分类号　　密级

博 士 学 位 论 文

题　　目：	发酵小米多肽的抗氧化与抗菌活性的研究
英文并列题目：	**Study on Antioxidant and Antimicrobial Activities of Peptides Derived from Fermented Foxtail Millet**
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专　　业：	食品科学
研 究 方 向：	分子营养
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学位授予日期：	**2014-05-29**

答辩委员会主席：夏文水

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二〇一四年六月

Study on Antioxidant and Antimicrobial Activities of Peptides Derived from Fermented Foxtail Millet

By

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A thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy (PhD)

In

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May 2014

DECLARATION

I, Amadou Issoufou do solemnly declare that this report entitled "Study on the Fermented Foxtail Millet-Derived Peptides with Antioxidant and Antimicrobial Activities", is my personal research work under the supervision of Professors Le Guo Wei and Shi Yong Hui in the the Institute of Food Nutrition and Functional Factors, School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu Province, P. R. China during the period of 2010 – 2014. I further declare that this work is original and has never been submitted to this university or elsewhere for the award of any academic qualification.

Signed:.....Date: 29 May 2014

Amadou Issoufou

CERTIFICATION

This is to certify that the research work presented in this report entitled "Study on the Fermented Foxtail Millet-Derived Peptides with Antioxidant and Antimicrobial Activities" by Mr Amadou Issoufou, for the award of Doctor of Philosophy (PhD) in Food Science based on the results of studies carried out by him in the Institute of Food Nutrition and Functional Factors, School of Food Science and Technology, Jiangnan University, Wuxi, P. R. China, under my guidance and supervision during the academic season 2010 – 2014. I further certify that neither the research report nor any other part thereof has been submitted previously for consideration of award of a degree and/or any other academic qualification here or elsewhere.

Signed: Guowei Le / Yonghui Shi Date: 29 May 2014.

Prof. **Le Guo Wei** / Prof. **Shi Yong Hui**

(Supervisors)

DEDICATION

This work is dedicated to:

~ My late parents and family members, may their souls rest in peace;

~ My wife, my pride and joy;

~ Orphans who find it so difficult to eat, even little to keep their body and soul together.

ACKNOWLEDGEMENT

My sincere gratitude goes to my research work supervisors, Prof. Le Guo Wei and Prof. Shi Yong Hui under whose diligent and impressive supervision this research was undertaken. Their advice direction, encouragement and guidance throughout the work were immense, and have made it a reality. To achieve this would not have been possible were it not for the opportunity and financial support granted to me by the Governments of the Republic of Niger and People's Republic of China; to them I remain grateful. I will forever remember the support and motivation of Ass. Prof. Sun Jin. I would also like to thank all the teachers and my lab mates for their help in ensuring a smooth run in the lab. Special thank go to my sisters Mariam Nalwoga and Dr Maureen J. Cheserek; my brothers Oumarou Hama Moutaleb, Dr Mohamed Tabita Kamara, Dr Amza Tidjani, Md Charles Ndawula, Dr Zakari M Mounir, Dr Checkna Daou, Hassane H Alkassoumi, Dr Taha AA Naji, Dr Mohammed AA Gasmalla, Dr Camel Lagnika and Dr Gounga E Mohamadou for their support and friendship.

I really appreciate the support, motivation, love and care given by my nephew, Oumarou A. Sara and my Jiangnan University friends. May they live long to reap the fruit of their labor.

Lastly, all glorifications go to Almighty Allah, the Merciful, and the Beneficent, who in His infinite mercy sustained me throughout. And to so many others, I say thank you. May Almighty Allah bless you all!

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LIST OF ABBREVIATIONS

AAR: amino acid residue

AMPs: antimicrobial peptides

BATH: bacterial adherence to hydrocarbons

CFU: colony-forming unit

CSH: cell surface hydrophobicity

DNA: deoxyribonucleic acid

DPPH: 1,1-diphenyl-2-picrylhydrazyl

DSC: differential scanning calorimetry

FFM: fermented foxtail millet flour

FFMB: fermented foxtail millet bran

FFMBP: fermented foxtail millet bran with added protease

FFMH: heat-moisture treated fermented foxtail millet flour

FFMP: fermented foxtail millet flour with added protease

FFMp: fractionated foxtail millet peptides

FFMp10: fermented foxtail millet meal peptide fraction

FM: foxtail millet flour

FMH: heat-moisture treated foxtail millet flour

HMT: heat-moisture treatment

HPLC: high performance liquid chromatography

HPLC: high-performance liquid chromatography

НΦ: hydrophobic index

LC-MS: liquid chromatography mass spectrometer

log P: partition coefficient of amino acids hydrophobicity contributions

Mp1, Mp2, Mp3 and Mp4: modified FFMp10 sequence

MW: molecular weight

$O^{2\bullet-}$: superoxide anion

PBS: phosphate buffered saline

PER: predicted protein efficiency ratio

RDS: rapidly digesting starch

RP-HPLC: reverse phase high-performance liquid chromatography

RS: resistant starch

RVA: rapid visco-analysis

SDS: slowly digesting starch

SE-HPLC: size-exclusion high performance liquid chromatography

SEM: scanning electron microscopy

TCA: trichloroacetic acid

TFA: trifluoroacetic acid

THAA: hydrophobic amino acids

XRD: x-ray diffraction

ABSTRACT

Millets are a major food source in arid and semi-arid parts of the world. Millets are good sources of energy. They provide protein, fatty acids, minerals, vitamins, dietary fibre and polyphenols. Typical millet protein contains high quantity of essential amino acids especially the sulphur containing amino acids (methionine and cysteine). Processing millet by milling removes the bran and germ layers that are rich in fibre and phytochemicals, causing significant loss. The millets are source of antioxidants, such as phenolic acids and glycated flavonoids. Millet foods are characterized to be potential prebiotic and can enhance the viability or functionality of probiotics with significant health benefits. Traditionally fermented foods and beverages obtained from millet or millet mixed with other cereals (corn and sorghum) include *koko* (millet porridge), *fura, mangishi, jandh, uji, burukutu, kunu-zaki, ogi* and *bushera* while *dambu, masvusvu* and roti are representatives of unfermented millet based products. Solid-state bioprocessing of foxtail millet by *Lactobacillus paracasei* Fn032 is a biotechnological strategy to produce fermented foxtail millet meal with more beneficial components.

The effect of *L. paracasei* Fn032 fermentation and heat-moisture treatment (HMT) on the physicochemical properties of foxtail millet (S*etaria italica*) flour was investigated. The results obtained showed a significant ($P < 0.05$) increase in proximate chemical composition in general after fermentation and HMT. Differential scanning calorimetry analysis showed high decomposition temperature (T_d) trend of 180.59 and 189.82^{o}C after HMT. However, there was significant ($P < 0.05$) enthalpy (ΔH) decrease. Flour digestion resulted in variation of slow digestible starch and resistant starch count from 6.83 to 18.42% and 7.61 to 22.68% respectively, after fermentation and HTM. Following this observation, it was ascertained that in X-ray diffraction; pasting viscosity and fluorescence spectrophotometry show greater HMT influenced on the flour components. Findings from the scanning electron microscopy analysis showed microstructure differences of the flours samples.

Evaluation of antioxidant, antimicrobial properties and nutritional values of water extracts from fermented foxtail millet flour and its bran with and without protease by *L. paracasei* Fn032 was done. Fermented foxtail millet flour with added protease extract showed higher scavenging ability on DPPH radicals as well as reducing power than

fermented foxtail millet flour and fermented foxtail millet bran extracts. Both extracts, fermented foxtail millet flour and fermented foxtail millet flour with protease, showed significant ($P < 0.05$) effectiveness inhibition abilities on microbial growth when compared with fermented foxtail millet bran extracts. Amino acid profile revealed that fermented foxtail millet flour with protease, with relatively strongest antioxidant, antimicrobial activity, also had the highest total hydrophobic amino acids content (51.39%) and hydrophobic index (8.47 Kj/moL amino acid residue). Moreover, fermented foxtail millet flour with protease revealed the highest protein content, predicted protein efficiency ratio, and protein digestibility. Molecular weight of the whole extracts varied from 180–5000 Da.

Purification by RP-HPLC and amino acid sequencing by LC-MS of peptides derived from foxtail millet (*Setaria italica*) meal fermented by *L. paracasei* Fn032 were carried out. The purified foxtail millet peptide fractions (FFMp) of Tyrosine/Leucine-rich (FFMp4=756.84, FFMp6=678.74 and FFMp10=678.87 Da) showed significant ($P < 0.05$) scavenging activities for DPPH, and superoxide anion radicals. FFMp peptides (synthesized) have shown fairly inhibition of the Escherichia coli ATCC 8099 growth. Furthermore, FFMp4 and FFMp6 peptides showed resistance to trypsin proteolysis while FFMp10 appeared to have partial hydrolysis.

The effect of chemically synthesized short peptides (6 residues), previously identified from a fermented foxtail millet meal fraction (FFMp10) on the cell surface hydrophobicity (CSH), biomass growth, DNA binding ability of *Escherichia coli* ATCC 8099 was verified. The proteolytic responses of these peptides were also tested using the high performance liquid chromatography. Changes made in the FFMp10 sequence were: Mp1, Mp2, Mp3 and Mp4 peptides sequences by Arg and Lys amino acids substitutions. The results revealed that all the peptides resisted to pepsin treatment for 1 h. However, Mp2, Mp3 and Mp4 showed no significant HPLC elution profile changes after the trypsin treatment. The values of CSH were significantly higher ($P < 0.05$) with FFMp10, Mp1 and Mp2 2 h treatment on bacteria, whereas, lowered with Mp3 and Mp4 treatment. Overall peptides treatment lowered the rate of bacterial biomass growth when compared to control. Slight *E. coli* ATCC 8099 DNA bindings were observed in lane 2, 3 and lane 3 respectively for Mp1 and Mp2 incubation.

Fermentation and heat moisture treatment methods present a possible way of changing or improving the physicochemical properties and add nutritional value to foxtail millet meal. Fermented foxtail millet flour extracts were relatively effective in the antioxidant, antimicrobial properties assayed. Indeed, it is feasible to derive natural antioxidants peptides along with antimicrobial activity and resistance to enzyme from fermented foxtail millet meal.

Keywords: Foxtail millet, Fermentation, *L. paracasei* Fn032, Short peptides, Antimicrobial, Antioxidant, Proteolytic resistance, Hydrophobicity, DNA binding

摘要

小米是世界上干旱和半干旱地区的主要的食物来源，可为人类提供蛋白质、 脂肪酸、 矿物质、 维生素、 膳食纤维以及多酚类物质。典型小米蛋白含有大量的人体必需氨基酸尤其是含硫氨基酸 （如蛋氨酸和胱氨酸）。小米加工过程中除去了富含纤维素和植物素的麸皮层和胚芽层，因此造成了小米的营养流失。另外，小米也是酚酸类和黄酮类抗氧化物质的主要来源。小米食品可作为一种潜在的益生元，它能够显著增强益生菌的活性和生物学功能。许多传统发酵食品和饮料来自小米或小米与其他谷物 （玉米和高粱）混合物，包括 *Koko* (小米稀饭)、 *Fura*、 *Mangishi*、 *Jandh*、宇治、 *Burukutu*、库努崎、奥吉和 *Bushera* 而 *Dambu*、 *Masvusvu* 和罗迪是典型的非发酵小米食品的代表。用副干酪乳酸杆菌 Fn032 对小米进行固态发酵是一种典型有效的生物技术处理方法。

本文研究了副干酪乳杆菌 Fn032 发酵和湿热处理对小米粉理化性质的影响。结果表明，发酵和 湿热处理方法能显著增强小米粉中功能性化学成分的含量($P < 0.05$）。差示扫描量热分析显示湿热处理 ，小米粉出现了较高的分解温度 (Td)，温度范围在 180.59 oC 到 189.82oC 之间，而热 焓量 (Δ H) 显著下降。发酵和湿热处理处理后，慢消化淀粉和抗性淀粉含量分别为 6.83 - 18.42%，以及 7.61- 22.68%。另外，x 射线衍射对糊化特性的分析及荧光分光光度计结果显示湿热处理对小米粉的组成有很大的影响。扫描电镜分析结果显示了小米粉样品微观结构的差异性。

对副干酪乳杆菌 Fn032 发酵以及蛋白酶处理的小米粉及其米糠水提物的抗氧化、抗菌性能及营养价值评价结果表明。发酵小米粉中添加蛋白酶后的水解提取物与发酵小米面粉和发酵米糠提取物相比有较高的 DPPH 自由基清除能力。小米发酵物中添加或不添加蛋白酶，与发酵米糠提取物相比，表现较强的抑菌能力。氨基酸图谱分析表明发酵小米中添加蛋白酶组，具有较强的抗氧化、 抗菌活性，其总疏水性氨基酸含量达到最高(51.39%) ，其疏水性指数为 （8.47 Kj/moL 氨基酸残基)。此外，蛋白酶水解的小米发酵物组蛋白质含量达到最高、有效提高蛋白比和蛋白质消化率。提取物的分子量分布范围为 180 – 5000 Da。

反相液相色谱纯化副干酪乳杆菌 Fn032 发酵小米肽，并通过液质联用测定分离产物的氨基酸序列。富含酪氨酸/亮氨酸的小米肽（FFMp） (FFMp4 = 756.84，FFMp6 = 678.74 和 FFMp10 = 678.87 Da) 有较强的 ($P < 0.05$) DPPH 和超氧阴离子自由基的清除能力。合成的 FFMp 多肽能够显著抑制大肠杆菌 ATCC 8099 的生长。此外，FFMp4 和 FFMp6 显示了抗胰蛋白酶水解能力，而 FFMp10 有部分被水解。

