**A Research Proposal**

on

# Isolation and Characterization of Bioactive Component from Quinoa Seeds

Submitted to


in partial fulfillment of the requirements for the award of degree of

**MASTER OF SCIENCE (M. Sc.)**

**IN**

**(FOOD TECHNOLOGY)**

Submitted by: **Bababode Kehinde** Supervised by: **Er. Poorva Sharma**

**FACULTY OF TECHNOLOGY AND SCIENCES**

**LOVELY PROFESSIONAL UNIVERSITY**
**PUNJAB**

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# CERTIFICATION

This is to certify that Bababode Kehinde has personally completed M.Sc. Pre- dissertation entitled '**ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPONENT FROM QUINOA SEEDS'** under my guidance and supervision. To the best of my knowledge, the present work is the result of her original investigation and study. No part of pre-dissertation has ever been submitted for any other purpose at any university.

The project report is appropriate for the submission and partial fulfillment of the conditions for the evaluation leading to the award of the degree of Master of Food Science and Technology.

**Signature of Supervisor**

Er. Poorva Sharma

Assistant Professor,

School of Agriculture,

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# DECLARATION

I hereby declare that the work presented in the dissertation 1 entitled '**ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPONENT FROM QUINOA SEEDS'** is my own original work. The work has been carried out by me at School of Agriculture, Lovely Professional University, Phagwara, Punjab, India under the guidance of **Er. Poorva Sharma**, Assistant Professor (Food Technology) of School of Agriculture, Lovely Professional University, Phagwara, Punjab, India for the award of the degree of Master of Science in Food Technology.

Date: Bababode Kehinde

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I certify that the above statement made by the student is correct to the best of my knowledge and belief.

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# CHAPTER ONE

## 1.0 INTRODUCTION

### 1.1 Project Background

Bioactive peptides are defined as specific protein fragments that have a positive impact on the functioning or conditions of living beings, thereby improving their health (Korhonen and Pihlanto, 2006). The beneficial desirable effects of bioactive peptides are attributed to different properties found in peptides such as antimicrobial (Reddy *et al.,* 2004; Rajanbabu and Chen 2011), antioxidant (Sarmadi and Ismail, 2010), antithrombotic (Wang and Ng, 1999), anti-hypertensive (Erdmann *et al.*, 2008), immunomodulatory activities (Georgiev, 1990; Gauthier *et al.*, 2006), and probiotic potentials among others.

Bioactive peptides (BP) are organic substances synthesized by amino acids and joined by covalent bonds known as amide or peptide bonds. Although some BP exist free as a natural source, majority of the known BP are encrypted in the structure of the parent proteins and are released mainly by enzymatic reactions. Some BP have been synthesized chemically. BP have significant roles in human health by affecting the important body systems - digestive, endocrine, cardiovascular, immune, and nervous systems. BP are considered as the modern generation of biologically active regulators; they are effective in preventing both the oxidation and microbial degradation in foods and also improve the treatments of various diseases and disorders, thus increasing the quality of life. The growing interest in BP has motivated the scientific community and the food industry to researching and exploring the development of new food additives and functional products based on these peptides.

Peptides have proven to bear several useful properties for human health, including antimicrobial, antifungal, antiviral, and antitumor activities. These compounds are synthesized by almost all species of life. However, they are naturally produced in limited quantities. Consequently, researchers have tried to synthesize bioactive peptides to study their properties and applications in various areas.

They are versatile and besides having their applications in food preservation, peptides have also been incorporated into packaging materials. In food preservation, peptides can be integrated into materials to create antimicrobial packaging (Appendini and Hotchkiss, 2002). Through this method, antimicrobial packaging plays a functional role in maintaining the safety and quality of food, since the aim is to prolong food shelf life and to reduce bacterial growth on the product surface (Soares *et al.*, 2009a). This type of active packaging interacts with the product and/or the headspace inside to reduce, inhibit, or retard the growth and metabolic activities of microorganisms that may be present (Soares *et al.*, 2009b).

Food safety is having increasing concern of great importance worldwide. Recently, the costs of diseases caused by food borne pathogens was estimated to be about $152 billion in the United States (Scharff, 2010), and it is estimated that in the United States alone about 47.8 million illness cases, 128000 hospitalizations and 3000 deaths will be caused by food borne pathogens in 2011.

