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Developing Whey Protein Encapsulated Probiotics And Evaluating Environmental Listeria Using Whole-Genome Sequencing

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DEVELOPING WHEY PROTEIN ENCAPSULATED PROBIOTICS AND EVALUATING ENVIRONMENTAL *LISTERIA* USING WHOLE-GENOME SEQUENCING

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DISSERTATION ACCEPTANCE PAGE

Shayanti Minj

This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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It is with genuine gratitude and warm regard that I dedicate this dissertation to my loving family for their unconditional love and blessings throughout this journey,

and

to my advisor, whose has been a constant source of support and whose continued encouragement have pushed me to work hard for the things that I aspire to achieve.

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CONTENTS

ABBREVIATIONS

WPH: Whey Protein Hydrolysate

WPC: Whey Protein Concentrate

WPI: Whey Protein Isolate

SEM: Scanning Electron Microscopy

MRS: De Man, Rogosa and Sharpe Agar

CDC: Centers for Disease Control and Prevention

CFU: Colony Forming Units

PBS: Phosphate Buffered Saline

FDA: Food and Drug Administration

ACE: Angiotensin Converting Enzyme

ABTS: 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid

ATCC: American Type Culture Collection

MD: Maltodextrin

WPH10-MD: Whey Protein Hydrolysate10-Maltodextrin conjugate

RAST: Rapid Annotations Using Subsystems Technology

LAB: Lactic Acid Bacteria

ROS: Reactive Oxygen Species

MLST: Multi Locus Sequence Typing

WGS: Whole Genome Sequencing

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ABSTRACT

DEVELOPING A SPRAY-DRIED FORMULATION USING WHEY PROTEIN AND PROBIOTICS ENCAPSULATION, AND ASSESSING PERSISTENCE OF *LISTERIA* THROUGH WHOLE GENOME SEQUENCING

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In this study, a novel value-added dairy-based health formulation was developed using whey protein hydrolysate and probiotic organisms. In the first part of the study, different forms of whey proteins, concentrate WPC80, isolate WPI90, and hydrolysates WPH10, WPH15 and WPH20 were screened for bioactivities (antimicrobial, antioxidant, and antihypertensive activity). Hydrolysate WPH10, exhibiting the highest bioactivities was conjugated with maltodextrin. A batch of 2L conjugated solution was spray dried in a Niro drier with an inlet and outlet temperature of 200□C and 90□C, and alternatively, freeze dried at −80 °C under 50 mTorr vacuum. The bioactivities of the conjugated samples were then assessed. Overall, the hydrolysates showed significantly higher bioactivities as compared to concentrate and isolate. The conjugated WPH10 solution demonstrated higher antimicrobial, and antioxidant activity, whereas no significant difference in the antihypertensive activity was observed, as compared to WPH10 alone. Subsequent spray and freeze drying of the conjugate solution exhibited even higher antimicrobial, and antioxidant activities, while retaining the antihypertensive activity. Overall, the results indicate the ability of the WPH-maltodextrin to retain the bioactivities after conjugating with a reduced carbohydrate.

In the second part of the first study, conjugated whey protein hydrolysate (WPH10), was used as an encapsulant to entrap probiotic cultures; *Bifidobacterium animalis* subsp. *lactis* ATCC27536 and *Lactobacillus acidophilus* ATCC4356, through a spray drying process. Each culture was added in the conjugated solution at ratio of 1:1, with spiking level of 10 log CFU/mL. The mixture was spray dried in 2L batches using a Niro drier with an inlet and outlet temperature of 200□C and 90□C, respectively. Following drying, scanning electron microscopy was used to observe the particle structure of the WPH10 conjugate. Following, the dried formulation was stored in airtight bottles for 16 weeks at 4°, 25° and -18°C, to monitor cell viability, moisture, and functionality. From the micro images it was observed that subsequent drying, non-conjugated WPH10 presented link bridges between the powder particles, whereas in case of conjugated WPH10, the particles presented a round surface with pores on it. The mean counts in conjugated WPH10 matrix before and after drying were 10.59 log CFU/g and 8.98 log CFU/g, respectively. At the end of 10 weeks, the counts were 7.18 log CFU/g at 4°C and 7.87 log CFU/g at -18°C, whereas at 25°C the viability significantly decreased to 3.97 log CFU/g. At this point, the wetting time increased from 47min to 61min, and solubility decreased from 90% to 82%.

In the second study, whole-genome sequencing was done to determine the genotypic variants and associate with phenotypic expression in *Listeria* strains. Genomic DNA was extracted from the strains (*Listeria innocua* 634-2; *Listeria innocua* 634-34-S-5*; Listeria innocua* 634-34-S-6*; Listeria welshimeri* 634-3; *Listeria welshimeri* 634-253-S-5, and *Listeria monocytogenes* 315-S-1), isolated from the dairy processing environment and run through Whole-genome sequencing on the Ilumina MiSeq and

Nanopore sequencing platform. The genome was fed into the RAST database to develop the annotations. The results revealed 13 types of phenotypic responses related to the genotypic variants. Overall, the genotypic variants varied with the species and within the strains. Some of the common variants and features identified in the isolates were virulence (DNase, phage terminase, tRNA-Arg-ACG), cell signaling (NAG-IIA, NAG-IIB), phage immunity (CRISPR proteins), osmotic stress (*CadA*), oxidative stress (*YRKL*), and antibiotic resistance (*BaiE*, *Lde*). *L. monocytogenes* was positive for all the phenotypic characteristics, allowing it to be more persistent, whereas *L. innocua* and *L. welshimeri* isolates lacked variants for motility (*ActA*), biofilm formation (*AgD*), and acid tolerance (*AdiA*), making them more sporadic strains.

INTRODUCTION

This dissertation contains two studies. The first study represents development of a novel dairy-based value-added spray-dried formulation using whey protein hydrolysate and probiotics. The second study represents whole genome sequencing of *Listeria* organisms to assess their persistence in dairy processing environment.

Study I: Developing a spray-dried formulation using whey protein and probiotics encapsulation

With rising protein deficit among human population, there is an increase in consumer awareness toward a healthy diet and estimations are that the world demand for protein will double by 2050 (Westhoek et al., 2017). Whey protein hydrolysates (WPH) are greatly in demand for developing nutraceutical foods due to their unique health benefits associated with their consumption (Chen et al., 2006). These protein fractions, rich in bioactive peptides contain amino acids in a rapidly digestible form. They also possess specific bioactive properties such as antimicrobial, antioxidant, antihypertensive, opioid, and mineral binding (Hernandez-Ledesma et al., 2014). However, the incorporation of WPH into food formulations is not a trivial task since WPH possess lower stability during processing and subsequent storage (LaClair and Etzel, 2010). During heating and storage, the reactive sites are exposed, and the steric hindrance decreases, which increases the protein/protein interactions and thereby leads to aggregation (Adjonu et al., 2014). Recently, the stability of WPH has been addressed by several researchers, primarily by conjugating them with carbohydrates. Such preparations have helped in improving in physiological and functional properties, including thermal stability, solubility, emulsification capacity, water binding and antioxidant activity

(Zhang et al., 2014). During conjugation, the carbohydrate side chains interact with protein at the interface, limiting interactions between proteins and peptides. The WPH conjugates have been successfully spray dried, achieving greater stability of WPH along with diminishing its bitter taste. The WPH thus offers opportunity as an ingredient of choice for developing health formulations. Similarly, our health also depends on the gut microflora and thus, food products containing probiotics are receiving considerable attention. Probiotics exert several health benefits beyond the inherent basic nutrition (Schaafsman, 1996) and studies have suggested that these organisms produce certain bioactive metabolites, which could present potential benefits. Although, technological advancements have enabled the addition of probiotics to all types of food, maintaining stability of food matrix and viability of organisms during processing and storage has mostly been a challenge that limits the development of novel food formulations with probiotics. Microencapsulation technology has been widely used to increase the stability of probiotics with controlled release characteristics (Zanjhani et al., 2004). Whey based microencapsulation of probiotics has previously been investigated. However, such applications primarily focus on developing delivery systems for probiotic organisms alone. Hence, it may be useful to develop novel products, which not only catch attention of a wider consumer group, but also provide greater value addition in terms of the enhanced functionality of such nutritional products. The present study investigates the feasibility of developing a spray dried health formulation by using WPH-conjugate for entrapping probiotic organisms. Such a formulation with unique health benefits derived from both WPH and probiotic organisms could be used as a functional ingredient to develop other nutritional foods.

Study II: Assessing persistence of *Listeria* organisms through Whole Genome Sequencing

Listeria monocytogenes is a foodborne pathogen that can tolerate harsh conditions of food processing environments including a wide range of temperature (2°C - 45°C), low water activity (~0.93), wide range of pH (4.7 – 9.2) and varying NaCl concentrations (10% w/v) (Melero et al., 2019). It is an etiological agent of listeriosis and causes approximately 1600 illnesses and 260 deaths every year in US (Scallan et al., 2011) and according to US Department of Health and Human services Food and Drug administration, *Listeria* contamination in food industries have become one of the greatest challenges because of its ability to tolerate cold environment and moist environmental conditions in a food processing plant where it can persist for months to decades (Taylor and Stasiewicz, 2019). Such persistence is contributed by various mechanisms such as resistance to extrinsic stresses, tolerance to sub lethal concentrations of antimicrobial agents, formation of biofilms, equipment damage and inappropriate workflow between clean and dirty processing areas (Latorre et al., 2011; Muhterem-Uyar et al., 2015). This can increase the risks of cross contamination within food products.

Several dairy-related Listeria outbreaks have been reported worldwide since the first emergence in Southern California in 1985, where it was traced to consumption of Mexican-style cheese (Melo et al., 2015). Strains of *Listeria* are recurrently found in dairy processing environment such as floors, drains and equipment, notably in the cold and wet atmosphere of refrigerated premises, though these are routinely cleaned and disinfected. Good growth conditions are abundant in several hard to eliminate ‘harborage sites’ due to unhygienic design of equipment and premises or unhygienic or damaged

materials. Relative growth of *Listeria* vs. efficacy of cleaning and disinfection can influence their persistence in a dairy processing environment. A minimum initial bacterial load is thus necessary for *Listeria* to persist in a harborage site. Its persistence in the dairy processing environment has been reported, as the organism can form resistant biofilms leading to potential cross contamination of the product, especially from harborage sites. Hence, a genetic characterization of the isolates will help to elucidate the presence, spread and its persistence in dairy processing environments and throughout the food supply chain. The whole genome sequencing, in addition to culturing techniques, of resident isolates would provide in depth knowledge of the genetic sequences of these isolates, responsible for colonization and aggregation. The frequency of the sequences would thus help us to understand the attachment behavior of 'resident *Listeria*' to the surfaces in a dairy manufacturing environment.

To do so, in this study we acquired the 'resident strains' of *Listeria* from the dairy processing environments, and whole-genome sequencing was done to determine the genotypic variants and associated phenotypic expression in 10 different *Listeria* strains (industrial as well as ATCC). The genotypes were recognized and associated with the phenotypic characteristics such as biofilm forming abilities, pH, osmotic shock, and antimicrobial and sanitizing resistance *of Listeria* species that was related to their persistence in a dairy plant environment.

CHAPTER 1

LITERATURE REVIEW

STUDY I: Developing a spray-dried formulation using whey protein and probiotics encapsulation

Bovine milk is one of the most nutritious foods and is widely used for human consumption. It is one of the rich sources of nutrients that have several biological properties that impact the biochemical processes in our body, influences the development and functioning of specific organs, and protection from diseases. Milk provides a wide range of biologically active components such as bioactive proteins and peptides, oligosaccharides, immunoglobulins, and fats/lipids that protect against pathogens and illnesses on regular consumption.

Milk can be sourced from several milch animals including, cow, buffalo, goat, and sheep. Bovine milk contains approximately a total protein of 3.5%, fats, and essential vitamins, that support growth and development (Yalcin, 2006). It is a natural and rich source of well-balanced nutrients that show a diverse range of biofunctional properties. These properties are because of the presence of milk proteins/peptides, which support infant development, its growth, and confers health benefits beyond basic nutrition (Leppala, 2001). Besides, proteins extracted from milk are well characterized for their multiple functional characteristics and are utilized by several industries in food applications. The milk protein system is constituted majorly by two kinds of proteins: approximately 80% (w/w) casein, which is generally extracted from skim milk through precipitation using either an acid (isoelectric precipitation) or enzymes (rennet coagulation) and 20% whey, which is a leftover byproduct after the casein is extracted

(Madureira et al., 2007). Majorly whey portion of milk contains five fractions which altogether make up 85% of whey protein. These fractions include α-lactalbumin, β-lactoglobulin, glycomacropeptide, immunoglobulins, protease peptone, and serum albumin whereas the casein portion of milk contains β-casein, αs1-casein, αs2- casein, and κ-casein (Séverin et al*.,* 2005).

Proteins are macronutrients when consumed as supplements may exhibit favorable effects on growth metabolism and health (Chou et al., 2012; Bertenshaw et al., 2008). Several reports show that protein deficiency is one of the major health concerns globally (Gomes et al., 2009), and considering this condition, the introduction of dietary protein-rich supplements is of utmost importance. Some of the by-products from agricultural industries like, fruit pomace (Bhushan et al., 2008), soy extract (Katayama and Wilson, 2008), cereal brans (Pavlovich-Abril et al., 2016), and milk whey (Sousa et al., 2012), are increasingly getting popular as food ingredients with healthy components. This review focuses on exploiting the bioactive and functional properties of milk whey proteins.

Whey is a yellow-green colored liquid portion of milk, also called cheese serum is obtained after separation of the curd, during coagulation of milk using proteolytic enzymes or acids (Codex Alimentarius, 2011). It was considered as a major dairy waste for decades because of the disposal issues related to its high biological oxygen demand and high organic matter (Ahn et al., 2001). However, nowadays whey proteins are recognized as a potential source of nutrients and are exploited for its bioactive ingredients. Because of its high nutritional composition, it is used in several commercial food product applications and is significantly associated with the dairy industry (Walstra et al., 2006). Generally, fresh liquid whey from cheese-making is composed of 94.2%

water and 50% of the total solids of which 0.8% is whey proteins, 0.5% is minerals, 0.1% is fat and 4.3% is lactose, which is the main constituent (Almeida et al., 2013). However, the composition and the characteristics of whey may vary with the type of cattle, the diet of the animal, milk from which it is produced, processing techniques used, and other environmental factors (Park et al., 2007). Whey proteins are a form of globular proteins, containing a considerable number of α-helix patterns with evenly distributed hydrophilic and hydrophobic, and acidic and basic amino acids along their polypeptide chain (Evans, 1982). The major constituents of whey proteins include α-Lactalbumin (α-LA); β-Lactoglobulin (β-LG), bovine serum albumin (BSA), immunoglobulins (IG), bovine lactoferrin (BLF), bovine lactoperoxidase (LP), and minor amounts of glycomacropeptide (GMP). The composition of each constituent is shown in Table 1. However, the whey protein composition will vary based on the whey type i.e., sweet whey or acid whey, type of milk i.e., bovine, ovine or caprine, type of cattle feed, lactation stage, and the type of processing. Whey, acidic in nature will have a pH of approx. 5.1 and is generally produced through direct acidification whereas sweet whey has a pH of around 5.6 is produced through rennet-coagulation particularly during the cheese-making process (Pintado et al., 2001).

Whey protein derivatives: Concentrates, Isolates and Hydrolysates

With the rising popularity of healthy eating, there is a worldwide demand for food products formulated with high protein (Westhoek et al., 2017). The daily average protein intake for a sedentary person should be 0.8g per kg of body weight per day (g/kg/day) (Lemon, 1995). This amount of protein is required to maintain a positive nitrogen balance and healthy metabolic function in the body. There are various forms of supplemental

proteins available such as egg, soy, hemp, whey, casein. Among these, milk whey contains the maximum concentration of amino acids that are readily available and easy to digest, making it effectively incorporate into body cells (Smithers, 2008).

Besides, milk whey proteins are recognized as healthy ingredients because of their several advantages associated with their regular intake, including appetite control, exercise recovery, and promoting satiety (Shang et al., 2018). In recent years, several applications of membrane filtration have enabled the use of different whey protein components as food additives. Using selective membranes, after the milk is coagulated the whey protein is extracted in two main forms: whey protein concentrates (WPCs) (having ~34-89% protein) and whey protein isolates (WPIs) (having at least 90% protein) (Suarez et al., 2009; Wright et al., 2009). Passing the whey proteins through various processing treatments leads to the formation of whey products (Figure 1) with different qualitative and quantitative protein profiles, and minerals, lipids, and sugars. Application of selective membranes to fractionate whey proteins include ultrafiltration (UF) to concentrate proteins or the use of the diafiltration (DF) method to exclude the molecular compounds like minerals, lactose, and other low weight components. This leads to the production of whey protein concentrates (WPC) (Smithers, 2008). It is the most concentrated form of protein supplement, that has high calories and contains all the macro and micro-nutrients derived from the manufacturing process. However, based on the protein concentration, it can be of several types like WPC of 35%, 50%, 65%, and 80% (w/w) protein. When most of the components are removed i.e., the whey undergoes an additional purification step to eliminate or minimize the extraneous carbohydrates and fats to obtain a protein threshold of 90% (w/w), it is referred as whey protein isolate

(WPI). Though being a high-quality protein, the disadvantage of an isolated form of whey protein is that the purification leads to the elimination of some of the important micro-nutrients and protein fractions like lactoferrins, β-lactoglobulins, and immune-globulins.

The concentrates and isolates are composed of large intact protein structures, hence, during digestion, the enzymes in our digestive tract break down these proteins, targeting the amino acid bonds, to generate smaller peptides with amino acid sequences. To facilitate this process and make the protein absorption faster, the manufacturers pre-digest the protein to produce protein hydrolysates.

When whey protein concentrates or isolates are treated with acids, enzymes, or heat, the intact form of protein breaks down into peptides and amino acids leading to the formation of whey protein hydrolysates (WPH). These pre-digested forms of whey protein are effectively absorbed in the gut, and hydrolysates that are produced through enzymatic hydrolysis using protease enzyme, contains the identical amino acid composition to that of concentrate and isolate, thus on ingestion, can rapidly increase the amino acid concentration in the plasma as compared to intact forms of protein (Morifuji et al., 2010). The final composition of the hydrolysate largely depends on the type of process implied to break the proteins, the type of enzymes used, reaction or hydrolysis conditions applied, and the number of amino acid bonds that are targeted and broken. Therefore, the degree of hydrolysis is measured to determine the release of the amino acids. The greater the degree of hydrolysis, the smaller the amino acids per peptide, resulting in the generation of more bitter peptides (Buckley et al., 2010). However, all

these forms of proteins are enriched with several benefits and used as food additives to exhibit biological properties.

Biological properties of whey proteins associated with bioactive peptides

Biological properties of whey proteins (Figure 2) are widely recognized and have been increasingly exploited in scientific research studies and food applications by various industries. β-lactoglobulins contribute to 50% of the whey protein, which helps to bind minerals like zinc and calcium. It also has partial sequence homology to retinol-binding proteins. α-lactalbumin, on the other hand, is strongly advised to be added in the infant formulas or into foods to develop protein rich dietary intakes. Serum albumin can bind fatty acids and immunoglobulins like IgA, IgM, IgG1, and IgG2, which helps to develop passive immunity in consumers. Other protein fractions like lactoferrin are an iron-binding protein that increases the iron absorption in the digestive tract to inhibit enteric microorganisms and promote the growth of desirable microorganisms. It also modulates the immune system and is considered as the major non-specific disease resistance factor in the mammary gland. Lactoferricin is a peptide derived from Lactoferrin is used against intestinal pathogens. Lactoperoxidase is an enzyme with antibacterial properties that is used as a natural preservative to control acid development in milk during refrigerated storage (Walzem, 1999). Whey proteins are a rich source of essential amino acids like cysteine, branched-chain amino acids like leucine, isoleucine, and valine, and in bioactive peptides (Hulmi et al., 2010). Leucine plays an important role in regulating the synthesis of skeletal muscle protein and is 50-75% higher in whey proteins as compared to other sources (Chen et al., 2014). It is also high in Sulphur rich amino acids i.e., cysteine which is a precursor of glutathione (Bell, 2000). Glutathione is a non-enzymatic thiol obtained

from the diet, that acts as an antioxidant. It helps to protect from diseases by reducing the antioxidative stress and regulating the cellular processes (Trachootham et al., 2008). Glycomacropeptide (12%), released during rennet coagulation of cheese, is a casein-derived whey peptide that has many health benefits including satiety and phenylketonuria management (Neelima et al., 2013). Specific biological functions of the whey protein components are given in Table 2.

Depending on the protein concentration and characteristics, whey proteins are marketed in forms of whey protein concentrates, isolates, and hydrolysates (partially broken down through digestion) (Barth and Behnke, 1997). These derivatives have a broad range of biological functions including reducing oxidative stress, promoting muscle growth and synthesis, suppressing appetite, hypoglycemia, phenylketonuria management, reducing risks related to cardiovascular diseases, and protecting from ultra-violet (UV) radiation damage (Sousa et al., 2012).

Whey protein associated bioactive peptides

Isolated protein fragments, containing 2 to 20 amino acid residues, that influence health by delivering beneficial effects on body functions are referred to as bioactive peptides. Mellander in 1950 derived phosphorylated peptides from casein and showed an enhanced effect on rachitic infants in Vitamin-D independent bone calcification. Thereafter, numerous bioactive peptides have been isolated, identified, and studies (Korhonen and Pihlanto, 2004). BIOPEP consists of both sequence databases and tools for the evaluation of protein as precursors of bioactive peptides. Using this database, several peptides with biological functions have been identified (Dziuba et al., 2004). From which, ACE inhibitory peptides are the most identified ones (Clare et al., 2003;

Gobbetti et al., 2002; Ahn et al., 2000). However, other bioactive peptides with opioid, antioxidant, immunomodulatory, and anticancer properties have also been presented. Bioactive peptides can be isolated from different food proteins either through gastrointestinal digestion or through fermentation using proteolytic lactic acid bacteria. Depending on their amino acid chains, bioactive peptides, on ingestion may significantly affect the body functions related to the digestive, immune, cardiovascular, or nervous system. These amino acid sequences are specific to their actions in delivering health effects. For example, peptides exhibiting antioxidative, antimicrobial, ACE inhibition, and immunomodulation will possess specific known peptide sequence (Nongonierma and FitzGerald, 2015; Li-Chan, 2015; Fekete et al., 2013; Shimizu et al., 2004; Korhonen and Pihlanto, 2003). Some of these peptides also exhibit multi-functional activities (Meisel and FitzGerald, 2003). Hence, these bioactive peptides are recently being used in several food applications for the development of pharmaceutical, nutraceutical, and functional foods (Panchaud et al., 2012).

Manufacture of bioactive peptides from whey proteins

Bioactive peptides or biologically active peptides or are most produced through use of different enzymes through enzymatic hydrolysis. They can also be generated through food processing and microbial fermentation using proteolytic lactic acid bacteria.

Enzymatic hydrolysis of whey proteins

Bioactive peptides are mostly produced using different enzymes through enzymatic hydrolysis. Enzymes that are most widely used are proteases and they can be specific or non-specific to their target protein. Hydrolysis of whey proteins using enzymes is mostly preferred by food manufacturers due to their short reaction time, the specific site of

enzyme action, and the availability of wide sources of enzymes (from animal, plant, and microorganisms). Most used enzymes are trypsin, pepsin, chymotrypsin, bromelain, and they have their specific reactions conditions (temperature, pH, and time) (Agyei et al., 2011; Clemente et al., 2000). However, for the maximum activity, the type of enzymes to be used, enzyme: substrate ratio, and their reaction conditions should be optimized before the hydrolysis. The selection of enzymes is essential as it influences the cleavage site and patterns of the peptide bonds. Also, enzymatic modifications are known to produce peptides with more consistent molecular weights and improved functional and biological properties of the hydrolysates. Various proteases are commercially produced and used for generating bioactive peptides on a laboratory scale (Najafian and Babji, 2012). Sometimes, these enzymes, when used in combination, are shown to release more stable and effective peptides (Byun et al., 2001). Yamamoto et al., 2003, used enzymes to hydrolyze protein-rich food materials such as fish, milk, meat, cereal, eggs, and soybean to extract bioactive peptides. Those peptides exhibited properties like antihypertensive, opioid, immunomodulatory, antimicrobial, and mineral binding. Sarmadi and Ismail, 2010 showed that hydrolysis of β-conglycinin and glycinin using enzymes can lead to the production of active antioxidant peptides with R group amino acids. They also reported that digestion through enzymes can also produce bioactive peptides with a low molecular weight of below 1000Da.

Microbial Fermentation and Food processing of whey proteins

Food-grade bioactive peptides are mostly preferred to be produced through microbial fermentation using proteolytic lactic acid bacteria (LAB). These microorganisms are commonly found in our digestive system and are widely spread in nature. Lactic acid

bacteria are generally used in food fermentations because of their physiological significance and their role in influencing the texture and flavor of the product (Savijoki et al., 2006). During the fermentation process, these LAB also able to break down food proteins to produce biologically active peptides. Their proteolytic system comprises proteinases which break down the proteins to generate numerous oligopeptides (4-8 amino acids), an oligopeptides transport system, a route to let entry of nitrogen into cell and peptidases, which completely break down the accumulated peptides (Christensen et al., 1999). As compared to the enzymatic hydrolysis microbial fermentation is more economical and is recognized as safe. LAB being an efficient source of proteases require minimal nutrition and expresses proteases on the cell membrane which makes the enzyme extraction and purification convenient and cost-effective (Agyei et al., 2011).

Bioactive properties associated with the bioactive peptides isolated from whey proteins and derivatives

Antioxidant activity of the bioactive peptides

Oxidative stress in the body can lead to several disorders such as diabetes, cancer, cystic fibrosis, atherosclerosis, aging, and numerous other degenerative diseases. Whey protein is a precursor of the antioxidant glutathione exhibits antioxidant activity by suppressing the adverse effects of stress factors. The release of bioactive peptides from whey proteins is shown to raise the intracellular glutathione level and reduced the generation of in vitro interleukin IL-8 (cytokine responsible for mediating pathogenesis in the respiratory tract). (Piccolomini et al., 2012). Supplementation of pressurized whey (20g/day) for a month was shown to reduce C-reactive protein serum level in patients with cystic fibrosis (Lands et al., 2010). Whey protein hydrolysates produced from

alcalase enzyme treatment was found to contain two peptide fragments, P4 and P4c (a pentapeptide containing amino sequence of Val-His-Leu-Lys-Pro). These peptides exhibited antioxidant activity by significantly reducing hydrogen peroxide exposure to human lung fibroblast MRC-5 cells (Kong et al., 2012). A diet (MHN-02) formulated with antioxidants and whey peptides was tested for anti-inflammatory activity in rats. It was observed that the rats that received this diet showed higher survival (90%) as compared to the ones fed with the control (55%). This was due to the high superoxide dismutase activity (conversion of superoxide radicals to hydrogen peroxide and oxygen) and less pathological lesions in the MHN-02 diet group (Takayanagi et al., 2011). The role of whey protein derivatives in improving the glutathione synthesis in neurons and reducing the neuro-system disorders was studied (Ross et al., 2012). It was reported by Piccolomini et al., 2012, that whey protein isolates and native hydrolysates with antioxidant and anti-inflammatory peptides, when added to human epithelial colorectal adenocarcinoma Caco-2 cells, that was exposed to H_2O_2, both inhibited production of IL-8 and reactive oxygen species (ROS). However, the effect was comparatively higher for whey protein isolate treatment, which suggested that whey protein hydrolysates from isolates are more effective in alleviating inflammation and oxidative stress in intestinal cells. Besides, these activities were observed to elevate following hyperbaric treatment. In one of the studies, rats were subjected to a high concentration of iron followed by treating them with a placebo or whey protein diet to determine the effect on oxidative stress. After 6 weeks, the test animals showed an increase in lipid peroxidation and reduction in the radical scavenging activity. Whereas, in rats that were fed with a whey protein diet exhibited high blood glutathione level as compared to the control (iron

overload) group. This suggested the ability of the whey proteins to alter the high iron-induced DNA-damage and reduce ROS in cells (Kim et al., 2013). Pseudomonas aeruginosa is one of the known pathogens responsible for lung colonization and pulmonary infection, leading to difficulty in breathing (Lyczak et al., 2002). Kishta et al., 2013, studied the effect of whey protein in lowering the pulmonary infection and found that mice fed with a pressurized-whey protein diet showed a reduced level of oxidative stress, inflammation, and lung damage. The potential reason was the ability of the peptides to stimulate the leucocytes to kill the pathogens and protect the airway proteins from oxidation. A whey protein hydrolysate when administered in mice with paracetamol-induced hepato-nephrotoxicity, was found to increase the level of antioxidant enzymes like catalase, glutathione peroxidase, superoxide dismutase and reduced the production of thiobarbituric acid reactive substances (TBARS), and oxidative biomarkers like phosphatase, glutathione pyruvate transaminase and creatinine (Athira et al., 2013). It was also observed that peptide generated from chymotrypsin hydrolyzed whey protein exhibited higher ferrous chelating capacity and DPPH radical-scavenging activity as compared to whey protein isolate (Liu et al., 2014a).

Antihypertensive activity of the bioactive peptides

Bioactive peptides from whey protein concentrate, isolates, and hydrolysates having Angiotensin-Converting enzyme (ACE) inhibitory or antihypertensive activity are strongly associated with the renin-angiotensin system. Therefore, foods with antihypertensive peptides should be regularly consumed to control blood pressure and prevent cardiovascular disorders (Fitzgerald et al., 2003). ACE plays an important role in converting angiotensin I to angiotensin II (vasoconstrictor) in the rennin-angiotensin

system. Besides, it also degrades the bradykinin which is a potent vasodilator. Although structure-activity interaction of ACE-inhibitory peptides from milk proteins is not well defined, there is the possibility that peptide binding to ACE is accessed by the C-terminal tripeptide sequence of the substrate or competitive inhibitors, choosing hydrophobic (aromatic or branched side chains) amino acid residues at each of the three C-terminal positions (Haque and Chand, 2010).

Many whey proteins derived peptides have been described to demonstrate ACE inhibition activity. Whey protein hydrolysates (WPH) containing peptides derived from α-Lactalbumin (f 99-110) fraction is shown to demonstrate ACE inhibitory activity specifically in the sequences (f 99-108), (f 104-108), (f 105-110). It was reported that whey protein fraction (α-lactalbumin) (f 50-53) exhibited antihypertensive activity at IC_{50}= 733.3 μM. Other dipeptides that demonstrated similar ACE inhibition at IC_{50}=1522.6 μM and IC_{50}=349.1 μM include Tyr-Gly and Leu-Phe respectively (Mullaly et al., 11). Tripeptides (Try-Gly-Leu) (α-lactalbumin f 50-52) are also shown to demonstrate ACE-inhibition in the same range as that of the dipeptides. Whey protein hydrolysates derived from the β-Lactoglobulin chain consist of a mixture of peptides that are shown to demonstrate antihypertensive activity. Whey proteins treated with trypsin enzyme liberated peptides with moderate antihypertensive activity including β-Lg (f 22-25), (f 32-40), and (f 81-83). β-Lactoglobulin peptide (f 142-148) generated from trypsin as reported by Mullaly et al., 1997, exhibited higher ACE-inhibition effects with IC_{50}= 42.6 μM as compared to the peptides that showed ACE inhibition effects with a range of IC_{50}= 77-1682 μM. Studies from Philanto-Leppala, 2001, showed the most active antihypertensive whey protein peptide to be from α-Lactalbumin (f 104-108) with IC_{50}= 77 μM.

