Physiochemical And Microstructural Properties And Probiotic Survivability Of Symbiotic Almond Yogurt Using Polymerized Whey Protein As A Co-Gelation Agent

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PHYSIOCHEMICAL AND MICROSTRUCTURAL PROPERTIES AND PROBIOTIC SURVIVABILITY OF SYMBIOTIC ALMOND YOGURT USING POLYMERIZED WHEY PROTEIN AS A CO-GELATION AGENT

A Thesis Presented

By

Hao Shi

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of

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ABSTRACT

Almond milk-based products are becoming increasingly popular as milk product alternatives. In this study, a symbiotic almond yogurt containing probiotics and inulin as a prebiotic was developed using polymerized whey protein (PWP) as a gelling agent. Plant-based starter cultures YF-L02 (Lactobacillus delbrueckii subsp. bulgaricus, Streplococcus thermophilus, Lactobacillus acidophilus, Lactobacillus paracasei and Bifidobacterium animalis) were incubated in the almond slurry. The control and fortified (Calcium Citrate and Vitamin D) almond yogurt, were analyzed for chemical compositions, pH and viscosity changes and probiotics survivability for 10-week shelf-life. There were no significant differences between the control and fortified group in the pH, viscosity and probiotic survivability for 10 weeks test period. The pH of both groups decreased while the viscosity showed slightly increased during storage. In the final week of the study, the population of L. paracasei, Bifidobacterium could maintain above $10^6$ cfu/g; however, almond yogurts may not be a good medium for L. acidophilus whose population decreased rapidly over the first 4 weeks. Microstructure of almond yogurt was examined by scanning electron microscopy, indicating the gel structure was improved and strengthened by 0.6% Polymerized whey protein, 0.3% pectin and 0.05% xanthan gum. The results indicated that PWP may be a suitable gelation agent for formulating non-dairy fermented products, and meanwhile enhance the weak gel of almond yogurt. In conclusion, symbiotic almond yogurt might be able to consider as a functional food with viable probiotic population for therapeutic effects.

Key words: almond, yogurt, whey protein, symbiotic, microstructure
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CHAPTER 1 COMPREHENSIVE LITERATURE REVIEW

1.1 Introduction

Increasing modernization rises new demands for developing new products for people who have special needs in their lifestyles, for example reducing energy intake, delaying aging, addressing fatigue and stress, relieving pains and stress, and etc. In recent years, these changes and developments also involved the beverage sector, especially in plant-based beverages.

The global dairy alternatives market is expected to reach $41.06 billion by 2025 at a 16.7 % CAGR (Compound annual growth rate) from 2019 to 2025 (Grand View Research, 2019a). There are several factors that drive its development. One major functional requirement of plant-based beverage is alternating dairy milk in the diet for people who have problems with lactose intolerance, cow-milk allergy, calorie concerns and hypercholesterolemia (Sethi et al., 2016). Lactose intolerance is the biggest concern compared with other reasons. It was reported that the rates of lactose intolerance vary among ethnic origins. In adults, the prevalence of lactose intolerance in white Northern Europeans is generally below 17% (Finland and North France) with the lowest rates of 5 % occurring in Britain, while over 50 % of the population is hardly tolerant lactose in South America, Africa and Asia; in some Asian countries this rate is almost 100 % (Lomer et al., 2008). Besides, plant-based “milk” is more abundant and affordable in some countries where mammal milk is scarce and expensive (Mäkinen et al., 2016). Thus, it is reasonable to assume that the population in these regions with high rates of
lactose intolerance and low-income rates tends to increase their consumption of plant-based beverage.

Among all commercial plant-based beverages, especially almond beverages, have dramatically increased over 2013 (Dharmasena et al., 2015). Almond nuts are energy dense, providing a high portion of unsaturated fat, a good source of protein and abundant micronutrients such as calcium, magnesium, zinc etc. It is also sufficient in bioactive ingredients, vitamin E. Since almond products provide health benefits to human for these nutritional values, almond products, not merely almond nuts, but also the almond beverages and almond yogurts have been extensively developed.

However, there are still some problems limiting the growth of almond beverages and yogurts. Since the majority of the consumption is used for directly intaking, scare raw material supply is possible the key concern for the market grow. As a consequence, higher prices will negatively impact on its competition with conventional products. In addition, nutrients (protein and fiber contents) in almond milk significantly varies between brands because of the difference in product formulation. Also, majority of nutrients are lost in the extraction process. Some commercial almond beverages and yogurts contain excessive sugar and low protein, which is actually not suitable for those people looking to decrease caloric intake and manage weight. Moreover, what the most deficient aspect of plant-based beverages and yogurts, including almond beverages and yogurts, is its undesirable physical stability and texture, such as phase separation, whey off, grainy and chalky tastes. Therefore, related technologies are needed to address the following disadvantages. Polymerized whey protein (PWP) as a gelation agent in
fermented plant-based yogurts have been studied recently to improve the current texture and structure, such as corn yogurt, oat yogurt and coconut yogurt. In this study, the enhanced formulation of symbiotic almond-based yogurts containing starter cultures, inulin, and other ingredients will be developed by adding PWP to improve the texture and tastes.

1.2 Almond milk

1.2.1 The physical properties of almond milk

Almond milk, also called almond beverage, is a colloidal dispersion obtained by disintegrating almond and water. The oil drops and protein are dispersed together in almond milk, which suggests that they are dynamic thermally unstable. Due to the hydrophobicity properties, almond protein matrixes are easily flocculated, resulting in phase separation. Therefore, specific processes, such as homogenization and heat treatment, is necessary to carried about for its stabilization. Homogenization and heat treatment differently attribute to the stabilization of almond milk. It has been reported that high pressure homogenization (HPH) induce the flocculation of fat globules’ clusters and the uniform dispersion of agglomerates, changes the protein conformation and increase emulsion’s viscosity (Floury et al., 2000). Besides, HPH treatment also significantly reduce the size of fat globules and enhance partial protein solubility (Dhakal et al, 2014). While, heat treatment provokes almond protein denaturation and aggregation which give rise to the formation of a 3D network entrapping big aggregates of the small protein-lipid particles (Bernat et al, 2015a).
Considering the effects of HPH and temperature on almond beverages solubility, only when homogenized samples were submitted to thermal treatment and the proteins were denatured, did this help to stabilize the emulsions mainly due to a viscous effect. The emulsifying properties of the proteins treated by HPH were not suitable for stabilizing fat globules by interfacial protein adsorption. These facts show that heat treatment plays more effect on almond “milk” physical stability than HPH.

In order to produce stable product with reasonable price, scientists and industries intend to adapt low cost, high effective and environmentally friendly method in almond milk and yogurt manufacturing; however, HPH induces higher cost for the equipment and maintenance process. Thus, gelation agents and stabilizer with low use and cost might be a good way to replace and improve the conventional methods.

1.2.2 The role of almond beverage and almond yogurt in human nutrition

Almond beverage is the rapidest extending products among dairy milk and non-dairy milk in the recent ten years (Cherney & Haddon, 2017). There are several factors driven its demands, such as the changes of lifestyle, interest in alternative diets, and the special diet demands. What directly caused this shift from dairy to non-dairy milk are the intolerance of lactose, milk protein allergen, and health problems. Considering the role of almond beverages in human nutrition, it is reasonable to evaluate the nutritional properties of almond milk and almond yogurt.

- Energy

When comes to the energy intake in North America, obesity has been considered as major chronic disease as a consequence of overconsuming energy. For this property,
unsweetened almond beverages and almond yogurt may be suitable for people who are suffering from obesity and overweight, since they are less abundant in energy with a range of 12-25 kcal 100 ml-1 compared to bovine milk (34-61 kcal 100ml-1). Based on the recommendation of American Diabetes Association, some brands of almond beverages without additional sugars might meet the criteria for the people looking for low and medium GI foods (American Diabetes Association, 2014).

- Protein

   It is essential to evaluate the protein content in almond milk and almond yogurts for the functional properties of protein in human life. Almonds are a good source of protein, consisting of 12.1 % protein in nuts (Chen et al., 2006), nevertheless, the content of almond protein will be diluted by water in the extraction process, which cause almond milk has typically a lower protein content of approximately 0.76 g 100 mL-1 compared with exceed 3 g 100 mL-1 that in bovine milk. Moreover, some researches also showed the quality of protein in almond milk is considered insufficient and of “poor quality” based on the PDCAAS (protein digestibility-corrected amino acid score) when compared to the recommended FAO/WHO pattern for children aged 2-5 years for the low content of the limiting amino acids methionine and cysteine (Boye et al., 2012). In contrast, Kamil et al. (2012) suggested almond protein possess good digestibility and unusually high content of arginine according to True Protein Digestibility (TPD). Shortly, almond milk and almond yogurt are not appropriate as the primary source of protein for young children 2–5 years old. To amend the content and quality of protein while decreasing materials cost, pea protein and rice protein are commonly added in most commercial
almond beverages. When considering the amino acid needs of adults, almond beverages and yogurt may serve as a valuable alternative source of dietary proteins, when consumed as part of a balanced and varied diet rich in sulfur amino acids.

- **Lipids**

  Lipids not only play a vital role in ensuring adequate energy intake, but also provide essential fatty acids and fat-soluble vitamins. Almond beverages contain less fat 1.02 g/100 ml compared with 3.27 g/100ml fat of whole bovine milk and 1.98 g 100 ml-1 of 2% bovine milk separately (Chalupa-Krebzdak et al., 2018). Moreover, the compositions of fat in almond beverages and yogurt are better than that in bovine products, which has lower saturated fatty acid with relatively higher ratio of unsaturated fatty acid than bovine milk. According to USDA Food Composition Database (2018), the 1.98 g of fat 100 g-1 of 2% bovine milk is made up of 1.26 g of saturated fat, 0.56 g of mono-unsaturated fat, and 0.07 g of polyunsaturated fat. While, almond milk (100g) average contains 0.02 g saturated fat with 0.65 g monounsaturated and 0.66 g polyunsaturated fat. Numerous results from clinical studies demonstrated that almond consumption has positive effect on lipoprotein profile especially lowering Total Cholesterol and LDL cholesterol (Musa-Veloso et al, 2016; Spiller et al., 1998). Therefore, it indicated that almond milk and yogurt help maintain the level of healthy blood lipids and reduce the risk of heart disease.