The consumption of processed foods with chemical preservatives has led to increased consumer concern and the demand for more natural and minimally processed foods. As a result, researchers have shown a growing interest in natural antimicrobial agents such as certain peptides.

Peptides with antimicrobial potentials are used as the foremost chemical barrier against microbial attack, being synthesized in response to bacterial infections. They are synthesized by almost all species of life, from microorganisms, plants and animals, to humans (Georgiev, 1990; Hancock and Diamond, 2000). In animals, antimicrobial peptides are produced mainly in those tissues located in positions prone to exposure to adverse conditions such as skin, eyes, and lungs, which are more likely to be in contact with microorganisms (Zasloff, 2002; Papo and Shai, 2003).

Over 700 antimicrobial peptides have been reported, showing considerable variations with respect to their sequence, length, and structure (Papo and Shai, 2003). Antimicrobial peptides, being versatile, have found diverse applications, including those in biomedical devices, food processing equipment, and food preservation.

Quinoa (*Chenopodium quinoa Willd*) is an old crop of the Dicotyledoneae family of high biological value because of its quality protein content. It is a complete food regarded as one of he best sources of vegetable proteins with its protein content similar to that in milk and relatively higher than those present in staple cereals such as rice, wheat and maize though comparatively lower in gluten content (Nisar et al., 2017). Int is used as a flavor enhancer and maintenance of moisture content in baking flours (Vilche et al., 2003).

The use of amaranth and Qunoa as functional foods because of their nutritional composition was reported by Yamani and Lannes (2012). The presence of different types of essential vitamins and minerals and its protein content of high biological value made Sharma et al., (2015) categorize Quinoa as a pseudo-grain or pseudo-cereal. The presence of functional phytochemicals such as; phenolics, phytosterols, PUFAs (Poly Unsaturated Fatty Acids), amino acids, vitamins and minerals was also reportred by Alvez et al.,( 2010).

Quinoa has also been shown to be a potential source of functional peptides (Nongonierma, Le Maux, Dubrulle, Barre, & FitzGerald, 2015; Vilcacundoa et al., 2018)

## 1.2 Scope of the Project

The study attempts to isolate and characterize bioactive peptides from a rich protein source being a rich protein source and to characterize its functionalities.

The protein source that will be used is Quinoa seeds and two strains of probiotic microorganism will be used.

## 1.3 Objectives of the Project

A review of relevant literature revealed that isolation of bioactive peptides from various protein sources have been majorly by enzymatic hydrolysis. Furthermore, the isolation of functional peptides from quinoa as the protein source by fermentation has not been investigated.

The general objectives of the project include:

1. To optimize the extraction of milk from quinoa seeds.

2. To analyze the physicochemical properties of extracted flaxseed milk.

3. To check the proteolysis and hydrolysis of fermented milk.

4. To determine the proteolytic activity of probiotic strains.

5. To characterize the isolated peptides based on their functionalities.

# CHAPTER TWO

## 2.0 LITERATURE REVIEW

### 2.1 History of Bioactive peptide

The study of bioactive peptides dates back to 1902 when Starling and Bayliss discovered a substance secreted from the intestinal lining with the potential of stimulating the secretion of digestive enzymes by the pancreas (Baylis and Starling, 1902). The substance was named secretin and was found to be a peptide with its amino acid sequence determined. Several other peptides were discovered using a similar mechanism of purification and sequencing and the novel scientific concept of research was birthed (Schrader, Schulz-Knappe and Fricker, 2014) In 1950, bioactive peptides from a food source was first discovered by Mellender who found that phosphorylated casein peptides stimulated the bone calcification of rechetic infants without Vitamin D (Mellander *et al*., 1950). Over the years, considerable amount of research has been made on the isolation of bioactive peptides from various food sources.

