Contribution to the demonstration of the proof of the concept of the technological feasibility of using electro-activated whey as an ingredient and source of lactulose in the production of fermented dairy products

Thèse

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RÉSUMÉ

Le lactosérum est un coproduit de l'industrie de fabrication du fromage et de la caséine et se caractérise par une forte demande chimique et biologique en oxygène. Les énormes quantités de lactosérum générées dans le monde, sa composition particulière et son utilisation limitée dans l'industrie alimentaire rendent nécessaire la recherche d'autres moyens d'ajouter de la valeur à cet ingrédient en vue d'augmenter la rentabilité de la transformation du lait. Dans ce contexte, la technologie de l'électro-activation (EA) offre la possibilité de valoriser le lactosérum par la conversion *in situ* d'une partie du lactose en lactulose, un prébiotique bien connu et éprouvé. De plus, l'EA cathodique du lactosérum a montré une formation des bases de Schiff suite à la glycation avec différents sucres des protéines, des peptides et des acides aminés libres dans le processus d'électro-isomérisation du lactose en lactulose. Ces produits sont connus pour leur forte activité antioxydante. Ainsi, l'EA ouvre une possibilité de générer un ingrédient fonctionnel avec une valeur ajoutée significative. Dans ce contexte, l'objectif principal de ce projet de doctorat était d'étudier et de démontrer la faisabilité technologique de l'utilisation du lactosérum électro-activé comme ingrédient fonctionnel à haut potentiel prébiotique dans la production de différents produits laitiers fermentés.

La première étape de ce projet a été l'évaluation du comportement du lactosérum électro-activé dans la matrice de gel de lait fermenté. Une comparaison entre le pourcentage de matière grasse du lait, l'inoculum de lactosérum et le type de lactosérum a été effectuée. À cette fin, des échantillons de lait fermenté ont été préparés avec un ajout de 3, 6 et 9 % de lactosérum des deux types (électro-activé et non électro-activé). Il a été constaté que le lactosérum électro-activé prolongeait le temps d'obtention d'un pH de 4,6 en fonction de la quantité ajoutée. Ceci a été attribué à la capacité tampon plus élevée du lactosérum électroactivé; les résultats de l'acidité titrable ayant démontré des niveaux élevés de groupes acides libres. La microstructure du gel obtenu avec l'ajout du lactosérum électro-activé a montré une structure uniforme et moins poreuse, ce qui était en accord avec les résultats de la réduction de la synérèse. Pour confirmer ces résultats, un autre produit laitier fermenté avec un ajout de lactosérum électro-activé a été également développé.

Le kéfir enrichi de lactosérum électro-activé présentait également une phase de fermentation prolongée. Les particules de lactosérum EA ont été incorporées de manière homogène dans la matrice du gel de kéfir. Par conséquent, aucune synérèse n'était visible dans les

échantillons de kéfir additionnés de lactosérum EA à 9 %. De plus, les deux produits contenaient des niveaux élevés d'acides organiques (lactique, citrique, acétique, propionique et butyrique) lorsqu'ils étaient supplémentés avec du lactosérum EA. La production d'acide butyrique a été induite par l'ajout de lactosérum des deux types. L'analyse HPLC a révélé qu'environ 75-85% des niveaux initiaux de lactulose ont été conservés dans les produits avec du lactosérum EA après le processus de fermentation, ce qui démontre que la consommation de tels produits pourrait constituer une source de lactulose pour le consommateur.

La deuxième étape de cette recherche a été d'optimiser l'utilisation du lactosérum électro-activé en tant qu'ingrédient par son incorporation dans le produit qui convient à sa couleur et aux caractéristiques de la réaction de Maillard et des conjugués entre les matières azotées avec les sucres. Le lait fermenté cuit, Ryazhenka, a été testé comme une matrice alimentaire appropriée pour véhiculer le lactosérum EA enrichi en lactulose. L'extension du temps de fermentation a été moins importante pour ce produit. Ainsi, le Ryazhenka additionné de lactosérum à 9% a atteint un pH de 4,6 après 4 h de fermentation. Le produit additionné de lactosérum EA (9%) a atteint ce niveau après 6,5 h. De plus, le lactosérum EA a amélioré la capacité antioxydante de Ryazhenka. Au cours de cette étape, nous avons démontré par des tests *in vitro* que l'électro-activation du lactosérum peut diminuer l'allergénicité de la β-lactoglobuline de 19,52 mg/kg à 7,56 mg/kg, qui s'est stabilisée à 12,13 mg/kg après neutralisation. Comme le protocole de production de Ryazhenka comprend une étape de cuisson de 3 à 5 h à 97-100°C, on considère qu'il présente des taux d'allergénicité plus faibles en raison des changements de conformation des protéines induits par la chaleur. Ainsi, l'ajout de lactosérum électro-activé ne contribue pas à l'augmentation de l'allergénicité de ce produit.

Le troisième objectif de cette étude était de démontrer un potentiel prébiotique du lactosérum électro-activé en cultivant des bactéries probiotiques *Lactobacillus rhamnosus* subsp, *Lactobacillus rhamnosus* GG et *Lactobacillus acidophilus* ATCC4356. La densité optique (OD_{600}), le dénombrement sur plaques de Petri, la stabilité durant l'entreposage à 4 °C et la tolérance aux acides et à la bile des bactéries cultivées pendant 24 heures dans du lactosérum électro-activé ont été étudiés et comparés aux résultats obtenus par la culture sur du lactosérum, du lactosérum additionné de lactulose, du MRS et du MRS avec ajout de lactulose. Les valeurs OD_{600} les plus élevées (>2) ont été obtenues dans les biomasses de lactosérum EA pour toutes ces bactéries. Cependant, les numérations sur plaque de Petri n'ont

pas confirmé un nombre plus élevé de cellules bactériennes dans du lactosérum électro-activé. On peut donc conclure que le lactosérum électro-activé a probablement stimulé un métabolisme distinct chez les bactéries testées, ce qui est conforme à la définition des prébiotiques qui ont la particularité d'induire une stimulation de la croissance et/ou de l'activité des bactéries probiotiques afin de conférer des avantages pour la santé.

En résumé, cette recherche a validé la faisabilité technologique de l'utilisation du lactosérum électro-activé comme ingrédient dans la production de lait fermenté et source de lactulose qui reste stable durant l'entreposage pendant 14 jours à 4 °C. Également, ce projet a montré que le lactosérum électro-activé est un ingrédient fonctionnel prometteur pour une éventuelle utilisation potentielle comme additif alimentaire fonctionnel et prébiotique dans l'industrie laitière. De plus, il peut être utilisé comme agent protecteur pour améliorer la viabilité et l'activité des probiotiques.

ABSTRACT

Whey is a co-product of the cheese and casein-making industry and is characterized by a high chemical and biological oxygen demand. The generated worldwide huge quantities of whey, its particular composition and limited use in the food industry make it necessary to look for other ways to add value to this ingredient. In this context, electro-activation (EA) technology offers an opportunity to add value to whey by *in situ* conversion of part of lactose into lactulose which is a well-recognized prebiotic. Moreover, cathodic EA of whey showed a formation of Schiff bases following glycation of proteins, peptides and free amino acids in the process of lactose electro-isomerization into lactulose. These products are known to possess high antioxidant activity. Thus, EA opens a possibility to generate functional ingredients with significant added value. The main objective of this research was to prove the technological feasibility of the utilization of EA whey as a potential functional ingredient in the production of fermented dairy products.

The first step of this project was the assessment of the behaviour of electro-activated whey in a fermented milk gel matrix. A comparison between the percentage of milk fat, whey inoculum and whey type was conducted. For this purpose, fermented milk samples were prepared with an addition of 3%, 6%, and 9% whey of both types (electro-activated and non-activated). It was found that electro-activated whey prolonged the time of achievement of pH 4.6 depending on the amount added. This was attributed to the higher buffering capacity of electro-activated whey, as titratable acidity results demonstrated high levels of free acid groups. The microstructure of the gel with electro-activated whey demonstrated a uniform and less porous structure which was in accordance with the results of syneresis reduction. To confirm these results, another product with an addition of electro-activated whey was developed.

Electro-activated whey fortified kefir also had a prolonged lag phase of fermentation. EA whey particles are homogeneously incorporated in a kefir gel matrix. As a result, there was no syneresis visible in 9% EA whey added kefir samples. Moreover, both products contained high levels of organic acids (lactic, citric, acetic) when supplemented with EA whey. Production of butyric acid was induced by the addition of whey of both types. HPLC analysis revealed that approximately 75-85% of initial lactulose levels were conserved in the products with EA whey after the fermentation process.

The second step of this research was to optimize the utilization of electro-activated whey as an ingredient by its incorporation into the product that suits its colour and Maillard reaction conjugate characteristics. Ukrainian baked fermented milk, Ryazhenka, was tested as a suitable food matrix for carrying lactulose-enriched EA whey. The extension of fermentation time was less drastic for this product. Thus, 9% whey-added ryazhenka achieved a pH of 4.6 at 4h. EA whey-added product (9%) attained this level at 6.5 hours. Moreover, EA whey improved the antioxidant capacity of ryazhenka. During this step, we demonstrated *in vitro* that electro-activation of whey can diminish β-lactoglobulin allergenicity from 19.52 mg/kg to 7.56 mg/kg, which stabilized at 12.13 mg/kg after neutralization. As the production protocol of ryazhenka includes a 3-5h baking step at 97-100°C, it is considered to have lower allergenicity rates due to heat-induced conformational changes in the proteins. Thus, the addition of electro-activated whey does not contribute to an increase in the allergenicity of this product.

The third objective of this study was to demonstrate a prebiotic potential of electro-activated whey by culturing the following probiotic bacteria: *Lactobacillus spp., Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus* ATCC4356. Optical density (OD600), plate counts, storage stability and acid and bile tolerance of bacteria cultured for 24 hours in electro-activated whey were studied and compared to the results obtained by culturing on whey, whey with lactulose, MRS, and MRS with lactulose addition. The highest OD600 (>2) were obtained in EA whey biomasses for all bacteria. However, the plate counts have not confirmed a higher number of bacterial cells. Thus, it may be concluded that EA whey stimulates a distinct metabolism in tested bacteria. It complies with the definition of prebiotics, which requires stimulation of growth and/or activity of probiotic bacteria in order to confer health benefits.

To sum up, this research has validated the technological feasibility of using EA-whey as an ingredient in fermented milk production and showed that EA-whey is a promising component with potential use as a functional food additive and prebiotic in the dairy industry. Moreover, it can be used as a protective agent to enhance the viability and activity of probiotics.

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AEM: Anion Exchange Membrane
ANOVA: Analysis of the Variance
BCAA: Branched-chain amino acids
BOD: Biological Oxygen Demand
BSA: Bovine Serum Albumin
CEM: Cation Exchange Membrane
CFU: Colony Forming Units
COD: Chemical Oxygen Demand
DDPH: 2,2-Diphenyl-1-Picrylhydrazyl
Dia-NF: dia-nanofiltration
EA: electro-activation
EAW/EA whey: Electro-activated whey
ED: Electrodialysis
GDL: Glucono-delta lactone
GIT: Gastro-intestinal tract
GOS: Galactooligosaccharide
GRAS: Generally Recognized As Safe
HPLC: High-Performance Liquid Chromatography
IGs: Immunoglobulins
LA- transformation: Lobry de Bruyn- Alberda van Ekenstein transformation
MRPs: Maillard Reaction Products
MW: Molecular Weight
NF: Nanofiltration
ORP : Oxidation-reduction potential
SCFAs: Short-Chain Fatty Acids
SCP: Single-cell protein
SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SMP: Skim milk powder
UF: Ultrafiltration
WPC: Whey protein concentrate
WPI: Whey protein isolate
α-La: α-Lactalbumin
β-Lg: β-Lactoglobulin
κ – CN : κ- casein

DEDICATIONS

This work is dedicated to all the girls and women in the world in pursuit of education and their dreams.

“Everything in food is science. The only subjective part is when you eat it”
Alton Browns

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Special thanks to the reader of this thesis and appreciation of my work.

FOREWORD

The present thesis is submitted to the Faculty of Graduate and Postdoctoral Studies of Laval University to meet the requirements for obtaining the *Philosophiae Doctor es Sciences* (Ph. D) degree in Food Science and Technology at the Faculty of Agriculture and Food Sciences.

It consists of 7 chapters and is presented as a thesis with the insertion of articles that were submitted and published in scientific journals. The first chapter presents an immersion into a literature review on the topic. The second chapter is a description of the hypothesis and the research objectives of the research.

The third chapter presents the article entitled "Use of Electro-activated Whey as Ingredient in Fermented Milk Production: Proof of the Concept of the Technological Feasibility" published in "Journal of Food Science and Technology" under the authorship of Sabina Aidarbekova and Mohammed Aider.

The fourth chapter presents the second article entitled " Study of the Physico-chemical, Structural, Microbiological properties and Volatile Flavor Compounds Profile of Kefir Supplemented with Electro-activated Whey" published in the "International Dairy Journal". Authors: Sabina Aidarbekova and Mohammed Aider.

The fifth chapter presents the third article entitled "Production of Ryazhenka, a Traditional Ukrainian Fermented Baked Milk, by Using Electro-Activated Whey as Supplementing Ingredient and Source of Lactulose" published in “Food Bioscience”. Authors: Sabina Aidarbekova and Mohammed Aider.

The sixth chapter presents the results of the third objective of the research and is presented in a form of the fourth article entitled "Impact of Electro-activated Whey on Growth, Acid and Bile Resistance of *Lactobacillus rhamnosus* spp., *Lactobacillus rhamnosus* GG and *Lactobacillus acidophilus* ATCC 4356" submitted to publication to "International Dairy Journal". Authors: Sabina Aidarbekova and Mohammed Aider.

The seventh chapter draws the general conclusion of this work. The results obtained allowed us to meet the requirements of proving the research hypothesis.

INTRODUCTION

Cheese is a nutritious and delicious food that is popular worldwide. With ever-growing global consumption, its production is projected to reach 25.3 million metric tonnes by 2023 (Parashar et al., 2016). However, there is a gloomy side of cheese production, which is large amounts of generated polluting by-products. The amount of co-produced cheese whey exceeds the final product 9 times. Nevertheless, if relevant treatment is applied cheese whey can be transformed from gutter to gold, as its highly nutritious contents can be extracted to be used as functional food ingredients and supplements. Importance of cheese whey components, whey proteins and lactose and their derivatives touch nutraceutical, food and pharmaceutical industries.

Whey proteins are extracted from whey by a membrane, pressure-driven, and chromatographic technologies. Whey proteins have exceptional nutritional properties underlying in their rich content of essential amino acids, branched-chain (L-isoleucine, L-leucine, and L-valine) amino acids, and sulphur-containing amino acids (cysteine, methionine). Thus, whey proteins are known to promote bone growth and muscle strength, immunoregulatory, anti-inflammatory and antimicrobial properties (Krissansen, 2007). Lactose is a rather less desired product extracted from cheese whey, due to its low functionality stemming from low fermentability, low sweetening power, and low solubility. In addition to that ubiquitous utilization of lactose in consumer products is hindered by the high prevalence of lactose intolerance among the world's population (approximately 75%) (Mattar et al., 2012). However, the production of lactose derivatives such as galactooligosaccharides (GOS), lactulose, lactitol, lactobionic acid, lactosucrose, and epilactose extends its usability. GOS, lactitol and lactulose are recognized as prebiotics. Prebiotics are defined as 'nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health'(Roberfroid, 2007). Prebiotics are commonly added to fermented dairy products to support the growth and survival of probiotic bacteria. The combination of prebiotics and probiotics gave rise to the concept of synbiotic functional foods. Moreover, the addition of prebiotics can enhance functional properties of fermented dairy products, such as sensory, physicochemical, rheological,

and economic characteristics, due to their stabilizing and emulsifying properties (Mohammadi & Mortazavian, 2011). Several prebiotics are used as fat-replacers in low-fat fermented dairy products (Srisuvor et al., 2013).

In the study by (Özer et al., 2005) an effect of prebiotics inulin (0.5%, 1%) and lactulose (0.25%, 2.5%) was tested on viability and growth of *L. acidophilus* LA-5 and *B. bifidum* BB-02 in yogurt. Supplementation of these prebiotics significantly enhanced the survival of the probiotic bacteria on required therapeutic levels. Lactulose was more efficient in promoting the growth of *B. bifidum* BB-02 than inulin. Indeed, a bifidogenic effect of lactulose was extensively studied since its first establishment in 1957 (Ruszkowski & Witkowski, 2019). Its effect on yogurt's physicochemical properties was also broadly researched. (Heydari et al., 2018) reported that the addition of 3% lactulose reduced the storage modulus of yogurt gel and induced the highest degree of syneresis as compared to other prebiotics (inulin, hi-maize, lactitol, maltodextrin and β-glucan). A problem of weaker gel and higher syneresis at the addition of lactulose to yogurt formulation was also observed by (ben Moussa, Boulares, et al., 2019). In this regard, despite well-recognized health benefits of incorporation of prebiotics to fermented milk product matrix needs an exhaustive adjustment of technological compatibility for maintenance of desired physicochemical properties of the product.

A study by (Kareb et al., 2015) has proposed electro-activation of whey to obtain a new functional ingredient with a prebiotic property. Indeed, electro-activation of whey at 800 mA, for 60 minutes converts approximately 35% of lactose into lactulose (Kareb et al., 2015; Karim & Aider, 2020). The principle of this isomerization underlies in the accumulation of high numbers of OH^- ions in the cathodic compartment of the electro-activation reactor, which provides an alkaline media and proton acceptors that are required by lactulose LA-isomerization reaction. To our best knowledge, there were no studies conducted on the use of electro-activated whey as a functional ingredient in food products.

Thus, this Ph.D. research project aimed to study the technological feasibility of using lactulose-enriched electro-activated whey as a functional ingredient in the production of fermented dairy products. The obtained products would provide a good alternative of valorization of cheese whey through applying it in human nutrition.

1 CHAPTER 1 : LITERATURE REVIEW

1.1 Cheese whey

Cheese whey is a liquid left after the separation of casein curd. It as a yellowish colour caused by the presence of riboflavin (vitamin B2) (Chairunnisa et al., 2019). It is an abundant by-product of the cheese industry as 1 tonne of cheese generates as much as 9 tons of cheese whey (Palmieri et al., 2017). In 2020 alone Canada's cheese production reached 510000 metric tonnes (https://www.statista.com). And it was projected that global cheese production will reach 25.3 million metric tonnes by 2023 (Parashar et al., 2016).

Whey contains 55% of milk nutrients in a dissolved form. The high nutritional value makes whey a burdensome by-product. Rich in lactose and protein, whey has a high biological (30,000–50,000 ppm) and chemical oxygen demand (60,000–80,000 ppm) which can lead to algal bloom, depletion of oxygen in water streams, and soil etherification (Schmidt et al., 2020). Thus, multiple research and manufacturing practices were developed to escalate the utilization of this by-product and turn it into a co-product. However, these attempts are hindered by limited use of whey and its derivatives, high costs of valorization installations for smaller producers, and the inability to treat entire amounts of whey produced.

Cheese whey, otherwise called sweet whey for majority of cheesetypes (pH 6-7), is produced during the production of the majority of cheese varieties by rennet coagulation (hard, semi-hard cheese). As opposed to acid whey (pH <5) which is uniquely produced when the curd is separated after acidification of the milk gel, such as cottage cheese, Greek yogurt, and cream cheese (Nishanthi et al., 2017). The differences in the composition of acid and sweet whey are depicted in **Table 1.1.** Generally, whey dry matter consists of 70% lactose, 13% proteins and 12% minerals. Acid whey commonly contains higher levels of minerals and is lower in protein and lactose content. Thus, its salty and acid taste hampers its usage in the food industry (Lievore et al., 2015). According to (Kilara & Vaghela, 2004) 94% of whey produced in the USA was sweet whey, with only 6% represented by acid whey. In this regard, there is a bigger requirement for development of new feasible methods of transformation of sweet whey. Moreover, whey's legal generally recognized as safe (GRAS) status provides a basis for production of value-added food ingredients from whey.

Table 1-1: Differences in compositions of acid and sweet whey (Lievore et al., 2015; Onwulata & Huth, 2009)

	Sweet whey	**Acid whey**
Lactose (%)	64.3-74.6	61.4 - 70
Total protein (%)	11.1-16.6	7.9-10.7
Total ash (%)	7.1-10.7	11.4
Fat (%)	0.37-1.52	0.6
Titratable acidity	0.07-0.19	0.028-0.44
pH	6-7	<6

1.1.1 Cheese whey components

Whey contains 6-7% of dry matter and 93% of water. It retains half of milk nutrients, such as whey proteins (0.6-0.8% w/v), lactose (4.5-5% w/v), lipids (0.4-0.5% w/v) and mineral salts (0.6-1.0%), and appreciable amount of water-soluble vitamins (Batista et al., 2018).

1.1.1.1 Whey proteins

About 20% of proteins in milk are represented by compact globular proteins called whey proteins. The most abundant of these are lipocalin protein β-lactoglobulin (50%), calcium metallorotein α -lactalbumin (12%), immunoglobulins (10%), serum albumin (5%) and proteose peptones (0.23%) (Sedaghat Doost et al., 2019). Moreover, whey contains smaller proteins and enzymes, such as lactoperoxydase and lactoferrin. Furthermore, rennet clotting during cheese preparation fragments the C-terminal part of κ- casein molecule from residue 106 (Met) to 169 (C-terminal Val), which is known as glycomacropeptide. Glycomacropeptide comprises 20%-25% of rennet whey protein products (Madureira et al., 2007; Neelima et al., 2013).

Whey proteins are separated from whey by membrane filtration (ultrafiltration, diafiltration, microfiltration) and are represented by the products named whey protein isolate (WPI) (95-98% protein) and whey protein concentrate (WPC) (50-85% protein). Whey protein extraction leaves a by-product whey permeate (deproteinized whey). Due to their excellent nutritional qualities, whey protein products are used in sports drinks to improve muscle tissue

gain and relieve muscle pain. Also, whey proteins were reported to be effective antimicrobial, antiviral, antioxidant, hypolipidemic, immunomodulating agents as well as to prevent cancer, cardiovascular diseases and osteoporosis (González Siso, 1996; Madureira et al., 2007; Mollea et al., 2013). (Dalev, 1994) had also suggested the use of whey proteins in production of iron proteinates for treatment of iron deficiency in animals. A major oxidoreductase of milk lactoperoxydase and lactoferrin are also extracted from whey to be used as a natural preservative in milk products and antimicrobial agent in cosmetics.

Table 1-2: Several characteristics of whey proteins (Bosco et al., 2018; Madureira et al., 2007; Yalcin, 2006)

Whey protein	Approximate molecular weight, kDa	Number of amino acids	Suggested biological role
β-lactoglobulin	18362	162	Transport of retinol, palmitate, fatty acids, vitamin D and cholesterol; Calcium and zinc binding; Transfer of passive immunity to the newborn; Regulation of phosphorus metabolism in mammary gland; Precursor of bioactive peptides
α-lactalbumin	14147	123	Coenzyme for biosynthesis of lactose; Calcium carrier; High affinity to Zn^{+2}, Mn^{+2}, Cd^{+2}, Cu^{+2}, and Al^{+3};
Immunoglobulins (IgG1, IgG2, IgA, IgM)	150000 - 1000000	>500	Immunological function; Food grade antimicrobials;
Serum albumin	69000	583	Fatty acids, lipid, flavor compound binding;

			Immunomodulation;
Lactoferrin	78000 - 80000	698	Antimicrobial effect through iron binding; Antiviral effect; Antioxidant capacity; Wound healing; Antitoxin; Anti-inflammatory;
Lactoperoxidase	89000	612	Antimicrobial effect; Wound healing;
Glycomacropeptides	7500	64	Antimicrobial (effective against dental plaque); Antiviral; Immunomodulation;

1.1.1.1.1 Functional properties of whey proteins

Functional properties of the proteins describe their usability in food production and are defined by several physicochemical properties of the proteins, such as amino acid composition, order of amino acids, molecular size, conformation, net charge, and hydrophobicity (Abd El-Salam et al., 2009).

Main functional properties of food proteins are based on three types of interactions: (i) protein/water (solubility, water absorption, water retention, wettability, swelling, adhesion, dispersibility, viscosity); (ii) protein/protein (precipitation, gelation, texturation); (iii) protein/interface (emulsification, foaming) (Bouaouina et al., 2006). By functional properties of whey proteins, we mean the combination of functional properties of all its components. Even though the vastest type of whey proteins, β-lactoglobulin, has a primary role in functionality of whey protein mixtures. β-lactoglobulin is an amphiphilic globular protein that can adsorb at the water-oil and air-water interface, and has an excellent capacity to form gels. At the interface β-lactoglobulin partially unfolds and forms intermolecular associations by hydrophobic or S-S bridges (Bouaouina et al., 2006). The second most abundant whey protein, α-

lactalbumin has weak gel forming properties. However, it contributes to emulsification and stabilization by whey proteins. Along with β-lactoglobulin it forms films at the interface by forming S-S bridges.

Solubility is arguably the most important functional property. Whey proteins demonstrated high solubility over a range of pH2- pH9. Whey proteins are highly soluble even at their isoelectric points due to a large ratio of surface hydrophilic to hydrophobic residues (Dissanayake et al., 2013). This quality among others (high number of sulfhydryl groups, molecular flexibility, hydrophobicity, and surface activity) makes it a good foaming, texturizing, stabilizing, emulsifying, gelling, and water-binding agent to be used in food processing (sport beverages, confectionary, processed meat, salad dressings, artificial coffee creamers, nutritional protein drinks, creamed soups), as well as nanoparticles and edible film production (Dissanayake & Vasiljevic, 2009; Sedaghat Doost et al., 2019)

Other whey proteins also serve different purposes. For example, lactoferrin is used in chewing gum, mouthwash, toothpaste, cosmetics as well as in veterinary preparations and fish and animal feed. However, the addition of lactoferrin should be consulted with a recommended dose of 10 to 300 mg per day, excluding the amount of lactoferrin consumed by drinking milk. The functional properties of whey proteins can be further ameliorated by enzymatic hydrolysis and by forming the conjugates with carbohydrates (WPI-dextran, WPC-pectine) to obtain better gelling, foaming properties, and solubility (Abd El-Salam et al., 2009; González Siso, 1996).

The abovementioned functional properties are also present in other components, such as gelatin, hydrocolloids (guar gum, carrageenan, xanthan gum, locust bean gum), and starches. However, not many of them of them can pride themselves on having nutritional properties that whey has (Dissanayake et al., 2013)

1.1.1.1.2 Nutritional properties of whey proteins

Health properties of whey were mentioned as early as the III century BCE in Ancient Greece. In the Middle Ages it was ubiquitously used to soothe burns, and believed to improve vitality (Madureira et al., 2007). In current day literature it is stated that in comparison with caseins whey proteins have higher value of protein efficiency ratio (PER whey proteins 3.4, PER casein 2.8), and higher proportion of essential amino acids (lysine, threonine, methionine,

isoleucine). Biological value of whey proteins exceeds that of whole egg protein, and most plant proteins such as soya, peanuts, corn, and wheat gluten. Whey proteins contain a relatively high proportion of branched-chain (L-isoleucine, L-leucine, and L-valine) amino acids (BCAA, 26%) (Ha & Zemel, 2003). The sulphur-containing amino acids' (cysteine, methionine) content of whey proteins is higher than that of whole-milk proteins (1.35% versus 0.36%). These sulfur-containing amino acids are responsible for the role of whey in immunomodulation and antioxidant effect due to the modulation of the sulfur-containing tripeptide glutathione (Saleh et al., 2007). Lysine content is also higher in whey than in total milk-proteins (10.5% versus 7.75%). Another important advantage of whey proteins is abundance of essential amino acid leucine. Leucine is an important component in the translation initiation pathway of muscle protein synthesis (Ha & Zemel, 2003). Whey contains a surplus of all the amino acids overpassing the recommendations of FAO/WHO.

High nutritive value of whey proteins can be disrupted by heating at high temperatures (121°C, 83 min).

1.1.1.2 Lactose

Lactose is a disaccharide that is unique for mammalian milk. It comprises about 40% of milk solids and 75-80% of whey. Its isolation was first reported in 1633 by Fabrizio Bartoletti by evaporation of whey (Ugidos-Rodríguez et al., 2018). It is present in milk in a dissolved state; thus, all its content remains in whey after casein coagulation. This abundant whey component is unfairly considered inferior to other whey components.

Lactose (4-O-ß-D-galactopyranosyl-D-glucopyranose, $C_{12}H_{22}O_{11}$) is a disaccharide composed of glucose and galactose connected by a β (1→4) glycosidic bond. It is a reducing sugar since it has a free hemiacetal group. The unit of glucose can be hydrolysed by water, forming an open chain structure with a reactive carbonyl group which makes lactose reactive (i.e. for a Maillard reaction). Less than 0.1% of lactose is in the form of an open structure in a solution (Hettinga, 2019).

Because of the presence of a chiral carbon, lactose has 2 isomers: α- and β-anomers. They can be differentiated by a specific rotation of +89° (α-lactose) and +35° (β-lactose). **Figure 1.1** shows the structural formula of lactose with the indication of an anomeric carbon (C1) of

the hemiacetal group that freely changes orientation (mutarotation) from the α- to the β-form in a solution. Mutarotation is the rate of transformation between two spatial isomers (Hettinga, 2019). It depends on temperature, pH, presence of salts and other sugars. Temperature and pH play a role in the proportion of lactose forming an open chain structure, which is an intermediate step between transformation to α- or β-lactose. An equilibrium of two isomers in a solution of $[\alpha]^{20}_{D}$ =+55.3° (equivalent of 37.3% α-lactose and 62.7% β-lactose) is reached regardless of the initial forms. This equilibrium is dependent on the temperature of the solution (Gänzle et al., 2008).

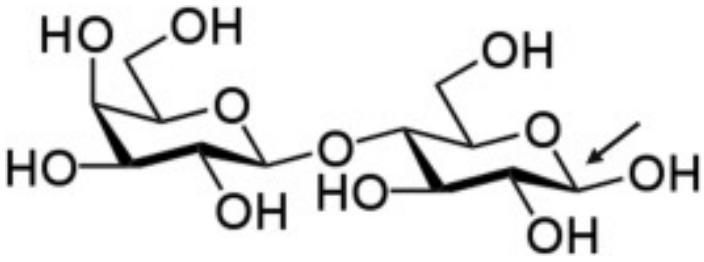

Figure 1-1: The spatial arrangement of lactose chemical structure. Arrow indicating the chiral carbon.

1.1.1.2.1 Solubility and crystallization of lactose

Solubility and crystallization are two main properties of lactose that define its usability. Those qualities are defined by the muto-isomers. Thus, α-lactose is less soluble and forms hard monohydrate crystals. It is the most common lactose isomer in food industry. Crystallization of α-lactose occurs in oversaturated solutions (saturation >2.1) at temperatures <93.5°C. β-lactose is more soluble and forms anhydrous crystals. Its crystals form in oversaturated solutions at the temperatures <93.5°C. At 20°C the solubility of α-lactose is 70g/L, whereas β-lactose dissolves at concentration of 500 g/L (Hettinga, 2019; Wong & Hartel, 2014). Mixture of equal proportions of α- and β-lactose has a noncrystaline or amorphous state. Compared to other polymorphs of lactose, in this state it is highly hygroscopic. The viscosity of amorphous lactose is temperature-dependent. Below the glass transition temperature the amorphous lactose solution forms a solid glass-like matter, and above this temperature, it becomes viscous. The glass transition temperature of lactose is defined by its concentration, relative humidity, and the presence of other milk components. Lactose spontaneously crystalizes at

oversaturation degree >2.1. In less saturated solutions, addition of fine lactose powder is also used for lactose crystallization. Lactose can be purified from cheese whey or permeate by crystallization. It is produced by the precipitation of proteins and minerals, slow crystallization, refinery, concentration, washing and air-drying of lactose (Carpin et al., 2016; MARWAHA & KENNEDY, 1988).

1.1.1.2.2 Lactose applicability and drawbacks in the food industry

The chief uses of lactose are pharmaceutical excipients and infant formulae. Human milk contains the highest level of lactose among other mammals. The difference between human milk (7%) and cow milk (4.6%) in infant formula is compensated by lactose addition to correct carbohydrate/protein ratio of cow milk to match breast milk (Walzem et al., 2002; Wong & Hartel, 2014). Plasticity, light flavour, and reduced sweetening power (20%-30% of sucrose) make lactose apt for use in pill tablets. Along with pharmaceutical products it is used in the food industry as a texture enhancer in confectionary, baked goods (cakes, biscuits), chocolate, soups, and sauces.

However, lactose's disadvantages outcompete its applicability. The limits of lactose's usability are low solubility and indigestibility in many individuals. At 25°C solubility of lactose is approximately 10% of that of sucrose (Gänzle et al., 2008; Hettinga, 2019). In addition to that, approximately three-quarters of the World's adult population (approximately 65%) have lactase non-persistency, which can cause bloating, osmatic diarrhea, and overall gastrointestinal discomfort at the consumption of milk products (Wiley, 2020). A tolerable level of lactose in these individuals is 12 g/per meal (Silanikove et al., 2015). In this regard, lactose derivatives, such as galacto-oligosaccharides, epilactose, lactulose, lactosucrose, and D-tagatose are more popular food additives, with well-documented prebiotic, indigestibility, and obesity prevention properties (Xiao et al., 2019).

1.1.1.2.3 Maillard reaction: whey proteins and lactose

Maillard reaction or nonenzymatic browning is a cascade of reactions that is initiated by interaction between reducing sugars and amine groups of proteins during food processing and storage. The products of Maillard reaction play an important role in different foods, such as coffee, bread, grilled meats. Maillard reaction results in specific flavour, color, and texture. In UHT milk formation of Maillard reaction products is a sign of deterioration during the

storage. However, it is not a disagreeable trait for all dairy products. Products such as dulce de leche, varenets, ryazhenka include the initiation of Maillard reaction during their production.

Maillard reaction is divided in early, intermediate, and advanced stages. In the early stage the condensation of free amino group with a reducing sugar and the formation of N-substituted glycosylamine takes place. Later, unstable Schiff's bases rearrange into Amadori products. Further Amadori products are degraded into sugar- amino compounds, such as deoxyosones, carboxymethyllysine. Finally, formation of pigmented sugar-protein polimers, mainly melanoidins occurs (Sedaghat Doost et al., 2019).

Given favorable conditions (temperature, water availability, substrate) whey is very prone for Maillard reaction as whey proteins are rich in lysine residues. It can also occur in whey protein concentrates as there is 5 to 51% of remaining lactose (Le et al., 2011). External conditions affect the final products of the reaction. (Cortés Yáñez et al., 2018) reported obtaining predominantly initial stage MRPs (no browning) in whey protein/lactose mixtures with water availability Aw=0.52 and intermediate and final stage MRPs occurred (browning) in liquid models. Maillard reaction improves heat stability, emulsifying properties, and antioxidant activity of whey protein (Oh et al., 2013). (Sedaghat Doost et al., 2019) suggested Maillard reaction to overcome critical heat, pH and ionic strength sensitivity, which would make WP suitable for infant formula processing conditions.

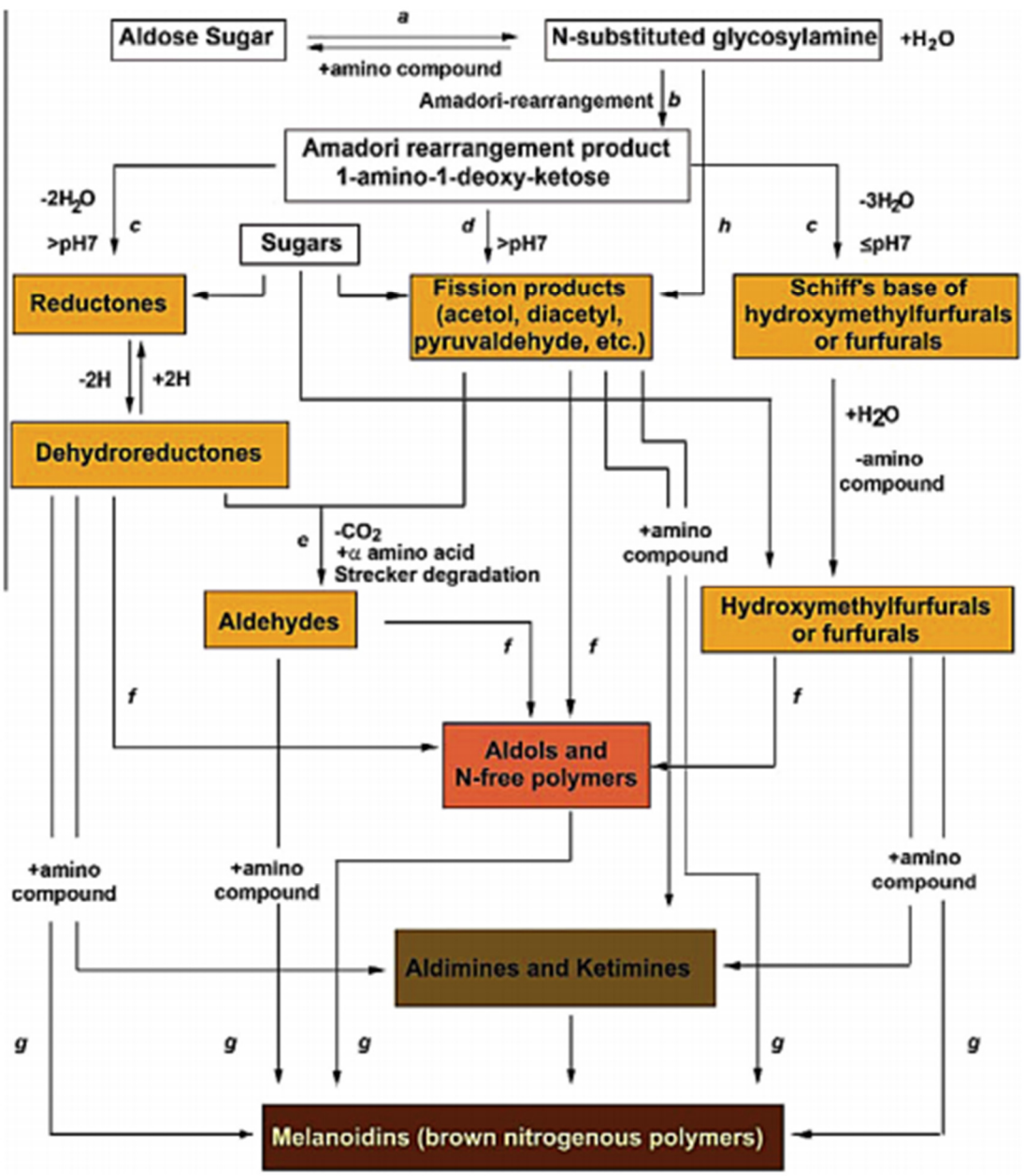

Figure 1-2: The Hodge diagram of Maillard reactions complex. Adapted from (Arena et al., 2017)

1.1.1.3 Whey minerals and other minor components of whey

Almost all of the milk minerals are present in whey after the separation of curd. Whey contains minerals such as calcium, magnesium, sodium and potassium as cations and phosphate, citrate, and chloride as anions. Whey minerals are considered one of its main defaults as they cause membrane fouling during membrane filtration treatment and give salty taste to the

products where it is added. For this reason, different demineralization techniques such as nanofiltration (NF), dia-nanofiltration (Dia-NF), ion exchange, electrodialysis (ED) or their combinations are common practices in dairy industry (Merkel et al., 2021).

Other minor compounds of cheese whey include citric and lactic acids (0.02-0.05%), non-protein nitrogen compounds (urea and uric acid), B group vitamins (Prazeres et al., 2012). Also, higher density lipids that are bound to lipoproteins remain in whey as residual lipids. Main lipid classes identified in whey lipids were triacylglycerols, phospholipids, diacylglycerols, free fatty acids, cholesterol esters, cholesterol, and monoacylglycerol. Residual lipids impair gelation properties of whey proteins and cause off flavors during the storage, thus special treatment for their removal is required (Abd El-Salam et al., 2009; Vaghela & Kilara, 1996).

1.1.2 Cheese whey environmental impact

Above-stated rich nutritional load of whey makes it a burdensome by-product and augments its negative impact on the environment. Whey has high chemical oxygen demand (50-102 g/L) and biochemical oxygen demand (27-60 g/L), which is 175 times higher than ordinary sewage effluent (Smithers, 2008; Yadav et al., 2015). Thus, it can disrupt a treatment process of municipal wastewater and requires a separate handling. Even on the level of a dairy plants, (Janczukowicz et al., 2008) have reported that whey should be treated separately from other dairy effluents, as whey contains complex components that are difficult to degrade. According to the study of (Janczukowicz et al., 2008) cheese whey wastewater is the least biodegradable dairy effluent. Both acid and sweet cheese whey were slowly metabolized by the biomass. In the course of five days 1 g of biomass had digested 0.13g (sweet whey) and 0.14g (acid whey) of the organic load (BOD), which is significantly lower of that of other dairy effluents. Some difficulties associated with whey biodegradability are rich organic load (COD on average 70 g COD l^{-1}), low alkalinity and rapid acidification, production of slimy exopolysaccharides by bacteria. The authors concluded that all dairy wastewaters can be mixed and treated in a same sludge, except for whey. Its low susceptibility to biodegradation requires its treatment in a separate installation (Janczukowicz et al., 2008).

Direct disposal of whey in the water bodies and soil threatens the environment with the issues as alteration of soil physicochemical qualities and causes excess in minerals, reduced dissolved oxygen content leading to death of aquatic life (MARWAHA & KENNEDY, 1988; Palmieri et al., 2017). Eutrophication of both soil and water body mainly results from excessive input of minerals such as phosphorus and nitrogen. If for soil this can cause lower crop yields, in water basin it cases a disruption of biodiversity by bloom of algae (i.e. cyanobacteria), and formation of large amounts of bacterial biofilms. The latter may reduce the quality of potable water. Thus, whey is a threat for human health and surrounding environment. A glowing example of deleterious effect of whey on an aquatic life is the case of spillage of acid whey in Ohio, US in 2008. This led to the death of more than 5400 wild animals (mostly fish) from depletion of dissolved oxygen. Crop kills were also reported to be associated with and change in redox potential of soil and saline deposits caused by whey disposal. According to (Papademas & Kotsaki, 2020; Ryan & Walsh, 2016) Each mm (10^3/ha) of whey applied to the soil resulted in 400-600 kg of salt deposit per hectare.

Pushed by this knowledge and raising public concern, most industrialized countries such as United States, Canada, Australia, New Zealand, and the European Union countries established strict control measurements regulating the disposal of whey to the environment (Smithers, 2008). This pushed the producers to seek alternative, environmentally friendly methods of whey treatment and valorization.

1.1.3 Predisposal treatment and valorization

Commercially available technologies in industrial wastewater treatment are represented by biological, physical-chemical and land treatments. Biological treatment includes activated sludge process, aerated and anaerobic lagoons, stabilization ponds, multistage biological systems, and fermentation. The disadvantages of lagoons and stabilization ponds are requirement for big space, bad odor and difficulty of sludge disposal. Anaerobic and aerobic digestion is more suitable for dairy wastewater treatment as its rich organic content promotes the growth of a biomass. Anaerobic digestion process can fuel itself as it allows the production of methane, as well as ethanol and organic acids (Hansen & Cheong, 2019; Marwaha & Kennedy, 1988).

Physico-chemical treatment methods include sedimentation, chemical precipitation, coagulation and flocculation, pH adjustment, reverse osmosis, nanofiltration, ion exchange, and adsorption. They are often used in combination with other methods.

The most common wastewater treatment in dairy industry is consisted of three stages. Primary step is the dissolved air floatation (DAF) technique that allows to remove fats, oils and greases, followed by biological secondary treatment. Tertiary treatment can be employed to remove phosphorus with the use of ferric sulphate or aluminium chloride (Ashekuzzaman et al., 2019; Hansen & Cheong, 2019). Also, preliminary chemical precipitation was suggested as an effective whey to handle cheese whey as being more economically feasible. Under several conditions cheese whey sludge can be used as an agricultural fertilizer (Carvalho et al., 2013). Food industry wastewater disposal methods vary depending on regulations of the countries. In Canada, Water System Effluent Regulation requires a secondary treatment of wastewaters (Government of Canada, 2012, 2017b). Secondary treatment is dedicated to removal of biodegradable organic matter and nutrients. The final quality of the effluent should reach $CBOD_5$ and TSS of 15 mg/L. **Table 1.4** depicts Canadian government requirements for industrial wastewater discharge in water bodies or soil.

Table 1-3: The stages of dairy wastewater treatment.

Primary	Screening, buffer tank, pH neutralization, sedimentation, flotation
Secondary	anaerobic reactor, aerated lagoons, activated sludge
Tertiary	Denitrification, phosphorus removal, membrane filtration, disinfection, constructed wetlands.

Table 1-4: Canadian government requirements for industrial wastewater discharge in water bodies or soil.

Average carbonaceous biochemical oxygen demand (CBOD)	Average suspended solids (SS)	Total residual chlorine (TRC)* (on average)	Maximal un-ionized ammonia (NH_3)** (expressed as nitrogen, at 15 °C ± 1 °C)
≤ 25 mg/L	≤ 25 mg/L	≤ 0.02 mg/L	< 1.25 mg/L

* TRC standard for systems < 5000 m^3/day comes into effect January 1, 2021. ** Un-ionized ammonia (NH_3) reporting requirements ended June 30, 2014, but the standard for NH_3 must still be met and can be determined through sampling.

The cost of wastewater treatment is largely associated with the need for constant energy supply (Rustum et al., 2020). According to Statistics Canada, $91.7 million was spent by food industry in 2009 on wastewater treatment. Half of the cost of dairy wastewater treatment is attributed to the sludge disposal (Ashekuzzaman et al., 2019).

In this regard, valorization of whey is the best way of its treatment. As bulk whey is difficult to handle and transport due to the high volumes of liquid and short shelf life, the first step of its valorization is drying by multiple effect evaporators or spray dryers. Whey powder or its other forms (demineralized whey, delactosed whey, deproteinated whey) are used as an animal feed and in several food products (bakery goods, beverages, yogurts, ice-creams, sauces) (Smithers, 2008). However, whey powder is a low return product with a little demand. Their widespread usage in food industry is hindered by high saline taste and small protein/sugar ratio (González Siso, 1996). Fractionation of whey provides higher value products such as whey proteins, lactose, and its derivatives. Fermentation technologies allow the production of value-added products such as bioethanol, biopolymers, single cell protein, probiotics, hydrogen, and methane (Yadav et al., 2015). Despite available options approximately 50% of world produced whey is still discarded as an effluent (Barba, 2021; Panesar et al.,

2007; Yadav et al., 2015). From utilized whey, 25% is transformed by afore-mentioned technologies, 30% is used in a form of whey powder, and 45% in liquid form (Kosseva et al., 2009). **Figure 1.3** depicts a schematic representation of whey-derived products.

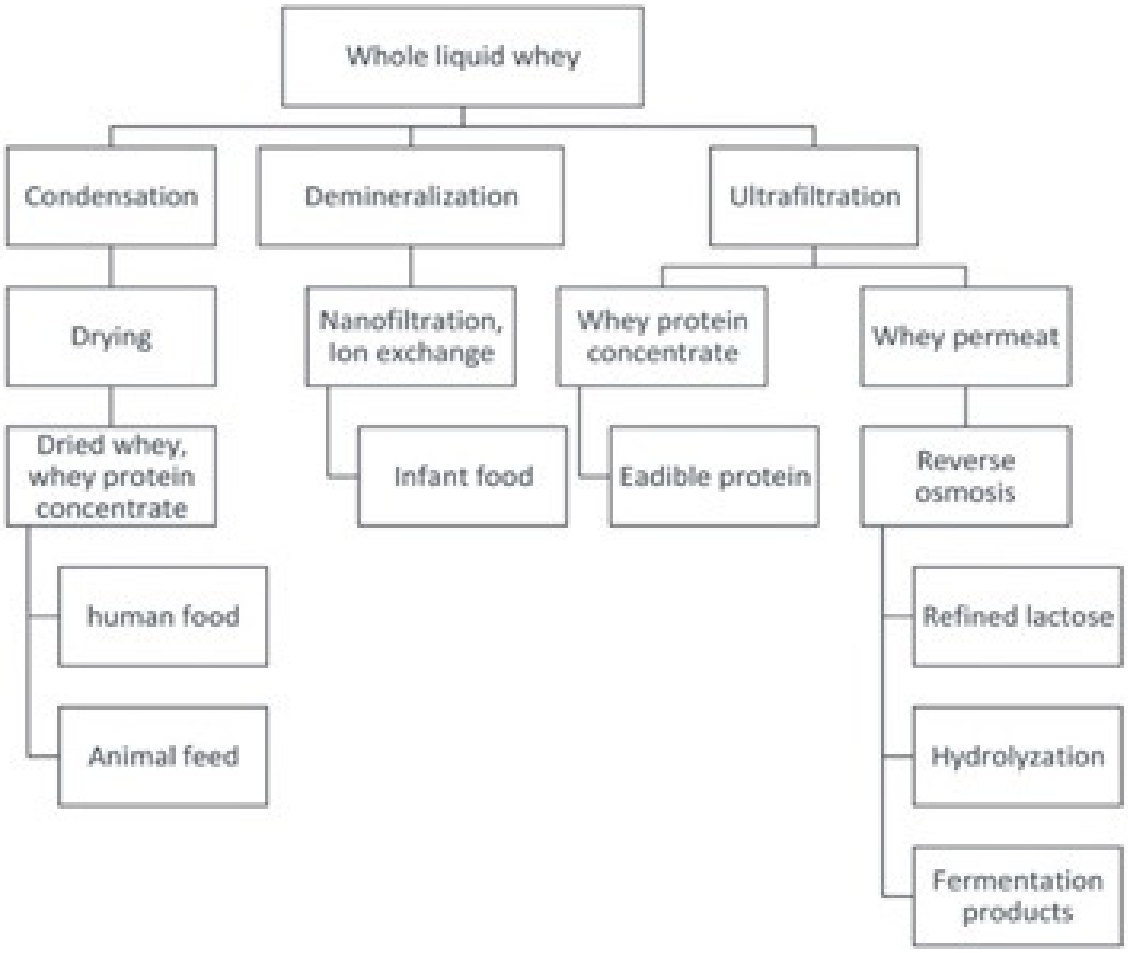

Figure 1-3: Schematic representation of whey valorization products. Adapted from (Hansen & Cheong, 2019)

In an idyllic picture all the components of whey could be valorized until the remain of so-called potable "cow water" (Smithers, 2008). However, despite the different techniques of whey valorization, the problem of whey is far from being solved. Without creating a commercial value for the whey or whey-containing products the investment in the technologies will not be justified. In addition to that, the proposed technologies need to be developed from bench-top to pilot and plant-scales.

1.1.3.1 Agriculture: land disposal and animal feed

Whey valorization technologies and municipal disposal are costly for smaller producers (<5000 kg cheese/year). Thus, pushing them to get rid of the whey by feeding it to local farm animals and land application (Hughes et al., 2019). Mostly whey is supplied into drinking water for monogastric animals (swine). Liquid whey is both prebiotic and protein source for animals. Thus, animal growth promoting effect of whey was attributed to immunomodulation and amelioration of intestinal microbiota of pigs (Kobayashi et al., 2011).

Undoubtedly, solution to whey problem of a farm by feeding it to animals has a beneficial ecological effect through avoiding the pollution of water or soil. Moreover, incorporation of whey, with its reach lactose content can improve an environmental footprint of the animals themselves. According to (Pierce et al., 2006) incorporation of lactose in pigs' finisher diets reduces urinal and fecal nitrogen excretion and ammonia emission. This effect was also attributed to selective promotion of microorganisms in the gut, which growth increases their nitrogen uptake. Decrease in ammonia excretion has also beneficial effect on malodors of a husbandry. Economical gain can also be established in a form of an avoided feed/fodder. An article on life cycle assessment of a small-scale cheese producer feeding by-product whey to pigs on the farm has assumed that on protein basis 10 L of whey corresponded to 1 kg of fodder (Canellada et al., 2018).

Whey products are also used as a feed for calves and sheep. The study on whey protein supplementation to basal feed of the ruminants concluded that lactose and whey proteins improve the fiber intake and crude protein decomposition in the rumen by propagation of specific bacteria and protozoa (S. B. Lee et al., 2019). In poultry as in swine husbandry, quality of proteins plays a crucial role. It was stated that animal proteins i.e. highly nutritive proteins of whey coupled with high B-vitamin complex, perform better than plant proteins in poultry nutrition (Georganas et al., 2020). Despite abovementioned benefits, excess in lactose and mineral consumption can cause health issues in animals. Therefore, supplementation of untreated whey should be strictly controlled and limited (Ryan & Walsh, 2016).

Throughout the history, whey was used as a fertilizer. However, the negative impact of introducing high-mineral whey to the land is discussed in previous sections. It is worthy to

mention that several cultures, such as tomatoes have moderate tolerance to soil salinity. Thus, this plant can be cultured on pre-treated or diluted whey wastewater (Prazeres et al., 2012).

1.1.3.2 Single cell protein production

Single cell protein or bioprotein is a microbial biomass cultivated on a carbon source that can be used in human food or animal feed. With increasing demand for proteins by growing human population, production of single cell proteins from food waste (starches, fruits peel, cellulose, wheat straw, sugarcane molasses) could be a sustainable solution (Oshoma & Eguakun-Owie, 2018). Moreover, the quality of this sort of protein has closer affinity to highly nutritious animal proteins, rather than plant proteins. The crude protein in single cell microorganisms can reach up to 40-80% (w/w) for dry cell weight. Besides high protein content, the advantages include short generation times, independence of environmental factors, and steady production and quality. The disadvantage of SCP could be the presence of nucleic acids, that can cause pathological physiological conditions in human bodies, such as renal calculi. In this regard, yeasts/fungi are preferred source of SCP, as their DNA is shorter than those of bacteria (Yadav et al., 2015). In addition to that, bigger size of yeasts and fungi makes it easier to yield the biomass. Yeasts/fungi are precipitated by centrifugation, plasmolyzed by heating at 85°C, and dried (González Siso, 1996). There are multiple microorganisms and algae that are Generally Accepted as Safe for human and animal consumption. However, a handful of them can metabolize cheese whey lactose: Bacteria - *Aeromonas hydrophylla,* fungi – *Penicillum cyclopium,* yeasts – *Candida intermedia, Saccharomyces cereviciae*, *Kluyveromyces lactis, Kluyveromyce fragilis, Torulopsis bovina* (Nasseri et al., 2011). The preferred substrate for these yeasts is whey permeate, as they cannot metabolize protein content of whole whey. Nutrisearch company has industrialized the production of *Saccharomyces cerevisiae* (bakery yeast) on cheese whey as a carbon source. The process, as other processes including bioconversion by yeasts, includes the step of lactose hydrolysis prior to fermentation (Gänzle et al., 2008). Single cell protein production reduced BOD of whey higher than ethanol production. Generally, production of SCP, ethanol, biogas and other metabolites reduces at least 75% of whey's BOD. However, resulting effluent is not completely harmless for disposal as remaining percentage of BOD is still unacceptable for direct disposal (González Siso, 1996; Mawson, 1994).