化学合成短肽（含有 6 个氨基酸残基），在大肠杆菌 ATCC 8099 细胞表面疏水性，微生物增长，与 DNA 的 结合水平等方面，与小米发酵肽 (FFMp10) 不同。通过高效液相色谱法也对多肽的蛋白水解能力进行了分析。FFMp10 的氨基酸序列是在 Mp1、 Mp2、 Mp3、 Mp4 多肽序列的基础上用 Arg 和 Lys 氨基酸替换。结果表明所有多肽对胃蛋白酶水解 1 h 均有抗性。而 Mp2、 Mp3 和 Mp4 高效液相色谱洗脱分析表明其对胰蛋白酶水解具有抗性。与 Mp3 和 Mp4 相比，Mp10、 Mp1 和 Mp2 处理大肠杆菌 2 小时，表现出较高的抑菌活性。与正常对照组相比，所有肽处理均能降低细菌增长。对 Mp1 和 Mp2 于大肠杆菌共培养分析的电泳图分析表明，肽能够与大肠杆菌 ATCC 8099 的 DNA 结合。

发酵及湿热处理方法对小米理化性质以及营养价值起到很好的改善作用。发酵的小米粉提取物有抗氧化、 抗菌活性。事实上，从发酵小米中提取具有天然抗氧化活性、抗菌活性、以及抗酶解活性的肽是可行的。

关键词：小米、 发酵、 L.副干酪乳杆菌 Fn032、 短肽、 抗菌、 抗氧化、 水解抵抗，疏水性，DNA 结合

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CHAPTER 1
General Introduction and Literature Review

1.1 Introduction

Cereal grains are the most important source of the world's food and have a significant role in the human diet throughout the world. Millets are one of the cereals asides the major wheat, rice, and maize. Millets are major food sources for millions of people, especially those who live in hot, dry areas of the world. They are grown mostly in marginal areas under agricultural conditions in which major cereals fail to give substantial yields (Adekunle, 2012). Millets are classified with maize, sorghum, and Coix (Job's tears) in the grass sub-family Panicoideae (Yang *et al.*, 2012). Millets are important foods in many underdeveloped countries because of their ability to grow under adverse weather conditions like limited rainfall. In contrast, millet is the major source of energy and protein for millions of people in Africa. It has been reported that millet has many nutritious and medical functions (Obilana & Manyasa, 2002; Yang *et al.*, 2012). It is a drought resistant crop and can be stored for a long time without insect damage (Adekunle, 2012); hence, it can be important during famine.

Discrepancies exist concerning classification of family millet, with some references giving the family name Gramineae, and others classifying it in the family Poaceae. There are many varieties of millets. The four major types are Pearl millet (*Pennisetum glaucum*), which comprises 40% of the world production, Foxtail millet (*Setaria italica*) (Yang *et al.*, 2012), Proso millet or white millet (*Panicum miliaceum*), and Finger Millet (*Eleusine coracana*). Pearl millet produces the largest seeds and it is the variety most commonly used for human consumption (Mariac *et al.*, 2006; ICRISAT, 2007). Minor millets include: Barnyard millet (*Echinochloa spp.*), Kodo millet (*Paspalum scrobiculatum*), Little millet (*Panicum sumatrense*), Guinea millet (*Brachiaria deflexa = Urochloa deflexa*), Browntop millet (*Urochloa ramosa = Brachiaria ramosa = Panicum ramosum*), Teff (*Eragrostis tef*) and fonio (*Digitaria exilis*) are also often called millets, as more rarely are sorghum (*Sorghum spp*.) and Job's tears (*Coix lacrima-jobi*) (ICRISAT, 2007; Adekunle, 2012). The world total production of millet grains at last count was 762712 metric tons and the top producer was India with an annual production of 334500 tons (43.85%) ((FAO, 2012), Table 1.1)).

Millets represent a unique biodiversity component in the agriculture and food security systems of millions of poor farmers in regions such as Sub-Saharan Africa. Pearl millet is an important food across the Sahel, although, India is the largest producer of pearl millet (Bhattacharjee *et al.*, 2007). Millets are often ground into flour, rolled into large balls, parboiled, and then consumed as porridge with milk; sometimes millets are prepared as beverages. Roti, made from pearl millet has been the primary food of farmers in Gujarat India (FAO-FAOSTAT, 2009). There is an emerging need for the world to feed its growing population, therefore, it is important to explore plants such as millets that are grown locally and consumed by low income households in places like India and the Sahel zone (Obiana, 2003). Cereals, in particular, millet based foods and beverages are known worldwide and are still part of the major diet in most African countries and India (Obilana & Manyasa, 2002; Amadou *et al.*, 2011). Millet-based fermented foods and beverages have been extensively studied; however, published research on millet and its food value and potential is limited. This review summarizes the nutritional composition of millets, some traditional millet-based foods and beverages as well as some health benefits, and the use of millets in the food industry.

Table 1.1 Top world millet grains producers (2010).

Rank	Country	Seed (tons)
1	India	334500
2	Niger	108798
3	Nigeria	59994
4	Mali	43878
5	Senegal	30995
6	China	26429
7	Burkina Faso	20428
8	Russian Fed.	20000
9	Chad	14775
10	Uganda	11750
11	Sudan	11000
World total		**762712***

*May include official, semiofficial or estimated data Source (FAO, 2012).

1.2 Nutritional composition of millet grains

Millets are unique among the cereals because of their richness in calcium, dietary fibre, polyphenols and protein (Devi *et al.*, 2011). Table 1.2 represent amino acids content in different types of millets. Millets generally contain significant amounts of essential amino acids particularly the sulphur containing amino acids (methionine and cysteine); they are also higher in fat content than maize, rice, and sorghum (Obilana & Manyasa, 2002).

Table 1.2 Amino acid profiles of different millet grains variety (Foxtail, Proso, Pearl and Finger millet).

Amino acids (g/100g)	**Foxtail millet** (defatted flour) (a)	**Proso millet** (Dehulled Grain) (b,c)	**Pearl millet** (true prolamine) (c)	**Finger millet** (native grain) (d)
Essential Amino Acid				
Isoleucine	4.59	4.1	5.1	4.3
Leucine	13.60	12.2	14.1	10.8
Lysine	1.59	1.5	0.5	2.2
Methionine	3.06	2.2	1.0	2.9
Phenylalanine	6.27	5.5	7.6	6.0
Threonine	3.68	3.0	3.3	4.3
Valine	5.81	5.4	4.2	6.3
Histidine	2.11	2.1	1.7	2.3
Tryptophan	NA	0.8	1.2	NA
Nonessential Amino Acid				
Alanine	9.30	10.9	8.1	6.1
Arginine	3.00	3.2	0.9	3.4
Aspartic acid	7.71	6.2	6.2	5.7
Cystine	0.45	NA	0.8	NA
Glutamic Acid	22.00	21.3	22.8	23.2
Glycine	2.91	2.1	0.7	3.3
Serine	4.56	6.3	5.4	5.3
Tyrosine	2.44	4.0	2.7	3.6
Proline	5.54	7.3	8.2	9.9
***PER[(b)]**	0.80	1.10	1.60	2.00

*Protein Efficiency Ratio (PER). NA: not available. **References**: (a) (Kamara *et al*., 2009); (b) (Bagdi *et al*., 2011); (c) (Saldivar, 2003); (d) (Devi *et al*., 2011).

In general, cereal proteins including millets are limited in lysine and tryptophan content and vary with cultivar. However, most cereals contain the essential amino acids as well as vitamins and minerals (FAO-FAOSTAT, 2009; Devi *et al.*, 2011). Plant nutrients are largely used in the food industry, and cereal grains constitute a major source of dietary nutrients worldwide (Izadi *et al.*, 2012; Amadou *et al.*, 2013). Modification of a protein is usually realized by physical, chemical, biological such as fermentation or an enzymatic treatment, which changes its structure and consequently its physicochemical and functional properties (Lestienne *et al.*, 2007; Amadou *et al.*, 2011; Amadou *et al.*, 2013). Table 1.3 represent the content of different varieties of millet, foxtail, fonio, proso, pearl and finger millets.

Badau *et al.* (2005) reported that the phytic acid content of the unmalted pearl millet grain ranged from 2.91% to 3.30%. The total dietary fibre (22.0%) of finger millet grain were reported relatively higher than that of many other cereal grains (e.g. 12.6%, 4.6% and 12.8% respectively for wheat, rice, maize and sorghum (Shobana & Malleshi, 2007; Siwela *et al.*, 2010). However, the dietary fibre content in pearl millet ranges between 8 to 9% (Taylor, 2004).

Table 1.3 Proximate composition of millet grain varieties

Component (g/100g, dry basis)	**Foxtail millet** flour	**Fonio** whole grain	**Proso millet** dehulled grain	**Pearl millet** whole grain	**Finger millet** native grain
Protein	11.50	9–11	11.58	14.8	8.2
Ash	0.47	1–1.1	NA	1.64	2.7
Fat	2.38	3.3–3.8	4.9	4.86	1.8
Total CHO*	75.2	84–86	80.1	59.8	83.3
Crude fiber	NA	NA	0.7	12.19	3.5
References	(Kamara *et al.*, 2009)	(Vodouhe *et al.*, 2003)	(Bagdi *et al.*, 2011)	(Taylor *et al.*, 2010.)	(Devi *et al.*, 2011)

*Carbohydrate (CHO). NA: not available.

Bagdi *et al.* (2011) analyzed the composition of free and bound lipids in proso millet (*Panicum miliaceum*) flours and brans. In the free lipids, hydrocarbons, sterol esters, triacylglycerols, diacylglycerols, and free fatty acids were present (Bagdi *et al.*, 2011). The predominant fatty acids in the free lipids were linoleic, oleic, and palmitic acids, though, in the

bound lipids, monogalactosyl diacylglycerols, digalactosyl diacylglycerols, phosphatidylethanolamine, phosphatidyl serine, and phosphatidyl choline were tentatively identified (Bagdi *et al.*, 2011). Saldivar (2003) studied the content of total lipids, lipid classes and fatty acids composition in small millets, such as foxtail millet (*Setaria italica*), proso millet (*Panicum miliaceum*), and finger millet (*Eleusine coracana*). They reported the total lipid content in the foxtail, proso, and finger millets ranged from 5.2 to 11.0% (dry basis), while it ranges from 5.1 to 8.3% in the little, kodo, and barnyard millets (Saldivar, 2003). In addition, examination of some millet cultivars from Tunisia and Mauritania by Ibrahima *et al.* (2004) showed that the fatty acid profile of the millet lipid is mainly characterized by the presence of high levels of linoleic, oleic and palmitic acid. However, palmitoleic acid (C16:1), stearic acid (C18:0) and linolenic acid (C18:3) were less represented. Other fatty acids are identified in trace amounts (arachidic acid C20:0, behenic acid C22:0, erucic acid C22:1). The work of Liang *et al.* (2010) presented the general properties of foxtail millet oil and its fatty acid profile. It is apparent that millet oil could be a good source of natural oil rich in linoleic acid and tocopherols (Liang *et al.*, 2010; Amadou *et al.*, 2011). The main polyphenols in cereals are phenolic acids and tannins, whilst flavonoids are present in small quantities; they act as antioxidant and play many roles in the body immune system defence (Chandrasekara & Shahidi, 2010; Devi *et al.*, 2011). Whole cereal grains are considered a rich source of fibre. However, foods from grains have marked differences in the amount and type of dietary fibre (Shukla & Srivastava, 2011). The dietary fibre content in cereal-based food varies greatly, depending on the extent of milling. Finger millet shows relatively higher than other cereals carbohydrate (72%) comprises of starch as the main constituent and the non-starchy polysaccharides which amounts to 15–20% of the seed matter as an unavailable carbohydrate dietary fibre content and complements which are the health benefits of the millet (Devi *et al.*, 2011). The main function of dietary carbohydrate is to supply energy (Saleh *et al.*, 2013). Millets are good sources of magnesium and phosphorus. Magnesium has the ability to help reduce the effects of migraine and heart attacks, while, phosphorus is an essential component of adenosine triphosphate (ATP) a precursor to energy in the body (Badau *et al.*, 2005; Liang *et al.*, 2010; Devi *et al.*, 2011).

1.3 Millet in probiotic and prebiotics foods

Probiotics aid the existing flora, or help repopulate the colon when bacteria levels are reduced by antibiotics, chemotherapy or disease. FAO/WHO stated that probiotics are "lives microorganisms which when administered in adequate amounts confer a health benefit on the host" though, this should also specify genus, species and strain level, as well as a safety assessment. Most of probiotic foods generate fatty acids, vitamins and other vital nutrients that improve the body's resistance against pathogens microorganisms (FAO/WHO, 2001; Abd El-Salam *et al.*, 2012). Fermented foods are thus, important to human beings all over the world with between 20 to 40% of food supply being from fermented foods (Rivera-Espinoza & Gallardo-Navarro, 2010; Amadou *et al.*, 2011; Anukam & Reid, 2009). Lei *et al.* (2006) reported an interesting intervention study in Northern Ghana using spontaneously fermented millet product as a natural probiotic treatment for diarrhea in young children.

Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one, or a limited number of bacteria in the colon (Laminu *et al.*, 2011; Abd El-Salam *et al.*, 2012). Food ingredients classified as prebiotics must not be hydrolyzed or absorbed in the upper gastrointestinal tract, need to be a selective substrate for one, or a limited number of colonic bacteria, must alter the microbiota in the colon to a healthier composition and should induce luminal, or systematic effects that are beneficial to the host health (Lei & Jakobsen, 2004). Cereals, in particular millet-based fermented foods and beverages (Table 1.4), have been extensively studied and form a major part of the diet in most African countries (Rivera-Espinoza & Gallardo-Navarro, 2010).