### 2.2 Description of Bioactive Peptides

Bioactive Peptides (BP) (Shahidi and Zhong, 2008; Sharma et al., 2011; Walther and Sieber, 2011) have been defined as specific protein fragments that have a positive impact on body functions or conditions and may influence health (Kitts and Weiler, 2003). Currently, there are more than 1500 different BP have been reported in a database named 'Biopep' (Singh *et al.*, 2014). BP are organic substances synthesized by amino acids and joined by covalent bonds also known as amide or peptide bonds, whereas proteins are polypeptides with a greater molecular weight (MW). BP and proteins play important roles in the metabolism of living organisms and,

by extension, in human health. They perform hormone or drug-like activities and can be categorized based on their mode of action as antimicrobial, antithrombotic, antihypertensive, opioid, immunomodulatory, mineral binding and anti-oxidative respectively. The amino acid configuration (composition and sequence) determines the activity of the peptides once that they are released from the precursor protein where they are encrypted. Natural processes within the body are regulated by the interaction of specific amino acid sequences that form part of proteins (Fields *et al.*, 2009). Proteins can be classified as endogenous if they are derived from amino acids synthesis within an organism, or exogenous if obtained through the diet or from an external origin to an organism, and they represent one of the primary components of the food. Proteins derived from plant and animal origins are potential sources of a wide range of BP coded in their structure (Carrasco-Castilla *et al.*, 2012; Bhat *et al.*, 2015a). Although the relationship between structure and functional properties is not yet well established, many BP share some similar structural features that include a peptide residue length ranging from 2–20 amino acids (Moller *et al.*, 2008), and the presence of hydrophobic amino acids in addition to proline, lysine or arginine groups. BP have also proved to be resistant and stable to the action of digestion peptidases (Kitts and Weiler, 2003). BP are also considered as new, generational biologically active regulators that can prevent, for example, oxidation and microbial degradation in foods. They are also applicable in the treatment of various medical conditions, thus increasing the quality of life (Lemes *et al.*, 2016). Recently, functional foods (Haque *et al.*, 2008), therapeutic diets, and nutraceuticals (Moldes *et al.*, 2017) have received much attention, particularly for the desirable impact that they can have on human health and their delicate use in the prevention of certain diseases. Consequently, considerable interest and attention has been devoted to the production and properties of BP the past few years. Despite the fact that BP have been identified

and isolated from several natural sources, and their activities investigated in many disciplines, the present review is mainly concerned with BP in the context of different food matrices.

## 2.3 Sources of Bioactive Peptides

Of all the macronutrients available in foods, peptides and proteins are of uttermost importance, because of their functionality in supplying the required raw materials for protein biosynthesis and represent a source of energy (Walther and Sieber, 2011; Dziuba and Dziuba, 2014). In addition, they are an integral part of the intricate series of organic transformations that occur during the handling, processing and storage of foods that ultimately contribute to their organoleptic and sensory characteristics. Besides their nutritional importance, food proteins, and peptides exhibit distinct biological activities (Hartmann and Meisel, 2007; Moller *et al.*, 2008). BP are majorly encoded inside bioactive proteins (Meisel and Bockelmann, 1999). Bovine milk (Torres-Llanez*et al.*, 2005; Korhonen, \2009; Léonil, 2014; Mohanty *et al.*, 2015; Mohanty *et al.*, 2016), cheese (Pritchard *et al.*, 2010), beans, eggs and dairy products are by far the greatest sources of bioactive proteins and peptides derived from foods. However, they can also be obtained from other animal sources such as bovine blood (Przybylski et al., 2016), gelatin (Lassoued *et al.*, 2015), meat, various fish species such as tuna, sardine, herring and salmon. Some plant sources of BP and proteins are wheat (Kumagai, 2010), maize, soy (Singh *et al.*, 2014), rice (Selamassakul *et al.*, 2016), mushrooms, pumpkin, sorghum (Moller *et al.*, 2008), and amaranth (Silva-Sanchez *et al.*, 2008). In vivo, encrypted and bound peptides can be released during gastrointestinal (GI) digestion by proteolytic enzymes such as trypsin or by microbial enzymes. In vitro, BP can also be liberated during food processing or ripening by microbial enzymes (e.g. *Lactobacillus helveticus*) (Gobbetti *et al.*, 2002; Meisel, 2005; Korhonen and

Pihlanto, 2006; Korhonen, 2007; Dziuba and Dziuba, 2014). BP have been discovered and isolated from animal and plants sources and are abundantly present in protein hydrolysates and fermented dairy products. Currently, BP and nutraceutical proteins are being produced to improve human health by preventing or alleviating diverse and chronic medical conditions such as coronary heart disease, stroke, hypertension, cancer, obesity, diabetes, and osteoporosis (Gilani *et al.*, 2008; Boelsma and Kloek, 2009).