1.1.3.3 Fermentation for ethanol and biogas production

Most common yeasts used in alcohol beverage production, *Saccharomyces cerevisiae*, lacks both lactose permease system and the intracellular enzyme for lactose hydrolysis. Whereas yeasts of *Kluyveromyces* species have an ability to ferment whey lactose into ethanol. *Kluyveromyces marxianus* is the first-choice microorganism in whey-ethanol fermentation. Other microorganisms examined for ethanol fermentation from whey are *Escherichia coli*, *Candida pseudotropicalis*, and genetically modified *Saccharomyces cerevisiae* (Hughes et al., 2019). Conversion of whey lactose to ethanol is a two-step process. First, lactose needs to be hydrolyzed to glucose and galactose, then the uptake of these monosaccharides drives the fermentation. Ethanol fermentation after hydrolysis follows two phases. Conversion of glucose to pyruvate is the first phase. Glycolysis generates ATP for intracellular energy transfer. Galactose is enzymatically converted to glucose 6-phosphate. As a result, 4 ATP molecules, 4 pyruvate molecules, and the reduction of NAD^+ to NADH occur. At the second stage pyruvate is converted to ethanol and NAD^+ is restored (Hughes et al., 2019). *Kluyveromyces marxianus* can ferment more that 95% of whey lactose, with 80-85% conversion efficiency (González Siso, 1996). However, in nonconcentrated whey low lactose levels (5-6%) allow the maximal production of ethanol of 2-3%, which is only suitable for beer production (Hughes et al., 2019; Risner et al., 2018). A prior concentration of whey or whey permeate by reverse osmosis or ultrafiltration significantly increases the cost of ethanol production (Parashar et al., 2016). Nevertheless, the concentrated substrate does not resolve the problem, as ethanol production remains limited by low osmotic tolerance of *K. marxianus* to increased concentration of ethanol and other solutes. Inhibition of further ethanol production is achieved at concentrations of 45-52 g/l or 5.5-6.5% (v/v) (Hughes et al., 2019).

(Kargi & Ozmihci, 2006) suggested to avoid costly pre-concentration of whey by fermentation of cheese whey powder to ethanol. The authors also outlined the sufficiency of sodium and phosphorus levels in whey for ethanol fermentation by *K. marxianus*, as addition of the minerals did not affect the final yield.

Another drawback of whey to ethanol fermentation is contamination of whey by lactic acid bacteria, which compete with yeast biomass for the carbon source and produce acids that are

toxic for yeasts. Contamination can lead to the yield loss of 27% to complete misfunctioning of the fermentation process (Parashar et al., 2016).

From environmental viewpoint, production of ethanol reduces the BOD of whey for approximately 75%. In addition to that GRAS status of *K. marxianus* permits the use of yeast biomass for animal feed or human food (Hughes et al., 2019). Despite the existing technologies, optimization of the production of ethanol from whey is an ongoing process (Risner et al., 2018).

Anaerobic digestion is a widespread technology in animal husbandry and agro-industrial wastewater treatment. The produced biogas can be used for the generation of heat and electricity. Moreover, this method is suitable for smaller plants as microorganisms can convert organic load to methane and carbon dioxide in several hours, as opposed to collecting the waste whey for longer periods of time. Anaerobic digestion mostly takes place at mesophilic (35°C) or thermophilic (55°C) conditions. It has two stages, (1) hydrolysis of complex compounds by facultative and anaerobic bacteria and (2) uptake of nutrients and production of methane and carbon dioxide by strict anaerobes (Antonelli et al., 2016). However, whey has several disadvantages in terms of biogas production including contamination of whey by lactic acid bacteria, and therefore acidification and production of bacterial metabolites. Low alkalinity (2.5 kg m^{-3} as $CaCO_3$) also hinders the granulation process and causes biomass washout (Kavacik & Topaloglu, 2010).

Majority of studies were conducted on production of biogas by co-digestion of nutrient source such as protein, fat, fiber, sugars, starch with animal manure, which brings environmental and economic benefits for farmers and producers. In a co-digestion process the content of nutrients, as well as the negative effects of toxic compounds, can be balanced, increasing the gas yield. Antonelli et al. (2016) (Antonelli et al., 2016) studied anaerobic digestion of whey inoculated by swine wastewater for production of methanol. At the temperatures of 32°C and 26°C, 270 and 171 L $(kg\text{-}sv)^{-1}$ of biogas was produced, with methane content of 63% and 61%. Mix of cow manure with whey for anaerobic digestion was studied by (Comino et al., 2009, 2012; Kavacik & Topaloglu, 2010) and poultry manure (Carlini et al., 2015). Liquid whey is used to dilute high total solids content of manure and increase biogas production. In poultry manure treatment it avoids using water for dilution. According

to (Hublin et al., 2012) co-digestion of whey with cow manure increased specific methane yields (0.35 to 0.80 m^3 biogas/kg of volatile solids) in comparison with manure only (0.20 to 0.30 m^3 biogas/kg of volatile solids). It needs to be mentioned that co-digestion process requires a thorough control of the balance of micro and macro nutrients, C:N ratio, pH, toxic compounds and total solids (Hublin et al., 2012)

As in the case of ethanol production the effluents from the anaerobic digestion are not suitable for disposal into water streams and still require secondary treatment. Nevertheless, fermented whey can be used as fertilizer due to retention of valuable components, such as sodium and phosphorus during co-digestion (Kavacik & Topaloglu, 2010).

1.1.3.4 Whey protein fractionation

A way to produce higher value-added products from whey lies in its fractionation. Recovery of proteins and their products is the major step of cheese whey valorization. Depending on the type of treatment or their combination used, various value-added whey products are produced: whey protein concentrates and isolates, reduced lactose whey, demineralized whey, hydrolyzed whey, whey permeate, individual whey proteins, protein hydrolysates, casein glycomacropeptide (Abd El-Salam et al., 2009).

Commercial approaches to separate whey proteins from permeate are ultrafiltration, diafiltration, microfiltration and ion exchange chromatography for more concentrated whey protein products (whey protein isolates). There exist the technologies allowing the separation of individual whey proteins such as α-LA and β-LG based on differential solubility at varying pH, temperature, and ionic strength (Abd El-Salam et al., 2009). Thermal processing under pH ranging from 3.0 to 4.6 selectively precipitates α-LA and bovine serum albumin, separating it from β-LG. For the process optimization desired pH can be achieved by adding citric acid, sodium citrate, or EDTA (Toro-Sierra et al., 2013).

Membrane technologies, chromatography, and precipitation also allows production of lactoferrin, lactoperoxydase, and glycomacropeptides (GMP). Even though chromatography is the major industrial procedure for produciton of individual proteins, its high operation cost makes up to 80% of the price of the final product (Aguero et al., 2017).

Ultrafiltration and nanofiltration are membrane methods based on the application of pressure and selective membrane separation, targeting the components with molecular weight of 1-1000 kDa (ultrafiltration) and 100-500 kDa (nanofiltration). Ultrafiltration is based on the principle of passage of low molecular weight components (lactose, salts, water) (Aguero et al., 2017). Ultrafiltration is prevalent in dairy industry, as it allows to avoid phase change as in case of evaporation. Ultrafiltration retains up to 65% of solid proteins. To get a higher concentration of proteins further treatment by diafiltration is performed. Along with electro-dialysis and ion-exchange resins the combination of ultrafiltration and diafiltration is also used for desalination of the whey protein product (Wen-qiong et al., 2019). Then, whey protein retentate is concentrated by reverse osmosis or evaporation, and spray dried. Microfiltration is another pressure- driven membrane technology that is used to separate bacteria, fats and large aggregates. The details of different membrane treatments are given in **Table 1.5**.

Overall, membrane methods provide cost and process efficiency, saving the initial quality of the proteins (no denaturation), and demineralization of the final products. To mention, high mineral content of whey hinders its widespread use in foods, including infant formulations. They are environmentally- friendly techniques as they do not require use of harmful chemicals (Wen-qiong et al., 2019) and have reduced energy requirements (Rezaei et al., 2014).

Table 1-5: Pressure- driven membrane processes for whey treatment.

Method	Membrane pores	Practical use
Microfiltration	< 1 mm	Removal of lipids, protein aggregates and microbes
Ultrafiltration	1-100 nm	Concentration of the molecules with a molecular weight between 10.0 and 1000.0 kD
Nanofiltration	0.1-1 nm	Demineralization, Lactose reduction
Reverse osmosis	0.1 nm	Concentration

Proteins extracted from whey can be further subjected to hydrolysis to produce value-added whey products - Whey Protein Hydrolysates (WPH). WPH is usually obtained by enzymatic hydrolysis of different levels (Patel, 2015).

In spite of vast applicability of membrane technologies in whey valorization, there is a major downside of this industrial practice. Formation of complexes by calcium, phosphorus, and other ion species and pore clogging by the proteins cause membrane fouling (Luján-Facundo et al., 2017). The main protein contributing to membrane fouling during whey processing is β-LG (Wen-qiong et al., 2019). Concentration polarization resulting from an accumulation of rejected solutes on the membrane surface further hinders the permeate flow. Both solutes and membrane material have an impact on fouling. Membrane characteristics such as hydrophobicity and chemical and electrostatic attractions promote fouling (Rezaei et al., 2014). An impressive amount of research was dedicated to studying this phenomenon. Industrial practices of membrane cleaning are available. Among them, acids and bases, surfactants, and enzymes (Popović et al., 2010). In general, the constant requirement for cleaning products and annual membrane replacement adds up to the cost of membrane treatment of whey (Wen-qiong et al., 2019). Thus, due to the higher cost, whey protein products lose the competition to cheaper protein sources such as soya.

A by- product of whey membrane filtering is water and water-soluble fractions of whey, such as lactose and minerals. Lactose represents 80% of whey permeate total solid content and is often neglected as compared to protein fractions of whey. However, protein recovery does not solve the pollutant problem of whey. Recent works have demonstrated that multiple high value-added lactose derivatives can be produced from whey permeate (Enteshari & Martínez-Monteagudo, 2020; Gutiérrez et al., 2012; Lindsay et al., 2018; Seki & Saito, 2012).

1.1.3.5 Lactose derivatives

Lactose can serve as a raw material for the production of various value-added derivatives, such as lactulose, lactobionic acid, lactitol, galactooligosaccharides, epilactose, lactosucrose, tagatose, sialyllactose (Gutiérrez et al., 2012; Seki & Saito, 2012). A complete list of lactose derivatives and methods is depicted in **figure 1.4.**

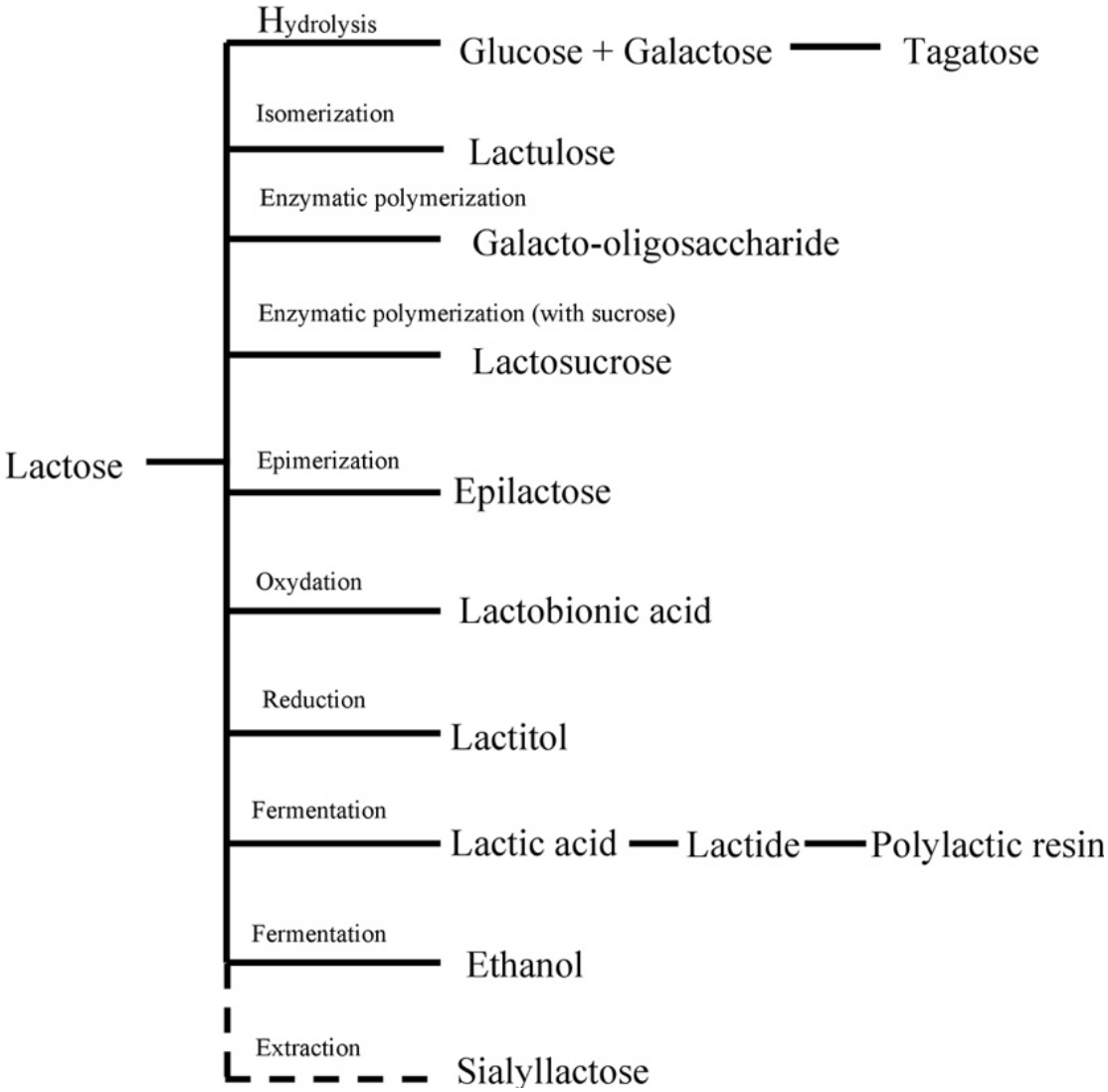

Figure 1-4: Lactose derivatives. Adapted from (Seki & Saito, 2012)

Production of lactic acid would be the most straightforward way of lactose conversion, as lactic acid bacteria (L. delbrueckii subsp. bulgaricus, L. acidophilus, L. casei, L. helveticus, etc.) readily ferment lactose and produce lactic acid. Lactic acid and its derivatives are applied in food, pharmaceutical, cosmetics, skincare, and leather industries (Panesar et al., 2007). However, purification steps, such as extraction, membrane separation, ion exchange and electrodialysis, distillation, of the resulting product increase the production cost (Castillo Martinez et al., 2013). Another way of whey lactose treatment is hydrolysis of lactose to its two component monosaccharides glucose and galactose. Majority of bacteria more readily metabolize the monosaccharides glucose and galactose. In addition to that, hydrolysis enhances the disadvantages of lactose in terms of sweetness and solubility, which makes it possible to replace saccharose and starch syrup in ice-cream and confectionary production (Chatzipaschali & Stamatis, 2012).

Galactooligosaccharides represent a group of non-digestible oligosaccharides that are produced by the use of galactosyl transferase enzyme (Seki & Saito, 2012). They are Generally recognized as safe (GRAS) and ubiquitous used in food industry as prebiotics.

Lactulose and lactitol (4-fl-galactopyranosyl-o-sorbitol) are synthetic derivatives of lactose. Lactulose is formed via chemical, enzymatic, or electro-chemical isomerization of galactose moiety of lactose to fructose. Lactulose is valuable in pharmacological industry as a laxative agent, and for the treatment of hepatic encephalopathy. In addition to that, it is a recognized bifidus factor. Meanwhile, lactitol is a product of chemical hydrogenetation of lactose (Gänzle et al., 2008). It is used along lactulose for treatment of hepatic encephalopathy and chronic constipation. Lactobionic acid (b-D-galactosyl-gluconic acid) and tagatose are relatively new compounds obtained from lactose treatment, thus their use in food industry is limited to their novelty. Yet, lactobionic acid is a primary component of the solutions for transplant organ cold storage, due to its ability to prevent cell swelling and oxidative stress. Lactobionic acid is produced by oxidation of lactose. While d-tagatose is a product of enzymatic isomerization of d-galactose. Tagatose and lactitol are known as low caloric sweeteners (Schaafsma, 2008).

Relative sweetness and physiological effects of lactose derivative sugars compared to lactose are summarized in **Table 1.6**.

Table 1-6: Relative sweetness and physiological properties of lactose derivatives. (Tiefenbacher, 2017).

Lactose derivative	Relative sweetness	Properties
Glucose	0.6-0.75	Fermentation
Galactose	0.5-0.7	Fermentation
Lactulose	0.4-0.6	Prebiotic
Lactitol	0.3-0.4	Low caloric sweetener
Tagatose	0.9	Low caloric sweetener
Epilactose	n/a	Prebiotic

It can be concluded that the prebiotic effect of lactose derivatives has a big market potential, due to a growing customer demand. Thus, ample research needs to be focused in this direction. It should be noted that despite of the diversity of possibilities of lactose conversion, it still represents a small proportion of the whey produced worldwide (Gänzle et al., 2008)

1.2 Electro-activation

1.2.1 Electro-activation technology development

The precursor of development of electro-activation technology was an observation made by Russian academician V. V. Petrov in 1802. While developing a high-voltage galvanic battery he noticed that emission of electrolysis gases near the electrodes was followed by acidification of water near the anode and alkalization of water near the cathode. V. V. Petrov introduced a porous membrane to separate two liquids and collect alkaline catholyte and acid anolyte. This phenomenon did not receive a name until 1832, when M. Faraday established the laws of electrolysis. In 1972 V.M. Bakhir noticed that electrically activated diluted solutions have properties differing from conventional ones. He was the first to introduce the term “electrochemical activation” in 1975. At first, electrochemical activation found its use in Oil industry (L. S. Kavruk, 2002; V.M. Bakhir, n.d.). From 1972, a group of scientists led by

V.M. Bakhir was developing a state-of-the-art technology of drilling mud treatment. By 1980, the technology was leveraged to other fields, giving rise to experiments with unipolar electrochemical activation of different colloidal systems. Ample research and development were done in the utilisation of electrochemical activation in the production of solutions with disinfectant properties and water purification (Aider et al., 2012; Bakhir V.M., n.d.; Liato et al., 2017). The reports of using electro-activated solutions in prevention and treatment of intestinal infections of young calves could also be found in scientific literature (A.A.Zakomyrdin, 2002; L. S. Kavruk, 2002).

Since 1990, commercial production of electro-activation apparatus has been carried out by NPO "EKRAN". Years of meticulous research, and design of optimal conditions gave rise to three widely used electro- activation systems: a water treatment installation "Izumrud", "STEL" devices producing electrochemically activated disinfectants and detergents, and "AQUACHLOR", "ECOCHLOR" devices for production of different electrolysis substances. These installations are being used in several hospitals and city water cleaning systems in the Russian Federation. The use of electro-activated solutions is approved by the ministry of agriculture of the Russian Federation (SanPin 2.1.4.1074-01, Standard of Russian Federation) and its product – anolyte is classified as class 4, i.e. minimal toxicity substance when ingested and applied to the skin by the Russian Standard GOST 12.1.007-76 (A.A.Zakomyrdin, 2002). Moreover, in Japan, electro-activated water (slightly acidic anodic water) is considered a safe food additive with bactericidal properties (Liato et al., 2015).

In recent years, research in the field of electrochemical activation in the food industry is gaining momentum. A significant contribution was made under the supervision of professor M. Aider in extraction of proteins from food industry sub-products (soya, canola, whey), lactulose production, and food conservation by application of electro-activation (Aider et al., 2012; Aït-Aissa & Aïder, 2014b; Cayemitte et al., 2021; Djouab & Aïder, 2019; Gerliani et al., 2020a, 2020b; Karim & Aider, 2020b; Koffi et al., 2014; Liato et al., 2017).

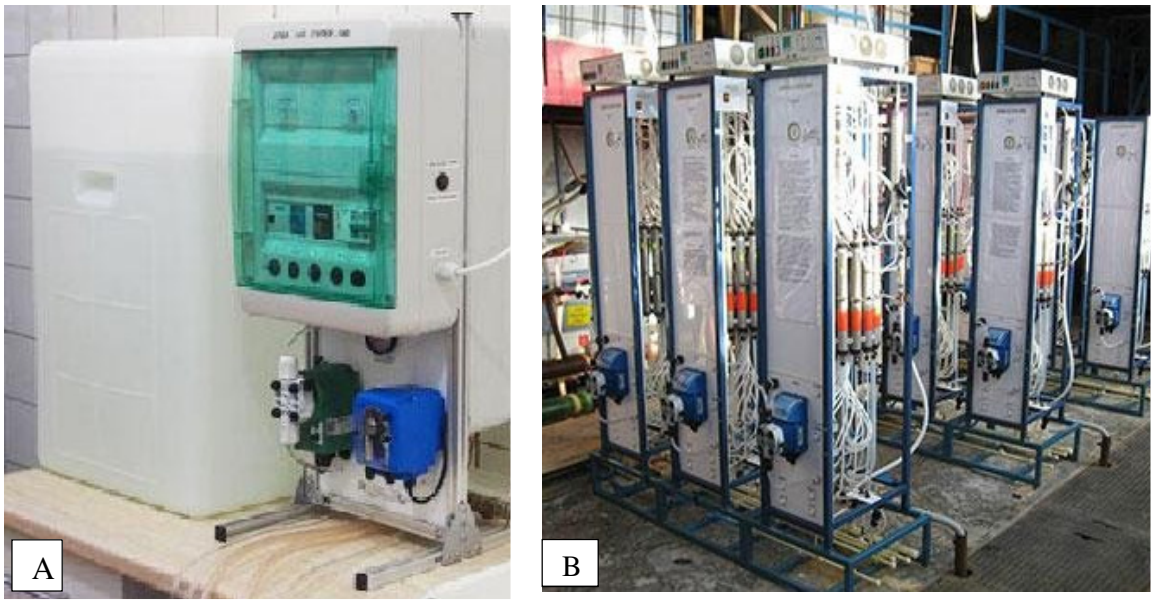

Figure 1-5: Electro-activation devices: (A) STEL and (B) Aquachlor (adapted from algiss.ru)

1.2.2 Principles of electro-activation reactions in aqueous solutions.

Electrochemical activation combines electrochemical and electro-physical effects on ions and molecules of water and solutes in the area of spatial charge at the electrode surface (Aider et al., 2012). It is accompanied by non-equilibrium charge transfer by electrons across the "electrode-electrolyte" interface under conditions of minimal heat production. Particularly, when water is treated by electric current which exceeds an electric potential of water decomposition (+1.25V), water turns into metastable state and undergoes electrochemical changes such as the level of electrons, pH, the redox potential, electrical conductivity, vapor equilibrium index, active acidity, crystallization temperature, and surface tension coefficient (Aider et al., 2012). The extent of changes in mentioned physical and chemical properties depends on several factors including the ratio of water volumes in different electric chambers, reactor configurations, material of the electrodes, solute concentration, temperature, electric voltage and processing time (Ignatov et al., 2015). The electric current in this case plays a role of an oxidizing or reducing agent. Electrical decomposition of water provokes formation of highly reactive valence-unsaturated radicals and an excess of potential energy (Kyianovskyi A, 2019). Thus, electro- activated solutions contain 3 groups of reactive substances: 1 – stable components (acids, bases), 2 – highly active metastable compounds (molecular ions and free

radicals), 3 – long-lasting quasi- stable structures formed at near-electrode area (hydrated ion shells, molecules, radicals). The second group elements define the redox potential of a solution.

Electro-activation consists of four major processes: (i) electrolysis, (ii) electrophoresis, (iii) electroflotation, (iv) electrocoagulation. Water electrolysis is a primary reaction characterized by water decomposition and liberation of H^+ and OH^- ions. Electrolysis takes place at the electrode surface, whereas the following three secondary reactions occur in the water solution, thus referred to as volume processes (Hsu, 2005). Electrophoresis is the movement of charged elements in the direction of the oppositely charged electrode. Further, these reactions lead to formation and liberation of gas bubbles and gaseous forms of H_2 at the cathode and O_2 at the anode (electroflotation). Electrocoagulation is the formation of colloidal aggregates, mostly provoked by changes in temperature and pH (Ignatov et al., 2015).

Unique qualities of electro-activated water were studied by Pastukhov and Morozor (Pastukhov & Morozov, 2000). The authors investigated vibrational energy modes of electro-activated water solution by Raman spectroscopy. They found a considerable difference between electro-activated and non-activated solutions at spectra between 700 and 2700 cm^{-1}. At this range electro-activated solutions demonstrated a striking intensity peak. Intensity in this region was reduced over time (slower decrease in anolyte) which corresponds to the relaxation, and when anolyte and catholyte were mixed at proportional volumes. It was suggested that this difference was caused by the excess of H^+ (anolyte) and OH^- (catholyte) ions in the absence of hydrated acid or alkali residues that could hinder mobility of electrons or protons. Previously it was stated that once complete relaxation is achieved, the distinct parameters of electro-activated solutions are equalized with corresponding non-activated solutions. The relaxation could take place from several minutes to several days. This relaxation state is an important point in proving the concept of the harmlessness of electro-activated solutions, as upon relaxation the water solution returns to its normal state.

In the case of electro-activated water, there were studies demonstrating mathematical modelling of the relaxation process, as well as pH and ORP of activated solutions. It was calculated that for open systems, water electro-activated in anolyte returns to its natural condition in 36 hours, and for catholyte in 48 hours. In closed systems relaxation was extended to

approximately 94 hours (Chushkin, 2016; Semenenko et al., 2020). The studies of (Kareb, Aider, et al., 2018) and (Aidarbekova & Aider, 2021) implied that electro-activated 10% whey solutions maintain their alkaline pH (11-12) during 48 hours of storage at room temperature in an open system. Thus, in order to exploit distinct characteristics of electro-activated solutions, parameters of electro-activation need to be configured to allow maximal elongation from the thermodynamically stable state.

1.2.3 Near electrode and electrolyte reactions

As it was mentioned before, electrochemical activation takes place at the electrode/electrolyte interface where the energy is the highest. Charged ions lose their charge and turn into decay products when they reach the electrode surface (Aït-Aissa & Aïder, 2014a).

Series of redox reactions take place on the surface of cathode and anode due to the flow of electrons (Petrushanko I. Yu., 2001). Water molecules obtain a resonant microcluster structure (Koffi et al., 2014). Physical properties such as differences in electrical potential, electrophoretic mobility and concentration of electroactive species give rise to two different layers at the electrode/electrolyte area. A so-called double-diffusive layer (EDL) is 0.1 μm in fresh water and thinner in more concentrated solutions. EDL is composed of 2 parallel spaces with contrary charged ions with overlapping of their electronic fields. Outside of EDL tension equals zero, thus EDL does not create an electric field in the entire solution, but divides it into two parts. Each zone has its own potential, and passing through EDL the potential changes, which is called potential difference (Zoski, 2007). The inner layer contains the ions bound to the electrode by electrostatic forces. In the outside layer ion species are less structured, yet less mobile than in bulk solution (Santos et al., 2013). The contact zone between two electric conductors is very important and electron transfer depends on the nature of the conductors, electric field potential, and reactor geometry (Aït-Aissa & Aïder, 2014a). The type of electrode reactions is dependent on the nature of the electrode and electrolyte, as well as environmental conditions such as temperature and presence of the mixtures. Generally, there are two chemical phenomena that take place during electrolysis of water - oxidation (gaining an electron) at the anode and reduction (losing an electron) at the cathode. Con-

sequently, it leads to acidification of the media in anodic and alkalinization in cathodic compartments (Aider et al., 2012). Electrode reactions are followed by liberation of $O_{2(gas)}$ at anode interface and $H_{2(gas)}$ at cathode interface.

Oxidation at anode (**Eq. 1.1**):

$$2H_2O \longrightarrow O_{2(gas)} + 4H^+ + 4e^- \qquad \textbf{(Eq. 1.1)}$$

$$4OH^-_{(aqueous)} \longrightarrow O_{2(gas)} + 2H_2O_{(liquid)} + 4e^-$$

Reduction at cathode (**Eq. 1.2**):

$$2H_2O_{(liquid)} + 2e^- \longrightarrow H_{2(gas)} + 2OH^-_{(aqueuous)} \qquad \textbf{(Eq. 1.2)}$$

$$2H^+ + 2e^- \longrightarrow H_{2(gas)}$$

However, water is a weak electrolyte. Electric conductivity of deionized water is 0.05µS/cm (at 25°C), to compare electric conductivity of whey is approximately 8 µS/cm. In this regard, mineral salts (NaCl, KCl, K_2SO_4) are used to facilitate electric current transfer.

Another important concept in electrolyte reactions is the number of ions transferred. The amount of electricity carried is determined by the concentration of ions and their electrophoretic mobility; when the concentrations of cations and anions are the same, their participation in the transfer of electricity depends only on their relative velocity. Since the velocities of cations and anions can be significantly different, the transfer numbers must also be different (Ye & Feng, 2010).

1.2.4 Catholyte and anolyte properties

Catholyte is an alkaline liquid, sometimes with white sediment due to the precipitation of certain solutes at high pH. Other changes in catholyte include a decrease in ORP (-200…-800), water conductivity and surface tension. Dissolved oxygen and nitrogen levels go down, instead of it other reactive species form: hydroxyl anion (OH^-), hydroperoxide anion (HO_2^-), molecular oxygen ion radical (O_2^-), oxygen anion (O^{2-}), peroxide anion, and metastable aqua

complexes of hydrogen superoxide, free hydrogen radical, free peroxide radical. Thus, the catholyte is saturated with reducers (Ignatov et al., 2015).

The catholyte reportedly has antioxidant and immune-stimulating properties, stimulates energy production (ATP), blood circulation and tissue regeneration, regulates carbohydrate and lipid metabolism, promotes DNA synthesis and cell growth and division (Petrushanko & Lobyshev, 2004; Toshka Petrova, 2020). It was also suggested that low ORP of catholyte favours the growth of anaerobes (Petrushanko & Lobyshev, 2004).

The properties of the anolyte are opposite to the catholyte: higher ORP (+500…+1100) and electrical conductivity. Increase in levels of dissolved oxygen and chlorine, and decrease in hydrogen amounts (Toropkov V.V., 1999). It is saturated by oxidizing compounds: hydroxyl radical, peroxide anion, singlet molecular oxygen, superoxide anion, ozone, atomic oxygen.

The anolyte is an acid liquid with antimicrobial properties. Regarding the cytotoxicity of this antimicrobial agent, it is classified as a substance of class 4, with the minimal toxicity within the class (GOST 12.1.007-76). Heating of the anolyte to 50□ improves its bactericidal activity by 30–100 % (Leonov, 1999).

1.2.5 Electro-activation reactor design

Depending on the field of application, purpose, and type of liquid, various electrolyzing reactors are available (Vrabie et al., 2020). The quality and stability of resulting activated solutions depends on electrolyzing system design. An intensity of electro-activation is dependent on electric current density and electrode polarization. Also, electrode surface area, which allows a contact with a bigger number of molecules plays a pivotal role (Kyianovskyi A .M, 2019) Another technical aspect is a requirement for low power consumption systems. These, and other parameters need to be taken into consideration while designing a reactor for electro-activation. There are several types of reactors developed through the evolution of electro-activation technology.

The first flow-through Electrochemical Cells were developed in 1989. It consisted of parallel flow electrochemical modules with external tubular and internal rod-shaped electrodes and tubular ultrafiltration diaphragm. It allowed to equally saturate the solution with metastable activated particles and unipolar water treatment (Tomilov A .P., 2002). Industrial production

and renovation of the system took place over the years: the number of industrially installed FEM-1 systems was 1000, FEM-2 -80000, and FEM-3 exceeded a million, and was used in production of multiple devices, including the "Redox" units for cleaning artificial kidney dialyzers, Ehatron units for poultry farms, and Exatron-K for straw saccharification (Bakhir V. M., 1999). The most recent electro-activation modules were released in 2012 under the name BM (Bakhir modules) and patented under the patent of RF №2012105962 (http://vbinstitute.org/equipment/elements/).

While continuous-flow type reactors are more functional for industrial settings, research laboratories rely on stationary type cells. Stationary electro-activation reactors are usually open systems containing different chambers separated by ion- exchange membranes, and electrodes (positive and negative) immersed into solution and connected to an external power source. The material from which the reactor cell is made should not react with the solutions. In multiple studies on electro-activation, the reactor was made from plexiglass (Aït-Aissa & Aïder, 2014a; Cayemitte et al., 2021; Gerliani et al., 2020; Karim & Aider, 2020). The strength of electric fields on the water molecules is maximal at the level most proximal to the electrode, reaching hundred thousand volts per cm. Thus, the influence of an electric current diminishes with the distance from the electrodes. (Aït-Aissa & Aïder, 2014a) obtained higher alkalinity rates in cathodic compartments with lowest distance between electrode and extremity of the compartment. Along with the distance, the factor of concentration of the solutes plays a role in distribution of electric field. Consequently, a reactor must be intended in a way to ensure the activation of micro- volumes of water in all the layers. (Aït Aissa & Aïder, 2014) conducted a series of experiments to define optimal reactor configuration for electro-activation of lactose solution. The factor of membrane surface area/electrode surface area ratio was found to be insignificant ($P > 0.05$) in catholyte alkalinization process. On the contrary, the effect of electric current intensity, time, and reactor configuration (i.e. membrane types placed between different compartments) was very pronounced. The authors concluded that the reactor configuration where cathodic compartment was separated from middle compartment by cation exchange membrane (CEM) permitted retention of high concentrations of hydroxyl ions, thus facilitating efficient lactulose alkaline isomerization.

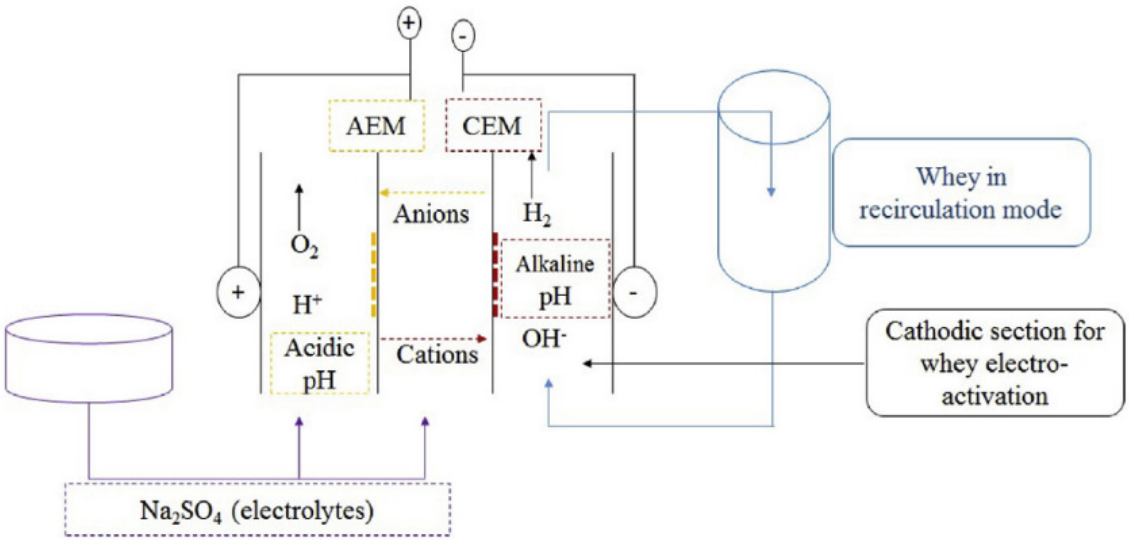

Figure 1-6: Schematic representation of whey electro- activation chamber. Adapted from (Kareb et al., 2017)

1.2.5.1 Membranes

Separation by mono- or bipolar membranes allows keeping different types of ions in different compartments of a reactor, thus preventing mutual neutralization of the solutions (Gerzhova & Aider, 2020). The membranes regulate the mass transfer of charged species. Monopolar ion exchange membranes allow the passage of specific ions – anions (anion-exchange membranes) or cations (cation-exchange membranes). The membranes can be made from organic polymers (polystyrene-co-divinylbenzene, polypropylene, polyethylene) and inorganic materials (zeolites, betonite, phosphate salts). However, the organic polymer membranes are more efficient (Xu, 2005).

The selectivity is ensured by specific charged groups fixed to polymeric matrix of the membrane: cation exchange membranes contain negatively charged fixed groups (- COO^-, - SO_3^-, - PO_3^{2-}, -$C_6H_4O^-$ –PO_3H^-), and anion exchange membranes contain positively changed groups (-NH_3^+, - NRH_2^+, –NR_2H^+, –NR_3^+, –PR_3^+, –SR_2^+) on the polymer matrix (Hassanvand et al., 2017; Xu, 2005). Essentially, the charge and concentration of the fixed groups influence the passage of ions through the membranes. Also, membrane thickness and pore sizes are important parameters. Multiple researchers used CMI-7000 (functional group Sulphonic Acid) and AMI-7001 (functional group Quaternary Ammonium) membranes (membranes international inc.) with standard thickness of about 0.45 ± 0.025 (Djouab & Aïder, 2019).

1.2.5.2 Electrodes

(Aït-Aissa & Aïder, 2014a) have reported that an electrode material played a significant ($P < 0.001$) role on pH of electro-activated solutions. The catholyte pH raised to 10.5 ± 0.27 when copper electrode and 9.25 ± 0.08 when titanium and type 304 stainless steel electrodes were used. There are a number of general requirements for the electrode material, such as good electrical conductivity, high catalytic activity, selectivity for the targeted electrochemical reaction, pH and temperature stability. It is crucial that electrode material does not interfere in the reaction. Thus, inert and insoluble materials are preferred.

According to (Ignatov et al., 2015), metals such as lead and cadmium are used for the cathodes as they require high electrical voltage and initiate production of reactive free radicals (Cl*, O*, OH*, HO2*) (Ignatov et al., 2016).Another study used RuO_2–IrO_2–TiO_2 anode and cathode with an active surface area of 40 cm^2. This type of electrodes was mentioned to resist fouling and corrosion, and provide high stability and electro- reactivity (Liato et al., 2015). Titanium coated with Ruthenium Iridium (anode) and 304 stainless steel (cathode) were reported to be used by multiple researchers (Aït Aissa & Aïder, 2014; Kareb, Aider, et al., 2018; Karim & Aider, 2020).

1.2.6 Electro-activation as a green technology

Electro-activation technology was named "green" technology by many researchers working with it (Djouab & Aïder, 2019; Gerliani et al., 2020b; Kareb et al., 2017). The notion of green technology is defined as "Technology whose use is intended to mitigate or reverse the effects of human activity on the environment". Taking into account the available data about electro-activation technology, the following points can outlined:

(i) Electro-activation is a reagentless technology. It allows production of highly acidic/alkaline solutions without an addition of acids or bases;

(ii) Its sustainability is supported by the fact that the reactor and electrodes are multi-use, and require a single-time installation;

(iii) Electro-activated solutions neutralize themselves (relaxation) with the time, thus, do not create toxic wastewaters.

To oppose it, the technology can be considered green, only if it uses renewable sources of energy, rather than fossil fuel-generated electricity (Natela Gerliani, 2019). However, it

should be considered that current technologies alternative to electro-activation also rely on electricity. For example, conventional chemical lactulose isomerization requires heating to increase the lactulose yield. Another sustainability challenge of electro-activation technology is a need of changing the membranes with certain frequency due to membrane fouling.

1.2.7 Electro-activation of whey

One of the most acute issues in dairy industry is treatment of whey in environmentally friendly manner. In this regard, several researchers have been working on valorization of whey through electro-activation (Aidarbekova & Aider, 2021; Kareb et al., 2015; Karim & Aider, 2020; Paladii Irina, 2021; Sprinchan et al., 2011).

A series of exhaustive studies were published by (Kareb, Aider, et al., 2018) on the development of a novel dairy functional ingredient by electro-activation of cheese whey. Functional ingredients are intended to produce a beneficial effect on consumers' health through certain physiological activities (Kruger & Mann, 2003). Indeed, the research of (Kareb, Aider, et al., 2018) has outlined antioxidant and prebiotic properties of electro-activated whey (Kareb, Champagne, et al., 2018). In addition to that, 25 biologically active peptides were identified in electro-activated whey. From them 19 are potential ACE (Angiotensin-converting enzyme) inhibitors, 1 is suggested to have anticancer, 1 antibacterial, 2 dipeptidyl-peptidase IV (DPP-IV) inhibition and 1 opioid agonist effect (Kareb et al., 2017). (Momen et al., 2022) studied the functional properties of whey proteins in electro-activated whey. The authors revealed that electro-activation had enhanced the functional properties of whey proteins. In particular, electro-activation of whey improved protein solubility at pH range 5-7 to almost 100%, compared to control whey with 75% solubility. The foaming of electro-activated whey was significantly higher than that of control whey. It exceeded the foaming indices previously obtained from WPI, and lentil proteins, and was comparable to egg white protein. The higher foaming and emulsifying capacities of electro-activated whey were attributed to higher solubility and formation of stronger viscoelastic film at air/water (water/oil) interface by protein aggregates (especially β-lactoglobulin) formed during the electro-activation.

As described for other electro-activated solutions, qualities of electro-activated whey are dependent on the electro-activation conditions. In the majority of studies, whey was electro-activated in 3 compartment reactors, with anodic, cathodic and middle compartments. The

electrolytes such as Na_2SO_4 and K_2SO_4 were used to fill anodic and middle compartments, while whey solution benefitted from alkalinization in cathodic chamber (Kareb et al., 2015; Karim & Aider, 2020b; Momen et al., 2022).

Despite all favorable changes in whey proteins, the main rationale for whey valorization through electro-activation is production of lactulose. Isomerization of lactose to lactulose is carried out by accumulation of hydroxide ions serving as proton acceptors in the reaction and formation of highly alkaline media. Moreover, excess internal potential energy in electro-activation reactor facilitates the isomerization reaction (Ryabtseva S.A., 2001). Other whey components such as minerals and proteins also participate in lactulose synthesis. LA-transformation is catalyzed by buffering minerals, so whey minerals may serve in accumulation of actively charged particles (Sprinchan et al., 2011). Moreover, mineral buffers support optimal pH of lactulose solutions. Organic acids and proteins contribute to Amadori transformation. However, higher protein concentrations can lead to formation of undesired products by Maillard reaction (Ryabtseva S.A., 2001).

Electro-activation of bulk whey containing 4.0 - 4.7% of lactose is a cheaper alternative of production of lactulose enriched ingredients as pure lactose is an expensive raw material for lactulose production. Moreover, direct electro-activation of cheese whey allows shortening of the process of whey valorization by lactulose production, as it does not require lactose purification steps.

The cost of electro-activation treatment of whey can be mainly estimated from the energy expenditures of the process. These calculations allow the development of the reactors with optimized energy consumption. In this regard, a parameter as global electric resistance of the reaction should be considered (Aït Aissa & Aïder, 2013). It is dependent on the conductivity of the solutions and is described by Ohm's law, which represents the ratio between the applied voltage U (V) and the current intensity I (A), as given in (**Eq. 1.3**). Another parameter to consider is membrane fouling that can increase the global resistance of the system (Koffi et al., 2014).

$$\boldsymbol{R = U/I} \quad \textbf{(Eq.1.3)}$$

1.3 Lactulose

1.3.1 General information on lactulose

Lactulose (4-0-β-D-galactopyranosyl-D-fructofuranose) is a synthetic disaccharide composed of galactose and fructose, which are linked via 1→4 glycosidic bond. It has the same formula as lactose $C_{12}H_{22}O_{11}$, and a molecular mass 342.30 g/mol (Aït-Aissa & Aïder, 2014b). Lactulose can be found in 5 isomeric forms, such as the fructose moiety in the form of an α or β pyranose, α or β-furanose, in an acyclic form (Aït Aissa & Aïder, 2013)

It was first synthesized by Montgomery and Hudson (1930) by heating lactose in a saturated calcium hydroxide solution. Lactulose is reported to spontaneously form during milk heat treatment (UHT milk) and storage (Y.-H. Cho et al., 2012). However, only small amounts of lactulose can be formed in the conditions of neutral (6.7) pH of milk. Approximately 0.3 g L1 of lactulose was formed in UHT-milk and 1.6 g L^{-1}in sterilized milk (Schuster-Wolff-Bühring et al., 2010).

A bifidogenic effect of lactulose was first described by Petuely in 1957 (Ruszkowski & Witkowski, 2019). The prebiotic effect of lactulose starts at 3g daily (Förster-Fromme et al., 2011; Ruszkowski & Witkowski, 2019). A full guide on dosages of lactulose and its benefits are depicted in Table 1.7. More than 99% of ingested lactulose reaches the colon to be fermented by microbiota. Lactulose metabolism by colonic microbiota was extensively studied by (Mao et al., 2014). The study identified the genes responsible for lactulose metabolism in 222 GIT bacterial strains. Transport and glycosidase encoding genes were not unique for lactulose, but common for all galactooligosaccharides (GOS). Thus, it was concluded that lactulose is considered as GOS with a lower degree of polymerization. A significant novelty of the study was a suggestion that lactulose is not a selective bifidogenic agent, as it can be fermented by *Cronobacter*, *Enterococcus*, *Klebsiella*, and *Pseudomonas* species. Other bacteria reported to utilize lactulose *in vitro* are *Faecalibacterium prausnitzii*, *Bacteroides oralis*, *Escherechi coli*, and *Clostridium perfringens*.

Colonic bacteria consuming lactulose produce organic acids (mostly lactic and acetic) and in some cases carbon dioxide. The acids cause osmotic fluid accumulation in the bowel, which is the basis of the laxative effect of lactulose (Panesar & Kumari, 2011). Besides prebiotic

and laxative effects, physiological effects of lactulose extend to the treatment of hepatic encephalopathy, prevention of colonic cancer and stimulation of increased mineral absorption. Hepatic encephalopathy is a condition in which high levels of toxic components, such as ammonia accumulate in the blood. It is a neurological dysfunction leading to hepatocellular failure and the inability to eliminate the toxins in the blood. Lactulose is applied as a common treatment of this condition for its ability to reduce ammonia levels by (i) the acidification of caecal content enhances the protonation of ammonia to ammonium ions, which remain within the colon and are excreted through defecation; (ii) the promotion of carbohydrate-metabolizing bacteria by lactulose intake results in the diminished activity of proteolytic bacteria, which contribute to ammonia production (Schuster-Wolff-Bühring et al., 2010). Moreover, lactulose could be potentially used in medical nutrition for patients with antimicrobial therapy prescriptions. It was reported that daily lactulose intake of 20 mg per kg of body weight helped to maintain "healthy" gut microbiota in patients on azithromycin therapy (10 mg per kg of body weight) (Nikolaou et al., 2020). Thus, the production of lactulose is of substantial interest for medical, pharmaceutical and food industries (Aït Aissa & Aïder, 2013).

Table 1-7: Doses and physiological effects of lactulose.

Dose of lactulose	**Physiological effect**	**Source**
5 g/day, during 5 days	Increased bifidobacterial and lactobacilli growth, Increased production of acetate, butyrate and lactate Increased NaOH consumption, Decrease in ammonia and BCFA.	(Bothe et al., 2017)
15- 40 g/day (adult)	Constipation treatment	(Schuster-Wolff-Bühring et al., 2010)

50-180 mL lactulose syrup daily (adult)	Hepatic encephalopathy treatment	(Schuster-Wolff-Bühring et al., 2010)
4 to 12 weeks of 30- 60 g/d lactulose	Modification of fecal and biliary bile acid compositions	(Ruszkowski & Witkowski, 2019)

1.3.2 Ways of lactulose production

Lactulose is a synthetic ketose sugar that can be produced from aldose lactose by two types of molecular rearrangement. The most common and most industrially utilized one is Lobry de Bryun – Alberda van Eikenstein (LA transfromation). This reaction requires lactose to be in acyclic form with an anomeric center. The first step of this transformation is formation of unstable enolic form. Dynamic hydrogen of enols initiates further transformation. In alkaline conditions C=O bond in the second carbon atom forms. The presence of proton acceptors causes redistribution of electron density. Thus, the glucose terminal transforms to fructose terminal (Aït-Aissa & Aïder, 2014b).

The second way of lactulose production is interaction of semiacetal hydroxyl of lactose with amines. Formation of lactosylamine by Amadouri transformation and cleavage to lactulose and amine. This reaction is difficult to control and has many secondary products as aminocarbonyl compounds (Ryabtseva S.A., 2001).

Catalysts used for chemical rearrangement of lactose molecules include carbonates (potassium and sodium), hydroxides (potassium, sodium, and calcium), magnesium oxide, tertiary amines, borates, and sodium aluminates. With the temperatures of 70–100 °C approximate lactulose yield is 20–33% (Nooshkam et al., 2018). However, as a consequence of heating and further molecular rearrangements, lactulose undergoes formation of unwanted secondary

products, such as galactose, formic and iso-saccharinic acids. Higher concentrations of lactose do not ensure an increase in lactulose yields as there is less availability of proton acceptors. Moreover, according to (Ryabtseva S.A., 2001) the concentration of lactose in the initial solution higher than 20% speeds up the formation of secondary products. In general, the best conditions of lactulose conversion are created at equimolar concentrations of catalyst and lactose.

Other catalysts used for lactose to lactulose isomerization are sulphites and phosphates. Reducing capability of these salts protects disaccharides from oxidation and allows the use of higher concentrations of lactose (60-65%) and higher temperatures (80-100□) (Aït-Aissa & Aïder, 2014b).

Higher yields of lactulose (70-80%) were obtained with the use of complex agents such as aluminates and borates, as the latter can form insoluble complexes with lactulose at alkaline pH, and protect lactulose from degradation. The principle of this reaction is as following: carbohydrates containing cis-α-glycolic groups react with boric acid and its salts, and form stable negatively- charged complexes of two types. These complexes protect lactulose from autocatalysis and re-isomerization. Also, interaction of tetraborate with lactose leads to destabilization of the lactose molecule and facilitates rearrangement. However, this process is difficult to control industrially and it poses a substantial problem of removing the catalyzer (Ryabtseva S.A., 2001).

Another group of catalysts belongs to heterogenous natural catalysts, represented by calcium carbonate- based eggshell and oyster shell, as well as limestone and sepolites. Their advantage is considerable reduction of energy use, and waste production, as they can be easily separated by centrifugation. However, this method did not find its industrial application (Schuster-Wolff-Bühring et al., 2010).

The final steps of chemical isomerization of lactulose are neutralization, catalysts removal, deionization and lactulose purification from other sugars (Sitanggang et al., 2016). Removal of the catalyst is conventionally carried out by anion and cation exchange resins. This treatment allows production of the final syrup containing 60-70% dry matter, from which 42-48% is lactulose (Ryabtseva S.A., 2001). Another way of purification is by ion- exchange electrolysis (calcium hydroxide-based isomerization). After isomerization and purification steps,

lactulose syrup is neutralized by addition of organic acids (0.115-0.125% citric acid) to prevent autocatalytic decay of lactulose. Aluminates are removed by sedimentation or membrane technologies coupled with centrifugation. Further, lactulose can be separated from non-isomerized lactose by difference in solubility in alcohol solutions (Schuster-Wolff-Bühring et al., 2010). Due to formation of secondary coloring products, lactulose solution should be subjected to decoloration step (Ryabtseva S.A., 2001).

Table 1-8: Downstream processes of lactulose production. Adapted from (Schuster-Wolff-Bühring et al., 2010)

Process step	Effect
Acidification	Disintegration of lactose-chelate complex Precipitation of chelating agents Precipitation of alkalizers as salts
Concentration, cooling, separation	Precipitation of chelating agents Crystalization of lactose
Chromotography	Removal of salts Removal of boric acid Removal of lactulose
Extraction with alcohol	Separation of lactose

Table 1-9: List of catalysts used for chemical synthesis of lactulose. (Nooshkam et al., 2018; Ryabtseva S.A., 2001; Sitanggang et al., 2016).

Type pf the catalyst	Chemical agents	Conditions	Lactulose yield
Hydroxides	Sodium, calcium, potassium	pH11±0.2, temperature 70±2°C, time - 20±5 min	27-29%
Sulfites and phosphates		2.1-8.6% of phosphates; temperature 104°C; Time 20- 240 min	20%
Aluminates and borates	boric acid+ sodium hydroxide	pH 10.5- 11.5; temperature 70-75°C; time 4h	up to 87%
Natural calcium carbonate- based by- products and sepolites	egg and oyster shell, limestone	pH 6.2-6.5; temperature 100°C; time 1 min	18-25%

Thus, above mentioned data outlines a visible disadvantage of lactulose production by the use of chemical catalysts in hot-alkaline conditions, which is a tedious process of catalyst removal and purification of the final product. These downstream steps cause significant energy and water consumption (Schmidt et al., 2020). There is also a question of the purity of raw material - lactose, as impurities of whey or whey permeate result in undesirable side reactions, lowering lactulose yield and increasing processing costs (Nooshkam et al., 2018). In this regard, lactulose production by enzymatic transglycosylation may be viewed as a solution to these issues. β-glycosidases catalyze lactose hydrolysis reaction, and the galactosyl

moiety is transferred to fructose as an acceptor. An amino acid sequence of galactosidases is the defining factor of the reaction, as it affects its ability to accept nucleophiles of non-aqueous nature and to transfer the galactosyl moiety to the C4 position of fructose (Nooshkam et al., 2018). The yields obtained by this method vary between 4-44%, which is still outcompeted by chemical isomerization methods (Schuster-Wolff-Bühring et al., 2010). This reaction requires the addition of high amounts of fructose to serve as nucleophilic acceptor, which leads to considerable amounts of unreacted fructose in the final product. Coupled with the high price for the enzymes, this fact makes the process economically unfeasible for industrial scale.

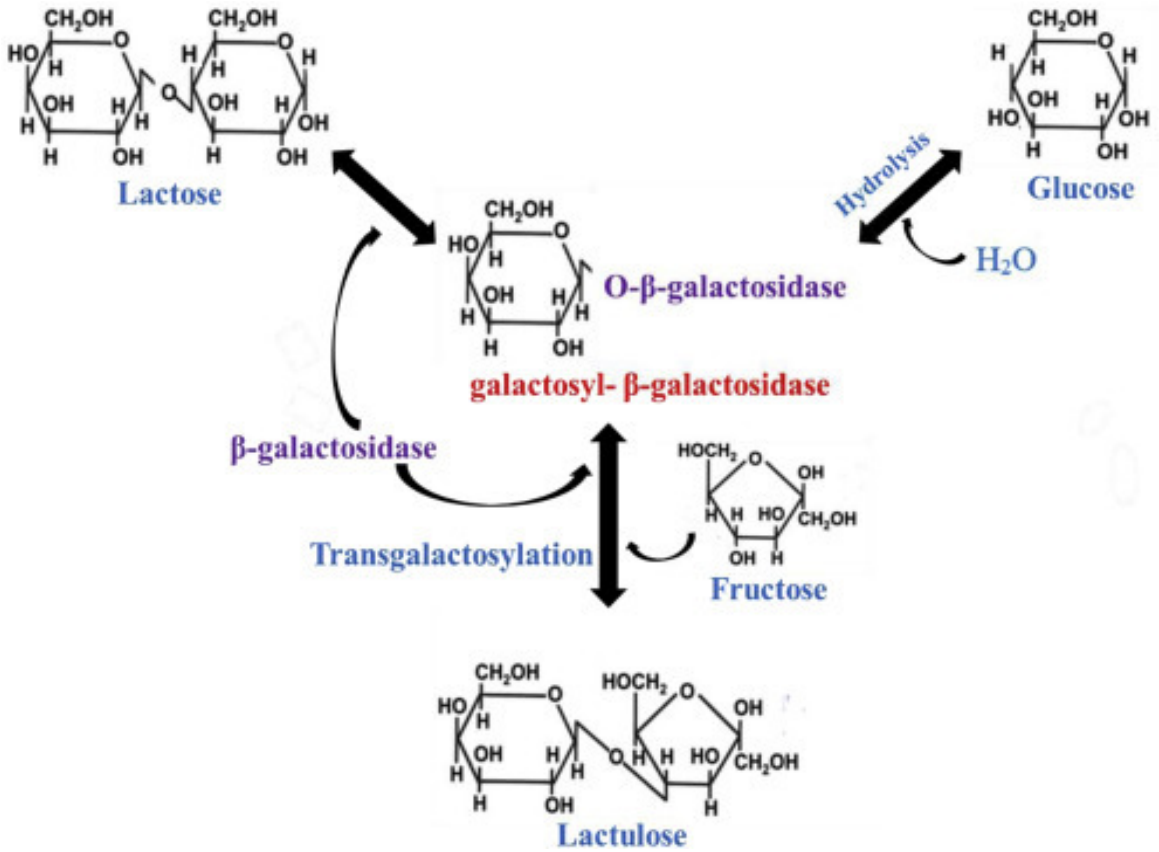

Figure 1-7: Enzymatic transgalactosylation of lactose to lactulose. Adapted from (Nooshkam et al., 2018).