Table 1.4 Traditional process fermented/unfermented millet foods and beverages

Product	Strain	Usage	References
Ben-saalga	LAB	gruel	(Tou *et al.*, 2006; Songre-Ouattara *et al.*, 2009; Rivera-Espinoza & Gallardo-Navarro, 2010)
Burkutu	*S. cerevisiae, S. chavelieri, Leuconostoc mesenteroides, Candida, Acetobacter, G. candidum*	beer	(Nzelibe & Nwasike, 1995; Iwuoha & Eke, 1996; Oi & Kitabatake, 2003; Songre-Ouattara *et al.*, 2009;

			Haggblade & Holzapfel, 1993)
Bushera	*Lactococcus, Leuconostoc, Enterococcus* and *Streptococcus*. *L. brevis*	gruel	(Muyanja *et al.*, 2003; Prado *et al.*, 2008)
Dambu	Unknown	dumpling	(Nkama *et al.*, 1999; Agu *et al.*, 2008)
Fura	*Enterobacter, bacillus, Staphylococcus, Fusarium culmorum, A. orizae, A. niger, A. flavus A. parasiticus and Mucor racemosus*	porridge	(Jideani *et al.*, 2001; Jideani *et al.*, 2001; Inyang & Zakari, 2008)
Jandh	LAB, yeast and Mold	beer	(Karki, 1986; Tamang *et al.*, 1988; Dahl, 1997)
Koko	*L. fermentum, L. salivarius, Enterobacter clocae, Acinetobacter, L. platarum, L. brevis, S. cerevisiae,C. mycoderma, W. confusa*	porridge	(Campbell-Platt, 1994; Lei & Jakobsen, 2004)
Kunu-zaki	LAB, yeasts	paste	(Efiuvwevwere & Akona, 1995; Akoma *et al.*, 2002; Oranusi *et al.*, 2003; Agarry *et al.*, 2010)
Mangisi	LAB	beer	(Benhura & Chingombe, 1989; Zvauya *et al.*, 1997; Gadaga *et al.*, 1999)
Masvusvu	LAB, Yeasts	mash	(Efiuvwevwere & Akona, 1995; Zvauya *et al.*, 1997; Akoma *et al.*, 2006)
Ogi	*L. plantarum, L. fermentum, Le. mesenteroides,* and *S. Cerevisiae ,C. mycoderma, Corynebacterium, Aerobacter, Rhodotorula, Cephalosporium, Fusarium, A. and Penicillium*	paste	(Akinrele, 1970; Odunfa, 1985; Marero *et al.*, 1988; Inyang & Idoko, 2006)

Roti	Unknown	bread	(Dahl, 1997; Wickramasinghe *et al.*, 2005)
Togwa	*L. plantarum, L. brevis, L. fermentum, L. cellobiosus, W. confuse* and *P. pentosaceus*.	gruel	(Mugula *et al.*, 2003; Oi & Kitabatake, 2003; Prado *et al.*, 2008)
Uji	LAB	porridge	(Mbugua, 1984; Masha *et al.*, 1998; Onyango *et al.*, 2003; Onyango *et al.*, 2004; Onyango *et al.*, 2004)

LAB: lactic acid bacteria, A: *Aspergillus*, C: *Candida*, E: *Endomycopsis*, G: *Geothrichum*, L: *Lactobacillus*, Lc: *lactococcus*, le: *Leuconostoc* , P: *Pediococcus*, R: *Rhizopus*, S: *Saccharomyces*, St: *Streptococcus*, W: *Weissella*

1.4 Traditional fermented millet foods and beverages

Malt: The malting process, which involves soaking, germination, and drying, transforms grains into malt through high enzyme activity. Millet and sorghum malt production is a traditional practice in Africa, where malt is used in lactic acid- and alcoholic-fermented beverages and infant food production (Marero *et al.*, 1998; Uvere *et al.*, 2000; Badau, 2006; Hashemi *et al.*, 2012). Malting induces important beneficial biochemical changes in the millet grain. Moreover, soaking generates grain softening and increases water absorption. Enzymes produced during germination are responsible for hydrolysis of starch and proteins, which makes sugar and peptides/amino acids directly available. Furthermore, proteolytic enzymes improve the availability of limiting amino acids such as lysine, methionine, and tryptophan (Mahgoub & Elhag, 1998; Badau *et al.*, 2005; Akoma *et al.*, 2006). These attributes depend on the type and quality of grain. Traditional malting processes in many developing countries involve three main operations: soaking, germination, and drying. The duration and conditions of each operation are highly variable, resulting in highly variable malt and derived product quality. Total aerobic germ, coliform, yeast, and filamentous fungus levels in malt derived from traditional processes can be higher than the limits recommended by the Codex Alimentarius Commission (Hounhouigan *et al.*, 2006). Like other cereals, millets are susceptible to fungal growth and mycotoxin production under certain environmental conditions. Mycotoxins not only threaten consumer health but also are a major threat to malt quality (Sohrabvandi *et al.*, 2010).

Koko: *Koko* is a millet porridge that is consumed daily by many people in West Africa as lunch or an in-between meal. *Koko* is produced by steeping pearl millet overnight, discarding the steep water, wet-milling the millet grains together with spices (usually ginger, chili pepper, black pepper, and cloves), and adding water to the milled materials to make a thick slurry. The slurry is then sieved, fermented, and sedimented for 2–3 h. The liquid top layer is decanted and boiled for 1–2 h, and finally, the sedimented bottom layer is added until the desired consistency is obtained. The whole process generally starts in the evening with steeping of the millet grain, and the final product is ready for consumption around noon the following day. *Koko* can be sold as porridge in plastic bags or bowls and is normally consumed with added sugar. The predominate microorganisms in *koko* are *Weisella confusa*, *Lactobacillus fermentum*, and *L. salivarius* (Campbell-Platt, 1994; Lei & Jakobsen, 2004).

Fura: Pearl millet is an important food for millions of people inhabiting the semi-arid tropics and is a major source of calories in developing regions of the world (FAO/ICRISAT, 1996). The Sahel is a region that borders semi-arid and arid areas of Africa north of the equator. The staple food in the Sahel region is *fura* made from millet flour. Millet grain is slightly moistened with water and ground in a locally fabricated disc attrition mill. The hull is removed from the grain after drying in the sun, and the grain is ground using a hammer mill and sieved. Pearl millet flour is mixed with powdered black pepper, powdered ginger, and water (95°C) in a mortar and kneaded into a smooth dough with a pestle. The dough is hand molded into balls, placed inside a cooking pan containing boiling water, and cooked for 30 min at atmospheric pressure. The balls are kneaded again while still hot until a smooth, slightly elastic mass is obtained. The dough is then molded into balls of *fura*. The stiff dough produced is reconstituted to a porridge-like consistency with sour milk (Jideani & Wedzicha, 1995; Jideani *et al.*, 2010). In recent years a company in Abuja, Nigeria, specializing in the production of powdered (instant) *fura* has made *fura* available in supermarkets (Jideani & Wedzicha, 1995; Jideani *et al.*, 2010).

Mangisi: Fermented beverages constitute a major part of the diet of rural African families, serving as alcoholic beverages and weaning foods, in addition to their role in social functions and ceremonies (Michodjèhoun-Mestre *et al.*, 2005; Vieira-Dalodé, 2008). *Mangisi* is a sweet-sour beverage made from naturally fermented millet mash (Zvauya *et al.*, 1997). Preparation varies in different regions of sub-Saharan Africa such as Zimbabwe and Uganda. In one variation, finger millet is malted and then milled, and the flour is mixed with water. The mixture is slowly heated

for 80 min to near boiling. The resulting product is a mash (*masvusvu*) that is cooled, diluted, strained, and allowed to stand for several hours during which spontaneous fermentation takes place, producing *mangisi* (Zvauya *et al.*, 1997). Another variation involves malting and milling finger millet, mixing the flour with water, and boiling the mixture for 1–2 h. The *masvusvu* is cooled, diluted, and allowed to stand overnight. On the second day more malt flour is added, and the mixture is left to ferment until the third day, when the coarse solids are strained off. The mixture is returned to the fermentation vessel, and the *mangisi* is then ready for consumption (Benhura & Chingombe, 1989). Gadaga *et al.* (1999) reported that the product contains more alcohol due to the addition of extra malt to the brew on the second day, which could serve as an additional source of inoculum, and also to the longer fermentation time.

***Jandh*:** Traditional fermented foods are generally specific to certain geographic regions and particular communities. *Jandh* (a type of beer), a slightly acidic and sweet beverage, is a major traditional alcoholic product of Nepal (Dahal *et al.*, 2005). *Jandh* is a fermentation product of finger millet (*koko* or *marua*), which is sometimes supplemented with a small amount of wheat or corn (Tamang *et al.*, 1988). *Jandh* is prepared as follows. Millet seeds are softened using steam and then spread on leaves (preferably banana leaves). *Murcha*, the starter culture, is powdered and sprinkled on the boiled and cooled seeds. After thorough mixing, the seeds are piled in a heap and kept for 24 h at ambient temperature. Next, they are usually placed in an earthen pot and covered with leaves and straw. (In urban areas, the seeds are allowed to ferment in polyethylene bags.) If air leaks into the fermentation substrate, the product becomes sour (Karki, 1986). In the case of millet, after fermentation the seeds are kneaded to remove the seed coats. The grits are then placed in bamboo vessels with water (cold or hot depending on the season). After 10 min, the beverage is ready to drink. This liquor is believed to be a beneficial tonic, especially for postnatal women (Karki, 1986; Dahal *et al.*, 2005).

Uji: *Uji* is a thin, lactic acid-fermented porridge that is widely consumed in East Africa (Kenya, Uganda, and Tanzania) and is referred to as *togwa* and *obusera* in Tanzania and Uganda, respectively. It is prepared by lactic acid fermentation of cereal (maize, finger millet, or sorghum) and cassava flours mixed in different combinations and proportions. The fermentation inocula are derived by a technique referred to as backslopping. The most popular 1:1 combinations are maize and sorghum, maize and finger millet, cassava and finger millet, and cassava and sorghum (Onyango *et al.*, 2003; Onyango *et al.*, 2004). Adults consume *uji* as a refreshing beverage, and

children consume *uji* as their principal weaning food (Onyango *et al.*, 2004). *Lactobacillus plantarum* is the predominant species of lactobacilli in typical *uji* fermentation and is responsible for the high lactic acid levels and subsequent sour flavor of *uji*. Other species present include *Pediococcus acidilactici, P. pentocaceus, L. paracasei subsp. paracasei, L. fermentum, L. cellobiosus,* and *L. buchneri* (Mbugua, 1984; Masha *et al.*, 1998).

Burukutu and Pito: In many countries millet has been used successfully as a substitute for barley. For example, grains such as finger millet have been used in sub-Saharan Africa and India as major ingredients in the traditional manufacture of malt (Nzelibe & Nwasike, 1995; Songre-Ouattara *et al.*, 2009). Traditionally, African beers differ from Western beer types in several ways: they are often sour, less carbonated, and have no hops. African beers are consumed unrefined, including unfermented substrates and microorganisms (Haggblade & Holzapfel, 1993). *Pito* and *burukutu* are brewed concurrently by fermenting malted or germinated single cereal grains such as millet or a mixture of cereal grains into a brownish suspension or liquor (Iwuoha & Eke, 1996; Badau, 2006). *Burukutu* is a popular alcoholic beverage among the peoples of sub-Saharan Africa.

Kunu-zaki: *Kunun-zaki* is a fermented nonalcoholic cereal-based beverage. It is a popular refreshing beverage in areas of the Sahel such as northern Nigeria, Niger, and Tchad. *Kunun-zaki* production is essentially a home-based industry, and at present, there is no large-scale factory production. Efiuvwevwere & Akona (1995) studied the microbiology of the *kunun-zaki* fermentation process and reported *that Lactobacillus fermentum* and *L. leichmannii* were predominant at the end of the fermentation period. Akoma *et al.* (2002) described the production of four types of *kunun-zaki* using combinations of millet or millet and wheat, with or without the addition of ground malted rice, fermented for 6 h. Agarry *et al.* (2010) outlined how *kunun-zaki* could be produced using developed starter culture (controlled fermentation), natural (uncontrolled) fermentation, and different combinations of millet, wheat, malted rice, and starter culture. For the control experiment, uncooked cereal starch (previously sterilized) was mixed thoroughly with hydrolyzed cereal starch before addition to gelatinized cereal starch. This mixture was incubated at ambient temperature (without addition of starter culture) for 6 h to establish whether fermentation could take place. The authors claimed *kunun-zaki* produced with the addition of starter culture to either millet and malted rice or millet, wheat, and malted rice had several advantages (flavor, aroma, appearance, and overall acceptability) over other products. However, in the Sahel, the quality of traditional food products such *kunun-zaki* has always depended on the

skill of local producers and the season in which a product is made (Oranusi *et al.*, 2003). Other fermented millet beverages include *braza* and *darassum*, which are made in Romania and Mongolia, respectively (von Mollendorff *et al.*, 2006).

Ogi: *Ogi* is a porridge prepared from fermented millet, sorghum, or maize paste or cake in West Africa. *Ogi* is often sold as a wet cake wrapped in leaves or polyethylene bags. Gelatinized *ogi* is called pap and is mainly used as a traditional infant weaning food, as well as a breakfast meal for many adults. In many parts of Africa, children are fed mashed adult foods or gelatinized cereal flour slurries to complement breast milk from 4 to 6 months of age. These slurries absorb a large quantity of water and swell greatly when mixed either with cold or hot water. Traditional and industrialized methods for manufacturing *ogi* have been reported (Banigo *et al.*, 1974). Malting and fermentation techniques can be used to modify the starch content of the cereals so they do not thicken and, therefore, do not require dilutions. Other benefits from good manufacturing processes include the inhibition of pathogens throughout the fermentation process (Marero *et al.*, 1988). Akinrele (1970) showed that *Lactobacillus plantarum, Corynebacterium spp., Aerobacter spp., Candida mycoderma, Saccharomyces cervisiae, Rhodotorula spp., Cephalosporium spp., Fusarium spp., Aspergillus spp.*, and *Penicillium spp.* are the major organisms responsible for the fermentation and nutritional improvement of *ogi*. Odunfa, (1985) identified *Lactobacillus plantarum* as the predominant organism in *ogi* fermentation responsible for lactic acid production (Inyang & Idoko, 2006). Lactic, acetic, butyric, and formic acids give *ogi* its characteristic aroma and sour flavor (Inyang & Idoko, 2006; Amadou *et al.*, 2011). Light colored *ogi* with a mild sour flavor is reportedly preferred by consumers (Akinrele, 1970; Inyang & Idoko, 2006).