## 2.4 Probiotic Microorganisms

Probiotics are defined as living microorganisms, which when administered in adequate, prescribed amounts, are beneficial to the health of the host. Health benefits have mainly been attributed to specific probiotic strains of the following genera: *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, *Enterococcus*, *Streptococcus*, *Pediococ cus*, *Leuconostoc*, *Bacillus*, *Escherichia coli*. The human microbiota is currently getting a lot of attention and research has already proven that alteration of this microbiota may have far-reaching consequences. One of the possible routes for correcting dysbiosis is by consuming probiotics. The credibility of specific health claims of probiotics and their quality and safety must be established through science-based clinical studies (Rijkers *et al.*, 2011). As probiotic properties have been discovered to be strain specific, accurate information about particular strains is also very important. On the other hand, it has also been discovered that the use of various probiotics for immunocompromised patients or patients with a leaky gut has also yielded infections, sepsis, fungemia, bacteraemia. Although the vast majority of probiotics that are used today are generally regarded as safe and beneficial for healthy individuals, there is need for proper caution in

selecting and monitoring of probiotics for patients and complete consideration of risk-benefit ratio before prescribing is highly recommended (Gruber *et al.*, 2013).

Probiotics are non-harmful bacteria that live in the intestines. Besides promoting healthy digestion and absorption of some nutrients, they also protect by crowding out pathogens, such as yeasts, other bacteria and viruses that may otherwise cause disease (Fijan and Šostar-Turk, 2012). Probiotics manifest a mutually advantageous symbiosis with the human gastrointestinal tract. They benefit from the ingested foods and the human body utilizes the byproducts of their life processes. Acidophilus is the most well known probiotic, but there are several thousands of other strains that offer health benefits (Relman, 2002).

It is scientifically established that certain species of microorganisms cause sickness and in extreme cases, death. Few examples of such deadly microorganisms include *Yersinia pestis*, influenza virus, AIDS/HIV virus, *Clostridium tetani*, *Mycobacterium tuberculosis* and *Vibrio cholerae*. Recently; several multi-drug resistant bacteria have been known to cause important health-care associated infections. Dangerous serotypes of these bacteria have led to the emergence of food-poisonings due to production of enterotoxins. Some of these bacteria are medically important and they include: methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), extended-spectrum beta-lactamase (ESBL) producing Enterobacteriaceae, multi-drug resistant *Pseudomonas aeruginosa*, multi-drug resistant *Mycobacterium tuberculosis* and Enterohemorrhagic *Escherichia coli* (EHEC) (Vieira, Teixeira and Martins, 2012)

Throughout the history of microbiology, most human studies have been focused on the disease-causing organisms associated with people; whilst fewer studies have examined the benefits of the resident bacteria (Savage, 1977). The biological system of microorganisms that live in/or on the human body have their biological importance. This biological system is known as the human microbiome. It has been known that the human body is inhabited by a minimum of 10 times more bacteria than the number of human cells in the body, and that majority of these bacteria are found in the human gastrointestinal tract (Ubeda and Pamer, 2012). The biological relationship between the host and the gut microbiota in human is symbiotic. However, commensal intestinal microbiota contributes extensively to the enhancing of the body's resistance against infections, differentiation of the host immune system, synthesis of certain nutrients such as vitamins, short-chain fatty acids and other low molecular mass molecules (Ramakrishna, 2013).

### 2.4.1 Lactobacilli

*Lactobacillus acidophilus* is the most popular probiotic and one of the most important for the health of the small intestine. Besides the lining of the intestine, acidophilus has the potential to take up residence in the vagina, cervix or urethra (Walter, 2013). Acidophilus inhibits the proliferation of pathogens, and produces such natural antibiotics as lactocidin and acidophilin, which enhance immunity. Acidophilus has anti-microbial effects against popular pathogens of public health concern such as *Staphylococcus aureus, Salmonella, Escherichia coli* and *Candida albicans* (Makarova *et al.*, 2006).