In recent years direct electrochemical transformation of lactose production was proposed as a reagentless method. The production of lactulose by using basic catholyte of electro-activation solutions is based on a hypothesis that hydroxide ions formed in the cathodic

compartment can serve as proton acceptors in the reaction of lactulose isomerization (Ryabtseva S.A., 2001). An excess of potential energy formed during electro-activation catalyzes the reaction. In addition to that, autonomous heating to 50-60□ is taking place due to the electrochemical reactions. (Karim & Aider, 2020a) studied different regimen of electro-activation of lactose. The authors obtained a maximal lactulose yield of 38% with the parameters 900 mA, 10% lactose solution, 40 minutes. It was concluded that in comparison with potassium hydroxide-induced isomerization, higher yields of lactulose were achieved in shorter periods by electro-activation. Moreover, the electro-activation method answers the main challenges of lactose/lactulose reactions, such as environmental sustainability, stable isomerization results, absence of the catalyst, and therefore downstream processing. Thus, lactulose produced by electro-activation can be used as a food ingredient.

1.3.3 Lactulose as food ingredient

A long history of lactulose as a food additive counts back to 1957, when it was introduced by Patuely as bifid factor. In the European Union, lactulose-containing products are labeled as "prebiotic" and "lowering colonic transit time" and in Japan lactulose is listed as a FOSHU (Food of Specified Health Use) functional ingredient (Schuster-Wolff-Bühring et al., 2010). (Olano & Corzo, 2009) mentioned that lactulose-enriched yogurts shortened gastric emptying and intestinal transit time, and were effective in treating constipation in children. In addition to nutritional benefits, lactulose added to fermented milk products may enhance their physical-industrial properties. (de Souza Oliveira et al., 2011) reported a decrease in fermentation time and maintenance of high numbers of probiotic bacteria in yogurt supplemented with lactulose (4% w/w). These results signify that the disaccharide was readily metabolized by used bacteria *L. acidophilus LAC4, L. rhamnosus LBA, Bifidobacterium animalis subsp. lactis BL 04, S. thermophilus,* and *L. delbrueckii subsp. bulgaricus LB340,* which increased the acidification rate. Similar results were obtained by (Özer et al., 2005) where the addition of prebiotics lactulose or inulin reduced the incubation time. The addition of 1.5% lactulose also resulted in higher viscosity in a synbiotic yogurt with probiotic strains *Lactobacillus rhamnosus* and *Lactobacillus reuteri* (Shaghaghi et al., 2013). In symbiotic yogurts containing *Lactobacillus casei* addition of lactulose-inulin complex resulted in lower syneresis and higher probiotic bacterial counts, than control (Aghajani & Pourahmad, 2012). In the study

of (ben Moussa, Marwa, et al., 2019) addition of 6% lactulose recued yogurt syneresis to 6.5%. A reduced syneresis and augmented viscosity can be explained by contribution of hydrophilic sugars to improving a water holding capacity of the gel and increase in total dry matter (ben Moussa, Boulares, et al., 2019). To better understand the effect of lactulose supplementation to fermented milk products, in the following section we will discuss formation and physical properties of acid milk gels.

1.4 Fermented milk products

Acid milk gels is a general name for products produced by acid coagulation of milk. They contain yogurts, kefir, sour cream, acidophilus milk, and other products varying among different cultures (ryazhenka, koumiss, matsoni, labneh, ayran, lassi etc.). Health benefits of consuming fermented dairy products, particularly associated with probiotic bacteria and their metabolites are well-known among the consumers. Consumption of fermented dairy products was associated with hypocholesterolemic and antiobesity effects and reduction of a risk of development of cardiovascular diseases, metabolic and immunological disorders (Companys et al., 2021; Ebringer et al., 2008; González et al., 2019). With 1.9 billion adults suffering from obesity, development of fermented dairy products with additional weight control benefit is a new lucrative niche in this market (Manzanarez-Quín et al., 2021). Overall, a growing demand of consumers for health–promoting functional products push the producers to constantly seek for new functional formula to attract health–conscious clients (Sarkar, 2019).

1.4.1 Formation of acid milk gels

Acidification of milk can be achieved by addition of starter cultures fermenting lactose to lactic acid or by direct supplementation of harmless, acid-liberating components such as glucono-δ-lactone (GDL), γ–galactono-lactone, tetramethyl-δ-mannono-lactone, and lactid. GDL-based gel formation is mostly used in milk acid gel studies due to its ability to mimic lactic acid bacterial fermentation and for providing the possibility of monitoring the kinetics of gel formation in a relatively convenient period which is different from instant acidification obtained by using strong acids such as HCl which moreover can cause the denaturation of casein micelles. Indeed, GDL acidification initiates slowly after supplementation of the acid

and the observed kinetics of acidification is somewhat similar to fermentation by starter cultures without drastic drop of the pH (Lucey, Tamehana, et al., 1998; Sadeghi et al., 2014). Degree of acidification is dependent on the initial concentration of GDL added. Compared to bacterial fermentation, higher permeability and whey separation in milk acidified with GDL was previously reported. This difference in gel microstructure is explained by the production of bacterial exopolysaccharides by starter bacteria. Another distinct characteristic of bacterial fermentation is higher storage modulus (G') due to the shorter ageing time near isoelectric point (Lucey, Tamehana, et al., 1998). Furthermore, milk acidified by bacterial cultures was claimed to have superior sensorial properties, less sourness and more typical yogurt-like aroma and taste (Trop, 1984), whereas GDL is associated with bitterness after some critical concentration added to the product. In both cases of gel formation, temperature plays a defining role in acidification speed (Baglio, 2014).

Caseins play a major role in acid gel formation as they are polyelectrolytes, which makes them very susceptible to pH changes. Caseins are groups of phosphoproteins assembled in micelles containing thousands of subunits and molecules with a diameter of 50-300 nm. Their self- assembly is associated with colloidal calcium phosphate and bound calcium (O'Kennedy, 2011). They are composed of αs1, αs2, β and κ- casein fractions held together by hydrophobic and electrostatic bonds and calcium phosphate bridges. Some characteristics of the main four casein subunits (αS1, αS2, β and κ-caseins) are given in Table 1.10 which demonstrates that high content in hydrophobic amino acid residues is one of the main characteristics of these molecules. Also, the presence of ester bound phosphate residues in high numbers is responsible for binding Ca^{2+}. Consequently, αs1, αs2, and β caseins are very sensitive to Ca^{2+}-induced precipitation. Whereas the prevalence of proline is the reason behind caseins' non-globular shape (Lucey & Horne, 2018; Roefs et al., 1990).

Table 1-10: Description of casein types, their percentage in casein micelle, molecular features and ester bound phosphate residues

Type of casein	Percentage in the micelle	Molecular features	Ester bound phosphate residues
α_{s1}	38	3 predominantly hydrophobic regions	8-9
α_{s2}	11	Contains hydrophobic regions	10-13
β	36	Hydrophilic N-terminal part and a hydrophobic C-terminal part	5
κ	13	C-terminal part of K-casein has a carbohydrate group, comprised of 3 or 4 hexose residues	1-2
Other (γ)	2	Formed by breakdown of main caseins	

The casein micelles are stable colloidal systems. In spite of its low amounts κ-caseins can stabilize 10 times its mass of other casein micelle particles (Fox & Brodkorb, 2008). Some authors attribute this effect to "hairy" hydrophobic glycopeptide projections on casein micelles providing entropic repulsions and effective bonding to the aqueous phase of milk (Lucey, 2016). However, there is no commonly accepted model of casein micelle, and as a consequence of a gelation process. There are three theories describing different parts of the process: the adhesive sphere, the percolation and the fractal models. The adhesive sphere theory represents the stability of particles in different environmental conditions. According to this theory, casein micelles are sterically stabilized by glycomacropeptide (GMP) which

is a part of κ-casein. The nature of GMP is a polyampholyte/ polyelectrolyte brush. This steric stabilization can be gradually disrupted when pH hits the point of acid dissociation constant of carboxylic groups. Deficiency of this model is that it excludes the stabilizing effect of electrostatic bounds and only relies on steric stabilization by κ-CN hairs situated on the surface of κ-casein. Meanwhile the percolation model shows the dynamics of gel formation. This model reviews the micelles distributed on a square lattice and interacting with their closest neighbors. These bonds are formed randomly and as the number of bonds increases the size of cluster increases accordingly. The aggregation continues until a certain threshold. This feature of percolation corresponds with gel formation where number of links increases till one point, a cluster is created and spans the container. At this point, not all fractions join the system-spanning cluster (Lucey and Singh 2003). This explains the increase of storage modulus after gelation point. Deficiency of this theory is that it suggested to be relevant only at the gelation point. In addition to that, it is unable to fully explain mechanical properties of acid gels (Horne 1999).

Fractal theory considers gel aggregation as spherical particles of radius 'a' moving by Brownian motion and aggregating once they bump into each other. The forming blocks can further aggregate among themselves and form bigger clusters (Andoyo et al., 2015; Lucey, 2002). Although this model has characterized semi-quantitative features in irreversibly aggregating systems, it does not consider any kinds of rearrangements, interpenetrations and a possibility of homogeneity of aggregates' size. As opposed to that, (Lucey, van Vliet, et al., 1997) found that acid casein gels could undergo rearrangement at the particle (or cluster) level during or after gelation. Some particles do not aggregate with the network and stay in the form of small particles.

Colloidal calcium phosphate (CCP) is also a significant part of the casein complex, known as calcium caseinate calcium phosphate complex. However, despite common belief it does not contribute to the integrity of a micelle but rather provides a storage of calcium and phosphate. Thus, the level of CCP practically does not influence gelation kinetics (Anema, 2009; Lucey, 2002). Gelation of milk is initiated by dissolution of calcium that affects weak bonds of κ- and β- caseins. Further, negative charge of κ-casein hairs on the surface of the micelles is disrupted due to lowering of pH and reduction of the electrostatic repulsions between the

micelles is an essential factor for their stabilization. H^+ ions deprive the dissociation of casein carboxyl groups and hydroxylic groups of phosphoric acid (phosphoserine groups). When pH reaches 5 the casein micelles are further destabilized due to cleavage of calcium phosphate and structural calcium. At the isoelectric point of casein of pH 4.4-4.6 casein particles aggregate and form chains, which captivate dispersing media and fat globules and other milk components, forming three-dimensional networks (Lucey, 2002; ROEFS et al., 1990; Sadeghi et al., 2014). As casein gels are dynamic, the stage of aggregation is followed by rearrangement of the gel network to achieve an equilibrium (Ercili-Cura et al., 2013). Overall, the gelation process is dependent on pH, temperature, mineral system, milk composition and bacterial cultures.

A gel has some mechanical and rheological properties, such as viscosity, elasticity, plasticity, and adhesiveness. Unlike other thermo-reversible types of food gels, casein gels are considered irreversible (Lucey, 2002). Furthermore, the formation process of a gel is not a final step regarding the particle arrangements. In casein gels, particle rearrangements were in detail reviewed by several authors (Lucey, van Vliet, et al., 1997; Mellema et al., 2000; van Vliet et al., 2004). Rearrangements appear on the level of sub- particles, particles, clusters and gel-liquid separation which is known as syneresis. They are caused by fusion of particles and continuous formation and breakage of physical cross-links between protein strands. It is reported that particles have a tendency to migrate towards denser regions, which results in large hollow spaces between dense regions connected by thin strands. In production terms, it leads to high level of whey separation in acid milk gel products.

1.4.2 Physical and structural properties of acid milk gels.

1.4.2.1 Viscoelastic properties (rheology) of acid milk gels

The rheological properties of acid gels of milk can be studied by dynamic oscillatory tests, with small or large amplitude (large amplitude oscillatory shear). Other instruments of measuring gel physical properties include texture profile analyzers and rotational viscometers (W. J. Lee & Lucey, 2003). Rheology tests measures three main variables: applied strain, stress response and a time at which these events take place. The main parameters studied in acid casein gels are storage (G') and loss (G'') moduli and the ratio of these two variables –

tangent of the phase angle (tan δ). G' indicates the energy that was stored (elastic properties) and G'' indicates the energy that was depleted (viscous properties). These parameters allow identification of the state of the gel: strong gel, weak gel, viscoelastic fluid, or pseudo gel. Thus, G' should exceed G'' to be considered a gel (Hundschell & Wagemans, 2019). Tangent of the phase variable demonstrates superiority of viscous or elastic characteristics. When it is higher than 1 it represents more vicious properties than elastic. The increase in tan δ demonstrates the increase in relaxation of bonds, which is the case when viscous properties start to dominate over elastic ones. These parameters depend on the number and strength of bonds between casein particles, particle distribution and structure, and described by the following formulas **(Eqs 1.4-1.6):**

$$G' = (\tau_0 / \gamma_0) * \cos \delta \qquad \textbf{(Eq. 1.4)}$$

$$G'' = (\tau_0 / \gamma_0) * \sin \delta \qquad \textbf{(Eq. 1.5)}$$

$$\tan \delta = G''/G' \qquad \textbf{(Eq. 1.6)}$$

where, τ_0 - amplitude of the shear stress, γ_0 - amplitude of the strain, δ - phase angle.

Rheological properties of acid milk gels were studied by numerous authors (Lakemond & van Vliet, 2008; W. J. Lee & Lucey, 2003; Lucey, 2002; Lucey, Tamehana, et al., 1998; Lucey, van Vliet, et al., 1997; Sadeghi et al., 2014), covering the effect of fat content and heat treatment (Anema, 2008). (Lucey et al., 1999) reported that heat treatment over 80°C shortens the gelation time and increases pH of gelation. Heating milk at the temperatures above 70°C causes denaturation of whey proteins, which further form complex via disulfide bond with κ-caseins at the surface of the micelle and free κ-caseins in serum (Mahomud et al., 2017). These complexes affect fermentation kinetics and lead to formation of stronger gels (Vasbinder & de Kruif, 2003). An increase of gelation pH can be explained by higher isoelectric point of major whey protein β-lactoglobulin (pI = 5.1). Similarly, higher gelation pH was observed in a study of partial κ-casein hydrolysis. In this case gelation starts at higher pH because of altering negative charge of casein micelles (Gastaldi et al., 2003). Controlled partial hydrolysis of κ-casein influenced micellar surface reactivity and shifted the balance in the benefit of hydrophobic interactions, which facilitated the aggregation of casein micelles.

Fat content of milk also affects storage modulus, with higher G' obtained by full fat milk gels (Lucey et al., 1999). Fat is present in milk in the form of globules, and contributes to the total solids of the gel. Thus, skim milk gels are expected to have weaker, more porous gels. The role of fat globules in gel formation are altered when the globules are subjected to mechanical treatment damaging their membranes (MFGM), as in case of milk homogenization. Milk fat globules reduced in size absorb proteins, such as caseins and whey proteins to the fat globule surface (Michalski et al., 2002). As a consequence, fat globules obtain structure-forming properties and are better integrated in gel network (Sfakianakis et al., 2015).

1.4.2.2 Microstructure of milk gels

Numerous studies were devoted to studying the milk acid gels' microstructure (Kalab and Harwalkar 1973, Lucey, Munro et al. 1998, Fiszman, Lluch et al. 1999, Sanchez, Zuniga-Lopez et al. 2000, LEE and LUCEY 2003). Milk acid gels represent networks of protein chains with heterogeneous structure which entrap liquid, encapsulated bacteria, EPS and fat particles. Altogether they form big conglomerates and holes. Specific terms describing the microstructure of the acid milk gels are explained in **Table 1.11** (Lucey, 2017; Lucey, Munro, et al., 1998).

Table 1-11: The terms used to describe casein acid gel microstructure.

Term	Definition
Branched/ high apparent interconnectivity	Appearance of regular, interlinked chains, strands and clusters in the network
Honeycombed	Appearance of pores with clearly defined 'cell walls' or boundaries
Tortuous	Microstructure that appears to consist of irregular or bent, clusters or strands of aggregated protein particles

Casein gel microstructure is not as variable as other globular protein gels. Nonetheless, various factors can affect its microstructure: milk homogenization, heat treatment, inoculum, and total solids content (Ong et al., 2011). (Lucey, Munro, et al., 1998) reported the differences in structure of acid gels induced by bacteria and GDL preheated to 30°C and 42°C. Prompt acidification by GDL tended to provide gels with less abundant thin cross-linkages, having wider inter-spaces, whereas bacterial type of acidification formed bigger clusters with thick strands. Production of exopolysaccharides and the presence of bacterial cells are a possible cause of this observation. Gels from unheated milks were observed to have "tortuous" clusters. While the microstructure of gels made of milk subjected to pre-heating of >80°C had more "brunched" arrangement (Lucey, Munro, et al., 1998; Vasbinder et al., 2004). This "branched" texture may be the result of severe heat treatment which induces denaturation of whey proteins, that serve in forming bridges between casein micelles and preventing too dense clustering of casein micelles. Scanning electron microscopy of heated milk has demonstrated that the size of casein micelle was increased as a result of interaction with whey proteins. Moreover, the heating duration influenced the degree of interaction between whey proteins and κ-casein. At slow heating, small β-lactoglobulin aggregates penetrated hairy layer of κ-casein (Mahomud et al., 2017). Methods other than heating used to manipulate the microstructure of milk gels include altering processing conditions and addition of hydrocolloids to increase the total solids content (Lucey, 2008)**.**

1.4.2.3 Permeability and syneresis in acid milk gels

Acid milk gels are heterogenous both on the level of individual particles and their distribution. Permeability of the gel refers to inhomogeneity of gel network (Lucey & Singh, 1997). These inhomogeneities can be studied by permeability tests. Higher permeability rates signify a coarser gel with bigger pores. The coefficient of permeability, B, can be identified by the equation previously described by Roefs et al (Roefs, de Groot-Mostert et al. 1990) and Lucey et al. (Lucey, Munro et al. 1998) , as shown in **equation 1.7.**

$$\boldsymbol{B = -\left[\ln\left(\frac{h_\infty - h_{t2}}{h_\infty - h_{t1}}\right)\right]\eta H / \left[\,\rho g (t_2 - t_1)\right]} \qquad \textbf{(Eq. 1.7)}$$

Where, h_∞ is height of whey in the reference tube, h_{t1} - height of whey in tube at time t_1, h_{t2} - height of whey in tube at time t_2, η – whey viscosity, H is the length of gel, ρ is the density of whey and g - acceleration due to gravity.

Several studies have demonstrated the range of permeability for acid milk gels (formed at 30°C) to be $\sim 1–2 \times 10–13$ m^2 (Bremer et al., 1989; Peng et al., 2009). Unlike other parameters, permeability is not greatly affected by severe heating. In the study of (Lucey, Munro, et al., 1998) permeability of acid gels formed by GDL from reconstituted milk with and without heating at different temperatures for different duration of time. There was no noticeable difference in pore sizes between gels made from of heated and unheated milk. Supposedly, heating only affected the thickness and orientation of the strands. Addition of solids and their type can influence the permeability of milk gels. (Peng et al., 2009) has reported that permeability was the highest in yogurts fortified with micellar casein (MC), and the lowest when skim milk powder and milk protein isolate added yogurts. These results were explained by the fact that MC-added samples maintained the pH of 5 – 4.6 for a longer duration, which allowed the higher rate of post-gelation rearrangements.

Another important physical parameter of acid milk gels is syneresis or wheying-off, which is one of the biggest physical defects of acid milk gels. It can occur as a result of damage of the gel structure or rearrangements of casein networks after their formation (Lucey, 2017). The released liquid appears as a consequence of shrinkage and inability of the protein aggregates to entrap all the liquid phase. Syneresis is common for non-reversible structures. Equilibrium between the pressure gradient in gel network and resistance to whey expulsion define the rate and extent of syneresis (WALSTRA et al., 1985). One dimensional syneresis is related to the flow of liquid through the network, which follows Darcy's law for laminar flow through a porous medium (Lucey, 2016) (**Eq. 1.8**):

$$\boldsymbol{\upsilon = Bp/\eta\chi} \qquad \textbf{(Eq. 1.8)}$$

where ν stands for superficial flow velocity of the liquid, B - permeability coefficient, η - viscosity of the liquid, p - pressure acting on the liquid, χ the distance over which the liquid must flow.

Heating of milk reduces the degree of syneresis (Castillo et al., 2006; W. J. Lee & Lucey, 2003). Indeed, in yogurt production, heating is the main industrial strategy of improving textural structure and decreasing the degree of syneresis. According to many authors (Anema, 2019; Chandan, 2017; Y. H. Cho et al., 1999; Famelart et al., 2004) heat denaturation of whey proteins results in firmer gels as denatured whey proteins (mainly β-lactoglobulin) associate with casein micelles through κ-casein by forming hydrophobic and intermolecular disulphide bonds. Depending on the conditions of heating, other types of bonds between β-lactoglobulin and κ-casein can form. Non-covalent bonding can occur on early stages of heating at low temperatures, and hydrophobic bonds at the early stages of aggregate formation (Anema, 2019). All factors affecting wheying-off in industrial settings are listed in **Table 1.12.**

Table 1-12: Factors affecting degree of syneresis.

Factors affecting syneresis	Example
High incubation temperatures	Gels formed at 40°C compared with 20°C are coarser and more prone to spontaneous syneresis
Excessive heat treatment of the mix	≥ 85°C for 30 min
Low total solids content of the mix	skimmed milk
Containers with sloping walls or not well positioned	causes stresses on the gel which may come away from the sides and shrink
Movement or agitation during or just after gel formation	any disturbances while the gel is still weak
Rapid rate of acidification	GDL type of acidification vs. bacterial fermentation
Low acid production	pH 4.9 compared with 4.6

Other means of syneresis control are addition of stabilizers and hydrocolloids (pectin, gelatin) and increasing of total solids content (WPC, WPI, caseinates). Fat percentage can also affect the degree of syneresis with zero fat fermented milk being prone to textural and physical defects due to lower solids content (Sandoval-Castilla et al., 2004). Compensation of the latter is usually on diary ingredients, such as skim milk powder, WPCs, WPIs, and sodium caseinate. However, the full amount of these ingredients needed to obtain solids content as in full fat milk can cause excessive firmness, grainy texture and powdery taste (Torres et al., 2018).

1.4.2.4 Strategies to improve gel properties

Several methods are industrially adapted to improve gel quality and survival of probiotic bacteria, and avoid the defects, such as syneresis and weak texture. However, there is no one-size-fit-all method. Thus, dairy science is constantly working on the new answers for the old questions.

1.4.2.4.1 Heat treatment

Majority of milk gels are subjected to high temperature (>85^{o}C) heating prior to the start of fermentation. A heating of milk to 85^{o}C for 30 minutes or 90^{o}C- 95^{o}C during 5-10 minutes is compulsory in yogurt production. High temperatures induce thermal denaturation of whey proteins and dissociation of casein micelles. Consequently, whey proteins participate in gel formation. During the acidification process, denatured whey proteins associate with casein micelles through κ-casein by forming hydrophobic and intermolecular disulfide bonds (Anema, 2019). Two main benefits of these association are stronger bonds and increasing of a gelation pH . The first one can be explained by denatured whey proteins acting as supplementary bridges between caseins. As for the second one, the isoelectric point of β-lactoglobulin (pI≈ 5.2), the most abundant whey protein, that is higher than that of caseins, thus precipitation occurs earlier (Famelart et al., 2004; Goulding et al., 2019). B-lactoglobulin is more prone to heat denaturation than α-lactoalbumin. According to (Lucey & Singh, 1997) heating milk at 90^{o}C for 30 minutes resulted in denaturation of > 90% of both types of whey proteins. In this study, authors claim that preliminary exposure of milk to high temperatures resulted in increased storage modulus (G'), which indicates firmer gels. This fact was confirmed by other studies (Lucey et al., 1999; Lucey, Munro, et al., 1998; Lucey, Teo, et al.,

1997) in which analysis of microstructure has shown that unheated milk not only forms weaker gels, but also has a less regular structure, whereas gels made of heated skim milks had a branched structure.

1.4.2.4.2 High pressure homogenization

In industrial settings raw milk is subjected to homogenization in order to rupture fat globules into smaller particles. This procedure prevents fat globules from rising to the surface and acting as inert filler in milk gels, due to their protein surface membranes. High pressure homogenization (HPH) technically resembles conventional homogenization. The sole difference is that conventional homogenization is performed at <30 MPa, while HPH goes up to 400 MPa (Loveday, Sarkar et al. 2013). Homogenization pressure going up to 1000 MPa is called ultra-high-pressure homogenization. Advantages of high-pressure homogenization are partial denaturation of whey proteins and inactivation of enzymes and bacteria. According to (Serra, Trujillo et al. 2007) pressure treatment of > 200 MPa causes milk to form firmer gels in comparison with high temperature treatment (90°C for 90s). This effect is attributed to denaturation of whey proteins and enhanced protein-protein and fat-protein interactions. Also, the reduction of size of protein particles and fat globules significantly affects the gel texture. Smaller fat globules tend to better incorporate into protein networks. In addition to that, pressure-induced partial denaturation of proteins liberates peptides and free amino acids that can serve as a nitrogen source for probiotic bacteria and enhance their survival and metabolism in fermented milk. HPH treatment was reported to cause an increase in production of lactic and acetic acids, and improve aromatic profile (R. Massoud, 2016). Ubiquitous application of this method is hindered by high costs of the equipment.

1.4.2.4.3 Ultrasonic processing

Ultrasonic processing is a relatively new technology that permits to destroy microorganisms and disrupt particles in a solution. It utilizes a high-intensity (10-1000 $W \cdot cm^{-2}$) sound waves at the frequencies of >18 kHz (Loveday et al., 2013). (Madadlou et al., 2010) studied rheological properties of casein acid gels formed from milk sonicated at 24 and 130 kHz for 0, 60 and 120 minutes. As the effect of sonication, gel formation was retarded. The structure of the gel was also distinct from control: firm gels, stronger connections and small particles. The most pronounced influence was noticed in samples sonicated for 120 minutes. This effect

is possibly attributed to whey protein denaturation and disruption of casein micelle (Lucey, 2008; Madadlou et al., 2010).

However, this method has its side issues. One of them is formation of free radicals. Local high temperatures generated at the time of bubble collapse cause the formation of highly reactive radicals (Zisu et al., 2010). Measures need to be taken to avoid oxidation of lipids and other susceptible components. Another disadvantage is complementary heating that occurs under ultrasonic processing conditions. These drawbacks can be minimized by sonication at frequencies as low as 20 KHz and application of radical scavengers (Ashokkumar et al., 2008). Moreover, ultrasonication causes rubbery and burned off-flavors in milk products, due to emissions of volatile compounds such as benzene, toluene, 1,3-butadiene, 5-methyl-1,3-cyclopentadiene, 1-hexene, 1-octene, 1-nonene, p-xylene, n-hexanal, n-heptanal, 2-butanone, acetone, dimethylsulfide and chlorophorm (Sfakianakis et al., 2015).

1.4.2.4.4 Transglutaminase (TGase)

Microbial transglutaminase is an enzyme that is used in dairy industry to improve food texture characteristics, such as viscosity, firmness, elasticity and degree of syneresis and prolong storage time (Kieliszek & Misiewicz, 2014; Lorenzen et al., 2002; Loveday et al., 2013). It catalyzes the formation of isopeptide bonds between proteins. In milk, caseins are more susceptible to cross-linking than whey proteins, due to their open and flexible structure with its readily accessible lysine and glutamine residues (Ardelean et al., 2013; Kieliszek & Misiewicz, 2014; Loveday et al., 2013). (Loveday et al., 2013) outlined two important applications of TGase in yoghurt manufacture: TGase treatment of milk prior to fermentation, or simultaneous supplementation of TGase and starter cultures, where crosslinking occurs during acidification. Treatment of milk with TGase prolongs the time of fermentation, while simultaneous fermentation by starter culture without inactivation of the enzyme does not cause any changes in fermentation time (Lorenzen et al., 2002). It is explained by the fact that when added concomitantly, starter bacteria have enough time to supply themselves with necessary amino acids before cross-linking takes place (Jaros et al., 2006). Unavailability of nitrogen sources due to formation of high-molecular-weight polymers may hinder survival of probiotic bacteria in this type of yogurts.

1.4.2.4.5 Fortification by dairy powders

In yogurt production, low total solids content can impair the gel texture and induce higher whey separation (Peng et al., 2009). Increasing a non-fat solids (SNF) content improves the firmness and viscosity of milk protein gels (Lucey, 2008). The SNF can be conventionally increased by their concentration by evaporation, ultrafiltration or reverse osmosis or fortification by whey protein concentrates, skim milk powder, Na and Ca caseinates, milk protein concentrates (Karam et al., 2013). In the study of (Damin et al., 2009) addition of sodium caseinate reduced the fermentation time of yogurts on a concentration-dependent manner. Supplementation of sodium caseinate has also significantly improved the firmness of the gel, while addition of SMP and WPC did not yield significant results.

Fortification by whey proteins coupled with heat treatment causes a high level of cross-linking in the acid milk gel resulting in higher viscosity and less syneresis. However, several authors suggest that association of whey proteins and κ-casein make the micelles more soluble and less prone to aggregation (Karam et al., 2013). A less expensive WPC is used more often than WPI, as in spite of higher protein contents of the latter, WPC provides the results comparable to WPI. The supplementation of whey proteins is also favorable due to their nutritional and functional properties.

Addition of SMP is a common practice in yogurt industry. It is preferred over whole milk powder (WMP), due to the oxidized taste caused by WMP. The rates of SMP added in yogurt range between 1-6%, with recommended amounts of 2-3%. This procedure gives yogurt its desirable characteristics such as viscosity and texture. Nonetheless, the use of SMP for increasing the dry matter can cause excessive acidity, due to high lactose content of the powder (Karam et al., 2013).

1.4.2.4.6 Exopolysaccharide Producing Cultures

Some starter culture bacteria have an ability to produce heteropolysaccharides called exopolysaccharides (EPS) contributing to the texture and water retention capacity of fermented milk gels (Amatayakul et al., 2006; Loveday et al., 2013). In dairy industry, EPS is a promising technique for improving the texture of the products. (Hassan et al., 2003) studied microstructure of acid gels made of EPS+ and EPS- Streptococcus thermophilus strains.

CLSM microscopy images revealed that EPS were situated in pores and not interacted with the proteins making up the network. The EPS containing gel microstructure was characterized by larger pores and thicker protein aggregate strands. EPS produced in a fermented milk can vary in the range 45-600 mg L^{-1} depending the bacterial strain and process temperature. Majority of dairy-used strains produce EPSs containing glucose, galactose, rhamnose, N-acetyl-galactosamine, N-acetyl-glucosamine (Mende et al., 2013). Two types of EPS produced by lactic acid bacteria are recognized: capsular layer enrobing the bacteria and "ropy" EPS (or free EPS) secreted to the environment (Lucey, 2008). Some strains are known to produce both types of EPS. Purification of microbial EPS and addition to GDL-induced milk gel has revealed that only ropy EPS contributed to the stiffness of the gel on concentration-dependent manner (Mende et al., 2013). Capsular EPS were not able to improve yogurt texture, however influenced sensory characteristics of the yogurt, improving acetaldehyde flavor (Guzel-Seydim et al., 2005). The effect of EPS on gel depends on whether they are integrated in gel networks with the proteins or occupy the pores of the networks. (Zhang & Zhang, 2012) hypothesized that as EPS are released to the gel gradually during fermentation, the balance of repulsive/attractive forces with milk proteins also vary with the time. Other factors influencing of EPS effective role as a thickening is the molecular mass and polymer stiffness of the EPS (Mende et al., 2013; Zhang & Zhang, 2012), as these parameters directly influence a water holding capacity of these hydrocolloids. Application of EPS-producing strains requires thorough investigation of various strains and their technological applicability in fermented dairy products.

CHAPTER 2: Research problematic, hypothesis and objectives

2.1. Research problematic

The scientific literature has clearly outlined that whey processing is a huge challenge because of its specific composition and high water content. Moreover, it represents one of the biggest environmental problems of the dairy industry. The massive amounts of whey generated each year require suitable and economically feasible treatments. Existing methods of whey valorization are far from offering a permanent solution. Thus, the industries are continuously seeking sustainable and efficient methods for turning whey into a valuable co-product that can be used as an ingredient in the food industry. Recently, electro-activation of whey was demonstrated as an efficient method to convert part of lactose into lactulose which is a recognized prebiotic. This treatment allows *in situ* reagentless lactulose formation by using whey without any pre-treatment of fractionation. Thus, electro-activated whey was suggested to be used as a lactulose-enriched functional food ingredient. Demand for the development of novel products with health-promoting properties is fueled by consumers worldwide. In this regard, fermented dairy products could benefit from this new development, as they are one of the most popular food matrices for functional ingredients. In this context, electro-activated whey can be used as ingredient in the development of novel dairy products, particularly beverages, which can serve as efficient vectors of lactulose for human nutrition. Moreover, to this day, no studies were performed on the behavior of electro-activated whey in fermented milk products.

2.2. Hypothesis

Considering the potential health-promoting, prebiotic and antioxidant properties of electro-activated whey, it can be used as a functional ingredient and source of lactulose in the production of novel fermented dairy products. As whey is naturally compatible with milk products, it has been hypothesized that it is technologically feasible to use electro-activated whey in the process of fermented dairy products production as a functional ingredient with a prebiotic and antioxidant effect.

2.3. General objective

The main objective of this research work was to study and to demonstrate the technological feasibility of using electro-activated whey as a functional ingredient and vector of lactulose in the process of fermented dairy products production and to understand its effect on different physico-chemical, textural, and microbiological parameters of the final products.

2.4. Specific objectives

To achieve the general objective of this project, four specific objectives were studied:

Specific objective # 1: To study the technological feasibility of the addition of electro-activated whey to milk in the process of fermented milk production and to study its physico-chemical properties by using a commercial starter culture composed of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* as the fermenting agent.

Specific objective # 2: To study the technological feasibility of using electro-activated whey as ingredient and source of lactulose in the technological process of kefir production and to evaluate the properties of the final product.

Specific objective # 3: To study the technological feasibility of using electro-activated whey as ingredient and source of lactulose in the technological process of Ryazheenka production, a popular traditional Russian-Ukrainian dairy beverage made from baked milk, and to evaluate the properties of the final product by using *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* as the starter culture.

Specific objective # 4: To study the impact of electro-activated whey on the growth of lactic acid bacteria with known probiotic effect.

CHAPTER 3: Use of electro- activated whey as ingredient in fermented milk production: proof of the concept of the technological feasibility

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RÉSUMÉ

Il a été signalé précédemment que l'électro-activation alcaline du lactosérum permettait de convertir 25 à 45 % du lactose en lactulose. La neutralisation après l'électro-activation n'a pas entraîné la perte du rendement en lactulose. Le lactulose est un prébiotique reconnu et est utilisé comme additif dans les produits laitiers fermentés. L'objectif de ce travail était d'étudier le comportement du lactosérum électro-activé utilisé comme ingrédient dans un lait fermenté. Pour réaliser cette étude, avant la fermentation, du lait (0, 1, 2 et 3% de fait) a été supplémenté avec du lactosérum électro-activé à 3, 6 et 9%. Différentes analyses ont été réalisées telles que la cinétique d'acidification, les paramètres texturaux et rhéologiques, la synérèse, la production d'acides organiques, les paramètres sensoriels et microbiologiques ont été évalués. De plus, l'effet du lactosérum électro-activé sur la microstructure du lait fermenté a été analysé par microscopie électronique à balayage. Les résultats obtenus ont montré que l'ajout de lactosérum électro-activé a prolongé le temps de fermentation. Les échantillons avec du lactosérum électro-activé avaient une microstructure plus homogène et moins poreuse et étaient caractérisés par une synérèse significativement réduite par rapport au lait ou au lait supplémenté en lactosérum. La présence de lactosérum électro-activé n'a pas entravé la croissance bactérienne et a déclenché une production supérieure d'acides organiques tels que les acides lactique, acétique, citrique, propionique et butyrique. L'analyse du lactulose résiduel pendant le stockage réfrigéré a montré qu'environ 50 % du lactulose restait dans le lait fermenté, ce qui suggère que la consommation de ce produit fournira une quantité suffisante de lactulose pour atteindre le côlon de l'hôte. Enfin, cette étude a clairement démontré la faisabilité de l'utilisation du lactosérum électro-activé comme ingrédient contenant du lactulose dans la fabrication de lait fermenté.

Mots clés: Lactosérum électro-activé; Ingrédient; Valeur ajoutée; Lait fermenté; Lactulose; Prébiotique.

ABSTRACT

Alkaline electro-activation of whey was previously reported to convert 25-45% of lactose into lactulose. Neutralization after electro-activation did not cause the loss of the lactulose yield. Lactulose is an established prebiotic and is used as an additive in fermented dairy products. The aim of this work was to study the behaviour of electro-activated whey used as an ingredient in a fermented milk. To carry out this study, prior to fermentation, milk (0, 1, 2 and 3% fact) was supplemented with 3, 6 and 9% electro-activated whey. Different analyses were performed such as acidification kinetics, textural, and rheological, syneresis, organic acids production, sensorial, and microbiological parameters were assessed. Furthermore, effect of electro-activated whey on the fermented milk microstructure was analyzed by scanning electron microscopy. The obtained results showed that addition of electro-activated whey prolonged time of fermentation. Samples with electro-activated whey had more homogenous and less porous microstructure and were characterized by a significantly reduced syneresis compared to milk or whey supplemented milk. Presence of electro-activated whey did not hinder bacterial growth and triggered superior production of organic acids such as lactic, acetic, citric, propionic and butyric acids. Analysis of residual lactulose during refrigerated storage showed that approximately 50% of lactulose remained in the fermented milk, suggesting that consumption of such product will provide sufficient amount of lactulose reaching the host colon. Finally, this study clearly demonstrated the feasibility of using electro-activated whey as a lactulose-containing ingredient in fermented milk manufacturing.

Keywords: Electro-activated whey; Ingredient; Added value; Fermented milk; Lactulose; Prebiotic.

3.1. INTRODUCTION

Preservation of milk by fermentation traces back to 7000 BC (Tamime, 2002). Early milk fermentation occurred spontaneously, as an effect of chymosin when milk was placed to a dish made from an animal stomach. Nowadays, the industrial production of fermented dairy products is well established and develops apace. At the same time, the growth of the market, constant development of new products, longer destinations for transportation and periods of storing challenges dairy products manufacturers to seek for better practices of keeping acceptability of textural and sensorial properties and consumer-appealing appearance of the products such as the absence of syneresis, as well as maintaining the number of beneficial bacteria ensuring claimed health benefits (Mattila-Sandholm, Myllärinen, Crittenden, Mogensen, Fondén, & Saarela, 2002). Other challenges are imposed by consumer preference shift towards healthy products, all-natural diets and environmentally friendly production practices, including the use of secondary resources such as whey in the case of dairy products.

Solutions to improve textural properties and appearance of fermented dairy products include increase of total dry matter content, heat denaturation of whey proteins to improve the water holding capacity of the proteins matrix, high pressure homogenization, use of microbial enzymes as well as special starter cultures producing exopolysaccharides with high texturizing capacity, and addition of hydrophilic hydrocolloids (Loveday, Sarkar, & Singh, 2013; Lucey, 2019). In many fermented dairy products, addition of hydrocolloids or thickening agents such as gelatin, carrageenan and starch are prevalent, however they do not correspond to the all-dairy composition of the final product. Moreover, some of them may meet restrictions as halal, kosher, or organic composition of the product (Salehi, 2021). The supplementation of whey proteins to achieve higher total solid content is another common practice which also provides nutritional and functional value to the fermented dairy products (Karam, Gaiani, Hosri, Burgain, & Scher, 2013). The other used strategy to improve the nutritional/functional value as well as the overall consumer perception of dairy products consists of using prebiotics as functional ingredients in the formulation of fermented dairy products to maintain the survival of probiotic bacteria as well as to contribute to a healthy host microbiota since prebiotics do not undergo hydrolysis until reaching the colon where they are metabolized by intestinal microbiota, especially probiotics such as lactic acid bacteria

and bifidobacteria. However, it is also important to mention that addition of some prebiotics can influence sensory attributes and physico-chemical properties of the fermented milk (Heydari, Amiri-Rigi, Ehsani, Mohammadifar, Khorshidian, Koushki, et al., 2018). The main prebiotic substances used in dairy products are fructo-oligosaccharides (FOS), soybean oligosaccharides (SOS), galactooligosaccharides (GOS), isomalto-oligosaccharides (IMO), xylo-oligosaccharides (XOS), inulin, and lactulose.

Recent studies have reported electro-activation as a new and highly promising method of a prebiotic lactulose production from whey, a secondary resource material of the cheese and casein industry (Kareb, Champagne, & Aïder, 2016a, 2016b). The technology of electro-activation suggests subjecting aqueous solutions to the effect of an external electric current in a modulated reactor by anion and cation exchange membranes, in order to exploit the solution alkalinity created at the cathode-solution interface following water electrolysis (Aider, Gnatko, Benali, Plutakhin, & Kastyuchik, 2012). This technology demonstrated vast applicability as alternative for protein extraction and functionalities modifications as well as lactose electro-isomerization into lactulose *in situ* from whey or whey permeate. Several studies have reported that electro-activation of whey leads to isomerization of significant proportion (25-45%) of lactose into lactulose, which is a well-established prebiotic (Akhtar, Mondor, & Aïder, 2018; Djouab & Aïder, 2019; Kareb, Champagne, & Aïder, 2016a). Kareb et al. (2018) obtained higher rates of probiotic bacteria growth when media was supplemented with electro-activated whey in comparison with untreated whey, lactulose, lactose, sucrose, glucose, and galactose. This bifidogenic effect was attributed to the lactulose-rich content of electro-activated whey, as well as the ability of electro-activated whey to protect bacterial membranes from lipid oxidation because the electro-activation process occurred under highly reducing conditions created in the cathodic compartment of the electro-activation reactor and maintained by the appropriate disposition of the cation exchange membrane facing the cathode. Moreover, based on their numerous results, the authors suggested electro-activated whey as a new functional dairy ingredient with a dual prebiotic and antioxidant potential (Kareb, Champagne, Jean, Gomaa, & Aïder, 2018).

Thus, the aim of this work was to study the behaviour of electro-activated whey used as an ingredient in a fermented milk. To carry out this study, prior to fermentation, milk (0,

1, 2 and 3% fact) was supplemented with 3, 6 and 9% electro-activated whey. Different analyses were performed such as acidification kinetics, textural, and rheological, syneresis, organic acids production, sensorial, and microbiological parameters were assessed. Furthermore, effect of electro-activated whey on the fermented milk microstructure was analyzed by scanning electron microscopy.

3.2.MATERIALS AND METHODS

3.2.1. Preparation of electro-activated whey powder

A 10% (w/w) cheese whey solution prepared from reconstituted commercial whey powder (Agropur Cooperative, Quebec, Canada) was used to prepare electro-activated whey. A schematic representation of electro-activation reactor is given in **Figure 3.1**. The compartments of the electro-activated reactor were filled as follows: whey solution was poured in cathodic compartment, 0.25 M K_2SO_4 solution in the middle and 0.1 M K_2SO_4 solution in the anodic compartments. The middle compartment was separated from the anodic and cathodic compartments by anion and cation exchange membranes, respectively. This reactor configuration allowed a creation of highly alkaline and reducing conditions in the cathodic compartments while inference of the anodic reactions was avoided. The electro-activation operating conditions were as follows: electric current intensity of 800 mA and electro-activation time of 60 min. After 60 min electro-activation, the whey solution was left for 48 h for relaxation. After that, the solution was neutralized to pH 7 by using 1 N HCl solution and then lyophilized. The lyophilized electro-activated powder was used for milk supplementation in the process of fermented milk production and studies.

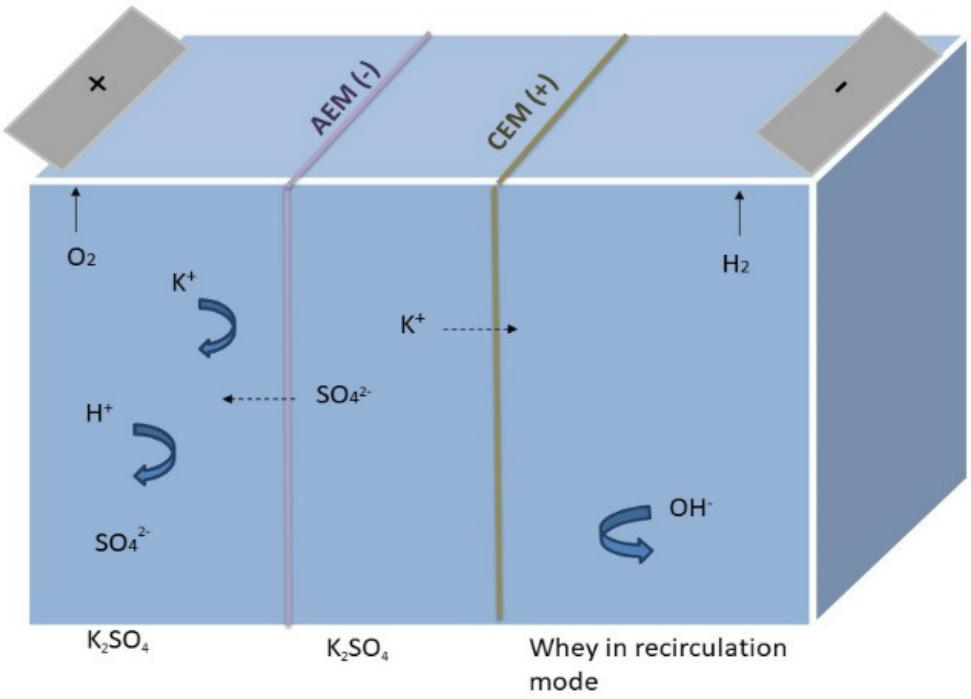


Figure 0-1: Schematic representation of the used configuration of the electro-activation reactor for whey electro-activation. AEM: Anion exchange membrane. CEM: Cation exchange membrane. (+): Anode. (-): Cathode.

3.2.2. Preparation of fermented milk

Commercial pasteurized milk of 0, 1, 2 and 3% fat content (Quebon, Quebec, Canada) was used. Commercial thermophilic starter culture Fromagex (Fromagex, Quebec, Canada) composed of a mixture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* was used as indicated by the manufacturer at an amount of 0.60 g/L of milk. Powders of whey and electro-activated whey were added to a pre-heated milk at a temperature of 42 ± 1 °C prior to the fermentation process at 3, 6 and 9% (w/w) as milk replacement. After adding the starter culture, each sample was poured into several 50 mL tubes and put in a temperature-controlled incubator (Jeio tech, MA, USA) set at 42 °C. To monitor the fermentation kinetics and process, each hour three tubes were analyzed.

3.2.3. Monitoring of the fermentation process

The pH of the fermenting samples was monitored by a pH-meter (Oakton pH 700) equipped with an electrode Oakton (Vernon Hills, IL). The corresponding titratable acidity was measured by using the titration method AOAC 947.05 (AOAC, 1990). Results of titratable acidity were interpreted in Dornic degrees which corresponds to 1 ml 0.1 N NaOH and represents the amount of 0.1 N NaOH solution needed for neutralization of milk product diluted in double amount distilled water or 100 g of product. The measurements were taken once per hour. Directly after reaching pH of 4.6-4.7, fermented milk samples were cooled to 4 °C and stored for 21 days. The pH and acidity measurements were monitored every 3 days for 21 days to measure the fermented samples post-acidification as a function of the production conditions.

3.2.4. Texture profile analysis

Fermentation of the samples was performed in containers with a diameter of 45 mm and analysed on a texture analyser TAxT2 (Texture technologies, Hamilton, USA). Compression test was performed with a cylinder 25 mm in diameter and 35 mm in height. The operation speed was 90 mm/min and the distance covered in the sample was 15 mm. Tested parameters were the firmness (g) indicating the force necessary to cause deformation, and adhesiveness (g.sec) which is the work of penetration of a cylinder for overcoming the force of attraction with the sample.

3.2.5. Rheological measurements

Milk gel formation was monitored by Ares G2-101 rheometer (TA Instruments, New Castle, DE, USA) by using the followings parameters: DIN geometry, cup 29.99 mm and cylinder 27.679 mm diameter, loading gap 140 mm. A 27 mL sample was introduced into the cups. Silicon oil was used to prevent evaporation. Oscillation frequency of 1 Hz and an applied strain 0.01 to 100% were used. The strain was chosen within the linear viscoelastic range of this type of gel network that was monitored experimentally. Measurements were taken from 10 points per decade. Parameters as loss modulus, storage modulus, and tangent of loss angle were measured. Storage modulus (G') higher than 1 Pa was taken as a point of the beginning of gel formation.

3.2.6. Produced syneresis measurement

Syneresis (free whey separation) was analyzed in accordance with reported method by Gauche et al. (2009) (Gauche, Tomazi, Barreto, Ogliari, & Bordignon-Luiz, 2009). A sample of 15 g of each fermented milk was weighted on analytical balance and centrifuged at 350xg for 10 min at 6 °C. Separated whey was weighted and total syneresis rate was calculated by using the following **equation 3.1**:

$$Syneresis = \left(\frac{mass\ of\ whey\ separated\ after\ centrifugation}{total\ mass\ of\ the\ sample}\right) * 100\% \text{ (Eq. 3-1)}$$

3.2.7. Microstructure analysis by scanning electron microscopy

A 10% (v/v) formaldehyde solution was used to fix the samples. After, fixed samples were washed with 0.1 M sodium cacodylate buffer (pH 7.3) for 20 min three times. The next steps were post-fixation with 1% osmium tetroxide for 90 min; second washing and dehydration in graded ethanol (50, 70, 95, and 100%) for 20 min each; soaking two times in hexamethyldisilazane for 20 min; and drying. Samples were metallized in a sputter coater and observed at 15 kV (Jeol JSM-6360LV Jeol Ltd., Tokyo, Japan).

3.2.8. Volatile aromatic compounds

Aromatic profile was evaluated by measuring the volatile aromatic compounds of the fermented milk samples by using the gas chromatographer Agilent G1530A and a mass spectrometer Agilent mass selective detector 5973 with multi-purpose sampler (Agilent technologies, USA). The GC Column DB wax 122-7062 was used at a temperature of 250 °C. Total flow 8.7 ml/min, split ratio 5:1, split flow 5.0 ml/min, injection 0.5µl, constant flow mode of 1 ml/min were used as the operating conditions.

3.2.9. Organic acids

Organic acids content was determined by high performance liquid chromatography (HPLC) by a Hitachi 7000 HPLC system series equipped with a UV detector set at 220 nm. The organic acids were separated on a Phenomenex Kinetex® 2.6 µm PS C18 Column (100 LC, 100 × 4.6 mm). A 20 mM potassium phosphate KH_2PO_4 solution at pH 1.59 was used as a mobile phase. The following operating parameters were used: flow rate of 0.3 mL/min, column temperature set at 30 °C, total elution time of 30 min; injected sample volume of 50 µL.

3.2.10. Total bacterial count

A sample of 1 mL of the fermented milk was diluted until 10^{-7} in peptone water. Dilutions were plated on MRS agar by surface distribution in a volume of 100 µl. Total bacterial counts of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* were calculated after 48 h incubation in anaerobic conditions at 37 °C.

3.2.11. Sensory analysis

Sensory analysis was performed by semi-trained panel. 20 panelists received a brief training on terms and traits needed to be described. Samples were warmed up to the room temperature. Each of 6 samples given to a panelist was assigned a random three-digit number. Following criteria were tested: appearance, taste, texture, and aftertaste. Samples were prepared from zero fat and 3% fat milk, with added 9% whey (w/w), 9% electro-activated whey (w/w) and milk without any additives served as a control. Each characteristic needed to be given a score from 0 to 10 based on the strength of appearance of the characteristic in the sample. 0: Not pronounced, 10: Very pronounced.

3.2.12. Statistical analysis

Analysis of the variance (completely randomised ANOVA) was performed by using SAS software (Version 9.1, SAS Institute, Cary, NC, USA). Samples were prepared considering following independent variables: milk fat, type of whey and whey concentration. Milk of each fat percentage without any additives served as control. All the samples were subjected to the analyses in thrice. In total 84 samples were tested for fermentation process (pH, titratable acidity), texture, post acidification, syneresis. Selected samples were subjected to rheological, sensorial, microbiological analyses, and microscopy. Mean values and standard error were used for comparisons. The Tukey's HSD test was used to compare means with 5% as significance level.

3.3. RESULTS AND DISCUSSION

3.3.1. Fermentation process

Fermentation process of dairy products represents a gradual drop of pH influenced principally by lactic acid production by starter cultures. However, other organic acids such as acetic, citric, and propionic are also produced. At pH 4.6 casein reaches its isoelectric point, undergo structural denaturation and precipitates; forming three-dimensional networks which captivate water, dispersing media, fat globules and other milk components (Lucey, 2017). The **Figure 3.2** depicts time-dependent patterns of pH changes during the fermentation process of defatted and 3% fat milk with an addition of whey and electro-activated whey at different concentrations. All the samples started at the point around the standard pH of milk 6.4-6.6. Duration of the fermentation process of the control samples was as predicted by the manufacturer of the starter culture (5-6 h). Addition of electro-activated whey slowed down the fermentation process to 2, 4 and 10 h for 3%, 6% and 9% concentration of electro-activated whey, respectively. These results of prolonged fermentation time at addition of lactulose-rich electro-activated whey are contrary to the study reported by Ben Moussa et al. (2019) where addition of 1.5% of lactulose to yogurt formulation induced a fermentation duration of 4 h, with a control sample being at 5 h (Ben Moussa, Boulares, Chouaibi, Mzoughi, & Hassouna, 2019). Other researchers have also obtained slight decrease in fermentation duration in yogurts fortified with different components, including moringa extract (Zhang, Jeong, Cheng, Bae, Seo, Petriello, et al., 2019), ulvan which is a cell wall polysaccharide (Shalaby M & Amin H, 2019) and chickpea (Chen, Singh, Bhargava, & Ramanathan, 2018). These differences can be attributed to different factors such as the type of the fermenting strains and the composition and conditions of the fermentation media.

In the present study, the delayed pH drop is argued to be a consequence of higher buffering capacity of the used electro-activated whey. In dairy products, buffering capacity is stemmed from the sum of compounds containing one or several acid–base groups. The compounds contributing to the buffering capacity of milk are salts, proteins, and organic acids (Salaün, Mietton, & Gaucheron, 2005) which only partially dissociate in aqueous media. Kim et al. (2018) have studied high-buffering yogurt prepared with addition of phosphates (Kim, Oh, & Imm, 2018). As a result, the high-buffering yogurt showed a higher water holding capacity,

more homogenous, less porous microstructure, and prolonged fermentation time. Kareb et al. (2016) investigated physical and chemical properties of electro-activated whey and reported an improved buffering capacity and higher negative redox potential (Kareb, Champagne, & Aïder, 2016). In this study, we performed a check fermentation study with 9% demineralized electro-activated whey. The obtained results showed that the removal of the salts reduced the time of fermentation from 16 to 12 h (data not shown). This result was correlated with the electro-activated whey buffering capacity.