Ben-saalga: *Ben-saalga* is a millet-based fermented gruel that is made in a large number of traditional production units in Burkina Faso (West Africa). Traditional cereal-based fermented foods are frequently used as complementary foods for infants and young children in Africa (Tou *et al.*, 2006; Rivera-Espinoza & Gallardo-Navarro, 2010). The daily quantity of millet usually processed into *ben-saalga* in a traditional production unit is around 7 kg. Processing includes the following main steps: washing and soaking of grain (pearl millet), grinding, kneading, sieving, settling, and cooking. Aromatic ingredients, such as ginger, black pepper, pepper, and mint, usually are added in small quantities prior to grinding depending on the tradition of the *ben-saalga* producer (Tou *et al.*, 2006; Songre-Ouattara *et al.*, 2009). During final cooking, the supernatant resulting from the settling step is collected and heated for 40 min to near boiling. Afterward, the

paste is added to the supernatant and boiled for 7 min. Tou *et al.* (2006) reported that the sour *ben-saalga* resulting from cooking the sour paste had inadequate nutritional characteristics with respect to the requirements for infants and young children.

Bushera: *Bushera* is the most common traditional beverage prepared in the western highlands of Uganda, where sorghum and millet are important staple and commercial crops. The product is consumed by both young children and adults. Numerous methods are used to prepare *bushera*. Flour made from germinated sorghum or millet grain is mixed with boiling water and left to cool to ambient temperature. Germinated millet or sorghum flour is then added, and the mixture is left to ferment at ambient temperature for 1–6 days. The lactic acid bacteria isolated from bushera generally are from five genera: *Lactobacillus, Lactococcus, Leuconostoc, Enterococcus*, and *Streptococcus*. *Lactobacillus brevis* is more frequently isolated than other species (Muyanja *et al.*, 2003; Prado *et al.*, 2008).

***Togwa*:** *Togwa* is a lactic acid-fermented traditional beverage consumed in Africa. In southern Tanzania, togwa is usually made from maize flour and finger millet malt. In this region, it is consumed by both adults and young children and is used as a refreshment as well as a weaning food (Oi & Kitabatake, 2003; Prado *et al.*, 2008). *Togwa* is prepared by cooking the cereal or cassava flour in water. After cooling, the starter culture (old *togwa*) and cereal flour from germinated grain are added. Fermentation is spontaneous and uncontrolled, resulting in a product with variable quality (López-García, 2008; Rivera-Espinoza & Gallardo-Navarro, 2010). The bacteria isolated from *togwa* include *Lactobacillus plantarum, L. brevis, L. fermentum, L. cellobiosus, Weissella confuse,* and *Pediococcus pentosaceus*. All of them are present throughout fermentation. The *L. plantarum* group is the predominant organism at the end of *togwa* fermentation and has been identified as the predominant organism at the end of several natural lactic acid fermentations (Mugula *et al.*, 2003; Amadou *et al.*, 2010).

1.5 Nonfermented millet foods and beverages

***Dambu*:** *Dambu* is a steamed, granulated dumpling generally made from millet, maize, or sorghum depending on availability. Moistened millet flour is blended with spices and steamed 30 min. The coarse particles are sprinkled into fermented milk, and sugar may be added to taste (Nkama *et al.*, 1999). *Dambu* is produced both at home and commercially. Most *dambu* producers use a traditional method involving a wooden mortar and pestle to dehull and mill the grain. The

traditional pounding process is time-consuming, which limits production in most African countries (Jideani *et al.*, 2010). Moreover, because the cereal flour spoils quickly and cannot be stored, it has to be milled daily as needed for use in *dambu*. Like fura, *dambu* (Jideani *et al.*, 2001; Agu *et al.*, 2008) has a limited shelf life (2 days) at tropical temperatures and due to a lack of proper packaging. Refrigerated storage conditions could prolong shelf life (Agu *et al.*, 2008).

Masvusvu: *Masvusvu* is a sweet beverage traditionally made from malted finger millet in many villages in Zimbabwe. As reported earlier (Zvauya *et al.*, 1997), *masvusvu* is unfermented *mangisi*. A mixture of water and malted millet meal is heated in an earthen pot and stirred slowly at intervals for 80 min until near boiling. The slurry mixture thickens, and the light-brown product is consumed as either a food or beverage. Released reducing sugars impart a sweet flavor. *Masvusvu* is also used as an adjunct during opaque beer brewing (Zvauya *et al.*, 1997). The preparation of *masvusvu* differs from that of either *kunun-zaki*, which is made from unmalted wet millet flour (Efiuvwevwere & Akona, 1995), or *gowé*, which is prepared from wet-milled malted sorghum flour (Vieira-Dalodé, 2008).

***Roti*:** There are many different variations of flatbreads found in many cultures across the globe. *Ragi* roti, known as finger millet roti, is an unleavened flatbread made from *ragi* flour (Dahl, 1997). Other *rotis* made from different grains are part of the daily diet of people in northern and central India. The most popular flatbread is roti made from *atta* flour (Indian whole-wheat flour) (López-García, 2008). The preparation of roti consists of mixing the *ragi* flour, chopped onions, chili, coriander leaves, grated coconut, and salt in a bowl. Water is added little by little until a dough ball is formed (it should not be too soft). The dough is divided into two parts and cooked on a griddle. The dough is patted into round on the griddle, a few drops of oil are added, and then the griddle is placed over a medium flame. The roti is covered with a lid, cooked for 4–5 min on one side, and then flipped to cook the other side for another 2–3 min (Wickramasinghe *et al.*, 2005).

1.6 Millet in the industry

Millets have good grain qualities suitable for processing. Processing of the grain for many end uses involves primary (wetting, dehulling and milling) and secondary (fermentation, malting, extrusion, glaking, popping and roasting) operations. Being a staple and consumed at household levels, processing must be considered at both traditional and industrial levels, involving small, medium and large-scale entrepreneurs (Obilana & Manyasa, 2002; Hamad, 2012). Dehulling is

not favorable to millets due to their small grains sizes. In addition, dehulling causes nutrients loss. All the Millets can be milled by hand grinding (household level) or machine milling (cottage, small-to-medium scale service and large scale industrial). Millet and sorghum malt production is a traditional practice in Africa, where malt is used in lactic acid- and alcoholic-fermented beverages and infant food production (Adekunle, 2012). Traditional malting processes in many developing countries involve three main operations: soaking, germination, and drying. The duration and conditions of each operation are highly variable, resulting in highly variable malt and derived product quality (Vidya *et al.*, 2013). *Burukutu* and *Pito* are traditional African beers differ from Western beer types in several ways: they are often sour less carbonated and have no hops; these beer are products of both at traditional and industrial level (Anukam & Reid, 2009; Amadou *et al.*, 2011). The emerging principal uses of millets as an industrial raw material include production of biscuits and confectionery, beverages, weaning foods and beer (Anukam & Reid, 2009; Laminu *et al.*, 2011). Grits, flour, and meals from cereals such as millet, sorghum, and corn are now common items in the market. Soft biscuits and cookies are being made using sorghum, maize and wheat composites, while cakes and non-wheat breads have become a subject of increasing scientific and technological enquiry, showing encouraging results (Akeredolu *et al.*, 2005; Laminu *et al.*, 2011; Vidya *et al.*, 2013).

In the infant weaning food sector, in spite of unlimited potential, progress has been slow, as the installed capacity for industrial malting is limited. Many brands of beer in the underdeveloped countries market have substantial content of local cereal such as millet to reduce the cost of imported barley. The industries are confronted with a number of problems which tend to diminish product qualities and affect overall utilization. For instance, in the nonalcoholic beverage and weaning food sectors, storage quality of the grain, nutritional losses after processing, high cost of imported equipment and variation among cultivars are some of the problems militating against improved utilization of millet in the developing countries (Akeredolu *et al.*, 2005; Laminu *et al.*, 2011; Adekunle, 2012). In a weaning process there is always the need to introduce soft, easily swallowed foods to supplement the infant's feeding early in life. A process weaning diet from pearl milletconophor nut flour was found to promote growth in a clinical experiment (Akeredolu *et al.*, 2005); whereas, weaning food blends prepared from fermented pearl millet/roasted cowpea in 70:30 and 60:40 ratios were reported to have resulted in lower levels of phytic acid and higher in vitro protein digestibility of the weaning food blends (Laminu *et al.*, 2011).

1.7 Some potential health benefits of millets

Studies has shown that diets rich in plant foods are protective against several degenerative diseases such as cancer, cardiovascular ailments, diabetes, metabolic syndrome, and Parkinson's disease (Chandrasekara & Shahidi, 2010; Beit-Yannai *et al.*, 2011; Amadou *et al.*, 2012; Saleh *et al.*, 2013). Millet is more than just an interesting alternative to the more common grains. The grain is also rich in phytochemicals, including phytic acid, which is believed to lower cholesterol, and phytate, which is associated with reduced cancer risk (Coulibaly *et al.*, 2011). These health benefits have been partly attributed to the wide variety of potential chemopreventive substances, called phytochemicals, including antioxidants present in high amounts in foods such as millets (Izadi *et al.*, 2012).

Millet is gluten-free, therefore an excellent option for people suffering from celiac diseases often irritated by the gluten content of wheat and other more common cereal grains. It is also useful for people who are suffering from atherosclerosis and diabetic heart disease (Gélinas *et al.*, 2008). Choi *et al.* (2005) and Park *et al.* (2008) reported that protein concentrate of Korean foxtail millet and proso millet significantly elevated plasma adiponectin and HDL cholesterol levels and caused major decreases in insulin levels relative to a casein diet in type 2 diabetic mice. Furthermore, proso millet also improved glycemic responses and plasma levels (Park *et al.*, 2008). In addition, proso millet protein concentrate has protective effects against D-galactosamin-induced liver injury in rats (Ito *et al.*, 2008). Choi *et al.* (2005) and Park *et al.* (2008) concluded that proso millet protein could be a potential therapeutic intervention in type 2 diabetes. Devi *et al.* (2011) review the nature of polyphenols and dietary fiber of finger millet and their role with respect to the health benefits associated with millet.

Much attention has been devoted to investigations of the nutraceutical and antioxidant properties of some major millet varieties, including finger millet, pearl millet, and foxtail millet. Chandrasekara & Shahidi (2010) reported in their studies on free-radical quenching activity of finger millet (*Eleusine coracana*), that nonprocessed brown finger millet had the highest radical quenching activity than the processed one and postulated that tannins and phytic acid were responsible for the activity (Devi *et al.*, 2011; Kamara *et al.*, 2012; Saleh *et al.*, 2013). Millets extract from the seed coat where reported to have shown high antibacterial and antifungal activity compared to whole flour extract due to high polyphenols content in seed coat (Viswanath *et al.*,

2009; Xu *et al.*, 2011). Free radical quenching potential of different millets kodo millet, finger millet, little millet, foxtail millet, barnyard millet (*kudiraivali*), great millet (*jowar*) and their white varieties were revealed to have significant antioxidant activity by 1, 1, Diphenyl -2-picrylhydrazyl (DPPH) method (Devi *et al.*, 2011; Amadou *et al.*, 2013; Saleh *et al.*, 2013). For foxtail millet, methanolic extracts of whole flour and bran-rich fraction exhibited a significantly higher ($P < 0.05$) radical-scavenging activity (44.62% and 51.80%, respectively) using a DPPH model system, and reducing power (0.381 and 0.455, respectively) at 2 mg, than the ethanol and water used for extraction (Suma & Urooj, 2012). On the other hand, 50% ethanol extract from defatted foxtail millet bran was found to be the best-promoting phenolic compound with substantial antioxidant activity (Amadou *et al.*, 2011). In addition, defatted foxtail millet protein hydrolysates also exhibited antioxidant potency (Kamara *et al.*, 2012).

Millet grain fractions and extracts were found to have antimicrobial activity. In one study, seed protein extracts of pearl millet, sorghum, Japanese barnyard millet, foxtail millet, samai millet, and proso millet were evaluated in vitro for their ability to inhibit the growth of Rhizoctonia solani, Macrophomina phaseolina, and Fusarium oxysporum. Protein extracts of pearl millet were highly effective in inhibiting the growth of all 3 examined phytopathogenic fungi. The results indicated that the 23-kDa thaumatin-like proteins (TLPs) were predominantly expressed in the seeds and inflorescence of pearl millet (Radhajeyalakshmi *et al.*, 2003). In another study, phenolic acids from finger millet milled fractions (whole flour, seed coat, 3%, 5%, and 7%) were isolated. In addition, a novel antifungal peptide with high potency was isolated from foxtail millet seeds (Xu *et al.*, 2011).

Thus, millet may serve as a natural source of antioxidants and antimicrobials in food applications and as a nutraceutical and functional food ingredient in health promotion and disease risk reduction. However, more studies in animal models and with human subjects are needed to verify their activity and health benefits.

1.8 Problem statement and justification

In the past few years, there has been increasing interest in research about fermented grains to produce bioactive peptides. Generally, they are hidden within polypeptide chains of large proteins. They are dormant, but are released during proteolysis in the gastrointestinal tract. Various

properties of these bioactive peptides include hypotensive, antithrombotic, opioid, antioxidant, and antimicrobial.