*Lactobacillus brevis*, abbreviated *L. brevis*, is a lactic acid synthesizing probiotic that is helpful in synthesizing Vitamins D and K.

*L. bulgaricus*, used in yogurt fermentation performs a protective role by producing lactic acid, which creates a friendly environment for other species (De Vuyst, 2014).

*L. plantarum* produces lactolin, another natural antibiotic. Plantarum can also produce L-lysine, an anti-viral amino acid. This organism destroys nitrate, promoting nitric oxide levels and decreases pathogens (Bauer, du Toit and Kossmann, 2010).

*L. rhamnosus* has a high tolerance to bile salts, surviving in less favourable conditions. This species have shown numerous benefits to the elderly and infants alike. *Rhamnosus* helps those that are lactose intolerant, protects the small intestine, and produces lactic acid in the large intestine (Taverniti *et al.*, 2006). Other lactobacilli strains include *L. fermentum, L. caucasicus, L helveticus, L. lactis, L. reuteri* and *L. casei.*

### 2.4.2 Bifidobacteria

*Bifidobacterium bifidum* is the most recognized specie of this category. Living and growing within the mucus lining of the large intestine and/or vaginal tract, bifidum prevents pathogenic bacteria and yeast from invasion (Sarkar, 2010). Bifidum creates favorable changes in pH levels by synthesizing lactic and acetic acids (Walker, 2013). Furthermore, this species increase absorption of iron, calcium, zinc and magnesium.

*Bifidobacterium* species have received particular attention and their study reveals many insights into the various potential therapeutic applications of probiotic bacteria (Didari *et al.*, 2006). These organisms predominate in the gastrointestinal tract of babies fed with human milk where they account for some 95% of the flora (Seale and Millar, 2014). Breast-fed infants have a much

lower rate of GI- tract infections in comparison with babies fed on bovine or other milks. The contrast between the two groups of neonates in developing countries is particularly noticeable. Bifidobacteria has been discovered to be responsible for the resistance of breast-fed infants to enteric infections. The striking predominance of these bacteria is due to selective agents in meconium (the sterile fluid in the GI-tract of human neonates), human colostrum and human milk (Walker, 2013). These selective factors are referred to as 'bifidus growth factors'.

*B. infantis* stimulates the production of cytokines that has positive effects on the immune system, and can destroy such pathogens as *Clostrida, Salmonella and Shigella.*

*B. longum* colonizes the large intestine. It fully occupies it and prevents unfriendly bacteria and yeast from taking residence. This can considerably decrease the frequency of gastrointestinal problems, such as diarrhea, and nausea during antibiotic use.

### 2.4.3 Other Strains

*Streptococcus thermophilus* is another probiotic used in yogurt production. Breaking down lactose to create lactase, the enzyme that digests milk sugars, this species can help those who are intolerant to lactose. Other strains of *Streptococcus* include *infantis, cremoris,* and *faecium*

Studies have proven *Enterococcus faecium* to be helpful for diarrhea, shortening duration of symptoms. It also destroys pathogenic microbes, such as rotavirus (Hadji-Sfaxi *et al.*, 2011). Studies have also shown this strain to lower LDL (or bad cholesterol) considerably. This organism has a high resistance to antibiotics. Although a transient guest, *Enterococcus faecium* is a welcome, harmless natural resident in the human body (Pieniz *et al.*, 2014). Popular probiotic microorganisms include the *Bifidobacterium* and *Lactobacillus* genera. Other bacteria and yeasts

e.g. *Saccharomyces boulardii* have also been used extensively (Thygesen, Glerup and Tarp, 2012). *Bifidobacterium* species and strains of Lb. acidophilus and Lb. casei are in frequent usage.