Titratable acidity measurements represent the total amount of protons coming from undissociated acids and free ones while pH is only an indicator of free H^+ ions concentration. In this regard, the results of titratable acidity varied from pH measurements (**Figure 3.3**). The results shown in **Figure 3.3** depict that samples with electro-activated whey had the levels of free acids comparable with those of controls. However, they were unable to drop the pH index and form a solid gel. This data also confirms a hypothesis of higher buffering capacity of electro-activated whey achieved at around pH 5.1. It is commonly accepted that lowering pH causes a decrease of net negative charge of the casein micelles. Colloidal calcium phosphate responsible for casein micelle integrity is completely dissolved at pH 5.1, whereas re-aggregation of the proteins in a network requires achieving the isoelectric point which at pH 4.6 for casein micelles (Lucey, 2017).

Even though it is apparent that fermentation time is a parameter of a great technological and economical importance, attempts to shorten the fermentation duration should not come at an expense of the gel and product quality. It was reported that a rapid fermentation causes an early dissolution of colloidal calcium phosphate and release of individual casein particles. This leads to formation of a premature gel with weaker bonds and bigger pores (Sah, Vasiljevic, McKechnie, & Donkor, 2016), which in its turn affect the ability of the protein network to hold water and by the way accelerates the syneresis phenomenon which presents a serious problem of the product quality and consumers acceptance. Thus, a realistic balance between the fermentation time and physical and quality parameters of the gel and the final product must be achieved adequately.

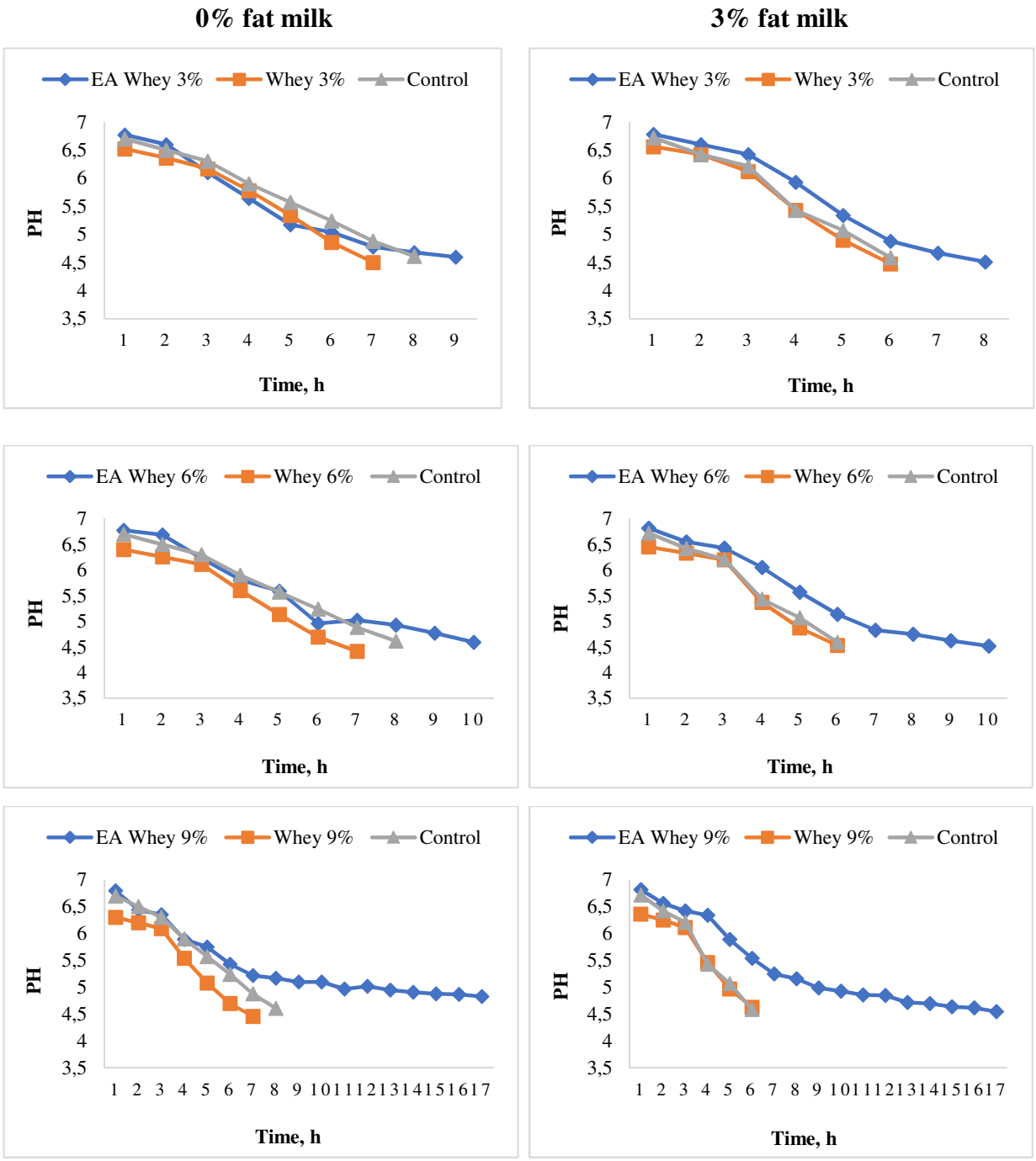


Figure 0-2: Evolution of pH during the fermentation process of defatted (0%) and 3% fat milk containing 3% (A), 6% (B) and 9% (C) of electro-activated whey (EA Whey), original (non-electro-activated) whey, and control sample (milk without any supplementation).

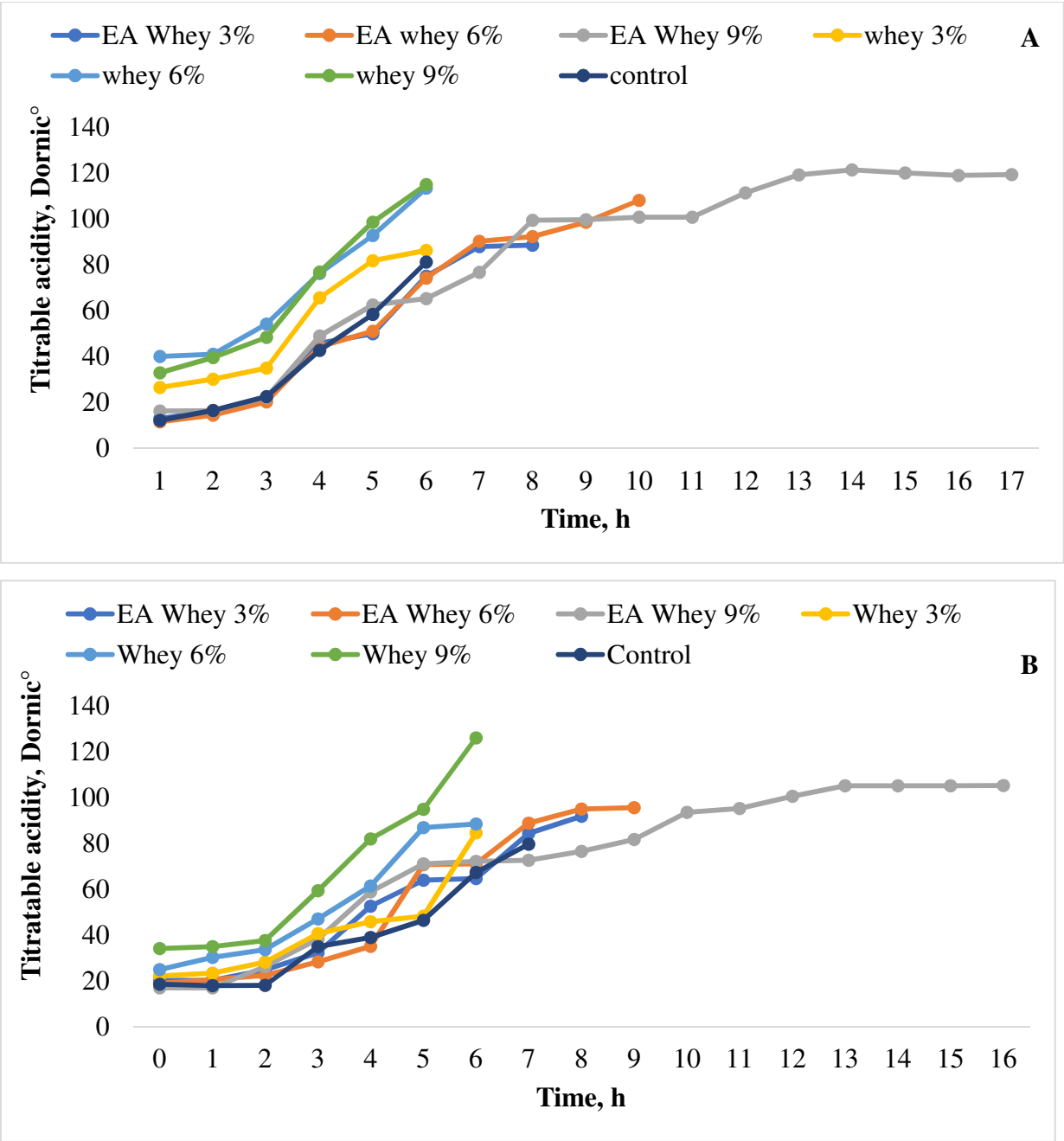


Figure 0-3: Titratable acidity of 3% fat (A) and 0% fat (B) milk during fermentation process. EA Whey: Electro-activated whey.

3.3.2. Rheological and textural properties

From a hydrodynamic point of view, as an effect of gelation, milk transforms from Newtonian fluid to a semi-solid form (Lucey, 2017). **Figure 3.4** represents the rheological properties of the fermented milk samples with whey and electro-activated whey added at 9% supplementation level. Gelation was taken as G' reaching 1 Pa. All samples formed the gel. Time of initiation of gel formation for samples supplemented with 9% whey was 3.7-3.8 h and for samples with 9% electro-activated whey it was 10.50-11 h. These results are in good agreement with the already obtained results for the fermentation process monitoring regarding pH evolution of the different samples supplemented with whey and electro-activated whey (EAW). Final storage modulus for 9% EAW supplemented milk sample was 18.6 Pa and for 9% whey added sample it was 19.94 Pa in a 3% fat milk. In the case where skimmed milk was used, G' of the sample with EAW was 2 times lower than samples with whey, showing its lower ability to form a gel.

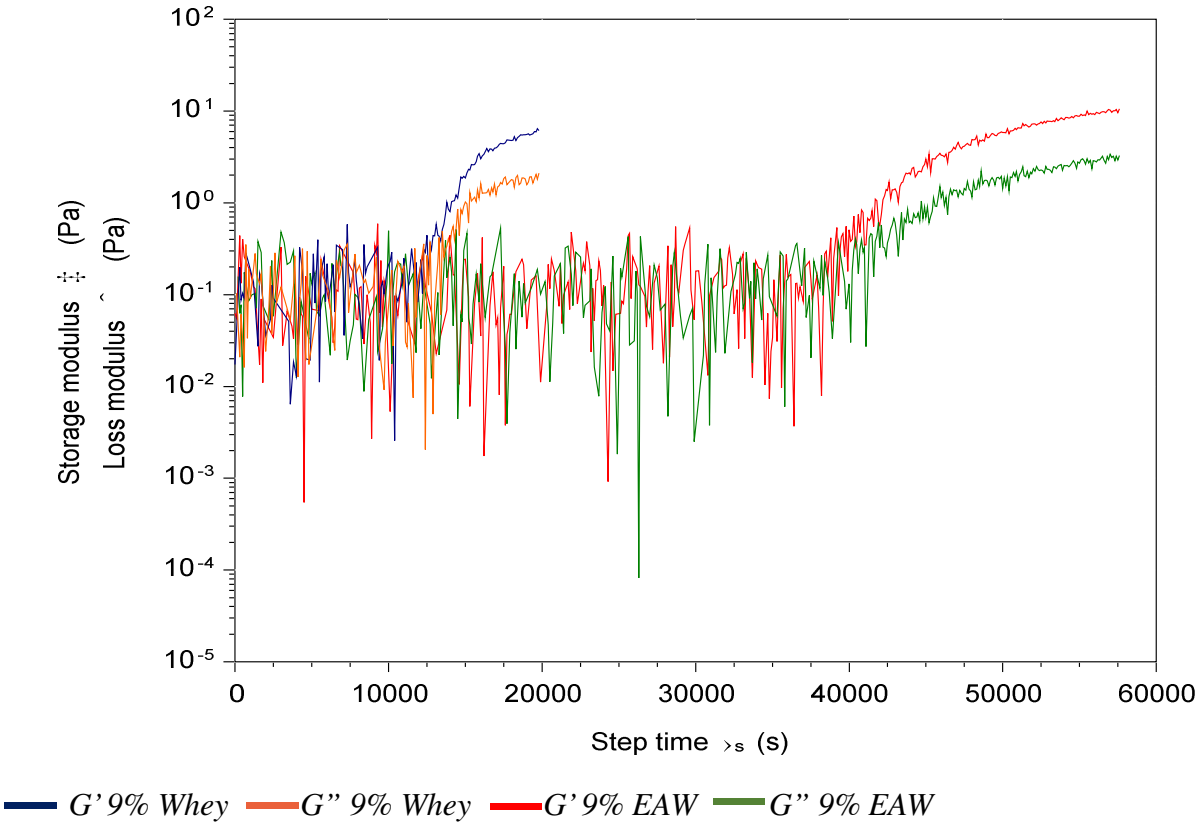


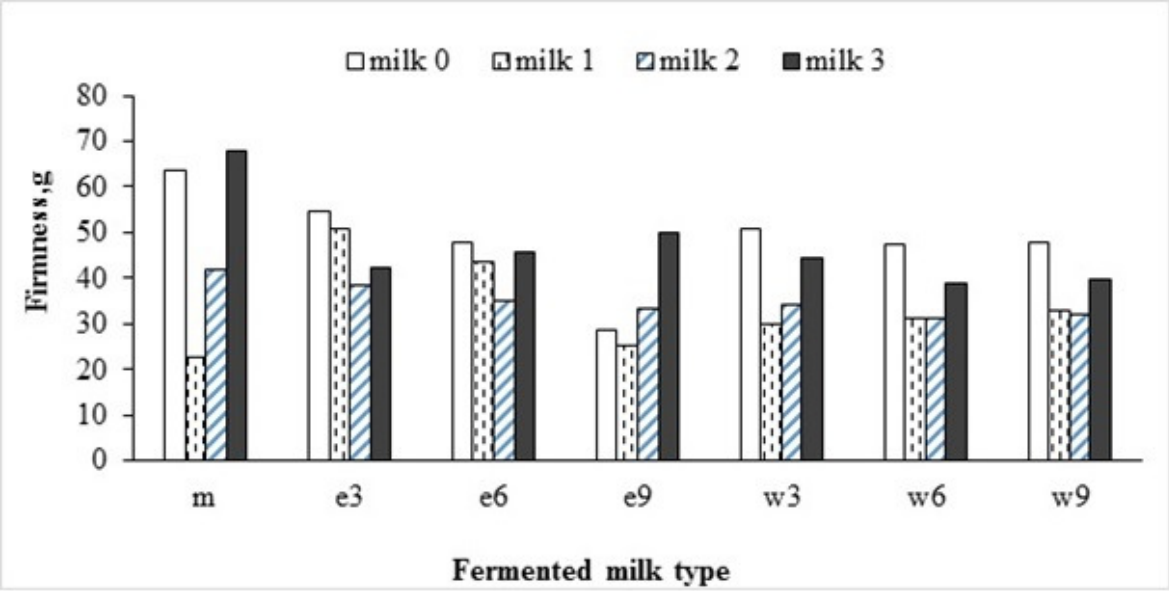
Figure 0-4: Rheological properties G', G" of fermented 3% fat milk supplemented with 9%

of non-electro-activated whey and of 9% electro-activated whey.

Structural parameters were tested using penetration tests and the results of textural properties of fermented milk samples with whey and electro-activated whey are shown in **Figure 3.5**. It should be noted that high levels of firmess and adhessiveness in skim milk control samples (without supplementation) is due to the significant separation of liquid phase, leaving a denser gel with shortened filaments. Fat content did not significantly affect the texture in all samples with electro-activated whey. The effect of addition of whey is more pronounced in 3%-fat milk with the highest 68 ± 1 g in the control and 50 ± 5 in 9% electro-activated whey added samples, and the lowest 39.5 ± 4 g for 9% whey added samples. However, only the control samples had significantly higher firmness which was attributeed to higher syneresis. Similar tendency was observed for adhesiveness in control sample reaching 537 ± 1 g.sec. Addition of 9% electro-activated whey resulted in higher adhessiveness 379 ± 66 g.sec in comparison with 9% whey 292 ± 31 in samples made from 3% fat milk, which indicates slightly higher attraction between the particles in the matrix.

Formation of Maillard reaction products in electro-activated whey could be contributed to textural properties of the samples. Sun et al. (2011) obtained less stiff gels as a result of WPI-dextran conjugation by Maillard reaction (Sun, Yu, Yang, Wang, Zhang, Zhang, et al., 2011). The authors explained the result by the fact that vast hydrophilic parts of polysaccharides reduce hydrophobic interactions between proteins and that high molecular sugars hinder the interactions of neighboring proteins. The emulsion stabilizing effect of Millard reaction products was also previously reported (Wooster & Augustin, 2006). Usually texture of milk gels is enhanced by addition of whey proteins concentrate or caseinate (Akalin, Unal, Dinkci, & Hayaloglu, 2012). It was reported that fortification with skimmed milk powder did not have similar effect, due to low protein content. In our study, whey powder is much lower in protein content than protein isolates. Thus, more significant effect on gel texture would not be expected. Whey proteins were studied to contribute to the texture in their heat-denatured form. According to many authors, heat denaturation of whey proteins results in firmer gels as denatured whey proteins associate with casein micelles through κ-casein by forming hydrophobic and intermolecular disulphide bonds (Brodkorb, Croguennec, Bouhallab, & Kehoe, 2016;

Lee & Lucey, 2010), whereas native whey proteins did not contribute to the texture (Blecker, Habib-Jiwan, & Karoui, 2012). In this study whey was not subjected to heating.



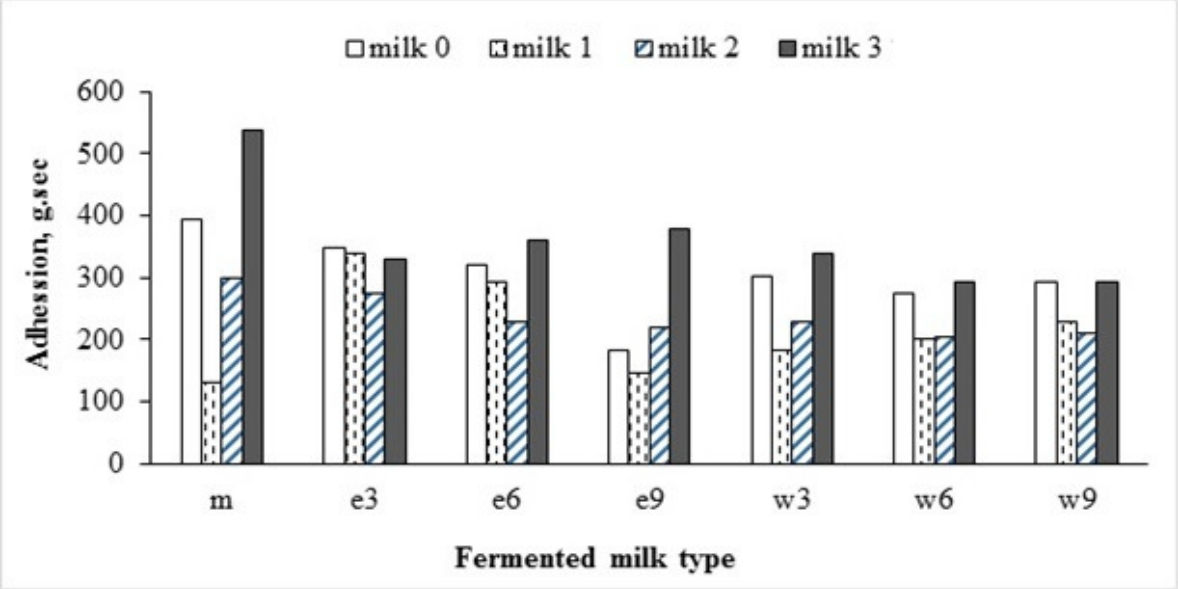


Figure 0-5: Firmness and adhesiveness properties of fermented milk samples made from milk with 0%, 1%, 2% and 3% fat and supplemented with electro-activated whey (e) and non-electro-activated whey (w) at concentrations of 3%, 6%, 9%. (m): control, (w3): 3% whey, (w).

3.3.3. Syneresis

In fermented dairy products, syneresis is a serious concern because it has highly negative impact on product quality, consumer perception and economic profitability. Syneresis is caused by shortening of the casein filaments, densification of the protein networks and release of the aqueous phase previously entrapped in the gel network. On the structural level, syneresis is caused by fusion of particles and continuous formation and breakage of physical cross-links between protein strands. It was reported that the gel particles tend to migrate towards denser regions, a phenomenon which results in large hollow spaces between dense regions connected by thin strands (T van Vliet, 2000; Ton Van Vliet, Lakemond, & Visschers, 2004). Moreover, from organoleptic consideration, syneresis is generally considered as an unappealing defect for the consumers (Basiri, Haidary, Shekarforoush, & Niakousari, 2018; Gilbert, Rioux, St-Gelais, & Turgeon, 2020). In the present study, **Figure 3.6** shows the degree of syneresis for 0% and 3% fat milk samples with varying concentrations and types of added whey and electro-activated whey. Analysis of the obtained data showed that the percentage of syneresis was characterized by a whey concentration dependent character. Samples supplemented with electro-activated whey displayed a visible superiority in preventing the aqueous phase separation during the refrigerated storage at 4 °C during 14 days. Moreover, analysis of the obtained results showed that the fat content of the used milk also had an influence on the degree of syneresis. Skim milk samples were especially prone to textural and physical defects due to lower solids content as previously described by other authors (Lucey, 2016; Sandoval-Castilla, Lobato-Calleros, Aguirre-Mandujano, & Vernon-Carter, 2004). Even if skimmed milk products are popular among consumers for their potential health benefits, nevertheless, the absence of fat globules results in dairy products with poor textural characteristics. It should be noted that the main industrial strategy of improving textural structure and decreasing the degree of syneresis in fermented dairy products such as yogurts consists of heat treatment of the milk. The procedure was not applied in the present study to avoid the masking effect of this treatment on any eventual syneresis. Other methods of syneresis control are addition of stabilizers and hydrocolloids such as pectin, gelatin, and carrageenan, and increasing of total solids content by adding whey proteins concentrates (WPC) and isolates (WPI), as well as caseinates. However, addition of hydrocolloids causes flavor impairments even at low concentrations (Lesme, Rannou, Famelart, Bouhallab, & Prost,

2020). In addition to that, utilization of hydrocolloids in plain yogurts is prohibited by legislation of some countries (Peng, Serra, Horne, & Lucey, 2009). This can be avoided by using dairy components to improve the physical properties of the end product. However, the excess of latter can lead to powdery taste and grainy texture. In the present study, the samples containing electro-activated whey did not develop any of these defects at visual analysis, while they were characterized by considerably lower of syneresis in comparison with whey-supplemented samples.

The study of Heydari et al. (2018) on direct supplementation of prebiotics to yogurt have demonstrated that the addition of lactulose as a prebiotic increased the degree of syneresis in yogurt, which is caused by the incompatibility of the used prebiotic with milk proteins and that the carbohydrate prebiotic dissolves in the milk aqueous phase (Heydari, Amiri-Rigi, Ehsani, Mohammadifar, Khorshidian, Koushki, et al., 2018). As opposed to that, the present study we shows that lactulose supplementation as a part of the used electro-activated whey demonstrated a promising result in terms of free aqueous phase formation. Moreover, other prebiotics, β-glucan and inulin were reported to increase the degree of syneresis, whereas Vasiljevic et al. (2007) suggested that long-chain polysaccharides having prebiotic effect interfere with the casein and hinder a strong network formation (Vasiljevic, Kealy, & Mishra, 2007). Thus, compared to the already reported information of the negative effect of adding some probiotics to fermented dairy products, the use of electro-activated whey as an ingredient up to 9% supplementation level in fermented milk production showed highly positive impact on the product quality, especially its ability of preventing syneresis.

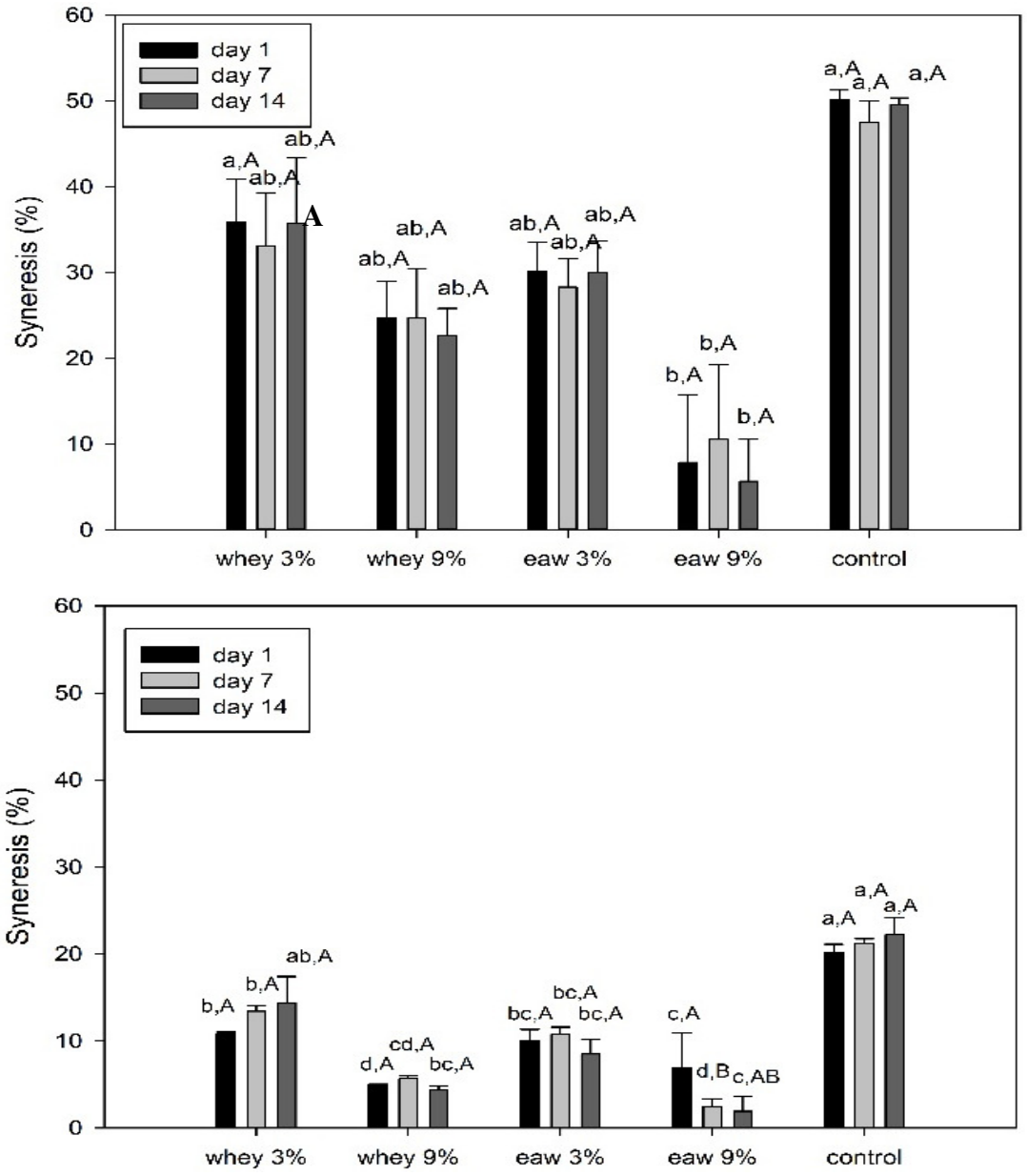


Figure 0-6: Degree of syneresis (%) in 0% fat milk (A) and 3% fat milk (B) supplemented with whey and electro-activated whey (eaw). Control is fermented milk without any whey added. Small letters indicate differences between treatments and capital letters between the days for the same treatment.

3.3.4. Microstructure

The results of scanning electron microscopy (SEM) of the fermented milk samples are shown in **Figure 3.7**. A common structure of unheated milk acid gels described as casein network forming a "tortuous" structures and pores of whey was described by many authors (Damin,

Alcântara, Nunes, & Oliveira, 2009; Marafon, Sumi, Granato, Alcântara, Tamime, & Nogueira de Oliveira, 2011; Ozcan-Yilsay, Lee, Horne, & Lucey, 2007; Sodini, Lucas, Tissier, & Corrieu, 2005). Similar structure could be clearly seen from the micrographs obtained in the present study. The scanning electron microscopy results showed that the fermented milk samples supplemented with 9% electro-activated whey were characterized by very small pores which can be seen as black areas in the SEM images, having more homogenous structure and smaller particle size in comparison with the fermented milk samples supplemented with whey or a control without any supplementation. The observed differences in the fermented milk samples microstructures are attributed to the interaction between the whey and the electro-activated whey with the casein micelles and the formed gel network during the fermentation process and the refrigerated storage. After electro-activation of whey, its main protein fractions, α-lactalbumin and β-lactoglobulin, undergo partial hydrolysis with a subsequent formation of smaller peptide fractions. Moreover, sugars-peptides and sugars-amino acids conjugates are formed because the electro-activation process occurred under highly alkaline and reducing conditions in the cathodic compartment of the electro-activation reactor. Thus, all these newly formed compounds may have higher affinity of binding to the casein micelles with high water holding capacity. In this case, the electro-activated whey integrated the formed gel network not only as filling material but by strong binding to different sites of proteins by means of different mechanisms such as hydrogen bonds as well as electrostatic interactions. Moreover, correlation of microstructure and syneresis was previously outlined by Gilbert et al. (2020) (Gilbert, Rioux, St-Gelais, & Turgeon, 2020) who reported that whey is situated in the voids between protein networks and is repulsed at later stages of gel rearrangement. In the present study, we observed smaller pores indicating a uniform distribution of the particles and formation of a structure with a higher water holding capacity (Lee & Lucey, 2010).

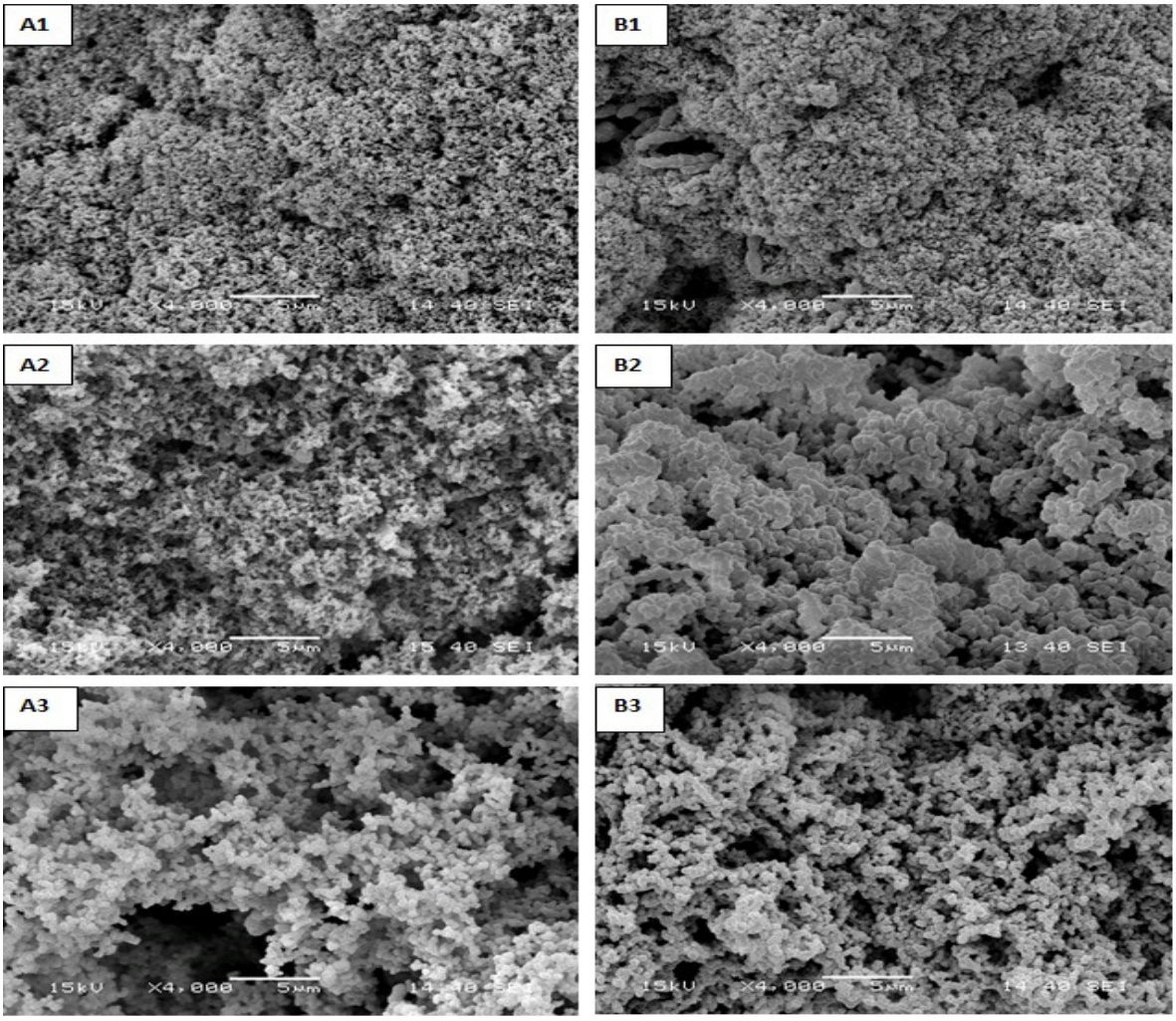


Figure 0-7: Microstructure of fermented milk samples. A: 0% fat milk, B: 3% fat milk. 1: with 9% electro-activated whey added, 2: with 9% non-electro-activated whey added, 3: control fermented milk.

3.3.5. pH and titratable acidity during refrigerated storage

It is known that acid tolerant starter bacteria continue to grow at a slower rate during the refrigerated storage. This growth results in further acidification of the fermented milk which leads to increase in hydrophobic and electrostatic interactions among proteins and formation of large agglomerates of casein micelles. Thus, this phenomenon does not only impair sensorial qualities of the product but induces reformation of a highly dense gel network with expulsed aqueous phase (Deshwal, Tiwari, Kumar, Raman, & Kadyan, 2021).

In the present study, the fermented milk samples supplemented with different concentrations of electro-activated and untreated (non-electro-activated) whey were stored at 4 °C directly after reaching a fermented product pH 4.6-4.7 for 21 days. The fermented milk samples pH and titratable acidity expressed in °Dornic were tested every 3 days. Evolution of pH and titratable acidity was not fat-dependent, thus only the results for the fermented skim milk samples are shown in **Tables 3.1-3.2** for pH and titratable acidity, respectively. Moreover, analysis of the obtained results showed that there was no statistical difference in pH and titratable acidity during the 3 weeks refrigerated storage for all samples. These data is in agreement with the results of Ben Moussa et al. (2019) in which it has been shown that addition of lactulose to yogurt formulation caused none significant changes in acidity levels during refrigerated storage (Ben Moussa, Boulares, Chouaibi, Mzoughi, & Hassouna, 2019). Also, Aghajani and Pourahmad (2012) reported that addition of lactulose to yogurt with starter culture and probiotic *Lactobacillus casei* did not cause significant post-fermentation acidification during 21 days of storage (Aghajani & Pourahmad, 2012). On contrast, the authors reported a significant reduction in pH when lactulose was added together with inulin.

Table 0-1: Evolution of pH during the storage of fermented skim milk samples.

	day 0	**day 3**	**day 6**	**day 9**	**day 12**	**day 15**	**day 18**	**day 21**
Whey 3%	4.57 ± 0.06	4.44 ± 0.03	4.46 ± 0.07	4.39 ± 0	4.41 ± 0.02	4.53 ± 0.1	4.47 ± 0.06	4.45 ± 0.06
Whey 6%	4.58 ± 0.05	4.48 ± 0.04	4.46 ± 0.03	4.41 ± 0.02	4.41 ± 0	4.55 ± 0.08	4.51 ± 0.05	4.44 ± 0.03
Whey 9%	4.57 ± 0.03	4.45 ± 0.01	4.45 ± 0	4.42 ± 0.03	4.4 ± 0.02	4.51 ± 0.04	4.5 ± 0.02	4.46 ± 0
EAW 3%	4.56 ± 0.03	4.55 ± 0.09	4.53 ± 0.01	4.51 ± 0.04	4.53 ± 0.01	4.66 ± 0.07	4.63 ± 0.06	4.57 ± 0.02
EAW 6%	4.52 ± 0.01	4.5 ± 0.01	4.52 ± 0.07	4.49 ± 0.09	4.49 ± 0.09	4.61 ± 0.02	4.59 ± 0.05	4.57 ± 0.07
EAW 9%	4.55 ± 0.04	4.65 ± 0.09	4.69 ± 0.03	4.49 ± 0.1	4.71 ± 0.02	4.75 ± 0.05	4.74 ± 0.03	4.74 ± 0.03
Control	4.7 ± 0.08	4.56 ± 0.05	4.51 ± 0.07	4.46 ± 0.03	4.41 ± 0.04	4.42 ± 0.03	4.5 ± 0.08	4.52 ± 0.08

EAW: Electro-activated whey. Control: Milk without any supplementation.

Table 0-2: Evolution of titratable acidity (Dornic°) during the storage of fermented 3% fat milk samples.

	day 0	**day 3**	**day 6**	**day 9**	**day 12**	**day 15**	**day 18**	**day 21**
Whey 3%	100.5 ± 8.9	111.1 ± 12	114.8 ± 2.3	104.9 ± 3.5	113.5 ± 9.9	129.1 ± 1.2	124.7 ± 1.6	120 ± 15.4
Whey 6%	109.4 ± 0.2	119.6 ± 11.3	114.2 ± 2.8	138.5 ± 7.6	161 ± 19.5	127.6 ± 10	139.9 ± 5.3	135.5 ± 8.8
Whey 9%	116.2 ± 17.9	164.5 ± 7.3	189.3 ± 11.5	164.9 ± 12.8	181.4 ± 17.9	173.8 ± 18.5	158.6 ± 3.4	161.1 ± 7.6
EAW 3%	96.6 ± 7.5	105.8 ± 9.7	101.2 ± 9.6	94.9 ± 2.6	113.2 ± 5.5	118.2 ± 0.6	118.2 ± 2.3	109.4 ± 0.2
EAW 6%	148.1 ± 22.7	136.1 ± 15.9	129.6 ± 2.6	137.2 ± 7.2	134.6 ± 18.8	144.6 ± 8.8	162.1 ± 13.9	132.1 ± 8.5
EAW 9%	123.2 ± 7.6	149.1 ± 10.1	147.5 ± 14.5	161.5 ± 11.7	129.7 ± 0.2	139.7 ± 0.7	154.6 ± 14.4	136.8 ± 1.7
Control	61.5 ± 11.7	93.8 ± 7.8	85.9 ± 2.6	76.7 ± 4	91.5 ± 2.3	83.3 ± 12.7	119.7 ± 5.2	119 ± 6.9

EAW: Electro-activated whey. Control: Milk without any supplementation.

3.3.6. Total bacterial count

The viable cell counts of *Lactobacillus delbrueckii* subsp. *bulgaricus* and S*treptococcus thermophilus* are given in **Figure 3.8**. Overall, all the samples reached the conformity by the number of ferment bacteria per portion of a product. It is commonly accepted that synergistic proto-cooperation of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* is responsible for the initiation of acid milk gels formation. Initially, due to its proteolytic ability, *Lactobacillus delbrueckii* subsp. *bulgaricus* liberates different amino acids such as valine, leucine, histidine, and methionine, supporting the growth of *Streptococcus thermophilus*. The latter being more oxygen tolerant proliferates at high rates and produces formic acid. It was suggested that formic acid is the main metabolite produced by *Streptococcus thermophilus* promoting the growth of *Lactobacillus delbrueckii* subsp. *bulgaricus.*

At lower pH, *Lactobacillus delbrueckii* subsp. *bulgaricus* grow at high rate (Horiuchi & Sasaki, 2012; Routray & Mishra, 2011). To verify that prolonged fermentation is not a factor of inability of bacteria to reproduce in the presence of electro-activated whey, we performed bacterial plate counts at the final points of fermentation for different samples: 5 h for the control and 16 h for the samples supplemented with 9% electro-activated whey because pH 4.6-4-7 was reached at these periods, respectively. These results are in accordance with the data obtained by Kareb et al. (2018) where electro-activated whey was actively metabolized by the tested lactic acid bacteria. Media enriched with electro-activated whey showed that the bacterial growth was higher than in the media containing lactose, lactulose, whey, and sucrose. The authors reported that starter cultures reached the highest optical density when they were grown on electro-activated whey and this result was attributed to electro-activated whey composition and its very good antioxidant capacity (Kareb, Champagne, Jean, Gomaa, & Aïder, 2018).

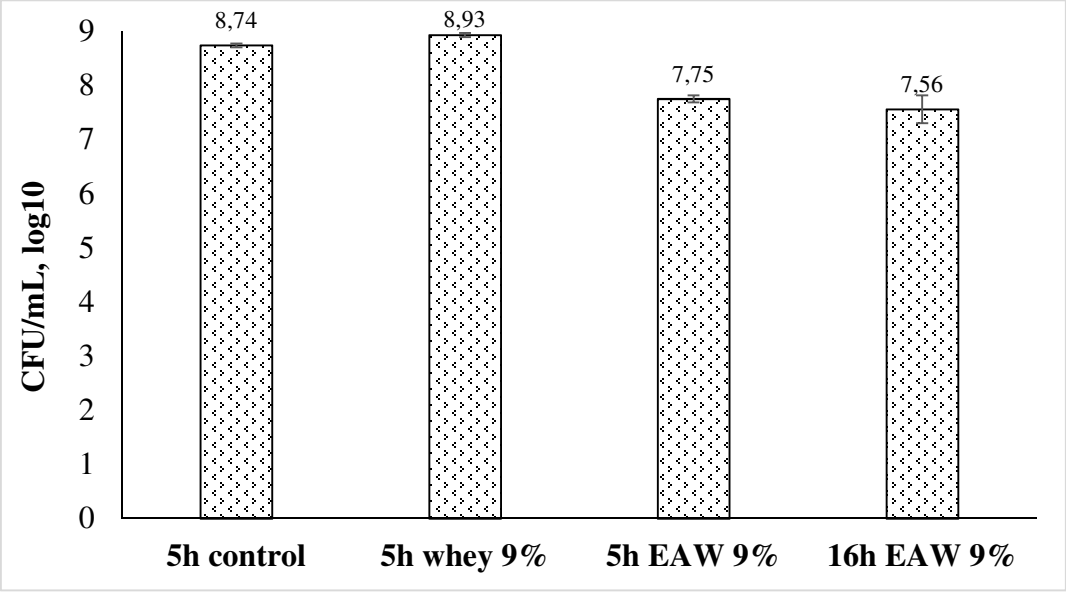


Figure 0-8: Colony forming units (CFU) of lactic acid bacteria in 1 mL of fermented milk. EAW: Electro-activated whey.

3.3.7. Organic acids production by starter cultures

The health promoting effect of fermented milk products is attributed to the presence of probiotic bacteria and their metabolites among which organic acids are particularly of importance. Moreover, one of the most important bioactive compounds of carbohydrate metabolism by lactic acid bacteria are short chain fatty acids such as butyric acid, a group of molecules containing from two to six carbon atoms. It is commonly recognized that production of organic acids and some short chain fatty acids (SCFA) is strain and substrate dependent. Most common organic acids and SCFA in fermented milk products are butyric, acetic, citric and propionic acids. Acetic acid was noted to influence the appetite, and propionic to take part in the reduction of liponeogenesis and cholesterol synthesis (Annunziata, Arnone, Ciampaglia, Tenore, & Novellino, 2020). In the present study, acetic and propionic acids HPLC corresponding peaks were the highest in the fermented milk samples supplemented with electro-activated whey. Lactic acid is the main product of lactose metabolism by lactic acid bacteria. At the time point of 5 h, samples containing 9% electro-activated whey demonstrated significantly higher amount of lactic acid (Table 3.3). Butyric acid production was induced by addition of whey and electro-activated whey. Vaseji et al. (2012) studied butyric acid production in yogurts with conventional starter cultures and probiotic added yogurts. As

a result, probiotic containing yogurts had significantly higher levels of butyric acid. Furthermore, it is suggested that lipolytic activity of probiotic bacteria is involved in production of butyric acid from milk fat (Vaseji, Mojgani, Amirinia, & Iranmanesh, 2012).

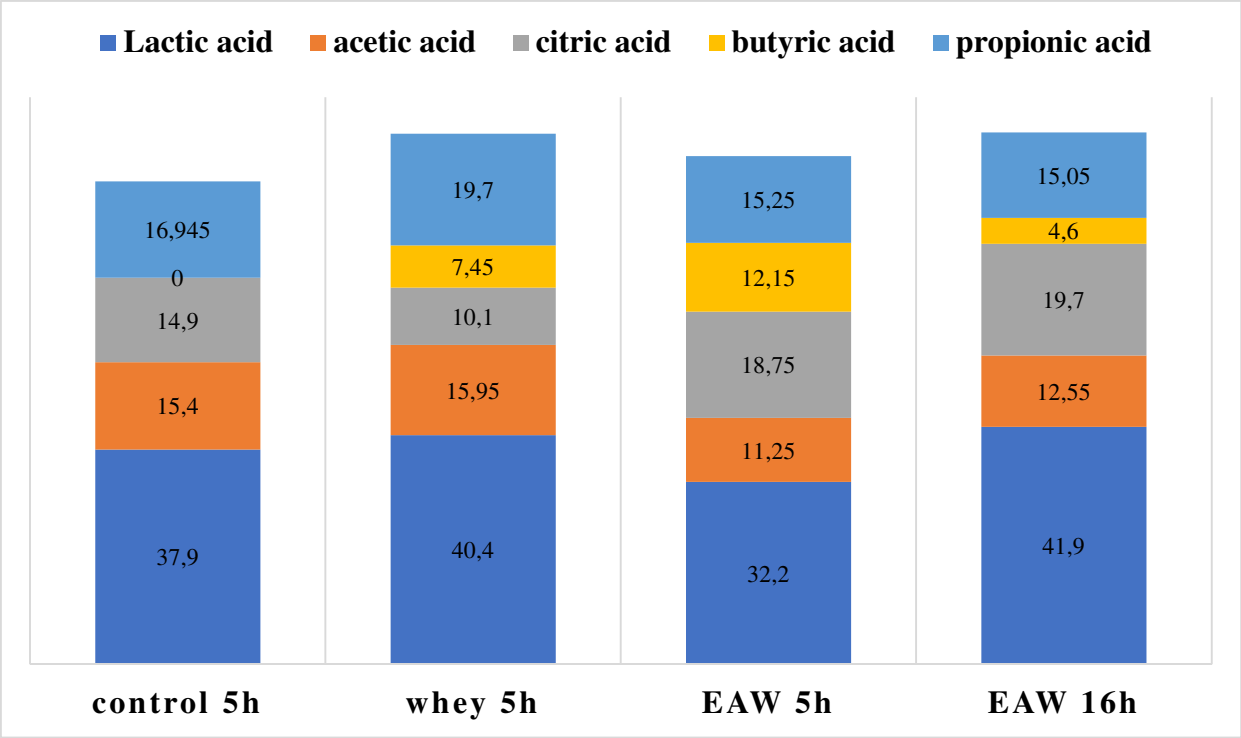


Figure 0-9: Ratios of the different organic acids contained in the fermented milk samples supplemented with 9% whey and 9% electro-activated whey (EAW).

Table 0-3: Concentration of lactic, acetic and citric acids in fermented milk samples supplemented with 9% whey and electro-activated whey (EAW).

	Average content, mg/L		
	Lactic acid	**Acetic acid**	**Citric acid**
Control 5h	2647.97 ± 124.10	1599.77 ± 108.09	1108.61 ± 225.79
Whey 5h	5036.68 ± 107.79	2941.79 ± 119.43	1333.16 ± 65.39
EAW 5h	5949.47 ± 61.18	3099.64 ± 641.36	3799.39 ± 120.34
EAW 16h	8346.47 ± 160.77	3665.37 ± 35.53	4268.14 ± 78.81

3.3.8. Aromatic profile

Over 90 volatile compounds were identified to be associated with fermented dairy products such as yogurts in many previous studies. Formation and quantity of this metabolites is strain-specific, and depends on other factors such as fermentation temperature and composition of the growth media (Routray & Mishra, 2011). According to Beshkova et al. (1998), the majority of yogurt aroma compounds are already present in fresh milk such as aldehydes, ketones, and alcohols. In this study, the main volatile compounds identified in fermented milk samples were 2,3-Butanedione (diacetyl), 2-Butanone, 3-hydroxy- (acetoin), 2-Propanone, 1-hydroxy- (acetol), 2-Furancarboxaldehyde, 5-methyl-, furfural, 2-Furanmethanol, ethanol, acetic acid, propionic acid, formic acid, urea, butyrolactone, 2-Cyclopenten-1-one, 2-hydroxy- (Beshkova, Simova, Frengova, & Simov, 1998). The main metabolite of lactic acid bacteria fermentation is lactic acid which has low volatility. The same is applied to other organic acids such as propionic and formic acids. Even though they largely contribute to fermented dairy products typical sour and acidic taste. Moreover, the main aroma forming compounds of yogurts are diacetyl and acetaldehyde. In the present study, diacetyl was detected only in the fermented milk samples containing whey and electro-activated whey. However, its absence in the control sample could be related to the level of the component which is lower than the detection threshold of the used GC method. Diacetyl and acetoin are ketones associated with buttery, fresh milk aroma of fermented milk products. The method used in this study did not permit the detection of acetaldehyde in all the samples. According to Cheng

(2010), its concentration in yogurt is approximately 8 and 40 ppm and it is the main contributor to yogurt's green apple-like tart flavor. Another suggestion that acetaldehyde was rapidly converted to ethanol by an active production of alcohol dehydrogenase enzyme by the bacteria composing the used starter culture. Also, 2-furanmethanol is an intermediate product of Maillard reaction that could be formed due to sugar and protein degradation in milk (Cheng, 2010). This analysis had a qualitative character to distinguish the difference in volatile compound production of samples with addition of electro-activated whey. Even though there were a higher number of peaks in samples with added whey and electro-activated whey, not all the peaks were identified. There can also be a difference in the amounts of organic compounds such as different organic acids and short chain fatty acids (SCFA).

3.3.9. Sensory analysis

Figure 3.10 represents the results of the sensory analysis test of the fermented milk samples prepared from 3% fat milk and which was performed with 20 panelists. In the case of visual whey separation defatted fermented milk samples had a significant disadvantage (results not shown). Indeed, low fat dairy products are often associated with weak textural properties and large degree of syneresis as a consequence of their lower total dry matter content which some time is somewhat compensated by addition of skim milk powder, whey protein concentrates (WPC), as well as sodium caseinate. The full amount of these ingredients needed to obtain solids content as in 3% fat milk usually causes excessive firmness, grainy texture, and powdery taste (Sandoval-Castilla, Lobato-Calleros, Aguirre-Mandujano, & Vernon-Carter, 2004). In the present study, it was remarkable that addition of electro-activated whey reduced the whey separation (syneresis) in zero fat fermented milk, a fact which is also confirmed by the above-mentioned data on syneresis measurement. This quality raises the potential of using electro-activated whey in low fat dairy fermented products to enhance their overall quality and consumer acceptability. The texture of the samples containing electro-activated whey appeared ropier and less firm than samples with untreated whey and control. At addition of electro-activated whey, the graininess was reduced in the zero fat fermented milk samples in comparison with control. Overall flavor perceptions had a milk fat dependent pattern. However, it is difficult to judge the qualities of fermented milk samples with added electro-activated whey, due to the unusual baked taste and yellowish color that appeared as a result

of Maillard reactions that occurred during whey electro-activation process. Thus, respondents asked to leave a comment have mentioned that electro-activated whey samples had some slight saltier taste, and this could interfere with their judgement of the flavor. This inconvenience can be overcome in the future by demineralization of the electro-activated whey during the processing.

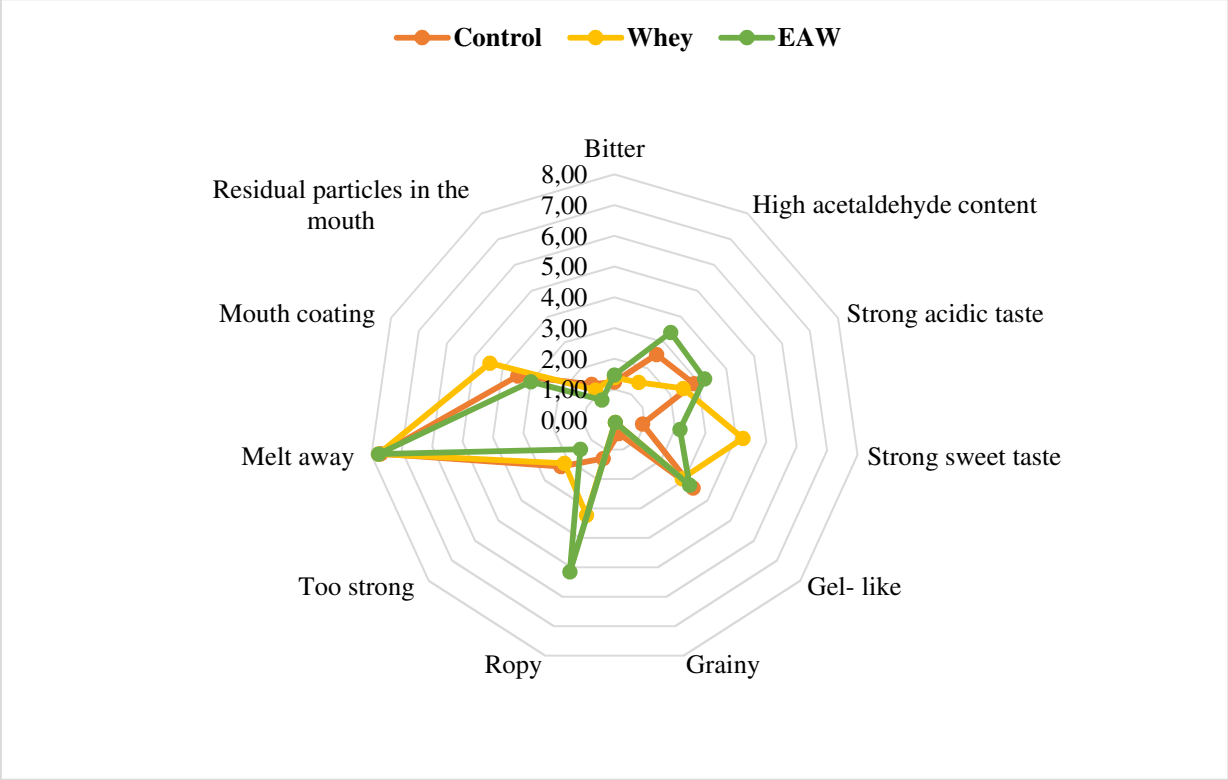


Figure 0-10: Sensory evaluation of the samples prepared from 3% fat milk with 9% electro-activated whey, 9% untreated whey and control.