In China, because of their potential contribution to national food security, millets as a food resource have been relatively neglected contrary to the African nations where millet is major staple food, but are now receiving increasing attention from agriculture and food security policymakers.

Beside the limited published literature on foxtail millet variety a lot are yet to be done to promote the potential nutraceutical value of it.

No studies have been yet focused on fermented foxtail millet meal (FFM) by *Lactobacillus paracasei* Fn032 for the characterization of bioactive peptides. In the present study, foxtail millet meal (FM) has been fermented with *L. paracasei* Fn032 to identified and purified bioactive peptides. The fermented foxtail millet meal extracts were evaluated for potential antimicrobial antioxidants activities, furthermore, peptides were purified and characterized and tested against *Escherichia coli* ATCC 8099.

1.9 Objectives of this Study

- Effect of fermentation by *Lactobacillus paracasei* Fn032 and heat-moisture treatment on the physicochemical properties of foxtail millet (*Setaria italica)* flour

- Evaluation of antimicrobial, antioxidant activities and nutritional values of fermented foxtail millet meal extracts by *Lactobacillus paracasei* Fn032

- Purification and characterization of foxtail millet-derived peptides with antioxidant and antimicrobial activities

- Antimicrobial effects of modified FFMp10 chemically synthesized short peptides on *Escherichia coli* ATCC 8099 hydrophobicity

1.10 References

Abd El-Salam MH, Hippen AR, Salem MM, Assem FM & El-Aassar M (2012) Survival of probiotic Lactobacillus casei and Enterococcus fecium in Domiati cheese of high conjugated linoleic acid content. *Emirates Journal of Food and Agriculture* 24: 98–104.

Adekunle AA (2012) *Agricultural innovation in sub-saharan africa: experiences from multiple-stakeholder approaches*. Ghana.

Agarry OO, Nkama I & Akoma O (2010) Production of kunun-zaki a Nigerian fermented cereal beverage) using starter culture. *International Research Journal of Microbiology* 1: 018-025.

Agu HO, Anosike AN & Jideani IA (2008) Physicochemical and microbial qualities of dambu produced from different cereal grains. *Pakistan Journal of Nutrition* 7: 21-26.

Akeredolu IA, Addo A. A. & Akeredolu O. A. (2005) Clinical evaluation of pearl millet Technol conophor weaning mix as supplementary food for Nigerian children. *Brazilian Archives of Biology and Technology* 48: 531-536.

Akinrele IA (1970) Fermentation studies on maize during the preparation of a traditional african starch-cake food. *Journal of the Science of Food and Agriculture* 21: 619-625.

Akoma O, Jiya EA, Akumka DD & Mshelia E (2006) Influence of malting on the nutritional characteristics of kunun-zaki. *African Journal Biotechnology* 5: 996-1000.

Akoma O, Onuoha SA, Ajiboye MO, Akoma AO & Alawoki AM (2002) The nutritional and sensory quality characteristics of kunun-zaki produced with the addition of hydrolytic enzymes from malted rice (*Oryza sativa*). *The Journal Food and Technology in Africa* 7: 34-26.

Amadou I, Gbadamosi OS & Le GW (2011) Millet-based traditional processed foods and beverages—A review. *Cereal Food World* 56: 115-121.

Amadou I, Le GW & Shi YH (2012) Effect of boiling on the cytotoxic and antioxidant properties of aqueous fruit extract of desert date, *Balanites aegyptiaca* (L) Delile. *Tropical Journal of Pharmaceutical Research* 13: 437- 444.

Amadou I, Gounga ME & Le GW (2013) Millets: Nutritional composition, some health benefits and processing — A review. *Emirates Journal of Food and Agriculture* 25: 501-508.

Amadou I, Le GW & Shi YH (2013) Evaluation of Antimicrobial, Antioxidant Activities, and Nutritional Values of Fermented Foxtail Millet Extracts by *Lactobacillus paracasei* Fn032. *International Journal of Food Properties* 16: 1179-1190.

Amadou I, Amza T, She YH & Le GW (2011) Chemical analysis and antioxidant properties of foxtail millet bran extracts. *Songklanakarin Journal of Science and Technology* 33: 509-515.

Amadou I, Kmara MT, Amza T, Foh MBK & Le GW (2010) Physicochemical and Nutritional Analysis of Fermented Soybean Protein Meal by *Lactobacillus plantarum* Lp6. *Word Journal of Dairy and Food Sciences* 5: 114-118.

Amadou I, Le GW, Shi YH, Gbadamosi OS, Kamara MT & Jin S (2011) Optimized *Lactobacillus plantarum* Lp6 solid-state fermentation and proteolytic hydrolysis improve some nutritional attributes of soybean protein meal. *Journal of Food Biochemistry* 35: 1686-1694.

Amadou I., Gounga E. M. & Le G. W. (2013) Millets: Nutritional composition, some health benefits and processing — A review. *Emirates Journal of Food and Agriculture* 25: 501-508.

Anukam KC & Reid G (2009) African traditional fermented foods and probiotics. *Journal of Medecinal Food* 12: 1177-1184.

Anukam KC & Reid G (2009) African traditional fermented foods and probiotics *Journal of Medecinal Food* 12: 1177-1184.

Badau MH (2006) Microorganisms associated with pearl millet cultivars at various malting stages. *Internet Journal of Food Safety* 8: 66-72

Badau MH, Nkama I & Jideani IA (2005) Phytic acid content and hydrochloric acid extractability of minerals in pearl millet as affected by germination time and cultivar. *Food Chemistry* 92: 425-435.

Bagdi A, Balázs G, Schmidt J, Szatmári M, Schoenlechner R, Berghofer E & Tömösközia S (2011) Protein characterization and nutrient composition of Hungarian proso millet varieties and the effect of decortication. *Acta Alimentaria* 40: 128-141.

Banigo EOI, deMan JM & Duitschaever CL (1974) Utilization of high lysine corn for the manufacture of ogi using a new improved processing system. *Cereal Chemistry* 51: 559-572.

Beit-Yannai E, Ben-Shabat S, Goldschmidt N, Chapagain BP, Liu RH & Wiesman Z (2011) Antiproliferative activity of steroidal saponins from Balanites aegyptiaca—An in vitro study. *Phytochemistry Letters* 4: 43-47.

Benhura MA & Chingombe A (1989) Traditional brewing methods in Zimbabwe. *Zimbabwe Science News* 23: 69-70.

Bhattacharjee R, Khairwal IS, Bramel P & Reddy KN (2007) Establishment of a pearl millet [*Pennisetum glaucum* (L.) R. Br.] core collection based on geographical distribution and quantitative traits. *Euphytica* 155: 35-45.

Campbell-Platt G (1994) Fermented foods — a world perspective. *Food Research International* 27: 253-257.

Chandrasekara A & Shahidi F (2010) Content of insoluble bound phenolics in millets and their contribution to antioxidant capacity. *Journal of Agricultural and Food Chemistry* 58: 6706-6714.

Choi YY, Osada K, Ito Y, Nagasawa T, Choi MR & Nishizawa N (2005) Effects of dietary protein of Korean foxtail millet on plasma adiponectin, HDL-cholesterol, and insulin levels in genetically type 2 diabetic mice. *Bioscience, Biotechnology, and Biochemistry* 69: 31-37.

Coulibaly A, Kouakou B & Chen J. (2011) Phytic acid in cereal grains: structure, healthy or harmful ways to reduce phytic acid in cereal grains and their effects on nutritional quality. *American Journal of Plant Nutrition and Fertilization Technology* 1-22.

Dahal NR, Karki TB, Swamylingappa B, Li Q & Gu G (2005) Traditional Foods and Beverages of Nepal—A Review. *Food Reviews International* 21: 1-25.

Dahl MS (1997) *India (Countries of the World)*. Capstone Press, MN, Mankato.

Devi P, Vijayabharathi R, Sathyabama S, Malleshi N & Priyadarisini V (2011) Health benefits of finger millet (*Eleusine coracana* L.) polyphenols and dietary fiber: a review. *Journal of Food Science and Technology* 1-20.

Efiuvwevwere BJ & Akona O (1995) The microbiology of *kunun-zaki* a cereal beverage from northern Nigeria, during the fermentation (production) process. *World Journal of Microbiology & Biotechnology* 11: 491-493.

FAO-FAOSTAT (2009) database archieve. ed., http://faostat.fao.org,2009

FAO (2012) Economic and Social Department: The Statistical Division. ed., Food and Agricultural Organization, Rome, Italy.

FAO/ICRISAT (1996) The World Sorghum and Millet Economies: Facts. ed. United Nations Food and Agriculture Organization, Rome, and International Crops Research Institute for the Semi-Arid Tropics, , Andhra Pradesh, India.

FAO/WHO (2001) Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. ed., Food and Agriculture Organization of the United Nations and World Health Organization Expert Consultation Report, Cordoba, Argentina.

Gadaga TH, Mutukumira AN, Narvhus JA & Feresu SB (1999) A review of traditional fermented foods and beverages of Zimbabwe. *International Journal of Food Microbiology* 53: 1-11.

Gélinas P, McKinnon CM, Mena MC & Méndez E (2008) Gluten contamination of cereal foods in Canada. *International Journal of Food Science & Technology* 43: 1245-1252.

Haggblade S & Holzapfel WH (1993) *African alcoholic beverages*. Marcel Dekker, New York.

Hamad S. H. (2012) The microbial quality of processed date fruits collected from a factory in Al-Hofuf City, Kingdom of Saudi Arabia. *Emirates Journal of Food and Agriculture* 24: 105-112.

Hashemi M, Shojaosadati S, Razavi S, Mousavi S, Khajeh K & Safari M (2012) The Efficiency of Temperature-Shift Strategy to Improve the Production of α-Amylase by *Bacillus sp*. in a Solid-State Fermentation System. *Food and Bioprocess Technology* 5: 1093-1099.

Hounhouigan I, Bougouma B, Mouquet-Rivier C, Fliedel G, Mestres C, Noel JM, Triande E & Monteiro G (2006) Production of good quality sorghum or millet malts for semi-industrial food production in West Africa. In: Traditional Grains Review ed. Institute for Food and Biotechnology, Gothenburg, Sweden.

Ibrahima O, Dhifi W, Raies A & Marzouk B (2004) Study of the variability of lipids in some millet cultivars from Tunisia and Mauritania. *Rivista Italiana Sostanze Grasse* 81: 112–116.

ICRISAT (2007) International Crops Research Institute for the Semi-Arid Tropics, 2007 annual report. ed. International Crops Research Institute for the Semi-Arid Tropics.

Inyang CU & Zakari UM (2008) Effect of germination and fermentation of pearl millet on proximate, chemical and sensory properties of instant "*fura*"—A Nigerian cereal food. *Pakistan Journal of Nutrition* 7: 9-12.

Inyang CU, & Idoko CA (2006) Assessment of the quality of ogi made from malted millet. *African Journal of Biotechnology* 5: 2334-2337.

Ito K, Ozasa H, Noda Y, Arii S & Horikawa S (2008) Effects of free radical scavenger on acute liver injury induced by D-galactosamine and lipopolysaccharide in rats. *Hepatology Research* 38: 194-201.

Iwuoha CI & Eke OS (1996) Nigerian indigenous fermented foods: their traditional process operation, inherent problems, improvements and current status. *Food Research International* 29: 527-540.

Izadi Z, Nasirpour A, Izadi M & Izadi T (2012) Reducing blood cholesterol by a healthy diet *International Food Research Journal* 19: 29-37.

Jideani VA & Wedzicha BL (1995) Shelf life and predominating microflora of a non-fermented pearl millet dough. *Journal of Food Science* 11: 83-93.

Jideani VA, Oloruntoba RH & Jideani IA (2010) Optimization of Fura Production Using Response Surface Methodology. *International Journal of Food Properties* 13: 272-281.

Jideani VA, Nkama I, Agbo EB & Jideani IA (2001) Survey of fura production in some northern states of Nigeria. *Plant Foods for Human Nutrition* 56: 23-36.

Jideani VA, Nkama I, Agbo EB & Jideani IA (2001) Survey of fura production in some northern states of Nigeria. *Plant Foods Human Nutrition* 56: 23-36.

Kamara MT, Amadou I & Zhou HM (2012) Antioxidant properties of fractionated foxtail millet protein hydrolysate. *International Food Research Journal* 19: 207-213.

Kamara MT, Zhou HM, Zhu KX, Amadou I & Tarawalie F (2009) Comparative study of chemical composition and physicochemical properties of two varieties of defatted foxtail millet flour grown in China. *American Journal of Food Technology* 4 255-267.

Karki T (1986) Some Nepalese Fermented Foods and Beverages: Traditional Foods, Some Products and Technologies. ed. Central Food Technology and Research Institute, Mysore, India.

Laminu HH, Modu S & Numan AI (2011) Production, in vitro protein digestibility, phytate content and acceptability of weaning foods prepared from pearl millet (*Pennisetum typhoideum*) and cowpea (Vigna unguiculata). *International Journal of Nutrition and Metabolism* 3: 109-113.

Lei V & Jakobsen M (2004) Microbiological characterization and probiotic potential of koko and koko sour water, African spontaneously fermented millet porridge and drink. *Journal of Applied Microbiology* 96: 384-397.

Lei V, Friis H & Michaelsen KF (2006) Spontaneously fermented millet product as a natural probiotic treatment for diarrhoea in young children: An intervention study in Northern Ghana. *International Journal of Food Microbiology* 110: 246-253.

Lestienne I, Buisson M, Lullien-Pellerin V, Picq C & Trèche S (2007) Losses of nutrients and anti-nutritional factors during abrasive decortication of two pearl millet cultivars (*Pennisetum glaucum*). *Food Chemistry* 100: 1316-1323.

Liang S, Yang G & Ma Y (2010) Chemical characteristics and fatty acid profile of foxtail millet bran oil. *Journal of the American Oil Chemists' Society* 87: 63-67.

