dekker; 2004. pp. 616-646. DOI: 0-8493-7236-4

[10] Shete BS, Shinkar NP. Dairy industry wastewater sources, characteristics & its effects on environment. International Journal of Current Engineering and Technology. 2013;**3**(5): 1611-1615. ISSN: 2277-4106

[11] Shivsharan VS, Kulkarni SW, Wani M. Physicochemical cahracterization of dairy effluents. International Journal of Life science and Pharma Research. 2013;**2**(2):182-191. ISSN: 2250-3137

[12] Şengil A, Özacar M. Treatment of dairy wastewaters by electrocoagulation using mild steel electrodes. Journal of Hazardous Materials. 2006;**B137**:1197-1205. ISSN: 2250-3137

[13] Cristian O. Characteristics of the Untreated Wastewater Produced by Food Industry. Analele Universităţii din Oradea, Fascicula: Protecţia Mediului; 2010. p. XV

[14] Deshannavar UB, Basavaraj RK, Naik NM. High rate digestion of dairy industry effluent by upflow anaerobic fixed-bed reactor. Journal of Chemical and Pharmaceutical Research. 2012;**4**(6):2895-2899. ISSN: 0975-7384

[15] Tawfika A, Sobheyb M, Badawya M. Treatment of a combined dairy and domestic wastewater in an up-flow anaerobic sludge blanket (UASB) reactor followed by activated sludge (AS system). Desalination. 2008;**227**(1-3):167-177. DOI: 10.1016/j.desal.2007.06.023

[16] Tawfika A, Sobheyb M, Badawya M. Treatment of a combined dairy and domestic wastewater in an up-flow anaerobic sludge blanket (UASB) reactor followed by activated sludge. Desalination. 2008;**227**(1-3):167-177. DOI: 10.1016/j.desal.2007.06.023

[17] Qazi JI, Nadeem M, Baig SS, Baig S, Syed Q. Anaerobic fixed film biotreatment of dairy wastewater. Middle-East Journal of Scientific Research. 2011;**8**(3):590-593. ISSN: 1990-9233

[18] Bulletin of the International Dairy Federation. The World Dairy Report No: 485/216; 2016

[19] SKKY: Su Kirliligi Kontrol Yonetmeligi (Water Pollution and Control Regulation). T.C. Basbakanlik Mevzuati Gelistirme ve Yayin Genel Mudurlugu. 2004. p. 54. Available from: http://mevzuat.basbakanlik.gov.tr

[20] Demirel B, Yenigün O, Onay TT. Anaerobic treatment of dairy wastewater: A review. Process Biochemistry. 2005;**40**:2583-2595. DOI: 10.1016/j.procbio.2004.12.015

[21] Marshall KR, Harper WJ. Treatment of wastes from the dairy industry. In: Barnes D, Forster CF, Hrudey SE, editors. Surveys in Industrial Wastewater Treatment. Vol. 1. Boston: Pitman Advanced Publishing Program; 1984. pp. 296-376

[22] Dabhi YM. Physicochemical treatment of dairy plant wastewater using ferrous sulphate and ferric chloride coagulants. International Journal of Basic and Applied Chemical Sciences. 2013;**3**(4):9-14

[23] Karpati A, Bencze L, Boszeki J. New phisico-chemical pretreatment of dairy effluents. In: Proceedings of International Symposium on Waste Management Problems in Agro Industries; İstanbul. 1989. pp. 121-128

[24] Lolei M, Alidadi H, Nekonam G, Kor Y. Study of the coagulation process in wastewater treatment of dairy industries. International Journal of Environmental Health Engineering. 2013;**2**(5):17-21. DOI: 10.4103/2277-9183.132684

[25] Hamdani A, Chennaoui M, Assobhei O, Mountadar M. Dairy effluent characterization and treatment by coagulation decantation. Le Lait. 2004;**84**(3):317-328

[26] Rusten B, Eikebrokk B, Thorvaldsen G. Coagulation as pretreatment of food industry wastewater. Water Science and Technology. 1990;**22**:1-8

[27] Blanc F, Navia R. Treatment of dairy wastewater by chemical coagulation. In: Proceedings of the 45th Industrial Waste Conference; Pardue University, USA. pp. 681-689

[28] Rao M, Bhole AG. Removal of organic matter from dairy industry wastewater using low-cost adsorbents. Journal of Indian Chemical Engineer. Section A. 2002;**44**(1):25-28

[29] Kurzbaum E, Shalom OD. The potential of phosphate removal from dairy wastewater ad municipal wastewater effluents using a lanthanum modified bentonite. Applied Clay Science. 2016;**123**:182-186. DOI: 10.1016/j.clay.2016.01.038

[30] Vourch M, Balannec B, Chaufer B, Dorange G. Treatment of dairy industry wastewater by reverse osmosis for water reuse. Desalination. 2008;**219**:190-202. DOI: 10.1016/j.desal.2007.05.013

[31] Suarez A, Fidalgo T, Riera FA. Recovery of dairy wastewaters by reverse osmosis. Production of boiler water. Separation and Purification Technology. 2014;**133**:204-211. DOI: 10.1016/j.seppur.2014.06.041

[32] Sarkar B, Chakrabarti PP, Vijaykumar A, Kale V. Wastewater treatment in dairy industries: Possibility of reuse. Desalination. 2005;**195**:141-152. DOI: 10.1016/j.desal.2005.11.015