3.3.10. Residual lactulose during storage

In addition to conventional beneficial effect of fermented milk products, the claimed functional health effect of electro-activated whey supplemented products is the presence of lactulose which is a proven prebiotic in the clinically important levels in a 100 g portion. According to Ben Moussa et al. (2019), consumption of 2 g per day of lactulose stimulates an increased production of short-chain fatty acids by consumer's microbiota, 5 g per day is necessary to ensure an evident prebiotic effect, and 7.5 g is enough to decrease ammonia levels

in the body (Ben Moussa, Boulares, Chouaibi, Mzoughi, & Hassouna, 2019). In the present study, we evaluated the consumption of lactulose by the starter cultures used in milk fermented products with an addition of 3% and 9% electro-activated whey (EAW). Initial concentrations of lactulose in samples were 6885 mg/Land 6805 mg/L in skimmed and 3% fat milk supplemented with 3% EAW, and 21343 mg/L and 21364 mg/L in samples supplemented with 9% EAW. In the end of the regular fermentation process to pH 4.6-4.7, the levels of lactulose in 3% EAW supplemented samples were 84% in skimmed fermented milk and 74% in 3% fat milk fermented samples of the initial concentration. In the fermented milk samples supplemented with 9% EAW, the remained lactulose was 64% in skimmed fermented milk and 61% in the 3% fat fermented milk. Storage period affected the 9% EAW fermented milk samples as the levels of lactulose dropped in the first week of storage with a 64% and 30% diminishing levels. In the 3% EAW supplemented samples, only a loss of 8% and 16% was recorded from the level corresponding to the end of fermentation. Indeed, the catabolism of lactulose varies significantly among bacterial genera, and even different strains. The study of Sahota et al. (1982) have indicated that Lactobacillus species were metabolizing more than 20% of available lactulose, while Streptococcus species were able of utilizing only 8.8% of lactulose (Sahota, Bramley, & Menzies, 1982). When studying the response of *Lactobacillus acidophilus* in the mixtures of lactose and lactulose, the preference was given to a dominant substrate, thus, in presence of lactose during fermentation, its utilization might prevail that of lactulose (Bohačenko, Pinkrová, Peroutková, & Pechačová, 2007).

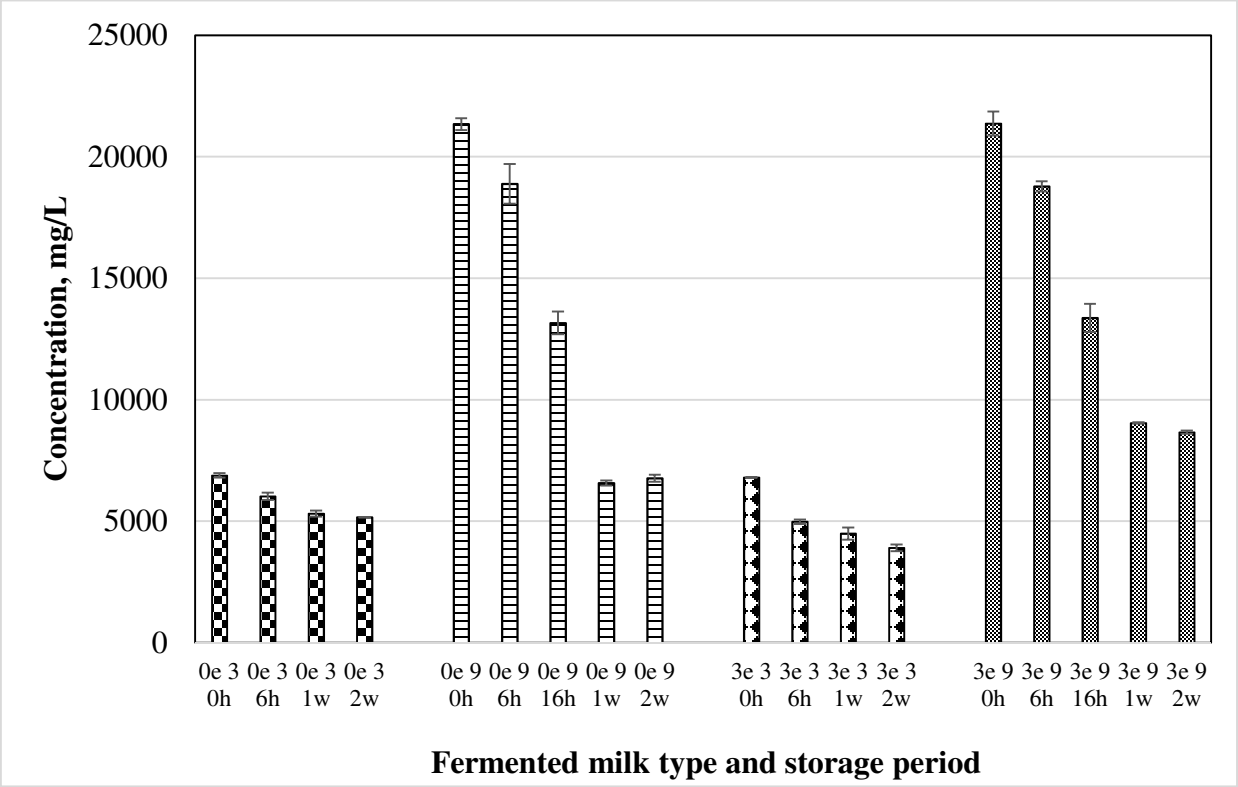


Figure 0-11: Residual lactulose in the fermented milk samples as a function of the storage period and product type. 0e: 0% fat milk + electro-activated whey at 3 and 9% supplementation level, 3e: 3% fat milk + electro-activated whey at 3 and 9% supplementation level.

Given all above-described characteristics of the fermented milk products supplemented with electro-activated whey, we suggest this technology for production of set-style drinkable fermented dairy products. Current technology of drinkable yogurt production includes the steps of mixing and breaking the gel to obtain less viscous product. Utilization of electro-activated whey in the formula will let avoiding this step, as it leads to formation of more ropy type of gels. Fermentation can be performed directly in the set style. Moreover, this study has shown that electro-activated whey supplemented fermented milk products have a potential of prolonged storage period. Remarkable formation of different organic acids and short chain fatty acids along with the proven prebiotic properties of lactulose may have beneficial effect on consumer health. Another noteworthy quality proven by this study was the ability of electro-activated whey powder to serve as a zero-fat product texture enhancer. Further optimization

of the technological process of incorporation of electro-activated whey for better utilization of its beneficial properties are however necessary.

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CHAPTER 4: Study of the Physico-chemical, Structural, Microbiological properties and Volatile Flavor Compounds Profile of Kefir Supplemented with Electro-activated Whey

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RÉSUMÉ

L'objectif de ce travail était d'étudier les propriétés physico-chimiques, microbiologiques et structurelles, ainsi que les composés de saveur volatile du kéfir complété par du lactosérum électro-activé à un niveau de 3-9%. La cinétique de fermentation a montré un retard pour atteindre un pH de 4,6 dans le kéfir avec du lactosérum électro-activé. La synérèse était absente dans le kéfir avec du lactosérum électro-activé. Le kéfir avec 3% de lactosérum électro-activé a montré un comportement pseudo-plastique non-newtonien avec un caractère d'amincissement par cisaillement, tandis que le kéfir supplémenté à 9% avait un comportement pseudo-plastique newtonien. Le profil minéral a montré une teneur plus élevée en K^+ dans le kéfir avec du lactosérum électro-activé, ce qui est dû à la teneur élevée en cet ingrédient. La microscopie à balayage électronique a révélé une structure fine homogène du kéfir avec du lactosérum électro-activé par rapport au contrôle ou au kéfir supplémenté en lactosérum. Cette structure indique le piégeage des particules de lactosérum électro-activé dans la matrice du kéfir qui est un produit laitier liquide avec des particules colloïdales dispersées. L'analyse GC a montré un profil aromatique volatile riche du kéfir avec du lactosérum électro-activé.

Mots-clés: Kéfir; lactosérum électro-activé; prébiotique; valorisation intégrale du lactosérum.

ABSTRACT

The aim of this work was to study the physico-chemical, microbiological, structural properties, and the volatile flavor compounds of kefir supplemented with electro-activated whey at 3-9% level. Fermentation kinetics showed a delayed time to reach pH 4.6 in kefir with electro-activated whey. Syneresis was absent in the kefir with electro-activated whey. Kefir with 3% electro-activated whey showed non-Newtonian pseudoplastic behaviour with shear thinning character, whereas the 9% supplemented kefir had a Newtonian pseudoplastic behaviour. Mineral profile showed higher K^+ content in kefir with electro-activated whey which was due to its high content in this ingredient. Electron scanning microscopy revealed a homogeneous fine structure of kefir with electro-activated whey compared to the control or whey supplemented kefir. This structure indicated entrapment of electro-activated whey particles in the kefir matrix which is a liquid dairy product with dispersed colloidal particles. GC analysis showed rich volatile aromatic profile of kefir with electro-activated whey.

Keywords: Kefir; electro-activated whey; prebiotic; whey integral valorization.

4.1. INTRODUCTION

A worldwide annual production of cheese has been growing for the last five years from 19.52 million metric tonnes in 2015 reaching 21.3 million metric tonnes in 2020. Cheese whey is an abundant co-product (effluent) of cheese industry. It is estimated that ratio of cheese to whey produced from 10 tons of milk is 1:9. Whey cannot be directly disposed due to its rich organic content that can cause eutrophication by depletion of dissolved oxygen and high oxygen consumption in water-living organisms, reduction of redox potential, increase in salinity and waterproofing of soil due to the build-up of organic components (Bosco, Carletto, & Marmo, 2018; Ryan & Walsh, 2016). Lactose comprising 70% of whey solids is the principal contributor to its pollutant effect (Rocha & Guerra, 2020).

As a response to a growing cheese production and stricter environmental control measures, valorization of whey is a still worldwide important topic in research and industry. Half of whey produced worldwide is used to manufacture various food and feed products. Approximately 60% of this whey is used in bulk form as an animal feed and for biomass growth purposes (Spalatelu, 2012). Whey is valorized by fractionation to whey proteins (by combining microfiltration with ultrafiltration-diafiltration, lactose and its derivatives such as lacitol, lactulose, lactobionic acid, and galactooligosaccharides (Bansal & Bhandari, 2016; Bosco, et al., 2018; Onwulata & Huth, 2009; Rocha & Guerra, 2020). However, both whey and lactose have a low production and nutritional value for the food industry. Bulk whey is difficult to incorporate into foods due to several drawbacks. Its main component lactose has a relative sweetness of 0.16 making it a weak sweetener. Thus, it is limited to use for texture enhancement through added total solids and sugar replacement when needed to reduce sweetness, for example in confectionaries. Also, lactose has a weak fermentable power. It is not fermented by many microorganisms including yeasts (Pasotti, et al., 2017). Another issue is the high prevalence of lactase insufficiency in world's population causing lactose intolerance. Moreover, whey derived from cheese making has relatively high sodium salt content which in its turn has negative impact on both nutritional, techno-functional and organoleptic properties of supplemented foods.

Overall trend for healthy nutritional products is increasing worldwide (García-Burgos, Moreno-Fernández, Alférez, Díaz-Castro, & López-Aliaga, 2020). Fermented dairy products

such as kefir, yogurts or simply fermented milk are amongst the most common functional foods because of their health beneficial impact; particularly on the human microbiota. Kefir is a fermented dairy product originating from Caucasus Mountains and very popular in Eastern Europe and Central Asia with a gaining popularity worldwide. It has an acidic tart and natural carbonation flavour and is popular for its refreshing and therapeutic properties; particularly, a positive effect on gastro-intestinal conditions. It is probably the most versatile dairy beverage in terms of its microbial composition because the kefir grain is a symbiotic composition of different strains of bacteria and yeasts with a predominance of lactic acid bacteria. Indeed, the melting pot of microorganisms including a large variety of lactic acid bacteria and beneficial yeasts stands behind its outstanding health promoting properties (Bengoa, Iraporda, Garrote, & Abraham, 2019). It was reported to have cholesterol-lowering, immune-modulating, anti-carcinogenic, anti-stress, anti-allergenic, anti-asthmatic, and anti-diabetic effects (Dimitreli, Petridis, Kapageridis, & Mixiou, 2019; D. H. Kim, Jeong, Song, & Seo, 2018). In addition to that, up to 30% of kefir lactose is hydrolyzed by its own microbiota, making it acceptable by consumers with lactose intolerance (Turkmen, 2017). According to the kefir technological process, it is allowed to increase its total dry matter up to 20%. Thus, whey and its derivatives such as electro-activated whey can be used as ingredient for this purpose. Unfortunately, the use of whey is limited due to the aforementioned limitations of using whey. To overcome these limitations, whey can be substituted by the electro-activated whey which is a whey-derived ingredient obtained following its whole transformation into a value-added product containing lactulose which is a prebiotic with already demonstrated potency, and proteins hydrolysates characterized by high solubility and good techno-functional properties (Kareb, 2018).

Electro-activation has recently been demonstrated as an effective method to convert lactose into lactulose *in situ* of a bulk whey. The yield of lactulose from whey following electro-activation treatment reached 37-40%. It is achieved by creation of alkaline conditions in a catholyte compartment of an electro-activation reactor (Aider & Halleux, 2007; Aider & Gimenez-Vidal, 2012). The method is based on water electrolysis at the interface of electrodes and solution. It does not include addition of catalysts or heating. It employs nontoxic, accessible electrolytes that do not require further purification steps. The principal reactions taking place in the reactor are oxidation in anodic (O_2 gas), and reduction in the

cathodic compartments (H_2 gas) (Aider, Gnatko, Benali, Plutakhin, & Kastyuchik, 2012; Aït-Aissa & Aïder, 2014). Lactulose has a higher degree of sweetness than lactose (0.6-0.8 relative to sucrose). Moreover, since 1950 it is recognized as a bifidus factor; a component with growth stimulating effect on lactic acid bacteria and bifidobacteria. It is a recognized prebiotic owning to the presence of non-hydrolysable β-glycosidic bond and resistance to thermal-acidic conditions. Lactulose enriched fermented dairy products were demonstrated to be effective in constipation treatment and overall improvement of health conditions (Pranami, Sharma, & Pathak, 2017; Treepongkaruna, et al., 2014). In this context, whey treatment by electro-activation and addition of electro-activated whey into dairy beverages such as kefir can be a big contribution to lessen the environmental burden of whey disposal, to increase the use of whey for human nutrition and to increase the profitability of the dairy industry. Moreover, it could bring a commercial value and nutritive benefits to the consumers, as innovative functional dairy products having evident advantage in conquering the market.

Electro-activation was applied to whey in order to improve its quality and use as whole ingredient in the dairy industry and in fermented dairy products specifically. Following electro-activation, it is possible to transform a part of lactose (up to 40%) into lactulose which is a recognized prebiotic. So, in the final fermented product, lactulose is partly used by the starter culture and part of this prebiotic remains unmodified. Thus, by consuming this product, the consumer will have the opportunity to be provided by a prebiotic which will be very beneficial for the host microbiota and its overall health.

Thus, the aim of this work is to study the feasibility of using electro-activated whey, rich in lactulose, as an ingredient for the supplementation of kefir up to 9% level and to study the impact of the electro-activated whey on the physico-chemical, microbiological, structural, and volatile flavor compounds profile of the fermented product. To the best of our knowledge, no studies were performed on incorporation of electro-activated whey to kefir.

4.2.MATERIALS AND METHODS

4.2.1. Electro-activated whey production

Sweet whey powder (75% lactose, 12% total proteins, 7% ash, moisture content less than 5%) was graciously obtained from Agropur Cooperative (St-Hubert, Quebec, Canada). Whey solution (10%, w/w) was electro-activated in a reactor containing 3 compartments: anodic, cathodic, and central. Whey solution was placed in the cathodic compartment which is connected to the electric current generator through a food-grade stainless electrode used as a cathode. Other two compartments were filled with 0.1 M and 0.25 M K_2SO_4 solutions, respectively. A ruthenium-iridium coated titanium was connected to the electric generator and served as the anode. The central compartment was separated from the anodic and cathodic compartments by anion and cation exchange membranes, respectively. A schematic representation of the electro-activation reactor is given in **Figure 4.1**. Electro-activation was performed at 800 mA during 60 min. Further, electro-activated solution was left at ambient temperature (22 ± °C) for 48 h for relaxation. After 48 h, the electro-activated whey solution was neutralized to reach a pH 7.0 by careful addition of 1 N HCl. Electro-activated whey powder was obtained by freeze drying and was used for further analyses and kefir supplementation.

4.2.2. HPLC analyses of electro-activated whey

The HPLC system (Water, Millipore Corp., Milford, MA, USA) was used to determine the sugar contents in the samples (that were collected from the cathodic compartment and reaction medium of whey solutions during the EA). The HPLC system was equipped with a carbohydrate analysis column (Waters Sugar Pak-I, 300 × 6.5 mm^2, Waters Co.) and a refractive index detector (Hitachi, model: L-7490). The column temperature was set at 90 □, and an isocratic mobile phase (a solution of 50 mg/L Ca-ethylenediamine tetra-acetic acid) was used at a flow rate of 0.5 mL/min. The analysis was then performed by injecting 50 µL of sample and setting the operating time to 30 min/sample. Finally, the identification and quantification of different sugars (lactose, lactulose, glucose, galactose, and fructose) were achieved by matching their retention times with the standard solutions.

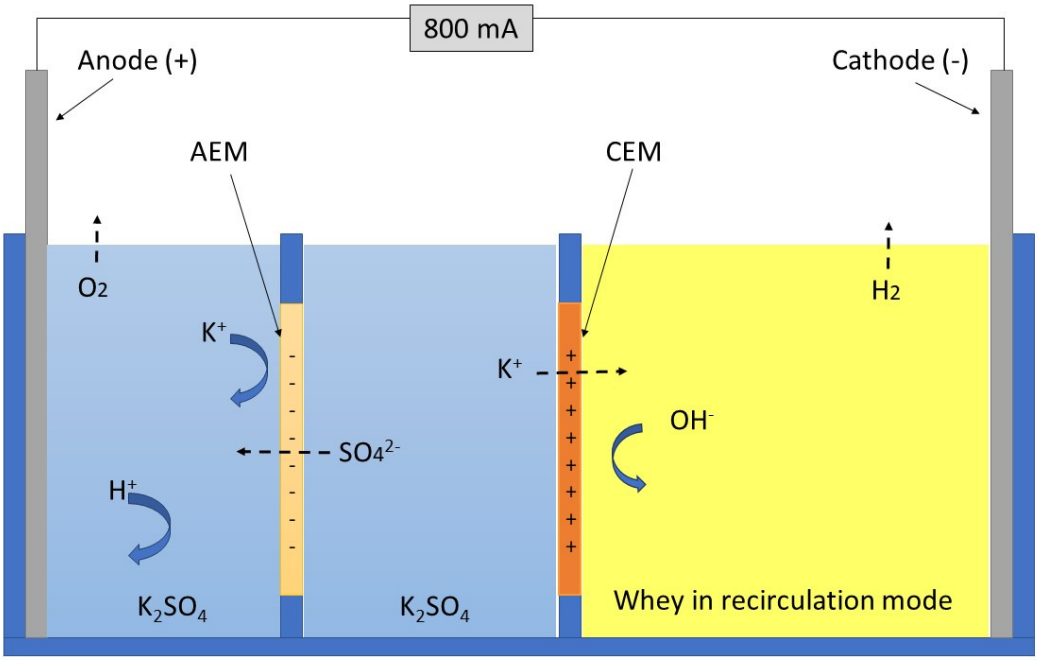


Figure 0-1: Schematic representation of a reactor for whey electro-activation.

4.2.3. Circular dichroism of electro-activated whey

Circular dichroism was observed using a spectropolarimeter Jasco J-815-150S (Jasco Corp., Tokyo, Japan). Whey and electro-activated whey samples at ambient temperature (22 ± 1 °C) were poured in 0.01 mm quartz cuvettes. Circular dichroism spectra were recorded from 260 to 190 nm. The following parameters were used: standard bandwidth, data pitch 0.1 nm, data integration time 1 sec, and scanning speed 100 nm/min. Average of 10 accumulating reads was taken for a final result.

4.2.4. Zeta potential measurement and particle size distribution

Zeta potential and particle size distribution of two types of whey; initial and electro-activated whey, were measured by using the Zetasizer 2000 instrument (Malvern Instruments Ltd., UK). Zeta potential was analyzed in the pH range from 3 to 7 since it is the range of the consumed foods by humans. The pH was adjusted manually by using citrate or phosphate

buffers.

4.2.5. Fermentation process of kefir

All kefir samples were prepared from commercial pasteurized milk (Quebon, Natrel, Quebec, Canada). The used commercial milk was of 0 and 3% fat content and 3.6 ± 0.23% total protein. A lyophilized commercial starter culture Choozit Kefir DC Lyo 1000 L including a mixture of lactic acid bacteria and yeasts (Danisco, Denmark) was added as recommended by the manufacturer directly to milk which was previously warmed up to 30 °C. The milk was then supplemented with whey and electro-activated whey powders at concentrations of 3% and 9% (w/w). The fermentation process was carried out under controlled constant temperature in a microbiological incubator (Jeio tech, Billerica, MA, USA) at 30 °C until the pH value of 4.6-4.7 was reached. During kefir fermentation, pH was measured by using an Oakton pH-electrode (Vernon Hills, IL, USA) connected to an Oakton pH 700 pH-meter. Titratable acidity was determined by a titration method, according to the AOAC 947.05 method (AOAC, 1990). The results were expressed in Dornic degrees corresponding to the volume of 0.1 N NaOH solution needed for neutralization of fermented milk product diluted in double amount with distilled water or 100 g of the titrated product. Both measurements (pH and titratable acidity) were performed every hour to monitor the fermentation process.

4.2.6. Kefir proximate analysis

Kefir samples were analyzed for total solids, protein, lipids, and minerals. Total solids were measured by the standard gravimetric method in a vacuum dryer at 60 °C. Protein content was determined by the combustion method on a MicroN Cube analyser (Elementar, Rhein-Main, Germany). Lipid content was analyzed by modified Mojonnier method of extraction with ether. Minerals were determined by ICP-MS.

4.2.7. Syneresis

Degree of syneresis was analyzed as follows: a sample of 15 g of each fermented product was weighted with high precision on analytical balance and then centrifuged at 350xg for 10 min at 6 °C. After that, the separated whey was weighted and the total syneresis rate

of the kefir samples was calculated using the following formula (**Eq. 4.1**) (Gauche, Tomazi, Barreto, Ogliari, & Bordignon-Luiz, 2009):

$$\boldsymbol{Syneresis} = \left(\frac{\boldsymbol{mass\ of\ whey\ separated\ after\ centrifugation}}{\boldsymbol{total mass\ of\ the\ sample}}\right) \cdot \mathbf{100}\% \quad \textbf{(Eq. 4.1)}$$

4.2.8. Apparent viscosity

Apparent viscosity properties were measured by AR-G2 rheometer (TA Instruments Ltd, West Sussex, UK). Parameters were chosen according to Vimercati et al. (2020) with some modifications. Peltier steel DIN concentric cylinder with a diameter of 28 mm and length of 42 mm, and cup diameter of 30.4 mm were used. Analyses were conducted at a temperature of 18 °C. The samples were subjected to shear stress increasing linearly from 0.03 to 30.0 1/s. Results were interpreted by applying Power law model (Vimercati, et al., 2020).

4.2.9. Microstructure by scanning electron microscopy

The samples were fixed with 10% (v/v) formaldehyde and thrice washed in 0.1 M sodium cacodylate buffer with a pH of 7.3 for 20 min. Next step was post-fixation with 1% osmium tetroxide for 90 min, followed by the second washing and dehydration in graded ethanol (50, 70, 95, 100%) for 20 min each. Samples were soaked in hexamethyldisilazane two times for 20 min and dried. Samples were then metallized in a sputter coater and observed at 15 kV by using a JEOL electron microscope JEOL JSM-6360LV model (JEOL Ltd., Tokyo, Japan).

4.2.10. Colorimetry

Color of kefir samples was tested on Colorimeter Minolta CR-300 using L*C*H° absolute chromaticity measuring system. Fifteen mL samples were placed in a glass tube and read thrice. An average of three reads was taken. The results were interpreted as following: L* represents lightness of the sample from 0 (black) to 100 (white) (Mapari et al., 2006), C* stands for chroma and indicates saturation of the color which depends on the amount of pigment, and H° (hue angle) shows the shade of the sample. At H° = 0 the color is red, H° =

90 yellow, H° = 180 green and H° = 270 blue.

4.2.11. Bacteriological analysis

Aliquots of 1 mL of kefir samples were serially diluted until 10^{-7} in peptone water. Aliquots of 100 µL of diluted samples were inoculated to MRS agar surface (Difco™ Lactobacilli MRS Agar, BD (VWR, Montreal, Canada). Total lactic acid bacteria counts of kefir grains were calculated after 48 h incubation in anaerobic conditions at 30 °C.

4.2.12. Organic acids and sugars analysis by high performance liquid chromatography (HPLC)

Organic acids were measured by HPLC using Hitachi L-7000 (Hitachi High-Tech, Japan) system equipped with a Hitachi-7400 UV detector at a wavelength of 220 nm. Organic acids were separated by using a Phenomenex Kinetex®, 2.6 µm PS C18, 100 □ LC Column (100 × 4.6 mm). The column was maintained at a temperature of 30 □. A solution of 20 mM potassium phosphate KH_2PO_4 (pH 1.59) was used as a mobile phase with a flow rate of 0.3 mL/min. Each sample was injected at a volume of 50 µL and ran for 30 min.

Sugars were analysed by HPLC on a Hitachi L-7000 (Hitachi High-Tech, Japan) system connected to a refractive index detector (Model 2487, Waters, Milford, MA, USA). Sugars were separated by using a Sugar Pak-I 300 × 6.5 mm^2 column (Waters Corp., Milford, MA, USA). Column was heated and maintained at a temperature of 85 °C. The flow rate was set at 0.5 mL/min and the elution was fixed at 30 min. Results were interpreted by comparing retention times to those of the standard solutions.

4.2.13. Aromatic profile of kefir

The aromatic profile of different kefir samples was estimated by measuring the volatile compounds by gas chromatography on GC Agilent G1530A, and MS Agilent mass selective detector 5973 equipped with multi-purpose sampler (Agilent technologies, Santa Clara, CA, USA). The method was based on the following parameters: temperature 250 °C, total flow 8.7 ml/min, split ratio 5:1, split flow 5.0 ml/min, injection volume 0.5 µl, constant flow mode, flow 1 ml/min. Column DB wax 122-7062 was used. The aromatic compounds were identified by comparing the retention times of the obtained picks to those of the standard

compounds.

4.2.14. Statistical analysis

Analysis of the variance (completely randomised ANOVA) was performed by using SAS software (Version 9.1, SAS Institute, Cary, NC, USA). All experiments were carried out at least in triplicate and mean values ± std were used for different comparisons. The Tukey's HSD test was used to compare the mean values at 95% confidence level ($p < 0.05$).

4.3. RESULTS AND DISCUSSION

4.3.1. Properties of the electro-activated whey

According to the HPLC results, electro-activation of 10% whey solution accommodated conversion of 37% of whey lactose into lactulose. The obtained results indicated that the obtained lactulose after electro-activation, and 48 h relaxation remained at a statistically similar level after neutralization to pH 7.0 with HCl (**Table 4.1**). Thus, the result supports the fact that lactose electro-isomerisation into lactulose is irreversible when produced as aforementioned. This result is comparable with conventional production of lactulose, yield of which is dependent on the chemical catalysts used: about 30% by using alkaline catalysts, and 87% in complexing with alkaline catalysts such as aluminates, borates (Schuster-Wolff-Bühring, Fischer, & Hinrichs, 2010). However, this yield by complexing with alkaline catalysts gradually decreases due to lactulose decomposition into secondary products, a phenomenon which was not observed with electro-activation technology. The requirement for the presence of catalysts such as calcium hydroxide, sodium hydroxide, magnesium oxide is the reason behind cumbersome purification steps. Moreover, chemical isomerization is difficult to carry out directly from whey (Aït-Aissa & Aïder, 2014; Nooshkam, Babazadeh, & Jooyandeh, 2018). Another method of lactulose synthesis is based on using bacterial β-galactosidases, which hydrolyze lactose into monosaccharides and form lactulose through rapid trans-galactosylation mechanism at the presence of galactosyl acceptor (fructose) (Nooshkam, et al., 2018; Wang, Yang, Hua, Zhao, & Zhang, 2013). The productivity of lactulose by this method is hindered by requirement of high amounts of fructose for inducing the conversion. As a result, high levels of unreacted fructose accumulate in the solution. Even though this method provides more ecofriendly alternative of lactulose synthesis, it still loses to chemical isomerisation in terms of yield. In this regard, electro-activation has both advantages, being a green technology and producing high levels of lactulose.

Table 0-1: Sugar contents (%) of whey and electro-activated whey before and after neutralisation.

	Lactose	Lactulose	Glucose	Galactose	Fructose
EAW pH 11	56.75 ± 1.43	37.49 ± 1.04	-	4.58 ± 0.13	1.19 ± 0.02
EAW pH 7.0	59.88 ± 0.18	30.42 ± 0.31	3.80 ± 0.16	4.88 ± 0.21	1.03 ± 0.01
Whey pH 5.6	96.25 ± 1.15	-	0.98 ± 0.08	2.78 ± 0.06	-

(-): None detected by HPLC. EAW: Electro-activated whey.

**Glucose was not detected because it undergoes further isomerization to galactose at pH 11-12 (Djouab and Aider, 2018).*

4.3.2. Circular dichroism

It was previously reported that main changes in electro-activated whey are attributed to its sugar content (Kareb, 2018; Karim & Aider, 2020). To analyse the changes on the level of secondary structure of proteins in electro-activated whey, circular dichroism was performed. Illustration on the **Figure 4.2** depicts circular dichroism graphs at the wavelength of 190- 260 nm of whey and electro-activated whey before and after neutralisation. Taking a heterogeneous nature of whey proteins, all curves' shapes corresponded to a random coil. In non-treated whey, minimum point at 208 nm indicates the presence of α-helices; however, the overall shape indicates the mix of other structures, including β-sheets. Electro-activated whey has the same pattern but is more unstructured. The study of Tomczyńska-Mleko et al. (2018) confirms that increasing the pH to 11 reduced the share of the organised structures and leads to a corresponding increase in the unstructured particles to 60% (Tomczyńska-Mleko, et al., 2018). The study showed that the proteins do not gain their previous state even after neutralisation.

Detailed analysis of the obtained circular dichroism (CD) results indicated that the secondary structure was affected by the electro-activation treatment. Indeed, between 190 and 250 nm, normally we evaluate only the secondary structure (far UV spectra), whereas for the tertiary structure, the CD spectra must be recorded between 250 nm and 350 nm (near UV spectra). The observed far UV CD spectra is too similar of the α-lactalbumin and β-lactoglobulin. The untreated whey presented a minimum peak around 205 and 220 (typical for α-lactalbumin and β-lactoglobulin), suggesting a secondary structure rich in α-helix structure. A small peak around 210 nm is observed, indicating the presence of a β-sheet structure or the α-helix secondary peak. However, the CD signal corresponding to α-helix secondary peak must be normally more pronounced than the one observed in the present study. This may be due to the heterogeneity of the whey and electro-activated whey (EA whey). The EA whey neutralized to pH 7.0 and the none neutralized have almost the same spectra with an increased peak around 200 nm, suggesting probably more disordered structure with loss of the α-helix and the β-sheet structures having their typical minima peak around 210-220 nm. The none-neutralized electro-activated whey has apparently more disordered structure. The alkaline pH could play a crucial role for the secondary structure which can be attributed to several factors among which it is possible to cite the followings: The electrostatic repulsions between side chains probably created following the EA treatment could contribute to this observed change in the secondary structure; and specifically in the shift observed of the initial minimum peak toward 200 nm. The far-UV CD spectrum reflects the secondary structure of the protein and arises from the peptide bond absorption bands and the inherent chirality of the polypeptide chain. Also, tryptophan in side chains and disulfide bonds can also contribute to the observed far-UV spectra.

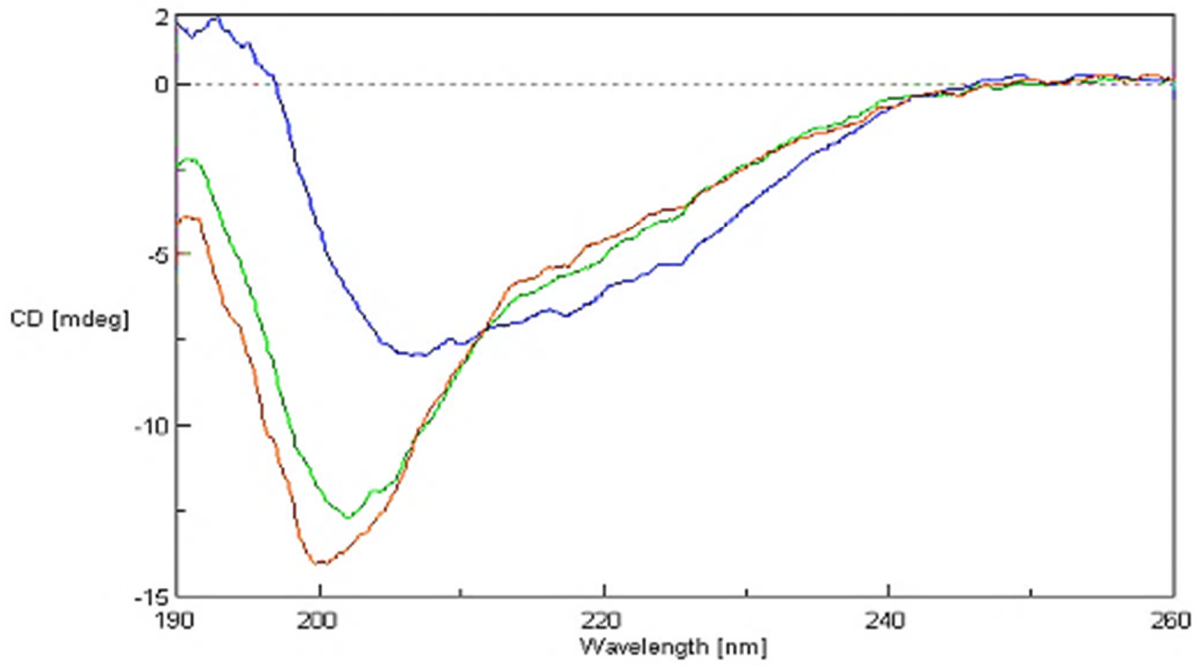


Figure 0-2: Circular dichroism of whey and electro-activated whey proteins at the wavelength 190 nm to 260 nm.

4.3.3. ζ-potential determination and particle size distribution

ζ-potential and mean particle size distribution of the used whey and its derived electro-activated whey are shown in **Figures 4.3A,B**. The effect of pH on ζ-potential of the electro-activated whey and the initial (non-electro-activated whey) is shown in **Figure 4.3A**. ζ-potential is the indicator of the particle surface charge that corresponds to physical stability of a protein suspension against aggregation. The particles with smaller negative or positive ζ-potential are prone to flocculation and aggregation due to inter-molecular and Van der Waals attractions (Cano-Sarmiento, et al., 2018; Salgin, Salgin, & Bahadir, 2012). At pH 3, ζ-potential of whey (4.07 mV) and electro-activated whey (-4.6 mV) showed similar absolute values with different charges. This change in surface electric charge could be explained by

anionic reactive particles (OH^-, H^-, O_2^-, HO_2^-) of electro-activated whey that bound to positively charged sites of proteins, causing a decrease of the net electric charge of the particles and leading to possible protein aggregation and formation of bigger particles (Beirami-Serizkani, Hojjati, & Jooyandeh, 2021). At higher pH values, electro-activated whey (EAW) was characterised by higher negative charge compared to whey, which implies formation of more stable particles in solution. Similar trend was reported by Souza et al. (2012) who conducted a study on microparticles containing whey proteins and pectin where pectin/whey protein complex reached high negative charge in aqueous solution. The negativity of the charge was found to be dependent on the carbohydrate-proteins ratio. Higher protein concentration implied domination of the positive charge, whereas electrostatic interaction between proteins and negatively charged carbohydrates covered positive sites of the proteins (Souza, et al., 2012). Further increase in negative charge with increasing pH could be explained by the fact that proteins are more unfolded at higher pH, making more binding sites available for anions (Gunasekaran, Ko, & Xiao, 2007). At pH between 3 and 4, the 10% whey solution reached an isoelectric point (pI). As it was predicted, at pH < pI the protein charge is positive whereas it is negative at pH > pI (Azarikia & Abbasi, 2016). Electro-activation treatment of 10% whey solution induced a shift of the pI value of the whey proteins to pH < 3, implying the stability of the solution at low pH levels characteristic of highly acidic media. The aforementioned factors can also explain distribution of particle size of the electro-activated whey, which was more homogeneous than that of non-treated whey. Thus, being characterized by small particle size distribution, the electro-activated whey is easily entrapped in the pores of the forming gel during kefir fermentation. This result will be later discussed in correlation with the microstructure of the produced kefir as a function of the added whey and electro-activated whey. Regarding the particle size distribution (**Figure 4.3B**), it can be seen that electro-activation yielded whey proteins with smaller particle size and more homogeneous distribution of the particles in regard to their size. This is the results of the high alkalinity of the medium to which whey was submitted and which had hydrolysing effect on proteins and generation of smaller molecules.

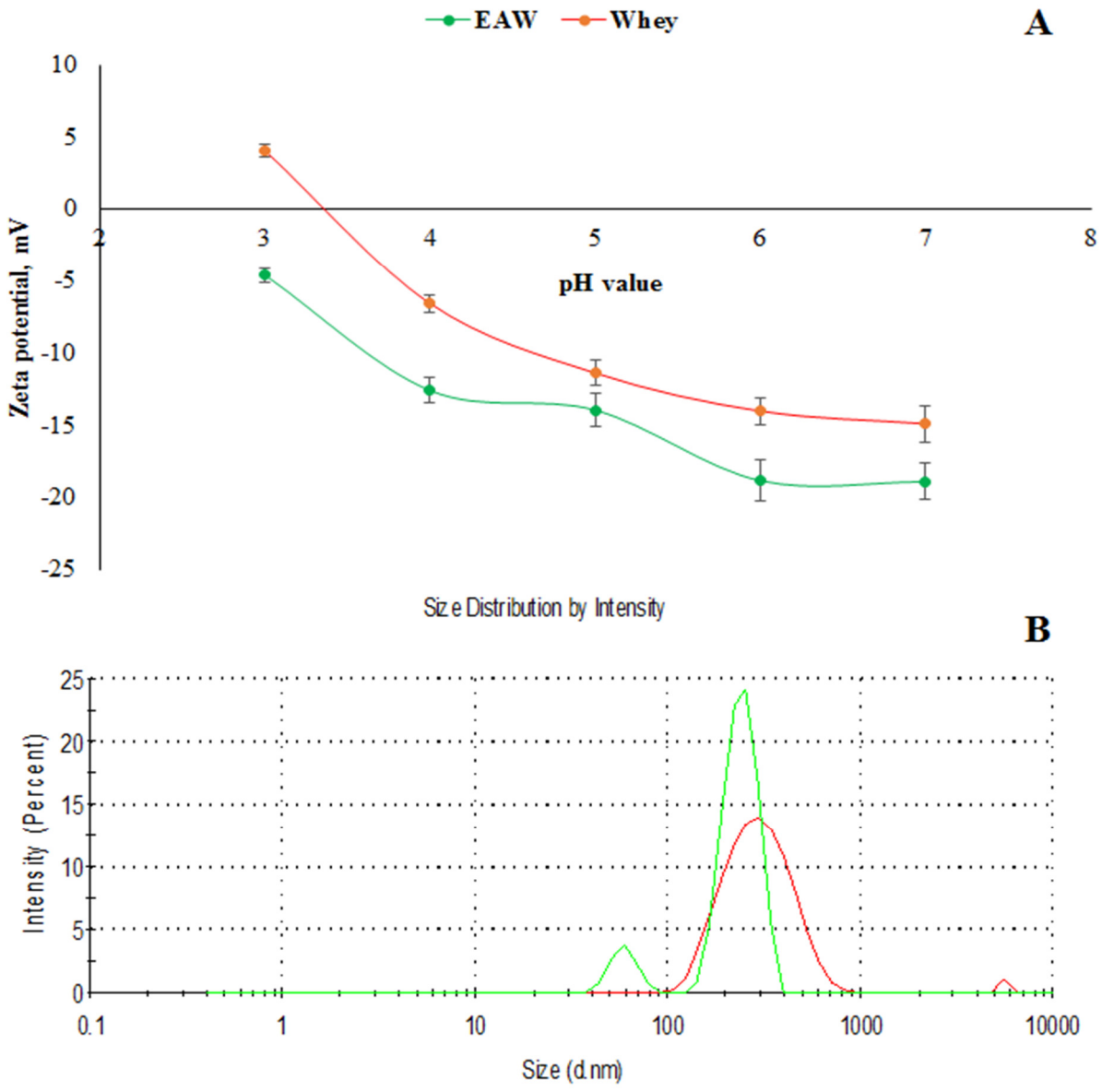


Figure 0-3: Zeta potential (A) and Particle size distribution (B) of electro-activated whey (EAW) (green line) and none-electro-activated whey (red line).

4.3.4. pH and titratable acidity during the fermentation process of kefir

Fermentation kinetics of kefir samples was assessed by measuring pH and titratable acidity every hour until pH of 4.6-4.7 was reached (visible gel formation) (**Figure 4.4**). The results showed a prolongation of the fermentation time at a supplementation level of 9% with

whey and electro-activated whey. Control kefir samples (without any supplementation) reached the desirable pH level after 9 h of fermentation. Compared to non-treated whey in the same concentrations, addition of 3% electro-activated whey retarded the lag phase of fermentation to 1 h and addition of 9% electro-activated whey by 10 h. In this context, it has been hypothesised that the buffering capacity of electro-activated whey could be the major factor contributing to maintaining stable pH in these samples, and that this buffering capacity was due to the induced modifications of proteins during the electro-activation process by forming high amount of ionisable acid or base groups responsible for the buffering capacity. The influence of the higher potassium content in electro-activated whey could also serve as a possible partial explanation of this prolongation. In the study of Kim et al. (2018b), high buffering yogurts were produced by addition of phosphate salts to milk (Kim, Oh, & Imm, 2018). As a result, pH drop was prolonged from 8 to 12 h. Another explanation of fermentation kinetics may underlie in water availability for kefir bacterial cultures. In the present study, statistical analysis to correlate the milk fat content with the milk acidification during the fermentation process indicated that the percentage of milk fat did not influence acid production and pH drop. Indeed, it was previously stated that milk fat globules do not interact with water thus do not influence water availability. However, addition of whey and electro-activated whey increases total solids amount, consequently reducing water availability (decreasing a_w). It was reported that for proper growth, kefir lactic acid bacteria require a water activity (a_w) above 0.95 (Goncu, Celikel, Guler-Akin, & Akin, 2017). Nonetheless, timespans of electro-activated whey enriched kefir samples comply with industrial production practices as 20-24 h fermentation period was reported by many authors (Bensmira, Nsabimana, & Jiang, 2010; Glibowski & Kowalska, 2012; Kök-Taş, Seydim, Özer, & Guzel-Seydim, 2013; Turkmen, 2017).

Total titratable acidity was the highest at biggest concentration of the added whey and electro-activated whey. These results were expected as availability of additional substrate promotes bacterial activity. Our findings are in accordance with the study of Gunenc et al. (2017) where kefir samples were produced with an addition of germinated and non-germinated wrinkled lentils. Authors reported higher titratable acidity levels at the end of fermentation of kefir with whole germinated lentils. This effect was also demonstrated to be substrate and concentration specific (Gunenc, Yeung, Lavergne, Bertinato, & Hosseinian, 2017).

The present work only envisioned to study the feasibility of addition of electro-activated whey into kefir in a comparative aspect with a none-electro-activated whey.

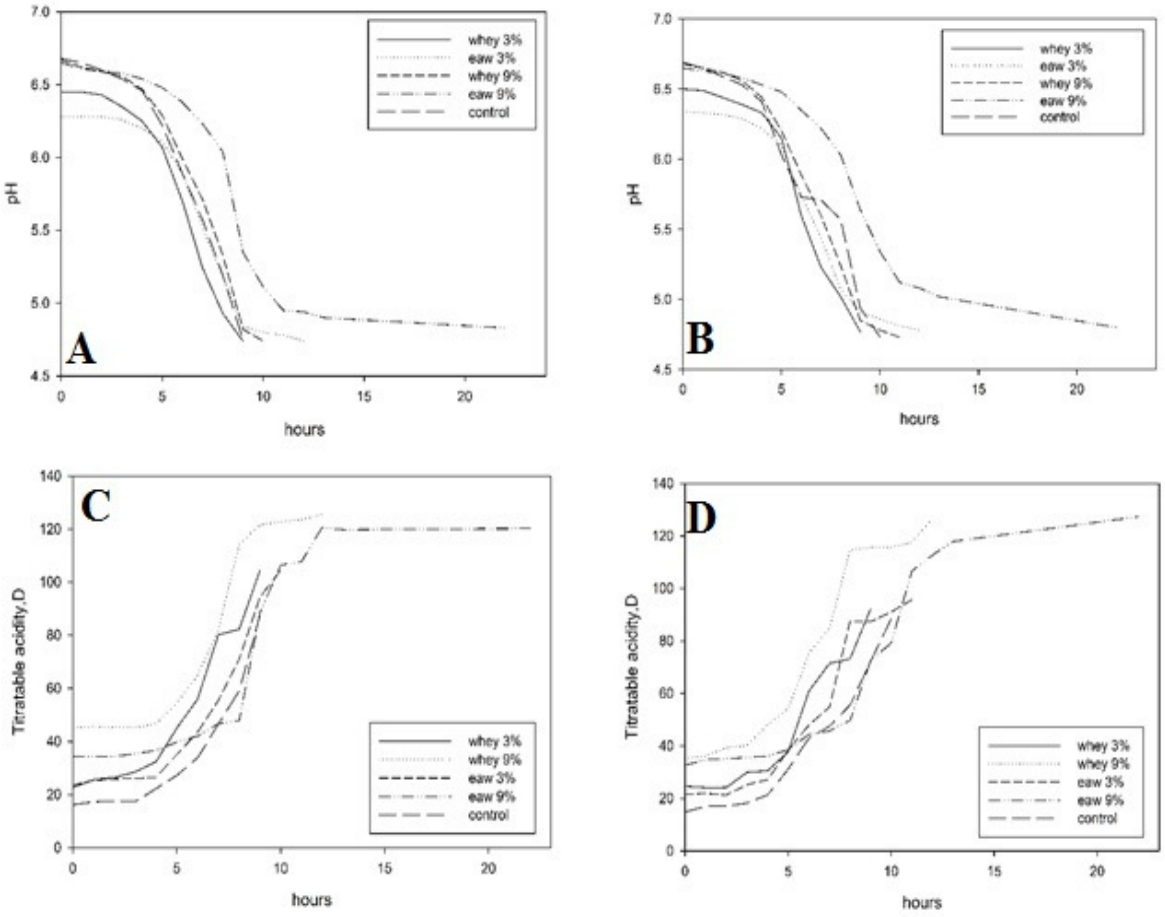


Figure 0-4: Fermentation kinetics of kefir samples with added electro-activated and non-treated whey. A: pH of 0% fat fermented milk. B: pH of 3% fat fermented milk. C: Titratable acidity of 0% fat fermented milk. D: Titratable acidity of 3% fat fermented milk.

4.3.5. Chemical composition of kefir samples

Addition of whey powder had increased total solids content in the supplemented kefir samples. Table 4.2 depicts an approximate chemical composition of different kefir samples. According to Russian Academy of Medical Sciences, defatted and full fat kefir should contain 91.4% and 88.9% of water. Our control samples (without addition of whey) comply with this requirement. Type of whey did not affect main components such as total solids, proteins, ash, and fat. Whey supplementation enhanced the levels of calcium, magnesium, phosphorus,

sodium, and potassium which comprise the main minerals found in whey, since whey contains almost all milk minerals among of which 2/3 calcium, 1/2 inorganic phosphate, 1/3 magnesium and around 10% of citrates are found in associated form with casein micelles. The remaining amounts are solubilized in a continuous aqueous phase, together with potassium, sodium, and chlorine (Gaucheron, 2005). Potassium and sodium are responsible for salt equilibrium of milk. Potassium and sodium phosphates and citrates regulate the amount of ionized calcium, which influences the size and stability of casein micelles.

Potassium amount in kefir samples was specific to the type of the added whey (**Table 4.3**). Electro-activated whey enriched the kefir samples with potassium which was 1.7 times greater than the supplemented kefir with non-treated whey and 4.7 times greater than control samples (non-supplemented kefir). This can be explained by the presence of the additional potassium ions which electro-migrated from the central compartment of the electro-activation reactor toward the cathodic compartment where whey electro-activation occurred. Indeed, potassium sulfate was used as conducting electrolyte in the central compartment during the electro-activation process as an electricity carrier and its dissociation together with the adjacent cation exchange membrane was favourable for its migration to the electro-activated whey. However, proximate analysis of the electro-activated whey supplemented kefir indicated that acceptable levels of potassium in foods were not exceeded. According to the American Society for Nutrition, an adequate potassium intake for people above 14 years old is 4.7 g/day. To compare, 100 g of dark chocolate, contain 3.5 times more potassium (830 mg) than the highest potassium level reached in our kefir samples (full fat kefir sample with 9% electro-activated whey) (Lanham-New, Lambert, & Frassetto, 2012). Furthermore, it has been reported that potassium supplementation to fermentation media has a positive effect on growth of several lactic acid bacteria (Boyaval, 1989; Russell & Diez-Gonzalez, 1998).This cation plays roles in bacterial osmotic regulation and cell homeostasis as well as in activation of intracellular enzymes. Acid-tolerant lactic acid bacteria utilize potassium as a counteraction for fermentation acid anions and regulation of internal pH. Thus, these bacteria maintain high internal potassium concentrations (Epstein, 2003).

Table 0-2: Proximate chemical composition of kefir samples, %.

Sample codes	Total solids	Total protein	Ash	Fat
0% fat milk + 3% whey	11.74 ± 0.13	4.00 ± 0.96	0.75 ± 0.35	0.10 ± 0.01
0% fat milk + 3% EAW	11.51 ± 0.14	3.57 ± 0.16	1.50 ± 0.01	0.10 ± 0.01
0% fat milk + 9% whey	16.61 ± 0.11	3.90 ± 0.47	1.50 ± 1.40	0.10 ± 0.01
0% fat milk 9% EAW	16.77 ± 0.15	3.96 ± 0.27	1.75 ± 1.01	0.10 ± 0.01
Control-1: 0% fat milk	9.56 ± 0.18	3.80 ± 0.95	1.00 ± 0.70	0.10 ± 0.01
3% fat milk + 3% whey	14.37 ± 0.17	3.45 ± 0.08	1.25 ± 0.35	3.00 ± 0.01
3% fat milk + 3% EAW	14.64 ± 0.11	3.45 ± 0.04	1.50 ± 0.70	3.10 ± 0.01
3% fat milk + 9% whey	19.91 ± 0.13	3.93 ± 0.54	1.25 ± 1.01	2.90 ± 0.01
3% fat milk + 9% EAW	19.51 ± 0.13	3.80 ± 0.09	1.75 ± 1.01	2.90 ± 0.00
Control-2: 3% fat milk	12.09 ± 0.25	3.10 ± 1.45	1.25 ± 0.35	3.25 ± 0.01

EAW: Electro-activated whey

Table 0-3: Mineral content of kefir samples (ppm) as a function of the amount of the supplementation level.

Samples	Minerals (ppm)						
	Ca	**Fe**	**K**	**Mg**	**p**	**Na**	**Zn**
0% fat milk + 3% whey	46.34 ± 1.00	0.06 ± 0.05	68.76 ± 1.53	5.16 ± 0.14	39.43 ± 1.78	23.07 ± 2.81	0.15 ± 0.00
0% fat milk + 3% EAW	46.30 ± 0.29	0.02 ± 0.00	110.26 ± 0.45	4.86 ± 0.02	37.61 ± 0.19	25.73 ± 1.28	0.17 ± 0.01
0% fat milk + 9% whey	58.52 ± 4.24	0.01 ± 0.00	133.06 ± 0.29	8.08 ± 0.43	51.47 ± 1.63	36.63 ± 3.19	0.17 ± 0.01
0% fat milk + 9% EAW	54.89 ± 0.95	0.05 ± 0.00	233.96 ± 4.47	7.22 ± 0.22	48.95 ± 0.85	48.37 ± 0.48	0.34 ± 0.03
Control-1: 0% fat milk	42.92 ± 0.85	0.03 ± 0.02	48.92 ± 1.29	4.05 ± 0.02	34.52 ± 1.42	18.99 ± 2.69	0.14 ± 0.01
3% fat milk + 3% whey	46.53 ± 1.54	0.02 ± 0.00	71.51 ± 0.67	5.31 ± 0.17	38.39 ± 0.51	22.67 ± 1.51	0.17 ± 0.02
3% fat milk	46.93 ± 0.19	0.03 ± 0.00	112.93 ± 3.61	5.21 ± 0.09	38.32 ± 0.23	27.09 ± 2.56	0.21 ± 0.01

+ 3% EAW							
3% fat milk + 9% whey	54.92 ± 0.84	0.04 ± 0.02	138.91 ± 4.09	8.03 ± 0.18	55.86 ± 3.56	41.13 ± 3.02	0.28 ± 0.11
3% fat milk + 9% EAW	54.24 ± 0.77	0.14 ± 0.06	235.32 ± 2.97	6.99 ± 0.1	55.62 ± 0.15	43.07 ± 0.61	0.18 ± 0.01
Control-2: 3% fat milk	41.65 ± 0.21	0.05 ± 0.04	50.40 ± 3.09	4.07 ± 0.12	34.85 ± 2.99	17.13 ± 0.71	0.17 ± 0.02

1

4.3.6. Kefir apparent viscosity

All kefir samples, except for the sample with 9% electro-activated whey, have demonstrated a non-Newtonian pseudoplastic behaviour with a shear thinning character ($R^2 > 0.99$). In Power law model, shear stress is equal to consistency coefficient multiplied by shear rate in the power of n, as shown in **Eq. 4.2** (Whittingstall, 2005).

$$\sigma = k.\dot{\gamma}^n \qquad \textbf{(Eq. 4.2)}$$

Where: κ is the consistency coefficient, $\dot{\gamma}$ is the (shear rate) ^{n}.

Table 4.4 represents apparent consistency coefficients of kefir samples made by using 0% fat milk and 3% fat milk supplemented with 3 and 9% whey or electro-activated whey (EAW). The shear rate range of 10-100 1/s represents shears associated with swallowing, chewing, and pouring a product (Mezger, 2014). Thus, the apparent viscosity at the closest shear rate point to 10 is given to demonstrate this parameter.

Table 0-4: Consistency coefficient and apparent viscosity of kefir samples.