- **Dietary fiber**

  Almonds are a good source of dietary fiber (13.4%), providing approximately 12% of the daily recommended amount of fiber per serving (240 g) (Chen et al., 2006).
This insoluble fiber plays an important role in reducing intestinal transit time and might be involved in the mechanism in which almonds decrease low density lipid-cholesterol LDL-C (Bennekum et al., 2005; Salas-Salvadó et al., 2006). It also possesses potential prebiotic effects to promote the survival of probiotics (Liu et al, 2014). The population of Bifidobacterium spp. and lactobacillus spp. were increased in human’s host as well as suppressing the growth of Clostridium perfringens when almond products were added in the daily diet. It echoed another study that almonds were a novel source of prebiotics which can increase the population of Bifidobacterium and Eubacterium rectale by Mandalari et al (2008). Unfortunately, although almonds contain rich dietary fiber, almond beverage only contains 1 g fiber per 8 oz (Schuster et al., 2018) because the majority of fiber is lost during the manufacturing process. The relatively low amount of dietary fiber might critically influence the effect of dietary fiber in almond beverages and yogurts. Nevertheless, almond beverages and yogurt still have potential functions of improving lipid profile and balancing intestinal microbiota environment by optimized the process technology and formulation.

- **Antioxidants**

  Almonds are rich in antioxidants, mainly \( \alpha \)-tocopherol and polyphenolics. Almond beverage contains 6.33 mg 100 g-1 vitamin E in the form of \( \alpha \)-tocopherol that is equivalent to 42 % of the 15 mg recommended daily amount (Chalupa-Krebzdak et al., 2018; National institutes of Health, 2018). Almond-derived antioxidants have been shown to increase body’s defense capacity, reduce fatty acid peroxidation and postprandial oxidative damage to protein (Jalali-Khanabadi et al., 2008). Particularly, \( \alpha \)-
tocopherol is the functional bioactive form of vitamin E that has a powerful effect on against free radicals to prevent coronary heart disease, cancer and eye disorders.

Compared with bovine milk, almond beverages as well as yogurt are able to protect the body against oxidative reactions and free radicals.

- **Minerals**

  Almonds are a good source of minerals for people, providing more than 20% daily value of manganese and calcium, and 10-20% of magnesium, copper, and phosphorus (Chen et al., 2006). The difference in mineral contents between almonds and almond beverage is rather large, which is also due to the manufacturing process. What the most concerned compound in dietary is calcium, especially the contents and bioavailability, since it plays an important role in strength of bone and teeth, muscle contraction and blood clotting. Owing to people’s health demands, commercial almond milk is usually fortified with calcium carbonate (Zhao et al., 2005). Calcium fortification in almond beverages and yogurt not only can increase the content of calcium, but also can improve bioavailability of calcium in almond milk.

  In conclusion, almond beverages arise as alternatives of dairy products for people who have special diet demands, such as diabetes, lactose intolerant and dairy allergy. Since the original almond beverages has lower energy than bovine milk without lactose, it is an appropriate beverage for diabetes and people who aren’t able to tolerate lactose. The content of protein in almond beverages diversities with brands due to the product formulation and ratio of dilution. Almond milk may be not suitable to act as a major protein resource for infants and children, while it is a good alternative for dairy products
for the sake of health conditions and balanced diets. Almond beverages contain lower total lipid, higher dietary fiber and unsaturated fatty acid contents than bovine milk, which makes it compatible to cow’s milk. They also have advantages in antioxidants, such as vitamin E and polyphenols, preventing people from oxygen damage, free radicals to reduce the risk of chronic diseases. In order to make up for the mineral lost in the manufacturing process, additional minerals especially calcium usually be fortified in almond beverages to provide the essential micronutrients for humans. Almond nuts and beverages expand people’s dietary choices and are a good replacement for conventional milk, even though it still has some limitations compared with bovine milk. But, in the future, these shortages are more likely to overcome with the improvement of manufacturing technology and process.

1.3 Whey protein

1.3.1 Introduction of whey protein

Whey protein is derived from whey which is a transparent green-yellowish liquid obtained from cheese and Greek yogurt manufacturing. Based on protein contents and its purities, whey protein can be classified into two main groups, whey protein concentrates (WPC) and whey protein isolates (WPI), containing up to 80 % and 90 % of proteins separately (Guo, 2019). WPI was concentrated from WPC by micro-filtration to remove extra fat. Whey protein is a heterogeneous mixture, which mainly consists of β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum (BSA), lactoferrin (Lf) and immunoglobulins (Ig) according to its abundance in whey protein (Smithers et al., 1996).
1.3.2 Nutritional properties of whey protein

It has been well-known that whey protein and its derivatives have therapeutic potential in human health and disease. Additionally, whey protein has a perfect protein quality based on the protein digestibility corrected amino acid score (PDCAAS). The PACAAS of whey protein is 1.00, compatible with egg and milk protein, which means whey protein can be highly digested and utilized by the body (US Dairy Export Council, 2007). The health benefits of whey protein generally include as follows (Guo, 2019):

- act as a sports supplement to help energy and muscle recovery, improve physical performance.
- manage satiety and control weight, prevent diabetes
- perform antimicrobial activities and help wound cure
- reduce the rate of cardiovascular disease and prevent cancer
- fight aging and play antioxidant properties

1.3.3 Introduction of polymerized whey protein

Polymerized whey protein (PWP), also named as pre-heated whey protein, heat-denatured whey protein or whey protein nanoparticle. When submitted to heat treatment, whey protein aggregates are formed which are intermediates between monomer proteins and the insoluble gel network (Walsh, 2014). Therefore, PWP shows functional properties such as gelation, emulsification, hydrophobic ligands binding ability and filming properties. It has not only been applied in food industries such as gelling agents, stabilizers, microencapsulation walls, and coating material to enhance the texture and
quality in various foods but also can be used to manufacture many bio-based products.
For example, wood coating/finishing, wood adhesives, office glues, and tissue adhesives.

1.3.4 The functional properties of β-lactoglobulin

β-LG is the richest component in whey protein, which makes up 50-60% of the total whey protein. It is a water-soluble and small molecule with the weight range from 18.20 to 18.49 KDa, containing 162 total amino acid residues. It has seven different genetic variants, two of them are the most common in industrial preparations, β-LG A and β-LG B, with different physical and chemical behaviors such as thermal stability, charge state, the reactivity of certain groups and heat-induced aggregation behaviors (Schokker et al., 1999).

β-LG protein is predominant by β-sheet. The twisted antiparallel β-sheet forms the cone-shaped barrel with the protective α-helix, which provides potential hydrophobic binding sites that enable β-LG interacts with various hydrophobic ligands in alkaline conditions. Therefore, these properties of β-LG make it become a member of the lipocalin family, play a role in ligand binding (Kontopidis et al., 2004). Most of hydrophobic and aromatic amino acid side chains are buried in the β-LG core and are not able to cleavage by enzymes; thus, it is rarely hydrolyzed by pepsin due to its compact structure (Hernández-Ledesma et al., 2006). The inner structure of unfolded β-LG is stabilized by disulfide bonds. Each β-LG has 5 cysteines, 2 disulfide bonds (SS) are formed leaving one free cysteine (–SH) (Guo, 2019). Therefore, the existing SH/SS interchange reactions and SH/SH oxidation reactions contribute to β-LG dominant polymerization and gel formation.
1.3.5 The applications of polymerized whey protein

- Food thickener/gelling agent

A thickening agent is a substance that can increase the viscosity without substantially changing other properties. PWP can be added in the food as a thickening agent due to its larger molecule size and higher viscosity. PWP also exhibits the characteristics of hydrocolloids that can gel and solidify fluid products, for example, cold-set gelling property. Firstly, the pH of whey protein solution is increased above its isoelectric point to prevent denaturation for the sake of inner aggregates electrostatic repulsion. Then, the solution is heat-treated at a certain temperature to aggregate. After cooling, the aggregates remain soluble and would normally form a gel by adding minerals or decreasing pH (Alting et al, 2004). Therefore, PWP might play a significant role in the food system to develop set-type products as a gelling agent. With the ability to trapping water and small molecules in the fermentation, PWP is increasingly popular in the dairy industry, because it considerably decreases the syneresis during storage and improves the texture of dairy and non-dairy products, including cow yogurts, goat yogurts, and plant-based yogurts.

- Food stabilizer /emulsifier

Whey protein is an amphiphilic compound, showing both hydrophilic and hydrophobic properties, making it able to form finely dispersed emulsion droplets in the oil-in-water system. The mechanism is that β-LG and α-LA adsorb oil-water interfaces and stabilize emulsions (Guo, 2019).

- Fat or dairy replacer
Fat plays an important role in food processing as well as taste and texture. However, because of high energy intake, people tend to use fat replaced to meet their functional properties by providing less energy. WPC acts as an ideal fat replacer that reduces overall food intake and induces satiety.

- **Hydrophobic nutraceuticals carriers**

  Some organic chemicals have bioactivity with poor stability due to its structure. Most of them are low in water solubility and sensitive to the environment, such as temperature, light and oxygen. Potential hydrophobic binding sites in β-LG make whey protein having the ability to strongly interact with various hydrophobic ligands such as fatty acid, hemin, ellipticine, aromatic hydrocarbons and carcinogenic hydrocarbons. Therefore, whey protein is a suitable carrier for these hydrophobic ligands.