López-García R (2008) Amaranth: An ancient whole grain from Mexico. *Cereal Foods World* 53: 155-156.

Mahgoub SEO & Elhag SA (1998) Effect of milling, soaking, malting, heat-treatment and fermentation on phytate level of flour Sudanese sorghum cultivars. *Food Chemistry* 4: 77-80.

Marero LM, Payumo EM, Aguinaldo AR & Homma S (1988) Nutritional characteristics of weaning foods prepared from germinated cereals and legumes. *Journal of Food Science* 53: 1399-1402.

Marero LM, Pagumo EM, Aguinaldo AR & Homma S (1998) Nutritional characteristics of weaning foods prepared from germinated cereals and legumes. *Journal of Food Science* 53: 1399-1405.

Mariac C, Luong V, Kapran I, Mamadou A, Sagnard F, Deu M, Chantereau J, Gerard B, Ndjeunga J, Bezancon G, Pham JL & Vigouroux Y (2006) Diversity of wild and cultivated pearl millet accessions (*Pennisetum glaucum* [L.] R. Br.) in Niger assessed by microsatellite markers. *Theoretical and Applied Genetics* 114: 49-58.

Masha GGK, Ipsen R, Petersen MA & Jakobsen M (1998) Microbiological, rheological and aromaticcharacteristics of fermented *Uji* (an East African Sour Porridge). *World Journal of Microbiology and Biotechnology* 14: 451-456.

Mbugua SK (1984) Isolation and characterization of lactic acid bacteria during the traditional fermentation of uji. *East African Agricultural and Forestry Journal* 50: 36-43.

Michodjèhoun-Mestre L, Hounhouigan DJ, Dossou J & Mestre C (2005) Physical, chemical and microbiological changes during natural fermentation of gowé, a fermented beverage from sprouted or nonsprouted sorghum (*Sorghum bicolor*) in West-Africa. *African Journal Biotechnology* 4: 487-496.

Mugula JK, Narvhus JA & Sorhaug T (2003) Use of starter cultures of lactic acid bacteria and yeasts in the preparation of togwa, a Tanzanian fermented food. *International Journal of Food Microbiology* 83: 307-318.

Muyanja CM, Narvhus JA, Treimo J & Langsrud T (2003) Isolation, characterisation and identification of lactic acid bacteria from bushera: a Ugandan traditional fermented beverage. *International Journal of Food Microbiology* 80: 201-210.

Nkama I, Angarawai IA & Badau MH (1999) Survey Report on Food Technology of Millet. ed. Lake Chad Research Institute, Maiduguri, Nigeria.

Nzelibe HC & Nwasike CC (1995) The brewing potential of "Acha" (*Digitaria exilis*) malt compared with pearl millet (*Pennisetum typhoides*) malts and sorghum (*Sorghum bicolor*) malts. *Journal of the Institute of Brewing* 101: 345-350.

Obiana AB (2003) Overview: importance of millets in Africa.

Obilana AB & Manyasa E (2002) *Millets. Pages 177-217 in: Pseudo Cereals and Less Common Cereals*. Springer-Verlag, New York.Odunfa SA (1985) *African fermentation foods*. Elsevier Publishers, London.

Oi Y & Kitabatake N (2003) Chemical composition of an East African traditional beverage, togwa. *Journal of Agricultural and Food Chemistry* 51: 7024-7028.

Onyango C, Okoth MW & Mbugua SK (2003) The pasting behaviour of lactic-fermented and dried *uji* (an East African sour porridge). *Journal of the Science of Food and Agriculture* 83: 1412-1418.

Onyango C, Noetzold H, Bley T & Henle T (2004) Proximate composition and digestibility of fermented and extruded uji from maize–finger millet blend. *LWT - Food Science and Technology* 37: 827-832.

Onyango C, Bley T, Raddatz H & Henle T (2004) Flavour compounds in backslop fermented *uji* (an East African sour porridge). *European Food Research and Technology* 218: 579-583.

Oranusi SU, Umoh VJ & Kwaga JKP (2003) Hazards and critical control points of kunun-zaki, a non-alcoholic beverage in Northern Nigeria. *Food Microbiology* 20: 127-132.

Park KO, Ito Y, Nagasawa T, Choi MR & Nishizawa N (2008) Effects of dietary Korean proso-millet protein on plasma adiponectin, HDL cholesterol, insulin levels, and gene expression in obese type 2 diabetic mice. *Bioscience, Biotechnology, and Biochemistry* 72: 2918-2925.

Prado FC, Parada JL, Pandey A & Soccol CR (2008) Trends in non-dairy probiotic beverages. *Food Research International* 41: 111-123.

Radhajeyalakshmi R, Yamunarani K, Seetharaman K & Velazhahan R (2003) Existence of Thaumatin-like Proteins (TLPs) in Seeds of Cereals. *Acta Phytopathologica et Entomologica Hungarica* 38: 251-257.

Rivera-Espinoza Y & Gallardo-Navarro Y (2010) Non-dairy probiotic products. *Food Microbiology* 27: 1-11.

Saldivar S (2003) *Cereals: dietary importance*. Academic Press, Agosto, London.

Saleh ASM, Zhang Q, Chen J & Shen Q (2013) Millet Grains: Nutritional Quality, Processing, and Potential Health Benefits. *Comprehensive Reviews in Food Science and Food Safety* 12: 281-295.

Shobana S & Malleshi NG (2007) Preparation and functional properties of decorticated finger millet (*Eleusine coracana*). *Journal of Food Engineering* 79: 529-538.

Shukla K & Srivastava S (2011) Evaluation of finger millet incorporated noodles for nutritive value and glycemic index. *Journal of Food Science and Technology* 1-8.

Siwela M, Taylor JRN, de Milliano WAJ & Duodu KG (2010) Influence of phenolics in finger millet on grain and malt fungal load, and malt quality. *Food Chemistry* 121: 443-449.

Sohrabvandi S, Mousavi SM, Razavi SH, Mortazavian AM & Rezaei K (2010) Alcohol-free Beer: *Methods of Production, Sensorial Defects, and Healthful Effects*. *Food Reviews International* 26: 335-352.

Songre-Ouattara LT, Mouquet-Rivier C, Icard-Verniere C, Rochette I, Diawara B & Guyot JP (2009) Potential of amylolytic lactic acid bacteria to replace the use of malt for partial starch hydrolysis to produce African fermented pearl millet gruel fortified with groundnut. *International Journal of Food Microbiology* 130: 258-264.

Suma PF & Urooj A (2012) Antioxidant activity of extracts from foxtail millet (*Setaria italica*). *Journal of Food Science and Technology* 49: 500-504.

Tamang JP, Sarkar PK & Hesseltine CW (1988) Traditional fermented foods and beverages of Darjeeling and Sikkim–a review. *Journal of the Science of Food and Agriculture* 44: 375-385.

Taylor JRN (2004) *Millet*: Pearl. In: C. Wrigley, H. Corke and C. E. Walker (Eds.). Encyclopedia of Grain Science (Vol. 2), Elsevier: London. pp. 253–261.

Taylor JRN, Barrion SC & Rooney LW (2010.) Pearl millet—New developments in an ancient food grains. *Cereal Foods World* 55: 16-19.

Tou EH, Guyot JP, Mouquet-Rivier C, Rochette I, Counil E, Traore AS & Treche S (2006) Study through surveys and fermentation kinetics of the traditional processing of pearl millet (*Pennisetum glaucum*) into ben-saalga, a fermented gruel from Burkina Faso. *International Journal of Food Microbiology* 106: 52-60.

Uvere PO, Adenuga OD & Mordi C (2000) The effect of germination and killing on the cyanogenic potential, amylase and alcohol levels of sorghum malts used for burkutu production. *Journal of the Science of Food and Agriculture* 80: 352 - 358.

Vidya S, Ravi R & Bhattacharya S (2013) Effect of thermal treatment on selected cereals and millets flour doughs and their baking quality. *Food and Bioprocess Technology* 6: 1218-1227.

Vieira-Dalodé G, Madodé YE, Hounhouigan J, Jespersen L & Jakobsen M (2008) Use of starter cultures of lactic acid bacteria and yeasts as inoculum enrichment for the production of gowé, a sour beverage from Benin. *African Journal of Microbilology Research* 2: 179-186.

Viswanath V, Urooj A & Malleshi NG (2009) Evaluation of antioxidant and antimicrobial properties of finger millet polyphenols (*Eleusine coracana*). *Food Chemistry* 114: 340-346.

Vodouhe SR, Zannou A & Dako EA (2003) Actes du premier atelier sur la diversité génétique du fonio (*Digitaria exilis stapf*) en Afrique de l'Ouest,Conakry, Guinée, 4-6 Aout 1998. ed. 73. IPGRI. Vi, Rome (Italie).

von Mollendorff JW, Todorov SD & Dicks LM (2006) Comparison of bacteriocins produced by lactic-acid bacteria isolated from boza, a cereal-based fermented beverage from the Balkan Peninsula. *Current Microbiology* 53: 209-216.

Wickramasinghe P, Rajah CS, Lowe J & Benson A (2005) *The Food of India*. Murdoch Books UK Ltd, London.

Xu W, Wei L, Qu W, Liang Z, Wang J, Peng X, Zhang Y & Huang K (2011) A novel antifungal peptide from foxtail millet seeds. *Journal of the Science of Food and Agriculture* 91: 1630-1637.

Yang X, Wan Z, Perry L, Lu H, Wang Q, Hao C, Li J, Xie F, Yu J, Cui T, Wang T, Li M & Ge Q (2012) Early millet use in northern China. *Proceedings of the National Academy of Sciences of the United States of America* 109: 3726–3730.

Zvauya R, Mygochi T & Parawira W (1997) Microbial and biochemical changes occurring during production of masvusvu and mangisi, traditional Zimbabwean beverages. *Plant Foods for Human Nutrition* 51: 43-51.

CHAPTER 2

Effect of Fermentation by *Lactobacillus paracasei* Fn032 and Heat-Moisture Treatment on the Physicochemical Properties of Foxtail Millet (*Setaria italica*) Flour

2.1 Introduction

Millet is related to sorghum and belongs to the Poaceae (formerly known as Gramineae) plant family. There are many varieties of millet, but the four major types are pearl millet (*Pennisetum glaucum*), which comprises 40% of worldwide production; foxtail millet (*Setaria italica*); proso or white millet (*Panicum miliaceum*); and finger millet (*Eleusine coracana*) (Léder, 2004, Adekunle, 2012). Millets are major food sources for millions of people, especially those who live in hot, dry areas of the world. They are grown mostly in marginal areas under agricultural conditions in which major cereals fail to give substantial yields (Adekunle, 2012). Foxtail millet is the one of widely planted species of millet in most parts of East Asia, especially in northern China, where the seed is ground and used for porridge. It has been cultivated in China since way back in the sixth millennium BC. China is ranked world number three in millet production behind India and Nigeria (Léder, 2004), foxtail millet was also named Italian millet, German millet, Chinese millet, and Hungarian millet (Yang *et al.*, 2012).

There has been a significant increase in the search for gluten-free cereal diet formulations to suit the medical demands of people with celiac metabolic defects. Millet feed formulations therefore present a perfect solution to above stated problem. Millet grains are often grinded into flour or transform into products similar to those of sorghum such as thick porridges, thin porridges, steam-cooked products, fermented breads, unfermented breads, boiled rice-like products, alcoholic beverages, non-alcoholic beverages and snacks (Léder, 2004; Amadou *et al.*, 2011); however, the most common and simplest food prepared from foxtail millet in China is porridge. Fermentation is one of most commonly used method in preparing millet base food products and it has been shown to further increase nutrient content and to reduce anti-nutrients (Amadou *et al.*, 2011; Osman, 2011; Amadou *et al.*, 2013; Amadou *et al.*, 2013), furthermore, fermentation induces physical and chemical changes of the products (Lu *et al.*, 2005; Yang & Tao, 2008; Osman, 2011; Natarajan & Rajendran, 2012; Peralta-Contreras *et al.*, 2013).

Heat-moisture treatment (HMT) at high temperatures (84 – 120^{o}C) and intermediate moisture content for 15 min to 16 h modifies the physiochemical properties of starch without destroying the granule structure, studies showed that all millets are similar to rice in composition and nutritional value (Gunaratne & Hoover, 2002; Léder, 2004; Lin *et al.*, 2011). The digestibility of starch in cereals seeds may be affected by non-starch components, such as protein and cell-wall matrices which can entrap starch granules, and lipids thus forming complex (Syahariza *et al.*, 2013). Moreover, Englyst *et al.* (1992) stated that starch products containing a portion that digests rapidly is known as rapidly digesting starch (RDS), a portion that digests slowly as slowly digesting starch (SDS) and a fraction that is resistant to digestion to be resistant starch (RS) which have potential physiological benefits similar to that of dietary fiber.

Though numerous studies have been conducted on the changes in physiochemical properties of starch, and the effect of fermentation on the flour structure; furthermore, studies have shown that the shelf-life of grain extracted flour can also be enhanced using heat moisture treatment (Gunaratne & Hoover, 2002; Osman, 2011; Chung *et al.*, 2012; Correia & Beirão-da-Costa, 2012; Rodríguez-Damian *et al.*, 2012). There might be interactions between fermentation and HMT whereas, the variation in the morphology is attributed to the biological origin of the grain genotype (Singh *et al.*, 2010); however, effect of HTM and fermentation by *L. paracasei* Fn032 information on the foxtail millet (*Setaria italic*) flour is yet to be known to the readers. Therefore, this study aims to evaluate the effect of fermentation by *L. paracasei* Fn032 and heat-moisture treatment on the physical and chemical selected characteristics of flour from foxtail millet.