Sample	Consistency coefficient ($Pa.s^n$)	Apparent viscosity at shear rate of 10.9 1/s (Pa.s)
Control kefir (3% fat milk)	2.30 ± 0.17^{a}	0.35 ± 0.07^{a}
Kefir from 0% fat milk + 3% whey	1.74 ± 0.15^{ab}	0.35 ± 0.07^{a}
Kefir from 0% fat milk + 9% whey	0.69 ± 0.59^{bc}	0.20 ± 0.14^{ab}
Kefir from 3% fat milk + 3% EAW	0.33 ± 0.19^{c}	0.05 ± 0.07^{ab}
Kefir from 3% fat milk + 9% EAW	0.005 ± 0.00^{c}	0.005 ± 0.001^{b}

EAW: Electro-activated whey

Like most fermented drinkable dairy products, kefir's technological process requires a stirring step. Mixing causes the breakage of some bonds and formation of casein aggregates leading to a phase separation upon aging (Paraskevopoulou, et al., 2003). Newtonian nature of the kefir supplemented with 9% electro-activated whey allows avoiding the stirring step

and keeping intact the product initial structure. To stabilize caseins in drinkable fermented dairy products from phase separation after stirring, several macromolecules such as gelatine and different polysaccharides such as guar gum, pectin, and xanthan gum are used. Some authors reported experiments with xanthan gum and they outlined that this macromolecule could stabilize casein micelles and fat globules and prevent the formation of strong gels (Paraskevopoulou, et al., 2003). Also, Kravtchenko et al. (2013) have noted that high methoxyl pectin in concentrations above the so-called ``critical pectin level`` prevents casein flocculation by steric repulsion forces (Kravtchenko, Parker, & Trespoey, 2013). In the case of the present study, interaction of electro-activated whey components (whey proteins or protein-carbohydrate conjugates) with milk caseins could hinder formation of a structure with high apparent viscosity.

4.3.7. Kefir color

Generally, milk has white color owing to the light scattering capacity of its colloidal system formed mainly of casein micelles. Electro-activated whey, however, has a light brownish-yellowish color as a result of the Maillard reactions occurring in the electro-activation reactor between reducing sugars (lactose, lactulose, and galactose) and proteins, peptides or free amino acids. In the study of the chemical properties and composition of electro-activated whey, Kareb et al. (2017) have reported the formation of Schiff bases, products of the intermediate steps of Maillard reactions (Kareb, Gomaa, Champagne, Jean, & Aïder, 2017). The authors have also indicated the antioxidant capacity of Schiff bases. Accordingly, this feature of the electro-activated whey shifts the samples with its addition closer to the H° index of 90°, which corresponds to the yellowish color. *L* value corresponds to the clarity or whiteness of the product, and higher scores indicate the color closer to standard white. In this case, control kefir samples without any addition of whey or electro-activated whey scored higher in the spectrum, followed by kefir supplemented with none-electro-activated whey. The saturation index C° was increasing respective to the concentration and the added whey or electro-activated whey, respectively, having the highest saturation for samples with 9% electro-activated whey, lower for kefir with 3% none-electro-activated whey, and the lowest in control samples without any supplementation (**Table 4.5**). Thus, these results indicate that addition of electro-activated whey to kefir to enhance its eventual health benefits by being a

good vector of a prebiotic lactulose is technologically feasible since its effect on the end product color was in the limit of the acceptance from appearance point of view.

Table 0-5: Color characteristics of kefir samples in L, C, H spectrum.

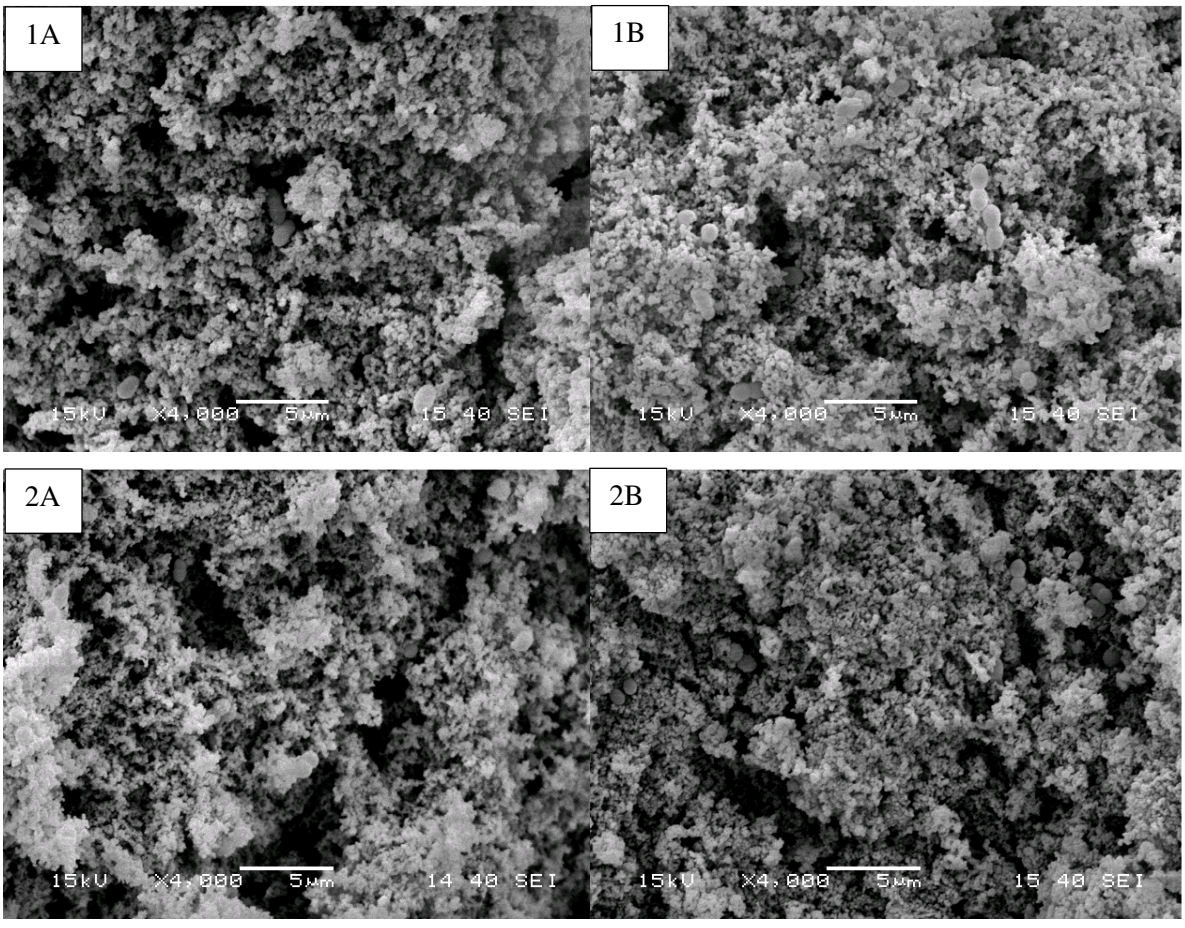
Kefir sample	*L*	*C°*	*H°*
0% fat milk + 3% whey	78.92 ± 1.23 [a,b]	3.63 ± 0.08 [e]	150.1 ± 1.8 [a]
0% fat milk + 9% whey	76.62 ± 0.08 [c]	4.92 ± 0.11 [d]	155.8 ± 1.0 [a]
0% fat milk + 3% EAW	77.31 ± 0.24 [c]	7.12 ± 0.13 [c]	114.6 ± 1.3 [b]
0% fat milk + 9% EAW	72.89 ± 0.12 [d]	13 ± 0.09 [a]	97 ± 0.1 [c]
0% fat milk	79.22 ± 0.61 [a,b]	2.76 ± 0.15 [e]	158.9 ± 0.3 [a]
3% fat milk + 3% whey	83.01 ± 2.08 [a]	5.79 ± 0.36 [c]	119 ± 3.4 [b]
3% fat milk + 9% whey	83.25 ± 0.52 [a]	6.18 ± 0.31 [c]	117.9 ± 0.8 [b]
3% fat milk + 3% EAW	82.68 ± 0.19 [a]	9.06 ± 0.15 [b]	99.3 ± 1.1 [c]
3%fat milk + 9% EAW	79.5 ± 0.23 [a,b]	14.31 ± 0.07 [a]	90.4 ± 0.1 [c,d]
3% fat milk	84.45 ± 1.17 [a]	5.34 ± 0.36 [c]	117.2 ± 1.4 [b]

EAW: Electro-activated whey

4.3.8. Kefir microstructure by scanning electron microscopy

The microstructure of the kefir samples as a function of the added whey and electro-activated whey is shown in **Figure 4.5**. In this study, electro micrograms obtained by scanning electron microscopy of kefir structure supplemented with electro-activated whey (EAW) demonstrated a less porous and more homogeneous structure than the kefir samples added with non-electro-activated whey (**Figs. 4.5-3A and 3B**). This microstructure can be attributed to the smaller particle size of electro-activated whey and their more homogeneous distribution which can easily fill in the pores of the formed kefir structure. Both kefir samples made by using EAW and made from milk with 0% and 3% fat content exhibited dense structures, indicating that addition of EAW can potentially serve as texture enhancer, particularly when defatted milk is used. Control and samples with 9% added whey had spongy structures with larger voids shown by the dark areas on the micrograms, which is common for milk gels (Marafon, et al., 2011; Ozcan-Yilsay, Lee, Horne, & Lucey, 2007). These voids contain the milk serum phase which is prone to be expelled and lead to phase separation or syneresis.

Several studies were conducted on microstructure of acid milk gels fortified with different components. Esperito-Santo et al. (2013) studied structural properties of a probiotic yogurt fortified with passion fruit (Espírito-Santo, et al., 2013). The results reveled more dense cross-links and less porous structure in fortified samples in comparison with control. The passion fruit fibers were firmly incorporated into casein gel matrix. Oppositely, Guggisberg et al. (2009) tested addition of a prebiotic inulin into acid milk matrix of defatted and whole milk. The addition of inulin in different concentrations (0, 1, 2, and 4%) did not visibly influence the microstructure. Samples with higher inulin concentrations had slightly bigger pores and less cohesive protein structure (Guggisberg, Cuthbert-Steven, Piccinali, Bütikofer, & Eberhard, 2009). Other studies have also shown that the use of whey protein in a dairy matrix has a simple filling role, which does not seem to be the case in the present study where it appears that the electro-activated whey is firmly inserted in the kefir network in a quasi-continuous phase.



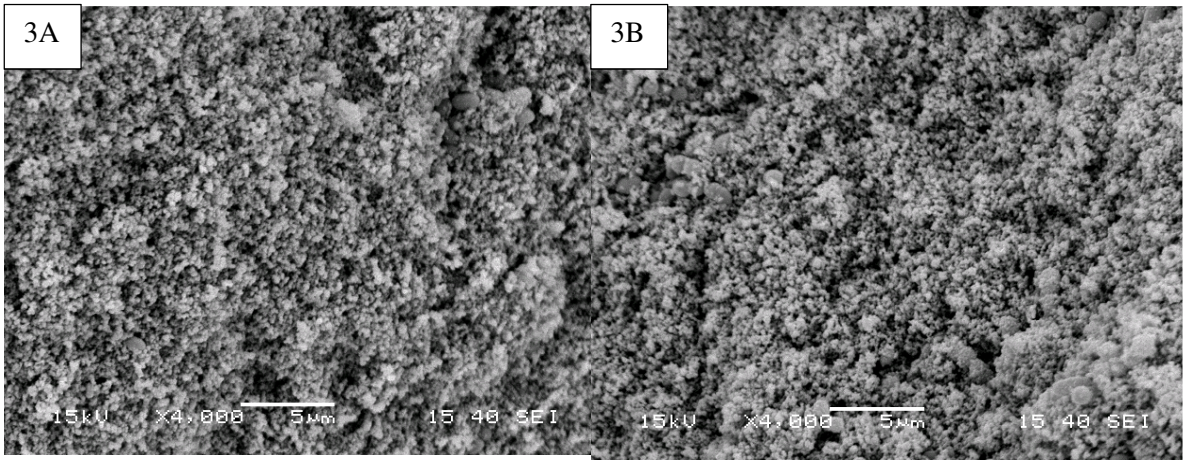


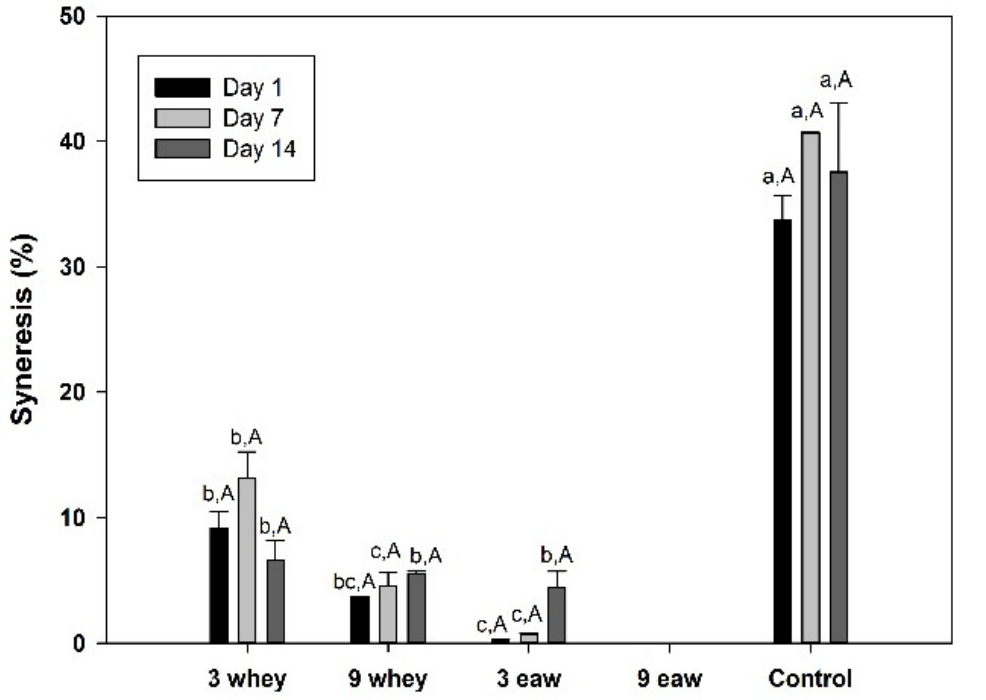
Figure 0-5: Scanning electron microscopy microstructure (x4000) of the kefir samples. A: 0% milk. B: 3% milk. 1: control. 2: Kefir with 9% whey. 3: kefir with 9% EAW

4.3.9. Syneresis of kefir samples

Syneresis or phase separation is one of the most apparent features for the consumer preference. It was previously reported that addition of hydrocolloids reduces syneresis in fermented milk products such as yogurt, kefir, and ayran (Beirami-Serizkani, et al., 2021).

In the present study, syneresis degree in 0% fat milk was significantly affected by addition of whey of both types (non-electro-activated and electro-activated), and their concentration ($p < 0.05$) (**Figure 4.6**), whereas the storage period did not significantly affect the syneresis. Samples supplemented with 9% electro-activated whey did not show any syneresis during all the tested storage period. Thus, they were not represented in the **Figure 4.6** since their syneresis value was zero, whereas the other samples (control and those supplemented by whey) showed different syneresis values. The same is applied to the 3% fat milk supplemented with 9% whey in the first day. Generally, 3% fat milk was less prone to syneresis due to the higher percentage of total solids (Yüksel & Erdem, 2010). The kefir samples made with 3% fat milk were not discriminated by the type of whey (electro-activated and none-electro-activated), but only its concentration. However, the difference from the control was noticed only on day 1 of storage. These findings corroborate with the previous study by Pourbaba et al. (2021) where physical-chemical properties of probiotics (*Lactobacillus acidophilus* LA-5, L. paracasei 431, *Bifidobacterium lactis* BB-12) and lactulose (2.5%, 5%) enriched novel kefir were examined (Pourbaba, Anvar, Pourahmad, & Ahari, 2021). The re-

sults revealed that higher concentrations of lactulose reduced syneresis due to higher fermentative activity in those samples. However, higher fermentative activity had a negative impact on the sensory properties of the kefirs by the increased acidity. Addition of isomaltooligosaccharide to 0% fat kefir in the study of Mei et al. (2017) had decreased the degree of syneresis 3-fold in comparison with control. Based on this and other characteristics, it was suggested that isomaltooligosaccharide in the concentrations less than 1% could serve as a fat replacer in 0% fat kefir (Mei, Feng, & Li, 2017). In our study, addition of electro-activated whey to defatted milk has also significantly reduced degree of syneresis in comparison with both controls. Thus, we suggest potential application of electro-activated whey as an enhancer of water holding capacity in 0% fat drinkable fermented dairy products such as kefir. The results of this study demonstrate a clear relation between electro-activated whey particle size, small porous microstructure and lower syneresis, in addition to the fact that in some kefir samples, addition of electro-activated whey permitted to completely avoid syneresis.



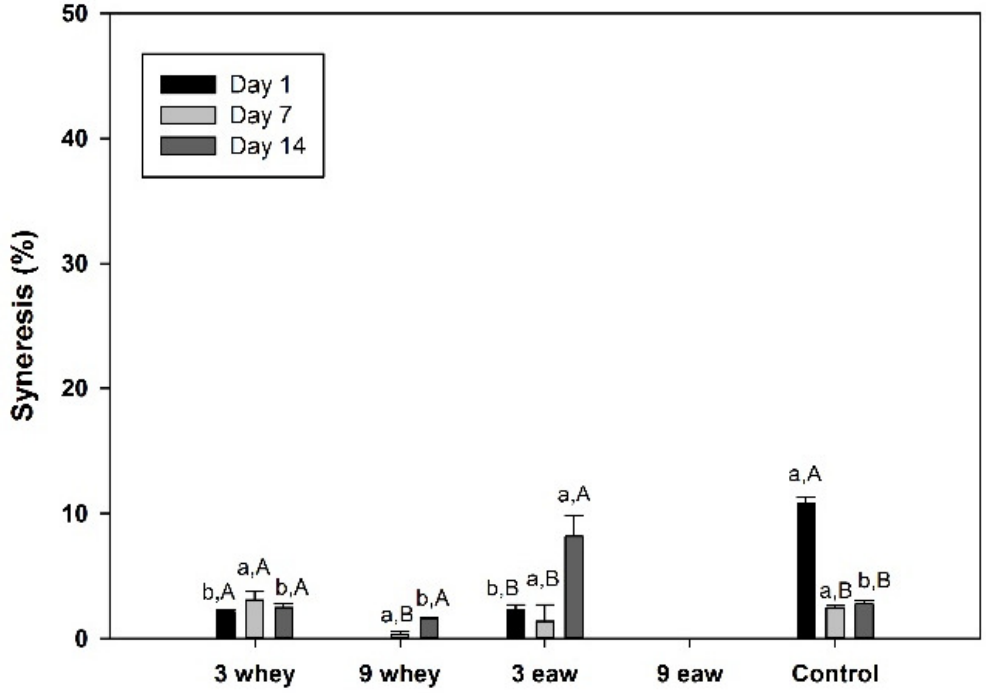


Figure 0-6: Degree of induced syneresis in kefir samples (first figure 0% fat milk, second figure 3% fat milk). Capital letters represent comparison between days for the same sample; lower case letters comparison between samples at the same day.

4.3.10. Total bacterial count and stability of bacteria during cold storage at 4 °C

Codex Alimentarius Commission guidelines for products containing lactic acid bacteria requires bacterial counts greater than 10^7 CFU per portion (generally of 100 g) (Rosa et al., 2017). In the present study, all the types of kefir complied with this requirement during the period of 2 weeks storage at 4 °C (**Figure 4.7 A,B**). The **Figure 4.7A** represents bacterial counts of kefir at 9, 12 and 22 h of fermentation. Even if a delayed pH decrease during fermentation in the kefir samples supplemented with EA whey was observed, higher bacterial counts at earlier hours of fermentation was recorded. The used kefir starter contains wide variety of microorganisms, including kefir yeasts, *Lactococcus lactis* subsp., *Leuconostoc* subsp., *Lactobacillus* subsp., and *Streptococcus thermophilus*. By this test, we assessed the growth of lactic acid bacteria. However, bifidogenic effect of lactulose-rich electro-activated whey could not be fully assessed, as prebiotics are more readily metabolised by probiotic bacteria rather than starter cultures. Delgado- Fernandez et al. (2019) studied the effect of addition of lactulose-derived oligosaccharides, galacto-oligosaccharides and lactulose in concentrations of 2 and 4% on the viability of probiotic bacteria in kefir samples (Delgado-Fernández, Corzo, Lizasoain, Olano, & Moreno, 2019). As a result, type and concentration of the prebiotics did not play a significant role in bacterial growth. The authors explain this observation by the presence of lactose in sufficient amount in the growth medium which is metabolised by the Embden-Meyerhof-Parnas pathway (EMP) by all the bacteria present in the kefir samples. Similarly, Pourbaba et al. (2021) did not find a significant difference in probiotic growth in novel kefir samples supplemented with 2.5 and 5% lactulose (Pourbaba, et al., 2021). Moreover, storage during 14 days did not affect survival of the probiotics, a result which is in good agreement with the findings of the present study (**Figure 4.7B**).

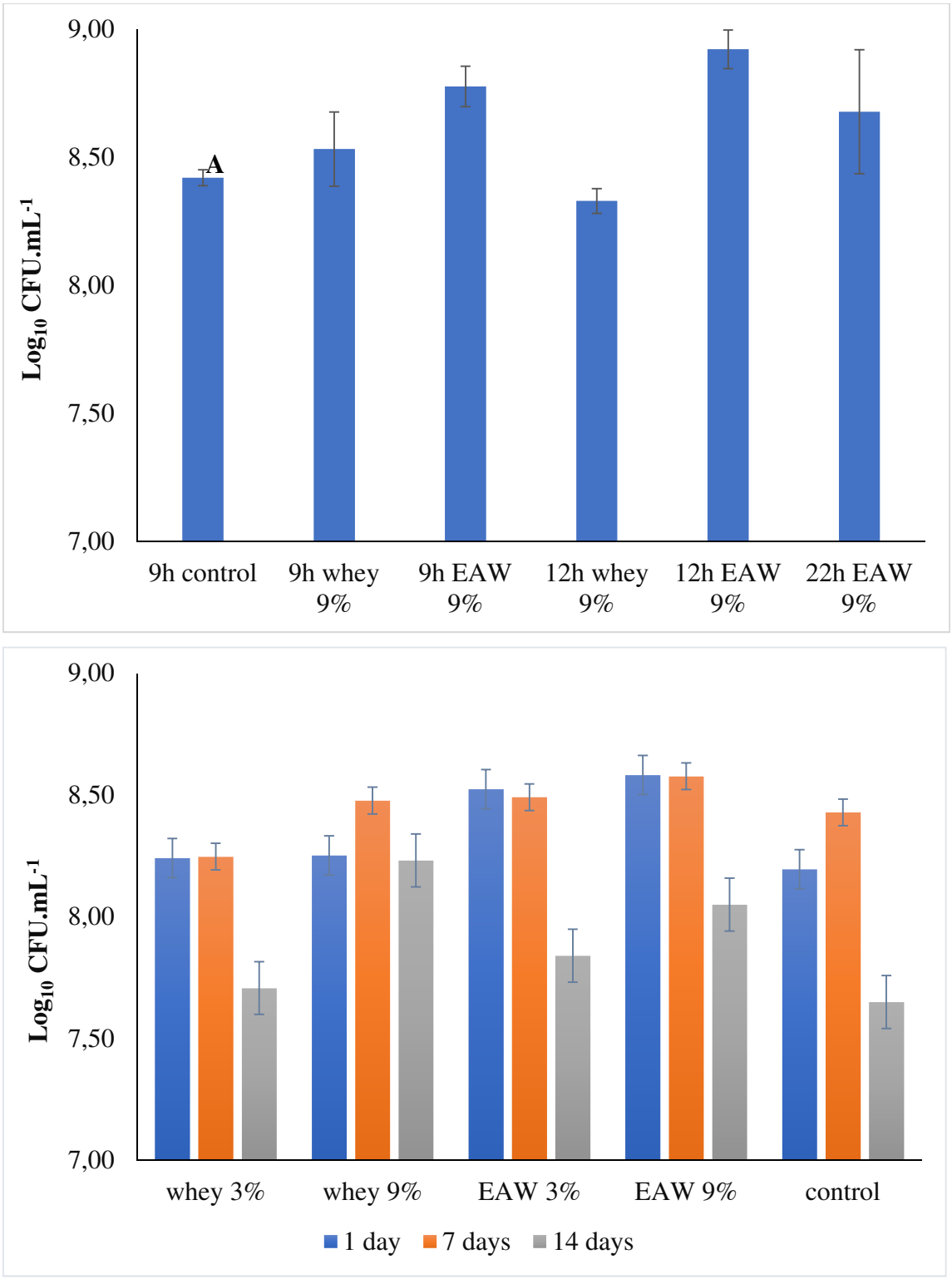


Figure 0-7: Lactobacilli counts (Log10 CFU.mL^{-1}) during fermentation (A) and during cold storage (B).

4.3.11. Organic acids produced during fermentation of kefir

The production of lactic acid, acetic acid, citric acid, butyric acid, and propionic acid by kefir cultures during milk fermentation is shown in Table 4.6. Organic acids such as lactic acid, propionic acid, acetic, and citric acids have an antimicrobial effect due to their liposolubility at acidic pH (Özcelik, Kuley, & Özogul, 2016). Samples containing electro-activated whey generated higher concentrations of lactic, acetic, and citric acids. A production of butyric acid was triggered by addition of whey and electro-activated whey. Acetic and lactic acids, in their turn, can be utilized by gut microbiota to produce butyric acid (Hernandez-Hernandez, et al., 2012). Butyric acid plays a special role in colon defense function and serves as an energy source for colonocytes, promotes production of mucins, antimicrobial peptides, and tight junction proteins that decrease epithelial permeability (Peng, Li, Green, Holzman, & Lin, 2009). In addition to that, butyric acid was reported to inhibit virulence of pathogenic bacteria by direct enhancement of its virulence genes (Guilloteau, et al., 2010). Multiple studies have also reported an anti-cancer properties of butyrate, which is reached by induction of apoptosis in human colonic carcinoma cells (Chen, Zhao, & Vitetta, 2019; Han, Bennett, Ahmed, Whelan, & Donohoe, 2018; McNabney & Henagan, 2017). Data shown in Table 4.6 demonstrates a high production capacity of three main metabolites of lactic acid bacterial fermentation in samples with electro-activated whey. This effect might be attributed to presence of lactulose in electro-activated whey which is a recognized prebiotic with growth and activity-stimulating effects. It was previously reported that supplementation of 2 g of lactulose increased short-chain fatty acid amount in the intestine (Ben Moussa, Boulares, Chouaibi, Mzoughi, & Hassouna, 2019).

Table 0-6: Amounts of short chain fatty acids in kefir samples during fermentation.

	Organic acids ($mg.L^{-1}$)		
Kefir sample	**Lactic acid**	**Acetic acid**	**Citric acid**
Control	2568.62 ± 30.77^{c}	1296.16 ± 192.06^{b}	821.02 ± 410.51^{b}
Kefir with Whey at 9 h	4430.00 ± 527.18^{bc}	2301.84 ± 59.73^{b}	1064.96 ± 14.21^{b}
Kefir with EAW at 9 h	7633.22 ± 193.19^{a}	4046.01 ± 11.40^{a}	1933.02 ± 37.99^{a}
Kefir with Whey at 12 h	4655.12 ± 78.24^{b}	2380.79 ± 53.49^{b}	1186.93 ± 117.25^{b}
Kefir with EAW at 12 h	7548.70 ± 701.93^{a}	4018.53 ± 448.92^{a}	2090.48 ± 30.27^{a}
Kefir with EAW at 22 h	7836.45 ± 59.20^{a}	4057.51 ± 194.36^{a}	1579.22 ± 255.63^{ab}

**mean values ± SE, Tukey HSD. EAW: Electro-activated whey. $p < 0.05$*

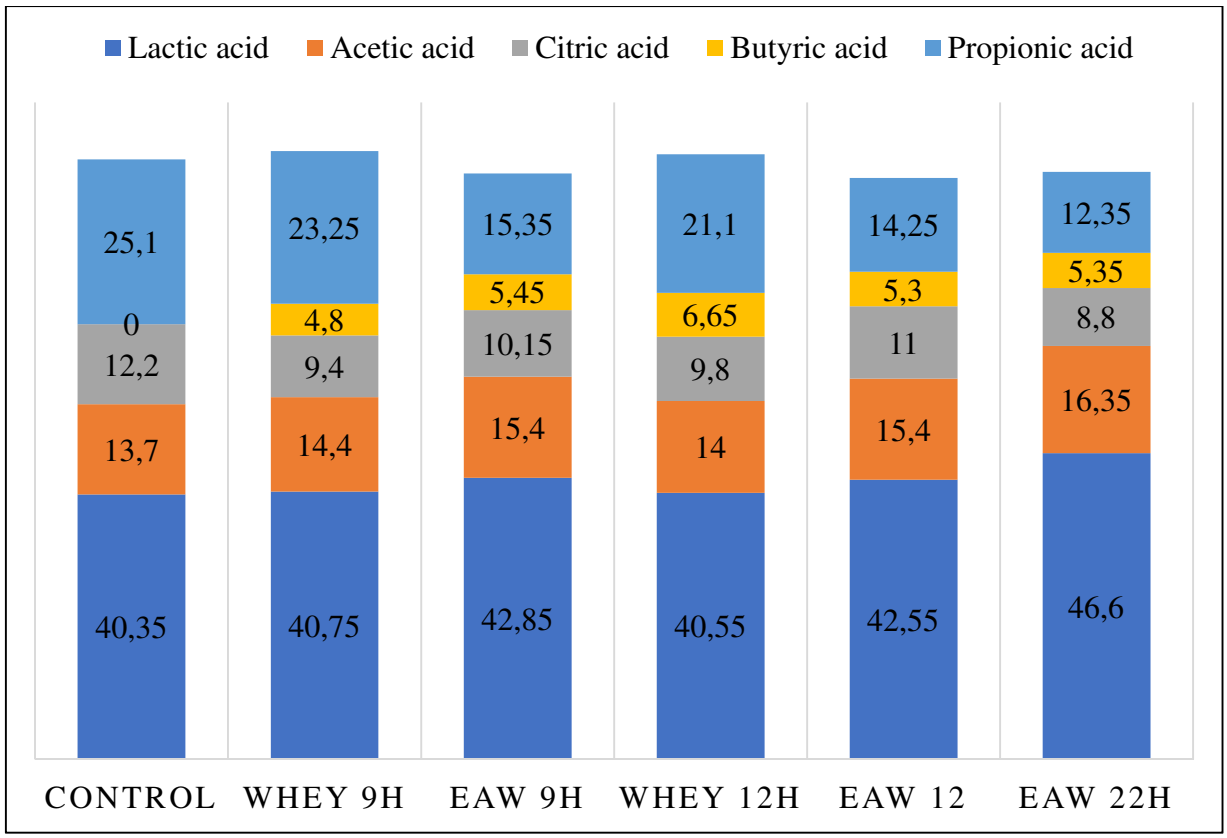


Figure 0-8: Percentage of organic acids produced during kefir fermentation process.

4.3.12. Volatile aroma compounds

Aroma compounds identified by GS-MS in kefir samples are given in **Table 4.7**. Kefir is a product of both homofermentative and heterofermentative fermentation by *Lactococcus lactis* subsp., *Leuconostoc* subsp., *Lactobacillus* subsp., *Streptococcus thermophilus* and kefir yeasts such as *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*. Homofermentative microorganisms metabolise lactose by Embden–Meyerhof–Parnas pathway to pyruvate. Further, the pyruvate serves as a substrate for the generation of 2 molecules of lactate per glucose unity and diacetyl with acetaldehyde from the remaining pyruvate. Heterofermentative fermentation is known to generate 1 molecule of lactate, ethanol, and CO_2 (Magalhães, et al., 2011). In this study, the volatile aromatic compounds did not vary between samples. Typical aroma components of fermented dairy products, such as acetoin, diacetyl, and alcohol were expressed in all the samples. Another product of heterofermentative pathway, acetic acid, had the highest peak in all trials. The presence of acetic acid in the fermented dairy beverages was shown to enhance antimicrobial capacities against pathogenic bacteria

in such products (Magalhães, et al., 2011). As shown in the previous section, the levels of citrate decreased in the end of fermentation of 9% EAW supplemented kefir samples. It could be utilized as a substrate for a production of acetoin and diacetyl by some lactic acid bacteria. Addition of whey affected formation of more caramel-like and sweet flavor. Contrary to other studies, (Beshkova, Simova, Frengova, Simov, & Dimitrov, 2003; Güzel-Seydim, Seydim, Greene, & Bodine, 2000; Kök-Taş, et al., 2013), no acetaldehyde was identified in this study. This can be due to the extraction method or by the difference in the starter cultures composition. According to Beshkova et al. (2003), the strain *L. bulgaricus* HP1 is principally responsible for acetaldehyde production in kefir samples (Beshkova, et al., 2003).

Table 0-7: Volatile aroma compounds in kefir samples.

Component name	Component type	Odor	Retention time (min)
Ethyl acetone (2-Pentanone)	Ketone	Brandy-like	17.163
Acetoin (2-Butanone, 3-hydroxy-)	Ketone	Woody, yogurty	31.712
Acetol (2-Propanone, 1-hydroxy-)	Ketone	Sweet	32.407
Diacetyl (2,3-Butanedione)	Ketone	Buttery	17.173
2-Cyclopentene-1,4-dione	Ketone	-	44.783
2-Cyclopenten-1-one, 2-hydroxy-	Ketone	caramellike	44.776
2-Propanone, 1-(acetyloxy)-	Ketone	Fruity	39.097
2-Furancarboxaldehyde, 5-(hydroxymethyl)-	Aldehyde	chamomile flowers	76.456
Furfural	Aldehyde	Sweet, burnt, nutty, fatty aroma, pungent	39.486
2-Furancarboxaldehyde, 5-methyl-	Aldehyde	Caramel- like	44.185
2(5H)-Furanone	Ether	Caramel sweet	51.474
Butyrolactone	Ether	Nutty, curry	46.837
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	Ether	Caramellike	68.038
2(5H)-Furanone, 3-methyl-	Ether	Maple, caramellike	44.488
2,5-Dimethyl-4-hydroxy-3(2H)-furanone*	Ether	Strawberry, caramellike	60.402
2-Pyrrolidinone, 1-methyl-**	Ether	fishy	48.992
2(5H)-Furanone, 5-methyl-	Ether	Caramellike	37.192
2-Furanmethanol	Alcohol	burnt, sweet, bitter	47.119
2-Cyclohexen-1-ol	Alcohol	Terpiniod aroma	53.17
Maltol	Alcohol	Malt, Toasted, Fruity	58.713
Formic acid	Acid	Pungent, vinegar	40.904
Acetic acid	Acid	Pungent, vinegar	38.524
2,3-Butanediol	Alcohol	Creamy	42.388

Urea, N,N-dimethyl-	Organic compound	-	62.891
Carbon dioxide		-	9.659
Cyclopentyl acetylene*	Aromatic hydrocar-bons	-	26.634

*Only whey (both types) added samples

** Only electro-activated whey samples

4.3.13. Residual lactulose in kefir

The levels of the residual lactulose after fermentation and during 2 weeks of storage at 4 °C were evaluated by HPLC-MS and the obtained results are shown in **Figure 4.9**. This result is important from the nutritional and functional point of view since lactulose enriched food products possess remarkable health benefits for the consumers. Indeed, lactulose is used to treat health disorders such as hepatic encephalopathy, liver cirrhosis, chronic constipation, and chronic kidney disease (Gluud, Vilstrup, & Morgan, 2016; Nooshkam, et al., 2018; Schuster-Wolff-Bühring, et al., 2010). It is also a well-recognized prebiotic used in infant formulae to promote the growth of bifidobacteria. Daily consumption of 2 g of lactulose increases the intestinal level of short chain fatty acids; ingestion of 5 g/day was reported to induce a bifidogenic effect and 7.5 g/day decreases ammonia levels in colon (Aguirre, Jonkers, Troost, Roeselers, & Venema, 2014; Ben Moussa, et al., 2019). In the present study, lactulose was introduced to kefir samples as a constituent of the electro-activated whey. Initially, the kefir samples made from 0% and 3% fat milk, and containing 3% EAW had an average of 6784 and 7240 mg/L of lactulose, respectively, which corresponds to the levels of lactulose in EAW-supplemented milk for kefir production. Kefir samples prepared with 9% EAW had 19907 mg/l and 20369 mg/L of lactulose at the beginning of fermentation. During the fermentation of kefir containing 3% EAW, 18% and 24% of lactulose was metabolised in the 3% fat and defatted milk samples used for kefir production. In samples with 9% EAW,

77% of initial lactulose remained in 0% fat milk, and 55% in 3% fat milk. Storage did not cause a sharp decrease of lactulose levels, which remained at levels of 2797 mg/L and 4179 mg/L in 3% EAW supplemented samples, and 11682 mg/L and 10198 mg/L in kefir containing 9% EAW. These findings suggest that lactulose is still available for conferring health benefits after consumption.

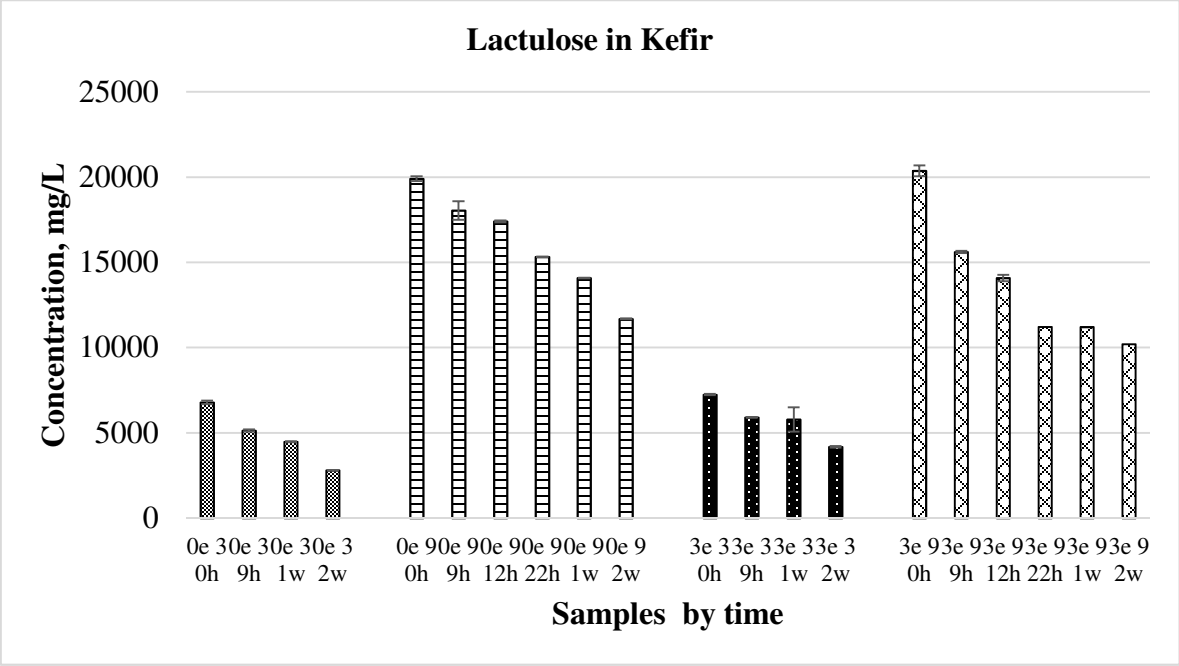


Figure 0-9: Concentration of lactulose in kefir samples during fermentation and storage. 0e3 : Kefir made from skimmed milk+ 3% EAW or 9% EAW; 3e3: Kefir made from full fat milk+ 3% EAW or 9% EAW;

4.4. CONCLUSION

In this study, the technological feasibility of incorporating electro-activated whey in the production of kefir was demonstrated. The obtained results permitted to demonstrate the proof of the concept of kefir production by using electro-activated whey as ingredient of supplementation. Moreover, kefir of adequate physico-chemical, structure, microbiological and volatile profile characteristics was successfully produced. This study demonstrated that supplementation of milk with electro-activated whey at 9% level permitted to completely eliminate syneresis during cold storage at 4 °C. Moreover, kefir with 3% electro-activated whey had a low viscosity and showed a non-Newtonian pseudoplastic behaviour with shear thinning character. From technological point of view, this characteristic is very important since it will be possible to produce drinkable kefir without need of any mechanical mixing, a fact which is favourable to save energy and to simplify the technological process. Scanning electron microscopy analysis showed a fine homogeneous microstructure. Microbiological analysis of kefir showed high bacterial count with at least 10^7 CFU/mL of kefir (corresponding to 10^9 CFU per portion of 100 g). GC analysis of the volatile compounds showed good flavour profile, characteristic of fermented dairy products. Moreover, HPLC analyses of the sugars during the storage period of kefir at 4 °C indicated that at least 50% of the initial lactulose, a proven prebiotic, was still detected in kefir after 14 days, suggesting that this product can be classified as functional kefir since it contains a proven and recognised prebiotic.

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CHAPTER 5: Production of Ryazhenka, a Traditional Ukrainian Fermented Baked Milk, by Using Electro-activated Whey as Supplementing Ingredient and Source of Lactulose

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RÉSUMÉ

Dans ce travail, le lactosérum électro-activé (LÉA) a été utilisé comme ingrédient dans la préparation du Ryazhenka, un lait cuit fermenté, à un niveau de supplémentation de 3 et 9%. La cinétique de fermentation, la viscosité, la couleur, la capacité antioxydante et les profils protéiques du produit final ont été étudiés. De plus, l'effet de l'électro-activation sur l'allergénicité de la lactoglobuline a été évalué par un test ELISA spécifique. Les résultats ont montré que l'ajout de lactosérum électro-activé comme ingrédient de complémentation est technologiquement faisable dans le processus de production de Ryazhenka. La cinétique de fermentation a montré que ce processus était dans l'intervalle technologiquement accepté même si l'ajout de lactosérum électro-activé retardait la phase de fermentation. L'étude de la viscosité a montré que l'ajout de LÉA a permis d'obtenir un produit final avec une bonne suivabilité correspondant à une bonne consistance de boissons laitières buvables. De plus, l'ajout de LÉA a empêché la synergie pendant le stockage réfrigéré à 4°C et a enrichi le produit en lactulose qui est un prébiotique reconnu. L'évaluation chromatique du produit final a montré une compatibilité de l' LÉA avec le Ryazhenka qui est connu pour sa couleur spécifique jaune-brunâtre. L'ajout de LÉA a amélioré de manière significative la capacité antioxydante du Ryazhenka avec des valeurs de IC_{50}DPPH = 2,54 ± 0,02 et IC_{50}PFRAP = 244,03 ± 28,83 pour le Ryazhenka produit en ajoutant 9% de LÉA au lait après le processus de cuisson et avant la fermentation. De plus, en utilisant un test ELISA spécifique à la β-lactoglobuline, cette étude a montré une diminution significative de l'allergénicité du lactosérum et de la β-lactoglobuline pure de 95%. Cette diminution a également été observée dans le Ryazhenka supplémenté avec 9% d'EAW mais à un niveau inférieur (66%). Ainsi, on peut conclure que le lactosérum électro-activé peut être considéré comme un ingrédient fonctionnel prometteur contenant un lactulose prébiotique comme ingrédient fonctionnel dans l'industrie laitière.

Mots clés : Lactosérum électro-activé; Ryazhenka; Lactulose; Prébiotique; Réduction de l'allergénicité.

ABSTRACT

Electro-activated whey (EA whey) was used as ingredient in the preparation of Ryazhenka, a fermented baked milk, at a supplementation level of 3 and 9%. Fermentation kinetics, viscosity, color, antioxidant capacity, and protein profiles of the final product were studied. Moreover, effect of electro-activation on β-lactoglobulin allergenicity was evaluated by a specific ELISA test. The results showed that addition of electro-activated whey as supplementing ingredient is technologically feasible in the process of Ryazhenka production. The fermentation kinetics showed that this process was in the technologically accepted interval even if the addition of EAW delayed the fermentation phase. The viscosity study showed that addition of EAW permitted to obtain a final product with good flowability corresponding to good consistency of drinkable dairy beverages. Moreover, addition of EAW prevented syneresis during the refrigerated storage at 4 °C and enriched the product with lactulose which is a recognised prebiotic. The chromatic evaluation of the final product showed a compatibility of EAW with Ryazhenka which is known for its specific yellowish-brownish color. Addition or EAW significantly improved the antioxidant capacity of the Ryazhenka with values of IC_{50}DPPH = 2.54 ± 0.02 and IC_{50}PFRAP = 244.03 ± 28.83 for the Ryazhenka produced by adding 9% EAW to the milk after the baking process and prior to fermentation. Moreover, by using a β-lactoglobulin specific ELISA test, this study showed a significant decrease of whey and pure β-lactoglobulin allergenicity by 95%. This decrease was also observed in Ryazhenka supplemented with 9% EAW but at lower level. Thus, it can be concluded that electro-activated whey can be considered as a promising functional ingredient containing a prebiotic lactulose as functional ingredient in the dairy industry.

Keywords: Electro-activated whey; Ryazhenka; Lactulose; Prebiotic; Allergenicity reduction.

5.1. INTRODUCTION

Ryazhenka is a fermented dairy drinkable product made from heated milk at 90-95 °C during 3-5 h. It is characterized by a creamy texture, pleasant caramel-like, umami, baked taste, and brownish color. It originates from Ukraine and is very popular among Commonwealth of Independent Countries of the former USRR (Rozhkova, Moiseenko, Glazunova, Begunova, & Fedorova, 2020). Even though a Russian version of this fermented drink carries a name Varenets, in scientific literature, Ryazhenka is commonly referred to as a Russian baked fermented milk or brown yogurt. According to Li et al. (2020), it was also introduced to Chinese market in recent years because of its health benefits and pleasant organoleptic properties (Li, Zheng, yu Kwok, Zhang, & Sun, 2020). Ryazhenka's peculiarity owes to its manufacturing process. More particularly, to the presence of the milk heating step prior to fermentation by common starter culture bacteria composed of a mixture of *Lactobacillus delbrueckii* subsp. *bulgaricus and Streptococcus thermophilus*. The heating at 90-95 °C for 3-5 h is commonly accepted to generate the specific smell, taste and texture of the fermented final product. According to Russian legislation the term Ryazhenka cannot be applied if heating of less than 3 h (GOST 31455-2012). Also, minimal duration of 3 h is necessary to obtain specific brownish color and cooked taste.

Whey is a valuable by-product of cheese industry. Its valorization is largely relied on production of whey protein isolates (90-98% protein) and whey protein concentrates (25-89% protein) by membrane filtration technologies such as microfiltration and ultrafiltration combined with a diafiltration procedure (Renhe & Corredig, 2018; Schopf & Kulozik, 2021). Indeed, whey proteins have a great nutritional value, high level of bioaccessibility and good techno-functional properties. With the protein efficiency ratio (PER) of 3.4 they outcompete caseins, whole egg protein, and plant-derived proteins such as soybean, peanut, corn, and wheat. Whey is rich in branched amino acids (L-isoleucine, L-leucine, and L-valine), sulfur-containing amino acids (cysteine, methionine) and several essential amino acids (lysine, threonine, methionine, isoleucine) (Ha & Zemel, 2003). Moreover, whey contains higher levels of lysine than whole milk proteins. Several authors have also reported antioxidant, antihypertensive, hypolipidemic, antiviral, and antimicrobial capacities of whey proteins (Madureira, Pereira, Gomes, Pintado, & Xavier Malcata, 2007; Patel, 2015; Saleh, El-

Garawany, Assem, & El-Shibiny, 2007). Nevertheless, the largest proportion of whey components is represented by lactose, which is often neglected or limited in terms of valorization. Lactose has low nutritive value, low sweetening power, low solubility and fermentability (Hettinga, 2019). In this regard, the recent development of valorization of whey and lactose through its *in situ* electro-chemical conversion to a prebiotic lactulose, a disaccharide composed of galactose and fructose, provides a promising alternative for whey valorization to a value-added ingredient possessing a prebiotic potential. This technology could lead to preparation of highly nutritious functional fermented dairy beverages containing whey proteins, lactulose, and remarkably lower in lactose load, due to its utilization during fermentation.

Electro-activation of aqueous solutions is based on subjecting the solutions to an external electric field under specific operating conditions and reactor configuration by using appropriate ion-exchange membranes. As result of appropriate electro-activation reactor configuration, excess accumulation of OH^- ions in cathodic compartment and H^+ ions in anodic compartment can be obtained, creating, respectively, highly alkaline (pH 10-13) and highly acidic (pH 2-3) media (Aider, Gnatko, Benali, Plutakhin, & Kastyuchik, 2012). The alkalinity in the cathodic compartment is a suitable medium for lactose isomerization into lactulose directly in situ of whey. Lactulose is a disaccharide (4-0-β-D-galactopyranosyl-D-fructofuranose) formed during the isomerization of the glucose moiety of lactose molecule into fructose (Nath et al., 2016). Chemical synthesis of lactulose can be achieved by two ways: (i) through the reaction of Lobry de Bruyn–Alberda van Ekenstein, which is characterized by the formation of the enolic intermediate shape of lactose and epilactose, and conversion of glucose moiety to fructose in alkaline media, and (ii) a reaction of lactose with ammonia or amines to form lactosylamine, which further undergoes an Amadori rearrangement and is hydrolysis to obtain lactulose (Aït Aissa & Aïder, 2013). Because Amadori conversion is a reaction which is difficult to control (synthesis of intermediates and by-products of the reaction, mainly aminocarbonyl compounds) the first way is the common industrial practice. However, even the common alkaline lactulose synthesis practices have considerable drawbacks. They depend on type of the chemical catalyst used (hydroxides, sulfites, phosphates, aluminates, borates), and generally include the requirement in high concentrations of the latter and hot-alkaline conditions. As a consequence, the following steps of processing are required: acidification, decolorization, demineralization, and separation (Nooshkam,

Babazadeh, & Jooyandeh, 2018). Moreover, this way of lactulose synthesis needs pure lactose as a raw material. As opposed to that, electro-activation permits isomerization of lactose directly in bulk solution of whey. Alkaline media created by electro-activation of whey solution can be considered as a reagent-free method of lactulose formation through Lobry de Bruyn-Alberda van Ekenstein pathway (LA-rearrangement) since there is no need of using catalysts. The main requirement for LA-rearrangement is the presence of proton acceptors which is achieved directly in the cathodic compartment of the electro-activation reactor where high concentration of OH^- ions serve this purpose. The efficacy of lactulose synthesis by electro-activation directly in whey solution has been proven by several studies (Kareb, Champagne, & Aïder, 2016; Karim & Aider, 2020). Also, according to Aït-Aissa and Aider (2013), electro-activation method is superior to chemical isomerization not only in terms of eco-efficiency, but it can also produce higher yields through the excess internal potential energy of the activated solution for which the required activation energy for the isomerization reaction is significantly lowered in comparison with chemical method (Aït Aissa & Aïder, 2013). Also, from the technological point of view, the addition of electro-activated whey to milk as a functional ingredient in the process of fermented dairy products production was previously studied by Aidarbekova and Aider (2021a,b). These studies demonstrated that the levels of residual lactulose remaining in the products after 2 weeks of cold storage at 4 °C storage is up to 9 g/L in a fermented milk and 11 g/L in kefir, which proves the concept of the functionality of these products since lactulose is a well-recognised prebiotic (Aidarbekova & Aider, 2021a, 2021b).

Hence, the aim of this study is to prove the feasibility of using electro-activated whey as a functional ingredient rich in lactulose and having high antioxidant capacity in the technological process of Ryazhenka production. Also, specifically, this study is aimed to validate the hypothesis that electro-activation treatment of whey can significantly reduce its allergenicity by affecting the involved fraction, β-lactoglobulin, in this nutritional concern.

5.2. MATERIALS AND METHODS

5.2.1. Electro-activated whey preparation

Commercial whey (Agropur Cooperative, Quebec, Canada) was dissolved in distilled water to prepare a 10% (w/w) solution. This solution was electro-activated in a cathodic compartment of the used electro-activation reactor under an electric current intensity of 800 mA for 60 min. The anodic compartment was filled with 0.1 M K_2SO_4, and the central compartment with 0.25 M K_2SO_4 solutions, respectively. After 60 min of electro-activation, the resulting electro-activated whey was left at ambient temperature (23 ± 1 °C) during a relaxation period of 48 h and then neutralized to pH 7. After that, the neutralized electro-activated whey was frozen at -40 °C for a subsequent freeze-drying to obtain a powder which was used in the present study. Three batches of powders were used. The detailed procedure of whey electro-activation is described in the following studies (Aidarbekova & Aider, 2021a, 2021b).

5.2.2. Preparation of Ryazhenka

For the process of Ryazhenka production, a 2% fat UHT commercial milk (Grand pre, Quebec, Canada) was used without any supplementary treatment since it is a sterilized milk with a standardized fat content. The first step consisted of heating milk up to 95 °C and its maintaining at this temperature during 4 h in a water bath (OLS 200-1, Grant Instruments, UK). Milk was then cooled down to 42 °C and a thermophilic starter culture containing *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* was added as indicated by the manufacturer in the amount of 60 mg/L (Chr. Hansen YF-L812, Fromagex, Rimouski, Quebec, Canada). Electro-activated and non-electro-activated whey were added in the concentrations of 3 and 9% (w/w). The fermentation process was carried out at 40 °C in a thermostatic incubator (Jeio tech, MA, USA). After the fermentation completed, the samples were gently stirred and stored at 4 °C. The residual lactulose in Ryazhenka supplemented with electro-activated whey (EAW), as indicator of the prebiotic character of the product, was determined by HPLC on a Waters system equipped with refractive index detector according to the method of (Kareb et al., 2016).

5.2.3. Fermentation kinetics

The fermentation process was monitored by measuring the pH and titratable acidity

which considered as the parameters reflecting the fermentation kinetics. Oakton 7000 pH-meter equipped with a pH-electrode was used in these tests (Oakton Instruments, Vernon Hills, IL, USA). A standard AOAC 947.05 method for measuring titratable acidity in milk products was used as described in (Fabro et al., 2006). Measurements were taken every hour until the desired final pH was reached (pH 4.6 ± 0.1).

5.2.4. Apparent viscosity and syneresis during refrigerated storage

Apparent viscosity of the Ryazhenka samples was measured by using AR-G2 rheometer (TA instruments, DA, USA). Peltier steel DIN concentric cylinder with a diameter of 28 mm and length of 42 mm, and cup diameter of 30.4 mm were used. The temperature of the samples was 18 °C, which corresponds to average temperature of product consumption. The samples were subjected to shear stress linearly from 0.03 to 30.0 1/s. Results were interpreted by a Power law model. Consistency indices were taken as apparent viscosity parameters (Aidarbekova & Aider, 2021a).

Syneresis of the Ryazhenka during the 14 days refrigerated storage at 4 °C was determined by using the following procedure: 15 g sample of the fermented Ryazhenka samples supplemented with whey and electro-activated whey (EAW) was weighted with high precision on analytical balance and centrifuged at 350xg during 10 min at 4 °C. After that, the separated whey was carefully sucked with a syringe and weighted. The total syneresis was calculated in % as the ratio of the separated liquid fraction to the initial sample weight (Dönmez, Mogol, & Gökmen, 2017).

5.2.5. Determination of Potassium Ferricyanide Reducing Antioxidant Power (PFRAP) of Ryazhenka

The reducing power of Ryazhenka was measured as follows: samples of 1 mL with the concentrations 1, 5, 10, 20, and 25% were mixed with 2.5 mL of 0.2 mole/L sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide (VWR, Montreal, Canada). The mixture was incubated in water bath at 50 °C for 20 min and cooled to ambient temperature (22 ± 1 °C). Further, 2.5 mL of 10% trichloroacetic acid was added (Fisher-Canada, Ottawa, Canada). The mixture was centrifuged at 5000 × g for 10 min. Then, 2.5 mL of supernatant was mixed with an equal volume of distilled water and 0.5 mL of 0.1% ferric

chloride (VWR, Montreal Canada). Time of incubation of the reaction mixture was set to 10 min. The absorbance at 700 nm with a Bio-Rad xMark microplate spectrophotometer (Agilent Technologies, Palo Alto, CA, USA) was recorded (Kareb, Gomaa, Champagne, Jean, & Aïder, 2017).