- **Microencapsulating agent**

  Microencapsulation is an effective and novel technology generally applied in protecting some target substances, such as small molecules and protein, cells of bacteria, yeast and animal organ. It is a process in which tiny molecules are surrounded by a coating material as a wall; thus, protein-based microcapsules have been produced by whey protein containing sensitive ingredients to control its release and improve stability. For example, whey protein was reported to successfully used in microencapsulation of oil, probiotic, bioactive substances and vitamin as a microencapsulation agent (Guo, 2019).

- **Films and Coating**
Films and coats protect some products against oxygen and moisture, thereby it can extend the shelf-life by reducing respiration, water loss, and oxidation reaction. Whey protein-based cast films are formed by whey protein coordinating with plasticizer, antioxidants, antimicrobial agents or probiotics; then foods, such as fruits, seafood, nuts, and meat, which are immersed in the film solution for a certain time to ensure total coverage of the entire surface with good adherence and perfect integrity.

Besides, whey protein can also be applied in other fields, including wood glue and adhesive, office glue and tissue adhesive.

1.3.6 Interaction with other polysaccharides

Whey protein not only stabilizes the system by itself but also can interact with other stabilizers such as pectin, xanthan gum, lecithin, and others.

- Pectin

Pectin is an anionic polysaccharide, containing low methoxyl (LM) and high methoxyl (HM) groups. It exhibits negative charges on the carboxylate group in the acid environment so that it can interact with whey protein containing positive charges on amino acid via electrostatic attraction. Although the mixture of LM pectin and whey protein shows different behaviors at different pH levels, there are sufficient studies that had confirmed that the combination of pectin and PWP can improve the gel structure. The low pH suppresses the electrostatic repulsion between LM pectin molecules, leading to strong intermolecular interactions between WPI and LM pectin molecules. In short, LM pectin contributes to the building of the network that acts as the structural framework to support the gel system and WPI provides the stabilization of the networks through
connecting the junction zone (Wijaya et al., 2017). WPI-beet pectin conjugates stabilized emulsion showed substantial to improve physical stability, including decreased droplet size and more homogenous droplet size distribution (Xu et al., 2012).

- **Xanthan gum**

  Xanthan gum (XG) is an excellent stabilizer and has been used to stabilize the emulsion by increasing emulsion viscosity. At acidic conditions, whey protein interacts spontaneously with the carboxylate groups of xanthan gum through electrostatic attraction (Guo, 2019).

- **Other emulsifiers with whey protein**

  Lecithin is an important natural stabilizer whose amphiphilic molecular structure, containing both a lipophilic part and hydrophilic group, provides its excellent emulsification properties. This is because “The improved stability of emulsion by using both whey protein and lecithin as emulsifiers was attributed to the interaction between lecithin and whey protein on the surface, which formed compact adsorption to response external deformations” (Wang et al., 2017). It was reported that adding 0.2%-2% lecithin can reduce the droplet size and interfacial viscoelastic properties of peony seed oil emulsion stabilized by WPI (Guo, 2019).

1.4 **Probiotics**

1.4.1 Definition of probiotics and categories

  The term “probiotics” refers to “live microorganisms”, which can alter the microflora in an intestinal compartment of the host and bring beneficial health effects by suppressing the growth of harmful bacteria when in a certain number. They must be able
to survive and colonize in the gastrointestinal tract under low pH conditions and resistant to bile and gastric juices (FAO/WHO, 2002). According to European Food and Feed Cultures Association and International Dairy Federation, there is a detailed list of microorganisms that have a long history of safety in food. For example, the most common probiotics that have been used and pose health characteristics are the lactic acid bacteria, including *Bifidobacterium*, *Lactobacillus* and *Lactococcus*. All of them were categorized as GRAS (generally recognized as safe), which means fewer health risks could be found for the host.

1.4.2 Health effect of probiotics

Although some health benefits provided by probiotics have been documented and established, it is still worth noting that these effects are very stain specific. That means comprehensive health benefits are coordinated with mixed probiotic bacteria rather than universal stains.

Shah et al (2007) organized the established health benefits of probiotic organisms with the alleviation of lactose intolerance, prevention, and reduction of symptoms of rotavirus and antibiotic associated diarrhea. However, there are still some potential benefits need to further confirm because of limitation of study samples and unclear mechanism. For example, prevention of allergy (atopic eczema, food allergy) and inflammatory bowel diseases, reduction of risk associated with mutagenicity and carcinogenicity, and stimulation of the immune system.
These effects are most likely achieved by creating a restrictive physiological environment to suppress the growth of potentially pathogenic microorganisms. For instants (Shah et al, 2017),

- lowering pH of the environment created by the production of organic acids.
- competitive exclusion with pathogenic bacteria, such as *Escherichia coli*, *Salmonella* and *Clostridium spp.* These probiotics can prior to adhering to the intestinal epithelium and thereby prevent infection.
- elaborate cellular modulation and humoral immune system, which fundamentally enhance the ability of host to resist external pathogens.

1.4.3 Survivability of probiotics

Survivability of probiotics is an important consideration for the functional health properties, because they must survive and maintain at a certain therapeutic level during the transition process in the gastrointestinal tract for the ideal health benefits. Therefore, it is necessary to investigate conditions that dominate the survivability of probiotics and improve some technologies.

In a delivery process (i.e. manufacturing process, package, and shipping, etc.), the viability and activity of probiotic culture generally will be exposed to different environmental stress factors, such as water activity, presence of oxygen, temperature, and acidity (Shah, 2007). The viability of probiotics in the food matrix also depends on strain selected, interactions between microbial species present, production of hydrogen peroxide, inner nutrients and ingredients of products and conditions of fermentation (inoculation level, fermentation time and temperature).
• Oxygen

The presence of oxygen has a detrimental effect on the viability of probiotics, which might express the toxicity in two ways. Firstly, some probiotics are relatively sensitive to oxygen, as a result of the intracellular accumulation of hydrogen peroxide and consequently the death of cell (Dave & Shah, 1997c, 1997b, 1997a). Secondly, oxygen would be utilized by yogurt cultures to produce some substances which directly affect probiotic (Talwalkar & Kailasapathy, 2004a). It is known that *L. delbrueckii ssp. bulgaricus* impacts on the growth of other probiotics due to hydrogen peroxide in the presence of oxygen (Talwalkar & Kailasapathy, 2004b).

Probiotics show different sensibility of oxygen. Strains of *L. acidophilus* and *Bifidobacterium spp.* are microaerophilic and anaerobic, respectively, which means *L. acidophilus* could semi-tolerant oxygen while *Bifidobacterium* will be inhibited in aerobic conditions. Both of them lack an electron-transport chain, which results in the incomplete reduction of oxygen to hydrogen peroxide (Talwalkar & Kailasapathy, 2004). Additionally, they are free from catalase, resulting in capably converting hydrogen peroxide to water. It is worth to note that *Bifidobacterium* is generally more susceptible to the harmful presence of oxygen than *L. acidophilus* (Ruiz et al., 2011). *L. paracasei* was considered as oxygen-tolerant anaerobes that can grow well in aerobic conditions.

• Acidity

The optimal pH for *L. acidophilus* is around 6, however, it has the ability to resist changes in cytoplasmic pH and maintain stability under acidic conditions (Wang et al., 2005). Therefore, its wide range makes it more acidic tolerant than *Bifidobacterium spp.*
whose growth will be significantly suppressed below pH 5.0. Although the acidic
tolerance of *Bifidobacterium* is low, there are still some exceptions in terms of cultivation
conditions, trains and species. For example, *Bifidobacterium animalis subsp. lactis* has
the highest acid tolerance and thus suitable to apply in the lower acidic food.

- Culture starter interaction

  Starter cultures antagonism negatively affect the growth of probiotic strains due to
  the production of inhibitory compounds. *L. delbrueckii ssp. bulgaricus* may affect
  the survival of *L. acidophilus* and *Bifidobacterium* because of acid and hydrogen peroxide
  produced in the fermentation process (Vinderola et al., 2002). Therefore, the survival of
  probiotic organism is potentially improved by removing *L. delbrueckii ssp. bulgaricus*
  from some starter cultures (Bâati et al., 2000). By contrast, it may support the growth of
  *Bifidobacterium*, which contributes to its proteolytic nature by releasing essential amino
  acids (Walsh et al, 2010).
### Table 1 The environment effects on the growth of probiotics

<table>
<thead>
<tr>
<th>Strains</th>
<th>Temperature</th>
<th>pH</th>
<th>Gaseous environment</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. acidophilus</em></td>
<td>The optimal growth temperature is 37°C and that it was able to grow at 22°C, whereas growth was not observed at 15°C (Bâati et al., 2000)</td>
<td>The optimal pH for <em>L. acidophilus</em> is around 6, but it can survive at the acidic conditions. pH conditions below pH 4.5 during fermentation were detrimental to bacterial resistance during frozen storage (Bâati et al., 2000).</td>
<td>It can survive in aerobic or anaerobic conditions. However, it is usually adapted anaerobiosis incubation when antibiotics are added due to the respiratory mechanism, but it is still not completely confirmed yet (Talwalkar &amp; Kailasapathy, 2004).</td>
</tr>
<tr>
<td><em>Bifidobacterium</em></td>
<td>The optimal growth temperature ranges between 36–38°C and 41–43°C for human and animal origin strains, respectively (Mortazavian et al., 2007).</td>
<td>The optimal growth pH is around neutrality (6.5–7.0), but some stains survive well at pH 3.5–4.0 (Mortazavian et al., 2007).</td>
<td>They are strict anaerobes and only a few species, such as <em>Bifidobacterium animalis subsp. lactis</em>, <em>Bifidobacterium boum</em>, <em>Bifidobacterium thermophilum</em>, <em>Bifidobacterium dentium</em> and <em>Bifidobacterium psychraerophilum</em>, can tolerate a microaerophilic environment (Ahn et al., 2001).</td>
</tr>
<tr>
<td><em>L. paracasei</em></td>
<td>The optimal temperature of <em>L. paracasei</em> is 37°C (Tharmaraj &amp; Shah, 2003a). It has been reported that 73% of <em>L. paracasei</em> were able to grow at 10 °C (Dave &amp; Shah, 1997a). Only a few species can survive at 45 °C (Reale et al., 2015).</td>
<td><em>L. paracasei</em> exhibited a good ability to grow at pH 5.5 -pH 6.5. 15 strains isolated mainly from dairy products showed a low ability to grow at pH 4.5. Two strains isolated from the human body and ripened cheese, had a good growth at pH 3.5 (Reale et al., 2015).</td>
<td>For many strains, the presence of oxygen might enhance growth compared to anaerobic cultivation, while for some specific <em>L. paracasei</em> strains aerobiosis impaired growth (Zotta et al., 2014).</td>
</tr>
</tbody>
</table>
1.4.4 Enumeration methods of probiotics