2.2 Materials and Methods

2.2.1 Materials

Foxtail millet meal was obtained from Anhui Yanzhifang Food Co., Ltd (Hefei, China). *L. paracasei* Fn032 strain was obtained from the State Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University (Wuxi, China). The *L. paracasei* Fn032 were maintained as glycerol stocks and stored at -70°C and were diluted in series for at least 3 times prior to being used in this study. All the chemical reagents used were of analytical grade.

2.2.2 Fermentation and sample preparation

An *L. paracasei* Fn032 25 µL aliquot was mixed aseptically with 23 mL double distilled water and 25 g defatted foxtail millet flour (10^7 CFU/g) in a 140 mm × 200 mm polyethylene bag vacuum sealed and then allowed to undergo solid-state fermentation at 37ºC for 48 h. The fermented foxtail millet flour (FFM) was vacuum oven dried (DZF-1B, Botai, Co. Ltd, Zhejiang, China) milled and then the flour was packed in polythene bags, sealed and kept in the freezer until required for analysis.

2.2.3 Heat-moisture treatment

Heat-moisture treatment was performed as described by Gunaratne & Hoover (2002) with slight modifications there by forming heat-moisture-treated foxtail millet flour (FM) and fermented foxtail millet flour (FFM). The moisture content of the samples was adjusted to approximately 22% by dispersing the samples in an appropriate amount of distilled water, then sealed under different stainless steel containers and equilibrated at 4°C overnight. The sealed samples were heated under a thermostatically controlled convection oven to 100°C for 16 h and then cooled at room temperature (28°C). The contents were removed from the containers and dried at 30°C until a uniform moisture content of approximate ~8% was attained. Both FMH and FFMH samples were ground and sieved using 100 mm mesh sieve.

2.2.4 Physicochemical analyses

The moisture content of foxtail millet flour samples was determined by drying in an air-oven at 105±1ºC until a constant weight was obtained. Ash content was determined by incinerating at 550ºC until the constant weight was achieved. Based on the total nitrogen content, the sample protein content was determined at conversion factor of 6.25 using Kjeldahl's method. Carbohydrate percentage content was obtained by taking the difference between the total percentage (100%) and the sum of moisture, sugar, tannins, crude protein, and ash contents. The above methods were determined according to AOAC (2000) methods and the pH was measured using pH-meter FE20 Mettler-Toledo Instruments (Shanghai, China).

2.2.5 Differential scanning calorimetry

The thermal properties of foxtail millet flour meal were investigated using a TA Q100-DSC thermal analyzer (TA Instruments, New Castle, DE) equipped with a thermal analysis data system.

Dry weight flour samples of 2.0-3.0 mg were loaded into hermetically sealed aluminum pans and then gradually heated from 20 to 120°C at a rate of 10°C/min. An empty sealed pan was used as a reference. The onset temperature (T_0), Peak or decomposition temperature (T_d) of the characteristic transitions, denaturation enthalpy-change (ΔH) and width at half peak height of endothermic peaks ($\Delta T_{1/2}$) were all recorded.

2.2.6 X-ray diffraction analysis

X-ray powder diffraction analysis (Shimadzu Lab XRD-6000) was used to examine the crystalline property of foxtail millet flour samples with scanning region of the two (θ) angles varying from 3° to 64° which covered all the significant diffraction peaks of starch crystallites.

2.2.7 Scanning electron microscopy (SEM)

SEM analysis was carried out using a scanning electron microscope (Quanta-200 FEI, Netherland). The samples were gold coated before being loaded to the scanning electron microscopy. The coated samples were loaded into the system and the image was viewed under 5.0 Kv potential using a secondary electron image. The image was captured using 11.1 mm Ricoh Camera of 600x Mag.

2.2.8 *In vitro* starch digestibility

The starch fractions due to rapidly digestible starch (RDS), slowly digestible starch (SDS), resistant starch (RS) and the total starch (TS) were determined. Samples dispensed into capped tubes and incubated at 37°C with invertase, pancreatic α-amylase and amyloglucosidase under a water bath shaker as described by Englyst *et al.* (1992). The formed supernatant was analyzed at 0 min, 20 min, and 120 min to determine the glucose content levels. The glucose data obtained was used to calculate the content of various starch types using the following formulas:

Total starch (TS) = (total glucose (TG) − free glucose (FG)) × 0.9
Rapid digestible starch (RDS) = (G20 − FG) × 0.9
Slow digestible starch (SDS) = (G120 − G20) × 0.9
Resistant starch (RS) = TS − (RDS + SDS)

Where, FG, G20 and G120 (mg) represent the amount of glucose in the supernatant due to analysis was recorded at 0 min, 20 min and 120 min respectively. The Total glucose content was determined by addition of 3, 5-dinitrosalicylic acid for 30 min after complete hydrolysis of starch into glucose in the presence of perchloric acid.

2.2.9 Pasting properties

Pasting characteristics were determined using a rapid visco-analyzer (RVA) (New port scientific, RVA TECMASTER, Australia). 3 g of each sample (moisture content as shown in Table 2.1) were mixed with 25 mL of distilled water thoroughly mixed and the canister was well fitter into the RVA as recommended.

2.2.10 Fluorescence measurements

Fluorescence spectra were recorded using a Hitachi F-7000 fluorescence spectrometer (Hitachi High Technologies, Tokyo, Japan) with a 700-voltage xenon lamp at room temperature (26 ± 2^{o}C). Foxtail millet flours were packed to uniformity by means of a few gentle shocks of the powder cell upon a wood surface. The scanning ranges were 275 nm and 280-600 nm for excitation and emission respectively. The spectral bandwidths of excitation and emission slits were set at 2.5 and 5.0 nm, respectively; using a scanning speed of 30 000 nm/min.

2.2.11 Statistical analysis

All experiments were conducted in triplicates and statistical analysis was performed using IBM-SPSS Inc. software (version 19.0). One-way analysis of variance (ANOVA) was used to determine significant differences between means, with the significance level taken at ($P < 0.05$). Duncan test was used to perform multiple comparisons between means, with the significance level $P < 0.05$.

2.3 Results and Discussion

2.3.1 Physicochemical compositions

The changes in physicochemical compositions and pH due to fermentation and heat moisture treatment effects on foxtail millet flour are presented in Table 2.1. Moisture content of the samples ranged from 11.38 to 13.31%. The pH reading of foxtail millet flour (FM) was 5.93 however due

to fermentation effects the pH value of fermented foxtail millet flour (FFM) dropped to 3.87. There was no significant ($P < 0.05$) pH variation effect on foxtail millet flour (FM) due to heat-moisture treatment (HMT). This is similar to the research findings by Fasasi *et al.* (2007) that reported the role played by pH in the traditionally processed African breadfruit seed (*Treculia africana*) flour. There was a significant ($P < 0.05$) increase in crude protein content from 12.02 to 13.51% in FM and FFM respectively however a slight decrease in crude protein content was observed in HMT flour samples. The protein content increase during fermentation can be attributed to microbial synthesis of proteins from metabolic intermediates during their growth cycles; moreover, the *L. paracasei* Fn032 strain growth in the sample might have also contributed in the obvious increase of protein content quantification after fermentation. This is in line with Elyas *et al.* (2002) work, significant increase in protein content of two pearl millet cultivars were observed as a result of fermentation whereas, Ibrahim *et al.* (2005) showed changes in protein fractions during fermentation of sorghum cultivar. After fermentation the total carbohydrate content in FM (83.77%) significantly ($P < 0.05$) decreased to 74.02% (FFM).

Table 2.1 Chemical Compositions of Fermentation and Heat-Moisture Treated Foxtail Millet Flour.

Analysis (%)	**FM**	**FFM**	**FMH**	**FFMH**
Moisture	12.27±0.55ab	13.31±0.60b	12.04±0.55a	11.38±0.62a
Protein	12.02±0.68a	13.54±0.47b	11.80±0.24a	13.08±0.15b
Soluble sugar	2.54±0.14a	3.58±0.68a	2.79±0.50a	3.33±0.49a
Tannins	0.14±0.00a	0.23±0.23a	0.09±0.08a	0.23±0.08a
Ash	0.89±0.09a	1.02±0.07a	0.88±0.02a	1.04±0.14a
Carbohydrate	72.14±0.22b	68.32±1.02a	72.40±2.12b	70.94±2.00a
pH	5.93±0.43b	3.87±0.16a	5.89±0.11b	3.99±0.11a
RDS	1.43±0.47a	3.76±0.79a	12.46±1.56b	15.92±2.60c
SDS	6.83±0.74a	13.66±0.87c	8.65±1.01b	18.42±0.67d
RS	7.61±1.62a	11.82±1.13b	19.25±1.21c	22.68±0.78d

Values are means ± standard deviation of three determinations. Rows with different letters indicate statistical differences ($P < 0.05$). Values followed by the same letter (a, b, c, d) in the same row are not significantly different ($P < 0.05$). RDS: rapidly digestible starch, SDS: slowly digestible starch, RS: resistant starch.

In accordance to the trends observed by Osman (2011) in pearl millet study. Though, *L. paracasei* Fn032 fermentation of foxtail millet flour did increase the protein content in the samples; contrary, no significant changes ($P < 0.05$) were found in soluble sugars, tannins and ash content after HMT application (Table 2.1). HMT induced the physicochemical property changes on flour and enhanced starch yield (Wongsagonsup *et al.*, 2008; Chung *et al.*, 2012). This was in accordance to research study findings on the quantification or determination of starch and sugar levels in five minor millets due to *in vitro* α-amylase starch digestion (Krishnakumari & Thayumanavan, 1995).

2.3.2 Thermal characteristics

Findings of thermal property changes on foxtail millet flour samples due to fermentation and HMT are shown in Table 2.2. Both HMT and fermentation by *L. paracasei* Fn032 significantly ($P < 0.05$) induced changes in the thermal characteristics of foxtail millet flour samples. After fermentation, there was a slight increase in the decomposition temperature (T_d) from 167.80 - 168.76^{o}C and an enthalpy (ΔH) decrease from 141.03 - 51.50 J/g. Moreover after HMT, T_d augment to 180.59 and 189.82^{o}C respectively for FMH and FFMH, and ΔH showed similar trends with sharp decrease (Table 2.2). Contrary trends were observed in fermented rice flours (Lu *et al.*, 2005) this could be attributed to the growth of lactic acid bacteria (*L. paracasei* Fn032) releasing more proteolytic enzymes that break down the grain cell walls releasing more starch hence increased crystalline structural proportions within the sample. The increase in T_o of heat-moisture treated foxtail millet flour samples has been attributed to structural changes within the starch granules (Table 2.2). This may also be attributed to the transformation of the inter-crystalline parts into amorphous phases. Lower enthalpy values were observed in FM as compared to fermented foxtail millet flour (FFM). This pattern may be due to the fact that the intact granules and HMT induced aggregates that had tendency of undergoing retro-gradation (Table 2). Similarly, (Gunaratne & Hoover, 2002); (Rodríguez-Damian *et al.*, 2012); (Wongsagonsup *et al.*, 2008) reported that due to HMT induced crystalline disruption; there was a decrease in the retro-gradation enthalpy levels in unripe banana HMT treated flour.

Table 2.2 Thermal Properties of Fermentation and Heat Moisture Treated Foxtail Millet Flour.

Samples	FM	FFM	FMH	FFMH
T_0 (°C)	89.13± 1.13a	121.44± 1.04c	110.32± 2.32b	144.89± 2.11d
T_d (°C)	167.80± 2.70a	168.76± 1.25a	180.59±2.60b	189.82±0.84c
ΔH (J/g)	141.03± 1.55d	51.50± 0.52b	56.02± 1.02c	11.08± 1.09a
$\Delta T_{1/2}$ (°C)	37.27± 2.01d	8.52± 0.50a	32.86± 2.13c	27.71± 0.73b

T0: Onset temperature denaturation; Td: Thermal decomposition temperature; Δ*H*: Enthalpy changes of the endotherm; Δ*T*1/2: Width at half peak height of endothermic peak. Values are means ± standard deviation of three determinations. Rows with different letters indicate statistical differences ($P < 0.05$). Values followed by the same letter (a, b, c, d) in the same row are not significantly different ($P < 0.05$).

2.3.3 X-ray diffraction

The X-ray diffraction patterns and intensities of the samples are shown in Figure 2.1. The foxtail millet flour samples subjected to fermentation and HMT showed the A-type X-ray diffraction pattern. It was reported that the 'A' type X-ray diffraction patterns are mainly shown by cereal starches while the 'B' type patterns represents the true crystalline forms of starch (Buleon *et al.*, 1998); a fourth 'V' type pattern arises sometimes from complexes formed between amylose and numerous polar organic molecules (Buleon *et al.*, 1998; Wongsagonsup *et al.*, 2008). Similar trends were reported in fermented rice flour research studies (Lu *et al.*, 2005).