5.2.6. Determination of 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity of Ryazhenka

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of Ryazhenka was measured according to Kareb et al. (2017) as follows: 0.1 mM DPPH solution was freshly prepared in methanol by dissolving 3.94 mg of DPPH powder in 100 mL of methanol. Then, 250 µL of the Ryazhenka samples in the concentrations of 1, 5, 10, 20, and 25% were added to 1 mL of DPPH solution. The mixtures were vigorously shaken and left in a dark place at ambient temperature (22 ± 1 °C) for 30 min. Then, the mixture was centrifuged at 5000 × g for 10 min. The absorbance of the supernatant at 517 nm was measured using a Bio-Rad xMark microplate spectrophotometer (Agilent Technologies, Palo Alto, CA, USA). Trolox standard curve was prepared for quantitative results (Kareb et al., 2017).

5.2.7. Determination of chelating activity on Fe^{2+} of Ryazhenka

Ferrous ions (Fe^{2+}) chelating activity of Ryazhenka was tested as described by Kareb et al. (2017) with some modifications. Briefly, 250 µL of Ryazhenka sample was diluted in 2.5 mL distilled water. Solutions of ferrozine and $FeCl_2$ were prepared by dissolving 24.6 (ferrozine) mg and 2.52 mg ($FeCl_2$) in 10 mL distilled water. Diluted Ryazhenka samples were then mixed with 50 µL of 2 mM $FeCl_2$ and 100 µL of 5 mM Ferrozine and incubated for 10 min at ambient temperature (22 ± 1 °C). After, the mixture was centrifuged at 5000 × g for 5 min and the absorbance at 562 nm was measured using a Bio-Rad xMark microplate spectrophotometer (Agilent Technologies, Palo Alto, CA, USA). Controls were performed by the addition of distilled water instead of a Ryazhenka sample. The following formula (**Eq. 5.1**) was used to interpret the results in a % of $FeCl_2$ chelating activity. An average of three measurements were taken (Kareb et al., 2017).

$$Fe^{2+}_{chalating\ ability} = \frac{A_{control} - A_{sample}}{A_{control}} \cdot 100\% \quad \textbf{(Eq. 5.1)}$$

Where: $A_{control}$ and A_{sample} are the absorbance of the control and experimental sample, respectively.

5.2.8. Measurement of browning intensity of Ryazhenka

Amount of intermediate and final products of Maillard reaction products of Ryazhenka samples were assessed by measuring the absorbance at 294 and 420 nm using a Bio-Rad xMark microplate spectrophotometer (Agilent Technologies, Palo Alto, CA, USA). Samples were diluted 10-fold and 200-fold with 100 mM phosphate buffer with a pH 7.4.

5.2.9. Colorimetry

Colorimetric analysis was conducted by using a Colorimeter Minolta CR-300 (Konica Minolta sensing Americas, USA) in the L*a*b scale absolute chromaticity measuring system. Where L* is the lightness index, a* is the red-green index, and b* is the blue-yellow index. The ΔE parameter representing the total color difference between the samples was calculated by the following formula **(Eq. 5.2):**

$$\Delta E = \sqrt{(L2 - L1)^2 + (a2 - a1)^2 + (b2 - b1)^2} \qquad \textbf{(Eq. 5.2)}$$

5.2.10. Sensorial test

A total of 30 semi-trained panelists were selected to perform sensorial tests on novel Ryazhenka product with an addition of electro-activated whey. Five samples with different whey and electro-activated whey concentration were blinded with three-digit codes. Consumer acceptance test using hedonic scale of 9 points was used. Tested parameters were aroma, color, taste, consistency, and overall appreciation. The selected parameters were scored as follows (from 1 to 9):

(1) Extremely dislike. (2) Very much dislike. (3) Moderately dislike. (4) Slightly dislike. (5) Neither dislike nor dislike. (6) Slightly like. (7) Moderately like. (8) Like very much. (9) Extremely like.

5.2.11. Proteins profile analysis by SDS-PAGE electrophoresis

To study the protein profile of Ryazhenka samples, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with and without β-mercaptoethanol (β-ME)

(reducing and non-reducing conditions) was performed using mini-protein electrophoresis system (Bio-Rad Laboratories, Hercules, Canada) according to the Laemmeli method. Briefly, the 20% (w/v) diluted Ryazhenka samples were mixed in a 1:1 ratio with the buffer (with and without addition of β-mercaptoethanol). The mixtures were heated at 95 °C for 5 min and loaded into the gels. The electric current was adjusted at 30 mA (per 2 gels). Next step, the separated proteins bands were left in coloration solution (Coomassie Brilliant Blue R- 250) for 1 h, followed by the initial decoloration for 30 min, and secondary decoloration overnight.

5.2.12. Analysis of free amino acids by UPLC-UV florescence

The free amino acids composition of the non-treated whey and electro-activated whey (EAW) was carried out by the method of derivatization with 6-aminoquinolyl-N-hydroxy-succinimidyl carbamate. The Waters Acquity UPLC system (Milford, MA) was used and it equipped with UPLC AccQ·Tag Ultra Column (2.1 mm × 100 mm) and with an Acquity tunable UV and fluorescence detectors as described in an AccQ·Tag Ultra kit. The analysed samples were centrifuged during 5 min at 4500× g under refrigerated conditions at 4°C. The standards and analysed samples derivatization of amino acids were prepared by mixing 60 µL of AccQ·Tag borate buffer, 10 µL of samples, 10 µL of internal standard (Norvaline at 100 µM), and 20 µL of AccQ·Tag. Ultra-pure reagents were added into a recovery vial subsequently. The mixture was vortexed immediately for 5 s and then allowed to rest for 1 min before heating at 55°C during 10 min. Five microliters of the solution obtained was injected into the Waters HPLC system and monitored using both UV and florescence set at 260 and 473 nm, respectively (Kareb et al., 2016).

5.2.13. Allergenicity test by ELISA

For this test, whey was freshly prepared from 0% ultrafiltered milk following acid precipitation of casein and centrifugation of the whey at 20 000 x g during 30 min to eliminate residual casein. A 0.3% β-lactoglobulin solution was prepared by dissolving β-lactoglobulin powder in distilled water. After that, these solutions were electro-activated and neutralized by the same procedure of electro-activated whey preparation. Then, none-electro-activated fresh whey, electro-activated whey (EAW) without neutralization (EAW at pH 11), electro-

activated neutralized whey (pH 7) solutions and Ryazhenka samples were analyzed for allergenicity. RIDASCREEN®FAST β-lactoglobulin kit (R-Biopharm, Darmstadt, Germany) for sandwich enzyme immunoassay for quantitative determination of β-lactoglobulin was used to measure the allergenicity of the aforementioned samples. All samples were prepared using allergen extraction buffer containing 15 mL of additive 1 (A-AEP) and extractor 2. Then, 1 mL of the tested sample was mixed with extractor 2 and heated for 10 min at 100 °C in water bath. Then, 15 mL of A-AEP heated to 60 °C was added to the samples. ELISA was performed in microwells with 100 µL of each standard and sample in duplicate. Wells were incubated for 10 min at ambient temperature (22 ± 1 °C) and washed thrice with a provided in the used kit wash buffer. After that, 100 µL conjugate added to the wells, followed by a 10 min incubation and triplicate wash steps. Wells with 100 µL of chromogen were incubated in the dark for 10 min, after which 100 µL of stop solution was added to the wells. Readings were done on Bio-Rad xMark microplate spectrophotometer (Agilent Technologies, Palo Alto, CA, USA) and were performed within 10 min after the addition of the stop solution.

5.2.14. Statistical tests

Experiments we performed in triplicate. Average ± standard deviation (SD) indices are given in the tables. Completely randomised ANOVA and Tukey's HSD test were performed to define significant difference. Statistix 9 software was used to perform statistical tests.

5.3.RESULTS AND DISCUSSION

5.3.1. Fermentation kinetics

In this study, fermentation kinetics was drawn based on the rate of acidification of milk samples reflected by two parameters: pH (**Figure 5.1**) and total titratable acidity (**Figure 5.2**). Addition of electro-activated whey (EAW) increased initial pH of the samples (0 h) as it has a neutral pH 7, whereas the addition of the non-electro-activated commercial whey acidified the initial media. To avoid this bias, **Table 5.1** represents the drop of the pH unit values of the different fermenting samples per hour of fermentation. Even though, the fermentation time of 3% electro-activated whey added samples lasted 1 hour longer than 3% whey, it can be seen that the pH of the former dropped for 1.45 units and the latter for 1.39 units at 3 h of fermentation. The similar trend is observed when 9% EAW and 9% whey supplemented samples are compared. Thus, the changes of initial pH of the solution could have an influence in prolongation of the lag phase during the fermentation of Ryazhenka samples with electro-activated whey.

Aidabekova and Aider (2021) have previously outlined the extended fermentation time of fermented milk following an addition of electro-activated whey (Aidarbekova & Aider, 2021a). As opposed to fermented milk, Ryazhenka is suggestibly a more suitable matrix for electro-activated whey, due to the affinity in denatured whey proteins, lactose hydrolysis, and partial lactulose formation, as well as the presence of Maillard reaction products in both heated milk and electro-activated whey. Influence of Maillard reaction on gelation of whey proteins was described by (Spotti et al., 2019) in the study of gelation of WPI/dextran complex with induced Maillard reaction products. The authors reported an inhibition of gel formation and weaker gels due to Maillard reactions. It was attributed to reduction of surface hydrophobicity of the conjugated systems. Electro-activated whey can also be described as a conjugated system between whey proteins and different sugars such as lactose, lactulose, and galactose.

Another point to consider in discussing the prolonged fermentation time when electro-activated whey (EAW) was added to milk prior to its fermentation is the higher buffering capacity of EAW. Buffering capacity of milk is recognized to be an effect of compounds with one or several acid-base groups, such as phosphates, citrates, lactates, carbonates, acetates,

and propionates. The presence of several acidic and basic amino acids also ensures a buffering capacity of whey proteins at pH 3-4 and pH 8, respectively (Salaün, Mietton, & Gaucheron, 2005). Also, Pescuma et al. (2010) suggested that fermented milk enriched with whey protein concentrates improved the post-acidification stability due to high buffering capacity of whey proteins (Pescuma, Hébert, Mozzi, & Font de Valdez, 2010). Moreover, Kareb et al. (2018) has reported higher buffering capacity of whey after electro-activation. This phenomenon can be described as following: in electro-activated solutions, catholyte absorbs CO_2 from the atmosphere and forms carbonates and bicarbonates. Carbonate buffering is prevalent in human bloodstream, water-containing materials, and soil. Thus, subsequent decarbonization of electro-activated solution, or electro-activation in a closed reactor could solve the problem of high buffering capacity of electro-activated whey products (Kareb, Champagne, Jean, Gomaa, & Aïder, 2018).

Production of free acids was superior in Ryazhenka samples supplemented with 9% whey of and electro-activated whey (EAW): whey 9% 113.09 ± 4.98, EAW 9% 113.73 ± 7.95. These results are related to the increased dry matter and substrate availability for bacteria. Commonly, 1 molecule of glucose forms 2 molecules of lactic acid during a homo-fermentation process by starter cultures (Prado et al., 2015). According to Russian food standards (GOST 31455-2012), variation of titratable acidity levels in Ryazhenka products vary between 70 °D and 110 °D. The obtained results in the present study demonstrated that the difference in levels of titratable acidity at 4 h of fermentation were insignificant for 3% (85.15 ± 1.04) and 9% EAW (86.31 ± 3.59) supplemented Ryazhenka. As 4 h represent the end of the fermentation of a 3% EAW supplemented samples, this observation confirms hypothesis of the effect of the high buffering capacity of the samples with higher concentration of EAW added.

Table 0-1: The units of pH drop during the fermentation process of Ryazhenka samples.

Fermentation time, h	Whey 3%	Whey 9%	EAW 3%	EAW 9%	Control
0	0.00	0.00	0.00	0.00	0.00
1	0.21	0.11	0.22	0.15	0.28
2	0.56	0.29	0.52	0.27	0.72
3	0.61	0.63	0.71	0.47	0.53
4		0.27	0.24	0.34	
5				0.26	
6				0.21	
7				0.09	

EAW: Electro-activated whey.

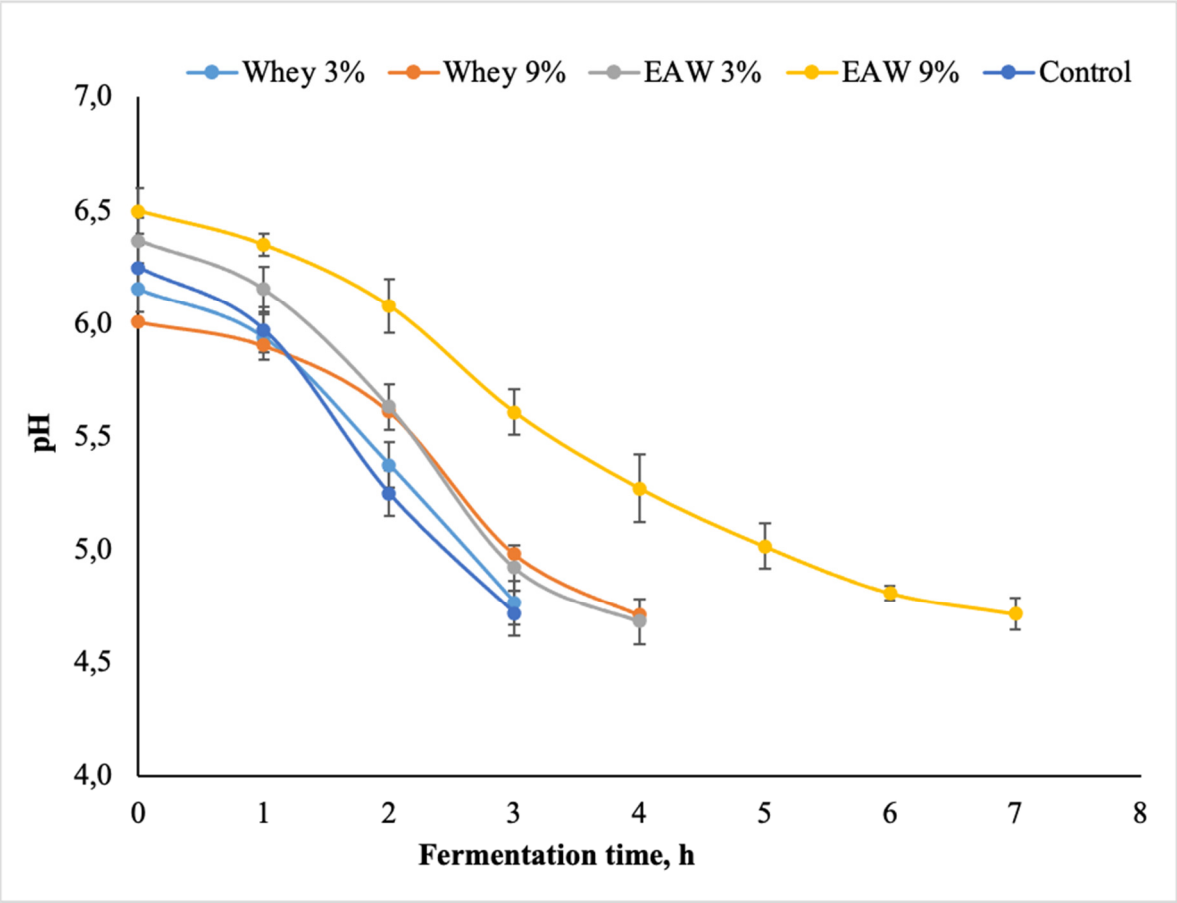

Figure 0-1: Evolution of the pH during the fermentation process of Ryazhenka. Samples coded as follows: Whey 3%: Ryazhenka supplemented with 3% whey. Whey 9%: Ryazhenka supplemented with 9% whey. EAW 3%: Ryazhenka supplemented with 3% electro-activated whey (EAW).

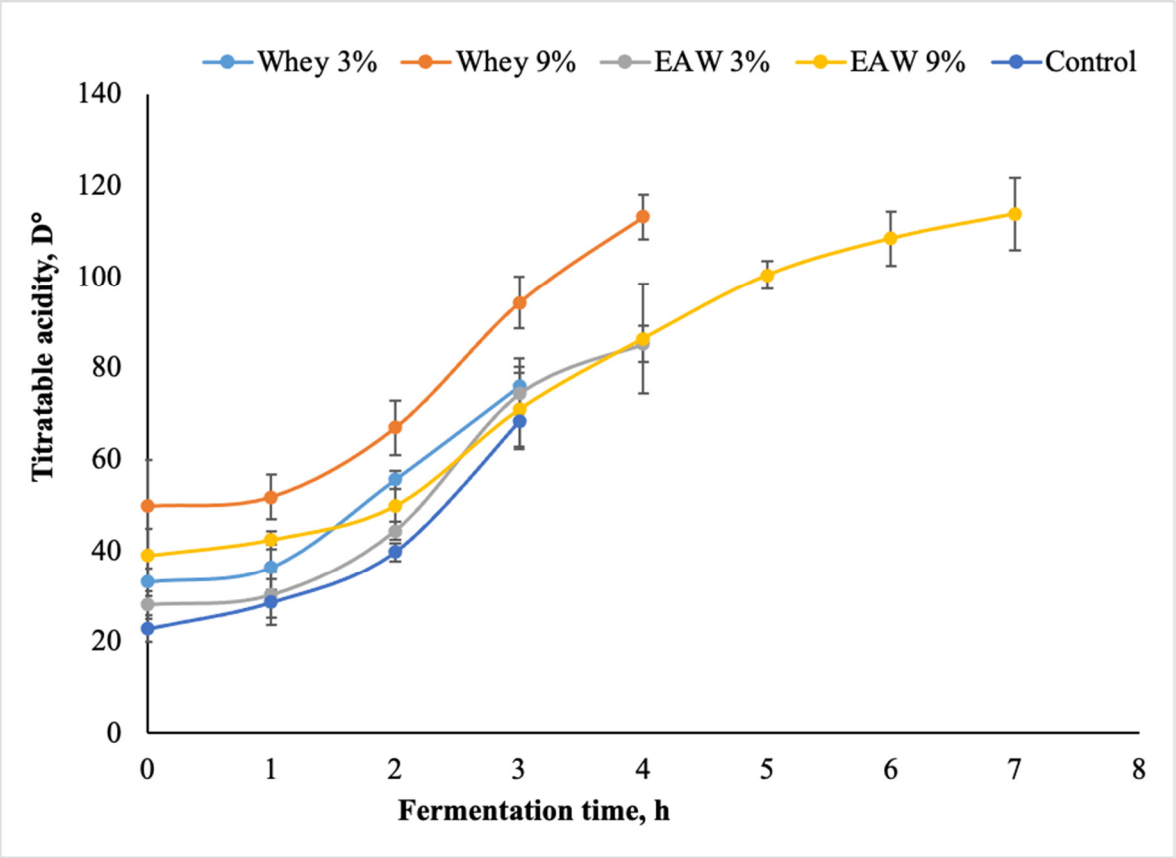

Figure 0-2: Evolution of the titratable acidity during the fermentation process of Ryazhenka. Samples coded as follows: Whey 3%: Ryazhenka supplemented with 3% whey. Whey 9%: Ryazhenka supplemented with 9% whey. EAW 3%: Ryazhenka supplemented with 3% electro-activated whey (EAW). EAW 9%: Ryazhenka supplemented with 9% electro-activated whey (EAW).

5.3.2. Color, apparent viscosity of Ryazhenka and syneresis

According to the obtained results of the colorimetric analysis, all Ryazhenka samples, including those without electro-activated whey had slightly brownish color. The L^*, a^*, b^* and ΔE values are shown in **Table 5.3**. The ΔE values of the samples were calculated using a corresponding formula and were taken as a difference in color perception from the control. According to this color parameter, when $\Delta E < 1$, the difference in color is not perceptible by the human eye, ΔE = 1-2 indicates that the color difference is perceptible at close observation, and when ΔE = 2-10, the difference between the samples colors is perceptible at a glance. The Ryazhenka sample with the highest concentration of electro-activated whey (9%) was

characterized by the highest ΔE index of 5.04. Samples with non-electro-activated whey (3% and 9%) and electro-activated whey (3%) had a ΔE index ranging between 1.2 to 1.45, indicating no perceptible significant difference for the consumer. Moreover, the statistical comparison of these samples indicated that there was no significant difference ($p > 0.05$) in samples with 3% supplementation level. However, it is important to mention that even if the supplemented sample with 9% EAW had the highest ΔE value ($\Delta E = 5.04$), the product corresponded to the desired feature for such product which is traditionnaly made from baked milk in clay jars.

Regarding the final product consistency, this parameter was evaluated according to its apparent viscosity. In this study, we applied a gentle manual stirring step before cooling, and the apparent viscosity was measured at day-1 after fermentation was achieved. The obtained results showed that the apparent viscosity differed as a function of type of added ingredient; whey versus electro-activated whey (EAW). Thus, the obtained results showed that samples with electro-activated whey had significantly lower apparent viscosity (**Table 5.3**) indicating that this product is more suitable as a drinkable fermented dairy product, whereas the results showed that the other samples correspond to high viscosity drinkable dairy products such as yogurts, as described in the literature (Ménard et al., 2018). Regarding syneresis during the refrigerated storage at 4 °C during 14 day, it was not observed for Ryazhenka samples supplemented with electro-activated whey (EAW), whereas this phenomenon was visible in the other samples (control and with whey added).

As previously indicted, Ryazhenka is a fermented dairy product made from baked milk at 90-95 °C during 3-5 h holding. Thus, this particular heating step in Ryazhenka production process causes an evaporation of water and some concentration of the milk dry matter (Antontceva, Sorokin, Sedykh, Krasnikova, & Shamtsyan, 2019). Consequently, the final product is generally characterized by relatively high apparent viscosity, as shown by the results obtained after applying a shear stress of 10 1/s and the subsequent corresponding state to the swallowing flow by the consumers (Mezger, 2006). Also, from the organoleptic point of view, Ryazhenka is one of the most appropriate fermented milk products regarding the affinity of the color with the added electro-activated whey characterized by a yellowish-brownish color. Indeed, the milk baking step in Ryazhenka production process leads to the development of browning nitrogen-containing high molecular weight compounds known as

melanoidins (Aljahdali & Carbonero, 2019). These compounds are generally formed at final stages of the Maillard reactions (MR) between the reducing sugars such as lactose, lactulose, glucose, and galactose with the free amino acid side chains of proteins, peptides and free amino acids; especially the ε- amino group of lysine residue. In addition to color characteristics, products of intermediate and final stages of Maillard reactions formed through the Strecker degradation play a role in specific flavor and aroma development in the final product (Aljahdali & Carbonero, 2019). The number of aroma compounds associated with Maillard reaction products exceeds 3500, among which one can find different ketones, aldehydes, sulphide compounds, and organic acids. Most of these compounds are developed in the intermediate stages of the Maillard reactions (Sunds, Rauh, Sørensen, & Larsen, 2018). Also, it is important to highlight that in the present study, the spectrophotometric test used did not differentiate between initial UHT milk and Ryazhenka samples after 4 h heating on the level of the MRPs, as shown by the absorbance at the wavelengths of 294 nm and 420 nm (**Table 2**). This result can be associated with the presence of UHT milk production which favoured the formation of MRPs because the UHT is generally conducted at a very high temperature of ≈140 °C with a holding time of few seconds followed by a cooling step which is not instantaneously. Indeed, Aktag et al. (2019) reported the presence of dicarbonyl compounds (3-Deoxyglucosone, 3- Deoxygalactosone, 1- Deoxyglucosone, Glucosone, Galactosone, Diacetyl, Methylglyoxal, Glyoxal) and furosine in different brands of UHT milk (Aktag, Hamzalıoğlu, & Gökmen, 2019). It was also noted that the progress of Maillard reaction in UHT milk is time-dependent and that the cooling period must be considered in this process. According to Rauh et al. (2015), Maillard reactions are the major deteriorating factor of UHT milk as it makes lysine unavailable. Thus, inaccessibility of lysine could hinder the further formation of MR products during the preparation of different Ryazhenka samples in the present study (Rauh et al., 2015).

Table 0-2: Absorption of Ryazhenka samples and UHT milk at 294 nm and 420 nm.

Samples	Intermediate Maillard reaction products (294 nm)	Browning index (420 nm)
Milk + 3%whey	1.31 ± 0.04^{a}	1.37 ± 0.04^{a}
Milk + 9%whey	1.28 ± 0.03^{a}	1.32 ± 0.04^{a}
Milk + 3% EAW	1.28 ± 0.03^{a}	1.34 ± 0.06^{a}
Milk + 3% EAW	1.27 ± 0.03^{a}	1.39 ± 0.07^{a}
Control	1.30 ± 0.02^{a}	1.42 ± 0.02^{a}
UHT milk	1.28 ± 0.01^{a}	1.46 ± 0.02^{a}

Table 0-3: Color and apparent viscosity of Ryazhenka samples.

	Color				Viscosity	
Sample	*L**	*a**	*b**	*ΔE*	Apparent viscosity (Pa.s)	Viscosity at 10.9 1/s
Milk + 3%whey	79.56 ± 0.20^{a}	-0.26 ± 0.15^{a}	13.26 ± 0.09^{a}	1.43^{a}	4.01 ± 1.06^{ab}	0.95 ± 0.07^{a}
Milk + 9%whey	77.48 ± 0.23^{a}	-0.63 ± 0.30^{a}	12.89 ± 0.4^{a}	1.35^{a}	2.91 ± 0.16^{ab}	0.8 ± 0.00^{ab}
Milk + 3%EAW	77.44 ± 0.22^{a}	0.16 ± 0.09^{a}	14.37 ± 0.03^{a}	1.21^{a}	2.545 ± 0.13^{c}	0.8 ± 0.00^{ab}
Milk + 9%EAW	75.50 ± 0.14^{a}	1.20 ± 0.13^{a}	17.61 ± 0.06^{a}	5.04^{b}	2.525 ± 0.67^{c}	0.6 ± 0.14^{b}
Control	78.26 ± 0.18^{a}	0.30 ± 0.04^{a}	13.49 ± 0.08^{a}	0	5.725 ± 0.98^{a}	0.95 ± 0.07^{a}

EAW: Electro-activated whey. Similar superscript letters indicate no significant difference at 95% confidence level.

5.3.3. Antioxidant capacity and ferrous ions chelating activity

Antioxidants are the molecules, ions or relatively stable radicals that are capable of slowing down an oxidation potential (activity) of oxidizing molecules. In the food industry, the demand for food stuffs with antioxidant properties has grown with the establishment of connection between oxidative stress and many diseases such as atherosclerosis, neurodegenerative diseases, diabetes myelitis, chronic inflammation and ageing (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012; Elias, Kellerby, & Decker, 2008; Gjorgievski, Tomovska, Dimitrovska, Makarijoski, & Shariati, 2013). Moreover, antioxidants contribute to the stability of foodstuffs against deterioration (Elias et al., 2008).

In the present study, there was a significant effect of the type of the added ingredient to Ryazhenka; whey versus electro-activated whey (EAW) as well as the control sample made by using only milk without supplementation (**Figure 5.3**). Antioxidant potency of electro-activated whey supplemented Ryazhenla samples was significantly ($p < 0.001$) higher than the antioxidant potency of the Ryazhenla samples supplemented with the non-electro-activated whey (whey) and the control (no supplementation). **Table 5.4** shows the IC_{50} values, which corresponds to the amount of the antioxidant substance needed to scavenge 50% of the radicals. The obtained results showed that the electro-activated whey scavenging activity was concentration-dependent, indicating the highest results in the Ryazhenka samples supplemented with 9% electro-activated whey (EAW). This result is in good agreement with the already reported information by Kareb et al. who demonstrated that electro-activation of whey generates a derivative product with significantly enhanced antioxidant activity (Kareb et al., 2017).

The studies of the antioxidant capacity of Ryazhenka are scarce in the scientific literature. According to a comparative study of Basov and Bykov (2013), Ryazhenka has the highest antioxidant capacity among all the tested fermented dairy products they tested: Ryazhenka, kefir, yogurt, and sour cream. By using different biophysical methods such as chemiluminescence and amperometry, the authors measured the antioxidant capacity of Ryazhenka and they expressed the results in ascorbic acid equivalents (mg/L VitC). They have obtained a result of 4.2697 ± 0.0155 mg/L VitC for Ryazhenla, while kefir had 3.1507 ± 0.0567 mg/L VitC, and yogurt 2.0852 ± 0.0289 mg/L VitC equivalent (Basov & Bykov, 2013). As microbiologically, Ryazhenka and yogurt can be made by using the same starter cultures

composed of *Streptococcus Thermophilus* and *Lactobacillus delbrueckii* subsp. *Bulgaricus* mixture, it is assumed that the main attribute to superior antioxidant capacity of Ryazhenka is attributed to the effect of the milk heating procedure which yields a formation of intermediate antioxidant Maillard reaction products. Indeed, increase of the antioxidant effect of milk heated at 70-130°C for 30 min was partially attributed to the unraveling of sulfhydryl groups of whey proteins, along with iron binding capacity of lactoferrin and bovine serum albumin, as well as the free radical scavenging activity of some amino acids such as tyrosine and cysteine (Tong, Sasaki, McClements, & Decker, 2000). Sulfhydryls are well-recognized free radical scavengers and remain the most important antioxidant agents of milk. Also, the authors had stated that antioxidant effect of whey proteins is concentration dependent. Another work studied commercial dulce de leche products regarding the induced antioxidant activity by the Maillard reaction products (MRPs). To some extent, dulce de leche has similarities with Ryazhenka as it is also made from a cooked condensed sweetened milk. Thus, owing to this process resulting of Maillard reaction products with evident antioxidant activity, this process is also responsible of its unique flavor and yellowish-brownish color. Moreover, many researchers reported that the stages of Maillard reactions were dependent on temperature and water availability, and browning products occurred in liquid systems heated at least at 60 °C. Moreover, antioxidant activity was attributed to the second and final stage of the MRPs. Tested dulce de leche samples demonstrated very low lysine contents signifying their inclusion in sugar-protein complex formation (Cortés Yáñez, Gagneten, Leiva, & Malec, 2018). Also, Kareb et al. (2018) mentioned that electro-activation significantly improved antioxidant capacity of whey. It could be due to protein unfolding due to high alkaline pH, and exposure of sulfhydryl groups as well as formation of the products of intermediate stage of Maillard reactions, Schiff bases, that have induced the observed antioxidant effect of electro-activated whey (Kareb et al., 2018). Another study reported an effect of Maillard reactions on improvement of antioxidant activity of whey proteins through a formation of MRPs that serve as hydrogen donors for free radical scavenging. In addition to that, Maillard reactions improve several functional properties such as heat stability and emulsifying properties of whey proteins (Oh et al., 2013). Finally, there are also several points of consideration related to the technology of electro-activation itself: aqueous solutions in catholyte have strong re-

ducing capacity steaming from nascent hydrogen (H*) which is formed during water electrolysis from the hydronium ions because in electro-activation, cathodes are usually submitted to high electrical current intensity (Ignatov et al., 2015). Moreover, whey possesses a strong iron-binding capacity mainly owing to the presence of lactoferrin (Chiang & Chang, 2005). In addition to iron, lactoferrin binds other metallic cations such as Zn^{2+}, Cr^{3+}, Cu^{2+}, Co^{3+} and Mn^{3+}. Maillard reaction products in milk are also recognized to form various soluble and insoluble complexes with metal cations and they are known as strong chelating agents (Tamanna & Mahmood, 2015).

Table 0-4: Antioxidant potency of Ryazhenka samples with added whey.

Samples	**DPPH IC_{50} (mg/mL)**	**PFRAP IC_{50} (mg/mL)**
Standard	2.67	65.30
Milk + 3% whey	4.21 ± 0.08^{a}	510.25 ± 97.38^{ab}
Milk + 9% whey	4.08 ± 0.07^{a}	360.08 ± 73.02^{bc}
Milk + 3% EAW	3.13 ± 0.03^{b}	348.20 ± 41.65^{bc}
Milk + 9% EAW	2.54 ± 0.02^{c}	244.03 ± 28.83^{c}
Control	3.83 ± 0.03^{d}	491.99 ± 8.58^{ab}

EAW: Electro-activated whey

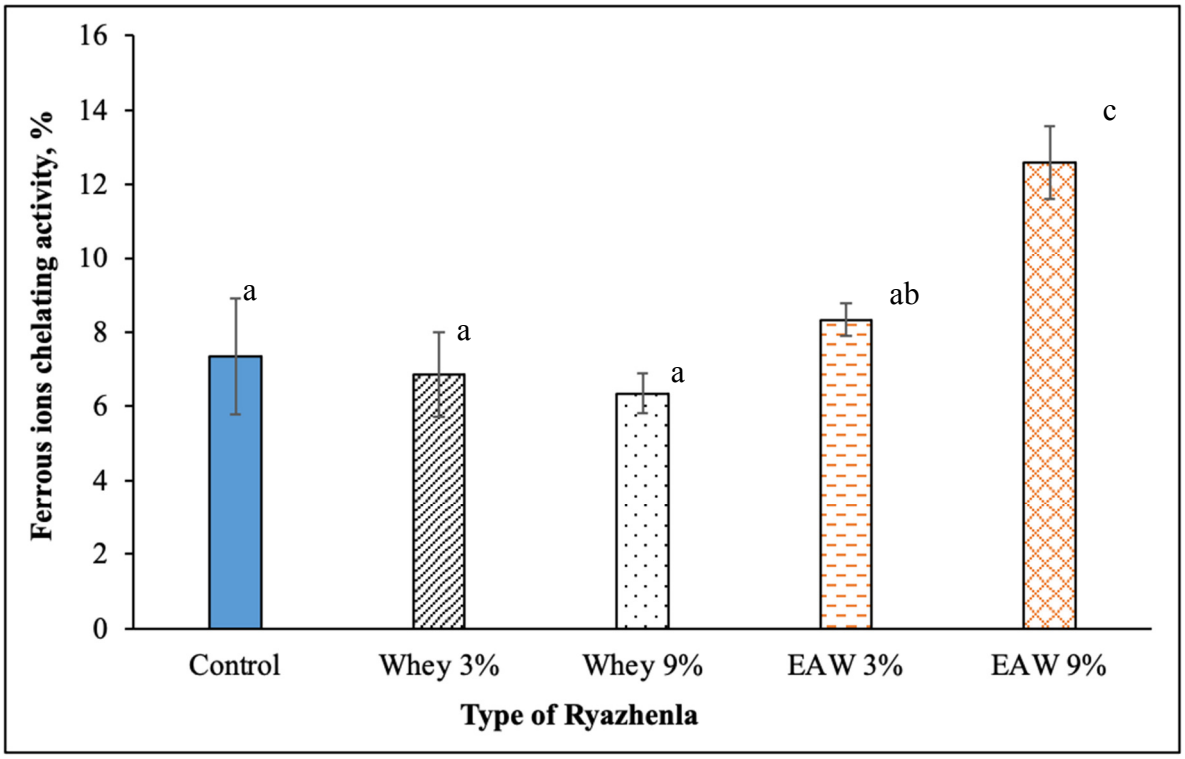

Figure 0-3: Ferrous ion chelating activity of Ryazhenka samples as a function the added concentration (3, 9%) of whey (Whey) or electro-activated whey (EAW). Similar superscript letters indicate no significant difference at 95% confidence level.

5.3.4. Protein and amino acids profile of electro-activated whey added Ryazhenka

The protein profile of Ryazhenka samples was studied by the SDS-PAGE electrophoresis. The main protein fractions in all samples were α_{s1}-casein (29-30kDa), β-casein (28.8 kDa), and less expressed κ-casein (20.5-20.8 kDa). Whey proteins α-lactalbumin (10-11.1kDa) and β-lactoglobulin (15.3-15.6 kDa) had the most pronounced bands when enriched with 9% whey and least in control with no added whey (**Figure 5.4**). Electro-activated whey (EAW) supplemented Ryazhenka samples were characterized by less pronounced bounds of aforementioned whey proteins which could be due to their partial hydrolysis during the electro-activated process under strong cathodic alkaline conditions. Indeed, it was previously reported by Kareb et al. (2016) that during 60 min of electro-activation α-lactalbumin and β-lactoglobulin bands visibly diminished due to a partial hydrolysis (Kareb et al., 2016). The study by Aidarbekova and Aider (2021) had demonstrated by a circular dichroism analysis

that high alkaline conditions in the cathodic compartment of the used electro-activation reactor increase the number of unstructured protein particles in whey. Moreover, the occurred partial hydrolysis of whey proteins could make the formed small fractions more available for bacterial uptake during fermentation.

Also, from a nutritional point of view, this protein hydrolysis during electro-activation of whey make the generated smaller whey protein fractions more bioavailable to the consumers (Aidarbekova & Aider, 2021b). Whey has highly nutritious amino acid content, with all essential amino acids in high concentrations compared to several plant proteins. It makes electro-activated whey a potent functional ingredient in preparation of nutritious beverages. Moreover, electro-activation permits to convert part of lactose into lactulose which is a sugar with high prebiotic potential. Thus, the reduced lactose content in electro-activated whey by converting 30-40% into lactulose is a very promising and highly nutritious food additive. **Table 5.5** shows amino acids content of whey after electro-activation. Completely randomized ANOVA with Tukey's HSD test demonstrated that electro-activation only caused the significant difference on the level of lysine. This outcome is supported by the fact that lysine residue actively reacts with milk sugar to form Maillard reaction products.

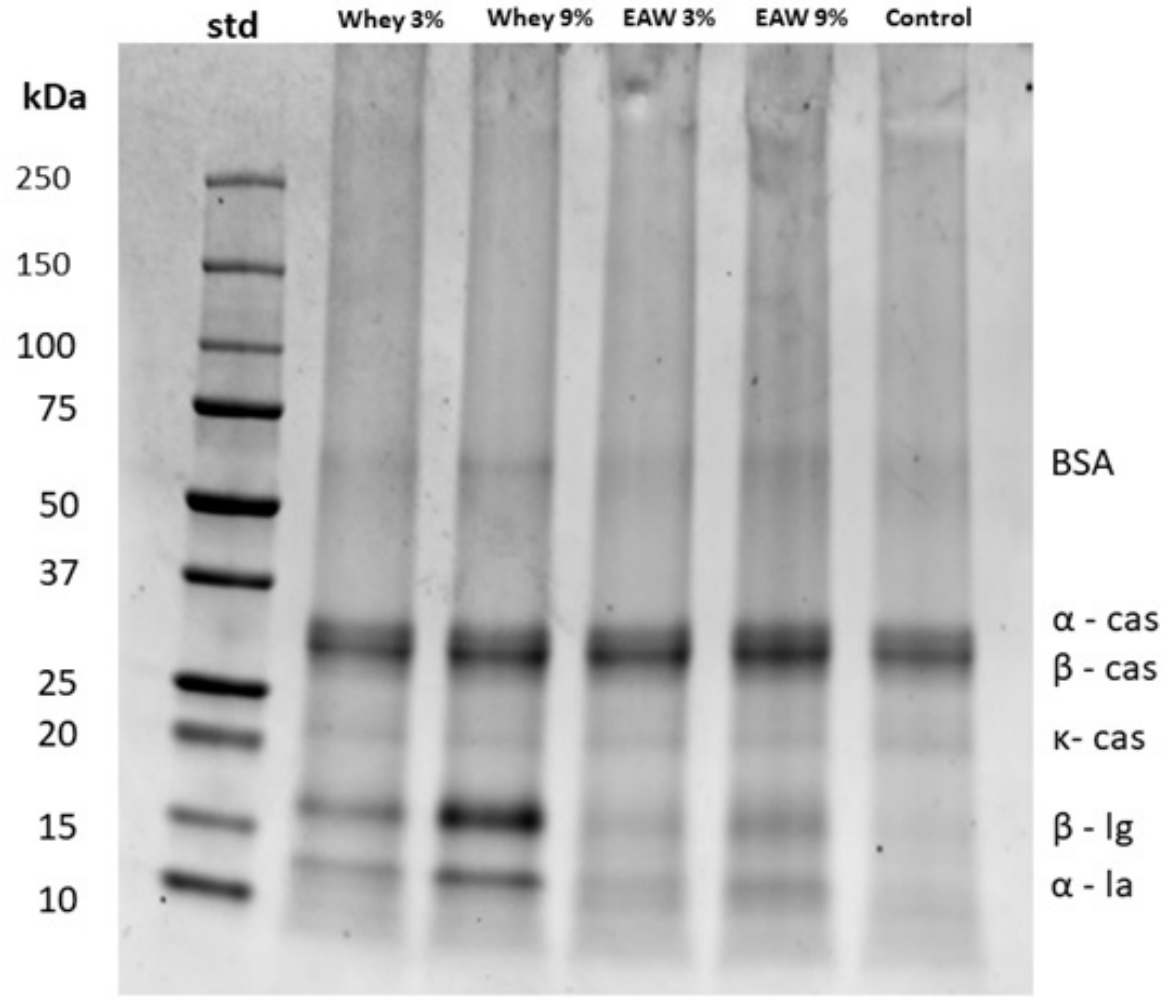

Figure 0-4: SDS-PAGE (+β-mercaptoethanol) of Ryazhenka samples with different concentrations of whey of 2 types. Ryazhenka without added whey served as control. Whey3%: Milk + 3% whey. Whey9%: Milk + 9% whey. EAW3%: Milk + 3% electro-activated whey (EAW). EAW9%: Milk+ 9% whey. EAW3%: Milk + 3% electro-activated whey (EAW). EAW9%: Milk + 9% electro-activated whey (EAW).

Table 0-5: Amino acid content of non-activated (Whey) and electro-activated whey (EAW).

Amino acid	EAW	Whey
Arg	20.49 ± 3.64[a]	27.23 ± 2.67[a]
Gln	1.17 ± 0.26[a]	1.46 ± 0.16[a]
Ser	3.58 ± 0.65[a]	5.12 ± 2.52[a]
Asn	0.91 ± 0.27[a]	0.93 ± 0.21[a]
Gly	46.90 ± 7.26[a]	25.0 ± 1.28[b]
Thr	5.62 ± 0.63[a]	4.41 ± 3.71[a]
Ala	16.29 ± 5.17[a]	21.92 ± 3.96[a]
Met	1.37 ± 0.11[a]	1.56 ± 0.03[a]
Pro	153.87 ± 20.19[a]	205.13 ± 33.11[b]
Asp	40.07 ± 7.07[a]	49.45 ± 1.25[a]
Lys	12.35 ± 1.2[a]	15.16 ± 0.14[a]
His	10.56 ± 0.64[a]	10.72 ± 1.02[a]
Val	2.95 ± 0.94[a]	3.88 ± 0.45[a]
Glu	298.64 ± 52.06[a]	364.01 ± 3.92[a]
Leu	3.61 ± 0.71[a]	4.62 ± 0.09[a]
Phe	15.69 ± 1.7[a]	17.12 ± 0.51[a]
Tyr	16.51 ± 2.14[a]	19.29 ± 0.65[a]

Comparison was made between EAW and whey for each amino acid and similar super-script letter indicate no significant difference at 95% confidence level.

5.3.5. Impact of electro-activation on allergenicity of β-lactoglobulin by ELISA assay

In the present study, the results regarding an eventual impact of electro-activation or milk heating on the allergenic character of whey, electro-activated whey and the fermented final product Ryazhenka was expressed through β-lactoglobulin content in the evaluated samples (**Table 5.6**). In the freshly prepared whey following acid precipitation and centrifugation of casein, the β-lactoglobulin concentration was estimated at 19.52 mg/kg and was statistically similar to that of a 0.3% β-lactoglobulin solution which was used as the positive control (19.10 mg/kg). Following the electro-activation treatment, as described in the methodology, the β-lactoglobulin concentration in the electro-activated whey decreased down to 7.56 and 12.13 mg/kg in the none-neutralized (pH 11) and neutralized electro-activated whey, respectively. This decrease corresponded to a potentially reduced allergenicity by 38% and 61%, respectively. When a 0.3% β-lactoglobulin solution was subjected to similar electro-activation conditions, the potentially reduced allergenicity was estimated to 92.7% and 95.3% for the none-neutralized and neutralized electro-activated β-lactoglobulin solution, respectively. In the Ryazhenka samples prepared by adding 3 whey or electro-activated whey, there was no significant difference between the mean values of β-lactoglobulin content as determined by the used ELISA test. This result is realistic and logic because the product was made by fermenting milk has a similar β-lactoglobulin content as the tested fresh whey or a 0.3% β-lactoglobulin solution. Interestingly, the detailed statistical analysis of the obtained results showed that heating did not affect β-lactoglobulin content and its eventual allergenic potential in this product. When 9% electro-activated whey was used as a supplementing ingredient in the process of Ryazhenka production, a slight decrease of β-lactoglobulin content was observed (**Table 5.6**). These results corroborate with the results of (Aidarbekova & Aider, 2021b) where circular dichroism of electro-activated whey showed a highly significant presence of unstructured coils after electro-activation, which partially decreased after neutralization of pH from 12 to 7.

Taheri-Kafrani et al. (2009) showed that denaturation (loss of tertiary and secondary structures) of β-lactoglobulin is associated with a weaker binding capacity of IgE from cow milk allergy patients. The authors showed that moderate glycation of β-lactoglobulin resulting from Maillard reaction has only a small effect on its recognition by IgE, whereas a high degree of glycation has a highly significant masking effect on the recognition of epitopes.

This finding demonstrated the importance of ε-amino groups of lysines in the definition of epitopes recognized by immunoglobulins IgE (Taheri-Kafrani et al., 2009). Even if the study of Taheri-Kafrani et al. (2009) was aimed to induce a denaturation by heating, the information they reported is in good agreement with our previous results where it has been shown that electro-activation induced denaturation and glycation of proteins with reducing sugars present in the electro-activated whey such as lactose, lactulose and galactose. Moreover, the study of Aidarbekova and Aider (2021) showed by a circular dichroism analysis that electro-activation affect β-lactoglobulin conformation and structure (Aidarbekova & Aider, 2021a). Also, it was reported that at pH > 7, β- lactoglobulin forms small aggregates due to the increase in reactivity of sulfhydryl groups (Abd El-Salam, El-Shibiny, & Salem, 2009). This particularity could have some impact on its eventual allergenicity. In spite of many health benefits of whey proteins, the major whey protein fraction β-lactoglobulin is a common allergen. Several studies have confirmed the diminishing effect of heating and fermentation by bacteria on antigenicity of bovine milk (Barbosa et al., 2017; Bu, Luo, Zhang, & Chen, 2010; Pescuma et al., 2010; Yao et al., 2014). In the study of Bu et al. (2010), fermentation by *L. bulgaricus* and *S. thermophilus,* and *L. helveticus* reduced the antigenicity of α-lactalbumin by 53-87% and β-lactoglobulin by 95% (Bu et al., 2010). According to Abd El-Salam et al. (2009), heat treatment of milk at 63 °C for 20s is enough to induce the changes in physico-chemical properties of whey proteins, such as hydrophobicity and conformational alterations (Abd El-Salam et al., 2009). Regarding the present study, this aforementioned information allows us to conclude that the Ryazhenka beverage has reduced allergenicity. Moreover, our results are confirmed by Gomes-Santos et al. (2015) who reported that hydrolyzed whey protein did not develop the allergy symptoms in β- lactoglobulin sensitized mice (Gomes-Santos et al., 2015). Taking into account the fact that cow's milk allergy affects approximately 2.5% of the young children, these findings could prove electro-activation treatment for use in infant formulae (Bøgh, Barkholt, & Madsen, 2015).

Table 0-6: Allergenicity associated to β-lactoglobulin as a function of the type of sample.

Sample	Absorbance		Concentration
	Mean value	CV	(mg/kg)
Whey	2.722	0.2	19.52
EAW pH 7	2.564	3.9	12.13
EAW pH 11	2.347	5.5	7.56
β-lactoglobulin	**2.716**	**0.4**	**19.10**
EA- β-lactoglobulin pH 7	**1.266**	**1.8**	**1.39**
EA- β-lactoglobulin pH 11	**1.165**	**4.3**	**0.897**
Ryazhenka+ whey 3%	2.728	1.2	19.95
Ryazhenka+ whey 9%	2.695	0.7	17.82
Ryazhenka+ EAW 3%	2.701	0.4	18.13
Ryazhenka+ EAW 9%	2.696	0.2	17.82
Ryazhenka control	2.692	0.4	17.58

CV: Coefficient of variation = ratio of the standard deviation to the mean value.

5.3.6. Sensory evaluation of Ryazhenka

Sensory qualities of Ryazhenka samples varied vastly due to the higher levels of minerals and free acids in 9% whey added samples (whey and electro-activated whey). It was represented in the commentaries of the participants, where consumers preferring more salty and spicy foods gave higher notes to 9% whey added samples, and less preference to milder taste of control and 3% whey added samples. Majority of traits (aroma, color, taste, appearance, overall appreciation, intention to buy) for control, Ryazhenka+ 3% whey and Ryazhenka+ 3% EAW received on average the same points, leaving behind 9% whey added samples (**Figure 5.5**). The average point of none of the samples received the highest mark but stayed on the level 6 (slightly like). The reason could be an unfamiliarity with the product.

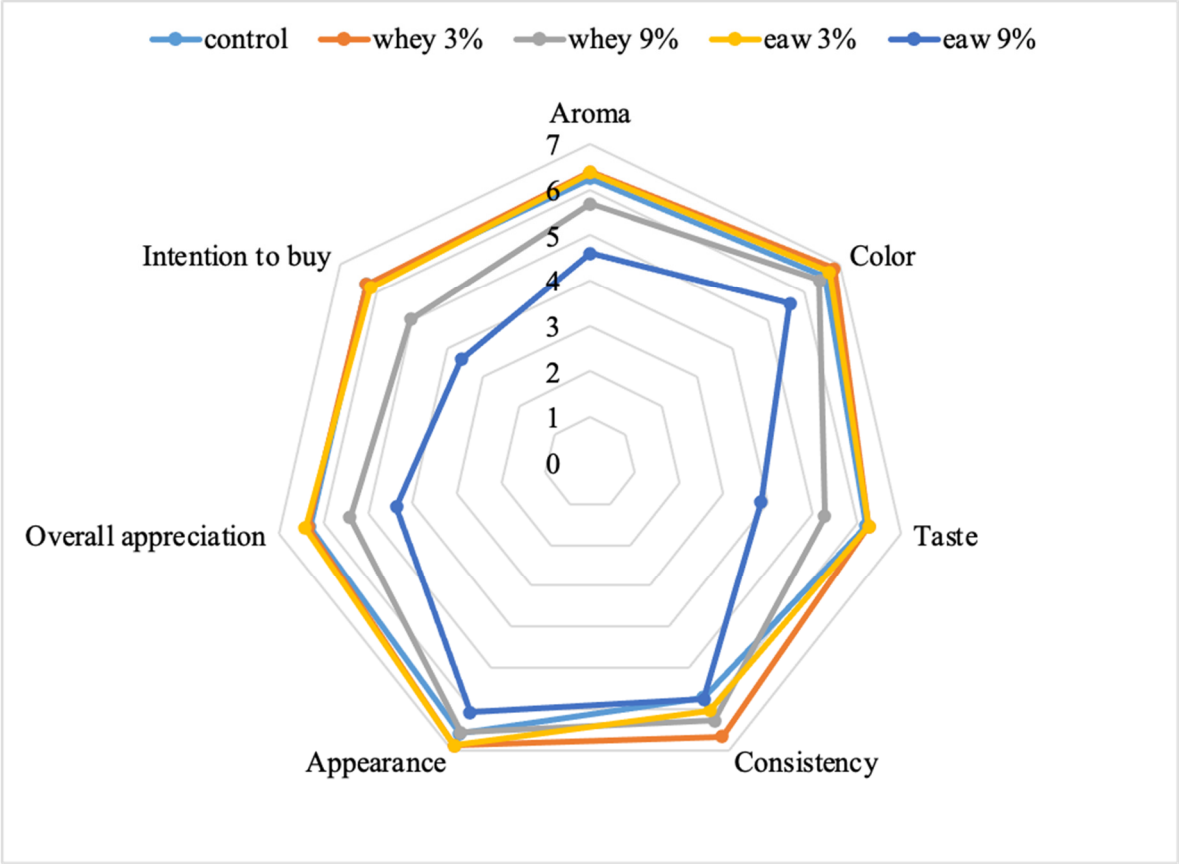

Figure 0-5: Sensory characteristics of Ryazhenka samples. Samples were coded as follows: whey 3%: Milk + 3% whey. whey 9%: Milk + 9% whey. eaw 3%: Milk + 3% electro-activated whey (EAW). eaw 9%: Milk + 9% electro-activated whey (EAW). control is Ryazhenka made from milk without any addition.

5.4.CONCLUSION

The present study demonstrated that the process of Ryazhenka production, a fermented baked milk, is feasible by using electro-activated whey (EAW) as supplementing ingredient at concentrations of 3 and 9%. The fermentation kinetics of the EAW supplemented Ryazhenka was different from the product supplemented with whey with a delayed lag-phase. Syneresis was not observed in the EAW supplemented Ryazhenka during a refrigerated storage at 4 °C. The use of EAW as supplementing ingredient was also compatible with the final product from the chromatic point of view since the process of milk baking prior

to fermentation induces a formation of a yellowish-brownish color in milk. Sensory evaluation of the produced Ryazhenka showed a good product acceptability by the panel list. Also, this study showed that electro-activation has highly significant effect on β-lactoglobulin allergenicity and the used ELISA specific test showed a reduction of up to 95% of the initial allergenic potential. Addition of EAW to Ryazhenka at a level of 9% also had an effect on the final product β-lactoglobulin content, and consequently on its allergenic potential. Finally, it can be concluded that this study contributed to enhance the potential of using electro-activated whey as a lactulose-rich ingredient in the technological process or Ryazhenka production yielding a final product with good organoleptic acceptability and enhanced antioxidant capacity.

Ryazhenka samples prepared with 9% EAW contained 2.79 ± 0.12 g/L of lactulose at the beginning of fermentation. During the fermentation of Ryazhenka, 23% of lactulose was metabolized. At the end of the 14 days storage period, lactulose content in the Ryazhenka supplemented with 9% electro-activated whey was around 53% of its initial content prior to fermentation. The lactulose content in the 14 days stored Ryazhenka was 1.39 ± 0.07 g/L, suggesting that lactulose is still available for conferring prebiotic health benefits to the consumer.

5.5.ACKNOWLEDGMENTS

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CHAPTER 6: Impact of Electro-activated Whey on Growth, Acid and Bile Resistance of *Lactobacillus rhamnosus spp.*, *Lactobacillus rhamnosus* GG and *Lactobacillus acidophilus* ATCC4356

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RÉSUMÉ

Le but de ce travail était d'étudier l'impact du lactosérum électro-activé, un ingrédient contenant du lactulose, sur la croissance et la survie de *Lactobacillus rhamnosus spp, Lactobacillus rhamnosus* GG et *Lactobacillus acidophilus* ATCC4356 dans des milieux acides et contenant des sels biliaires. Le lactosérum électro-activé a été comparé au lactosérum et au MRS seuls et complétés par du lactulose. Les résultats ont montré que l'OD_{600} était la plus élevée pour toutes ces bactéries lorsqu'elles étaient cultivées dans le milieu électro-activé. En même temps, les résultats obtenus ont montré que la phase de croissance Lag était la plus retardée dans ce milieu. Les résultats de la DO_{600} ont été vérifiés par la méthode d'ensemencement des bactéries sur une gélose nutritive. Les données obtenues ont montré que pour chaque bactérie donnée, aucune différence significative n'a été observée en fonction des résultats CFU/mL. Ainsi, il a été suggéré que le lactosérum électro-activé pourrait avoir un effet significatif sur l'aptitude des bactéries en augmentant leur activité, même à une population équivalente dans chaque milieu. Une étude de la stabilité des bactéries probiotiques pendant 14 jours de stockage réfrigéré à pH 4,6 et en présence de sels biliaires a révélé que le substrat de croissance n'a pas affecté de manière significative la survie bactérienne pendant cette période de stockage et que toutes les bactéries probiotiques testées sont restées proches de 10^9 UFC/mL.