Almond yogurt was incubated with commercial plant-based starter cultures (YF-L02) provided by Chr. Hansen, containing 5 different probiotics, such as *L. acidophilus*, *L. paracasei*, *Bifidobacterium species*, *L. bulgaricus* and *S. thermophilus*. Yogurt bacterium, such as *S. thermophilus* and *Lactobacillus delbrueckii ssp. bulgaricus*, produce acid in the fermentation process, and are not able to survive and colonize in the gastrointestinal tract. Therefore, *L. acidophilus*, *L. paracasei*, *Bifidobacterium species* are introduced in almond yogurts as probiotics. A number of factors have been claimed to affect the viability of probiotics in yogurts preceding, it is important that probiotic organisms maintain at a sufficient viable level throughout the product shelf life when they are consumed. To successfully monitor and assess the viability of probiotics in the storage period, selective enumeration methods should be able to count *L. acidophilus*, *Bifidobacterium* and *L. paracasei* differentially. Several media have been proposed for selective enumerating *L. acidophilus* and *Bifidobacterium* in yogurt culture organisms (*S. thermophilus* and *Lactobacillus delbrueckii ssp. bulgaricus*) or in their pure cultures (Table 2). Table 2 also described some selective enumeration of *L. paracasei* in the presence of other probiotic and yogurt bacterium.
### Table 2: Methods reviews for selective enumeration of *L. acidophilus*, *Bifidobacterium* and *L. paracasei*.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Media</th>
<th>Incubation conditions</th>
<th>Inhibitory agent</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>L. acidophilus</strong></td>
<td>T-MRS agar</td>
<td>37 ºC 3 days</td>
<td>Trehalose</td>
<td>Round creamy colony</td>
</tr>
<tr>
<td></td>
<td>Bile-MRS</td>
<td>37 ºC 3 days</td>
<td>Bile</td>
<td>Irregular white colonies</td>
</tr>
<tr>
<td></td>
<td>G-MRS</td>
<td>37 ºC 3 days</td>
<td>Galactose</td>
<td>Round creamy colony</td>
</tr>
<tr>
<td></td>
<td>MRS-Sorbitol</td>
<td>37 ºC 3 days</td>
<td>Sorbitol</td>
<td>Only small, rough, brownish colony (0.1-0.5 mm)</td>
</tr>
<tr>
<td></td>
<td>MRS pH 5.2</td>
<td>43 ºC 3 days</td>
<td>pH and temperature</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Basal agar-maltose</td>
<td>43 ºC 3 days</td>
<td>Maltose</td>
<td></td>
</tr>
<tr>
<td><strong>Bifidobacterium</strong></td>
<td>LP-MRS</td>
<td>37 ºC 3 days</td>
<td>Lithium chloride and sodium propionate</td>
<td>Small, round, creamy colonies.</td>
</tr>
<tr>
<td></td>
<td>G-MRS</td>
<td>37 ºC 3 days</td>
<td>Galactose</td>
<td>Round creamy colony</td>
</tr>
<tr>
<td></td>
<td>NNL-MRS</td>
<td>37 ºC 3 days</td>
<td>Nalidixic acid, Neomycin sulfate, Lithium chloride.</td>
<td>1 mm, white, smooth, shiny</td>
</tr>
<tr>
<td></td>
<td>NPNL-MRS</td>
<td>37 ºC 3 days</td>
<td>Nalidixic acid, paromomycin sulfate, Neomycin sulfate, Lithium chloride.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NKB-MRS</td>
<td>37 ºC 3 days</td>
<td>Nalidixic acid, Kanamycin sulfate, Polimixin B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BSM</td>
<td>37 ºC 3 days</td>
<td>Mixed additives</td>
<td>Round, purple and smooth colonies</td>
</tr>
<tr>
<td>L. paracasei</td>
<td>MRS pH 6.2</td>
<td>37°C 3 days anaerobic</td>
<td>20 μg/ml vancomycin</td>
<td>Large creamy and white colonies</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
<td>-----------------------</td>
<td>---------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>MRS pH 6.2</td>
<td>37°C 3 days anaerobic</td>
<td>0.5 μg/ml cefotaxime</td>
<td>--</td>
<td>All large, white creamy and smooth colonies</td>
</tr>
<tr>
<td>MRS pH 5.4</td>
<td>37°C 3 days anaerobic</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>MRS pH 6.2</td>
<td>21-22°C 3 days aerobic</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
</tbody>
</table>

Reference medium was MRS agar. References: Tharmaraj & Shah, 2003; Vinderola & Reinheimer, 1999.
There are some effective antibiotics are ready to add in the media for enumerating the single strain (see Table 2). For example, MRS-clindamycin-ciprofloxacin (MRS-CC) agar is commonly used for the selective enumeration of presumptive *L. acidophilus*, which has been published as international standards by the International Organization for Standardization (ISO). Clindamycin and ciprofloxacin both inhibit the growth of the most common microorganisms used in dairy products, such as *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus delbrueckii* subsp. *lactis*, *Streptococcus thermophilus*, *Bifidobacterium*, *Lactobacillus casei*, *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus rhamnosus* *Lactobacillus reuteri* and *Leuconostoc species*. *L. acidophilus* may not be effectively single enumerated when high amounts of *L. rhamnosus*, *L. paracasei* or *L. fermentum* presenting (Süle et al., 2014a). However, *L. acidophilus* could be separately counted by their unique morphologies, which *L. acidophilus* shows small (0.1-0.5mm), grayish, rough irregular colonies while *L. paracasei* is 2.0 mm, round, white colonies.

*Bifidobacterium* strains were reported to highly resist mupirocin which can inhibit the growth of lactic acid bacteria (LAB) strains (Rada & Koc, 2000). Recently, most studies have indicated that designed media agar containing mupirocin suitably isolates and selectively enumerates *Bifidobacterium* in fermented dairy products with other LAB. For example, Wilkins-Chalgren agar containing 100 mg/L of mupirocin (Rada & Koc, 2000), and Trans-oligosaccharide propionate agar supplemented with 50 mg/L of mupirocin (Süle et al., 2014). Compared with other agars, BSM agar with higher
selective is suitable for enumerating *Bifidobacterium* from *Lactobacillus* and *Streptococcus* strains (Sigma-Aldrich, 2019). It shows differentiated purple-brown colonies on this medium with easy operation.

Vancomycin and cefotaxime can both suppress other lactic acid bacteria in starter culture (Björneholm et al., 2002). The growth of *L. paracasei* in some yogurt product was reported to slightly suppressed on MRS with 1 μg/ml cefotaxime. *L. paracasei* would be enumerated together with *L. acidophilus* and *Bifidobacterium* on MRS media in pH 5.4, while it is possible to separately identify by *L. acidophilus* and *Bifidobacterium* because of different colony morphologies (Björneholm et al., 2002). *L. paracasei* was large creamy and white, while *L. acidophilus* was smaller flat greyish/white. *Bifidobacterium* was shiny white transparent and very small. Compared with other three ways, MRS agar aerobically incubated at ambient temperature is relatively easy operative with good selectivity. *L. paracasei* can grow well in aerobic conditions while *Bifidobacterium* is strictly inhibited in the existence of oxygen. The growth of *L. acidophilus* was suppressed when inoculated in the 21 °C.

### 1.5 Prebiotics

#### 1.5.1 Definition of prebiotics and categories

Prebiotics are derived from dietary fibers which are edible carbohydrate polymers (≥10 units) naturally occurring in foods. Some of them are neither digested or absorbed in the human small intestine and have beneficial physiological effects. However, not all fibers can be classified as prebiotics; prebiotics have specific definition: “prebiotics are nondigestible ingredients that modulates compositions and/or activity of the gut
microbiota by its metabolization by microorganisms in the gut, thus conferring a beneficial physiological effect on the host” (Bindels et al, 2015). Additionally, there are three criteria proposed by Gibson et al (2004) used to determine whether food ingredients can be classified as prebiotics:

- Resistant to gastric acidity and hydrolysis by mammalian enzymes and GI absorption
- Can be fermented by intestinal microflora
- Selectively stimulates the growth and/or activity of intestinal bacteria associated with health and wellbeing

Based on the aforementioned criteria, there are three confirmed prebiotics that has been used in the industries, such as lactulose, inulin-type fructans, Trans-galactooligosaccharides (TOS) (Carlson et al., 2018).

1.5.2 Functional properties of prebiotics

Prebiotics have been used as energy sources for gut microbiota to maintain and restore the balance of gut microflora by increasing the probiotics such as *Bifidobacterium* and *Lactobacilli* and consequently inhibit the growth of pathogens. Additionally, prebiotics are also of importance to modify the gut environment by decreasing pH in the fermentation process (Davani-Davari et al., 2019). It has been reported that the composition and population of the gut microbiota will be altered with pH decreasing from 6.5 to 5.5 (Belenguer et al., 2006). Acids are the fermentation products of prebiotics with probiotic, which will decrease the pH of the gut. This pH alternative can influence suppress acid-sensitive species and promote butyrate production. Moreover, prebiotics
also have functional properties on human health maintenance and protection against disorders as following.