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**Table 1.** Bioactive peptides and their functions.

| Peptide | Function |
|---|---|
| **Antimicrobial** | |
| LRLKKYKVPQL | Interacts with bacteria to cause inhibition. |
| PGTAVFK | Causes bacteria and yeast membrane destruction. |
| KVGIN, KVAGT, VRT, PGDL, LPMH, EKF, IRL | Inhibits *Listeria ivanovii* and *E. coli* growth. |
| Lp-Def$_1$ | Interacts with and impairs mitochondrial functions in *C. albicans*. |
| Maize α-hairpinins | Binds to microbial DNA to cause cell death. |
| **Antihypertensive** | |
| IVWY, FQ, VVG. IVWY, VAE, WTFR<br>IPYKLRP, PYKLRP, YKLRP, GILRP<br>VPP, IPP<br>GAAGGAF<br>LIVTQ, LIVT<br>LLKPY<br>AHLL<br>FISNHAY<br>AAATP<br>LGL, SFVTT<br>IIT | Inhibit ACE in thoracic aorta tissue and suppress angiotensin II-mediated vasoconstriction.<br>Competitively bind and inhibit ACE and results in blood pressure reduction |
| ADVFNPR, VVLYK, LPILR, VIGPR | Lower endothelia-1 levels significantly |
| **Anti-type 2 diabetes mellitus** | |
| PPL<br>YP, LP, IPI, VPL, IPA, IPAVF<br>PGVGGPLGPIGPCTE, CAYNTERPVDRIR, PACCGPTISRPG<br>GPAE, GPGA<br>MHQPPQPL, AWPQYL, SPTVMFPPQSVL, VMFPPQSVL, AWPQYL and INNQFLPYPY<br>ILAP, LLAP, MAGVAHI<br>IP, MP, VP, LP<br>LKPTPEGDL, LPYPY, IPIQY and WR | Inhibits dipeptidyl peptidase-IV |
| **Immunomodulatory** | |
| GFLRRIRPKLKT | Significantly inhibits LPS-induced nuclear translocation of NF-κB/p65, inhibits IL-1β and enhances TNF-α release. |
| St20 | Inhibits human T lymphocyte surface marker CD69 expression and cytokine IL-2 secretion. St20 also inhibits TNF-α and IFN-γ secretion in the activated human T lymphocytes. |
| PTGADY | Significantly increases the production of IL-2, IL-4, and IL-6. |

A = alanine, R = arginine, N = asparagine, D = aspartic acid, C = cysteine, E = glutamic acid, Q = glutamine, G = glycine, H = histidine, I = isoleucine, L = leucine, K = lysine, M = methionine, F = phenylalanine, P = proline, S = serine, T = threonine, W = tryptophan, Y = tyrosine, V = valine. LDL: Low-density lipoprotein, IL: Interleukin, TNFα: tumor necrosis factor alpha, DPPH: 2,2-diphenyl-1-picrylhydrazyl,

**Source: Daliri, Deog and Lee, 2017**

### 2.4.4 Health Benefits of Probiotics

According to the Food and Agriculture Organisation of the United Nations (FAO) and the World Health Organisation (WHO) probiotics are defined as live microorganisms, which when administered in adequate amounts, confer a health benefit on the host. This definition of probiotics is also adopted by the International Scientific Association for Probiotics and Prebiotics (ISAPP) and is used in most scientific publications. However, this definition is not accepted by European Food Safety Authority (EFSA) or the U.S. Food and Drug Administration (FDA) currently because they insist that the health claim incorporated in the definition is not measurable due to the fact that commercial markets have outpaced the ability of science to substantiate the evidence.

A wide range of benefits have been attributed to the ingestion of probiotics which includes;

- Adequate prevention of traveler's diarrhea
- Prevention of the possible outgrowth of spores of *Clostridium botulinum* in the GI-tract, the associated toxin production and a possible cause of sudden infant death syndrome (SID)
- Enhancing the immune system, improving resistance to infection and improving the general health status
- Effective protection against certain types of cancer
- Considerable lowering of serum cholesterol levels and reducing the incidence of coronary heart disease
- Prevention and treatment of peptic ulcer disease
- Treatment of intractable diarrhoea following antibiotic therapy
- Reducing inflammation caused by allergies and improved lactose digestion and reduction in intestinal bloating, flatulence and discomfort

## 2.4 Quinoa Seeds as a Rich Protein Source and Functional Food

Quinoa is described as a pseudo-cereal consumed by the South American Andean culture basically as a staple food. Over the years, the production and marketing of Quinoa seeds has increased progressively gaining attention worldwide. This has been related to its high biological value and also because it is free of gluten. Futhermore, the presence of several phytochemicals such as flavonoids, saponins and phenolic acids which makes it biologically functional avails it of its remarkable advantage over other plant foods (Vilcacundo & HernándezLedesma, 2017; Vilcacundo et al., 2018)