Neutrase enzyme hydrolysis of cheese whey protein generated a mixture of peptides, that was shown to exhibit strong antihypertensive or ACE-inhibition activity. ACE is responsible for regulating several biological processes and is strongly associated with cardiovascular disorders, hence, the role of whey proteins in inhibiting the ACE enzyme is relevant (Eliseeva, 2001). ACE-inhibitory bioactive peptides are generally below 1kDa and hold 38% of the total protein content in the whey protein hydrolysate (Estevez et al., 2012).

Opioid activity of the bioactive peptides

Bovine whey protein fractions like α-lactalbumin (f 50-53) and β-lactoglobulin (f 102-105) contain certain peptides that exert opioid activity. These peptides are referred to as α- and β- lactorphins (Chiba and Yoshikawa, 1986). These peptides have an affinity towards the opiate receptor, inhibited by naloxone. These peptides have an amino acid sequence of Tyr-Gly-Gly-Phe in their N-terminal and exhibit their activity to the target cell by binding to the specific opiate receptors. The presence of tyrosine residue at the N-terminal and the aromatic amino acids at the other positions play an important role in forming the peptide structure motif that perfectly binds to the opiate receptors (Teschemacher et al., 1994). These receptors play role in several physiological responses like μ-receptor for emotions and reduction of intestinal motility, κ- receptor for food consumption and sedation, σ-receptor for emotional behavior.

Several process treatments can be applied to generate lactorphins from the bovine whey proteins. α-Lactalbumin when treated with enzymes like pepsin, liberates α-lactorphin through proteolysis whereas when β-Lactoglobulin is treated with pepsin followed by trypsin or with a combination of trypsin and chymotrypsin, yields β-lactorphin. Further, hydrolysis of β-Lactoglobulin (f 146-149) using chymotrypsin alone,

led to the production of β-lactotensin (His-Ile-Arg-Leu). Considering receptor binding affinity, the α-lactorphin exhibits weak but consistent affinity whereas the β-lactorphin exhibits non-opioid activity when tested on the ileum of the guinea pig. Overall, these peptides belong to μ-type receptor ligands which displayed low receptor binding affinity towards opioid receptors. Both these peptides, when added in micromolar concentrations, were found to inhibit 3H-naxolone from binding to the receptor sites. In contrast, the morphine, which is a standard opioid peptide was found to inhibit 3H-naxolone in the range of IC50=23±13nM nanomolar concentrations (Paakkari et al., 1994). Approximately 0.9 g/L of α-Lactalbumin and 3.0 g/L of β-Lactoglobulin is present in bovine milk, which contributes to the production of 30mg of α-lactorphin and 90mg of β-lactorphin. During *in vitro* digestion of milk, these peptides get released to exhibit *in vitro* opioid effects. For hydrolysates, it was observed that the release of lactorphins at concentrations of 5-14% was sufficient to exhibit opioid activity *in vitro*.

Antidiabetic property of the bioactive peptides

Diabetes is one of the critical health issues that causes several disorders including vision loss, angiopathy, and blood flow restriction leading to tissue hypoxia and ulcers with reduced healing (Casqueiro et al., 2012). Consumption of hypoglycemia chemical drugs with a controlled diet can help to treat Type-2 diabetes. Dietary supplements with added whey proteins have been shown to demonstrate anti-diabetic effect by reducing the serum blood glucose level in healthy individuals, improve muscle mass, and increase the secretion of satiety hormones (cholecystokinin, leptin, and glucagon-like peptide 1(GLP-1)) and reduce the release of ghrelin (the hormone responsible for hunger) (Sousa et al., 2012). It was observed that the presence of cysteine, plays an important role in treating

glycemia and controlling inflammation in people with diabetes (Jain, 2012). A study from Badr et al., 2012a, showed the effects of whey protein on Type-I diabetes-induced wounds in a mouse model. It was found that whey proteins significantly lowered the diabetic inflammations and wounds by restricting the production of inflammatory cytokines and expression of chemokines (MIP-1α, MIP-2, KC, CX3CL1, TGF-β), as compared to the untreated mice. Salehi et al., 2012, investigated the effects of whey protein and found that increase in the levels of insulin and amino acids like valine, leucine, isoleucine, threonine, lysine are the major causes for the antidiabetic activity. Whey protein derivatives (isolate and hydrolysate) when supplemented in a fat-rich diet were found to improve the secretion of insulin leading to the lowering of postprandial triglyceride responses in Type-2 diabetes subjects (Mortensen et al., 2012). After feeding rats with a diet rich in whey protein hydrolysate for a month, an increase in the leucine content and insulin level was observed (Toedebusch et al., 2012). Results showed that the whey protein is metabolized in the gut and as result peptides and amino acids are released which are responsible for inducing the insulin level along with the secretion of the gut and incretin gastric hormones. When whey protein action was investigated for reducing the glucose concentration, it was observed that the protein was able to strongly lower the levels of plasma glucose, insulin, and C-peptide. However, it increased the levels of GLP-1 and PYY, which tells that consumption of whey protein before a meal can lower the post-meal glycemia by both insulin-dependent and insulin-independent pathways (Akhavan et al., 2014). Tong et al., 2014 demonstrated the effect of both whey protein and its hydrolysate fraction to exhibit anti-diabetic effects by improving the insulin resistance in rat subjects.

Anticancer activity of the bioactive peptides

Intake of whey protein has been shown in several studies to exhibit beneficial effects on cancer patients. Reports have stated that whey protein hydrolysates confer improved anticancer effect as compared to other forms of whey protein. A study showed that rats with colon cancer, when fed with a whey protein hydrolysate demonstrated a reduction in the appearance of macroscopic and microscopic tumors as compared to the rats belonged to the control group that fed with un-hydrolyzed whey protein (Attahallah et al., 2012). Whey protein was also reviewed for anticancer properties against the melanoma B16F10 cell model, and it was observed that expression of Caspase-3 increased significantly in the media containing whey protein isolate (Castro et al., 2009). Caspase-3 is known to play an important role in mediating apoptotic cell death (Takata et al., 2001). In a 48-year-old Caucasian female, when whey protein at a dosage of 10g (three times daily) was administered in combination with a weekly injection of testosterone enanthate before and during chemotherapy, an improvement in the lean body mass, physical movement, and overall quality of life were observed (Dillon et al., 2012). Another study demonstrated the protective effect of whey protein hydrolysate on rat pheochromocytoma PC 12 cells with oxidative damage. A 20-30% increase in the cell viability was observed at a dosage level of 100-400µg hydrolysate/mL as compared to the ones that were incubated with an infusion of H_2O_2. This suggests the potentiality of the whey protein hydrolysates to exhibit antioxidant activities (Zhang et al., 2012).

Immunomodulatory activity of the bioactive peptides

Whey protein derivatives in form of concentrates are known to improve innate mucosal immunity and deliver protection from immune disorders (Perez-Cano et al.,

2007). Public concern for atopic dermatitis (a condition where the skin is swollen, scaly with itchy rashes) is continuously increasing worldwide with infants being more susceptible to it. Recently, a meta-analysis showed that infants fed with a hydrolyzed form of whey protein developed reduced symptoms of atopic dermatitis as compared to the control subjects that were given with plain bovine milk (Alexander et al., 2010). These results suggested that diets included with whey protein might play an effective role to protect the infants from atopic dermatitis. Another study in mice models showed reduced levels of the plasma (Interleukin) IL-1α, IL-1β, IL-10, (Tumor Necrosis Factor) TNF- α, ROS (Reactive Oxygen species), and cholesterol after they were treated with whey protein concentrate and examined for blood parameters, plasma cytokine profiles, proliferation, and migration of immune cells (Badr et al., 2012b). Mice subjects given with the whey protein diet displayed significantly improved concentration of IL-2, IL-4, IL-7, IL-8, and glutathione. Besides, an improvement in the response of leucocytes, macrophages, and monocytes to different antigens was observed. As compared to the control group, in the treated group it was observed that the cytokine CC chemokine ligand-21 (CCL-21) and CXC chemokine ligand-12 (CXCL12) can attract the immune cells and migrate the B-cells, T cells, and dendritic cells towards them (Badr et al., 2012b). The bioactive effect of the whey protein isolate was determined against psoriasis (a skin condition with thick skin, dry scales, and red patches) and an intake dose of 20g/day was provided to the patients. It was found that the glutathione level increased and following the inflammation due to psoriasis decreased with the consecutive intake of whey protein (Prussick et al., 2013).

Muscle protein synthesis by bioactive peptides

Heavyweight exercise, resistance training, eccentric (muscle lengthening), concentric (muscle shortening) and isometric (muscle non-lengthening) exercises can cause skeletal muscle tear and damage and possibly can lead to internal inflammation (due to the production of inflammatory muscle protein markers) (Morton et al., 2009). Resistance training (heavy weightlifting) can lead to the accumulation of oxidation products in blood plasma resulting in leukocyte functionality (Freidenreich and Volek, 2012). Regular intake of whey protein supplements containing amino acids like hydrolysates has been reported to repair muscle damage. Ingestion of β-hydroxy- β-methyl butyrate, generated from leucine has been shown to improve muscle recovery. Expression of glucose transporter in skeletal muscle in form of cell-surface glucose transporter 4 (GLUT-4) is known to control the rate of glucose transport in the cell membrane, in response to insulin and muscle contraction. Hence, whey protein was investigated for its capability to accumulate GLUT-4 in the membrane which can lead to a reduction of glucose entrapment in the muscles. The major amino acid and bioactive peptide in the whey hydrolysate contributing to the process was found to be L-isoleucine and L-leucyl -L-isoleucine, respectively (Morato et al., 2013). The effects of whey protein on muscle functionalities like contraction, elasticity, extension's ability, and excitability as compared to a casein-diet were reviewed. It was observed that whey protein fed rats with isometric and concentric exercise injury recovered faster as compared to the ones fed with casein (Martin et al., 2013). Whey protein beverage supplemented with different doses of leucine and branched-chain amino acids was shown to stimulate the synthesis of myofibrillar protein both at high and low protein content and at 5g leucine content (Churchward-Venne et al., 2014). Leucine contributes to 10% of the total whey protein

amino acid and therefore, is more important for muscle protein synthesis and recovery. In a study, whey protein hydrolysate was consumed for 12-week, and it was observed that protein played role in reducing the muscle damage markers like creatine kinase and lactate dehydrogenase (Lollo et al., 2014). As compared to soy protein, it was found that whey protein supplements are more effective in expressing leucine in subjects undergoing resistance training exercises (Volek et al., 2013).

Identification of bioactive peptides isolated from whey proteins and derivatives

Bioactive properties of the peptides are determined based on their amino acid sequence and molecular weight. Mostly the peptides are of short-chain length with 2 to 6 amino acid sequences, however, some peptides with high molecular weight are made of 30 amino acids. Hence, to isolate these peptides, firstly they can be passed through an ultra-filtration membrane of varying molecular weight such as 10kDa, 5kDa, or 3kDa (Power et al., 2014). Roblet et al., 2012 recovered peptides from a soy-protein hydrolysate through ultra-filtration and screened them for their bioactive properties. He found an improvement in the bioactivities following fractionation. Another technique that has been commonly used for separating and purifying these bioactive peptides is High-Performance Liquid Chromatography (HPLC). Other methods such as Sodium dodecyl sulfate-polyacrylic gel electrophoresis (SDS-PAGE) and ultra-centrifugation, are also implied for the characterizing the protein and identifying the amino acid composition of the peptides. Recently, several other methods like electrospray ionization (ESI), mass spectrometry (MS), matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) are being used to isolate, identify, and characterize the bioactive peptides. Among these methods, mass spectrometry has been used to generate the peptide profile and

determine the molecular mass and amino acid sequences of the protein hydrolysates. It is also used to analyze the protein conformational changes and protein degradation products (del Mar Contreras et al., 2008). The peptides generated can be concentrated using ultra-filtration and size exclusion chromatography. And, to know the protein structure-based functional properties, reverse phase HPLC is used to segregate the peptides depending on their hydrophobic properties (Pownall et al., 2010).

Haileselassie et al., 1999, extracted peptides from an enzyme-modified cheese. The peptides were then passed through reverse phase HPLC using a Delta Pak C18 column. In total 8 peaks were generated (one from neutrase digest, five from neutrase debitrase digest, and two from microbial enzyme from Lactobacillus digest). These peptides were purified and identified through API mass spectrometry. In another study from Chiang et al., 2006, antihypertensive peptides were extracted from soy milk and fractionated into four parts (A-D) using a size exclusion chromatography. It was found that among the fractions obtained, B showed the highest antihypertensive property. Using proteolytic fermentation, three antimicrobial peptides were generated, and these peptides were later separated using reverse-phase HPLC. The fractionated peptides were identified for their properties using a mass spectrometry combined with MALDI-TOF (Hayes et al., 2006). Electrospray LC-MS was used to purify and identify peptide from buckwheat protein that exhibited ACE inhibitory activity. A UHPLC-Q-TOF MS/MS method was developed to identify peptides from Mactraveneriformis hydrolysate. Four nucleobases and one nucleoside were in total identified through the de novo sequencing based on the MS/MS fragmentation (Liu et al., 2015). Also, there are several databases such as BIOPEP bank,

that are available that can be used to generate information related to protein and peptides (Minkiewiez, 2008).

Functional properties of whey proteins

Functional properties of proteins refer to the physicochemical properties that play important role in imparting specific behavior and performance to proteins when added in food systems. The properties of whey proteins include thermal stability, hydration, gelling, and emulsification properties, that influence the final quality of foods. These properties vary with the interaction among protein-protein or with other food components and are strongly affected during preparation, processing, storage, and consumption of the foods. Some of the processing conditions, extrinsic and intrinsic parameters that influence the functionalities of whey proteins are shown in Table 4. Whey proteins and derivatives vary in their composition and hence, possess different functional properties. As a result, they are used in different food applications.

Thermal denaturation of whey proteins

The thermal processing of food is greatly influenced by processing as well as compositional factors. Processing parameters include temperature, pH, ionic strength, rate of heating and compositional factors include lactose and protein content. Foods with whey proteins when exposed to mild heating (40°C), leads to denaturation of β-lactoglobulin whereas heating beyond that (50°C to 60°C) leads to unfolding and exposure of the thiol group (Kella and Kinsella, 1988). During the cooling of β-lactoglobulin, in the presence of calcium, the protein-protein linkages are formed via disulfide bonding and entropic forces leading to aggregation (Haque et al., 1987). At low pH of <3, denaturation of whey proteins can develop unique physicochemical and

functional behavior. (Modler and Harwalkar, 1981) reported that whey protein concentrates obtained through ultra-filtration at pH 2.5-3, yielded proteins with different viscosity and gelling properties before or after heat treatment. Heating treatment at 90°C for 15min at pH 2.5-3, led to isoelectric precipitation of protein, that was very viscous with reduced solubility and increased setting to form a soft coagulum (Modler and Emmons, 1977). Several research have also been done on the unfolding of whey proteins following heat treatment. Investigations using differential scanning calorimetry (DSC) have shown the entire thermodynamics of the unfolding of whey proteins. During heating (20°C to 110°C) of α-Lactalbumin and β-Lactoglobulin at a concentration of 3-9% and pH 7, they are reported to have transition temperature (*Tt*) of 65°C and 73°C at 10°C per minute. And the *Tt* increased with the increase in the rate of heating. However, it was observed that increasing the pH from 6.4 to 7.3 increased the denaturation of the β-Lg and reduced the *Tt* from 79°C to 74°C (Ruegg et al., 1977). This suggests the ability of the whey proteins to unfold at increased pH and electrostatic repulsive forces within the polypeptides. At pH beyond 7, the thiol group of whey proteins gets exposed and as a result increases the thiol-disulfide interchange reaction (Kella and Kinsella, 1988). Hence, increasing the ionic strength conceal the exposed thiol groups, thereby increasing the hydrophobic interactions. Some of these protein fractions can reverse their denaturation (renaturation) depending on the pH. α-Lactalbumin, being a small protein with four disulfide bonds can reverse their denaturation by 80-90% at 3-9% concentrations (Ruegg et al., 1977). Kronman et al., 1981 reported that when pH is lowered to 3.75 or below, aggregates of α-Lactalbumin are formed following heat treatment and when pH is adjusted to 5.2 or above, the protein aggregation is reversed.

Acidic pH leads to exposure of apolar amino residues that facilitates protein-protein aggregation, leading to the unfolding of α-La. Protein aggregation is also dependent on bound calcium content and hence, removal of calcium during acidification leads to protein coagulation. The subjection of α-La to reduced pH of 3 removes bound calcium from the molecule making it more susceptible to irreversible heat-induced protein denaturation.

Hydration and solubility of whey proteins

The property of proteins to interact with water in different systems describes the solubility or hydration properties of whey proteins. It is considered as one of the important factors in whey protein preparations. Several physical and chemical characteristics influence the hydration properties of whey proteins. Physical parameters include protein particle size, shape, agglomeration state, and nature of porosity, and chemical parameters include protein surface net charge, hydrophobicity, and hydrophilicity (Kinsella, 1984a). Hydration properties play role in optimizing both processing and storage conditions (Kinsella and Fox, 1986). For example, whey protein powders obtained through controlled spray drying conditions to yield particle sizes of 150-200μm is most effective in improving the hydration properties (Neff and Morris, 1968). Determining the amino acid composition can help to estimate the water-binding properties of pure proteins. Besides, other factors like protein structural conformation, polarity, ionic strength, pH, and temperature also influence the water-binding capacity of the proteins. Determining the solubility of whey proteins can help to decide their specific applications to food products such as beverages. After precipitation of casein at isoelectric pH 4.6, the whey protein fraction remains soluble in the supernatant. Hence,

reduced solubility at pH 4.6 is usually used to analyze the extent of protein denaturation (Guy et al., 1967). Whey protein concentrates (WPCs) at concentrations from 5 to 100% display a wide range of solubilities, and this is due to their different production methods. To obtain an improved and consistent solubility, whey proteins and derivatives must be produced under processing conditions that lead to minimal heat denaturation and aggregation of protein components. Currently, the spray drying technique is largely being used to produce different whey protein powder forms. Therefore, the conditions associated with the spray drying method can be controlled and optimized to produce powders with enhanced solubility. However, in some cases, the proteins get partially denatured before the spray drying step and this mostly affects the functional performance of the whey protein products. The effect of pH largely influences the hydration or solubility of the whey proteins by altering the net charge of proteins. Proteins possessing a net positive or negative charge tend to dissolve in water as compared to the proteins having minimal net charge (for example in the isoelectric point). However, under certain conditions, whey proteins remain in the soluble form at their isoelectric pH. Ionic strength also varies according to ion species and valency. In the presence of salts, β-Lactoglobulin remains soluble even at pH 4-5. Heat and some processing treatments often lead to protein denaturation and aggregation, thereby reducing their solubility. However, the addition of salts like sodium chloride at 0.01M concentration has been shown to improve the solubility of β-Lactoglobulin when heat-treated at 80°C for 15min. Increasing the salt concentration was effective in increasing the solubility of the whey protein fractions by reducing the aggregation, and at 0.5M concentration, the protein precipitation completely inhibited (Townend and Gyuricsek, 1974).

Gelation ability of whey proteins

Whey proteins have the capacity to form gels with different properties varying from soft, smooth curds to viscous, rubbery, and stiff gels. Their properties differ based on hardness, cohesiveness, color, stickiness, and mouthfeel (Kornhorst and Mangino, 1985). Gels formed from whey protein concentrate appear stiff transparent gels to curd like opaque gels. Whey proteins with reduced protein concentrations and low ionic strength form translucent grey weak gels. Mild heating of whey proteins can lead to specific protein-protein interactions leading to the formation of gels whereas, extensive thermal processing can lead to the formation of coagulation or curd-like gels. During gelation, a structural network is formed within proteins in which the water is entrapped leading to reduced syneresis. The shape of the gel formed its color, mechanical strength, and elastic properties play an important role during food applications. Whey proteins undergo conformational changes when heat treated beyond its critical temperature, leading to form aggregates. These aggregates when cooled set to form a soft to firm, clear to opaque viscous gels. The gel formation varies depending on the type of protein, concentration, temperature, pH, and calcium (Mulvihill and Kinsella, 1988). Heat-induced gels are formed in two steps, wherein the first step, the protein exposed to heat undergoes conformational changes through protein-protein interactions and unfolding of polypeptide segments leading to the formation of a structural network (Bernal and Jelen, 1985). However, a balance between the repulsive and the attractive forces are necessary to form a three-dimensional network. In certain cases, limited protein unfolding during heating leads to exposure of hydrophobic residues, which later associate with cooling. This forms a gel network based on the number of interactive sites, their reactivity, and the amount of

the repulsive forces between the solute molecules. Hence, they are highly affected by pH and ionic strength. In protein gels containing serum albumin, approximately 1-2 molecules of protein arrange themselves to form a highly dense, viscous network depending on the degree of cross-linking (Clark et al., 1981). In another study, gels obtained from whey fraction, β-Lg at high and low ionic strengths, contained protein aggregates in a form of a continuous branching network (Harwalkar and Kalab, 1985b), whereas whey proteins with lysozymes formed gels containing straight, rod-like protein molecules arranged in small, irregular clusters with a bead-like structural network (Clark et al., 1981).

Emulsification property of whey proteins

Emulsions are referred to as heterogeneous systems formed by dispersing one or more phases in a continuous phase and they can be stabilized by incorporating surface-active agents, that are amphiphilic in nature i.e., they have an affinity for both the dispersed phases. The main intend to add an emulsifier is to reduce the interfacial tension and facilitate diffusion of the discontinuous phase. Emulsion systems stabilized using a protein, have an interfacial membrane formed around the oil droplet to avoid any formation of coalescence, flocculation, creaming, or oiling-off. However, to be an effective emulsifier, the protein must be soluble and able to lower the interfacial tension at the oil/water interface. Hence, factors such as pH, salts, protein concentration, and temperature play an important role in protein adsorption onto the surface of the fat globule, influencing the emulsifying properties of the whey proteins (Shimizu et al., 1981). Emulsions formed of whey protein-coconut oil displayed reduced creaming stability with enhanced viscosity and protein adsorption at pH 5. This suggests that the

electrostatic nature of proteins largely influences the emulsion stability (Yamauchi et al., 1980). Besides, the availability of the hydrophobic groups surrounding the fat globules also plays role in protein adsorption. Studies suggest that whey protein-based emulsions can be improved through the partial unfolding of proteins during emulsion formation like while homogenization (Tornberg, 1978). Whey protein concentrates (WPCs) enriched with α-La and β-Lg were analyzed for their emulsifying properties in an oil/water emulsion. WPC alone and β-Lg added WPC demonstrated similar emulsifying properties which tell that origin and whey processing to have little to no effect on the emulsifying properties. WPC with added α-La demonstrated moderate emulsifying capacity with reduced stability, indicating β-Lg enriched whey proteins to be more effective emulsifiers (Slack et al., 1986). In another study, emulsions formed of whey protein-coconut oil contained more protein at pH 5 as compared to pH 7 at the interfacial surface. At pH 9, β-Lg was strongly adsorbed and found to predominating whereas, at pH 3, α-La associated protein adsorption increased. This suggests that protein adsorption and their emulsifying properties are extensively affected by the pH of the medium. At alkaline pH, molecular expansion of β-Lg led to more adsorption whereas, at acidic pH, α-La was found to adsorb more readily probably due to the reduced stabilizing effect of bound calcium (Shimizu et al., 1981). The stability of emulsions and the role of proteins as emulsifying agents were found to be correlated with the surface hydrophobicity when examined through fluorescent probe cis-parinaric acid (Kato et al., 1983). Reduction in the surface hydrophobicity significantly lowered the emulsifying capacity of the β-Lg and serum albumin (BSA), in proportion with the extent of thermal denaturation. This tells that both being hydrophobic in nature, BSA and β-Lg underwent a conformational change during

heating, leading to the reduced affinity of apolar residues to hydrophobic sites. Emulsions with added WPCs also vary with the composition such as lipid content, ash, and sulfhydryl content and is often used to predict the emulsifying capacity of various WPC samples in aerated emulsions.

Improvement in the functionality through conjugation

Whey proteins are one of the nutritionally beneficial systems enriched with amino acids and bioactive peptides and hence, popularly used in developing functional foods. Though whey protein products are greatly in demand for developing nutraceutical foods due to their several health benefits associated with their bioactive properties such as antimicrobial, antioxidant, antihypertensive, opioid, and mineral binding (Hernandez-Ledesma et al., 2014). However, the incorporation of WPH into food formulations is not a trivial task because of their heat stability issues (LaClair and Etzel, 2010). To ensure product safety and shelf stability, most liquid foods and medical beverages undergo thermal treatments, and this can cause whey proteins to denature irreversibly to form gels and aggregates. Besides, due to poor emulsification properties, the use of hydrolyzed proteins in developing formulated powders can be significantly affected, because of increased stickiness of powder particles during the spray drying process. Moreover, the use of whey proteins with high protein levels can lead to extensive fouling and blockage of the equipment and pipelines. Sedimentation and gelation of denatured proteins, particularly during long-term storage show signs of unwanted sensory attributes in the final product. Developing value-added products using whey proteins can create a lot of technical limitations, leading some producers to switch to some other protein types.

However, such proteins may differ in terms of amino acid profile and digestion benefits of whey proteins.

Recently, the stability of whey proteins has been addressed by several researchers, primarily by conjugating them with carbohydrates. Heating of whey proteins or peptides with reducing carbohydrates leads to a series of chemical reactions, referred to as Maillard reaction and it is during the early stages of heating, where a covalent bridge is formed between protein and carbohydrate molecules, a conjugated protein is formed (Kato, 2002). Conjugation of whey proteins with reducing sugars via the Maillard reaction process (i.e., glycation) is an advancing area of interest, with some previous reports showing improvement in the physiological, nutritional, and functional properties, including thermal stability, solubility, emulsification capacity, water binding and antioxidant activity of the whey protein/peptide-based ingredients (Zhang et al., 2010).

However, the main challenge with conjugation is that it can occur naturally or can be initiated, which significantly influences the characteristics of the food products in terms of physical, chemical, biological, and organoleptic properties. Furthermore, conjugation of whey proteins with reducing sugars via the Maillard reaction is a very complex reaction process involving a series of processing parameters including pH, temperature, and time. Hence, scaling up of the process present serious challenges as an insufficient binding of the proteins to carbohydrates can lead to coagulation of proteins and an uncontrolled Maillard reaction can lead to the formation of unwanted or adverse effects, such as the formation of products that leads to the generation of off-flavors and toxic compounds) (Oliver et al., 2006). Therefore, for wider applications in developing value-added health ingredients, the effect of the different process parameters on the Maillard

reaction, and their impact on the functional and health properties of the proteins need to be studied.

Current applications of whey proteins and its derivatives

Role of whey proteins and derivatives as food additives

In food applications, whey proteins and derivatives are gaining attention due to their immense benefits owing to several functionalities including gelation, foaming, emulsification, solubility, and thermal stability. The addition of the whey proteins is known to improve the food sensory quality and enhance the texture. For example, whey proteins have been previously added to foods such as yogurt, bakery foods, energy bars, pasta, and beverages to influence the overall quality and nutrition of the foods. A study from Krzeminski et al., 2014 reported the effect of adding a complex of non-heat-treated whey protein and high methoxyl pectin in low-fat yogurt. The whey protein acted as a good fat-replacer and texturing agent for the yogurt. Another study showed the ability of the whey proteins to stabilize emulsions and improve the overall texture when added into whole-fat yogurt prepared from skim milk powder. When the droplets merger was used, it yielded whey protein agglomerates with high molecular weight and reduced emulsifying capacity, however, when passed through a high-pressure homogenizer at 20-100 MPa, it yielded a more stable emulsion (Kuhn and Cunha, 2012). Akahn et al., 2012 studied the effect of the addition of the milk-protein ingredients on the microstructure of probiotic yogurt (prepared with a combination of commercial starter culture and *Bifidobacterium lactis* Bb12) during a 28-day period refrigerated storage. One sample was added with sodium caseinate at the level of 2% and the other was added with whey protein concentrate at 2%. It was reported that the addition of sodium caseinate

transformed the firmness, adhesiveness, and the overall viscosity of the product, whereas the product added with whey protein demonstrated improved water holding capacity, viscous texture, and low syneresis as compared to the caseinate. Whey protein in combination with a plant protein was added into a date bar and the nutritional profile was optimized applying a response surface method (RSM) targeting the school children (Nadeem et al., 2012). An addition of 6.05% of whey protein concentrate (WPC) was found to be ideal. Several research are still ongoing to utilize whey proteins and their derivatives to develop nutraceutical and functional foods.

Benefits of combination of whey proteins and derivatives with other supplements

Extensive hydrolysis of whey proteins using enzymes can lead to the formation of bitter peptides, reducing their acceptability in food applications. Enzymatic hydrolysis breaks down the protein fractions like α-lactalbumin, β-lactoglobulin, serum albumin to generate whey protein hydrolysates containing bitter peptides. This bitter taste of the peptides is often masked using various inhibitors and some of these inhibitory compounds include sucralose, fructose, adenosine 5’ monophosphate, sucrose, adenosine 5’ monophosphate disodium, monosodium glutamate, sodium chloride, sodium gluconate, and sodium acetate (Leksrisompong et al., 2012). Several techniques involve identifying the bitter peptides and removing them to improve their sensory properties. Liu et al., 2014b identified four peptides contributing to bitterness in a whey protein hydrolysate. Fractionation techniques (ultra-filtration and chromatography) were used followed by LC-TOF-MS/MS (Liquid chromatography-time of flight-mass spectrometry) to identify the peptides and the constituent amino acids. Gad et al, 2011 reported an improvement in the antioxidant and metal chelating activities of the whey protein

concentrate (WPC) when supplemented with freshwater algae, spirulina in both in vitro and in vivo subjects using rat models.

Application of whey proteins can also be limited as some of its components like α-lactalbumin and β-lactoglobulin are associated with causing allergenicity, particularly in children. Some children are found to develop gastrointestinal problems (Kattan et al., 2011) or atopic dermatitis (Botteman and Detzel, 2015) or respiratory allergies (Hochwallner et al., 2014), or amphylactic reactions (Ameratunga and Woon, 2010) after ingestion of cow milk protein. Hence, it is important to assess the allergenicity risks before the administration of whey protein diets. One of the effective methods that were reported to reduce the allergenicity in whey proteins is heat treatment. Bu et al., 2009, analyzed the antigenicity of like α-lactalbumin and β-lactoglobulin in whey protein isolate through competitive ELISA (enzyme-linked immunosorbent assay) after exposing to heat treatment. It was observed that above 90°C, the antigenicity in the protein fraction decreased significantly. Treating whey proteins with enzymes are also known to reduce the allergenicity (Zhang et al., 2012). Whey protein concentrates were hydrolyzed with trypsin and fed to mice subjects. An increase in the secretion of the IFN-γ was observed in the subjects which suggest the ability of the hydrolysates to lower the allergenicity of the whey proteins (Duan et al., 2014).