[33] Andrade LH, Mendes FDS, Espindola JC, Amaral MCS. Nanofiltration as tertiary treatment for the reuse of dairy wastewater treated by membrane bioreactor. Separation and Purification Technology. 2014;**126**:21-29. DOI: 10.1016/j.seppur.2014.01.056

[34] Tchamango S, Njiki CPN, Ngameni E, Hadjiev D, Darchen A. Treatment of dairy effluents by electrocoagulation using aliminium electrodes. Science of the Total Environment. 2010;**408**:947-952. DOI: 10.1016/j.scitotenv.2009.10.026

[35] Melchiors MS, Piovesan M, Becegato VR, Becegato VA, Tambourgi EB, Paulino AT. Treatment of wastewater from the dairy industry using electroflocculation and solid whey recovery. Journal of Environmental Management. 2016;**182**:574-580. DOI: 10.1016/j.jenvman.2016.08.022

[36] Yavuz Y, Öcal E, Koparal AS, Öğütveren ÜB. Treatment of dairy industry wastewater by EC and EF processes using hybrid Fe-Al plate electrodes. Journal of Chemical Technology and Biotechnology. 2011;**86**(7):964-969. DOI: 10.1002/jctb.2607

[37] Markou V, Kontogianni MC, Frontistis Z, Tekerlekopoulou AG, Katsonous A, Vayenas D. Electrochemical treatment of biologically pre-treated dairy wastewater using dimensionally stable anodes. Journal of Environmental Management. 2017;**202**:217-224. DOI: 10.1016/j.jenvman.2017.07.046

[38] Yonar T, Sivrioğlu Ö. Electrochemical degradation of dairy effluent using novel Sn/Sb/Ni-Ti anodes. Journal of Physical Chemistry and Biophysics. **7**:2. In: 3rd International Conference on Electrochemistry; 10-11 July 2017; Berlin, Germany

[39] Bazrafshan E, Moein H, Mostafapour FK, Nakhaie S. Application of electrocoagulation process for dairy wastewater treatment. Journal of Chemistry. 2013;8:Article ID 640139. DOI: 10.1155/2013/640139

[40] Sharma D. Treatment of dairy waste water by electro coagulation using aluminum electrodes and settling, filtration studies. International Journal of ChemTech Research. 2014;**6**(1):591-599. ISSN: 0974-4290

[41] Vaccari DA, Li Y, Shammas NK. Chemical precipitation. In: Wang LK, Hung YT, Shammas NK, editors. Phsysicochemical Treatment Processes. Totowa, New Jersey: Humana Press Inc.; 2005. pp. 141-174. DOI: 10.1016/j.watres.2010.07.001

[42] Beneois K, Dahmani S, Berrebah K. Efficiency and limits of physicochemical treatment of dairy wastewater.(Case Study: Dairy industry in Western Algeria). International Journal of Chemical, Environmental and Biological Sciences. 2016;**4**(1):9-11. ISSN: 2320-4087

[43] Tzoupanas ND, Zouboulis AI. Coagulation-Flocculation processes in waster/wastewater treatment: The application of new generation of chemical reagents. In: Proceedings of the 6th International Conference on Heat Transfer, Thermal Engineering and Envıronment (HTE'08); 20-22 Aug 2008; Greece

[44] Aghili F, Ghoreyshi AA, Rahimpour A, Rahimnejad M. Enhanced treatment of pre-treated sour whey by PAC adsorption/ membrane process. Chemical Engineering and Processing. 2016;**99**:80-85. DOI: 10.1016/j.cep.2015.11.006

[45] Hung YT, Lo HH, Wang LK, Taricksa JR, Li KH. In: Wang LK, Hung YT, Shammas NK, editors. Granular activated carbon adsorption: Phsysicochemical treatment processes, Phsysicochemical Treatment Processes. Totowa, New Jersey: Humana Press Inc.; 2005. pp. 573-630. DOI: 10.1016/j.watres.2010.07.001

[46] Al-Jabari M. Kinetic mass transfer adsorption model for treating dairy wastewater with stone cutting solid waste. Environmental Technology & Innovation. 2017;**7**:21-29. DOI: 10.1016/j.eti.2016.11.004

[47] Vignesvaran S, Ngo HH, Chaudry DS, Hung YT. Physicochemical treatment processes for water reuse. In: Wang LK, Hung YT, Shammas NK, editors. Phsysicochemical Treatment Processes. Totowa, New Jersey: Humana Press Inc.; 2005. pp. 359-376. DOI: 10.1385/159259820x

[48] Kuswaha JP, Srivastava VC, Mall ID. Organics removal from dairy wastewater by electrochemical treatment and residue disposal. Separation and Purification Technology. 2010;**76**:198-205. DOI: 10.1016/j.watres.2010.07.001

[49] Chen JP, Chang SY, Hung YT. Electrolysis. In: Wang LK, Hung YT, Shammas NK, editors. Phsysicochemical Treatment Processes. Totowa, New Jersey: Humana Press Inc.; 2005. pp. 359-376. DOI: 10.1385/159259820x

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Technological innovations, customer expectations, and economical situations have been forcing the dairy industry to adapt to changes in technologies and products. The goal of this book is to present some new approaches on dairy processing. It will provide several applications on the use of some novel technologies in various dairy products, the improvement of functionalities and quality systems of dairy products, and the advances in dairy wastewater treatment. The book will be useful for both practicing professionals and researchers in the dairy field. I would like to send my sincere thanks to all the authors for their hard work and contributions.

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