Quinoa proteins, in addition to their high nutritional value have been found to possess some health benefits and also as a potential source of biologically functional peptides. In a study conducted by Aluko and Monu (2003), peptides enzymatically isolated using the alcalase enzyme were found to have the potential of scavenging the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical. More recently, quinoa hydrosylates prepared with papain were found to have a considerable scavenging effect on the peroxyl radical. (Nongonierma, Le Maux, Dubrulle, Barre, & FitzGerald, 2015)

Quinoa seeds are of high protein content of about 12-19% making them one richest sources of high biological value protein among other known grain crops (Przybylski, R. et al., 1994). The table below shows the amino acids of Quinoa proteins and their relative composition expressed in percentage.

Over the years, studies have been conducted relating to the characterization of bioactive peptides based on their health benefits. Aluko and Monu, 2003 in a study on the functionality and biological activities of protein hydrosylates fractionated from Quinoa seeds found the

hydrosylates to possess antioxidant potentials and antihypertensive health benefits by the inhibition of the angiotensin converting enzyme (ACE). Futher more, Takoa et al., (2005) in a study found that the protein-enriched fragment of Quinoa seeds had an invivo cholesterol-lowering effect in mice. Recently, Nongonierma et al., 2015 studied the DPPIV (dipeptidyl peptidase IV) inhibition and antioxidant potentials of quinoa protein hydrosylates isolated in vitro through the use of the papain enzyme. Their findings revealed that the hydrolyzed protein isolates showed considerable inhibition of DPPIV and scavenging of oxygen free radicals.

| Amino acid | Content (%) |
|---|---|
| Arginine | 14.53 |
| Leucine | 13.96 |
| Lysine | 12.97 |
| Isoleucine | 12.54 |
| Treonin | 8.97 |
| Valine | 7.84 |
| Phenyalanin | 6.84 |
| Tyrosine | 5.55 |
| Histidine | 5.27 |
| Cysteine | 4.70 |
| Methionin | 4.70 |
| Tryptophan | 2.14 |

**Table 2.2:** Amino acids of Quinoa proteins and their composition (Benaiges, M.A., 1997)

## 2.5 Production and Processing of Food Protein-Derived Bioactive Peptides

From review of relevant literature, conventional methods applied in the isolation of bioactive peptides have been by fermentation, enzymatic hydrolysis of food proteins, (Lee and Hur, 2017) or by chemical synthesis (which is mostly done for their purification and/or characterization). In some cases however, water extracts of mushrooms and some plant parts have proven to be potential sources of bioactive peptides (Geng *et al*., 2016).

### 2.5.1 Microbial fermentation

This involves culturing some microbes (bacteria or yeast) on potential protein substrates to hydrolyze the proteins with their enzymes as they grow. The growth of the bacteria or yeast causes the secretion of their proteolytic enzymes into the protein material to release peptides from the parent proteins. In most cases, the microorganism of choice is grown to its exponential phase in a broth at a temperature condition suitable for its growth. The cells are harvested, washed and then suspended in sterile distilled water usually containing dissolved glucose. The suspended cells are then used as a starter to inoculate a sterilized protein substrate (Aguilar-Toalá *et al.,* 2017; Rizello *et al.,* 2017). The extent of hydrolysis extensively depends on three factors – the strain used the type of protein and the fermentation time.

### 2.5.2 Enzymatic activity

The most common way to produce bioactive peptide is enzymatic hydrolysis. Digestive enzymes and combination of different proteinases including alcalase, chymotrypsin, pepsin and thermolysin as well as enzyme from bacterial and fungal source are used for the breakdown of protein.

# CHAPTER THREE

## 3.0 MATERIALS AND METHOD

### 3.1 Optimized Extraction of Quinoa Milk.

The extraction method that will be used is similar to the Cornell method applied by Abagoshu et al., 2015 in the extraction of soy milk from soy beans. The exception will be in the material (Quinoa seeds), the grounding temperature and the water proportion used.