Role of whey proteins and derivatives as encapsulating agents and coating materials

As consumers become more health-conscious, they are looking for natural ingredients rich in nutrients inside their foods and beverages (Nedovic et al., 2011). Hence processors are responding to this trend by continually incorporating healthful ingredients in foods or as supplements. Recently, bioactive compounds (example: vitamins, antioxidants,

minerals and ions, flavor, aroma compounds, lycopene, fats or enzymes or bacterial cells like probiotic microorganisms) have emerged as functional ingredients, leading to the production of novel formulations and value-added foods (de Vos et al., 2010). However, there are several challenges faced during the application of these bioactive molecules (Desai and Park, 2005). As a result, to overcome these challenges and considering the increasing demand for value-added novel ingredients in food, food manufacturers started implementing the process of encapsulation (Wandrey et al., 2009). These wide ranges of active compounds can be encapsulated or packaged in a carrier material composed of whey protein. The process of encapsulation involves the incorporation of any solid, liquid, or gaseous materials, including ingredients, enzymes, cells, or other molecules in different carrier materials to produce capsules of varying sizes (Nedovic et al., 2001). This facilitates transporting the agents at the delivery site and based on the strength of the carrier material, the core agents get released at various intervals. Besides, entrapping in a whey protein gel is known to reduce rancidity issues and augment stability. For instance, fortifying foods with iron presents numerous difficulties, and to address this problem, whey protein isolate was used, by utilizing its gelling properties. The isolate was exposed to cold-set gelation to form a matrix and following, iron was entrapped in it in the presence of ascorbate (Martin and de Jong, 2012). This led to improving the encapsulation efficiency of the whey protein to recover more iron and improve the in vitro bio-accessibility from 10% to 80%. The use of ascorbate contributed to strengthening the whey protein gel which led to increased recovery of iron and improved its release characteristics. Similarly, a whey protein concentrate was used as an encapsulant to entrap folic acid. A favorable interaction between the folic acid and the

protein matrix was observed, making it a suitable matrix for incorporating vitamins. When compared with a polymer (commercial resistant starch), WPC capsules imparted higher stability to folic acid (Perez-Masia et al., 2015). Whey protein encapsulants can also be formed in combination with other carrier materials such as carbohydrates and fats. A study by Gulseren et al., 2012, demonstrated the efficiency of the whey protein isolate nanoparticle when combined with and without methoxyl pectin. The results showed improved resistance to homogenization and overall stability of the encapsulants formed with pectin. Even during storage at pH 3, the nanoparticle suspension displayed higher interfacial pressures as compared to encapsulants without pectin. Such encapsulants can be potentially used as effective surfactants. An important benefit of the encapsulation process is to prevent the reaction of the core ingredient with other food components like, in the case of essential oils (Parris et al., 2005). Besides containing several compounds like phenols, alcohols, esters, ketones, and aldehydes, essential oils exhibit a wide spectrum of antimicrobial activity against bacteria, yeasts, and fungi. Hence, to confer stability inside a food matrix, such oils can be microencapsulated using whey protein derivatives as carrier material. For example, Mehyar et al., 2014 used WPI to encapsulate cardamom essential oil. It was found that the WPI microcapsules obtained had a spherical, regular, and smooth texture and during storage, it was able to retain the oil at 30% concentration.

In a study by Perez-Masia et al., 2015, a whey protein isolate was transformed into an edible film with ascorbic acid impregnated in it. The film was then assessed for the oxygen-scavenging property. It was observed that the tensile strength of the film improved with reduced oxygen permeability. This suggests the ability of the WPI films to

prevent oxygen diffusion and eliminate oxygen in the food systems, thereby enhancing the storage stability of the oxygen-sensitive products (Janjarasskul et al., 2011). Edible layering using whey proteins is also used to coat nuts to improve its shelf life by retarding the formation of rancidity in them. A study from Mehyar et al., 2012, showed the efficacy of a whey protein isolate to delay the oxidation and rancidity in walnuts and pine nuts. The nuts coated with WPI generated improved sensory characteristics throughout storage at 25°C for 12 days, as compared to uncoated nuts.

Whey protein-based packaging materials show great potential in replacing plastics and is one of the most promising biopolymers. Recently, whey proteins have been widely used in the field of active packaging to exploit their antimicrobial properties. Antimicrobial packaging refers to a form of active packaging with antimicrobial compounds infused in it. These compounds get released when used in edible films to impart improved shelf life of the product. Whey protein isolate (WPI) edible films with antimicrobial properties have been developed with infused essential oils (extracted from spices such as rosemary, oregano, and garlic). Previous studies show the efficiency of whey proteins to improve oxygen barrier properties and increase biodegradation when added in a compostable plastic film (Cinelli et al., 2014). Due to their high emulsification properties, whey proteins are also used in forming stable emulsions. Cheese is often fortified with vitamins to enhance its nutritional value. However, during the ripening period, the vitamins tend to degrade. Hence, to improve the retention of vitamins, Tippetts et al., 2012, studied the role of whey proteins to incorporate vitamin D3 in Cheddar cheese. They formed an oil-in-water emulsion by adding sodium caseinate, calcium caseinate, whey protein, and vitamin D3 to obtain the final dose of 280

IU/serving. The nano emulsions were stable and about 74-78% of vitamin D3 was retained in the product. In another study, Li et al., 2014, showed the ability of the whey protein isolates to generate stable nano emulsions under various thermal processes and ionic strengths. These emulsions were found to be stable under storage conditions even at higher protein concentrations without the addition of any polymers like gums and polysaccharides as a secondary layer. These results suggest the potentiality of the whey protein-based emulsions in the food and pharmaceutical industries.

Conclusion

From the findings above, it can be suggested that whey proteins and derivatives are functionally significant and have great potential in food applications. Most of the bioactive peptides isolated and purified from whey proteins have good antioxidant, antihypertensive, anticancer, antidiabetic, and hypocholesterolemic activity. After absorption, these peptides exert their action on specific target organs. Besides, the functional characteristics of the whey proteins also play an important role during applications in food systems. With the continuous expansion of the market for functional proteins, there is a need to develop simple cost-effective methods for the production, isolation, purification, and scalability of the whey proteins and peptides in huge amounts for the market. Recently, to meet the increasing demand from health-conscious consumers, food industries have started to explore protein blends (a mixture of proteins derived from various sources like casein, whey, plant, microbial sources, etc.) for developing protein-rich foods and beverages. However, to make the concept feasible, there are several technical and marketing challenges that are reported during the development process. Hence, these preparations should be tested in a simulated food

system followed by an actual food condition to validate the protein behavior and performance in commercial foods. Specific protocols should be designed regarding ingredient addition, temperature, pH, and other processing parameters. Such information is necessary to facilitate appropriate processing methods during manufacture to prevent compositional variation, the extent of protein denaturation, and other conformational changes.

The incorporation of whey protein into food formulations is not a trivial task because of their lower stability during processing and subsequent storage. While some studies have shown the protein ingredients produced through conjugation with maltodextrin to have greater functionality, the effect of conjugation on the bioactivities is not clearly established. Similarly, although, technological advancements have enabled the addition of probiotics to all types of food, maintaining stability of food matrix and viability of organisms during processing and storage has mostly been a challenge that limits the development of food formulations with probiotics. Whey based microencapsulation of probiotics has previously been investigated. However, such applications primarily focus on developing delivery systems for probiotic organisms alone. Hence, it may be useful to develop novel products, which not only catch attention of a wider consumer group, but also provide greater value addition in terms of the enhanced functionality of such nutritional products. The present study investigates the feasibility of developing a spray dried health formulation by using WPH-conjugate for entrapping probiotic organisms. Hence, the first objective was to develop whey protein conjugates and determine its ability to retain bioactive properties, and second, to use whey protein conjugate as an encapsulant material to entrap probiotics.

STUDY II: Assessing persistence of *Listeria* organisms through Whole Genome Sequencing

Listeria monocytogenes is a psychrotrophic food borne pathogen that can be transmitted to humans from food, causing listeriosis. It can cause mild to severe infections and have been found that people with immunocompromised conditions, pregnant women, elderly, and newborns are at greater risk for developing listeriosis with a lethality rate of 20 to 30% with severe infections (Buchanan et al., 2017). Although reports have shown a decline in the *Listeria* outbreaks over the past few years, several incidences of listeriosis have been declared leading to many food industries and governments to take necessary actions to visibly reduce the severe outbreaks. Numerous outbreaks were associated with the consumption of dairy products, particularly in ready-to-eat foods and soft variety of cheeses and is linked to both invasive and non-invasive listeriosis. In past five years, several listeriosis outbreaks in United States have been associated with soft cheese prepared from raw and pasteurized milk (Center for Disease control and Prevention, 2021) with recent outbreak in May 2021which was traced to Hispanic-style fresh and soft cheeses (Queso Fresco) made with pasteurized milk. It has been reported that dairy products like cheeses made from pasteurized milk displayed contamination with *L. monocytogenes* varying between less than 20 CFU/g (Little et al., 2008). Though pasteurization is considered as an effective treatment against food borne pathogens, organisms like *L. monocytogenes* can enter foods if produced under unsanitary conditions and can survive and grow under refrigerated temperatures.

Listeria monocytogenes being ubiquitous in nature, dairy food processors should take proper precautions and systematic approach towards food safety and follow proper

sanitation protocols. Acidity, osmotic and oxidative stress, low/high temperatures, presence of antimicrobials or bacteriocins, applications of decontamination and preservation technologies such as pulsed electric fields, high pressure and UV light are some of the stress conditions that are mostly encountered by *L. monocytogenes* along the food supply chain. The ability of *L. monocytogenes* to survive for long periods of time in a food or dairy processing facility is due to its resistance to these various environmental stress conditions, and its ability to attach to food contact surfaces and form resilient biofilms (Cruz and Fletcher, 2011). This makes the food processing environment more susceptible to *Listeria* contamination making it to be a major route to food product contamination (Perez-Rodriguez et al., 2008). Despite improvement in hygienic protocols, *L. monocytogenes* can grow at varying pH levels of 4.6 to 9.5, at low water activity of 0.92, and being psychrotrophic in nature it can grow and multiply below 0°C. It finds favorable growth conditions on floors, drains, and equipment surfaces particularly under cold and wet food industry atmosphere of refrigerated rooms (Carpentier and Cerf, 2011). And specific strains of *L. monocytogenes* have been consistently getting identified over many years in food processing facilities, which shows their persistent behavior in food processing environment. Some of the examples such as two separate strains of *L. monocytogenes* were found to be persistent in two different fish processing facilities over 6 years when analyzed through whole genome sequencing (Holch et al., 2013). In food processing plant *L. monocytogenes* was found to be persistent for 12 years which was linked to causing 29 cases and 4 deaths (Orsi et al., 2008). In another study by Vongkamjan et al., 2013, a *L. monocytogenes* strain was shown to be persistent for 11 years in a smoked fish processing facility when analyzed through ribotyping.

Hence, it is crucial to identify the genetic determinants and in association with the phenotypic characteristics which will help to understand the mechanisms utilized by the *Listeria* species to tolerate the stress conditions in food matrices and food processing environments. It has been reported that resistance of *Listeria* species to different stresses is interconnected, for example osmotic stress response by *L. monocytogenes* can provide cross protection to other stress conditions including heat, oxidation, ethanol, and extreme pH (Melo et al., 2015). It is also found that stress response demonstrated by two component systems *liaRS*, *lisRK*, *cesRK*, *agrCA*, and *virRS* (Pontinen et al., 2017) converge the stress signal on the level of SigB, which is the alternative sigma factor σ^B responsible for general stress response in *L. monocytogenes* and other Gram-positive microorganisms (Abram et al., 2008). SigB is shown to provide resistance to *L. monocytogenes* during oxidative stress (Chaturongakul and Boor, 2006), cold and freezing stress (Becker et al., 2000), osmotic stress (Fraser et al., 2003), and high acid and pressure stress (Wemekamp-Kamphuis et al., 2004). Presence of gene *clpL* protease has been found to play role in exerting tolerance to elevated temperatures and is reported that heat shock regulons namely class I and class II heat-shock genes and *recA* are expressed during exposure to high heat conditions (Van der Veen et al., 2007). Whereas at lower temperatures, genes such as *CspA*, *CspB*, and *CspD* are found to be playing role in survival of *L. monocytogenes* (Lee et al., 2012). Several mechanisms including GAD, ADI and AgDI systems are involved in maintaining internal pH under acid stress conditions (Soares and Knuckley, 2016), whereas in response to osmotic stress, *L. monocytogenes* can express *opuCABCD* operon for cartinine transport system, *gbuABC* for glycine betaine porter II system, and *betL* for glycine betaine uptake system (Chan et

al., 2007). In addition, it can express Csps genes *cspA* and *cspD* which provides resistance to elevated concentrations of salt as well as cold temperatures. Genes that are regulated to provide resistance to bacteriocins like nisin are *lmo2229*, *telA*, *mprF*, *anrAB*, and *dltABCD* (Bucur et al., 2018). Mutations in CtsR, a class III stress genes repressor with increased expression of *clpB*, *clpC*, *clpE*, and *clpP* have been linked to providing resistance to *L. monocytogenes* cells to high pressure processing (HPP) conditions (Van Boeijen et al., 2010). Whereas resistance to bactericidal effect of UV light is exhibited due to expression of gene *lmo0588* encoding for a putative photolyase. This gene helps the cell to repair injured by UV light (Gomez-Lopez et al., 2007). In *L. monocytogenes*, expression of genes such as *kat*, *perR*, *sigB*, *recA* responsible for oxidative stress has been correlated with biofilm formation. Synergistic effects have been observed for *kat* gene along with *sod* gene against toxic effects of hydrogen peroxide and superoxide anion radicals. During sublethal pulsed electric field (PEF) treatment, resistant *L. monocytogenes* strains is found to increase the expression of molecular chaperones such as GroEL, GroES, and DnaJ (Lado et al., 2004).

Most researchers have used genotypic evaluation through molecular typing methods to identify and distinguish persistent *Listeria* strains from the sporadic ones. Phenotypic studies of *Listeria* strains to identify and compare persistent strains from that of sporadic strains can produce inconsistent results particularly if the isolation method is carried out on different sampling dates. This can lead to identifying isolates that are indistinguishable or otherwise belong to the same strain when subtyping is used. Many researchers have reported the use of pulsed field gel electrophoresis for subtyping, however recently this technique has been replaced by whole genome sequencing (WGS),

which has helped to identify and compare *Listeria* strains through genotypic subtyping and overall, improve the listeriosis outbreak investigations (Moura et al., 2016). Being a cost-efficient technique, WGS is becoming a viable alternative for distinguishing and investigating *Listeria* contamination in food processing environments. In many studies, single nucleotide polymorphism (SNP)-based and multilocus sequence typing (MLST) allele based WGS have been used to analyze and investigate *Listeria* outbreaks (Jackson et al., 2016). In SNP-based analyses the SNPs present in the whole genome (entire genome with coding and non-coding regions) or the core genome (set of coding regions present in the strains). A whole genome MLST scheme targeting a specific 4797 pan genome loci was executed in PulseNet that was based on over 150 publicly available *L. monocytogenes* reference genomes (*Listeria monocytogenes* whole genome sequence typing, 2017). The entire coding loci specific to a set of closely related isolates can also be targeted which are not included in any pre-defined pan genome locus set in whole genome based MLST. It has been previously shown to trace food borne disease epidemiology of *Salmonella* (Leekitcharoenphon et al., 2014), *Escherichia coli* (Joensen et al., 2014), and *L. monocytogenes* (Gilmour et al., 2010).

Despite the improvement in the hygiene protocols, *L. monocytogenes* attaches to processing surfaces, improperly cleaned dairy equipment, floors and niches that are difficult to clean and become the potential source of milk and milk product contamination. Hence, related to these concerns it is important to know about the genetic expressions of the *Listeria* species responsible for their persistence behavior in food processing environments. In this study, whole genome sequencing (MiSeq and Nanopore sequencing) of the environmental *Listeria* strains, isolated from a dairy processing plant

was done to identify the expression of genes responsible for colonization and understand the resistance behavior of the strains to the dairy plant environment. Therefore, the first objective was to determine the genetic determinants of the *Listeria* isolates and associate with phenotypic responses and second to determine the genetic variation among the isolates that can influence their persistent nature.

CHAPTER 1 TABLES AND FIGURES

Table 1: Whey protein constituents and its composition [a]

Whey protein constituent	Concentration (g/L) [b, e]	Molecular weight in kDa [c, d]	Number of amino acid residues [c]
α-Lactalbumin	1.2	14175	123
β-Lactoglobulin	1.3	18277	162
Bovine serum albumin	0.4	66,267	582
Immunoglobulins (A, M and C)	0.7	25,000 (light chain) and 50,000-70,000 (heavy chain)	-
Bovine Lactoferrin	0.1	80,000	700
Glycomacropeptide	1.2	6700	64
Bovine Lactoperoxidase	0.03	70,000	612

[a] Madureira et al., 2007

[b] de Wit, 1998

[c] Eigel et al., 1984

[d] Brew et al., 1970

[e] Korhonen, 1995

Table 2: Biological activities of the major whey protein constituents [a]

Whey protein constituent	Biological activities	References
α-Lactalbumin	Anticancer activity	de Wit, 1998
	Lactose metabolism and synthesis	Markus et al., 2002
	Treatment of chronic stress-induced disease	Ganjam et al., 1997
β-Lactoglobulin	Transporter of retinol, fatty acids, palmitate, vitamin D and cholesterol	Puyol et al., 1991; Wu et al., 1999; Wang et al., 1997
	Increase in pregrastic esterase activity	Perez et al., 1992
	Mammary gland phosphorus synthesis and metabolism	Farrell et al., 1987
	Passive immunity transfer	Warme et al., 1974
Bovine serum albumin	Bind fatty acids	Walzem et al., 2002
	Anti-mutagenic activity	Bosselaers et al., 1994
	Anti-cancer activity	Laursen et al., 1990
	Immune system modulation through passive immunity	Mitra et al., 1995; Loimaranta et al., 1999
Immunoglobulins	Antimicrobial activity	Freedman et al., 1998

(A, M and C)	Antifungal activity	Okhyusen et al., 1998
	Opioid activity	Sharpe et al., 1994

[a] Madureira et al., 2007

Table 3: Processing conditions, extrinsic and intrinsic parameters affecting the functional properties of whey proteins [a]

Processing conditions	**Extrinsic parameters**	**Intrinsic parameters**
Heating	Temperature	Protein composition
Acidification	pH	Monomeric oligomeric
Counter ions	Oxidation-reduction	Protein blends
Ionic strength	potential	Rigidity/flexibility
Reducing conditions	Salts or ions	Hydrophobicity or
Drying	Water	hydrophilicity
Storage conditions	Carbohydrates	Surface charge
Modifications related to	Lipids,	Bound flavor ligands
physical, chemical,	Gums,	
enzymatic, and genetic	Surfactants	
	Tannins	

[a] Sourced from Kinsella and Whitehead, 1989

Figure 1: Production of whey protein derivatives

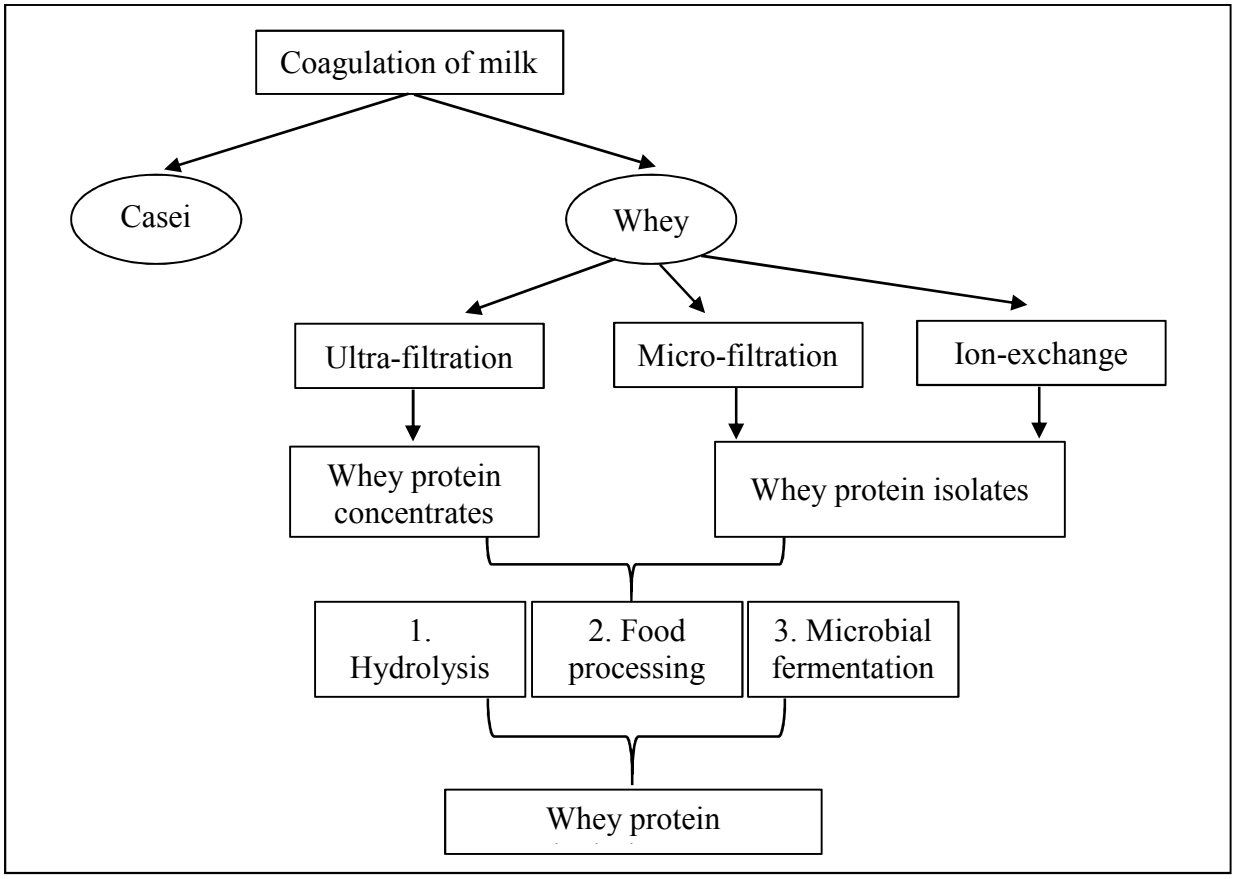
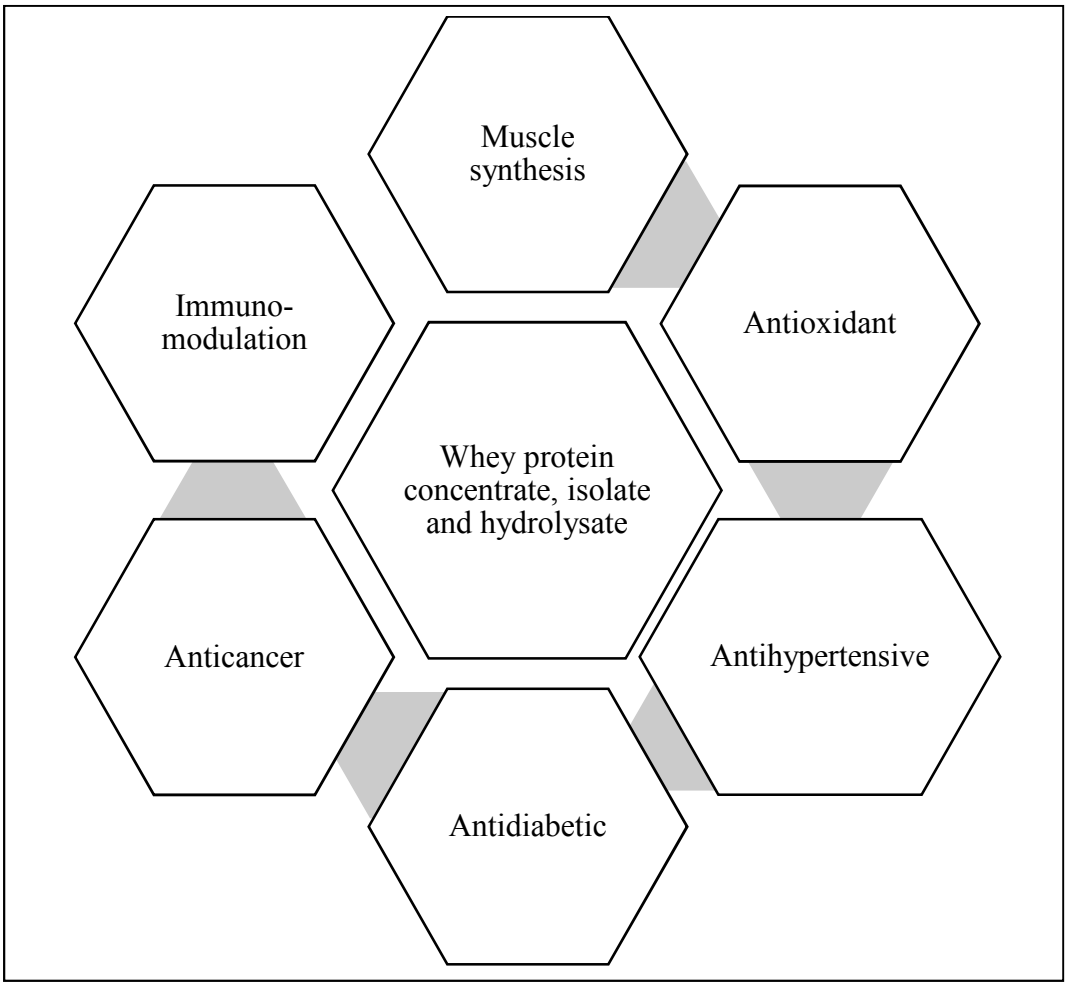


Figure 2: Biological properties of the whey protein derivatives



CHAPTER 2

EVALUATING THE EFFECT OF CONJUGATION ON THE BIOACTIVITIES OF WHEY PROTEIN HYDROLYSATES

ABSTRACT

In this study, the ability of a whey protein hydrolysate to exhibit the antimicrobial, antioxidant, and antihypertensive behavior after combining with a reducing carbohydrate was studied. Whey protein hydrolysates with varying degree of hydrolysis (WPH 10, WPH 15, WPH 20) were determined for their antimicrobial, antioxidant, and antihypertensive activities. Of these, hydrolysate (WPH 10) exhibited the highest antimicrobial activity (with 10-11.2 mm zone of inhibition) against tested microorganisms: *Listeria innocua*, *Staphylococcus aureus*, and *Bacillus coagulans*. In addition, the WPH 10 exhibited the highest antioxidant (866.56 TEAC μmol L^{-1}) and antihypertensive (67.52 %) attributes. Hence, based on the highest bioactivity, hydrolysate WPH 10 was selected for conjugation with maltodextrin and the effect of conjugation on the bioactivities was evaluated. The conjugated WPH 10 solution demonstrated higher antimicrobial (17.16 mm) and antioxidant activity (1044.37 TEAC μmol/L), whereas a slight decrease in the antihypertensive activity (65.4 %) was observed, as compared to WPH 10 alone. Conjugated solution was further spray dried in a Niro drier with an inlet and outlet temperature of 200□C and 90±5□C, and alternatively, freeze-dried at −80 °C under 50-mTorr vacuum. The dried WPH 10 conjugate exhibited even higher antimicrobial (18.5 mm) and antioxidant activity (1268.89 TEAC μmol/L), while retaining the antihypertensive activity (65.6 %). Overall,

the results indicate the ability of the WPH-maltodextrin to retain the bioactive behavior after combining with a reduced carbohydrate.

INTRODUCTION

With the rising popularity of healthy eating, there is a worldwide demand for food products formulated with high protein content (Westhoek et al. 2017). Milk whey proteins are recognized as healthy ingredients due to numerous benefits associated with their consumption, including promoting satiety, appetite control, and exercise recovery (Li et al. 2018). Selective fractionation of milk whey proteins results in two main forms: whey protein concentrate (WPCs) (containing ~34-89% protein) and whey protein isolate (WPIs) (containing at least 90% protein) (Wright et al. 2009). When whey proteins are subjected to enzymes or microbial fermentation, some of the amino bonds are broken, and this pre-digestion makes the hydrolyzed proteins to get absorbed rapidly in the body as compared to isolates or concentrates (Kresic et al. 2006). Enzymatic hydrolysis is preferred by food manufacturers due to their specific site of action and availability of wide sources of enzymes (from animals, plants, and microorganisms). Besides, the reaction conditions and the extent of the hydrolysis can be controlled to obtain the desirable nutritional and functional properties (Sandberg, 2011). Enzymatic hydrolysis can bring changes to the functional properties of the proteins depending on the type of enzyme used and its specificity, hydrolysis conditions, and the process of enzyme inactivation (Tavano, 2013). Whey proteins also possess biologically active peptides in their primary structure in an inactive form. Following hydrolysis, the peptides generated exhibits several health benefits to the gut (Daliri et al. 2017). Such hydrolysates, containing a short chain of amino acids, possess a large range of bioactive peptides

(Nongonierma et al. 2016), that may possess biological attributes like antimicrobial, antihypertensive, antioxidative, anticytotoxic, antidiabetic, immunomodulatory, opioid, and satiating activities (Fromentin et al. 2012). Hence, hydrolysates are incorporated in numerous dietary and nutritional products (including protein supplements for infants, athletes, and geriatric) (Sousa et al., 2014). Further, newer strategies are developed to expand its utilization that can help alleviate metabolic disorders (Patel, 2015). However, whey protein hydrolysates are susceptible to physical destabilizations like (denaturation, gelation, increased turbidity, foaming, and decreased solubility), specifically upon exposure to high heat treatment (Mulcahy et al. 2016). This is due to the increased interactions between protein molecules, or through changes in the surface charge of the proteins, that reduce the steric hindrance between them and thereby, leads to aggregation (Yu, 2011; Adjonu et al. 2013). Also, the exposure of the reactive sites (mainly free -SH groups) on protein/peptide molecules at the oil and serum phase results in reduced heat stability of whey protein hydrolysate-based emulsions (Drapala et al. 2016). Depending on the heat treatment and composition of the whey proteins, the interactions between a free -SH group and S-S bond of cysteine can lead to aggregation and formation of protein complexes (Considine et al. 2007; Wijayanti et al. 2014). The stability of whey hydrolysates was addressed primarily by conjugating them with carbohydrates (Yang et al. 2012). Conjugation of proteins with a reducing carbohydrate via Maillard reaction is an advancing area of interest, with some previous reports showing improvement in physiological and functional properties of hydrolysates, including thermal stability, solubility, emulsification capacity, water binding, and antioxidant activity (Zhang et al. 2010). It is during the early stages of heating that

conjugation occurs, where a covalent bridge is formed between protein and carbohydrate molecules. This interlinkage of the carbohydrate side chains with protein limits the aggregation between proteins and peptides, which in turn increases protein hydration and steric repulsion between proteins (Sosa et al. 2016).