Mots clés: Lactosérum électro-activé; Lactulose; Probiotiques; Croissance; Survie.

ABSTRACT

The aim of this work was to study the impact of electro-activated whey, an ingredient containing lactulose, on the growth and survival of *Lactobacillus rhamnosus spp.*, *Lactobacillus rhamnosus* GG and *Lactobacillus acidophilus* ATCC4356 in acidic and bile salts containing media. Electro-activated whey was compared to whey and MRS alone and supplemented with lactulose. The results showed that OD_{600} was the highest for all these bacteria when grown in the electro-activated medium. At the same time, the obtained results showed that the growth Lag-phase was the most delayed in this medium. The OD_{600} results were verified by the bacteria plating method on nutrient agar. The obtained data showed that for each given bacteria, no significant difference was observed according to the CFU/mL results. Thus, it has been suggested that electro-activated whey could have a significant effect of bacterial fitness by enhancing their activity even at an equivalent population in each medium. A study of the stability of the probiotic bacteria for 14 days refrigerated storage at pH 4.6 and in the presence of bile salts revealed that the growth substrate did not significantly affect bacterial survival during this storage period and that all the tested probiotic bacteria remained close to 10^9 CFU/mL.

Keywords: Electro-activated whey; Lactulose; Probiotics; Growth; Survival.

6.1. INTRODUCTION

Electro-activation was recently studied and developed as a significant contribution to improve whey valorization by enhancing its functionality by partial isomerization of lactose into lactulose, which is a well-known and recognized prebiotic (Aider & Halleux, 2007; Aider & Gimenez-Vidal, 2012; Kalathinathan, et al., 2021). Moreover, during the electro-activation process of whey, proteins, peptides, and amino acids' conjugates were formed with lactose, lactulose and galactose which significantly enhanced the antioxidant and functional properties of whey (Kareb, Gomaa, Champagne, Jean, & Aïder, 2017; Momen, Alavi, & Aider, 2022). Moreover, the technological feasibility of using electro-activated whey as an ingredient in different fermented dairy beverages was demonstrated (Aidarbekova & Aider, 2021).

Survival and active metabolism of probiotics such as lactic acid bacteria and bifidobacterial are affected by the environment composition and the presence of stress conditions such as low temperature, medium acidity, oxidation-reduction potential, bile salts and nutrients accessibility (Fijałkowski, Peitler, Rakoczy, & Żywicka, 2016; Naissinger da Silva, Tagliapietra, Flores, & Pereira dos Santos Richards, 2021). Recently, we demonstrated that following alkaline electro-activation, it is possible to produce whey, named electro-activated whey, which presents the potential to be used as a suitable growth medium to ensure viability and fitness of probiotics. The electro-activation process is conducted in the three-compartmental reactor which is modulated by anion and cation exchange membranes. In the cathodic compartment where electro-activation of whey occurs, highly reducing conditions are created with oxidation-reduction potential (ORP) of approximately -900 mV. Moreover, lactulose is formed in sufficient quantity and can be used as a carbon source with prebiotic properties. Also, intermediate Maillard reaction products (Schiff bases) are formed and are known to have good antioxidant properties (Kumar, Padmini, & Ponnuvel, 2017). Thus, it can be hypothesized that electro-activated whey can serve as a suitable carbon/nitrogen source and appropriate growth medium for probiotic bacteria under minimal stressing conditions.

Dairy products are good and most common vehicles of probiotics and prebiotics. Moreover, consumers are continuously seeking healthy products and are more aware of the positive impact of probiotic bacteria and prebiotics on health (Markowiak & Śliżewska, 2017) and mental health (Johnson, Thurairajasingam, Letchumanan, Chan, & Lee, 2021). In this

context, consuming probiotic-prebiotic enriched dairy products can be included in a strategy of improving a population's well-being. To achieve this goal, electro-activated whey can be considered a suitable ingredient for supplementing fermented dairy products since it contains a significant amount of lactulose, which is a well-recognized prebiotic, and it can contribute to improving the survival, growth and activity of different lactic acid bacteria and probiotics. Thus, a dual goal can be achieved which consists of improving the health benefits of fermented dairy products and increasing the overall economic profitability of the dairy industry by introducing a new ingredient with enhanced beneficial properties.

Thus, the aim of this work was to study the impact of electro-activated whey on the growth and survival of *Lactobacillus rhamnosus* spp., *Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus* ATCC4356.

6.2.MATERIALS AND METHPDS

6.2.1. Bacterial strains

Bifidobacterium animalis subsp. *lactis* Bb12, *Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus* ATCC4356 in the form of frozen powder were obtained from the bacterial strain collection of the Department of Food Science at Laval University. Each strain was twice revived prior to use in Man-Rogosa-Sharpe (MRS) broth (Merck, USA) (Kareb, Champagne, Jean, Gomaa, & Aïder, 2018).

6.2.2. Growth substrates

The growth of the studied bacteria was carried out on three different media: sweet whey (Whey), electro-activated whey (EAW) and MRS + Lactulose. Sweet whey powder was graciously provided by Agropur Cooperative (St-Hubert, Canada). Lactulose was purchased from Sigma-Aldrich (Oakville, Canada). The electro-activated whey was prepared as follows: a 10% whey solution (w/w) was electro-activated in the cathodic side of an electro-activation reactor containing three compartments: anodic, cathodic, and central which was separated from the anodic and cathodic sides by anion and cation exchange membrane, respectively. The whey solution was introduced in the cathodic compartment where reducing alkaline conditions were created while

the other two compartments were filled with 0.1 M and 0.25 M K_2SO_4 solutions to serve as electrolytes. After electro-activation, the whey solution was kept for relaxation during 48 h and then its pH was adjusted to 7 and freeze dried (Aidarbekova & Aider, 2021). Prior to use for bacterial growth, all media were filtered through sterile 0.45µm and 0.2µm filters. Sterility of the solutions used as growth media was verified by plating on nutrient media. Lactulose was added to whey and MRS broth to mimic the concentration of lactulose in the electro-activated whey since lactulose is a prebiotic and expected to have impact on bacterial growth.

6.2.3. Growth curves and bacterial enumeration

Initial number of bacteria in a suspension was calculated via a colony count and adjusted at OD_{600}. Each substrate was inoculated with 1% of each abovementioned cultures and transferred to sterile Vis-microplates (Sarstedt, Nümbrecht, Germany) in a volume of 250 µl and covered with 50 µl autoclaved sterilized mineral oil to ensure anaerobiosis. The plates

were then introduced to anaerobic jars containing an Anaerogen sachet (Oxoid, Nepean, Ontario, Canada). The microplates were incubated at 37 °C for 48 h. The OD_{600} was measured with an interval of 60 min and shaking step of a 3 s duration in a PowerWave XS2 microplate spectrophotometer reader (BioTek, Winooski, VT, USA). Bacterial cells were enumerated at 8, 16, 24 and 48 h by plate counting on MRS agar (Kareb, et al., 2018).

6.2.4. Tolerance to modeled gastrointestinal conditions

Aliquot of 1 mL of the tested probiotic bacteria after 24 h of fermentation was centrifuged at 8000 rpm during 15 min at 4 °C, as described by Hernandez-Hernandez et al. (2012). The pellet was then twice rinsed with PBS buffer and placed into a 1 mL PBS solution containing 0.3% bile extract (w/v) and saline solution with pH adjusted to 2.5. Probiotic bacterial cells were enumerated by plate counting before the exposure to stress conditions, and after 1 h of incubation at 37 °C in the mentioned stressing medium (Hernandez-Hernandez, et al., 2012). The survival rate was calculated by using the following equation (**Eq. 6.1**):

$$\%\ survival = \left(\frac{initial\ number\ of cells}{number\ of\ cells\ after\ exposure}\right) \cdot 100\% \qquad \textbf{(Eq. 6.1)}$$

6.2.5. SDS- Polyacrylamide gel electrophoresis

SDS-PAGE was conducted by using a mini-protein electrophoresis system (Bio-Rad Laboratories, Hercules, Canada) according to the Laemmli method. Non-diluted bacterial biomass was mixed in 1:1 ratio with the buffer with and without β-mercaptoethanol. The mixtures were then heated at 95 °C for 5 min and loaded into the gels. The electric current was set at 30 mA. After that, the gels were left in a coloration solution (Coomassie Brilliant Blue R- 250) for 1 h, followed by the initial decoloration for 30 min and the secondary overnight decoloration (Laemmli, 1970).

6.2.6. Statistical analysis

The results were expressed as means and standard errors of triple experiments. The software Statistix9 (Statistix, USA) was used to perform completely randomised analysis of variance (ANOVA) with confidence interval of 95% and Tukey's HSD test.

6.3.RESULTS AND DISCUSSION

6.3.1. Growth curves

The growth curves of the studied probiotic bacteria *Lactobacillus rhamnosus* GG (**Figure 6.1**), *Lactobacillus acidophilus* ATCC 4356 (**Figure 6.2**), *Lactobacillus rhamnosus* spp. (**Figure 6.3**) on different substrates were assessed. The MRS media was used as a control substrate which is suitable for the growth of the tested bacteria. Moreover, to identify whether lactulose which is contained in the EA whey is a contributor to its growth-promoting effect, MRS and whey were supplemented with lactulose (Lu in the graphs) in concentrations mimicking its content in the electro-activated whey. Moreover, to exclude the biasing effect of the brownish colour of EA whey, both media of whey and EA whey without bacterial inoculation were used as a control line to mark the absence of growth. To different extend, the obtained results showed that all three strains were able to grow in all the used substrates. As shown by the OD_{600} data, growth on EAW was characterized by the highest absorption values with a maximum value on 16, 18 and 24 h for *Lactobacillus rhamnosus* GG, *Lactobacillus rhamnosus* spp., and *Lactobacillus acidophilus* ATCC 4356, respectively. Interestingly, the addition of lactulose, which is a known prebiotic, to the MRS growth medium did not cause significant changes in the growth rates ($p < 0.05$). *L. rhamnosus* GG showed varying growth patterns, which were growth substrate dependent. The logarithmic growth phase in the EAW medium was retarded to 10 h in comparison with the MRS in which active bacterial proliferation started at 6 h from the start time point. Also, the obtained results showed that the highest cell density corresponding to the $OD_{600} \approx 2.1 \pm 0.01$ was observed when bacteria were grown in EAW medium from 20 to 48 h, whereas the non-electro-activated whey (Whey) promoted the maximal OD_{600} of 0.64 ± 0.02 and 0.78 ± 0.01 when it was supplemented with lactulose. Furthermore, an active growth of *Lactobacillus acidophilus* ATCC 4356 in the EAW medium was marked at 22 h by reaching the OD_{600} value of 2.1 ± 0.02. Also, this study showed a highly significant gap between the EAW and the other carbon sources for the growth of this probiotic bacterium, with OD_{600} of 1 ± 0.01 in the MRS and OD_{600} of 1.1 ± 0.01 in the case where lactulose was added to the MRS medium, as well as OD_{600} of 0.5 ± 0.01 and OD_{600} of 0.6 ± 0.02 in the whey and in the whey supplemented with lactulose (Whey+Lu). Moreover, the obtained results in the present study showed that *Lactobacillus rhamnosus* spp. has

equally reached a cell density corresponding to an OD_{600} of 2.1 on the EAW as a growth medium, followed by the MRS with OD_{600} 2 ± 0.05, MRS+lactulose (MRS+Lu) with OD_{600} 1.9 ± 0.00, whey supplemented with lactulose (Whey+Lu) with OD_{600} 0.8 ± 0.01, and the non-electro-activated whey (Whey) with an OD_{600} 0.7 ± 0.01 (**Figures 6.1-3**).

Specific growth rates representing the number of divisions of each cell per unit of time were defined from the appropriate models based on the growth kinetics on each substrate (Barragán, Sánchez, & Henao-Rojas, 2020). An exponential model was fit to the exponential growth phase with $R^2 > 0.95$. Doubling times of bacteria were derived from the following equations (Eqs. 2-3). The obtained results are summarized in **Table 6.1**.

$$\mu = \frac{lnOD2 - lnOD1}{t2 - t1} \qquad \textbf{(Eq. 6.2)}$$

$$Td = \frac{\ln(2)}{\mu} \qquad \textbf{(Eq. 6.3)}$$

The obtained data showed that all the tested bacterial strains showed a maximal generation doubling time on whey (Whey) and whey supplemented with lactulose (Whey+Lu). *L. Acidophilus* 4356 had the shortest doubling time of 2.2 h when the EAW was used as a growth substrate. Conversely, whey supplemented with lactulose (Whey+Lu) slowed down its growth.

Study of the growth of probiotic bacteria in electro-activated whey (EAW) was previously performed by Kareb et al. (2018) and the authors reported that the effect of EAW on probiotic growth was concentration dependent and strain specific (Kareb, et al., 2018). The authors monitored the highest OD_{600} for *L. rhamnosus* GG in media supplemented with 5% galactose (OD_{max} 1.9 ± 0.11) and 5% glucose (OD_{max} 1.85 ± 0.11), followed by 5% EAW (OD_{max} 1.45) and 5% whey (OD_{max} 1.03). Moreover, a strong bifidogenic effect of the media supplemented with 5% EAW was observed on *B. lactis* bb12 while obtaining a maximal cell density corresponding to OD_{max} 2.17 ± 0.10. This index significantly exceeded those of glucose, galactose, and whey. Lactulose (5% aqueous solution) as a sole carbon source produced the least biomass yields with OD_{max} 1.74 ± 0.11 and 0.51 ± 0.01 for *L. rhamnosus* GG and *B. lactis* bb12. Correspondingly, in the present study, the addition of lactulose to whey and MRS did not show any significant improvement of bacterial growth.

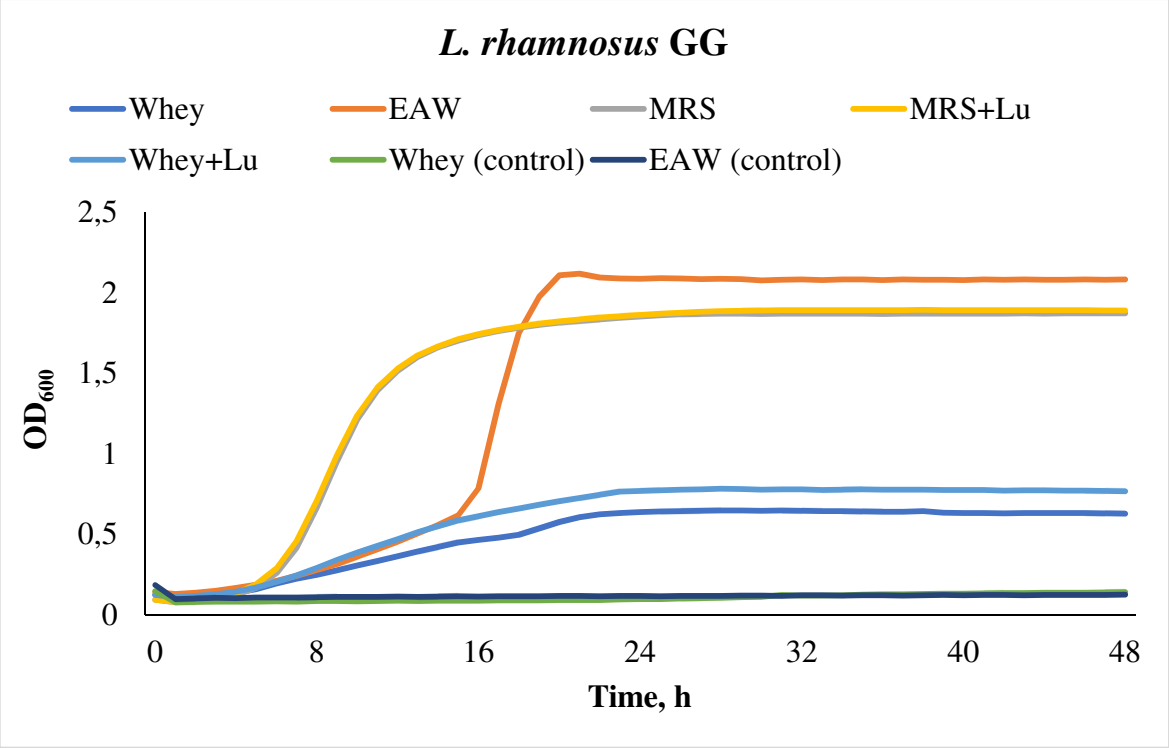

Figure 0-1: Growth curves of *Lactobacillus rhamnosus* GG on three carbon sources. Whey: Whey solution. EAW: Electro-activated whey solution. MRS broth. MRS+Lu: MRS broth+ lactulose. Whey+Lu: Whey+lactulose).

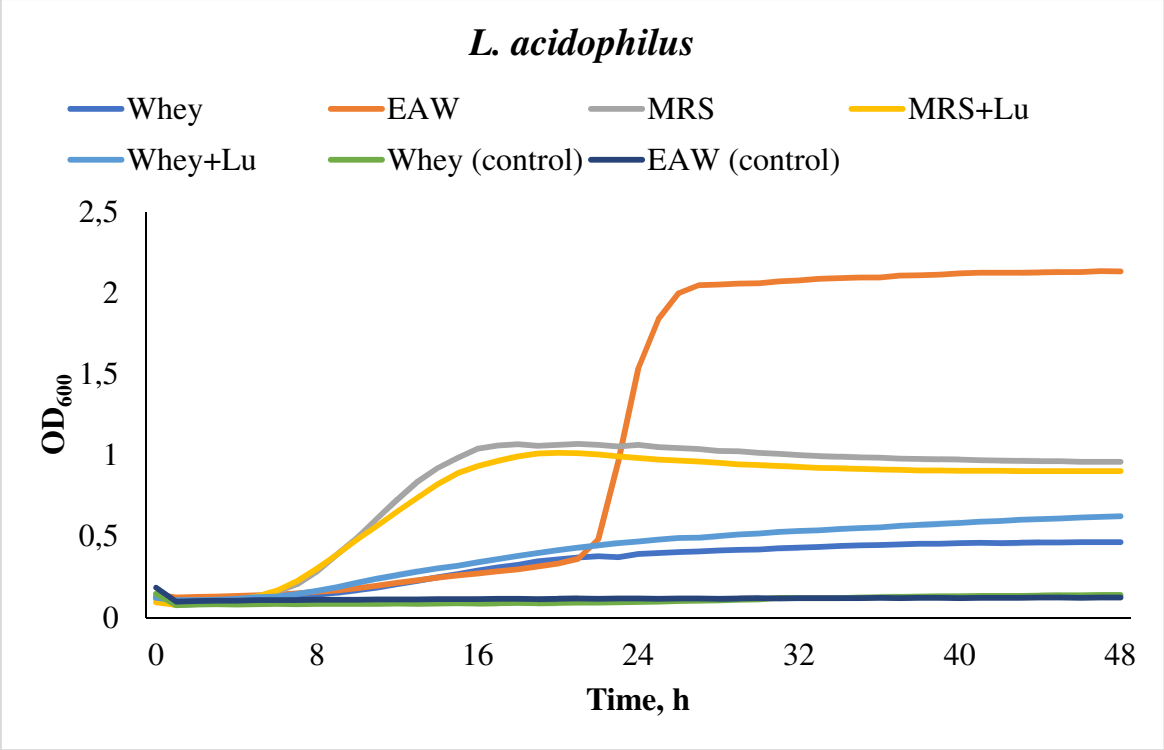

Figure 0-2: Growth curves of *Lactobacillus acidophilus* ATCC4356 on three carbon sources. Whey: Whey solution. EAW: Electro-activated whey solution. MRS broth. MRS+Lu: MRS broth+ lactulose. Whey+Lu: Whey+lactulose).

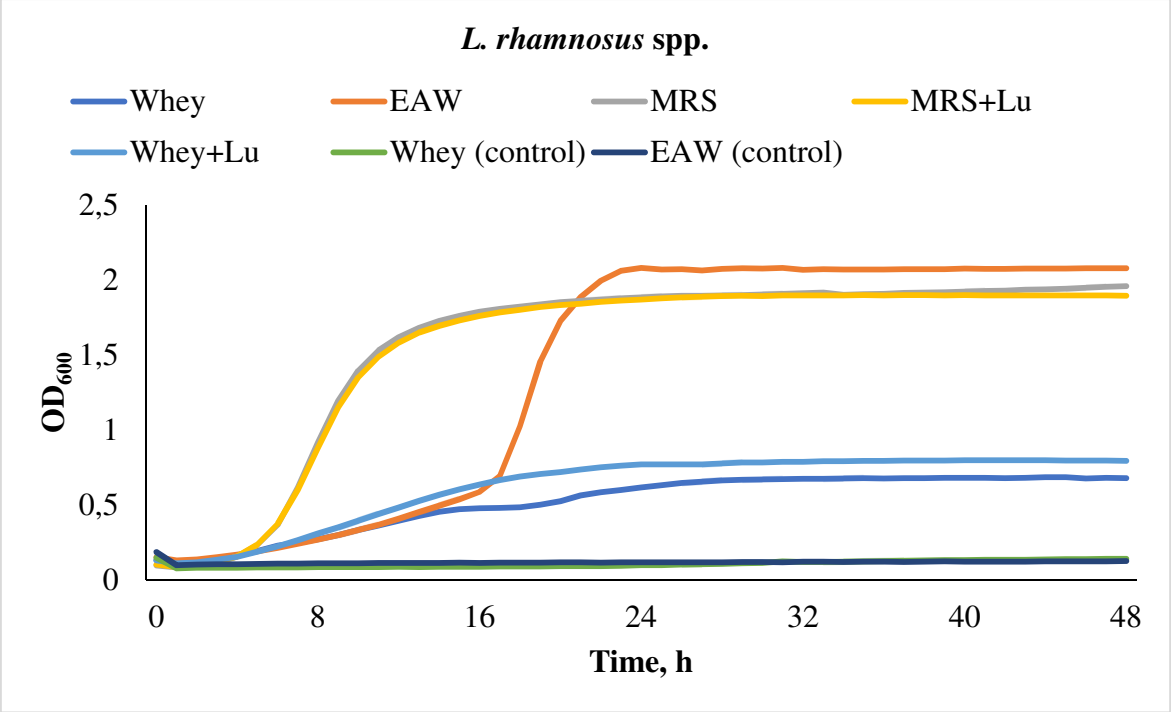

Figure 0-3: Growth curves of *L. rhamnosus* spp. on three carbon sources. Whey: Whey solution. EAW: Electro-activated whey solution. MRS broth. MRS+Lu: MRS broth+ lactulose. Whey+Lu: Whey+lactulose).

Table 0-1: Specific growth rate (μ (h^{-1})) and doubling time (T_d (h)) of probiotic bacteria grown on different substrates.

	μ (h^{-1})			**T_d (h)**		
	L. rhamnosus GG	*L. Acidophilus* 4356	*L. rhamnosus spp.*	*L. rhamnosus* GG	*L. Acidophilus* 4356	*L. rhamnosus spp.*
Whey	0.0715	0.0771	0.0724	9.7	9	9.6
EAW	0.1664	0.3157	0.1553	4.2	2.2	4.5
MRS	0.2585	0.1377	0.2796	2.7	5	2.5
MRS+Lu	0.2422	0.1424	0.2741	2.9	4.9	2.5
Whey+Lu	0.0914	0.0711	0.0987	7.6	9.8	7

EAW: Electro-activated whey. MRS+Lu: MRS + Lactuloase. Whey+Lu: Whey + Lactulose.

6.3.2. Bacterial counts

Growth of the tested bacteria on different substrates was evaluated by counting colony forming units (CFU) on MRS agar at different time intervals and the obtained results are shown in **Figures 6.4, 6.5, 6.6**. Overall, maximal cell count for all the strains and the used substrates was 8-9 log CFU.mL^{-1}, indicating a good growth susceptibility on these media as carbon and nitrogen sources. Thus, these results are in agreement with those obtained by Avonts et al. (2004) who reported an index of maximal cell count of 9 log CFU.mL^{-1} of lactic acid bacteria cultured on MRS which was used as a positive control in the present study (Avonts, Uytven, & Vuyst, 2004). The obtained results showed that at 16 h and 48h of culturing, there was no significant difference in bacterial growth for all the three tested bacterial strains and all substrates. Thus, the results of bacterial counts do not corroborate with the previous section, where absorption of bacteria cultured in electro-activated whey was higher than absorption of bacteria in other substrates, a study of the biomass protein content was conducted. The SDS-PAGE analysis revealed that the accumulated biomass in the EA whey grown bacterial cultures contained visibly higher amount of protein compounds (**Figure 6.7**). The bands corresponded to the protein profile of EA whey during 60 min: α-lactalbumin 14 kDa, β-lactoglobulin 18 kDa, immunoglobulins 70 kDa, and immunoglobulins 25-70 kDa.

The presence of these proteins and other peptides can be explained by the partial unraveling of whey protein complexes during electro-activation that permitted them to pass through the filters of 0.2 µm used as a cold sterilizing tool. However, their presence does not describe the higher absorption of bacteria cultured on EA whey, as the absorption of EA whey without bacteria was close to zero, as already demonstrated in **Figures 6.1-6.3**. Considering that electro-activated whey did contribute to the OD_{600}, and taking in consideration that the recorded OD_{600} values were the highest for the bacteria grown on EA whey, it was suggested that EA whey had significant impact on bacterial strains activity and was able to stimulate the production of specific metabolites at higher intensity than what was in the other media, including whey+lactulose (Whey+Lu) and MRS+lactulose (MRS+Lu). Indeed, this statement complies with the definition of a prebiotic effect of a given substance consisting of its ability to stimulate growth or activity of gastrointestinal microbiota (Davani-Davari, et al., 2019). However, the metabolic response of probiotic bacteria to the electro-activated whey needs to be further studied and explained by transcriptomic analyses to study if specific gene expression is associated with bacterial growth on electro-activated whey.

Also, the observed intensive and more active growth of the tested bacterial strains when grown in EA whey can be explained by the positive effect of this substrate on microbial fitness which is a concept used to explain the average reproductive success of a genotype in a specific culturing environment. Moreover, in terms of microbial growth, microbial fitness can be expressed through specific or general growth measurement indicators and competitive assays comparing different bacterial strains. In this context, microbial fitness can be described by terms such as vitality, which relates to an intact metabolic state and being relatively strong and active. Indeed, terminology pertaining to fitness such as viability and robustness have been used to compare different bacterial strains. So, the hypothesized higher bacterial activity during the fermentation process in the EA whey used as growth medium corroborates to a high extend with its bacterial fitness stimulating effect (Demain & Fang, 2000; Elena & Lenski, 2003). Furthermore, in recent studies, we reported that after electro-activation in the cathodic compartment of the used electro-activation reactor, the generated EA whey is characterized by high reducing capacity with an oxidation-reduction potential of -900 mV and strong antioxidant capacity. Thus, by using this ingredient as a carbon and

nitrogen source for bacterial growth, the resulted medium is less stressful because of its reducing character and antioxidant capacity; conditions which were favorable for bacterial activity enhancement (Kareb, et al., 2017).

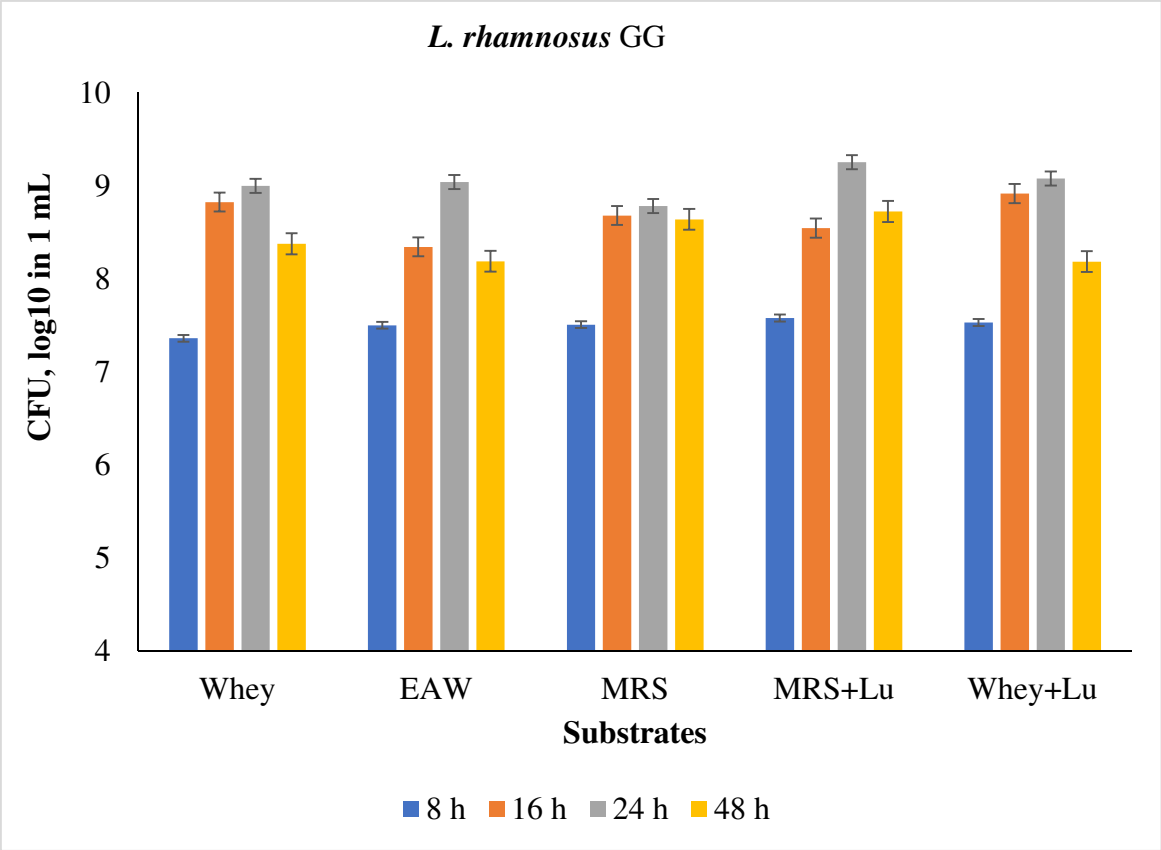

Figure 0-4: Plate counts of *L. rhamnosus* GG as function of the growth media. EAW: Electro-activated whey. MRS+Lu: MRS + Lactuloase. Whey+Lu: Whey + Lactulose.

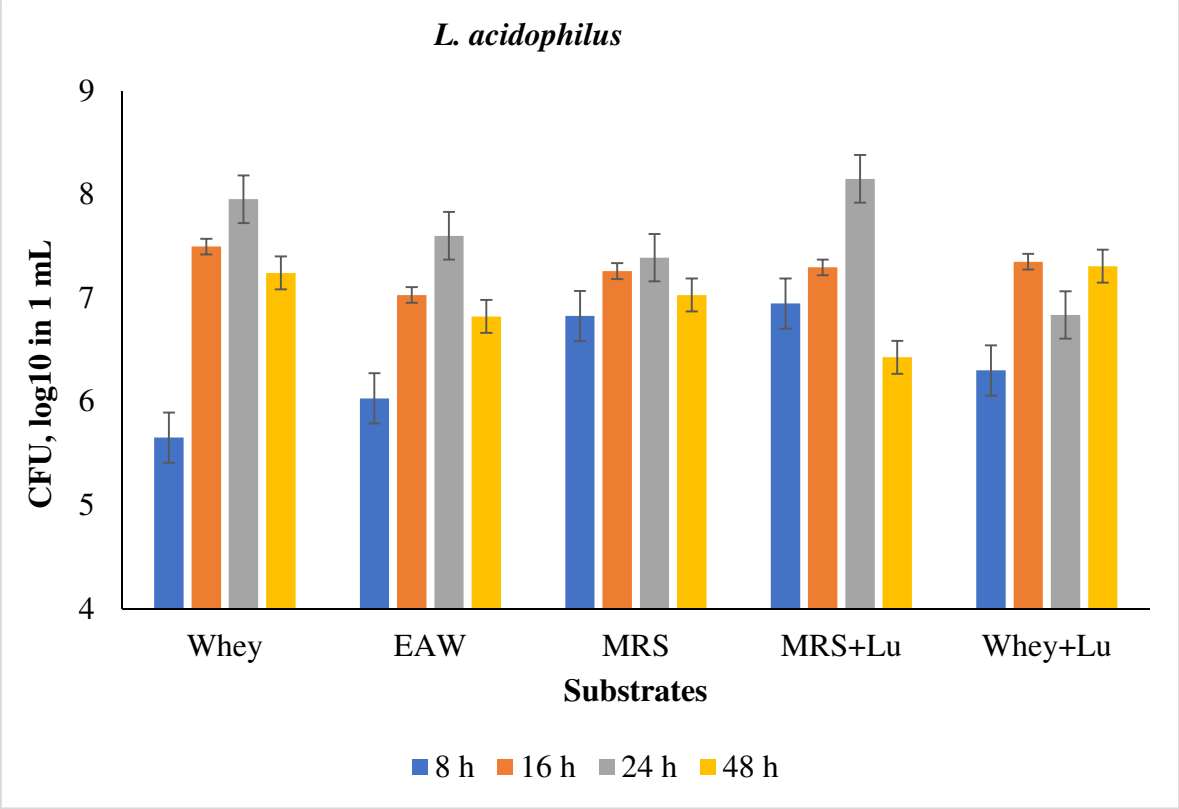

Figure 0-5: Plate counts of *L. acidophilus* 4356. EAW: Electro-activated whey. MRS+Lu: MRS + Lactuloase. Whey+Lu: Whey + Lactulose.

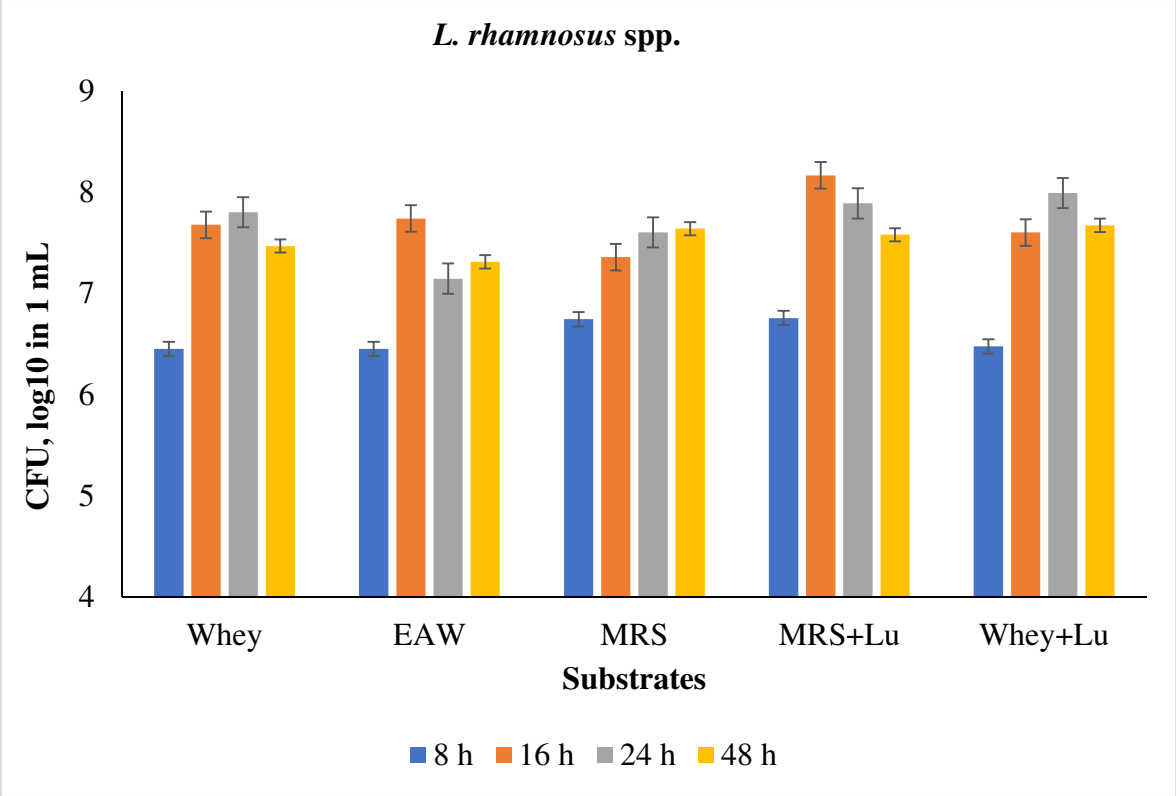

Figure 0-6: Plate counts of *L. rhamnosus* spp. EAW: Electro-activated whey. MRS+Lu: MRS + Lactuloase. Whey+Lu: Whey + Lactulose.

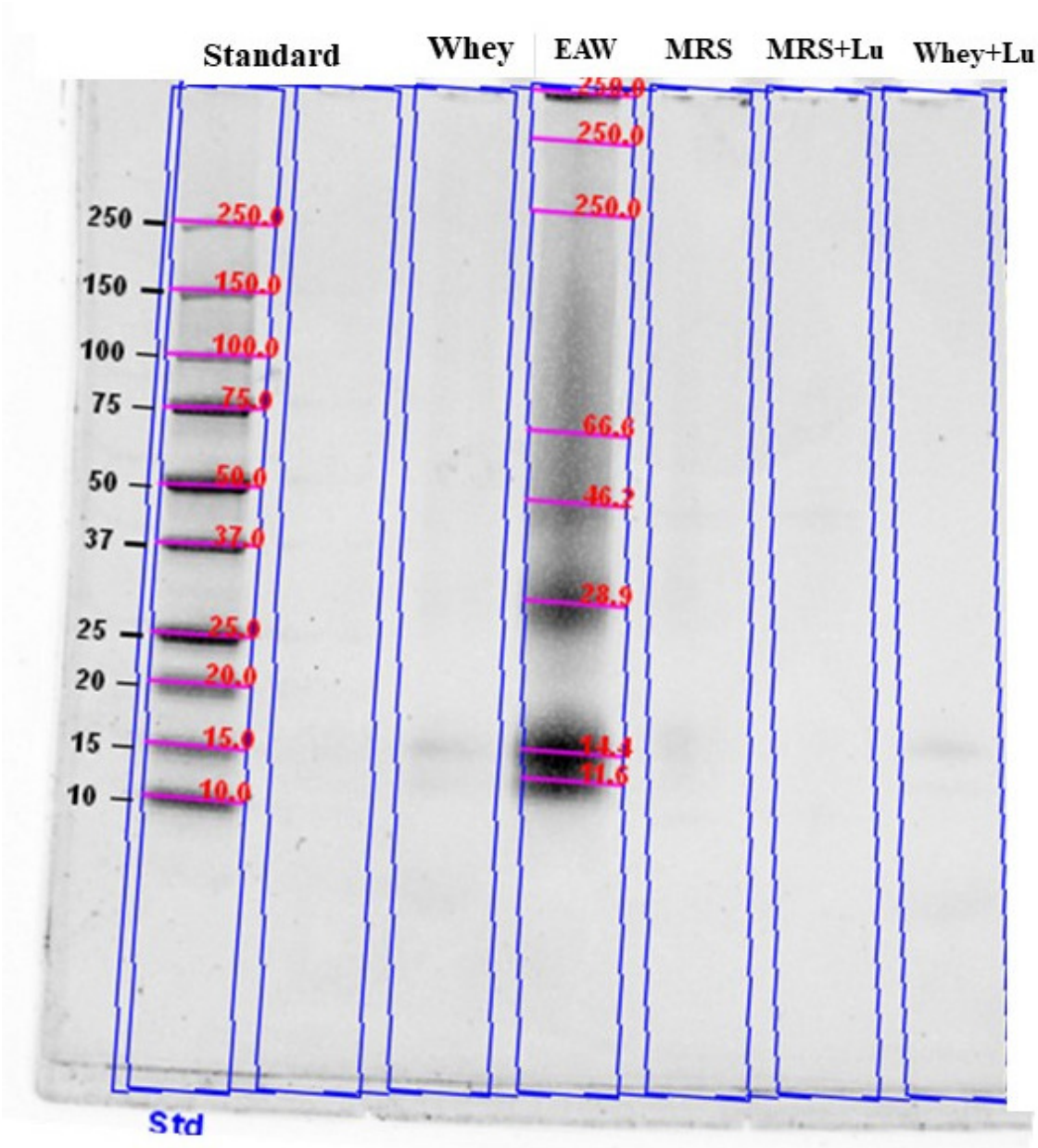

Figure 0-7: SDS-Polyacrylamide gel electrophoresis of the *L. rhamnosus* GG biomass grown in 5 different substrates: EAW: Electro-activated whey. MRS+Lu: MRS + Lactuloase. Whey+Lu: Whey + Lactulose.

6.3.3. Bacterial survival during refrigerated storage and acid/bile tolerance

Stability of probiotic bacteria during the storage is one of the main requirements for products enriched which probiotics. Canadian health authorities require at least 10^9 bacterial cells per serving, generally of 100 g (https://www.canada.ca/en/health-canada.html). To study the storage stability, probiotic bacteria were cultured in different substrates for 24 h and then introduced to model acid media imitating a fermented dairy product. MRS broth pH was adjusted to 4.6 by addition of lactic and acetic acids in the proportions present in fermented dairy beverages such as kefir. The results of bacterial counts during 14 days of refrigerated storage are given in **Table 6.2**. The results revealed that the substrate did not significantly affect bacterial survival during 2-week storage. In the timespan of 2 weeks, all tested bacteria remained in compliance with the requirements.

To confer health benefits to the host, probiotic bacteria should withstand the hostile conditions of the gastrointestinal tract. The key factors in acid tolerance of probiotic bacteria are the pH profile of H^+-ATPase and the composition of the cytoplasmic membrane. Thus, acid tolerance of bacteria can be enhanced by the media and cultivation conditions (Madureira, et al., 2005). Moreover, bile salts are known to selectively inhibit gram-positive bacteria, including probiotics (Ding & Shah, 2007). In the present study, two *L. rhamnosus* spp. strains were subjected to a low pH of 2.5 and bile salts at a concentration of 0.3% for 1 h to evaluate their survival ability during an eventual gastric transition where such conditions are encountered. The obtained results are given in **Table 6.3** and they show that *L. rhamnosus GG* had a higher acid survival rate when grown in MRS and MRS+lactulose, and statistically similar survival rates when grown in whey and whey supplemented with lactulose (Whey+Lu). EA whey cultured bacteria were statistically similar to both MRS and whey. Bile survival percentage of *L. rhamnosus GG* was the lowest in EA whey with an average value of 89.95 ± 7.27. However, it was not significantly different from the results obtained in whey, whey+lactulose (Whey+Lu) and MRS+lactulose (MRS+Lu). Other strain of *L. rhamnosus* spp. had a similar pattern of acid survival, hitting the highest survival percentage when cultured in MRS, as opposed to similar rates for whey-grown bacteria. This strain was less resistant to bile salts. Yet, bacteria grown in EA whey and whey provided higher tolerance to bile, with average values of survival percentage of 97.44 ± 4.91 and 96.23 ± 6.22, respectively.

Table 0-2: Plate counts of probiotic bacteria during refrigerated storage at 4°C in model media. Log10 CFU.mL^{-1}. All samples were at pH 4.6 at the initial moment of refrigerated storage. Average ± SE. HSD Tukey test.

Substrate	Whey	EA whey	MRS	MRS+Lu	Whey+Lu
L. rhamnosus GG					
1 day	7.52 ± 0.35[a]	7.3 ± 0.31[a]	7.45 ± 0.41 [a]	7.8 ± 0.19 [a]	7.53 ± 0.37 [a]
1 week	7.35 ± 0.13 [a,b]	7.01 ± 0.09 [b]	7.26 ± 0.06 [a,b]	7.7 ± 0.18 [a]	7.44 ± 0.17 [a,b]
2 weeks	7.24 ± 0.06 [a]	7.02 ± 0.06 [a]	7.28 ± 0.16 [a]	7.43 ± 0.07 [a]	7.31 ± 0.14 [a]
***L. acidophilus* 4356**					
1 day	6.13 ± 0.18 [a]	5.73 ± 0.22 [a]	5.83 ± 0.39 [a]	5.65 ± 0.19 [a]	6.15 ± 0.16 [a]
1 week	6.1 ± 0.18 [a]	5.55 ± 0.19 [a]	5.75 ± 0.34 [a]	5.31 ± 0.34 [a]	6.04 ± 0.28 [a]
2 weeks	5.44 ± 0.28 [a]	4.56 ± 0.37 [a]	4.62 ± 0.87 [a]	4.43 ± 0.76 [a]	4.96 ± 0.07 [a]
***L. rhamnosus spp*.**					
1 day	7.44 ± 0.28 [a]	7.18 ± 0.29 [a]	7.5 ± 0.31 [a]	7.38 ± 0.05 [a]	7.23 ± 0.19 [a]
1 week	7.3 ± 0.12 [a]	7.16 ± 0.3 [a]	7.49 ± 0.24 [a]	7.34 ± 0.09 [a]	7.22 ± 0.15 [a]
2 weeks	7.29 ± 0.21 [a]	6.71 ± 0.16 [a]	7.39 ± 0.42 [a]	7.15 ± 0.15 [a]	6.92 ± 0.37 [a]

EA-Whey: Electro-activated whey. MRS+Lu: MRS + Lactuloase. Whey+Lu: Whey + Lactulose.

Table 0-3 : Acid and bile survival of the tested bacterial stains (% at initial population). Average ± SE. HSD Tukey test.

% of survival	**Whey**	**EA whey**	**MRS**	**MRS+Lu**	**Whey+Lu**
Acid medium					
L. rhamnosus GG	56.81 ± 5.6^{b}	75.40 ± 11.87^{ab}	94.82 ± 1.41^{a}	94.57 ± 2.31^{a}	51.86 ± 8.23^{b}
L. rhamnosus spp.	68.10 ± 15.28^{a}	68.40 ± 20.02^{a}	96.74 ± 2.94^{a}	91.17 ± 2.96^{a}	66.58 ± 9.59^{a}
With bile added					
L. rhamnosus GG	95.34 ± 6.77^{a}	89.95 ± 7.27^{a}	92.17 ± 1.2^{a}	96.44 ± 6.57^{a}	96.01 ± 3.99^{a}
L. rhamnosus spp.	96.23 ± 6.22^{a}	97.44 ± 4.91^{a}	79.15 ± 22.34^{a}	72.42 ± 4.77^{a}	90.89 ± 6.57^{a}
Acid resistance (Log_{10}					
CFU/mL)	**Whey**	**EA whey**	**MRS**	**MRS+Lu**	**Whey+Lu**
L. rhamnosus GG (t = 0h)	8.8 ± 0.06^{a}	8.31 ± 0.27^{a}	8.44 ± 0.49^{a}	8.49 ± 0.18^{a}	8.76 ± 0.15^{a}
L. rhamnosus GG (t = 1h)	4.33 ± 0.43^{ab}	5.18 ± 1.03^{ab}	6.29 ± 0.42^{a}	6.32 ± 0.27^{a}	4 ± 0.58^{b}
L. rhamnosus spp. (t = 0h)	8.46 ± 0.06^{a}	8.42 ± 0.1^{a}	8.79 ± 0.01^{a}	8.76 ± 0.09^{a}	8.88 ± 0.01^{a}
L. rhamnosus spp. (t = 1h)	4.76 ± 0.94^{a}	4.86 ± 1.6^{a}	5.88 ± 0.22^{a}	5.73 ± 0.13^{a}	4.93 ± 0.76^{a}
Bile resistance (Log_{10}					
CFU/mL)	**Whey**	**EA whey**	**MRS**	**MRS+Lu**	**Whey+Lu**
L. rhamnosus GG (t = 0h)	8.81 ± 0.08^{a}	8.54 ± 0.2^{a}	7.67 ± 0.45^{a}	7.94 ± 0.42^{a}	8.5 ± 0.24^{a}
L. rhamnosus GG (t = 1h)	8.41 ± 0.63^{a}	7.79 ± 0.65^{a}	6.75 ± 0.15^{a}	7.82 ± 1.15^{a}	8.36 ± 0.71^{a}
L. rhamnosus spp. (t = 0h)	8.71 ± 0.12^{a}	8.53 ± 0.17^{a}	7.87 ± 0.46^{a}	7.83 ± 0.45^{a}	8.97 ± 0.06^{a}
L. rhamnosus spp. (t = 1h)	8.41 ± 0.38^{a}	8.32 ± 0.21^{a}	6.84 ± 1.82^{a}	7.22 ± 0.56^{a}	8.25 ± 0.58^{a}

EA-Whey: Electro-activated whey. MRS+Lu: MRS + Lactuloase. Whey+Lu: Whey + Lactulose.

6.4. CONCLUSION

In this study, the hypothesized prebiotic effect of EA whey was studied by using three commonly known representatives of intestinal beneficial microbiota *L. rhamnosus spp*., *Lactobacillus rhamnosus* GG and *Lactobacillus acidophilus* ATCC4356. The obtained results showed the effectiveness of culturing the selected bacterial strains in EA whey which served as a unique carbon and nitrogen source. Spectrophotometric analysis showed development of higher OD_{600} absorbance of these bacteria when cultivated in EA whey. Bacterial survival during refrigerated storage under model acidic conditions did not significantly differentiate between the strains and substrates consisting of whey, whey+lactulose, MRS, MRS+Lactulose and EA whey. The results also showed that resistance to bile salts was strain-medium dependent.

6.5. ACKNOWLEDGMENTS

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GENERAL CONCLUSIONS AND PERSPECTIVES

The dairy industry faces new challenges of this decade, which are environmentally friendly practices, zero wasteful production, added value to the by-products, as well as consumer preferences of all-natural and health-promoting products. This study has addressed all these issues, as the valorization of whey by electro-activation was proven to generate a functional ingredient that can be incorporated into a fermented milk matrix. The research project has significantly contributed to proving the evidence of technological feasibility of adding EA whey into fermented dairy products. It spotlighted some important aspects of the behavior of electro-activated whey in acid milk gels.

Production of lactulose- enriched whey by electro-activation and its potential role as a functional ingredient was previously described. However, a technological feasibility of using electro-activated whey in a food matrix was not elaborated. This thesis project focused on studying the technological aspects of adding EA whey into fermented dairy products and development of suitable functional product. Thus, the first objective (Chapter 3) of this project was to study the technological feasibility of fermented milk of the addition of EA whey to milk in the process of fermented milk production and study physico-chemical properties of the resulting product. It was demonstrated that electro-activated whey well incorporated and filled in the pores in the fermented milk matrix. Consequently, successful distribution of the EA whey in the gel network has reduced the syneresis of the products. Effect on syneresis reduction was dependent of the concentration of whey powder. As per nutritional characteristics, addition of 9% electro-activated whey induced the formation of the highest proportion of lactic, acetic and citric acids, as well as production of butyric acid. Moreover, 61-64% of lactulose remained after reaching the pH 4.6 in 9% electro-activated whey enriched products. This step has permitted to conclude that addition of electro-activated whey suits best the drinkable fermented dairy products, as it provokes the formation of ropier gel, which is visible more viscous.

The following objective (Chapter 4) was to study the technological feasibility of using electro-activated whey as ingredient and source of lactulose in the technological process of kefir production and to evaluate the properties of the final product. This step has proven the results

obtain in the previous study, as the characteristics of electro-activated whey-enriched kefir mirrored those of fermented milk. Eventually, apparent viscosity of kefir samples with 9% electro-activated whey was 0.005± 0.00 Pa.s^n for full fat kefir, at the control 2.30 ± 0.17 Pa.s^n. However, it did not significantly differ from 9% non-treated whey-added samples (0.69 ± 0.59 Pa.s^n). From technological point of view, this characteristic is very important since it will be possible to produce drinkable kefir without need of any mechanical mixing, a fact which is favourable to save energy and to simplify the technological process. In addition to that it was demonstrated that supplementation of milk with electro-activated whey at 9% level permitted to completely eliminate syneresis during cold storage at 4°C. The studied kefir had a high production capacity of three main metabolites of lactic acid bacterial fermentation in samples with electro-activated whey. In samples enriched with 9% electro-activated whey 77% of initial lactulose remained in skimmed and 55% in full fat milk after the fermentation. In both fermented milk products (fermented milk and kefir) the levels of remaining lactulose are sufficient to confer health benefits to the consumers. In addition to that bacterial counts remained on top of 10^7 CFU/mL of kefir which corresponds to the requirement of 10^9 CFU per portion of 100 g. In conclusion, at addition of electro-activated whey it was possible to obtain kefir of adequate physico-chemical and sensorial parameters. The main parameter that was visibly distinct during consumer preference tests was the brownish color of electro-activated whey-added products.

In this regard, the third objective of this project (Chapter 5) was devoted to development of Slavic fermented brown milk – Ryazhenka supplemented with electro-activated whey and study of its phisico-chemical characteristics and biological activity. As production of ryazhenka involves intensive milk heating step, the color characteristics of this product were compatible with electro-activated whey. Fermentation time of ryazhenka supplemented with 9% electro-activated whey declined to 6.5 hours (in comparison with fermented milk 16 hours and kefir 22 hours). Electro-activated whey enhanced antioxidant capacity of ryazhenka. 2.54 ± 0.02 mg/mL of 9% electro-activated whey enriched ryazhenka sample could inhibit 50% of DPPH, compared to 4.08 ± 0.07 mg/mL of ryazhenka with non-activated whey. Moreover, this study has demonstrated that electro-activation can reduce allergenicity of β-lactoglobulin, meaning that electro-activated whey has a potential to be used in infant formula and other sensible population.

The last objective (Chapter 6) aimed at studying prebiotic effect of lactulose-enriched electro-activated whey on common representatives of intestinal beneficial microbiota *L. rhamnosus spp*., *Lactobacillus rhamnosus* GG and *Lactobacillus acidophilus* ATCC4356. Optical density of all three bacteria (OD_{600} value) exceeded 2.1 at the end of active growth phase, whereas the maximal absorption for bacteria grown in non-activated whey reached $OD_{600} \approx$ 0.6-0.7. Despite OD_{600} values being twice as high, CFU counts did not underline any difference between the substrates. Thus, electro-activated whey was concluded to improve bacterial fitness and promote distinct metabolic activity.

In conclusion the confirmation of the hypothesis stating "it is technologically feasible to use electro-activated whey in the process of fermented dairy products production as a functional ingredient with a prebiotic and antioxidant effect" was met. This study has demonstrated that electro-activated whey could be used as a fat replacing and texture enhancing agent for drinkable dairy products with antioxidant and prebiotic capacity. Thus, electro-activation of whey can contribute to circular production in dairy industry.

This PhD project offers an insightful glimpse in the subject of utilization of electro-activated whey as a functional dairy ingredient. However, due to restricted time span, there is still a space for future investigations. Further technological adjustments and optimization of technological process are required to bring electro-activated whey-added fermented products to the industrial scale. In depth research can be conducted as for testing different technological parameters (starter cultures, temperature, milk initial treatment etc.) on described products as well as study of other matrices with electro-activated whey (for example cheese). Furthermore, an effect of electro-activated whey on probiotic bacteria needs to be further studied by genetical studies, in order to unveil the metabolic response of probiotic bacteria.

It would be interesting to study the addition of electro-activated whey to specialized medical nutrition products, and to study its physiological benefits, such as better recovery from antibiotic treatment. Another perspective would be development of a fermented dairy product with anti-obesity effect. Finally, the utilization of electro-activated whey in infant nutrition could bring additional ways of valorization of this valuable resource.

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