Quinoa grain (200g) will be soaked in water (1litre) at 27-35°C for a 6 h time period. Soaked grains will be drained and the outer seed coat will be dehulled mechanically followed by rinsing with tap water. The grins will be further grinded with water in varying ratios (1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8 and 1:9) in a high speed blender. The grain slurry will be subjected to indirect heating in water bath at 85°C for 45 min (Rekha et al., 2013) and then filtered through double layer cheese muslin cloth to separate the quinoa milk from residue.

The total solid of each grinding combination that produces Quinoa milk with total solids ranging between 9-11% is selected.

### 3.2 Evaluation of Physicochemical Properties

The extracted milk will then be analyzed for its physicochemical properties such as moisture content, protein, ash, total solids, pH, and titrable acidity.

The moisture content will be determined using the AOAC (2010) method. The protein content will be determined by the micro-Khedjal method (AOAC, 2007). The total solid content will be

determined by mathematically by subtraction of the moisture content.. The pH will be determined using an electronic pH meter. The titrable acidity will be determined by titrating 2ml of the milk with 0.1 N NaOH using phenolphthalein as an indicator to a reaction endpoint of pH value 8.3.

### 3.3 Bacterial Strains and Proteolytic Activity Examination

Some probiotic Lactic acid bacteria such as *Lactobacillus fermentum, Lactobacillus delbrueckii, Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus casei and Lactobacillus helveticus* will be acquired from Microbial Type Culture Collection (MTCC), Chandigarh, India. All procured strains will be kept at frozen condition in MRS agar broth with glycerol (15%).

The proteolytic potential of the above mentioned LAB strains will be examined by the methods applied by Beganovic *et al*., 2013 using skim milk agar for the assay. The well of the skim milk agar will be inoculated with a viable culture of probiotic strains and incubated at a temperature of 37°C for a period of 4h. The presence or absence of a clear zone around the inoculated agar well after the incubation period will be checked and recorded.

The dual plate assay method as described by Pescuma *et al*., (2010) will be used to check the compatibility of the bacterial strains with the proteolytic activity.

### 3.4 Seed Culture Preparation

The LAB strains with the highest proteolytic activities are purified under primary, secondary and tertiary seed (10%) cultures respectively. The spectrophotometric readings of the tertiary culture are taken at 610nm and centrifugation at 10000 rpm for 10 mins takes place. The pellet is then

collected, washed with sterile salt (0.85%,NaCl) and incubated at 37°C to be used for fermentation.

### 3.5 Quinoa Milk Fermentation

The washed and incubated cell pellet is then dissolved in equal volume of Quinoa milk and incubated at 37C for fermentation to take place.. during fermentation, at every 2h time interval, the pH, proteolytic activity, titrable acidity and total soluble solids will be checked. During fermentation, the proteolytic activity of the LAB will be checked using the *o*-phthalaldehyde (OPA) test. This test works on the principle that the α – Amino group released during protein hydrolysis reacts with 2-mercaptoethanol and *o*-phthalaldehyde to produce an adduct of strong absorption at 340nm. This absorption phenomenon is similar for all α – amino groups. When a combination of maximum proteolytic activity is obtained with a pH of less than 3, the fermentation is stopped.

### 3.6 Purification

After fermentation, the solution will be centrifuged at 10,000rpm for 10 mins and the supernatant passed through a syringe filter for purification.

### 3.7 Hydrolytic Assessment

The hydrolytic assessment will be evaluated by the SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) as suggested by Schagger and Von Jagow (1987), however, with some modifications. Both the fermented and non-fermented WPC (Whey protein Concentrate) media will be treated with the SDS (10%) for 10 mins at a temperature of 90C

centrifuged at 10,000 rpm for 10 min. Supernatant was collected and 2 µl of each sample was prepared separately in denaturing 4X buffer and heated at 100°C for 5min before electrophoresis. Gel was run in a Tris-glycine buffer at a constant current of 25 mA. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250.

### 3.8 Fractionation and Identification of Peptides

The bioactive peptides will be fractionated with a vivaspin centrifugal concentrator through the use of a different molecular weight membrane. Identification of active peptides will be carried out with the use of 2D gel electrophoresis, mass spectrometry and finally N terminal analysis (Makinen *et al*., 2014).

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