Hence, with evidence of whey protein hydrolysates when combined with a carbohydrate (whey protein hydrolysates-carbohydrate), to possess improved functional properties, it is important to analyze the bioactive behavior of whey protein hydrolysates-carbohydrate. In the present study, our objective was to determine the effect of whey protein-maltodextrin conjugation and drying process (spray drying and freeze drying) on the *in vitro* bioactive properties (antimicrobial, antioxidant, and antihypertensive) of a whey protein.

MATERIAL AND METHODS

Materials

Spray-dried samples of whey protein concentrate (WPC80) with protein content of 80.13%, whey protein isolate (WPI90) with protein content of 87.38%, and whey protein hydrolysates (WPH10, WPH15, and WPH20 with 10%, 15%, and 20% degree of hydrolysis and protein content of 89.98%, 83.78%, and 80.05% respectively) were obtained from Milk Specialties Global, MN, USA. The preparation of the hydrolysate samples is shown in Figure 1. The samples were diluted to 5% (w/v, protein) for assessing their antimicrobial, antioxidant, and antihypertensive behavior.

Determination of bioactivities of whey protein samples (WPI, WPC, and WPH)

Antimicrobial Activity

The antimicrobial activity was evaluated according to the methodology reported by Balouiri et al. (2016) against *Staphylococcus aureus* (ATCC 25923), *Listeria innocua* (ATCC 33090), *Salmonella enteritidis* (ATCC 13076), and *Bacillus coagulans* (ATCC 10545). The pure cultures were sub-cultured in BHI (Brain heart infusion) broth at 35 °C ±2 °C. A bacterial lawn of culture was prepared by spreading 100 µL of activated culture having 10^6 CFU/mL of each test organism on pre-poured nutrient agar plates with the help of a sterile spreader. Plates were held for 10min to let the culture absorb. Thereafter, five wells (6.0 mm each) were punched into agar plates and the wells were sealed with one drop of molten agar (0.8%) to prevent any leakage of the testing material from the bottom of the wells. Using a micropipette 100 µL of WPC80, WPI90, WPH10, WPH15, and WPH20 were poured individually onto each of the five wells. After 24h incubation, zones of growth inhibition (in mm) were measured.

Antioxidant Activity

The antioxidant activity was determined *in vitro* using the ABTS+ radical assay according to the methodology reported by Re et al. (1999); Tovar-Jimenez et al. (2017) with some modifications. This assay quantifies total radical scavenging capacity based on the ability of a given compound to scavenge a stable ABTS+ radical. The working solution was prepared by mixing 7 mmol/L of ABTS (2,2′-Azino-bis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) stock solution with 140 mmol/L of potassium persulfate and allowing the mixture to incubate in dark at room temperature for at least 12 h before use. Then, it was diluted with phosphate buffer saline (PBS) of 5 mmol/L (pH= 7) to adjust the absorbance at 734nm to 0.7 ± 0.02. An aliquot of 20 µL of WPC80, WPI90, WPH10, WPH15, and WPH20 was taken in a cuvette and to this 980

µL of PBS solution was added and mixed for 10s. A decrease in the absorbance at 734 nm was recorded after10 min using a spectrophotometer (Spectronic 200, ThermoFisher Scientific, USA). The results were expressed as Trolox equivalent antioxidant capacity (TEAC).

Antihypertensive activity

The antihypertensive activity of the whey protein samples was determined through ACE (angiotensin I-converting enzyme) inhibition assay as reported by Correa et al. (2011). This assay is based on the liberation of hippuric acid from hippuryl-L-histidyl-L-leucine (HHL) which is catalyzed by ACE. 20 µL of WPC80, WPI90, WPH10, WPH15, and WPH20 were mixed individually with 110 µl of 5 mM HHL solution and 100 µl of 0.1 M sodium borate buffer (pH =8.3). The reaction was initiated by the addition of 20 µL of the ACE (EC 3.4.15.1; from rabbit lung) enzyme (0.16mU per µL), and the mixture was incubated for 3 min at 37°C. The reaction was terminated by the addition of 150µL of 1N HCl. The hippuric acid liberated was extracted with 1 mL ethyl acetate by centrifugation followed by evaporation at 95°C for 10 min. The evaporated residue containing hippuric acid was dissolved in 1mL deionized water, and the absorbance of the solution was measured spectrophotometrically at 228. For blank, all components except ACE were added, and for the control, all components except sample were added. The extent of inhibition was calculated according to Equation (1):

$$\%\ ACE\ inhibition = \left(\frac{B - A}{B - C}\right) \times 100$$

Where A is the absorbance in the presence of ACE and sample; B is the absorbance without a sample (control), and C is the absorbance without ACE (blank). All experiments were conducted in triplicates.

Preparation of WPH-maltodextrin conjugate solution

The whey protein hydrolysate-carbohydrate solution was prepared through conjugation according to the protocol described by Mulcahy et al. (2016). For conjugation, WPH10 was selected among the whey protein samples, as it comparatively showed higher bioactivities. The WPH10 (5% w/v, protein) and maltodextrin (DE 10) (5% w/v) were blended in distilled water at room temperature under constant stirring. The pH of the solution was adjusted to 8.2 with 0.5N KOH and the mixture was let to hydrate for 18 h at 4°C. The pH of the mixture was readjusted to 8.2 with 0.5N KOH. Further, the solution was transferred to a 500 ml conical flask and heated at 90°C for 24 h in a shaking water bath. Samples of both control (WPH10) and WPH-maltodextrin conjugate were removed after 3, 5, 8 and 24 h interval and cooled immediately in iced water and pH was checked. At the end of the heating period, the conjugated solution was stored at 4□C for further analysis.

Spray drying and freeze-drying of WPH10-maltodextrin conjugate solution

Spray drying of the WPH10-maltodextrin (WPH10-MD) solution was carried out according to Yang et al. (2012), using a spray dryer (Niro Atomizer Versatile Utility Spray Dryer, Soeborg, Denmark). The WPH10-MD solution was fed into the dryer by a peristaltic pump at a flow rate of 110 mL min^{-1} at a compressed air pressure of 379.21 kPa. The inlet and outlet air temperatures were maintained at 200°C and 90±5°C, respectively. The spray-dried powder was collected in airtight bags and stored in a refrigerator until further analysis.

Similarly, freeze-drying of the WPH10-MD solution was carried out as per the protocol described by Ilango et al. 2016, using a laboratory-scale lyophilizer at a

condensing temperature of −80 °C and a vacuum pressure of 0.01 kPa. The powder was transferred in an airtight container and stored in a refrigerator until further analysis.

Antimicrobial, Antioxidant and Antihypertensive activity of WPH10- MD conjugate samples

In vitro antimicrobial, antioxidant, and ACE inhibitory activity of WPH10- MD conjugate solution, spray-dried and freeze-dried WPH10-MD conjugate powders were determined according to the methods described above in 2.2. Solutions of 5% (w/v, protein) of the conjugate samples were prepared for assessing the bioactive properties.

Amino acid profile

The spray dried sample of whey protein hydrolysate (WPH10) was sent to Experiment Station Chem Labs (ESCL), University of Missouri, Columbia for determining the amino acid composition and the total crude protein (CP) content. WPH10 was analyzed using HPLC system for determining the amino acid profile, according to the AOAC Official Method (2006). The total CP content was determined through Kjeldahl method, following 981.10 of the AOAC International, 2016. The nitrogen percent was multiplied by 6.25 (as a conversion factor) to calculate the protein content.

Statistical analysis

All experiments were performed in triplicates, and one-way ANOVA and Tukey's test was applied to differentiate between the mean values ($P < 0.05$). All figures with error bars were made using Sigma plot software V11 for windows (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Antimicrobial, antioxidant, and antihypertensive behavior of the whey protein samples

The data presented in Table 1 and Figure 2 shows the ability of the whey protein samples (WPI90, WPC80, WPH10, WPH15, and WPH20) to inhibit diverse microorganisms such as *S. aureus, L. innocua, S. enteritidis,* and *B. coagulans*. Among the tested samples, hydrolysate WPH10 showed significantly higher inhibition against *B. coagulans* and *L. innocua*. The inhibited organisms represent groups that can cause food poisoning and spoilage. Of significance is the *B. coagulans,* which is one of the spores forming bacteria (Bottone, 2010) and can cause spoilage of heat-treated products. Antimicrobial peptides derived from milk proteins usually show a broad range of activity against both gram-positive and gram-negative microorganisms of spoilage and health significance (Benkerroum, 2010). However, from our results, only Gram-positive bacteria were inhibited. No inhibition was observed against *S. enteridis* (data not shown), which may be due to the complex nature of the cell envelope of Gram-negative bacteria. As compared to Gram-positive bacteria, it is both structurally and functionally more complex, and these differences in compositions of bacterial membrane might have implications for the bacterial specificity and mode of action of these antibacterial peptides (Floris et al. 2003). For gram-positive bacteria, the peptides disperse across the peptidoglycan layer and destroy the cytoplasmic membrane whereas, in contrast, disruption of both inner and outer cytoplasmic membrane is required for gram-negative bacteria. Incapability to distort the outer layer leads to loss of antimicrobial activity (Li et al. 2017). The mechanism of antimicrobial activity of the peptides involves interaction

with membranes. The electrostatic bonding between the peptides and the bacterial membranes (negatively charged) is the primary stage of the pore formation process leading to cell death (Benkerroum et al. 2010). Most of the antimicrobial peptides are positively charged and comprises both hydrophobic and hydrophilic side (amphiphilic) that enables them to interact with the lipid membranes, leading to alter the electrochemical potential on the cell membrane, inducing a change in the cell morphology and cell damage (Lei et al. 2019). Therefore, the inhibitory activity of these WPH represents their application as natural antimicrobial agents and can overcome the disadvantages of using chemical drugs with side effects (Lei et al. 2019). In a previous study reported by Correa et al. (2011), the researchers observed the antimicrobial activity of hydrolyzed ovine milk caseinate against *Listeria monocytogenes* ATCC 15131, *Bacillus cereus* ATCC 9634, *Corynebacterium fine* NCTC 7547, *Staphylococcus aureus* ATCC 1901, *Salmonella enteritidis* ATCC 13076, and *Escherichia coli* ATCC 8739. Whey hydrolysates derived from enzymatic hydrolysis of whey concentrate using a combination of chymotrypsin and protease Eap1 exhibited antimicrobial activity against *Salmonella* sp. (Tovar-Jimenez et al. 2017). Similarly, cheese whey hydrolyzed using pepsin and rennet enzymes inhibited *B. subtilis* and *E. coli* (Elbarbary et al. 2019) and peptides generated by alkaline hydrolysis of goat whey showed antibacterial activity against all tested bacteria (*S. typhimurium* ATCC 14028, *S. aureus* ATCC 33018, *E. coli* ATCC 8739, and *B. cereus* ATCC 33018) (Osman et al. 2016).

Milk protein hydrolysates obtained through enzymatic hydrolysis are reported to have antioxidant activities. Free radical scavenging activities of the industrial samples of whey protein hydrolysates (WPH10, WPH15, and WPH20), whey protein isolate

(WPI90), and concentrate (WPC80) were determined using $ABTS^{+}$ radical assay. The $ABTS^{+}$ radical is reduced to a colorless compound in the presence of antioxidants with hydrogen donating or chain-breaking properties. The development of antioxidant activity concerning whey protein samples is shown in Figure 3. All the whey protein samples exhibited free radical scavenging activity, which confirms the fact that whey protein itself exhibits some antioxidant properties. Whey protein hydrolysates showed varying antioxidant activities (WPH10: 866.56 ± 1.13 TEAC μmol/L; WPH15: 683.26 ± 1.33 TEAC μmol/L; WPH20: 750.34 ± 1.91 TEAC μmol/L). The difference in the activity among the hydrolysates is commonly due to exposure of functional sites and specific peptides after enzymatic hydrolysis (Jin et al. 2016). Besides, it can also be attributed to several factors like enzyme: substrate (E/S) ratio, pH, temperature, and incubation times (Bamdad et al. 2017). Naik et al. (2013), analyzed the effect of E/S, pH, and temperature on ACE inhibition and antioxidant activities of ultrafiltration fractions (permeate and retentate) of WPH, produced through trypsin hydrolysis of WPC. It was observed that the low molecular weight peptides exhibited higher activities (ACE inhibition and antioxidant) as compared to other fractions. In another study, WPC was hydrolyzed using papain and pancreatin under various E/S ratios and evaluated for ACE inhibition activity. It was observed that irrespective of any membrane filtration process, the hydrolysate demonstrated higher ACE inhibition property at an E/S ratio of 0.5/100 (Silvestre et al. 2012). The difference in the antioxidant activity observed amongst individual unhydrolyzed samples (WPI90 and WPC80), might be due to the different tertiary structure of the protein. It has previously been reported that the primary structure of the protein as well as other factors, such as protein conformation, play important role in

exerting antioxidant activity (Gomez-Ruiz et al. 2008). Our findings are comparable to reports described by Gomez-Ruiz et al. (2008), in which the hydrolysates, similarly, demonstrated a higher antioxidant activity than the non-hydrolyzed whey protein. In a study conducted by Embiriekah et al. (2018), hydrolysates generated by pepsin and trypsin hydrolysis of whey concentrate expressed higher antioxidant activity as compared to non-hydrolyzed WPC, measured by the DPPH method. Also, Mohan et al. (2015) reported, pepsin hydrolysates possess antioxidant activity, irrespective of the degree of hydrolysis (DH). The improvement in the antioxidant activity can also be related to the specific amino acids that might have been exposed after hydrolysis. Hydrophobic amino acids such as Ala (A), Pro (P), Val (V), Leu (L), Phe (F), Ile (I), Met (M), Tyr (Y), Trp (W) have been associated with the radical scavenging activities of the peptides (Ren et al. 2008). Free radical scavenging activity, metal ion chelation, inhibition of lipid peroxidation, are some of the antioxidant mechanisms (Sarmadi & Ismail, 2010). Antioxidant activities might protect against oxidative damage related to oxidative stress in human disease conditions. These antioxidative properties may also be applied in preventing oxidation reactions (such as lipid peroxidation) that lead to the deterioration of foods (Zhang et al. 2010). Functional foods enriched with such natural antioxidants are captivating, since they could be utilized without any toxic effects associated with the use of synthetic compounds and might confer higher added nutritional value (Gomez-Ruiz et al. 2008).

Within the renin-angiotensin system, ACE converts angiotensin I to angiotensin II, which is a potent vasoconstrictor. Besides, ACE degrades the vasodilator peptide bradykinin. Hence, ACE inhibitors may potentially lower hypertension thus helping in

the prevention and treatment of cardiovascular diseases (Fitzgerald et al. 2004). Studies are being conducted for identifying ACE inhibiting peptides from different food sources that can act as natural alternatives to drugs (Chamata et al. 2020). Therefore, in this part of the study, ACE inhibitory activity of whey protein hydrolysates (WPH10, WPH15, and WPH20), whey protein isolate (WPI90), and whey protein concentrate (WPC80) were evaluated by ACE inhibition assay that is based on the hydrolysis of the substrate HHL by ACE. ACE inhibition activity concerning whey protein samples is shown in Figure 4. Whey protein hydrolysates (WPH10, WPH15, and WPH20) showed higher ACE inhibition with percent inhibition of 67.61±0.09%, 64.23±0.29%, and 65.49±0.22% respectively. This might be due to the exposure of reactive groups in the hydrolysates and could be attributed to the presence of hydrophobic (aromatic or branched side chains) amino acid residues at each of the three C-terminal positions (Haque & Chand, 2010). WPC80 showed the least percent ACE inhibition of 60.87±0.14%. Whey concentrate hydrolysate fractions obtained from Corolase PP hydrolysis demonstrated antioxidant as well as antihypertensive activity (O'Keeffe et al. 2017). Similarly, a UF fraction of a whey isolate hydrolysate showed both antioxidant and antihypertensive activities (O'Loughlin et al. 2014). Tavaras et al. (2012), reported that whey protein peptides derived from *Cynara cardunculus* proteinase hydrolysis had potent antihypertensive activity. A recent study by Baba et al. (2021) demonstrates the ability of whey protein hydrolysates generated from camel milk to exhibit antihypertensive properties. In another study by Jeewanthi et al. (2017), a ten-fold increase in the ACE-inhibition activity was observed after the hydrolysis of a whey protein concentrate (WPC-35). The structure-activity relationships of ACE-inhibitory peptides is well established, and it is reported

that peptides containing hydrophobic amino acid in the N-terminal and aromatic amino acid in the C-terminal can demonstrate high ACE-inhibition property (O'keefe et al. 2017).

Amino acid profile of whey protein hydrolysate (WPH10)

WPH10, demonstrating highest bioactivities was analyzed for amino acid composition. The analysis revealed total amino acid content of 92.31g/100g of sample as shown in Table 2. Among the essential amino acids, leucine was the major amino acid found while lysine was second. A significant amount of Threonine and Isoleucine was observed with a substantial amount of Valine and Phenylalanine were also found. Among the non-essential amino acids, glutamic acid was the major amino acid present followed by aspartic acid. Lower levels of proline, alanine, serine, glycine, cysteine, tyrosine, arginine, and tryptophan were observed.

Leucine is involved in protein metabolism and the translation initiation pathway of muscle protein synthesis. Lysine and leucine are also common components of antimicrobial peptides. According to Marshall & Arenas (2003), peptides that are cysteine-rich are thought to form pores or ion-permeable channels in the lipid bilayer of the bacterial membrane. Membrane-active cationic peptides can also enter the membrane due to the presence of multiple lysine and arginine residues. Lee et al. (2014) also demonstrated the role of phenylalanine and valine residues in the antimicrobial activity of Piscidin-1 (antibacterial peptide). Martin et al. (2015), treated hypertensive rats with a whey protein hydrolysate having isoleucine-tryptophan fraction over 14 weeks and found a significant decrease in plasma ACE activity. α-lactorphin (consisting of Tyr-Gly-Leu-Phe) released form whey protein during enzymatic hydrolysis showed a transient, dose-

dependent blood pressure lowering effect (Nurminen et al. 2000). Strongest antihypertensive activity was shown by tripeptide with amino acids (Ile-Pro-Ala), originated from β-lactoglobulin digests (Jakala & Vapaatalo, 2010). Whey protein peptide fractions consisting aromatic amino acids and hydrophobic amino acids might also attribute to achieve a higher metal chelation activity. Moreover, certain free amino acids, present in the WPI hydrolysates such as tyrosine, methionine, histidine, lysine, tryptophan, and proline might also contribute to the antioxidative activity.

Change in pH as an indicator of conjugation process

WPH10 was selected for conjugation with maltodextrin as it showed higher bioactivities as compared to other whey protein samples. WPH10-MD conjugate solution was prepared from blends of WPH10 (5% w/v, protein) and maltodextrin (5% w/v). The conjugation process was monitored through measuring the change in pH throughout the heating period. The results are demonstrated in Figure 5. Previous studies have shown that during preparation of conjugate, heating of the whey protein isolate/whey protein hydrolysate-maltodextrin solutions with pH 6.8, at 90 °C led to physical destabilization and separation of the samples; hence, the initial pH was adjusted to 8.2 to reduce these protein-protein/peptide interactions. The pH of the WPH control solution decreased slightly i.e., from pH 8.2 to 7.49 but not significantly ($P > 0.05$) during heating (90 °C for up to 24 h) but the pH of the WPH-MD solution decreased significantly ($P < 0.05$) from pH 8.2 to 6.77 on heating for 24 h. The decrease in pH may be to the production of organic acids (i.e., formic, or acetic) during the intermediate stages of the Maillard reaction and the consumption of N-terminal charged amino acids, such as the basic amino acid lysine (Jiang et al. 2012). This may also be attributed to reaction of proteins/peptides

with innate lactose present in the WPH during heating (Berg & Van Boekel, 1994). The decrease in pH of the WPH-MD solution after 24 h of heating at 90°C indicates the progression of Maillard reaction at a greater extent and rate.

Antimicrobial, antioxidant, and antihypertensive behavior of WPH10-MD conjugate samples

The antimicrobial activity of the WPH10-MD conjugate solution, spray-dried and freeze-dried WPH10-MD conjugate powder samples are shown in Table 1 and Figure 2. The WPH10-MD conjugate, in solution form or as spray-dried and freeze-dried powder samples, showed antimicrobial activity against all the bacteria tested. As compared to whey protein hydrolysate (WPH10) alone, WPH10-MD conjugate solution showed significantly higher antimicrobial activity, attributed to the antimicrobial activity of Maillard products, which get produced during the early stage of heating and due to the lower pH of WPH10-MD conjugate.

This also might be due to the release of free amino groups from the decomposition of Heyns compounds at the advanced stage of the Maillard reaction (Baisier and Labuza, 1992). Though the mechanism behind the antimicrobial activity of Maillard reaction products has not been completely understood, however, Rufian-Henares & Cueva (2009), identified antimicrobial activity in coffee melanoidins through metal-chelating of the membrane. Later, hydrogen peroxide (H_2O_2) was identified to be the antimicrobial component in the coffee brew (Mueller et al. 2011). Further, Song et al. (2018) found an increase in the extracellular and intracellular H_2O_2 concentration in *E. coli* cells when treated with HAHp (synthetic peptide) derived Maillard products. Further,

drying of the conjugates can help to enhance the bioavailability and make it possible to incorporate in different food matrices (Sarabandi et al. 2019).

The results from the antioxidant activity of WPH10-MD conjugate in solution, spray-dried, and freeze-dried WPH10-MD conjugate powder samples are shown in Figure 6. All the samples demonstrated radical scavenging activity indicating the presence of antioxidant peptides. However, as compared to whey protein hydrolysate alone, the WPH10-MD conjugate solution showed significantly higher antioxidant activity that might be attributed to the scavenging activity of Maillard reaction products (amadori compounds), which get produced during heating (reaction between protein and sugar). It is also suggested that Maillard reaction products can function as electron donors. Hydroxyl and pyrrole groups of advanced Maillard reaction products may play an important role in the scavenging activity (Yoshimura et al. 1997) and, are considered as effective electron donors to increase reducing property (Song et al. 2016). It has also been reported that Maillard reaction products such as free amino N groups and melanoidins play a role in enhancing the antioxidant activity of the protein conjugates through different mechanisms like scavenging of hydroxyl groups and proxy radicals (Liang & Kitts, 2014). On the other hand, the lysine and arginine side chains of proteins can react with amadori products to form advanced glycation products. These products are important in terms of nutritional and biological aspects.

Antihypertensive activity of WPH10-MD conjugate in solution, spray-dried, and freeze-dried WPH10-MD conjugate powder samples are shown in Figure 7. As compared to WPH10, there was a slight decrease in ACE inhibition activity of the WPH10-MD conjugate solution. This might be due to a decrease in available amino groups of protein

as they bind to sugar molecules during the Maillard reaction. Similar results were reported by Jiang & Brodkorb (2012), where a reduction in the ACE inhibition activity was observed after heating of bovine casein peptides with ribose and galactose. Wang et al. (2013) also reported that the consumption of peptides with ACE-inhibitory activity by the Maillard reaction might be the reason for the decrease in the ACE inhibitory activity. Subsequent spray drying and freeze drying, the WPH10 conjugate powders exhibited even higher antimicrobial and antioxidant activity while retaining the antihypertensive activity as compared to the WPH10 conjugate solution.

CONCLUSIONS

Functional proteins and peptides are now an important category in the food sector and many scientific reports have revealed that milk proteins exhibit biological properties apart from their established nutritive value. In this study, the effect of the conjugation of whey protein with carbohydrate, on the bioactivities was examined. Different forms of whey protein (WPC80, WPI90, WPH10, WPH15, and WPH20) were compared. Of which, whey protein hydrolysates overall presented higher bioactivities as compared to intact whey protein, attributable to the effect of the bioactive peptides released during hydrolysis. Among the hydrolysates, WPH10 was seen to exhibit higher bioactivities. And, hence, WPH10 was conjugated with maltodextrin and the effect of conjugation on the bioactivities was determined. In comparison to WPH10, conjugated WPH10 showed higher antimicrobial and antioxidant activities. A comparable effect in antihypertensive activity was observed after conjugation. Overall, it can be concluded that besides functionality, the bioactivities of whey proteins can also be enhanced by primarily conjugating them with reducing carbohydrates such as maltodextrin.

CHAPTER 2 TABLES AND FIGURES

Table 1. Antimicrobial activity of whey protein isolate (WPI90), whey protein concentrate (WPC80), whey protein hydrolysate (WPH10, WPH15, WPH20), and whey protein hydrolysate-maltodextrin conjugate (WPH10-MD).

Sample	**Zone of inhibition (mm)**		
	S. aureus	***L. innocua***	***B. coagulans***
WPI90	8.7±0.33[a]	8.6±0.33[a]	9.3 ± 0.33[a]
WPC80	8.2±0.33[a]	8.4±0.33[a]	8.6 ± 0.33[a]
WPH10	10.5±0.33[a]	11±0.57[b]	11.3 ± 0.33[b]
WPH15	9.2±0.33[a]	9.4±0.33[a]	9.6 ± 0.33[a]
WPH20	9.8±0.33[a]	9.6±0.33[a]	10.3 ± 0.33[a]
MD	N.I	N.I	N.I.
WPH10-MD conjugate solution	16.7±0.33[b]	17.2±0.33[c]	17.6 ± 0.33[c]
Spray-dried WPH10-MD conjugate	18.1±0.33[b]	18.4±0.33[c]	19 ± 0.57[c]
Freeze-dried WPH10-MD conjugate	18±0.57[b]	17.8±0.33[c]	18.6 ± 0.33[c]

WPI90 – whey protein isolate; WPC80 – whey protein isolate; WPH – whey protein hydrolysate (10, 15, and 20%); MD – maltodextrin; WPH10-MD – whey protein hydrolysate-maltodextrin conjugate; N.I. – no inhibition.

Mean ± standard deviation (n = 3) within treatments with different letters are (a–c) significantly different ($P < 0.05$) according to the Tukey test.

Table 2. Amino acid profile of WPH10 (per 100 grams of sample)

Amino acid	Quantity in grams/ 100grams of sample (w/w%)
1. Threonine	6.26
2. Valine	5.43
3. Methionine	1.99
4. Isoleucine	6.29
5. Leucine	9.75
6. Phenylalanine	2.85
7. Histidine	1.55
8. Lysine	8.37
9. Aspartic acid	9.94
10. Serine	3.67
11. Glutamic acid	16.07
12. Proline	5.37
13. Glycine	1.51
14. Alanine	4.64
15. Cysteine	2.29
16. Arginine	1.88
17. Tyrosine	2.69
18. Tryptophan	1.69
19. Hydroxyproline	0.00
20. Hydroxylysine	0.00
21. Ornithine	0.04

22. Lanthionine	0.00
23. Taurine	0.03
Total	92.31

Figure 1. Process flow chart for preparation of the whey protein hydrolysate (WPH) samples.

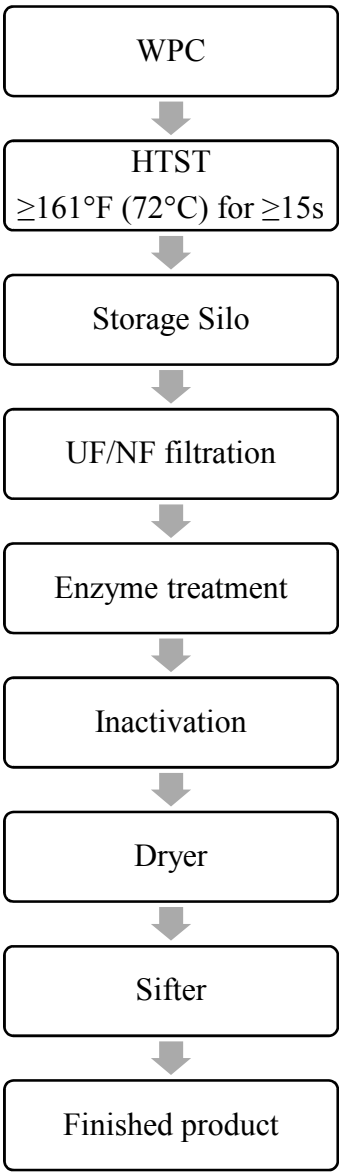
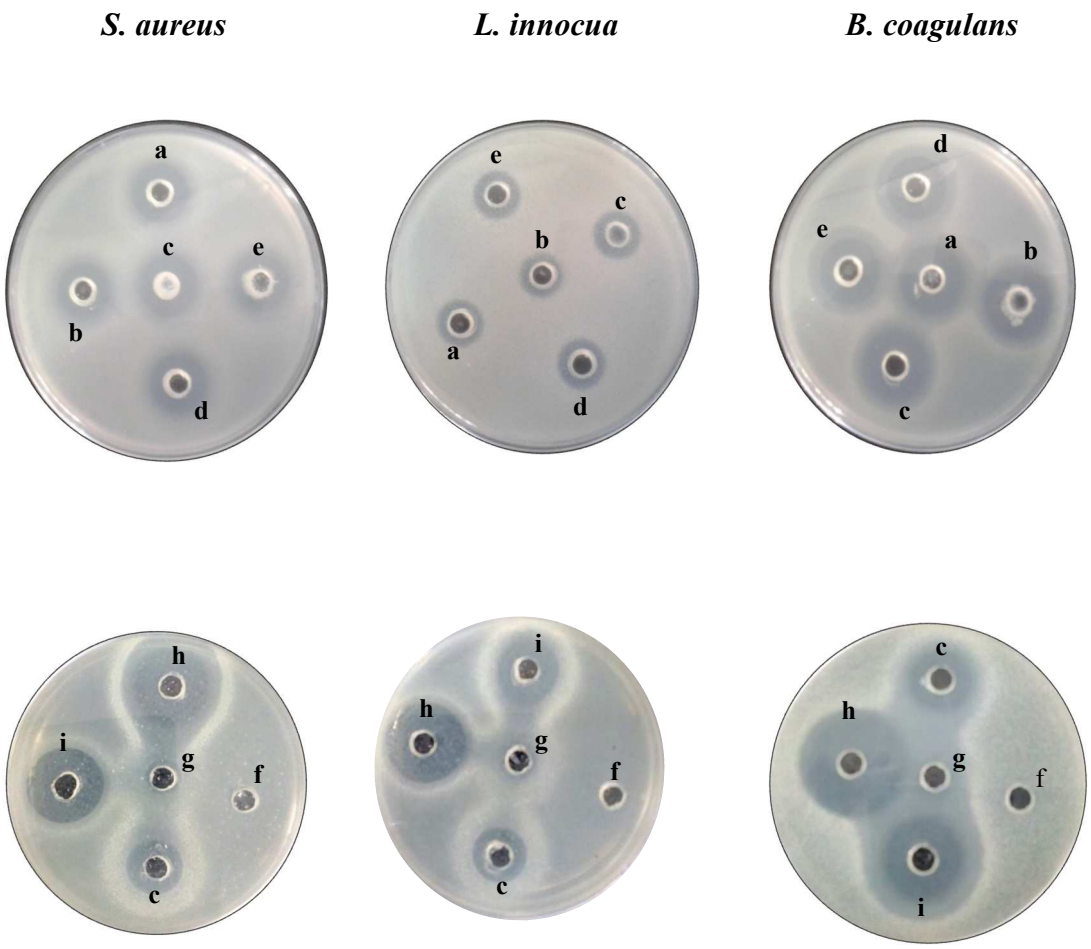


Figure 2. Antimicrobial activity of whey protein isolate (WPI90), whey protein concentrate (WPC80), whey protein hydrolysate (WPH10, WPH15, WPH20), and whey protein hydrolysate-maltodextrin conjugate (WPH10-MD).



a. WPI90 – whey protein isolate; b. WPC80 – whey protein isolate; c. WPH10– whey protein hydrolysate 10; d. WPH15- whey protein hydrolysate 15; e. WPH20- whey protein hydrolysate 20; f. MD – maltodextrin; g. WPH10-MD – whey protein hydrolysate-maltodextrin conjugate; h. Spray-dried WPH10-MD; i. Freeze-dried WPH10-MD

Figure 3. Trolox equivalent antioxidant capacity (TEAC) of whey protein isolate (WPI90), whey protein concentrate (WPC80), whey protein hydrolysate (WPH10, WPH15, and WPH20). Mean ± standard deviation (n = 3) within treatments with different letters are (a–c) significantly different ($P < 0.05$) according to the Tukey test.