- **Gastrointestinal disorders**

  Prebiotics have beneficial effects on gastrointestinal disorders, including inflammatory bowel syndrome (IBS) and Crohn’s disease (CD), antibiotic-associated diarrhoea, traveller’s diarrhoea and colon cancer (Gibson et al, 2004). The population of *Bifidobacterium* in people with IBS and CD is lower than the healthy people. It is reported that FOS and inulin have given positive results in promoting Bifidobacterium population and significantly reduce the disease severity probably by suppressing the growth of pathogens (Cummings et al., 2001; Gibson et al, 2010). Additionally, fermentation products of prebiotics, such as butyrate, have protective effects against the risk of cancer by reducing the proliferation rate in the colon. Consumption of an oligofructose and inulin mixture together with *Lactobacillus rhamnosus GG* and *Bifidobacterium lactis Bb-12* significantly reduced colorectal cell proliferation and genotoxicity and increased the intestinal barrier function (Rafter et al., 2007).

- **Immune system**

  The functions of improving immunity are contributed to the increasing population of beneficial microorganisms, such as *Lactobacillus* and *Bifidobacterium*, which can decrease the growth of harmful bacteria. Besides, prebiotics are also able to prevent pathogens bonding to the epithelium and help the expression of immunity molecules (Davani-Davari et al., 2019).

- **Nervous system**
Numerous research studied prebiotics could relieve patients suffering from Autism disorders by changing the population of *Clostridium perfringens* and *Bifidobacterium*, and hepatic encephalopathy by the fermented produced compounds (Lefranc-Millot et al., 2012).

- **Cardiovascular diseases (CVD)**

  Since CVD has become one of the major reasons causing people's death nowadays, many studies have investigated the connection of dietary fibers and prebiotics consumption on CVD (Carlson et al., 2018). Prebiotics have an indirectly positive effect on CVD by reducing the inflammatory compounds. It has revealed the consumption of prebiotics improve the lipid profile (Salas-Salvadó et al., 2006). It has shown that the consumption of beta-glucan has the potential to decrease the cholesterol and LDL, and FOS could reduce the blood triacylglycerol (TAG) level (Brighenti, 2007; Tiwari & Cummins, 2011).

- **Calcium absorption and bone health**

  Recent studies show that lactulose, TOS or inulin-type fructans enhance calcium absorption (Abrams et al., 2007). Crude fractions of chicory in diet improved bone parameters relative to native or reformulated inulin in rats (Demigné et al., 2008). However, not all prebiotics was observed having such phenomenon, such as FOS and GOS (Carlson et al, 2018).
Physiochemical and microstructural properties and probiotic survivability of symbiotic almond yogurt using polymerized whey protein as a gelation agent.

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2.1 Abstract

The increased demand for almond milk-based products with symbiotics is driven partially by the role of dairy product alternatives and their health benefits. The aim of this study was to develop a symbiotic almond yogurt that overcomes its undesirable textural profiles by using polymerized whey protein as a gelling agent. Plant-based starter cultures YF-L02 (Lactobacillus delbrueckii subsp. bulgaricus, Streplococcus thermophilus, Lactobacillus acidophilus, Lactobacillus paracasei and Bifidobacterium animalis) were incubated in the almond slurry with inulin as a prebiotic. Calcium citrate and Vitamin D were fortified in almond yogurt. Both groups with/without fortification were analyzed for chemical composition analysis, pH and viscosity changes and probiotic survivability for 10-week shelf-life tests. The gelation effects of PWP with pectin and xanthan gum in almond yogurt were compared through microstructure analysis. No difference in the pH, viscosity and probiotic survivability were observed between the control and fortified almond yogurts during 10-week tests. The pH of both groups decreased while the viscosity showed slightly increased during storage. The populations of L. paracasei, Bifidobacterium still remained above $10^6$ cfu/g in the final week; the population of L. acidophilus decreased dramatically over the first 4 weeks, especially control group. The microstructure of almond yogurt was examined by scanning electron microscopy, indicating the gel structure was strengthened by 0.6% polymerized whey protein, 0.3% pectin and 0.05% xanthan gum. In conclusion, PWP might be a proper gelation agent for formulating non-dairy fermented products and enhance the weak gel of almond yogurt.

Key words: almond, yogurt, whey protein, symbiotic, microstructure
2.2 Introduction

The emergence of the plant-based products market witnesses the rising demands for plant-based alternatives, including almond milk, soymilk, and rice milk. It indicates plant-based products have drawn considerable attentions, because of the increasing special health demands for people in modern society and the health promotion properties of plant-based products. The major factors pushing the development of plant-based products are milk allergy and lactose intolerance for the population who can’t tolerant dairy (Grand View Research, 2019a). Recently, functional properties, such as the low energy value, and scavenging oxidative radicals, also elaborate the consumption of plant-based beverages (Sethi et al., 2016). These factors contribute to the dramatically increased market size of almond milk since 2014, which has overtaken that of soymilk in U.S. and became the biggest segment among other dairy alternatives (Grand View Research, 2019b). It has been reported that almond milk is a good replacement for dairy milk in terms of low energy, abundant antioxidants and comparable lipid profile with dairy milk (Vanga & Raghavan, 2018). Almond milk is greatly preferred by people looking to reduce caloric intake and weight management. It only contains 40 calories per cup (240 ml), as compared to 169 calories of cow milk (Sethi et al., 2016). Almond milk is also rich in polyphenols which are able to reduce inflammation and oxidative stress (Chen et al., 2005). Additionally, the content of vitamin E and vitamin A in almond milk fulfill 10-50% and 10-30 % of the estimated average requirements (EAR), respectively, for an average adult human. Therefore, its high antioxidative ability shows powerful capacity in protecting against free-radical reactions and inhibiting the growth of cancer cells (Cases et al., 2005;
Klein et al., 2011). Next, the composition of lipid in almond milk is compatible with cow milk. Almond milk is free of cholesterol and saturated fat, therefore it is considered to benefit hypocholesterolemia and cardiovascular (Kamil & Chen, 2012).

However, although almond milk provides health advantages for consumers than conventional dairy product in mostly aspects, their taste and texture comprise the development of a commercially viable product. Therefore, technological issues urgently need to be addressed and overcome to produce almond milk-derived alternatives (e.g. almond yogurt) comparable to that of cow’s milk products in terms of appearance, texture, stability, sensory and nutritional value (Sethi et al., 2016). What the biggest problems for almond milk and almond yogurt are the unstable suspension and weak gel, separately. Because almond protein, belonging to the oleosin family, has low molecular weight and poor water solubility, which contributes to a poor stability of the obtained emulsions (Ahrens et al., 2005; Li & He, 2004). The high degree of hydrophobicity, as a consequence of its poor stability character, causes the protein flocculation and phase separation during the manufacturing process, which induce the formation of a weak gel in the fermentation of almond yogurt (Bernat at al., 2015a).

While considerable research has been devoted to the health properties of almond milk and almond yogurt, less attention has been paid to improve the physical texture of fermented almond yogurt. In order to address the challenges in regard to the textural properties, a possible solution is to add stabilizers in almond yogurts, such as pectin/xanthan gum and proteins. The supplementation of stabilizers not only improves the soupy and grandy texture, but also enhance the taste of almond yogurt (Dickinson, 2009).
Pectin, an anionic hydrocolloid, is able to interact with the protein molecules, thus, strengthening the protein gel structure and improve syneresis (Guo, 2019). It has also been reported that xanthan gum plays important role in preventing syneresis, increasing firmness, and keeping good consistency without gumminess in yogurts (El-Sayed et al., 2002). In addition, Schkoda et al (2001) illustrated that increasing protein concentration would be another way to improve the firmness and syneresis of yogurts. Particularly, studies of whey protein on improving yogurt qualities have been extensively investigated (Li & Guo, 2006; Walsh et al., 2010). The structure of β-lactoglobulin in whey protein can be manipulated to improve the gelation ability because the pH is subsequently reduced in the fermentation process as a result of lactic acid bacteria. This process is also known as “cold-induced gelation” (Alting et al., 2003). There are two steps in this process: 1), the pH of whey protein is increased above the pI of whey protein and then soluble aggregates are induced in the heating process; 2), after cooling, an acid-induced gel is formed as the pH gradually decreases below the pI of whey protein isolate (WPI). This modified whey protein is called polymerized whey protein (PWP), which is able to form a gel when decreasing the pH.

The gelling properties of PWP have been extensively investigated on improving textual properties in some fermented yogurts. Li et al. (2006) studied the effects of polymerized whey proteins on the consistency and water-holding properties of goat’s milk yogurt in which PWP increased viscosity and decreased the syneresis. PWP has also been used in plant-based yogurts, such as oat-based yogurts and corn-based yogurt, to enhance the gel structure (Walsh et al., 2010 & Wang et al., 2017). Thus, it is reasonable to
hypothesize that PWP could improve the textural properties of almond yogurt with forming a stronger gel structure.

The objective of this study was to develop symbiotic almond yogurt using PWP as a gelation agent and to evaluate the physio-chemical properties and probiotic survivability during a 10-week storage. Lastly, the microstructure of the yogurt was examined with various combinations of PWP with xanthan and pectin.