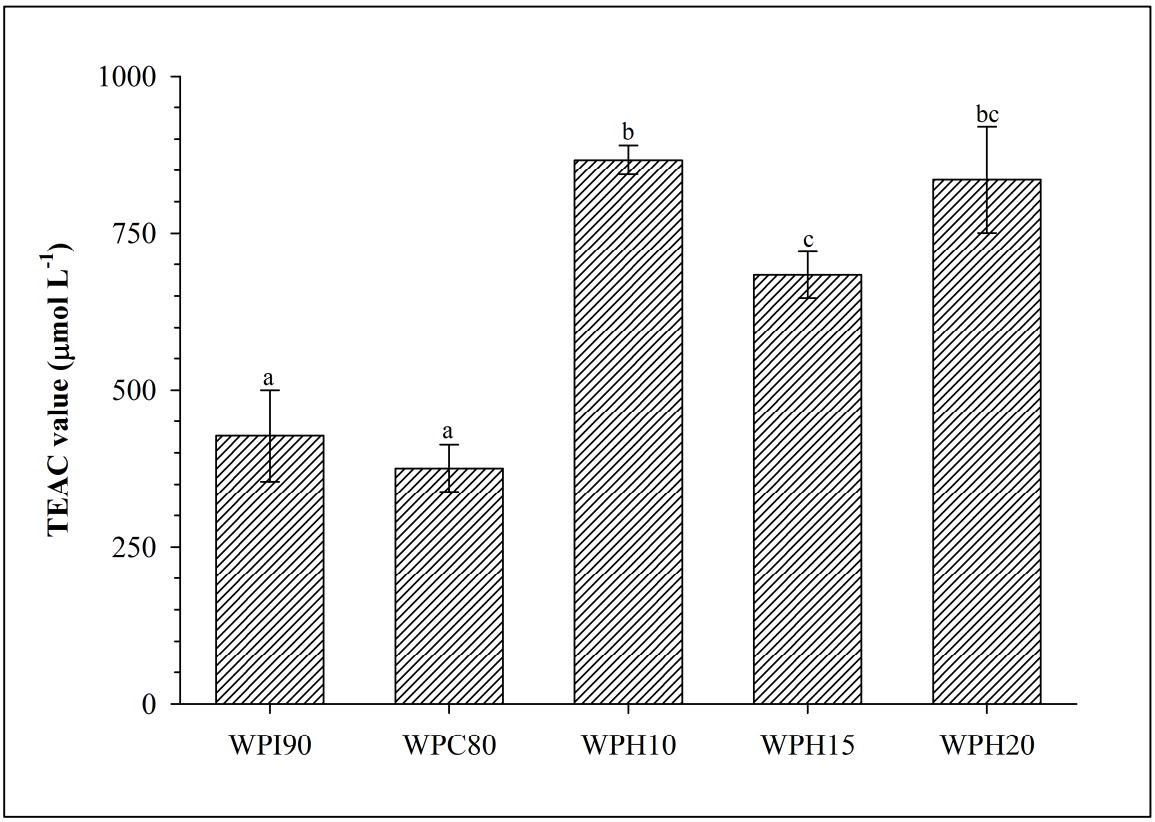


Figure 4. The inhibitory ability of angiotensin I-converting enzyme (ACE) of whey protein isolate (WPI90), whey protein concentrate (WPC80), and whey protein hydrolysate (WPH10, WPH15, WPH20). Mean ± standard deviation (n = 3) within treatments with different letters are (a–e) significantly different ($P < 0.05$) according to the Tukey test.

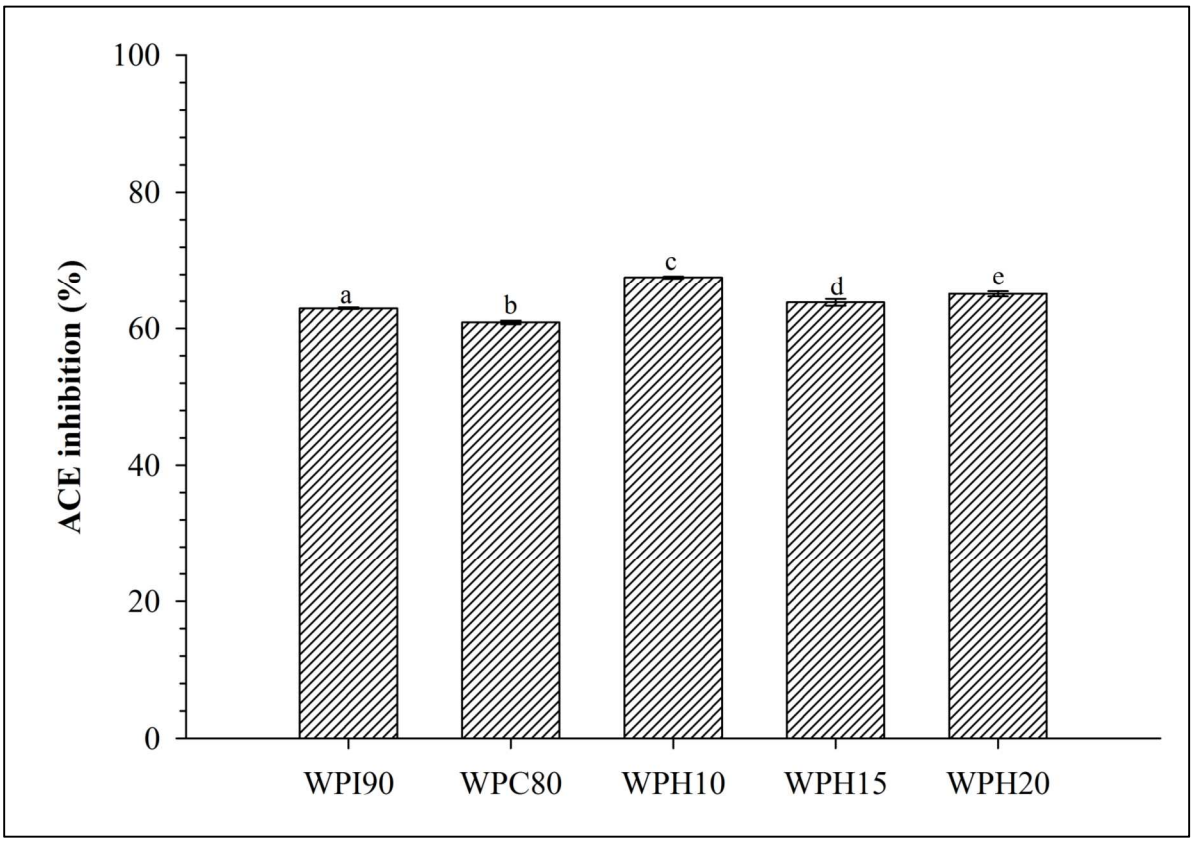


Figure 5. Changes in pH during the conjugation of whey protein hydrolysate (WPH10) with maltodextrin

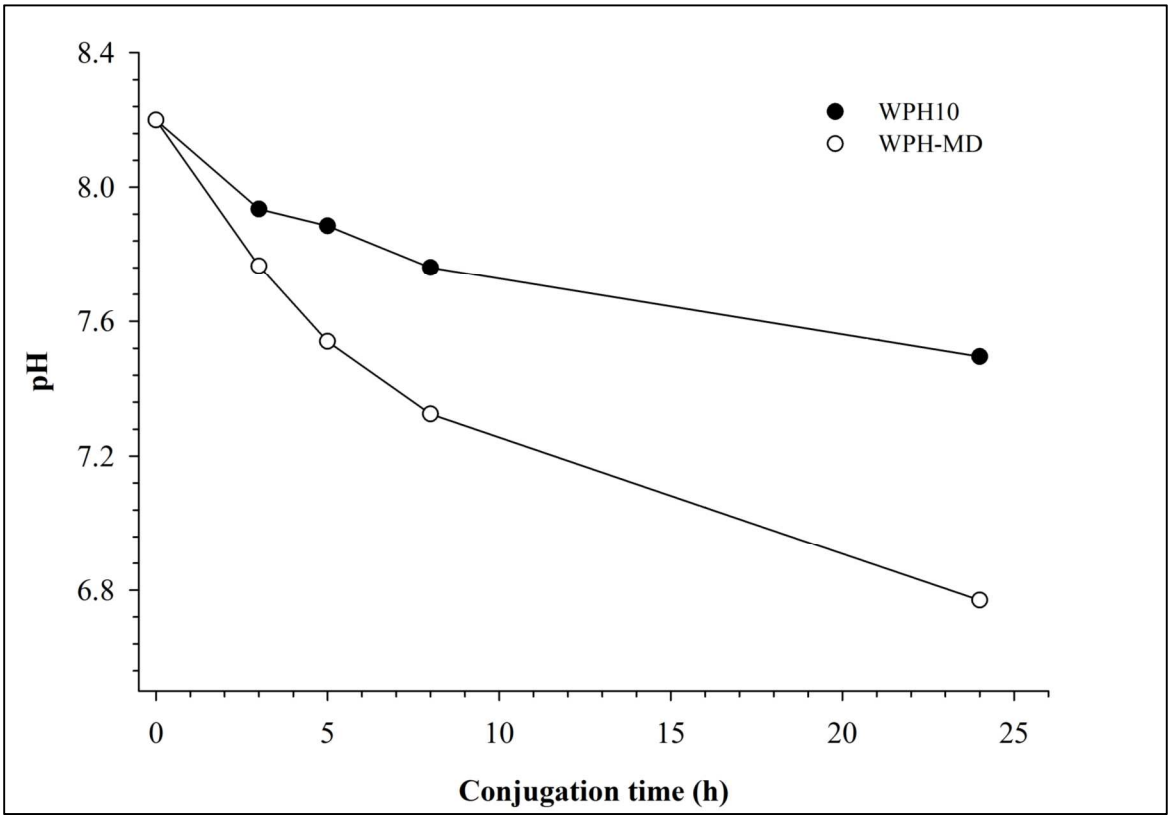


Figure 6. Trolox equivalent antioxidant capacity (TEAC) of maltodextrin (1), whey protein hydrolysate (WPH10) (2), whey protein hydrolysate-maltodextrin conjugate (3), spray-dried whey protein hydrolysate-maltodextrin conjugate (4), and freeze-dried whey protein hydrolysate-maltodextrin conjugate (5). Mean ± standard deviation (n = 3) within treatments with different letters are (a–c) significantly different ($P < 0.05$) according to the Tukey test.

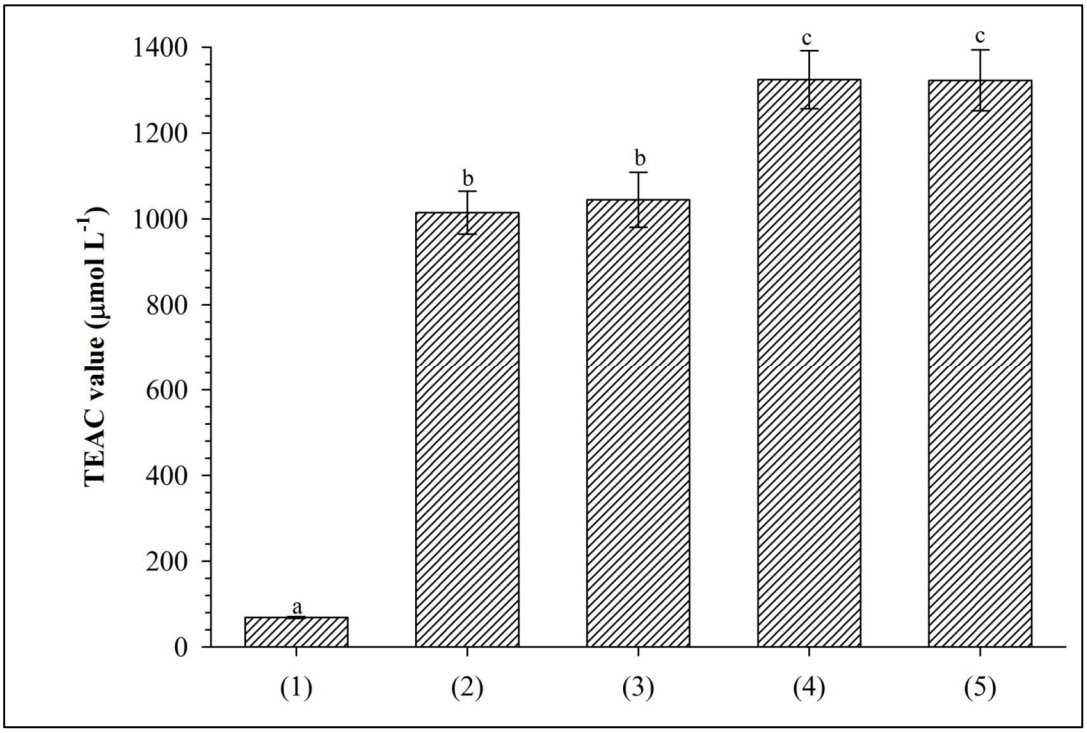
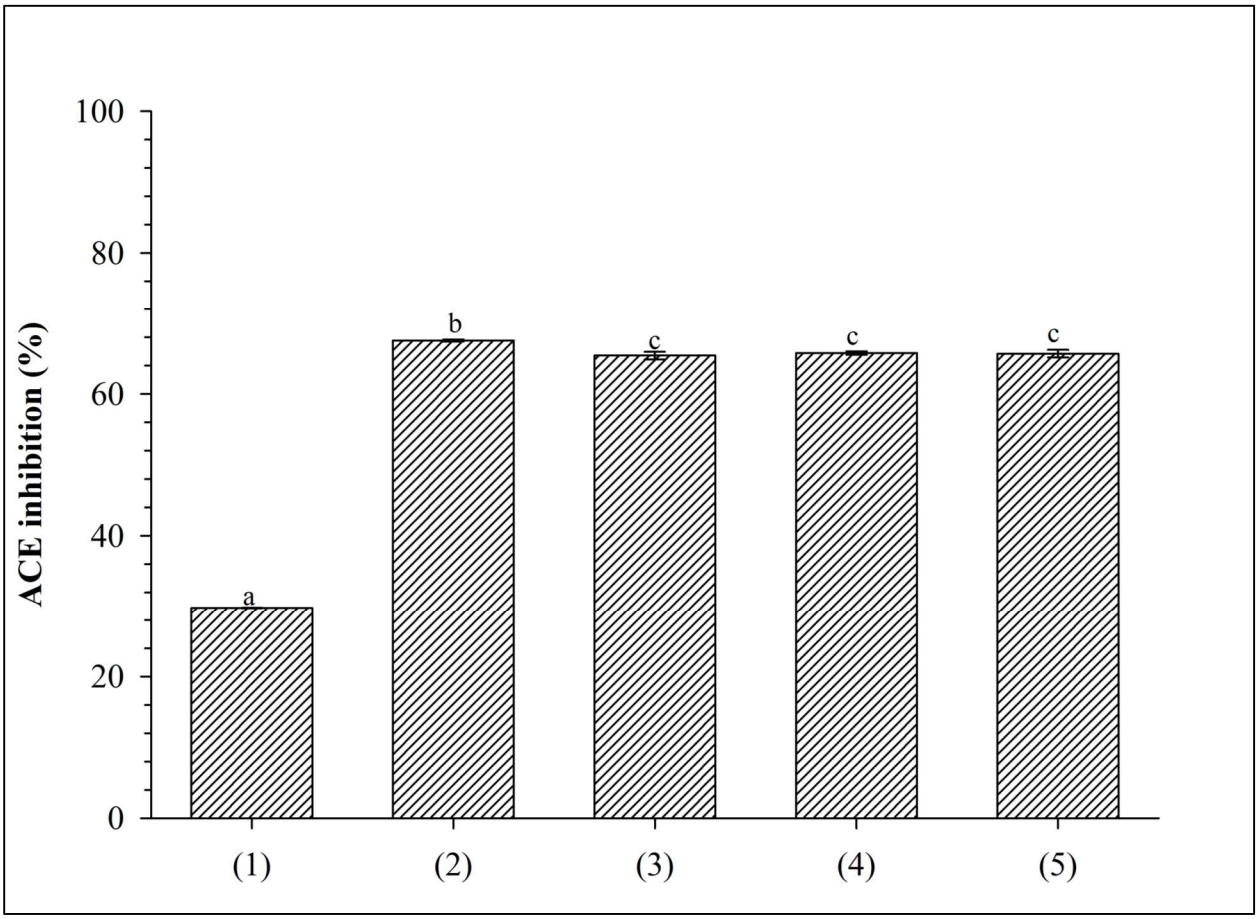


Figure 7. The inhibitory ability of angiotensin I-converting enzyme (ACE) of maltodextrin (1), whey protein hydrolysate (WPH10) (2), whey protein hydrolysate-maltodextrin conjugate (3), spray-dried whey protein hydrolysate-maltodextrin conjugate (4), and freeze-dried whey protein hydrolysate-maltodextrin conjugate (5). Mean ± standard deviation (n = 3) within treatments with different letters are (a–c) significantly different ($P < 0.05$) according to the Tukey test.



CHAPTER 3

DEVELOPMENT OF A SPRAY-DRIED CONJUGATED-WPH (WHEY PROTEIN HYDROLYSATE) POWDER WITH ENTRAPPED PROBIOTICS

ABSTRACT

Dairy foods enriched with probiotics are receiving considerable attention as modulators of the gut microbiota and host health. Microencapsulation technology has been widely used to increase the stability of probiotics. Whey based microencapsulation of probiotics has previously been investigated, however, due to thermal instability of the whey proteins, maintaining viability of the organisms has mostly been a challenge. Recently, the stability of the whey proteins has been addressed, primarily by conjugation. Hence, in this study conjugated whey protein hydrolysate (WPH10) was used as an encapsulant to entrap probiotics; *Bifidobacterium animalis* subsp. *lactis* ATCC27536 and *Lactobacillus acidophilus* ATCC4356, through spray drying. Cultures were mixed in the ratio of 1:1 at 10 log CFU/ mL and inoculated into the conjugate solution. The mixture was spray dried in 2L batches using a Niro drier with an inlet and outlet temperature of 200□C and 90±5□C, respectively. The final dried product was determined for cell viability and further stored for 12 weeks at 25, 4 and -18°C to monitor the viability and functionality. All experiments were conducted in triplicates and one-way ANOVA was applied to differentiate the mean values. Subsequent drying, the mean counts obtained before and after drying were 10.59±0.08 and 8.98±0.02 log CFU/g, respectively. At the end of 16 weeks, the counts were 7.18±0.08 log CFU/g at 4°C and 7.87±0.08 log CFU/g at -18°C, whereas at 25°C the counts dropped to 3.97±0.008 log CFU/g. At this point, the wetting time increased from 47±2min to 61±3min and solubility decreased from

90.7±0.13% to 82.2±0.4%. The conjugated WPH10, thus demonstrated as a carrier for probiotics and can be further used in food applications.

INTRODUCTION

According to FAO/WHO, probiotics are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (Culligan et al., 2009). They are active microorganisms, when ingested in certain amounts, modulates the gut microbiota to offer variety of therapeutic benefits. The two most common genera of probiotic microorganisms are *Bifidobacterium* spp. and *Lactobacillus* spp. (de Araújo Etchepare et al., 2020). To acquire the beneficial effects, it is recommended that, at the time of consumption, the foods containing probiotics should contain 10^6-10^7 live cells per mL or g of the product (Tripathi and Giri, 2014). It is also suggested that a daily intake of 10^8-10^9 cells per g is required to exert their physiological functions in the body (Knorr, 1998). However, several technological factors including temperature, pH, moisture, nutrient depletion, osmotic and oxidative stress poses considerable challenges in incorporation of these bacteria in different food matrices (Wilkinson, 2018). In addition, resistance to gastro-intestinal conditions has always been a major objection (de Araújo Etchepare et al., 2016). Hence, different approaches have been used to maintain the viability of the cultures including selection of resistant strains, use of oxygen-impermeable containers, incorporation of peptides and amino acids, and microencapsulation (Shah, 2000).

Microencapsulation technology has been widely known to increase the stability of probiotics with controlled release characteristics (Zanjani et al., 2014). Spray drying is one of most used techniques in microencapsulation process, due to its short time, low cost

and good stability and quality of end products (Solval, 2011; Felix et al., 2017). Compared to other conventional microencapsulation techniques, spray drying process can be easily scaled up to produce microcapsules in a continuous processing operation (I Re, 1998; McHugh 2018). A variety of encapsulants including alginate, gelatin, carrageenan, milk protein and pea protein concentrates and isolates, lipids have been studied (Ragavan and Das, 2018; Yasmin et al., 2018). However, large-scale production of gel beads, their large size diameters, dispersion, and requirement of transfer from organic solvents present serious difficulties in food applications (Picot and Lacroix, 2003). In case of milk whey proteins, though they have been a versatile nutritionally beneficial system, enriched with bioactive properties, major challenges related to their low thermal stability are faced. At high processing temperatures, whey proteins tend to destabilize leading to denaturation, gelation, foaming and decreased solubility (Adjonu et al., 2013). At high ionic strength, they are inclined to aggregation which causes sedimentation, during storage (Mulcahy et al., 2016).

Yang et al., 2012 addressed the stability of whey proteins, primarily by conjugating them with carbohydrates. Conjugation of proteins with a reducing carbohydrate via Maillard reaction is an advancing area of interest, with some previous reports showing improvement in physiological and functional properties, including thermal stability, solubility, emulsification capacity, water binding and antioxidant activity (Zhang et al., 2010). It is during the early stages of heating that conjugation occurs, where a covalent bridge is formed between protein and carbohydrate molecules. This interlinkage of the carbohydrate side chains with protein limits the aggregation between proteins and peptides, which in turn increases protein hydration and steric

repulsion between proteins (Sosa et al., 2016). Commonly used carbohydrate polymers are maltodextrin, β-cyclodextrin, corn syrup solids (CSS).

In view of the potential benefits of probiotics and conjugation, the intend of the present work was to study the effect of microencapsulation of probiotics in a conjugated whey protein on the viability and functionality of the product. In this study, conjugated whey protein hydrolysate (WPH10), which was identified in our previous study to have higher bioactive attributes, was used as an encapsulant to entrap probiotic cultures; *Bifidobacterium animalis* subsp. *lactis* ATCC27536 and *Lactobacillus acidophilus* ATCC4356, through a spray drying process. The product was stored at different temperature conditions to evaluate the viability of probiotic organisms and functional properties.

MATERIAL AND METHODS

Ingredients

Whey protein hydrolysate (WPH10) with 10% degree of hydrolysis and 89.98% of protein was procured from a commercial dairy plant. Maltodextrin with a dextrose equivalent (DE) of 10 was purchased from Sigma-Aldrich, USA.

Preparation of WPH10-maltodextrin conjugate

The WPH10-maltodextrin conjugate solution was prepared according to the protocol described by Mulcahy et al., 2016. WPH10 (5% w/v, protein) and maltodextrin (DE 10) (5% w/v) were dispersed in distilled water at room temperature and mixed under magnetic stirring for at least 2 h to ensure complete dissolution. The pH of the mixture was then adjusted to 8.2 with 0.5N KOH and allowed to hydrate for 18 h at 4°C. After

hydration, the pH was readjusted to 8.2 with 0.5N KOH. Further the solution was transferred to a conical flask and heated at 90°C for 24 h in a shaking water bath. At the end of heating period, the conjugated solution was cooled to room temperature for microencapsulation.

Probiotic cultures and inoculum preparation

Pure freeze-dried probiotic cultures (*Bifidobacterium animalis* subsp. *lactis* ATCC27536 and *Lactobacillus acidophilus* ATCC4356) were obtained from ATCC, USA. Fresh cultures were obtained after two respective transfers in MRS (de Man Rogosa Sharpe) broth supplemented with 0.05% (w/v) L-cysteine 37°C for 72 hours, under anaerobic conditions. Propagation of the cultures were continued, and the cells were harvested in their late log phase by centrifugation at 7700 rpm for 10min at 4°C. The cell pellets were washed twice in PBS (0.9% phosphate buffer saline) and suspended to achieve cell suspensions of 10 log CFU/mL. These cell suspensions were mixed in the ratio of 1:1 into the conjugated solution prior to microencapsulation.

Probiotic cells microencapsulation

The above inoculated whey protein hydrolysate-maltodextrin conjugate solution was spray dried in a pilot spray dryer (Niro Atomizer Versatile Utility Spray Dryer, Soeborg, Denmark). The solution was fed into the dryer by a peristaltic pump at a flow rate of 110 ml^{-min}, and the compressed air pressure of 55psi. The inlet air temperature and outlet air temperature were maintained at 200□C and 90±5□C, respectively. The obtained spray dried probiotic powder was collected and analyzed for viability of probiotic. WPH10 without maltodextrin was used as a control to represent the non-conjugated sample. Addition of maltodextrin without conjugation led to coagulation

during heating and spray drying process (shown in Figure 1) and hence, could not be used as a control for the encapsulation process.

Viability of probiotics and Encapsulation yield (EY)

The viability of the probiotic organisms was evaluated by standard pour plate technique. 1g of each powder sample was drawn out and serial dilutions were made using PBS (phosphate buffer saline). The diluted samples were plated on MRS agar supplemented with 0.05% (w/v) L-cysteine. The plates were incubated at 37 □C/ 72hrs under anaerobic conditions. Total count was taken in log CFU/g. Viable counts were taken before and after spray drying to determine the survival rate i.e., encapsulation yield (EY). It was calculated as follows:

$$EY = \frac{log\ N}{log\ N_0} \times 100$$

Where N_0 is the viable counts in log CFU/g of dry powder before spray drying and N represents the viable counts in log CFU/g of dry powder after spray drying.

Morphological analysis by scanning electron microscopy

The surface morphology of the WPH-MD conjugate entrapped probiotic powder was evaluated by scanning electron microscopy according to Yang et al., 2012 and Rosenberg et al., 1988. Spray dried powder samples were coated with 10 nm gold under vacuum by fine coat ion sputter. SEM was carried out at an accelerating voltage of 5 kV and the images were captured with magnifications of 5,000x and 10,000x.

Moisture determination

The moisture content of the spray dried probiotic powder was determined according to IDF, 26A. 1g of dry powder sample (A) was weighed into a porcelain dish. The dish was placed in hot air oven at 103□C for 4-5 h. At the end, the sample was drawn out, cooled and the weight was measured (B). The moisture content was calculated as follows:

$(\frac{A-B}{A}) \times 100$; where A- initial weight of sample; B- final weight of sample

Storage studies

The collected spray dried WPH10 conjugate encapsulated probiotic powder was packaged in airtight containers and stored at temperatures of −18, 4 and 25 °C. During storage, the samples were pulled out after 2, 4, 8, 12 and 16 weeks to monitor the cell viability, moisture content and functionality.

Functional properties

Solubility

The protein solubility of the WPH10-MD conjugate encapsulated probiotic powder was analyzed according to the method of Westergaard, 2004. In a beaker, 100 ml of water was taken at room temperature and 5g of sample (p) was added. The suspension was stirred for 30 minutes using a magnetic plate stirrer. 40 ml of the solution was drawn out in a tube, weighed, and centrifuged at 7000 x g for 10 min. The supernatant was transferred carefully in a pre-dried (1hr at 103±2□C, cooled in desiccators) and pre-weighed aluminum bowl. It was dried for 4-5 hours in an oven at 103±2□C, cooled and weighed (y).

% Solubility= $100-(\frac{y}{p} \times 100)$; where y-final weight after drying, p-initial weight of sample

Wettability

The method used was an extension of the International Dairy Federation (IDF) method (IDF, 1979). The method essentially measures the time for a given mass of powder to sink beneath the water surface, and this time is referred to as the wetting time. 250ml of water was weighed in a 600 ml dry beaker and heated to 50□C (assuring the inner side of the beaker, just above the water level remains dry). 10g of sample was taken and slowly added with the help of dry cone/aluminum foil (holding with stand) over the water. The time was recorded immediately after all the powder was added till all the powder particles wet completely.

Statistical analysis

The experiments were performed in triplicates, and one-way ANOVA was applied to differentiate between mean values. All figures with error bars were made using Sigma plot software V13 for windows 10 (SPSS Inc., Chicago, IL).

RESULT AND DISCUSSIONS

Morphology of the WPH conjugate encapsulated probiotic microcapsules

Following spray drying, the morphology of the whey protein hydrolysate (WPH10) before and after conjugation and after entrapment of the probiotic microorganisms in the conjugated WPH10 was analyzed through scanning electron microscopy. The images from SEM are presented in Figure 2. In the images of non-conjugated WPH10, a core- shell kind of powder particle can be clearly observed with

link bridges between the particles and some disruption of the structures after drying. This explains the high hygroscopicity and low thermal stability of the particles. However, in case of conjugated WPH10, a matrix type structure could be observed where the particles presented a round surface with numerous irregular pores on it. This confirms that subsequent conjugation, the particle structure modifies to a matrix type where the core material is homogenously distributed throughout the capsule (Su et al., 2008), making it suitable for encapsulation purposes. Further, the addition of maltodextrin decreased the hygroscopicity of the particles that restricted the formation of the link bridges. Similar observations were reported by Yang et al., 2012, where morphological analysis of a spray dried whey protein hydrolysate through SEM prior to conjugation showed a smooth surface with link bridges between the particles, and after conjugation with β-maltodextrin, the particles presented a matrix type surface with less link bridges and many pores on it. In Rocha et al., 2009, after conjugation of a casein protein hydrolysate with maltodextrin, similar matrix-type microspheres were produced.

After entrapment of probiotic cells in the conjugated WPH10, variable sizes microcapsules were produced in the range of (10- 20 μm in diameter). The images are displayed in Figure 3. Scanning electron micro images of the microencapsulated probiotic powder showed no free or non-encapsulated cells on the outside or on the surface of the microcapsules which indicates a successful entrapment of the probiotics with a high encapsulation efficiency. So, to confirm the powder particles were dispersed in distilled water (1g in 9mL of water) and observed under light microscope (image presented in Figure 3c). The images clearly show the probiotic cells around the broken conjugate particles and some inside the pores. Hence, this verify that the matrix structure of the

conjugated WPH10 is maintained even after the microencapsulation and shows that the cells remained entrapped within the carrier material. The observations can be comparable to those *Bifidobacterium infantis* entrapped microcapsules produced using caseinate-FOS-oil DGS emulsion (Crittenden et al., 2006). However, the microcapsules produced, provide advantages because of its smaller size as compared to beads produced using alginates, gelatin, or xanthan gums (typically > 100 μm in diameter) that impacts the mouthfeel and texture when added into food matrices (Sun and Griffiths, 2000; Krasaekoopt et al., 2004; Crittenden et al., 2006).

Viability of probiotics and encapsulation yield (EY)

The achievement of high viability of probiotic organisms throughout drying and storage period is challenging and is classified as one of the major problems in producing a commercial probiotic food (Mounsey and O'Riordan, 2008). For efficacy, the recommended viable probiotic counts in a probiotic food are 10^6-10^7 CFU/g (Tripathi and Giri, 2014). Table 1 and Figure 4 shows the viability of probiotic organisms in conjugated WPH10 matrix through spray drying and 16 weeks storage of the microcapsules at -18, 4 and 25°C. After spray drying, the probiotic cell counts obtained in conjugated WPH10 matrix were 8.98±0.02 log CFU/g with an encapsulation yield of 84.87±0.02%. However, when probiotic cells were encapsulated in non-conjugated WPH10 (control), under similar spray drying conditions, viable counts of 4.03±0.02 log CFU/g were achieved with an encapsulation yield of 37.94±0.17%. These results imply a good retention of the viability of probiotics in the conjugate carrier with a higher survival rate as compared to non-conjugated WPH10. Higher survival in the WPH10 conjugate is

considerably due to improved thermal resistance of the WPH10 and modification of the particle to a matrix type following conjugation with maltodextrin.

Microencapsulation through spray drying is the most preferred and commonly used methods because of its several advantages. Compared to other conventional techniques, it requires less time and can be easily scaled up in a continuous processing operation relatively at a low cost (Picot and Lacroix, 2004). However, numerous challenges are faced with whey proteins associated with heating factors, osmotic stresses due to dehydration and to encapsulate certain probiotic organisms such as *Bifidobacterium* spp. that are sensitive to high processing temperatures (Chavarri et al., 2012). Hence, the carrier material should be stable in terms of resistance to extreme drying conditions to provide protection to the probiotic cells and obtain powders with high numbers of viable cells in a form suitable for food applications.

Recently, the use of mixtures of whey proteins and carbohydrates or fats are being studied to enhance the stability of the whey proteins as a carrier. Use of maltodextrin has been previously reported to improve the stability after the spray drying encapsulation process because of its properties to improve the physiological properties like hygroscopicity, dissolution and thermal stability (Negrão-Murakami et al., 2017). It is also said that use of proteins and polysaccharide mixtures can help to link the proteins to protect the lactic acid bacteria (Nale et al., 2017) and can also be utilized as a dietary fiber by the organisms under gastrointestinal conditions (Salavati and Allenspach, 2017). Picot and Lacroix, 2004 reported that entrapment of *Bifidobacterium* spp. through spray drying in a heat denatured WPI carrier containing milk fat can be suitable in terms of cell viability as compared to WPI alone. Guerin et al., 2003 investigated viability of

probiotics entrapped in gel beads coated with WPI conjugated with pectin under gastrointestinal conditions. They observed that the resistance of the cells to acidic and bile conditions was enhanced with a reduction of less than 2 logs in case of coated gel beads. Comparatively, the survival of the cells in free form and in the uncoated gel beads was lower with 4-7 log reduction. In a study conducted by Oliveira et al., 2007, *Bifidobacterium* spp. and *Lactobacillus* spp. were encapsulated in casein/pectin matrix by coacervation followed by spray drying and they found that the wall material was efficient in protecting the microorganisms during drying and in the gastrointestinal conditions. In another study, *L. rhamnosus* GG was encapsulated @ 10^9 CFU/mL in a formulation prepared using whey protein isolate (WPI) and maltodextrin through spray drying and the results showed only 1log reduction of the bacteria with improved survival during storage conditions (Ying et al., 2012).

Viability of the probiotic microorganisms during storage conditions is an important criterion for foods containing probiotics. Therefore, the WPH10 conjugate entrapped probiotic microcapsules were stored 25°C, 4°C and -18°C for 16 weeks to monitor the cell viability and compared with non-conjugated. The results are shown in Figure 3. Throughout storage at 4 and -18°C, the probiotic counts were maintained, and at the end of storage period, a reduction of approx.1 log CFU/g was observed with final count of 7.18±0.008 and 7.87±0.008 log CFU/g at 4 and -18°C, respectively. At 25°C, the counts significantly dropped to 3.97±0.008 log CFU/g. In case of non-conjugated WPH10 (data shown in Figure 3) the probiotic counts sharply declined to 2.07±0.008 and 2.59±0.069 log CFU/g at 4 and -18°C respectively, whereas at 25°C storage, the counts declined to 2.39±0.061 log CFU/g at the end of 4 weeks and no counts were observed

after 8th week storage period. Overall, the survival of the organisms was better at 4 °C and at −18 °C at the end of 16 weeks than ambient temperatures (25°C). From these data, it was observed that the stability of the conjugated WPH10 was higher as compared to the non-conjugated WPH10 under storage conditions and survival of the cells was higher at 4 and -18°C as compared to 25°C. And the final concentration of 7 log CFU/g of viable cells are within the recommended levels of the probiotics in foods i.e., 10^6-10^7 CFU/g (Anekella and Orsat, 2013).

In a study, Fung et al., 2011, encapsulated *L. acidophilus* in dietary fibers through electrospinning and obtained a good survival when stored at 4°C for 21 days. Besides, similarly, the thermal resistance of the encapsulated probiotics indicated a possible protection of the cells when incorporated in heat-processed foods. Yasmin et al., 2018 encapsulated *Bifidobacterium longum* BL-05 and studied its survival at 4°C for 28 days. Storage at refrigeration temperatures exhibited a log reduction of 1.72 and 1.99 for all encapsulated treatments. This can be correlated with the present work in which multilayer microencapsulation using protein/carbohydrate mixtures significantly improved the survival of probiotics at 4°C during long-term storage.

The results obtained can also be related to Xu et al., 2016, where a Lactobacillus spp. was encapsulated in a pea protein isolate-alginate matrix and stored at -15°C. After 84 days of storage, the encapsulated bacteria showed highest survival rate as compared to all other samples. It was reported by Conrad et al., 2000 that at freezing temperatures cell death can occur due to formation of ice crystals that can cause structural damage to the cell membranes and result in change in the physiological state of the cells. However, in our studies, minimal log reduction at -18°C indicates the ability of the conjugated

WPH10 as a carrier to protect the cells at freezing temperatures. This makes it suitable to be added into frozen food or that requires freezing.

In relation to 25°C, the stability of the cells in conjugated WPH10 were maintained and the counts were within the recommended levels till 4 weeks of storage. Thus, providing a better protection as compared to non-conjugated WPH10, which presented sharp decline within 15 days of storage. This tells us that, at the analyzed temperature (25°C), it is difficult to maintain the viable counts and the loss of viability at ambient temperatures may be attributed to changes in the moisture content of the powder which can lead to cell membrane disruption and deactivation (Ilango et al., 2017). The increase in the moisture content with storage time could be associated with moisture absorption from the storage environment. Besides, crystallization of the lactose sugar could be one of the reasons, as some amount of the water gets released during the phase transition from the amorphous state to the crystalline state, (Pałacha & Sitkiewicz, 2008). Moreover, the viability of the bacteria during storage also depends on the sensibility of the bacteria to oxygen and the ability of encapsulating material as a protector. Following spray drying, the lipid oxidation during storage alters the composition of the lipid cell membrane. Other factors that impact the survival of the organisms during storage are the composition of the encapsulated powder, oxygen content and glass transition temperature. Hence, the viability of the organisms during ambient storage conditions could be enhanced by using effective desiccants or deoxidants, suitable packaging material and vacuum packaging.

Moisture determination

Determination of moisture content is one of the important factors to determine the shelf stability of the food products. It also affects the physical and chemical characteristics of a food which influences the freshness and storage stability of the food for a long period of time (Isengard, 2001). Both the non-conjugated and conjugated entrapped probiotic powders showed moisture contents of 0.91±0.08% and 0.64±0.14% i.e., below 5%, which is recommended in spray dried powders for preventing cell loss during storage of dried cultures (Peighambardoust et al., 2011). The outlet temperature and cubic effect of the outlet temperature had the greatest influence on the moisture content in the microcapsules. Therefore, higher outlet (90±5°C) and inlet temperature (200 °C) produced a powder with lower moisture content, thereby suggesting temperature to be the most important factor (Felix et al., 2017).

The functional properties of milk powder are important when the powders are used for recombination or in the manufacture of various food products. These functional properties include emulsification, foaming, water absorption, viscosity, gelation, and heat stability, which are essentially the manifestations of the physical and chemical properties of the milk.

Solubility

The ability of a powder to completely dissolve in water is termed as solubility (Gaiani et al., 2005). The protein solubility of the WPH10-MD encapsulated probiotic powder was analyzed according to the method of Westergaard (2004), with slight modifications and was compared with the control i.e., non-conjugated WPH10. The change in the solubility from initial day till 16 weeks of storage is displayed in Figure 5.

Following spray drying, the solubility of the WPH10-MD encapsulated probiotic powder, at the initial day of storage was 91.03±0.98 % whereas the solubility of WPH10 was 82.03±0.88%. The lower solubility of WPH10 powder could be attributed to the exhibition of hydrophobic residues and release of specific peptides during hydrolysis that promote peptide/protein-peptide aggregation (Creusot and Gruppen, 2007). Besides, during hydrolysis, peptides released have smaller molecular masses and less secondary structure which can limit the heat-induced changes (Chobert et al., 1988). In case of conjugated WPH10 encapsulated probiotic powder, conjugation offers an advantage. During the process of conjugation, the bulky dextran molecules get attached to the protein molecules which in turn increases the steric hindrance in between the molecules. This thereby increases the hydration of the protein (Mulcahy et al., 2016).

Further, both the powder samples were stored at three different temperatures (-18, 4 and 25°C). The samples were withdrawn after 2, 4, 6, 8 and 16 weeks and solubility were analyzed. After two weeks of storage the solubility of WPH-MD powder was (89.68±0.27% for -18°C, 90.23±0.99% for 4°C and 89.05±0.52% for 25°C) whereas for WPH10 powder, the solubility was (78.85±0.17% for -18°C, 79.3±0.2% for 4°C and 78.29±0.7% for 25°C). A slow but continuous decrease in solubility was observed in both the samples. For 8 weeks of storage, WPH-MD powder showed relatively good solubility at all the storage temperatures (>85%). At the end of 10^{th} week, the solubility of WPH-MD powder declined to 82.36±0.62% for -18°C, 83.1±0.99% for 4°C and 81.19±0.7% for 25°C and for WPH10 powder, the solubility declined to 69.41±0.24% for -18°C, 69.97±0.92 % for 4°C and 68.99±0.89% for 25°C. The decrease in solubility of the powder samples with storage time is possibly due to the formation of a complex matrix of

crosslinked proteins at the surface of the powder which eventually restricts water transport and subsequently retards the hydration of the powder particles (Anema et al., 2006). However, studies have also manifested, that the solubility will not decrease until a significant level of cross-linking of the protein molecules at the powder surface has occurred and this may explain why the solubility did not immediately decrease on storage. With storage time and temperature, the degree of crosslinking between proteins increases (Sharma et al., 2012). Hence, the solubility of the powder samples gradually decreased with time and the samples at room temperature exhibited lower solubility as compared to the samples stored at 4 and -18°C. Also, (Singh and Newstead, 1992), addressed that, increase in the moisture level of the milk powder samples during prolonged storage contributes to several changes in the milk protein structure which leads to decrease in solubility.

Wettability

Wettability of food powders is a result of a molecular interaction between solid and liquid phase and generally explained as the ability of the powder particles to overcome the surface tension caused by the water. The surface composition of powders plays an important role in the wetting process (Fang et al., 2008).

The wettability of the WPH10-MD encapsulated probiotic powder was analyzed according to the method of IDF, 1979, with slight modifications and was compared with the reference sample i.e., WPH10. The change in the wetting time from initial day till 16 weeks of storage is displayed in Figure 6. Following spray drying, the wetting time of the WPH10-MD encapsulated probiotic powder, at the initial day of storage was 47±2min whereas the wetting time of WPH10 was 53±2min. Both the powder samples showed

higher wetting time as compared to a commercial whole milk powder (WMP) and skim milk powder (SMP) (>1min) which can be explained by their high protein content as compared to WMP. In both cases, the powder particles became wet very quickly which turned instantly into lumps surrounded by the gelatinous layer. This layer greatly hindered the wetting of the powder inside the lumps. This phenomenon can be explained by the presence of high protein on the surface of powders. Similar observations have been reported by Silva and Mahony, 2016 in milk protein concentrate. WPH10-MD encapsulated probiotic powder showed less wetting time as compared to WPH10 powder which can be related to the effect of conjugation as linking of carbohydrate side chains with protein decreases the exposure of protein molecules on the particle surface (Mulcahy et al., 2016). Another possible explanation could be due to decrease in the hygroscopicity of the particles after addition of maltodextrin. Sharma et al., 2012 explained that if the particle surface is more hygroscopic, it dissolves too quickly which thickens the liquid and restricts further penetration of liquid into the powder mass.

Further, both the powder samples were stored at three different temperatures (-18, 4 and 25°C). The samples were withdrawn after 2, 4, 6, 8 and 16 weeks and wetting time were recorded. After two weeks of storage the wetting time of WPH-MD powder was (49±1min for -18°C, 48±1min for 4°C and 50±1min for 25°C) whereas for WPH10 powder, the wetting time was (56±1min for -18°C, 56±1min for 4°C and 59±1min for 25°C). A continuous increase in the wetting time was observed in both the samples and by the end of 16th week, the wetting time of WPH-MD powder increased to 61±1min for -18°C, 60min for 4°C and 63min for 25°C and for WPH10 powder, the wetting time increased to 71±1min for -18°C, 70±1min for 4°C and 72±1min for 25°C. The increase in

the wetting time with storage time can be attributed to the formation of a complex matrix of crosslinked proteins at the surface of the powder which eventually restricts water transport and subsequently retards the hydration of the powder particles (Anema et al., 2009). Swelling of the particles always results in a slower rate of wetting, which might even approach zero, as in the case of whey protein concentrate (WPC) (Kim and others, 2002).

CONCLUSIONS

The morphology of the conjugated WPH10 presented a matrix type microsphere and hence, was used for encapsulation of probiotic microorganisms. After microencapsulation through spray drying, the conjugated WPH10 demonstrated as a carrier for probiotic microorganisms with viable counts of 8.98 log CFU/g with an encapsulation yield of 84.87%. Hence, the survival rate was significantly higher in the conjugated WPH10 matrix as compared to non-conjugated WPH10. Besides, conjugation of the whey protein hydrolysate positively affected the viability of the probiotic cells under different storage temperatures (4°C, 25°C and -18°C). The microspheres were able to maintain and preserve the viability of the cells throughout the storage period at freezing (-18°C) and refrigeration temperatures (4°C). Conjugation with maltodextrin was effective in enhancing the functionality of the final spray dried product. With the improved solubility and wettability of conjugated whey protein hydrolysate (WPH10-MD) as compared to non-conjugated WPH10, it possesses great potential to be incorporated as a food ingredient. Overall, such probiotic powder formulation with improved functionality and having value added benefits from both WPH10 and probiotics beyond utilizing their individual benefits, can offer several food applications.

CHAPTER 3 TABLES AND FIGURES

Table 1. Viability of the probiotic microorganisms in the conjugated whey protein hydrolysate (WPH10-MD) after spray drying and its encapsulation efficiency in %.

Sample	Before Drying (log CFU/mL)	After drying (log CFU/g)	Encapsulation yield (%)
WPH10 encapsulated probiotic powder (control)	10.62±0.01[a]	4.03±0.02[c]	37.94±0.17[d]
WPH10-MD encapsulated probiotic powder	10.58±0.08[a]	8.98±0.02[b]	84.87±0.02[e]

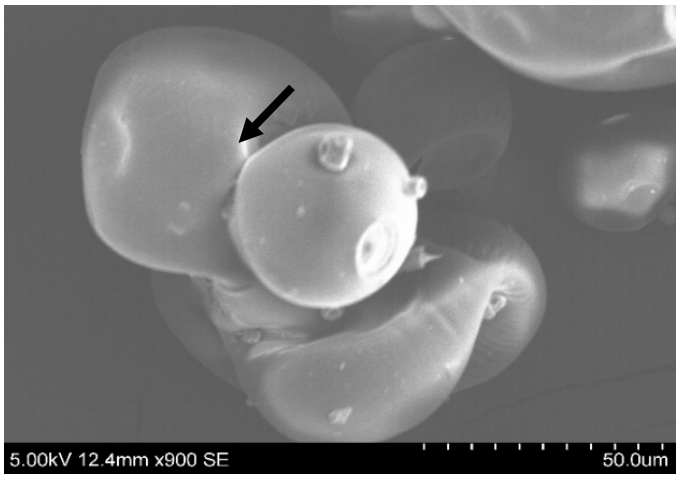
Mean ± standard deviation (n = 3)

(a-e): Values followed by same letter are not significantly different ($P<0.05$)

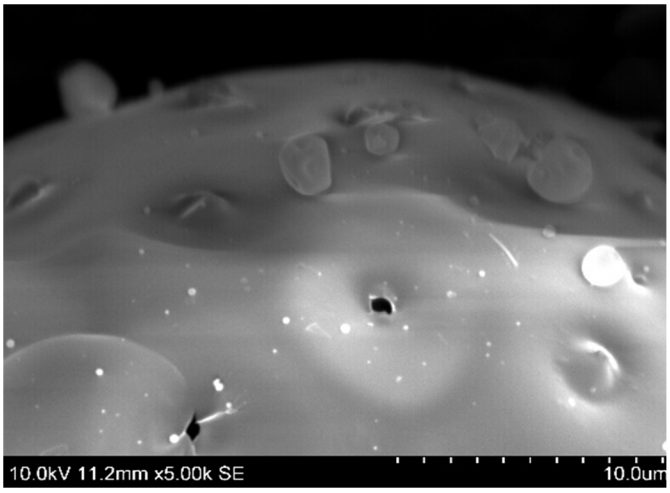
Figure 1. Conjugated WPH10 (left) and control non-conjugated WPH10 (right) after 24h of heating.

Figure 2. Scanning electron micro images of the powder particles with magnifications from 900-5k: a1 and a2. non-conjugated whey protein hydrolysate (WPH10), b1 and b2. maltodextrin conjugated whey protein hydrolysate (WPH10-MD).

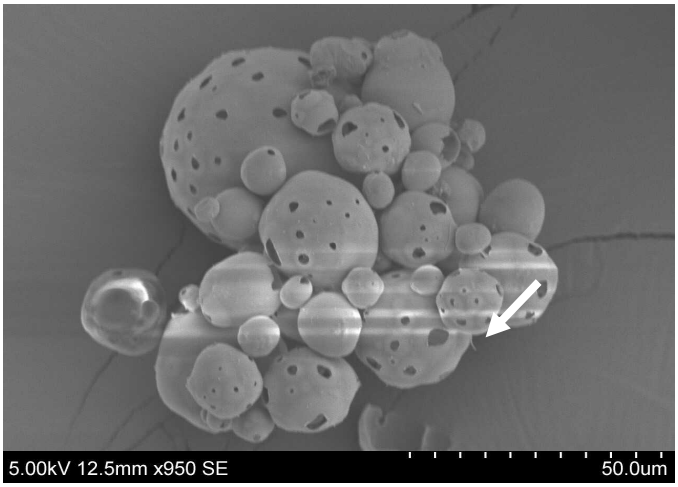
a1.



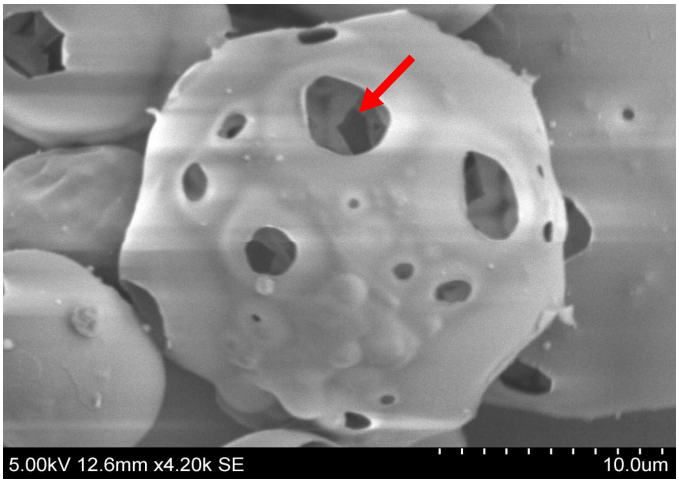
a2.



b1.



b2.



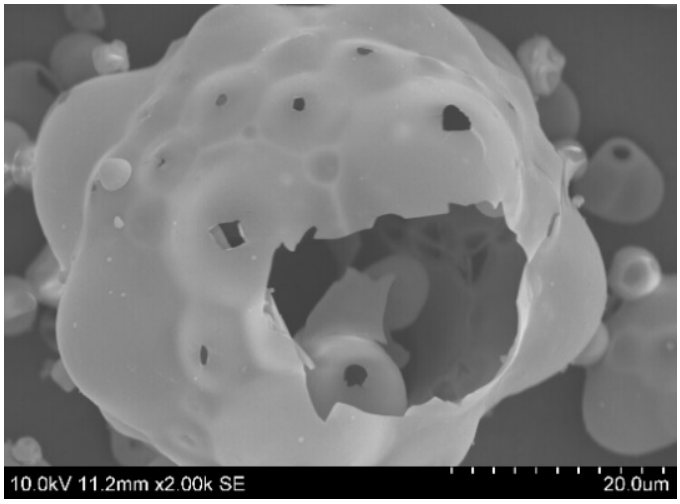
Black arrow represents the link bridges between WPH10 powder particles

White arrow represents the pores on the conjugated WPH10

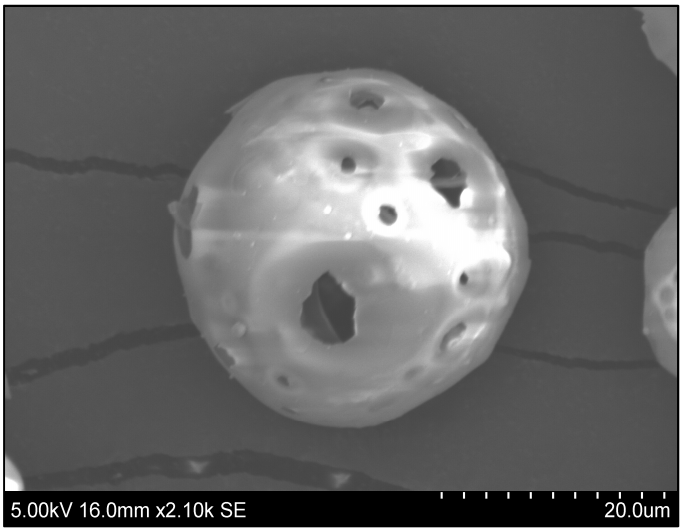
Red arrow represents the matrix structure that can be seen through the pores

Figure 3. Scanning electron micro images and light microscope images: a. non conjugated WPH10 entrapped probiotics powder, b. conjugate (WPH10-MD) entrapped probiotics powder particles, c. light microscope image of conjugate (WPH10-MD) entrapped probiotics powder.

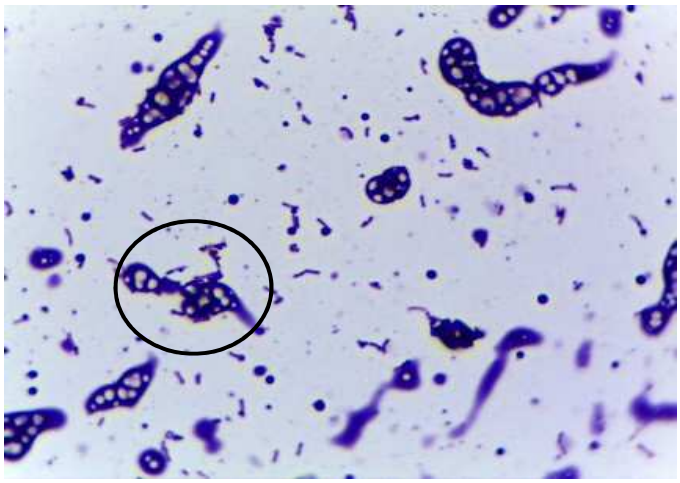
a.



b.



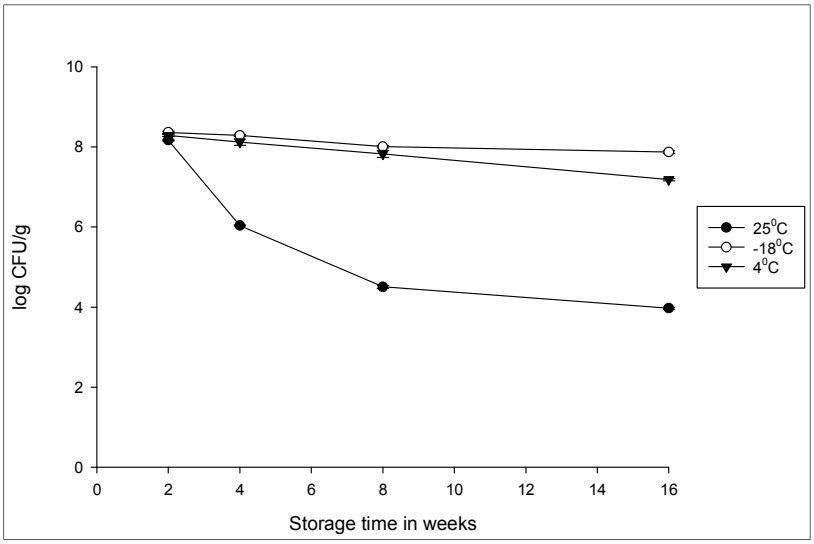
c.



Black circle in image c represents probiotic cells surrounding the broken conjugate powder particles

Figure 4. Viability of probiotic organisms in a. Conjugated (WPH10-MD) encapsulated probiotic powder as compared to b. Non-conjugated WPH10 entrapped probiotic powder and during storage at -18, 4 and 25°C for 16 weeks. Error bars represent standard error of the means.

a.



b.

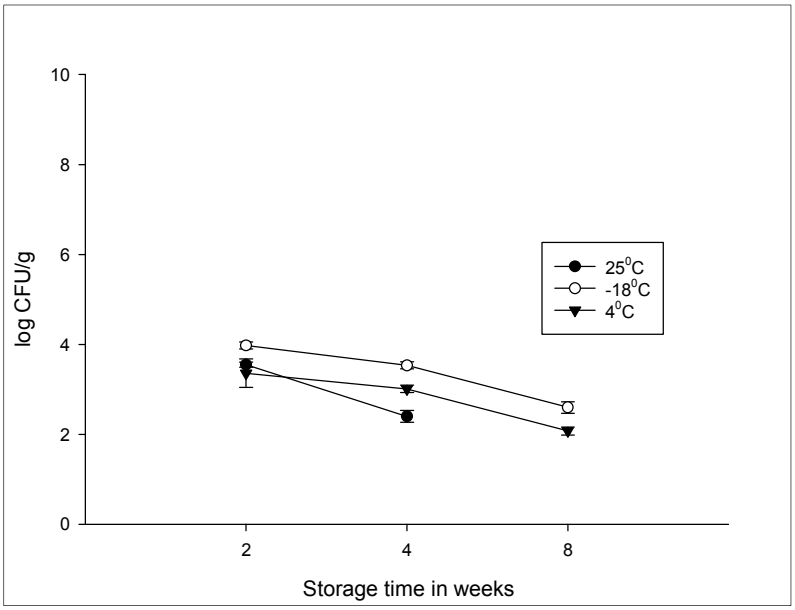
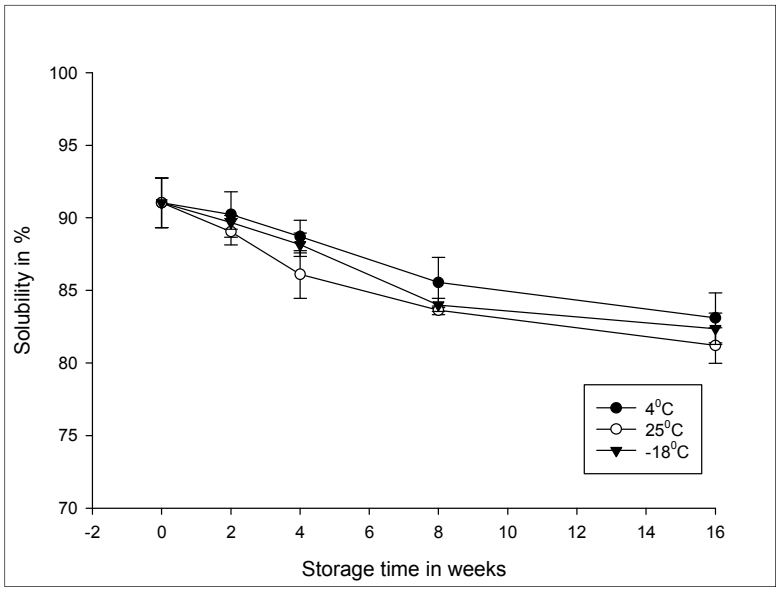


Figure 5. Solubility in % of a. Conjugated (WPH10-MD) encapsulated probiotic powder as compared to b. Non-conjugated WPH10 entrapped probiotic powder and during storage at -18, 4 and 25°C for 16 weeks. Error bars represent standard error of the means.

a.



b.