2.3 Materials and Methods

2.3.1 Materials

Raw blanched almonds and cane sugar (Domino) were purchased from a local market. Chicory inulin was obtained from BENEØ (Orafti®; Belgium). WPI was purchased from Fonterra (NZMP™; Auckland, New Zealand). Pectin was provided by CPKelco (GENUr LM-106; Atlanta, GA). Xanthan gum was purchased from Gluten Free You and Me LLC (Columbus, OH, USA). Sunflower lecithin was obtained from Farbest Brands (Park Ridge, NJ, USA). Maltodextrin was purchased from Bulk Supplement (Henderson, NV, USA). Almond protein was purchased from Noosh Brands (Simi Valley, CA, USA). Anhydrous dextrose (D-glucose) was purchased from Fisher Chemicals (Newington, NH, USA). Calcium citrate tribasic tetrahydrate was purchased from ACROS Organics (Morris Plains, NJ, USA). Vitamin D2 powder purchased from ACROS Organics (Morris Plains, NJ, USA). Starter cultures (YF-L02™) were provided by Chr. Hansen, containing *Streptococcus thermophilus, Lactobacillus bulgaricus* supplemented with *Lactobacillus acidophilus, Lactobacillus paracasei* and *Bifidobacterium animalis* (YF-L02™, Chr. Hansen, Milwaukee, WI, USA).
2.3.2 Methods

2.3.2.1 Polymerization of whey protein

According to the method of Wang et al. (2015), WPI (10% protein, w/v) was dissolved in water at room temperature and stored at 4°C overnight. The pH was increased to 7 (20°C) using 2 M sodium hydroxide before the solution was heated at 85°C for 30 min with constant stirring. The solution was then rapidly cooled to room temperature using ice water.

2.3.2.2 Almond milk preparation

Raw blanched almonds were weighted and then soaked in cold water with 0.5% sodium citrate overnight. Following, the almonds were drained and rinsed three times with cold water. The almonds were reweighed to measure the absorbed water. Almond milk was made at a 1:7 ratio of soaked almond to water. Six of seven parts water (by weight) were added and the mixture was homogenized in a blender (KitchenAid RNSB1570MS) three times for 1.5 min. This mixture was subsequently filtered with 4 layers of muslin cloth to remove solids. The remaining one-part water was used in dissolving hydrocolloids and making sugar syrup.

2.3.2.3 Optimization of production process

The final product formulation was optimized through a series of experiments in terms of PWP (0.4, 0.5, 0.6 % w/v), pectin (0, 0.2, 0.3% w/v), and xanthan gum (0, 0.05, 0.07 % w/v), as listed in Table 3.

The preparation of the symbiotic almond yogurt is shown in Figure 1. Almond slurry was obtained by mixing glucose (1 %), inulin (1%), maltodextrin (3%), almond
protein (0.7 %), lecithin (1%) as well as the optimizing amount of (0.4-0.6 %) PWP; then it was heated at 75°C for 5 min. Cane sugar (5 %, w/w) was blended with pectin and xanthan gum in the remaining one part of water and then heated at 85°C for 10 min to obtain complete dissolution. Next, the hot syrup was cooled to 41.5°C before starter cultures were added (0.01%).

2.3.2.4 Almond yogurt preparation

Following the preliminary studies, the optimized formulation was finalized. Batches were produced with all the ingredients following the optimized process with 0.6 % PWP, sugar syrup with pectin (0.3 %), and xanthan gum (0.05 %) (Figure 1). The mix was then divided into two parts before heating. A control (C) as well as the fortified portion (F) containing calcium citrate (250 mg/L) and vitamin D (133 mg/L) were prepared. Vitamin D powder was weighed (24 mg), dissolved in sunflower oil (92.5 g) and added 1 ml to the almond slurry (5 L). The amount of calcium citrate and vitamin D was calculated based on the 7.5 % and 20% daily value of recommended intake for an adult, respectively. The yogurt mixtures were filled into 8 oz cups (240 mL) and incubated at 41.5 °C for 5 hr. Three triplicates were conducted over the course of three different days. All samples were refrigerated at 4°C and stored for 10-week to evaluate the physio-chemical properties and probiotic survivability.

2.3.2.5 Physicochemical analyses

The samples were analyzed for chemical composition (protein, fat, total solids, and ash) using standard AOAC procedures (Association of Analytical Communities, 2012). Protein was analyzed by the Kjeldahl method. Total protein was calculated by
multiplying the protein nitrogen with the factor 6.25. Fat was determined by the Rose-Gottlieb Method. Total solids content was determined by drying samples in a forced-air oven (1350FM Horizontal Airflow Oven, 120°C for 4 hours). The ash content was determined by ignition at 540 °C in a muffle furnace (Isotemp®550, Fisher Scientific Co., Pittsburgh, PA, USA) overnight. Mineral contents were measured in an ash solution by ICP-OES (Avio 200, Perkin Elmer Optical Emission Spectrophotometer, Hopkinton, Massachusetts, USA). All analyses were measured in triplicate and the values are reported as the mean of three measurements.

The pH of the almond yogurt was measured weekly in triplicates using a pH meter (IQ Scientific Instruments Inc., San Diego, CA, USA). The apparent viscosity (mPa·S) was measured weekly using a Brookfield Viscometer (DV-I prime, Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA) at room temperature 20±1°C for 10 weeks. Viscosity was analyzed using spindle No. 3 at 100 rpm for 60 seconds.

2.3.2.6 Syneresis

Weighed almond yogurt (W1) was fermented in centrifuge cups for 5 hours and stored at 4°C overnight and then centrifuged at 2000 g at 4°C for 10 min (Sorvall RC-5 High Speed Refrigerated Centrifuge, Thermofisher Scientific, Waltham, MA, USA). The upper liquid supernatant was poured out and weighed (W2). The syneresis was calculated according to the following equation:

\[ \text{Syneresis (％)} = \frac{\text{W2}}{\text{W1}} \times 100 \]
2.3.2.7 Survivability of probiotics

Samples were randomly selected from both control and fortified yogurts on a weekly basis for the enumeration of the probiotics. The pour plate method was used to determine the survivability of *L. acidophilus* (ISO 20128:2006/IDF192), *L. paracasei* (CHR Hansen, 2009) and *Bifidobacterium animalis* (Bifidus Selective Medium (BSM) Agar, Sigma Aldrich, Milwaukee, WI, USA). *L. acidophilus* were enumerated by De Man Rogosa (MRS) agar with antibiotics clindamycin and ciprofloxacin. *Bifidobacterium* were enumerated in the commercial BSM agar with provided additives. Both *L. acidophilus* and *Bifidobacterium* were anaerobically incubated at 37°C for three days. The colonies of *L. acidophilus* appeared star-shaped, irregular and tiny; the colonies of *Bifidobacterium* were purple and round. All colonies were counted. *L. paracasei* colonies were aerobically incubated at 20°C in MRS agar for six days, and round and white colonies were counted.

2.3.2.8 Microstructure analysis

Almond yogurts were prepared and fermented in agar wells. Four groups of almond yogurts were prepared: (A) without stabilizers, (B) with PWP, (C) with PWP, pectin and xanthan gum, and (D) with PWP and pectin, xanthan gum plus calcium citrate and vitamin D, and incubated at 41.5°C for 5 hr. Agar wells were made from casting agar (2.5%) in dishes. A thin layer (2mm) of agar was sealed at the bottom of the wells. The samples were placed in the wells and sealed with thin agar disk and a beam of fresh agar liquid. Cubes with samples were cut from the plates. Scanning Electron Microscopy was carried out according to the method of Walsh et al. (2010). In brief, a buffer containing 2.5%
glutaraldehyde in 0.1 M sodium cacodylate was used to fix the agar cubes overnight at 4°C. The samples were then washed three times with the same buffer for 10 min each. After three rinses of diluted (50 mM) cacodylate buffer (pH 7.2), the samples were post fixed in 1.0% osmium tetroxide. The fixed samples were dehydrated in a graded series of ethanol to 100% (2 X), frozen in liquid nitrogen, and then fractured. The fractured pieces were mounted on aluminum scanning electron microscopy (SEM) and sputter coated with 3 nm of Cold/Palladium (Au/Pd). All samples were analyzed using JSM-6060 microscope (JEOL USA, Inc; Peabody, MA, USA) at 5 kV. Micrographs were taken at ×500 and ×3000 magnifications, labelling with number one (left) and two (right) respectively.

2.3.2.9 Statistical analysis

Data were analyzed using the SPSS statistical software version 25 (IBM SPSS Inc., Chicago, IL, USA). Chemical composition data of the almond yogurt samples were analyzed using One-Way ANOVA. The pH, viscosity, and probiotic survivability of the control and fortified groups were statistically analyzed using a Two-Way Repeated Measures ANOVA. Bonferroni post-tests were conducted to compare the means of the control and fortified batches at each individual week. Analysis of covariance was used to compare the trend over 10 weeks.

2.4 Results

2.4.1 Almond yogurt optimization

Almond slurry was made by optimized ratio of almonds and water at a ratio of 1:6 with the content of protein and fat being close to that of conventional yogurt (Table 4). The optimal formula was following: inulin added as a prebiotic at 1%, glucose (1%) as a carbon
source of the cultures, cane sugar (5% w/v) as a sweetener, an additional almond protein (0.7%), lecithin (1%) and maltodextrin (3%) added to increase the total solids and to stabilize the whole suspension, as well as the combination of PWP (0.6%), xanthan gum (0.05%) and pectin (0.3%). Fortification of calcium was chosen at the 7.5% DV (i.e., 1000 mg) for an adult rather than 15% DV fortified in commercial products, which amounts will able to maintain a stable and palatable product as coagulation occurs at a 15% DV level of fortification.

Neither xanthan gum nor pectin combined with PWP produced an overall good almond product in terms of viscosity, syneresis and taste. Almond yogurt with added xanthan gum and PWP not only had higher viscosity value (48.25±0.35mPa·S) than that with pectin and PWP (35.5±1.56 mPa·S), but also exhibited better water holding ability with lower syneresis (19.61±0.39%). Although the combination of PWP (0.4%-0.5%), pectin and xanthan gum increased the viscosity of almond yogurt, the texture was not greatly improved with lower syneresis. The syneresis was lowest with increasing PWP from 0.4% to 0.6% in the presence of xanthan gum (0.05%) and pectin (0.3%), and the viscosity was most desirable compared with the other combinations. In conclusion, almond milk yogurt supplemented with 0.6% polymerized whey protein, 0.3% pectin, and 0.05% xanthan gum offered a better viscosity and lower syneresis, as well as good consistency (Table 3).