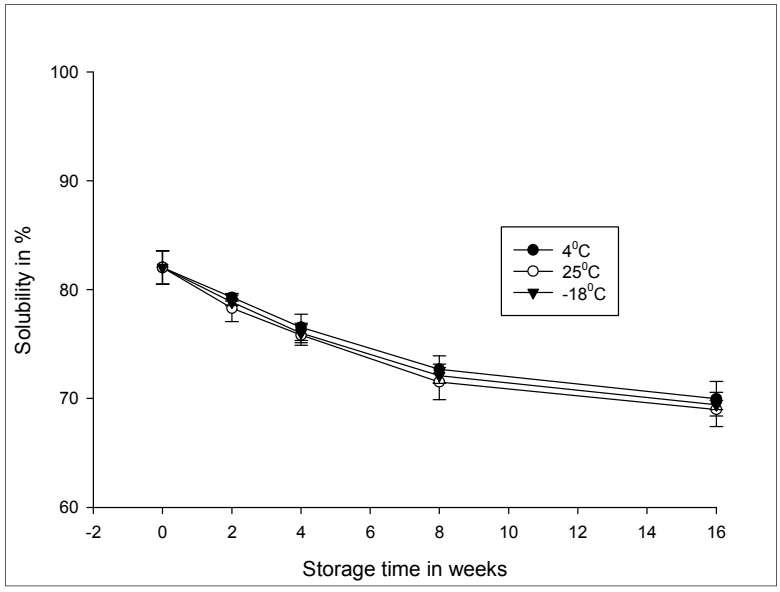
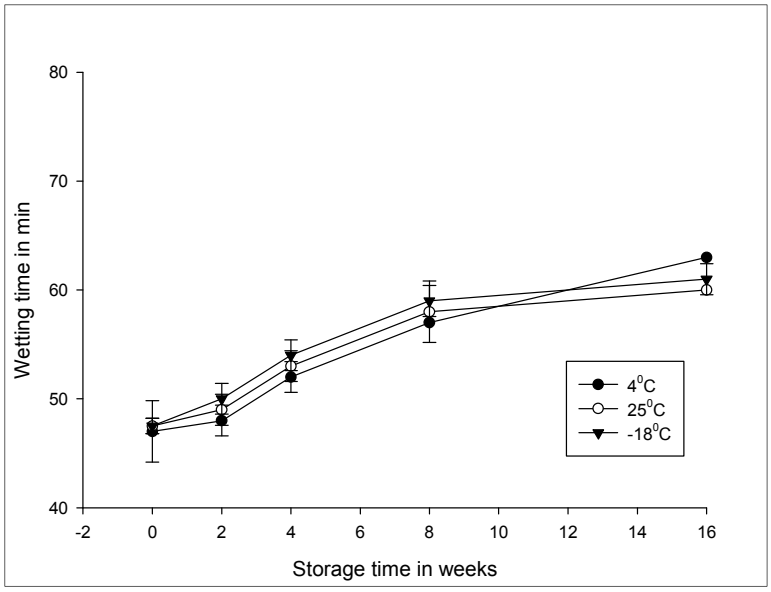
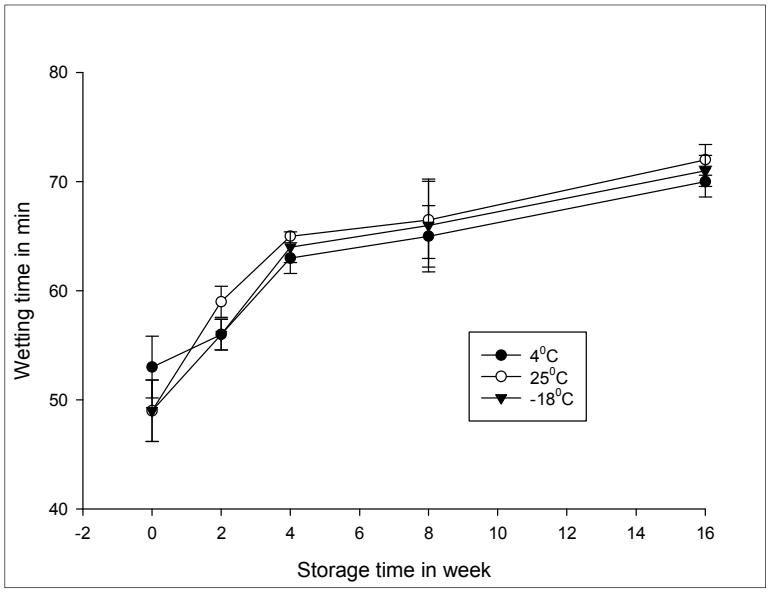


Figure 6. Wetting time in minutes of a. Conjugated (WPH10-MD) encapsulated probiotic powder as compared to b. Non-conjugated WPH10 entrapped probiotic powder and during storage at -18, 4 and 25°C for 16 weeks. Error bars represent standard error of the means.

a.



b.



CHAPTER 4
ASSESSING ENVIRONMENTAL PERSISTENCE OF *LISTERIA* USING WHOLE GENOME SEQUENCING

ABSTRACT

Listeria monocytogenes is a ubiquitous environmental bacterium and the causative agent of a serious foodborne illness, listeriosis. Despite improvements in hygiene protocols, some strains of *Listeria* can resist environmental stress conditions and can persist for long periods. This can pose a risk of product cross-contamination. Hence, to understand the persistent nature of the environmental *Listeria* isolates a genotypic evaluation is needed. In this study the genetic determinants of the *Listeria* isolates were determined using whole genome sequencing and associated with phenotypic responses. Following, the genetic variation among the isolates were identified that can play a role in influencing their persistent nature. *Six Listeria isolates* were obtained from the processing environment of a commercial dairy plant. The genomic DNA of the isolates was extracted using the Wizard Genomic DNA extraction kit. After the extraction, DNA libraries were created through PCR amplification. The DNA libraries were then run through whole-genome sequencing (WGS) on the Illumina MiSeq platform. Alternatively, WGS of the isolates was done using Nanopore sequencing for long reads of the DNA fragments through real-time sequencing using MinION. The genomes obtained were assembled using CLC Genomic workbench and the assembled genomes were fed into RAST database to develop the annotations. The results revealed at least 13 types of phenotypic responses related to the genotypic variants of the *Listeria* isolates. Among which the genetic determinants responsible for providing oxidative, osmotic, and antimicrobial resistances, acid tolerance, cell signaling, motility, and biofilm formation

could be associated with their resilient and sporadic nature of the *Listeria* isolates. When the isolates were compared for their genetic variation, it was observed that overall, the genetic determinants differed with the species and within the strains. It was found that Some of the common genes identified among species were virulence (DNase), oxidative (CadA) and osmotic stress (YRKL), cell signaling (NAG) and phage immunity (CRISPR). While *L. monocytogenes* was positive for all these phenotypic characteristics, which could be linked to their resistance to CIP conditions and its persistent nature, *L. innocua* and *L. welshimeri* isolates lacked variants for motility (ActA), biofilm formation (AgD), and acid tolerance (AdiA), making them less resilient to sanitization measures and environmental stress. Such information about the genes and their role in expressing phenotypic attributes can help create more robust interventions for the control of *Listeria* in a dairy plant environment.

INTRODUCTION

Listeria species are gram-positive, ubiquitous bacteria that can cause mild to highly fatal infection, listeriosis upon ingestion of foods contaminated with it (Melo et al., 2015). Among at least 10 types of *Listeria* species, *Listeria monocytogenes* is a major foodborne pathogen and a saprophyte, resilient to environmental stress conditions. It is considered an opportunistic pathogen that mainly affects people with underlying immune conditions such as pregnant women, elderly, and neonates. Although a wide variety of ready-to-eat foods can mediate listeriosis, dairy products are one of the common vehicles and approximately half of the outbreaks are linked to soft cheeses, milk, and other contaminated dairy products. The risk for *Listeria* outbreaks in soft-ripened cheeses is estimated to be 50-to-160- fold greater in cheeses made from unpasteurized/ raw milk

than cheeses produced from pasteurized milk (US FDA and Health Canada, 2015). Though pasteurization can eliminate *L. monocytogenes*, dairy products made from pasteurized milk can become contaminated due to inadequate hygiene practices after pasteurization. The first listeriosis outbreak in United States was in 1985 which was associated with Latin-style cheese (queso fresco and cotija cheeses) and was traced to have prepared from pasteurized milk (Ibarra-Sánchez et al., 2017). Recently in May 14'2021 CDC declared an outbreak of infections from *L. monocytogenes* that was traced to traced to Hispanic-style fresh and soft cheeses (Queso Fresco). There were 13 reported cases with 12 illnesses and 1 death (CDC, 2021).

Following it was reported that Cornell researchers have found five previously unknown and novel kinds of *Listeria* isolates, while examining the prevalence of *Listeria* in agricultural soil and water throughout the U.S which tells us the relevance of the different growth niches and how it plays an important role in survival of the *Listeria* organisms (Carlin et al., 2021). The ability of *L. monocytogenes* to survive for long periods of time in a food or dairy processing facility is due to its resistance to CIP conditions and other environmental stress conditions, and its ability to attach to food contact surfaces and form resilient biofilms (Cruz and Fletcher, 2011). *L. monocytogenes* ability to grow under varying pH levels (4.6-9.5), low water activity (0.92), and at low temperatures (0°C and below) make them favorable to grow on floors, drains and equipment surfaces particularly under cold and wet industry atmosphere of refrigerated rooms (Carpentier and Cerf, 2011). And specific strains of *L. monocytogenes* have been consistently getting identified over many years in food processing facilities, which shows their persistent behavior in food processing environment. This can pose a risk of product

cross-contamination, leading to possible outbreaks of listeriosis. Hence, identification of the genetic determinants associated with the phenotypic characteristics will help to understand the mechanisms utilized by the *Listeria* species to tolerate the stress conditions in food processing environments. It has been reported that resistance of *Listeria* species to different stresses is interconnected, for example osmotic stress response by *L. monocytogenes* can provide cross protection to other stress conditions including heat, oxidation, ethanol, and extreme pH (Melo et al., 2015). It is also found that stress response demonstrated by two component systems *liaRS*, *lisRK*, *cesRK*, *agrCA*, and *virRS* (Pontinen et al., 2017) converge the stress signal on the level of SigB, which is the alternative sigma factor σ^B responsible for general stress response in *L. monocytogenes* and other Gram-positive microorganisms (Abram et al., 2008). SigB is shown to provide resistance to *L. monocytogenes* during oxidative stress (Chaturongakul and Boor, 2006), cold and freezing stress (Becker et al., 2000), osmotic stress (Fraser et al., 2003), and high acid and pressure stress (Wemekamp-Kamphuis et al., 2004). The persistence of *Listeria* is also associated with its ability to form resilient biofilms which is supported by the presence of *comK* gene (Cherifi et al., 2018).

For these reasons, most researchers have used genotypic evaluation through whole genome sequencing (WGS) methods to identify and distinguish persistent *Listeria* strains which are present in the food processing environment for a long time, from the sporadic ones. Being rapid, sensitive, and accurate method, it is nowadays widely being used in investigating outbreaks, surveillance programs, and epidemiological studies (Gilchrist et al., 2015). Hence, to understand the persistent nature of the environmental *Listeria* isolates in dairy processing environment, a genotypic evaluation is needed. The

whole genome sequencing, in addition to culturing techniques, of resident isolates would provide in depth knowledge of the genetic sequences of the *Listeria* isolates, which would thus help to understand the persistent behavior of 'resident *Listeria*' to the surfaces in a dairy manufacturing environment.

This study involves investigating the genetic determinants and associated phenotypic characteristics of 10 *Listeria* isolates obtained *from the processing environment of a commercial dairy plant.* All the 10 isolates were screened for the genes encoding resistance to environmental stress conditions and their persistent behavior in a dairy manufacturing environment. Hence, the first objective was to identify the genetic determinants of the *Listeria* isolates and associated with phenotypic responses and second to determine the genetic variation among the isolates that can influence their persistent nature.

MATERIAL AND METHODS

Sourcing of materials

The following materials were procured for the DNA extraction: DNA extraction kit (Wizard® Genomic DNA Purification Kit #A1120) from Promega, Madison, WI, United States; Lysozyme (#L4919) from Sigma-Aldrich, St. Louis, MO, United States; 0.5M EDTA pH 8 (#PR-V4231) from Thermo Fisher Scientific, Waltham, MA, United States.

Selection of *Listeria* Isolates

Four isolates of Listeria were procured from ATCC: *Listeria welshimeri* Rocourt and Grimont ATCC 35897; *Listeria innocua* Seeliger ATCC BAA 680; *Listeria innocua* Seeliger ATCC 33090; *Listeria monocytogenes* Pirie ATCC 51414. Six industrial isolates

were procured that were previously isolated from the floors and drains of a dairy plant environment: 3 isolates of *Listeria innocua* 634-2; *Listeria innocua* 634-34-S-5*; Listeria innocua* 634-34-S-6*;* 2 isolates of *Listeria welshimeri* 634-3; *Listeria welshimeri* 634-253-S-5; and 1 isolate of *Listeria monocytogenes* 315-S-1.

All the isolates were activated by transferring two beads from the stock culture in Brain Heart Infusion, BHI (Oxoid, Thermo Scientific, UK) at 37°C for overnight. The activated culture was sub- cultured by transferring 1mL of the first activated culture to fresh BHI broth tubes. The tubes were then incubated at 37°C for overnight.

Whole-genome sequence analysis

For DNA isolation, activated *Listeria* cultures were centrifuged at 16,000×g for 2min to extract the cell pellet. The supernatant was removed, and the cell pellet was dissolved in 480µL of 50mM of EDTA (Ethylenediaminetetraacetic acid) (pH 8; Thermo Fisher Scientific, United States). Next, 60 µL of Lysozyme was added (10mg/mL, Sigma-Aldrich, United States) and the mixture was incubated at 37C for 60 min. Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, United States). The concentrations of DNA extracted were measured using Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, United States). The DNA concentrations were adjusted to 0.3 ng/µl and the DNA libraries were created with the Illumina Nextera XT or Nextera Flex Library Preparation protocol (Illumina, San Diego, CA, United States) as mentioned by the manufacturer. Following, the whole genome sequencing of the DNA libraries was performed in the Illumina MiSeq platform (Illumina, San Diego, CA, United States) using MiSeq Reagents Kits V2 chemistry with 2 X 250 paired-end chemistry (500 cycles). The quality of the raw reads was analyzed

through parameters such as cluster density (1200-1400 k/mm2) and percentage of cluster passing filters (>80%). Quality control, filtering, and trimming of raw reads was carried out using CLC Genomics Workbench vs 12.0.3 (Qiagen, Arhaus, Denmark). Assemblies were constructed using CLC Genomics Workbench vs 12.0.3 with a length fraction of 0.5, a similarity fraction of 0.8 and a minimum contig length of 3,000 bp. The assemblies were then annotated in RAST (Rapid Annotations using Subsystems Technology) Server. The subsystem produced a *subsystem spreadsheet* where the functional roles are connected to specific genes in specific genomes. The genes responsible for persistent behavior were identified here using WGS data of these isolates. In addition, the presence or absence of resistant genes, virulence factors, and biofilm associated genes were identified through BLAST-based approach (Lobo, 2008).

Alternatively, nanopore sequencing of the *Listeria* isolates were done for long reads of the DNA fragments through real-time sequencing using MinION (Oxford Nanopore Technologies, Oxford, UK) (Quick et al., 2015). 1ng of high-molecular weight input DNA was disintegrated through centrifugation at 5000 rpm using a Covaris G-tube (Covaris, Woburn, United States). The fragmented DNA was then repaired using NEB repair module (New England Biolabs, Ipswich, United States) and cleaned by adding SPRI beads in a ratio of 1:1 (beads to reaction mixture). End-repaired DNA was then A-tailored using the NEB A-tailing module. Subsequently, a sequence ready library was created using the gDNA sequencing kit according to the protocol provided by the MinION access program (MAP). Following, through the sample loading port, 150µL of the diluted library was loaded into the MinION flow cell, and sequencing was initiated for 72 h using the MinION control software, MinKNOW (version 0.45.2.6). The software

Guppy vs 4.4.2 was used for base calling on a GPU server. Assemblies were constructed using Flye vs 2.8.3 and polished with one round of Nanopolish vs 0.13.2 and another round of Racon vs 1.4.3. The resulting polished assemblies were annotated as described above using RAST.

Multi locus sequence typing of *Listeria* isolates using whole genome sequencing (WGS)

BIGSdb-Lm platform (https://bigsdb.pasteur.fr/listeria/listeria.html) was used in extracting the multi locus sequence typing (MLST) profiles of the *Listeria* isolates from the assemblies (Moura et al., 2017). The FASTA files were uploaded and allelic matches of whole genome assemblies were identified for all loci (https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_listeria_seqdef&page=sequenceQuery).

RESULT AND DISCUSSION

Genetic components and associated phenotypic responses in the *Listeria* isolates

Along the dairy processing environment, specifically during cleaning and sanitation, *Listeria* organisms encounter a wide range of stress factors, which affect their viability and activity. However, some *Listeria* species can survive or even replicate under such environmental stress conditions which lead to adhesion to food contact surfaces, colonization, and persistence of *Listeria* in various niches along the food chain (Bolocan et al., 2016). This allows to form potential reservoirs for product cross-contamination, and ultimately contributes to human infection (Sleator et al., 2009). Most research have explained the expression of different genetic factors to be involved during exposure of *Listeria* species to the environmental stress conditions. Adaptations of *Listeria* species to environmental stress conditions and antimicrobial agents in sub-lethal concentrations

have subsequent effect on antimicrobial resistance of *Listeria* organisms. Hence, an understanding of the principal mechanisms related to *Listeria* survival, antimicrobial resistance, virulence, and their persistence in extreme environmental conditions is important for better management and control of this pathogen, as well as for development of novel sanitation and antimicrobial agents. In this study, the results from whole genome sequencing (WGS) using MiSeq and Nanopore sequencing of the *Listeria* isolates revealed genotypic variants that could be associated with at least 13 types of phenotypic responses (Pathogenicity, biofilm formation, virulence, extreme pH tolerance, osmotic shock resistance, oxidative stress resistance, lysozyme resistance, heat shock resistance, cell signaling and extreme environment stress resistance, motility and attachment, antibiotics and toxic compounds resistance, immunity against bacteriophages, and sugar/carbohydrate metabolism (Figure 1). Among which the genetic determinants responsible for providing oxidative, osmotic, and antimicrobial resistances, acid tolerance, cell signaling, motility, and biofilm formation could be associated with their resilient and sporadic nature of the *Listeria* isolates.

The genetic determinants (putative arginine-ornithine antiporter gene *Arc*D, phospholipase C, glycoprotein *gp*160, and thiol-activated cytolysin) could be associated with playing a role in pathogenicity of the *Listeria* isolates. And some of the virulent genetic factors that were identified in the *Listeria* species were Putative DNase, protein acetyltransferase, phage terminase, *tRNA-Arg-ACG*, phage portal protein and *ECF* transporter. Arginine deiminase pathway (ADI) through metabolism of arginine is one of the common pathways utilized by microorganisms to resist or adapt to hostile environmental niches and host defenses (Xiong et al., 2014). It is a well-established

multi-enzyme pathway which plays a role in pathogenesis and is encoded by arc operon genes such as *arcA*, *arcB*, and *arcC* which hydrolyze arginine to orthinine producing ammonia, CO_2, and ATP (Fulde et al., 2014). Phospholipase C encoded by genes *plcC* or *plA* is an essential pathogenicity factor expressed by *L. monocytogenes* to cause murine cerebral listeriosis in host cells, and to protect itself from host cell immunity, it regulates its expression of genes for efficient pathogenicity (Marquis and Hager, 2000). It has been reported that the 107-residue polypeptide in *L. monocytogenes* encoded by *orfX* gene is like the viral protein (46% similarity in an overlap of 99 amino acids with the N-terminal end of glycoprotein gp160 of HIV type I). Hence, the presence of glycoprotein gp160 and indicating similar sequences suggest it to be remnants of a transducing bacteriophage (Boland et al., 2001). tRNA genes are usually found in bacterial genomes at the insertion sites of the pathogenicity islands (PAIs). Previously, a tRNA gene was found in *L. ivanovii* at the insertion site of virulence gene cluster, carrying many internalin genes which are one of the important virulence factors for host cell invasion (González-Zorn et al., 2000).

One of the defense mechanisms of *Listeria* species against disinfectants and antimicrobials is to form resilient biofilms. The presence of *Listeria* biofilms on food contact material surfaces can serve as a potential contamination in food processing pipeline. Genes that could be correlated with biofilm formation were agmatine deiminase *AgD*, *RmIA* (glucose 1 phosphate thymidylyltransferase), *RmID* (dTDP-4-dehydrorhamnose 3.5-epimerase), *uhpT* (dTDP- glucose 4,6-dehydratase), *RfbD* (dTDP-4-dehydrorhamnose reductase), and virulence positive regulatory factor *PrfA*. For motility and helping the *Listeria* isolates in attachment to surfaces, genes that could be

identified were *ActA* (actin-assembly inducing protein *ActA* precursor), and retron type RNA-directed DNA polymerase. In a study by Sudagidan et al., 2021, PCR results from all the *Listeria monocytogenes* strains isolated from smoked salmon and fish food contact surfaces were positive for biofilm-associated genetic components *hylA*, *iap*, *actA*, *plcA*, *plcB*, and *inlA-inlB-inlC-inlJ*. It has also been reported that presence of gene *prfA* has been associated with virulence. In addition, enzymes such as AgDs (agmatine deiminases), which catalyze the deamination of agmatine to N-carbamoyl putrescine and ammonia, are present in pathogenic bacteria such as *Streptococcus mutans, Pseudomonas aeruginosa, Listeria monocytogenes* and play a role in biofilm formation and mediate acid resistance (Williams et al., 2010).

In general, *Listeria* species cannot tolerate high acidic conditions and is not able to grow at a pH below 4.5 to 4.6 (McClure et al., 1989). However, the organisms can become highly tolerant to extreme acidic conditions because of stress hardening i.e., increased tolerance after adaptation to stressful environmental conditions. The ability of *L. monocytogenes* to modify its cellular physiology and become more resistant to stress conditions may counteract the effectiveness of acidification for food preservation or use of acids for cleaning and sanitation purposes. Under acid stress conditions, *Listeria* species can maintain its internal pH (pHi) through several mechanisms including the F_0F_1-ATPase; the arginine and agmatine deiminases (ADI and AgDI); the glutamatic acid decarboxylase (GAD) (Feehily et al., 2014; Lund et al., 2014). In our study, release of enzymes (carbamate kinase, arginine decarboxylase encoded by *AdiA*, and phospholipase C) could be associated with response acidic stress resistance of *Listeria* isolates. It has been reported that ADI and AgDI systems are involved in the response of *L.*

monocytogenes to extreme acidic conditions (Soares and Knuckley, 2016). ADI helps in importing arginine molecules to ornithine, CO_2, ammonia (NH_3), and ATP from the extracellular environment, in which the NH_3 is further protonated to NH_4, thereby increasing the internal pH (pHi) (Cotter and Hill, 2003). Similarly, in AgDI system, the agmatine is converted to putrescine and NH_3 to increase the internal cellular pH (Chen et al., 2011).

Under oxidative stress, high concentrations of oxygen radicals are generated which causes damage to the cell proteins, lipids, and nucleic acids and disturbs the normal redox state of cells, thereby leading to cell death. In bacterial cells, ROS (reactive oxygen species) containing oxygen on different redox states such as hydrogen peroxide, hydroxyl or peroxyl radical helps in activating enzymes such as superoxide dismutase, catalases, peroxides, and efflux pumps in response to the oxidative stress resistance (Archambaud et al., 2006). In our study, expression of enzymes such as transketolase encoded by *tkt*, flavodoxin 2, cadmium efflux system, and NAD(P)H oxidoreductase *YRKL* could be related to the oxidative stress resistance of the *Listeria* isolates. Oxidative stress resistance of *Listeria monocytogenes* was correlated with biofilm formation where four kinds of genes (*kat*, *perR*, *sigB*, and *recA*) were upregulated in Δ*sod* mutant of *L. monocytogenes* (Suo et al., 2014).

In foods, salts and sugars are added to improve the sensory properties and to increase the shelf life of the food. They are generally added at increased concentration to create an osmotic stress condition (Burgess et al., 2016), thereby challenging the bacterial cells for osmotic balance between the cytoplasm and extracellular environment (Bae et al., 2012). Hence, to combat the osmotic pressure and water loss, *Listeria* species can

accumulate osmolytes, also known as compatible solutes, such as cartinine, and glycine betaine in their cytoplasm. In response to which, there are certain expression of genes encoding for proteins involved in the transport of osmolytes (Bae et al., 2012). From our findings, the proteins, lysin decarboxylase encoded by *CadA*, teichoic acid biosynthesis protein, and glycine betaine encoded by gbuABC could be identified in the *Listeria* isolates. It has been reported that the main transport system encoded by opuCABCD operon, the glycine betaine porter II system encoded by gbuABC, and the sodium-motive force-dependent glycine betaine uptake system, encoded by betL (Chan et al., 2007).

Thermal treatments are one of the common strategies that are applied in most of the steps of dairy and food processing to eliminate or prevent outgrowth of food-borne pathogens. However, eliminating *Listeria* species completely becomes difficult due to their ability to survive and replicate at wide range of temperature (-0.4°C to 45°C) (Chan et al., 2007). Although, *Listeria* species are not able to manifest an extraordinary resistance to high temperatures, their ability to tolerate heat and of sub lethally injured cells to recover and grow during post-processing conditions creates a concern (Mackey et al., 1994; Sallami et al., 2006). Reports have shown expression of genes in *Listeria* that belong to specific SigB-dependent class II stress genes, and class I (*grpE*, *dnaK*, *dnaJ*, *groEL*, and *groES*) and class III (*ClpC*, *ClpP*, and *ClpE*) heat shock genes. From our findings, we could identify class III heat shock resistance genes that are responsible for encoding ATP-dependent proteases *ClpB*, and *ClpC*. These proteases are regulated by product of first gene of *clpC* operon i.e., repressor CtsR (Nair et al., 2000). Recently, it was observed that *clpL* protease played a considerable role in promoting heat tolerance to

L. monocytogenes AT3E. Further, inserting the *clpL* gene into *L. monocytogenes* sensitive strain increased its heat resistance characteristic (Pöntinen et al., 2017).

Survival of *L. monocytogenes* in environmental stress conditions is a complex process and involves various expression of genetic components through several mechanisms. Genetic determinants that could be associated with antimicrobials, lysozyme, antibiotics and other toxic compounds resistance were peptidoglycan N acetyl glucosamine deacetylase, bile acid 7-alpha dehydratase encoded by gene *BaiE*, cholylglycine hydrolase encoded by gene AFT97_06335, L-alanyl-gamma-D-glutamyl-L-diamino acid endopeptidase, cadmium resistance protein encoded by *cadA* genes, efflux pump *Lde*, metallo-beta-lactamase superfamily domain protein in prophage, and negative transcriptional regulator-copper transport operon gene. Efflux pumps encoded by *cadA* genes (*cadA1C1*, *cadA2C2*, *cadA3C3*, and *cadA4C4*) are some of the mechanisms for cadmium resistance in *Listeria* have been reported (Parsons et al., 2020). It has been observed that cadmium resistance in *L. monocytogenes* is mainly through horizontal gene transfer whereas arsenic resistance genes are encoded in chromosomes in *arsR1D2R2A2B1B2* which is carried together with gene *cadA4C4* on Listeria Genomic Island 2 (LGI2) (Parsons et al., 2020). Carnocyclin A (CCLA), produced by *Carnobacterium maltaromaticum* ATCC PTA-5313 is an antimicrobial peptide used mainly in ready to eat meat products to control the growth of *L. monocytogenes*. In response to it, cells of *Listeria* can express genes (*baiE*, *trn*, and *pykA*) to resist the exposure to CCLA and maintaining its metabolic functions. Recently in Abril et al., 2021, expression of metallo-beta-lactamases, MarR protein, M56 peptidases, and penicillin-binding proteins in *Listeria* isolates were related to be involved in response to

penicillin resistance. Role of cholylglycine hydrolase (Bile salt hydrolase; BSH) encoded by *bsh* gene has been associated with resistance to bile in all pathogenic *Listeria* species. The *bsh* gene in *L. monocytogenes* is known to be regulated by *PrfA* (transcriptional activator of virulence genes) (Dussurget et al., 2002).

Genetic components (phage terminase, N-acetyl glucosamine-specific IIA, N-acetyl glucosamine-specific IIB, N-acetyl glucosamine-specific IIC, hypothetical protein, and chromosome initiation inhibitor genes) could be related to cell signaling and providing resistance in extreme environmental stress conditions. It has been reported that NAG-A (N-acetyl glucosamine-IIA) in *Listeria* species plays a role in the deacetylation of N-acetyl-glucosamine-6-phosphate to glucosamine-6-phosphate and acetate. Presence of NAG-A domain in *L. monocytogenes* revealed two kinds of cytoplasmic putative proteins (*Lmo0956* and *Lmo2108*). These genes are necessary for the cytosolic steps of the amino-sugar-recycling pathway, and it has been observed that in *L. monocytogenes*, the cytoplasmic de-*N*-acetylase *Lmo0956* gene plays a role in cell wall peptidoglycan and teichoic acid biosynthesis. NAG is also reported to be essential for bacterial cell growth, cell division, sensitivity to cell wall hydrolases, and peptide antibiotics in *Listeria* (Popowska et al., 2011).

For sugar/carbohydrate metabolism in *Listeria* isolates, genetic components that could be associated are xylose isomerase, xyloside transporter, *XynT*, and xylulose kinase. The presence of xylose operon in *Listeria* species is required for sucrose synthesis and fermentation of D-xylose and α-methyl-D-mannoside to produce acid is one of the important biochemical properties in *Listeria*. It has been reported that xyloside

transporter genes such as *XynT* is involved in carbohydrate transport and metabolism in bacteria (Fieseler et al., 2012).

CRISPR-Cas systems are mostly present in bacteria to provide an adaptive immunity which protects them from bacteriophages and invading plasmids. Its function is controlled by CRISPR associated genes (*cas*) that encode a large family of proteins identical to 20-40 nucleotides long repeat sequences with nuclease or helicase activities and DNA and RNA binding domains. Cas genes can encode diverse proteins including helicase, nuclease, polymerase, or nucleotide binding activities, which are utilized for generating, maintaining, and processing CRISPR system. In our study, CRISPR-associated proteins (Cas1, Cas2, Cas3, Csn2), DNA primase/helicase, and phage terminase, were identified that could be associated in providing protection to the *Listeria* isolates against invading bacteriophages. Kuenne et al., 2013, identified CRISPR-Cas systems in *Listeria* where a small CRISPR RNA (*RliB*) was identified in *L. monocytogenes* strain EGD-e that contained five identical sequences interspaced by non-related spacer sequences of similar size.

Use of third generation (i.e., single-molecule) sequencing approaches including Nanopore sequencing (Oxford Nanopore Technologies, ONT) have the potential to revolutionize bacterial genome sequencing by producing long genome-reads (Pennisi, 2012). Besides, it has an advantage of being portable thereby providing sequence data in real time and enabling sequencing as a point-of-care test. In this study, from the Nanopore sequencing findings, several additional genetic determinants were found in the *Listeria* isolates, which were not previously identified through MiSeq sequencing (shown in Table 1). The genetic components were oligopeptide-binding protein *OppA*, ABC

transporter, glutamyl endopeptidase precursor (*blaSE*), phosphoenolpyruvate synthase, internalin like protein *Lmo2445*, pyrroline-5-carboxylate reductase, and ProG-like which was associated with virulence.

Proteins such as adenosyl nucleosidases, teichoic acid biosynthesis protein *GgaA*, permease protein *OpuBB*, LSU ribosomal protein *L34p*, RNA binding protein *Hfq*, L-alanyl-gamma-D-glutamyl-L-diamino acid endopeptidase, efflux pump *Lde*, and copper resistance protein D were found that could be associated with antibiotic and toxic compounds resistance in *Listeria* species.

Other components like cytoplasmic axial filament protein *CafA* was associated with motility and cell attachment in *Listeria* species, and L-proline glycine betaine ABC transport system permease protein *ProW*, *ADA* regulatory protein, and teichoic acid biosynthesis protein could be associated osmotic shock resistance.

Comparison of the *Listeria* isolates for their genetic variation

In all the *Listeria* species (pathogenic and non-pathogenic), many predicted genetic components including surface and secreted proteins, transcriptional regulators, and transporters were found which tells us their ability to survive in extreme and diverse environmental conditions. When the isolates were compared for their genetic variation, it was observed that overall, the genetic determinants differed with the species and within the strains (shown in Figure 1). And comparing genomic analyses *L. monocytogenes* with *L. innocua* and *L. welshimeri* also allowed us to recognize different classes of lost or deleted genes that in *L. innocua* and *L. welshimeri*.

It was found that some of the common genes identified among species were virulence (DNase), oxidative (*CadA*) and osmotic stress (*YRKL*), cell signaling (*NAG*)

and phage immunity (CRISPR). While *L. monocytogenes* contained most of the genetic components and was positive for all these phenotypic characteristics, which could be linked to their resistance to cleaning and sanitation protocols, and its persistent nature, *L. innocua* and *L. welshimeri* isolates lacked variants for motility (*ActA*), biofilm formation (*AgD*), and acid tolerance (*AdiA*), making them less resilient to sanitization measures and environmental stress. Most of the genes that were found in *L. monocytogenes* whereas absent in *L. welshimeri* and *L. innocua* indicates the possibility for gene loss or genes acquired by horizontal gene transfer (HGT) in *L. monocytogenes*. Specially, the presence of genes representing cluster regions of prophage, monocin, and transposon in *L. monocytogenes* could be a result of the horizontal gene transfer.

The genomic sequence in *L. monocytogenes* industrial isolate 315-S-1indicated an abundance of surface and transport proteins. Major surface proteins that were observed was *ActA*, playing a role in actin-based motility and virulence factor *PrfA*. Previously, an extensive analysis of *L. monocytogenes* EGDe genome was published showing 133 surface proteins, among which the largest proteins belonged to lipoproteins, followed by LPXTG motif. These proteins act as adhesins or invasins, promoting bacterial entry into eukaryotic cells, and contribute about one-third of the total proteins in pathogenic *Listeria* species. LPXTG motif proteins (*ActA* and *LmiA*) lacked in *L. innocua* and *L. welshimeri* industrial strains. Similar results were reported by Mariscotti et al., 2014 where two LPXTG proteins (Lmo1413 and Lmo2085) were found absent in non-pathogenic *Listeria* species. However, it was observed that the absent of these two proteins did not affect the cell invasion in *in vitro* infection models.

Similarly, biofilm formation, acid, and heat stress response genes (*AgD*, *AdiA*) were found to be absent in *L. welshimeri* and *L. innocua* industrial strains. For ADI pathway, Lmo0038 has been shown to be associated containing domains like peptidylarginine deiminase family (Gruening et al., 2006). Lmo0038 is previously reported to be found in pathogenic *Listeria* and plays a role in listerial pathogenesis. While a study by Chen et al., 2009, showed Lmo0038 to be conserved in *L. monocytogenes* and *L. ivanovii,* it was found to be absent in non-pathogenic *Listeria* strains (*L. welshimeri*, *L. innocua*, *L. grayi*, and *L. seeligeri*).

Some of the genetic components that were found to be common among the non-pathogenic industrial strains of *Listeria* (*L. welshimeri* and *L. innocua*) were tRNA-Arg-ACG, phage portal protein associated with virulence of the strains, teichoic acid biosynthesis protein associated with biofilm formation and osmotic shock resistance, and DNA primase/helicase associated with bacteriophage immunity. *L. innocua* and *L. welshimeri* are commonly found in food processing environments and may contribute to the dissemination of disinfectant resistance genes in listeriae, including *L. monocytogenes*.

Because of the presence of several common genetic components, *L. innocua* strains could be highly related to *L. monocytogenes*, whereas *L. welshimeri* was more distant. Biochemical properties such as acid production through fermentation of D-xylose and α-methyl-D-mannoside is used to differentiate *L. welshimeri* from other *Listeria* species. Some of the major virulence determinants in (phosphoribosyl transferase encoded by prs, ABC transporter, virulence regulatory factor PrfA) are found in *Listeria* pathogenicity island 1 or on chromosomal locus (Chakraborty et al., 2000). These factors

were present in *L. monocytogenes* and were missing in *L. welshimeri* and suggests the emergence of the species from gene loss and acquisition of novel plasmid genes from horizontal gene transfer.

Similarly, cadmium resistance genes (*cadA*) and ABC transporter was only observed in two strains of *L. innocua,* 1 strain of *L. welshimeri*, and *L. monocytogenes* strain. Resistance to quaternary ammonium disinfectant benzalkonium chloride (BC) using a plasmid borne disinfectant resistance cassette (*bcrABC*) is an important contributor to the ability of *Listeria* species to persist in the dairy and food processing plant environment and has been previously identified in *L. monocytogenes*. And, in a study by Katharios-Lanwermeyer et al., 2012, it was observed that the non-pathogenic species of *Listeria*, particularly *L. welshimeri* and *L. innocua* were able to horizontally transfer BC and cadmium resistance determinants to *L. monocytogenes* and other non-pathogenic listeriae. The results suggest that nonpathogenic *Listeria* species may behave as a reservoir for heavy metal and disinfectant resistant genes for other listeriae.

A major comparison of *L. welshimeri* with *L. innocua* and *L. monocytogenes* displayed the absence of three individual genes involved in the cell signaling, transport and metabolism of carbohydrates and amino acids. These genes encode for proteins of PTS (Phosphotransferase system)- N-acetylglucosamine-specific IIA, IIB, and IIC components. Previously, β-glucoside specific PTS system has been shown to mediate the repression of virulence genes in *L. monocytogenes* (Brehm et al., 1999). In addition, the presence of xylose utilization genes (*xynT*) was observed only in *L. welshimeri* industrial strains, which signifies a well-known characteristic phenotype of *L. welshimeri* and helps to differentiate them from other *Listeria* species. In *L. welshimeri* the xylose operon

consists of five genes, glycoside hydrolase (lwe0241) encoding for a putative α-xylosidase, xylose isomerase gene *xylA*, xylose proton symporter gene *xylP*, xylulose kinase gene *xylB* which is controlled by *xylR* protein. Hence, the presence of such uptake and utilization systems suggests *L. welshimeri* to have adapted a saprophytic strategy to survive in its natural environment (Hain et al., 2020).

When the industrial *Listeria* strains were compared with the control ATCC *Listeria* strains, some genetic determinants contributing to *Listeria* resistance in harsh conditions in the control isolates were missing. Comparing *L. welshimeri* industrial strain 634-3 with *L. welshimeri* ATCC 35897 revealed the presence of PTS system with β-glucoside-specific IIA, IIB, and IIC components in addition to PTS system with NAG-specific components in ATCC isolate. The *bvrABC* locus encoding a β-glucoside-specific PTS system has been previously shown to mediate virulence gene repression by β-glucosides in *L. monocytogenes* (Brehm et al., 1999). Whereas, genes encoding for arginine deiminase pathway (*ArcD*), ECF transporter, CRISPR-associated proteins (*Cas*), and cadmium resistance determinants (*Cad*) were absent in the ATCC isolate. These genetic components are mainly associated with resistance of *L. welshimeri* to extreme environmental conditions such as osmotic shock resistance, acid resistance, heavy metal resistance and bacteriophage immunity.

When *L. innocua* 634-2 was compared with *L. innocua* ATCC 33090 and *L. innocua* ATCC BAA 680, it was found that both the ATCC isolates contained surface proteins such as internalin like proteins which was absent in the industrial strain. These internalin like proteins acts as virulence factors and plays a crucial role in enhancing the pathogenicity of the *Listeria* species. In *L. innocua* 634-2, Phd-Doc toxin-antitoxin

systems were identified which was not found in the ATCC strains. Toxin-antitoxin (TA) systems were first discovered on low-copy number plasmids which consist of two genes encoding a toxin and an antitoxin. TA systems have also been associated with bacterial survival to antibiotic treatments by producing persister cells (small fraction of cells resistant to stress conditions) (Lewis, 2010). Other genes encoding for protein acetyltransferase, cadmium resistance, and copper homeostasis which confer to antibiotic and toxic elements resistance, were absent in the ATCC isolates.

Lastly, when industrial strain of *L. monocytogenes* 315-S-1 was compared to *L. monocytogenes* ATCC 51414, it was observed that both the strains had similar genetic components. Hence, no major difference in the genomes was observed, except for the absence of PTS system with β-glucosidase specific component in the industrial strain and ABC transporter and genes encoding for dTDP (deoxythymidine diphosphate) (rhamnose containing glycans) in the ATCC strain. Genes encoding for dTDP and ABC transporter protein OppA (ATP binding cassette transporters) have been associated with aggregation and forming biofilms in food processing environments (Zhu et al., 2008).

Multi locus sequence typing of *Listeria* isolates using whole genome sequencing (WGS)

The results of MLST analysis (containing sequence types, clonal complex, and lineage distribution) are summarized in Table 2. Among 6 industrial *Listeria* strains, 4 different sequence types (ST) could be detected using MLST. *L. monocytogenes* 315-S-1 belonged to ST-5 and lineage I with clonal complex (CC) representing CC5. In a recent study by Kurpas et al., 2020, 22.9% of the *L. monocytogenes* strains (PCR-serogroup II-b) recovered from food and food processing environment belonged to multi-locus

sequence type ST-5 and clonal complexes CC5. The strains were associated with benzalkonium chloride tolerance and identified with antimicrobial resistance genes. Both the *L. welshimeri* industrial strains 634-3 and 634-253-S5 belonged to sequence type ST-2688 and lineage *L. welshimeri*, representing CC2688. Among the three *L. innocua* industrial isolates, allelic profile of *L. innocua* 634-34-S6 could be detected which identified the strain to belong to sequence type ST-1489 and lineage *L. innocua*, with CC1489. In addition, allelic profiles of *L. welshimeri* ATCC 35897 and *L. innocua* ATCC 33090 could also be detected. *L. welshimeri* ATCC 35897 was identified as belonging to sequence type ST-129 and lineage *L. welshimeri*, representing CC129 whereas, *L. innocua* ATCC 33090 belonged to ST-139 and lineage *L. innocua* with CC139.

CONCLUSIONS

In conclusion, whole genome sequencing of the *Listeria* isolates identified genetic determinants that were correlated to at least 13 types of phenotypic characteristics. Some of the common variants and features identified in the isolates were virulence (DNase, phage terminase, tRNA-Arg-ACG), cell signaling (*NAG-IIA, NAG-IIB*), phage immunity (*Cas*), osmotic stress (*CadA*), oxidative stress (*YRKL*), and antibiotic resistance (*BaiE, Lde*). While *L. monocytogenes* was positive for all these phenotypic characteristics, *L. innocua* and *L. welshimeri* isolates lacked variants for motility (*ActA*), biofilm formation (*AgD*), and acid tolerance (*AdiA*), making them less resilient to environmental stress. Among which oxidative, osmotic, and antimicrobial resistances, acid tolerance, cell signaling, motility, and biofilm formation could be related with their resilient and sporadic nature of the *Listeria* isolates. Overall, this study provided an insight into their

persistent nature which is significant from a product cross-contamination and food safety perspective and such information about the genes and their role in expressing phenotypic attributes can help create more effective controlled strategy for the control of *Listeria* in a dairy processing environment.

CHAPTER 4 TABLES AND FIGURES

Table 1. Additional genetic components determined through Nanopore sequencing of the *Listeria* isolates

***Listeria* isolates**	**Phenotypic characteristics**	**Genetic components**
L. innocua 634-2	Virulence	Oligopeptide binding protein *OppA* ABC transporter
L. innocua 634-34-S5	Virulence	Oligopeptide binding protein *OppA* ABC transporter
	Antibiotic and toxic compounds resistance	Adenosyl nucleosides
L. innocua 634-34-S6	Osmotic shock resistance	Teichoic acid biosynthesis protein
	Bacteriophage immunity	Bipolar DNA helicase *HerA*
L. welshimeri 634-3	Virulence	Glutamyl endopeptidase precursor *blaSE*
	Osmotic shock resistance	Teichoic acid biosynthesis protein
	Cell signaling	NADPH: quinone oxidoreductase
L. welshimeri 634-253-S5	Virulence	Glutamyl endopeptidase precursor *blaSE*
	Antibiotic and toxic compounds resistance	Teichoic acid biosynthesis protein *GgA* Permease protein *OpuBB*

		LSU ribosomal protein *L34p*
	Osmotic shock resistance	Teichoic acid biosynthesis protein *GgA*
	Bacteriophage immunity	CRISPR protein *Cas6*
	Cell signaling	PTS system: N-acetylglucosamine specific IIA, IIB and IIC components
	Carbohydrate metabolism	Xyloside transporter *XynT*
L. monocytogenes 315-S1	Virulence	Oligopeptide binding protein *OppA*
	Antibiotic and toxic compounds resistance	Oligopeptide ABC transporter
	Bacteriophage immunity	Bipolar DNA helicase *HerA*
	Acid shock resistance	ADA regulatory protein
L. innocua ATCC BAA 680	Osmotic shock resistance	Teichoic acid biosynthesis protein *GgA*
	Cell signaling	PTS system: N-acetylglucosamine diacetylase
L. innocua ATCC 33090	Virulence	SSU ribosomal proteins (S14p and S6p) Internalin like protein *Lmo2445*
	Acid shock resistance	ABC transport system protein *ProW*
L. welshimeri ATCC 35897	Cell signaling	PTS system: N-beta-glucoside specific IIA, IIB and IIC components

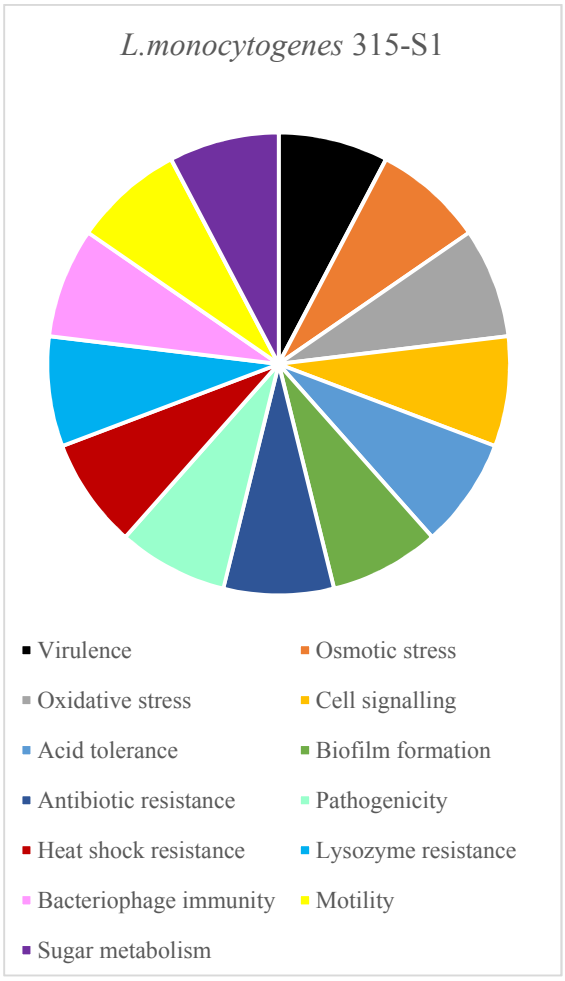
	Antibiotic and toxic compounds resistance	Copper resistance protein D Efflux pump *Lde*
	Carbohydrate metabolism	Xyloside transporter *XynT*
L. monocytogenes ATCC 51414	Virulence	Virulence regulatory factor *PrfA*
	Antibiotic and toxic compounds resistance	Bile acid hydrolysis *BaiE* Cadmium efflux system
	Cell signaling	PTS system: cellobiose specific IIC component

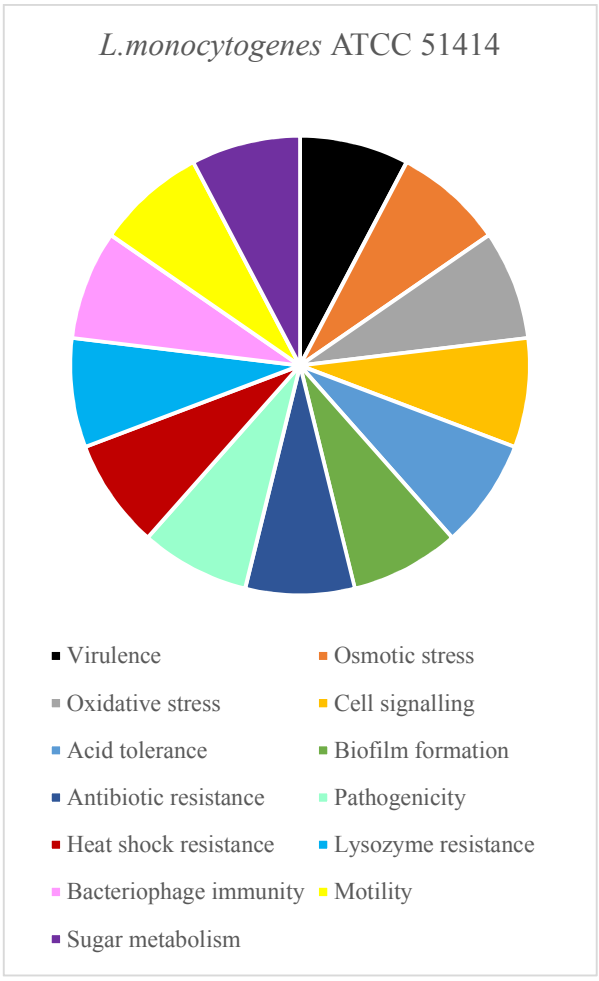
Table 2. Multi locus sequence typing (MLST) of the *Listeria* isolates

Figure 1. Identification of genetic components in *Listeria* isolates determined through MiSeq sequencing

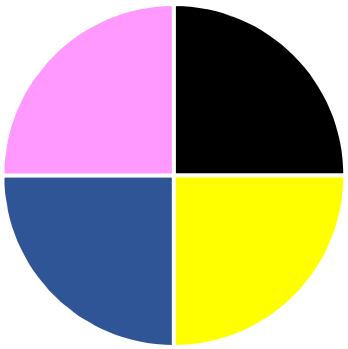
***Listeria* isolates**	**Sequence type (ST)**	**Clonal Complex (CC)**	**Lineage**
L. monocytogenes 315-S1	ST-5	CC5	I
L. welshimeri 634-3	ST-2688	CC2688	*L. welshimeri*
L. welshimeri 634-253-S5	ST-2688	CC2688	*L. welshimeri*
L. innocua 634-34-S6	ST-1489	CC1489	*L. innocua*
L. welshimeri ATCC 35897	ST-129	CC129	*L. welshimeri*
L. innocua ATCC 33090	ST-139	CC139	*L. innocua*

Figure 1. Genetic components in *Listeria* isolates identified through MiSeq sequencing





L.innocua 634-2



■ Virulence ■ Motility
■ Antibiotic resistance ■ Bacteriophage immunity

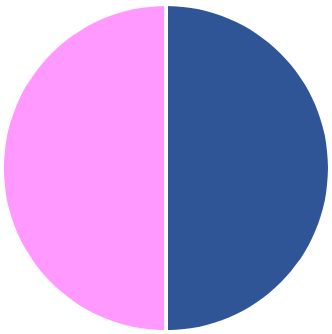
L.innocua ATCC BAA 680

■ Virulence ■ Cell signaling
■ Antibiotic resistance

L.innocua 634-34-S5

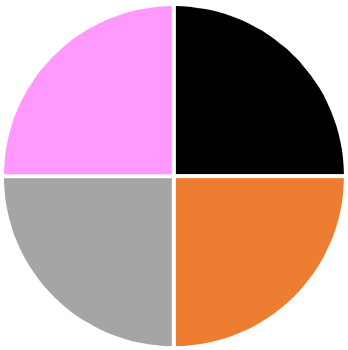
■ Virulence ■ Cell signalling
■ Antimicrobial resistance

L.innocua ATCC 33090

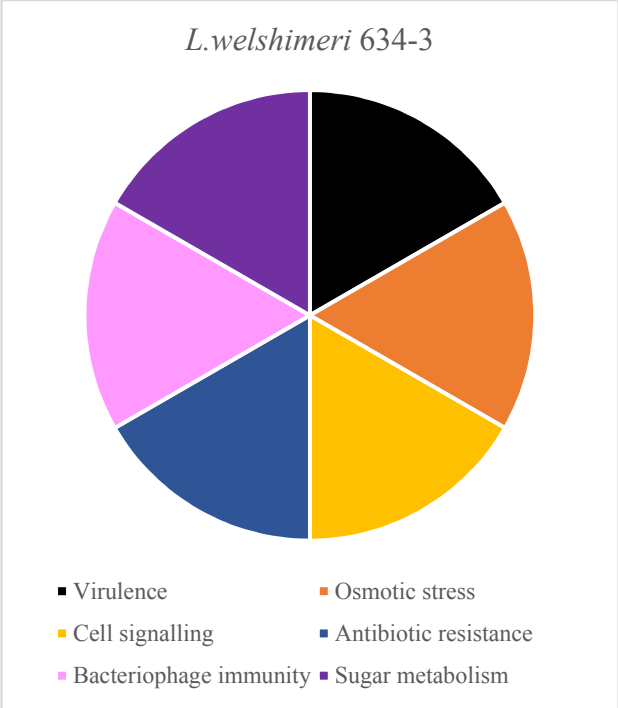


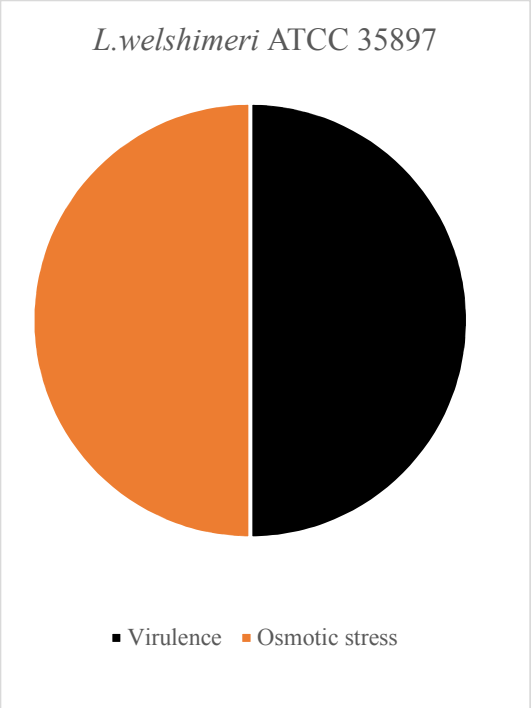
■ Antibiotic resistance
■ Bacteriophage immunity

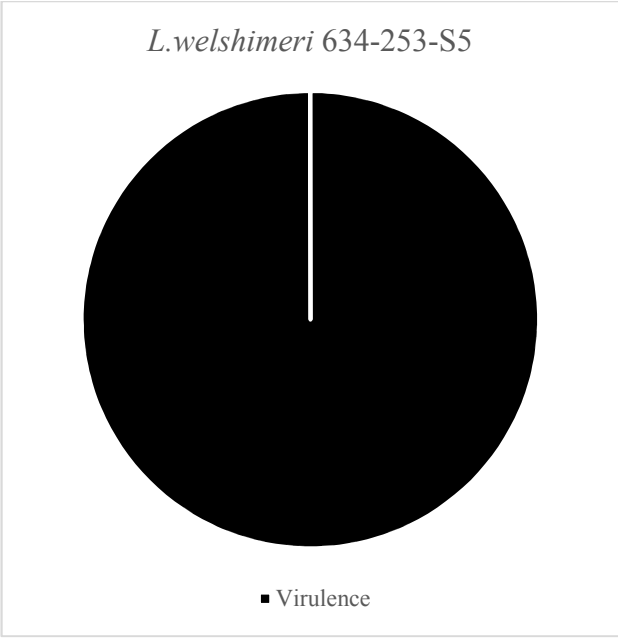
L.innocua 634-34-S6



■ Virulence ■ Osmotic stress
■ Oxidative stress ■ Bacteriophage immunity


L.welshimeri 634-3
Virulence
Osmotic stress
Cell signalling
Antibiotic resistance
Bacteriophage immunity
Sugar metabolism


L.welshimeri ATCC 35897
Virulence
Osmotic stress


L.welshimeri 634-253-S5
Virulence

SUMMARY AND CONCLUSIONS

In part I of the dissertation, a novel spray-dried dairy-based health formulation was developed using conjugated whey protein hydrolysate and probiotic microorganisms. In the phase I of this study, whey protein hydrolysate-maltodextrin conjugate was prepared, and the effect of conjugation on the bioactive attributes of the whey protein was determined. Different forms of whey protein (concentrate WPC80, isolate WPI90, and hydrolysates WPH10, WPH15, and WPH20) were screened for bioactive properties (antimicrobial by agar well assay, antioxidant by ABTS+ radical assay, and antihypertensive by ACE inhibition assay). Overall, the whey protein hydrolysate samples exhibited higher bioactive properties as compared to the intact form of whey proteins. Among which, whey protein hydrolysate WPH10, comprising a total amino acid content of 92.31g (per 100g of sample) exhibited the highest properties, and hence was conjugated with maltodextrin to obtain a thermally stable conjugated solution. The solution was spray dried, and alternatively freeze dried and bioactivities of the conjugated samples were assessed. The WPH10-maltodextrin conjugated solution exhibited higher antimicrobial, and antioxidant properties as compared to the non-conjugated WPH10, whereas a comparable effect on the antihypertensive activity was observed. Subsequent spray and freeze drying of the WPH10 conjugated solution, displayed even higher antimicrobial and antioxidant properties, while retaining the antihypertensive activity. Hence, the results indicate the ability of the conjugation of whey proteins with reducing carbohydrates such as maltodextrin, to retain and enhance the bioactive properties of whey proteins. This provides opportunities for application of conjugated whey proteins as a bioactive ingredient to develop functional foods and beverages.

As consumers become more health-conscious, they are looking for natural ingredients rich in nutrients inside their foods and beverages. Furthermore, the consumer mindset to follow a healthy lifestyle particularly in these testing times is further expected to enhance the demand for high nutrition diet. Hence, with increasing demand, it is essential to develop novel products with added value in terms of enhanced health benefits and functionality. In addition, such products can benefit all types of consumer groups. A common approach employed in the food industry to develop these kinds of value-added foods involves the addition of a healthy component and whey proteins and probiotics are one of the ingredients that are gaining rapid interest in this area.

Hence, in the phase II of this study, conjugated whey protein hydrolysate (WPH10-MD), which was identified in the previous study to possess higher bioactive properties, was used as an encapsulant to entrap a combination of probiotic microorganisms (*Bifidobacterium animalis* subsp. *lactis* ATCC 27536 and *Lactobacillus acidophilus* ATCC 4356) through spray drying process. Following, the dried formulation was stored in airtight bottles for 16 weeks at 4°, 25°, and -18°C, to monitor cell viability, moisture, and functionalities (solubility, wettability, and bulk density). Subsequent encapsulation, the mean probiotic counts (mixed population) was found to be 8.5 log CFU/g with an encapsulation yield of 84.87%, which was comparatively higher than non-conjugated WPH10. This tells the ability of the conjugated whey protein to be used as an encapsulant of probiotics. Besides, the viability of the probiotic cells was stable under different storage temperatures (4°C, 25°C and -18°C). When observed under scanning electron microscopy, the outer structure of the whey protein hydrolysate particles transformed into matrix-type microsphere i.e., a round continuous surface with formation

of irregular concavities, making the conjugated whey protein suitable as a carrier for probiotics. Conjugation with maltodextrin was also effective in enhancing the functionality (solubility and wettability) of the final spray dried product as compared to the non-conjugated whey protein. Hence, such value-added product will possess benefits from both whey proteins and probiotics and having even improved functionality, can offer opportunities as an ingredient for developing novel health formulations.

Regardless of the changes in food processing plant layout and cleaning and sanitization protocols, studies have shown that certain strains of *Listeria monocytogenes* are able to colonize in locations such as plant floor tiles and drains, and attribute to the resident microbial flora. Hence, in part II of the dissertation, a genetic evaluation using whole genome sequencing of the environmental *Listeria* strains was done to identify the genetic components contributing to their resistance to environment stress conditions and responsible for their persistent behavior in dairy and food processing environments. Genomic DNA was extracted from four ATCC isolates (*Listeria welshimeri* Rocourt and Grimont ATCC 35897; *Listeria innocua* Seeliger ATCC BAA 680; *Listeria innocua* Seeliger ATCC 33090; *Listeria monocytogenes* Pirie ATCC 51414) and six isolates previously isolated from dairy plant environment (*Listeria innocua* 634-2; *Listeria innocua* 634-34-S-5*; Listeria innocua* 634-34-S-6*; Listeria welshimeri* 634-3; *Listeria welshimeri* 634-253-S-5; and *Listeria monocytogenes* 315-S-1). Whole genome sequencing of the extracted DNA was done through Ilumina MiSeq and Nanopore sequencing platform. Annotations were developed using RAST database to determine the genetic determinants and related with the phenotypic responses. Following the genetic determinants were compared among and within the species. Overall, 13 types of

phenotypic environmental stress responses could be related to the genotypic variants of the *Listeria* isolates and the genotypic variants differed with the species and within the strains. Some of the common variants and environmental responses identified in the isolates were DNase, phage terminase, tRNA-Arg-ACG responsible for virulence of *Listeria*, NAG-IIA, NAG-IIB could be related to cell signaling and attachment, *CadA, YRKL, BaiE* and *Lde* responsible for osmotic stress, oxidative stress, and antibiotic stress resistances respectively. While *L. monocytogenes* was positive for all these phenotypic characteristics, *L. innocua* and *L. welshimeri* isolates lacked variants for motility (*ActA*), biofilm formation (*AgD*), and acid tolerance (*AdiA*), making them less resilient to environmental stress. When the industrial *Listeria* strains were compared with the control ATCC *Listeria* strains, some genetic determinants contributing to *Listeria* resistance in harsh conditions in the control isolates were missing. Such information about the genes and their role in expressing phenotypic attributes can help create more robust interventions for the control of *Listeria* in a dairy plant environment.

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