2.4.2 Chemical composition

The results of chemical composition were shown in Table 4. Protein contents of both yogurts were about 2.8%. The fat content of fortified almond yogurt (4.60 ± 0.32%)
was slightly higher (P= 0.026) compared to the control almond yogurt (3.47 ± 0.47%). There were no differences between the control and fortified yogurts in terms of protein, total solids and ash. The amount of calcium in fortified almond yogurt is 21.73 ± 0.63 mg/100g, significantly (p=0.021) higher than control group (17.77 ± 1.44 mg/100g). Except calcium, there is no significant difference between the two groups for other minerals, see Table 7.

2.4.3 Physical properties

• pH

There were no differences in pH between the control group and the fortified group during their 10-week shelf life. During the final week the pH of both samples decreased from the initial value of 4.3 to 4.1 (Figure 2).

• Viscosity

The changes in viscosity for both the control and fortified yogurt were small with 47.37 mPa•S and 41.22 mPa•S, respectively. Based on the analysis of covariance (Table 6 and Figure 3), the changes in viscosity showed increase trend statically insignificantly with 49.33 mPa•S and 57.63 mPa•S for the control and fortified samples in the last week. The differences were not significant for most of the testing (except week 6) (Figure 3).

• Syneresis

The syneresis of the control and fortified yogurts were 27.29 ± 0.6 % and 26.53± 0.65%, respectively, for fresh samples; however, after 14 days of storage, the syneresis of both yogurt types declined to 11.41± 1.71 % and 11.21± 1.17 %, respectively. There is no significant difference in syneresis for control and fortified yogurts.

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2.4.4 Probiotic Survivability

The population of *L. acidophilus* was $10^6$ cfu/g in the first week, then decreased quickly and was too low to count at week 4, particularly for the control yogurt (Figure 4).

The initial population of *L. paracasei* was about $10^7$ cfu/g in the first week and increased to approximately $10^{7.5}$ cfu/g after 10 weeks of storage (Figure 5).

The population of *Bifidobacterium* was above $10^7$ cfu/g in the first week, and gradually decreased over 10 weeks; yet, the population maintained at 6.5-7 cfu/g for both almond yogurts during the last week of storage.

There were no differences between the control and fortified products for *L. acidophilus* (*p*=0.922), *L. paracasei* (*p*=0.29) and *Bifidobacterium* (*p*=0.407) over 10-week study (Table 6).

2.4.5 Microstructure

The micrographs of the yogurt samples of the SEM are shown in Figure 7. The large void (solid arrow) was originally occupied by serum and fat. The presence of *S. thermophilus* (beaded strings) and three *Lactobacilli* species (rods) occasionally presented in the SEM images (A2 and D2). Although it is difficult to distinguish them from one another, yet, it still reflected the viable level of probiotic cells in the almond yogurt. The protein network was less open with smaller voids compared with A1 as a consequence of adding PWP, which made the structure (B) more compact and denser. The protein matrix was still relatively loose, compared with C and D almond yogurts with the combination of the PWP and stabilizers. The structure of C and D was similar with relatively well-defined and uniform structure.
2.5 Discussion

Since modern people have realized the importance of balanced diet, the portion of plant-resourced food are increasing in human’s diet. Plant-based alternatives are then rising to meet the current demands for customers, including both nutritional and functional properties. Almond yogurt, fermented by almond milk and probiotics, is considered a novel functional food, highly pleased for its nutritional properties, considering a good source of protein, fat and antioxidants (Chen et al., 2006; Fazilah et al., 2018). However, because of its hydrocolloid’s characteristic, almond milk, making by almond nut and water, needs undergo to optimize the formulation before making almond yogurt. Additionally, almond proteins (amandin) are large and oligomeric, showing poorer solubility and functionality; thus, it hardly handles the same technological processes possessed by their dairy counterparts (Day, 2013). Since the ability of gel formation plays vital role in the development and production of yogurts (Mäkinen et al., 2015), it is necessary to enhance the strength of gel to get a better texture property of almond yogurt.

Therefore, the present study proposed to develop novel almond-based yogurt exploiting the PWP to mimic the typical viscous structural properties of the commercial cow’s milk yogurt. The first adjustment to improve the solubility of almond protein in almond yogurt was achieved with adding pectin, xanthan gum and PWP. Both pectin and xanthan gum, widely used in food industry as thickening agents, were applied in almond milk to enhance the stability of emulsions. PWP, a soluble polymer with high molecular weight, has ability to form gel structure as lowering pH or adding salts (Alting et al.,
When whey protein and polysaccharides presenting together, both of them are negatively charged causing an electrostatic protein-polysaccharide repulsion when the solution pH was above iso-electric point (Ip 6.0) of whey protein. However, when the pH is lower than the protein pI, whey protein is protonated and interacts spontaneously with the carboxyl groups of polysaccharides through electrostatic attraction (Li & Zhong, 2016). As a result, the combination of xanthan gum, pectin and PWP showed superior gelation properties in the study of almond yogurt. XG with PWP formed a firmer gel which may entrap water and other soluble components in the network (El-Sayed et al., 2002).

Another solution to improve the solubility was adapting low temperature treatment (75°C). Studies have shown that heat treatment at 72°C did not show significant reduction in almond protein solubility, but 50% and 70% of the protein would be lost at 85°C and 99°C, respectively (Dhakal et al., 2014). Almond proteins exhibit high hydrophobic properties, so that the emulsifying properties of almond proteins are not suitable for stabilizing fat globules by interfacial protein adsorption (Dhakal et al., 2014). Almond proteins are easily coagulated during the heat process as a consequence of phase separation. The heat process lasted about 20 min to reach to setpoint and was maintained at 75°C for 5 min; thus, it was enough to pasteurize the almond milk for a total of 25 min. Higher temperature (90°C) was used to pasteurize the sugar syrup with xanthan gum and pectin, since pectin wouldn’t be completely dissolved at a certain high temperature.

The quality and texture properties were assessed by 10-week shelf like in terms of physical properties, structure analysis and probiotic survivability. Physical properties (pH,
viscosity and syneresis) are of important for almond yogurt in the shelf life, which directly related to the quality of products. pH and viscosity maintained relatively stable over the 10-week shelf life test. The presence of *L. bulgaricus* tend to continuously undergo metabiotic activities to produce acid, which causes slight decrease in pH (Li et al., 2012). Syneresis, also called “whey off”, is one of the important physical parameters to assess the texture of yogurt. The extent of syneresis in 14 days was 50% less than in fresh yogurt, suggesting that the water-holding capacity of the gel in almond yogurt was increased when storage time increased. A possible explanation might be the contracting effect on almond aggregated matrix that causes more released serum when the pH decreased during storage (Ghorbanzade et al., 2017). Similar results of syneresis in fermented yogurt during storage time have also been illustrated by other studies as well (Aryana et al., 2007; Estrada et al., 2011). Another possible reason might be lecithin. Adding lecithin to almond milk could not only maintain a stable system, but it also could absorb some water and mitigate its separation (Wang et al., 2017). Thus, the rate of syneresis was decrease because of the reduction of free water.

Scanning electron microscopy (SEM) is a useful method for assessing the structure of product products to help investigator analyze the factors affecting physical and textural properties. The micrographs of the yogurt indicated some details of the structure for different combination of PWP and polysaccharides. Without any stabilizers and PWP, the denser gel structure rarely formed with phase separation occurring in the fermentation process. The microstructure showed open, regular and honeycomb like structure, suggesting a weak gel formation during fermentation (A), which was induced by the poor
almond protein network as a result barely forming gel structure in the fermentation process. In contrast, the pores sizes became smaller and less regular with PWP added (B). This type of porous structure tends to trap the surrounding ingredients and immobilize liquid thereby increasing water-holding capacity. Although PWP formed gel structure bonding particles and the water-holding ability was increased as well (B), the matrices in the images did not aggregate compactly. It indicated that the gel strength was still relatively weak without xanthan and pectin. C and D both showed a tighter structure images when comparing with B. There was no apparent difference between C and D when calcium and vitamin D were added to the almond yogurt. These dense matrices were formed by the co-effect of the protein-protein and protein-polysaccharides interactions. Althing et al. (2013) suggested that large molecule polysaccharides, proteins or a mixture of both connect to immobilize solvents and solutes and filling materials. Therefore, the combination of PWP with xanthan and pectin could increase the elasticity and textural properties of the gel.

The evaluation of viable lactic acid bacteria (LAB) to ferment an almond milk-based yogurt is a critical test in the shelf life of new products within the dairy alternative field. Since the health benefits of probiotics are dosage-dependent, it has been suggested that the minimal population of probiotics in commercial probiotic products for clinical effect is $10^6$ cfu/g (Dave & Shah, 1998; Minelli & Benini, 2008). The YF-L02 starter cultures are used in this work, blending of yogurt bacteria (*Streptococcus thermophilus* and *L. bulgaricus*) with LAB (*L. acidophilus, L. paracasei and Bifidobacterium animalis*). The viability of each probiotic might be different, because different factors possess difference impact on the survivability of probiotics. This is reflected in the population of probiotics
during 10-week shelf life. There are several factors affecting the viability of probiotic cultures in yogurt, for example, strain types, interaction between species, production of hydrogen peroxide, pH, and oxygen. For *L. acidophilus*, the main factor for loss of viability over the first four weeks was the reduction of pH during storage. It has been reported that *L. acidophilus* cell population in yogurts decrease as a result of lactic and acetic acid accumulation produced by *L. delbrueckii ssp. bulgaricus* during freezing storage (Shah, 2000). Therefore, selection of appropriate strains on the basis of acid and bile tolerance will help to improve the viability of *L. acidophilus*.

Interaction with other species is another possible reason for the decrease of *L. acidophilus*. The existing of yogurt bacteria (*L. bulgaricus* and *S. thermophilus*) are essential in yogurt manufacturing providing the typical yogurt flavor. However, *L. bulgaricus* and *S. thermophilus* showed negative impacts on some strains of *L. acidophilus* (Ng et al., 2011). This might be due to hydrogen peroxide produced by *L. delbrueckii ssp. bulgaricus* in the presence of oxygen. Yogurt prepared with *L. delbrueckii ssp. bulgaricus* alone yielded seven- to nine-fold higher amounts of hydrogen peroxide than that prepared with *S. thermophilus* alone or with both species (Ng et al., 2011). *L. bulgaricus* had a stronger impact on survivability *L. acidophilus*. It indicated that *L. acidophilus* might be easily influenced by other stains in YF-L02 starter culture. Shortly, these possible explanations for the loss of *L. acidophilus* population in almond yogurt might provide practical information for food industry to further improve the viability of *L. acidophilus*.

*Bifidobacterium* are anaerobic in nature, which means oxygen is a critical factor to their survivability. The population of *Bifidobacterium* in almond yogurt was slightly
decreased during storage with the presence of oxygen. However, the changes of its population in 10 weeks were not considerable (Figure 6). The whey protein in the almond yogurt could provide abundant sulfur-containing amino acids (i.e., cysteine) to *Bifidobacterium* in support of its viable level. It has been reported that cysteine could provide nitrogen as a growth factor and while reducing the redox potential, both of which might favor the growth of anaerobic *Bifidobacterium* species (Dave & Shah, 1998).

It is important to highlight that the population of *L. paracasei* slightly increased in the 10-week shelf-life. Similar results were found by Tharmaraj et al. (2004) in the presence of different probiotics in fermented cheese-based onion. They suggested that *L. acidophilus* might benefit the growth and survival of *L. paracasei*, since the presence of *L. acidophilus* appears to overpower and nullify the antagonistic effects of *Bifidobacterium* on *L. paracasei*. Additionally, the higher acid resistance of *L. paracasei* might contribute to its better performance when the pH decreased from 4.45 to 4.2. *L. paracasei* were also found not adversely affected by any of the bacteria (i.e., *L. acidophilus*, *L. paracasei subsp. paracasei*, *L. rhamnosus*, *Bifidobacterium animalis*, and *Propionibacterium*) in any combinations (Tharmaraj et al., 2004).

The population of *Bifidobacterium* stably maintain above $10^7$ cfu/g and *L. paracasei* remained above $10^6$ cfu/g at the 10th week. These indicated that almond milk substrate contains components suitable for the supporting the growth of *Bifidobacterium* and *L. paracasei*. Therefore, the almond yogurt produced in our study is a suitable carrier for *Bifidobacterium* and *L. paracasei*, which could be considered a symbiotic product with its population of probiotics above $10^6$ cfu/g during 10-week storage. However, the
population of *L. acidophilus* dropped quickly, which showed it was not resilient in almond milk-based matrix within same storage condition.

### 2.6 Conclusions

A symbiotic almond yogurt was formulated using PWP as a gelation agent. The symbiotic almond yogurt has reasonable viscosity and low syneresis. The population of *L. acidophilus* dramatically declined in the first four weeks in the almond yogurt. However, the populations of *L. paracasei subsp paracasei* and *Bifidobacterium animal spp.* remained above $10^6$ cfu/g during 10-week storage period, especially the population of *L. paracasei* remained at $10^7$ cfu/g. The results indicated that the symbiotic almond yogurt might be a good vehicle for both probiotics and prebiotics.
<table>
<thead>
<tr>
<th>PWP (w/v%)</th>
<th>Pectin (w/v %)</th>
<th>Xanthan gum (w/v %)</th>
<th>pH</th>
<th>Viscosity (mPa·S)</th>
<th>Syneresis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>0</td>
<td>0.05</td>
<td>4.26</td>
<td>48.25 ± 0.35</td>
<td>19.61±0.39</td>
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<td>0.4</td>
<td>0.3</td>
<td>0</td>
<td>4.35</td>
<td>35.50 ± 1.56</td>
<td>38.03±0.40</td>
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<td>0.4</td>
<td>0.3</td>
<td>0.05</td>
<td>4.35</td>
<td>43.35 ± 1.91</td>
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<td>0.4</td>
<td>0.3</td>
<td>0.08</td>
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<td>0.5</td>
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<td>40.24±1.17</td>
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<td>0.6</td>
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<td>0.05</td>
<td>4.35</td>
<td>47.37 ± 5.86</td>
<td>26.91±0.53</td>
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<tr>
<td></td>
<td>Control</td>
<td>Fortified</td>
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<td></td>
<td></td>
</tr>
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<td>------------------</td>
<td>------------------</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Total solids</td>
<td>16.77 ± 0.457</td>
<td>16.66 ± 0.307</td>
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<tr>
<td>Protein</td>
<td>2.79 ± 0.006</td>
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<td>Fat</td>
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<td>4.60 ± 0.324     *</td>
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<td>Ash</td>
<td>0.22 ± 0.013</td>
<td>0.21 ± 0.007</td>
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Table 5 Statistical analysis of changes in probiotics, pH and viscosity by 2-way ANOVA (p< 0.05)

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>P value</th>
<th>Significance</th>
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<tr>
<td>L. acidophilus</td>
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<tr>
<td>Group</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>Week</td>
<td>0.007</td>
<td>**</td>
</tr>
<tr>
<td>Week*Group</td>
<td>0.922</td>
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<tr>
<td>L. paracasei</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group</td>
<td>0.993</td>
<td></td>
</tr>
<tr>
<td>Week</td>
<td>0.01</td>
<td>*</td>
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<tr>
<td>Week*Group</td>
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<tr>
<td>Bifidobacterium</td>
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<tr>
<td>Group</td>
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<td></td>
</tr>
<tr>
<td>Week</td>
<td>0.005</td>
<td>**</td>
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<tr>
<td>Week*Group</td>
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<tr>
<td>Group</td>
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<td></td>
</tr>
<tr>
<td>Week</td>
<td>0</td>
<td>***</td>
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<tr>
<td>Week*Group</td>
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<td>Viscosity</td>
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<tr>
<td>Group</td>
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<tr>
<td>Week</td>
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<td>***</td>
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<tr>
<td>Week*Group</td>
<td>0.1</td>
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</table>

*means p< 0.05; ** means p<0.01; *** means p< 0.001
Table 6 Covariance analysis of probiotics, pH and viscosity

| Strains        | Parameter estimates | Prob>|t| | Significance |
|----------------|---------------------|-----|-----|----------------|
|                | Group               | -0.058343 | 0.321 |                |
|                | week                | -0.10327 | <0.0001 | *** |
|                | Group * Week        | -0.017336 | 0.406 |                |
| Bifidobacterium| Group               | 0.004346 | 0.9247 |                |
|                | week                | 0.0542501 | 0.0012 | **             |
|                | Group * Week        | 0.0238551 | 0.1396 |                |
| L. paracasei   | Group               | 0.1597137 | 0.6516 |                |
|                | week                | -1.149592 | <0.0001 | *** |
|                | Group * Week        | 0.0709173 | 0.7765 |                |
| L. acidophilus | Group               | -0.013167 | 0.2116 |                |
|                | week                | -0.030889 | <0.0001 | *** |
|                | Group * Week        | -0.004566 | 0.2134 |                |
| pH             | Group               | -0.971467 | 0.3103 |                |
|                | week                | 0.7721373 | 0.023 | *               |
|                | Group * Week        | -0.455034 | 0.1738 |                |
| Viscosity      | Group               | -0.971467 | 0.3103 |                |
|                | week                | 0.7721373 | 0.023 | *               |
|                | Group * Week        | -0.455034 | 0.1738 |                |

*means p< 0.05; ** means p<0.01; *** means p< 0.001
<table>
<thead>
<tr>
<th>Substance</th>
<th>Control group</th>
<th>Fortified group</th>
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<tbody>
<tr>
<td>Ca</td>
<td>17.77 ± 1.44</td>
<td>21.73 ± 0.63</td>
</tr>
<tr>
<td>P</td>
<td>40.08 ± 1.98</td>
<td>39.01 ± 1.58</td>
</tr>
<tr>
<td>K</td>
<td>48.20 ± 4.14</td>
<td>45.80 ± 1.34</td>
</tr>
<tr>
<td>Mg</td>
<td>20.90 ± 1.50</td>
<td>19.59 ± 0.97</td>
</tr>
<tr>
<td>Na</td>
<td>10.58 ± 1.81</td>
<td>10.81 ± 0.88</td>
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<tr>
<td>Fe</td>
<td>0.58 ± 0.05</td>
<td>0.59 ± 0.10</td>
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Figure 1 Flow chart of the symbiotic almond yogurt preparation
Figure 2 Changes in pH during storage at 4 °C
Figure 3 Changes in viscosity during storage at 4 °C
Figure 4 Survivability of *L. acidophilus* during storage at 4 °C
Figure 5 Survivability of *L. paracasei* during storage at 4 ºC
Figure 6 Survivability of *Bifidobacterium* during storage at 4 °C
Figure 7 SEM micrographs of the symbiotic almond yogurts with different combination of PWP, pectin and xanthan gum. (A1/A2) Almond milk without PWP, pectin and xanthan gum. (B1/B2) Almond milk with PWP, no pectin and xanthan gum. (C1/C2) Almond milk with pectin, xanthan gum and PWP. (D1/D2) Fortified calcium and vitamin D almond with pectin, xanthan gum and PWP. Solid arrow=voids. ST= Streptococci. LA= Lactobacillus.
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