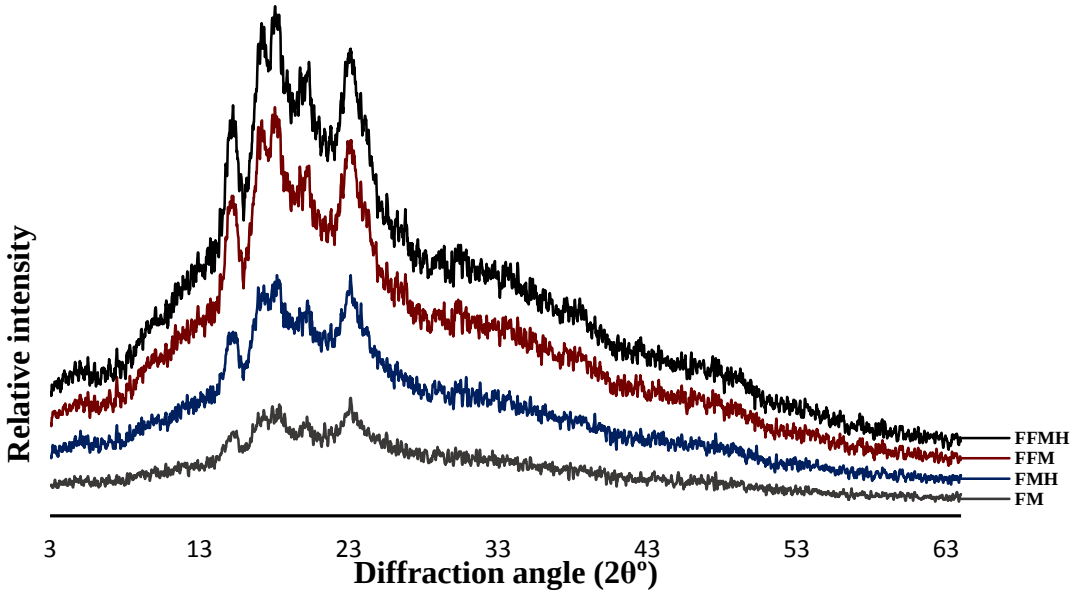


Figure 2.1 X-ray diffraction patterns of foxtail millet flour (FM), fermented foxtail millet flour (FFM), heat-moisture treated foxtail millet flour (FMH) and heat-moisture treated fermented foxtail millet flour (FFMH).

The main reflections for foxtail millet flour diffraction patterns were displayed at 2θ =14.8°, 16.6°, 17.8°, 19.7º and 23º however, fermentation and HMT treatment led to stronger diffraction intensities at the same peaks (FFM, FMH and FFMH) the appearance of a new crystalline peak at 2θ = 30° (Figure 2.1). After fermentation and HMT the FM 2θ = 17º peak was slightly split into two peaks (2θ = 16.6º and 2θ = 17.8º). Application of HMT may disrupt the crystalline region of starch and the double helical movement effect due to HMT could further disrupt the starch crystals thus, changing the overall crystalline orientation (Shih *et al.*, 2007; Rodríguez-Damian *et al.*, 2012). Parameters such as protein and fat have also been considered to have an influence on the starch properties such as crystallinity and viscosity. Therefore, the observed protein content trends after flour fermentation and after defatting might have led to the actual behavior in this report. This follows the trends suggested by Puncha-arnon & Uttapap (2013).

2.3.4 Starch digestibility

As shown in Table 2.1, FFMH revealed the highest values of starch fractions (15.92, 18.42 and 22.68%) while FM sample had the lowest RDS, SDS and RS values of 1.43, 6.83 and 7.61% respectively. Therefore, the study confirmed that both fermentation and HMT have a significant influence on the starch fractions (Table 2.1). Similar trends were reported by Lin *et al.* (2011) where resistant starch (RS) content in normal corn starch was observed to have increased from 23.3%, to 47.7% and 83.8% after heat moisture treatment, in addition, (Niba, 2003) also investigated the effect of HMT on the digestibility of maize, potato, cocoyam, yam, plantain and rice flours, reporting that the SDS content for all flours was increased compared with the native flours. In areas where foxtail millet is mainly cultivated, foxtail millet meal is used for making porridge and in due process the flour undergoes through all the above mentioned changes. Similarly, previous research studies have demonstrated that the RDS and glycemic index levels in processed food are highly correlated (Englyst *et al.*, 1992; Brennan, 2005). Whereas from the SDS structural perspective there was prolonged release of glucose from foxtail millet starch (FM and yet RS is resistant to digestion, thus, no glucose is available for glycemic response. However, fermentation by *L. paracasei* Fn032 and heat moisture treatment of foxtail millet further facilitate

the release of RS that are beneficial to health. Furthermore, SDS, RS or a combination of the two may contribute to formation of an improved nutritional quality of starch (Niba, 2003; Syahariza *et al.*, 2013). Fermentation exhibited minimal impact on the RDS level content though it had specific effects on the SDS and RS content (Table 2.1).

Singh *et al.* (2010) stated that morphological characteristics of starches from various botanical sources vary with the genotype such as size and shape of starch granules. The foxtail millet flour starch fractions (RS, SDS and RDS) increased after HMT (Niba, 2003; Lin *et al.*, 2011). Furthermore, fermentation causes proteolysis of the protein matrix surrounding the starch granules. Therefore starch granules are released from protein matrix and they are easily hydrolyzed by amylase, thus increasing the digestibility of the starch (Singh *et al.*, 2010). Although, the total starch concentration of foxtail millet flour increased.

2.3.5 Pasting characteristics

Trends of the pasting property changes due to fermentation and HMT effects on foxtail millet flour samples are presented in Figure 2.2. The RVA viscoamylogram of fermented samples FFM and FFMH showed higher peak viscosity, final viscosity, breakdown and setback of pasting properties than the non-fermented samples. The overlapped curves of samples (FM, FMH) and (FFM, FFMH) indicated that the changes in foxtail millet flour after fermentation was significant with increase in pasting characteristic profile (Figure 2.2); thus, HMT has not influenced the viscosity of samples. Though, researchers have demonstrated decreases in the peak viscosity, through viscosity, final viscosity, breakdown and setback after heat moisture treatment; while the pasting temperature may be increased depending on the treatment conditions (Rodríguez-Damian *et al.*, 2012; Puncha-arnon & Uttapap, 2013). Shih *et al.* (2007) reported a similar phenomenon in waxy rice flour after 20% moisture content. There was a sharp increase in peak viscosity, final pasting velocity and similar holding timing strength in all foxtail millet flour fermented samples. The significant increase in protein content after fermentation might have caused the shifting of viscosity. The observed changes after *L. paracasei* Fn032 fermentation concurrently decreased with breakdown and setback pasting properties of foxtail millet flour samples. These changes may have been due to the collapse in the starch crystal structural granules during the fermentation process. Therefore, breakdown plays a vital role in modifying the starch pasting properties; however, Yang & Tao

(2008) concluded that reduced breakdown viscosity may occur after lactic acid rice flour fermentation.

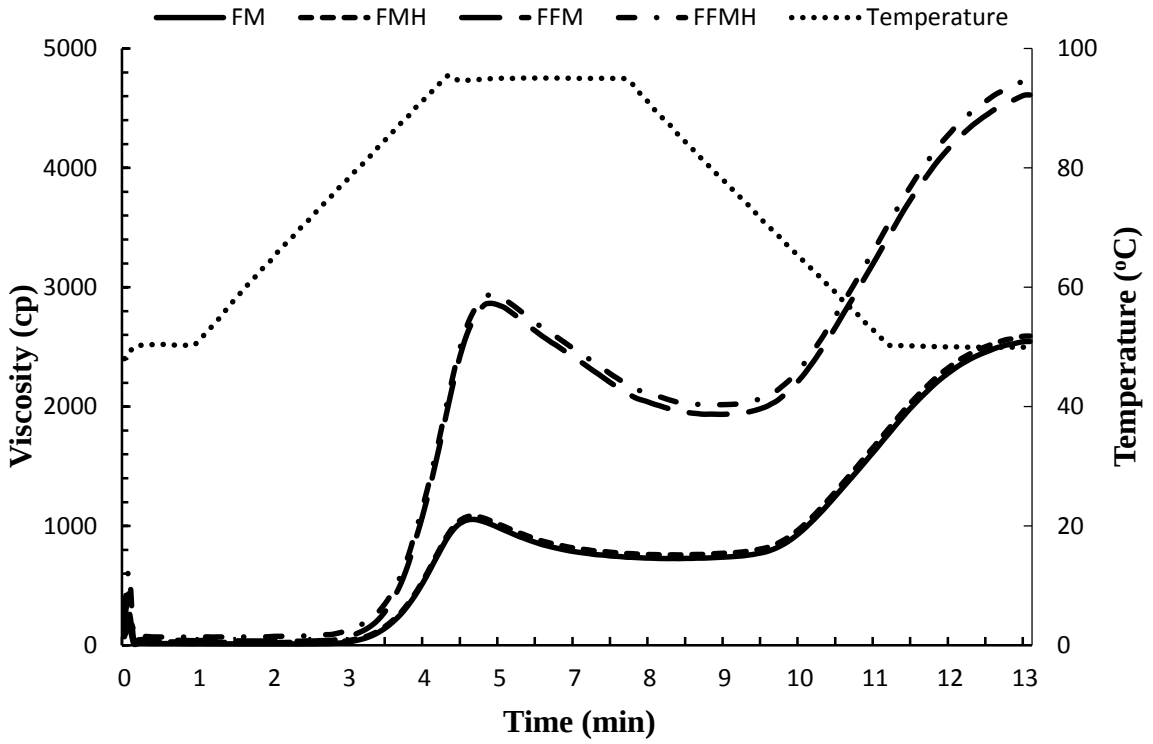


Figure 2.2 Effect of fermentation and heat-moisture treatment on pasting characteristics of foxtail millet flour (FM), fermented foxtail millet flour (FFM), heat-moisture treated foxtail millet flour (FMH) and heat-moisture treated fermented foxtail millet flour (FFMH).

2.3.6 Microstructural properties

According to obtained micrographs presented in Figure 2.3 the structure was consisted presumably of more or less larger granules except in the FFM sample. An irregular structural network was observed in the FM and FFMH consequently, HMT application was assumed to have played a double role in forming a regular shape in FMH sample. Moreover, FFM exhibited finer granules than its counterparts after fermentation, whereas heat moisture treatment turned flour sample (FFMH) with more disrupted and irregular starch granules shape. The samples structural differences clearly reflect the effects on foxtail millet flour meal due to fermentation and HMT that resulted into flour granule disruptions (Shih *et al.*, 2007; Ma *et al.*, 2011).

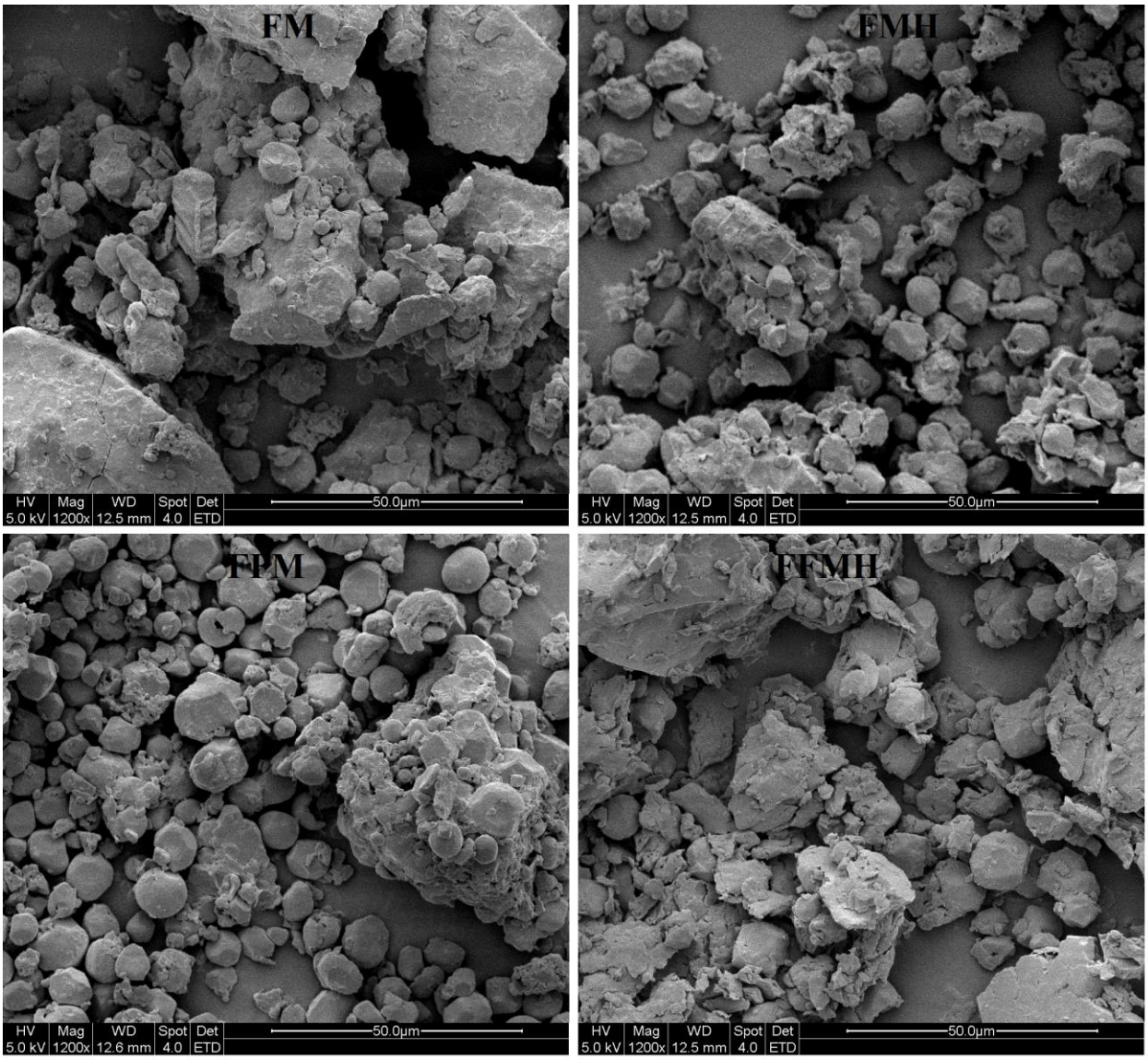


Figure 2.3 Scanning electron micrographs of foxtail millet flour (FM), fermented foxtail millet flour (FFM), heat-moisture treated foxtail millet flour (FMH) and heat-moisture treated fermented foxtail millet flour (FFMH).

Previous scanning electron microscope (SEM) studies on roasted flours showed that microstructure (in terms of starch granule and protein matrix) to be similar to that of raw flour samples however, SEM analysis of pre-boiled flours did not show presence of intact starch granules (Ma *et al.*, 2011; Rodríguez-Damian *et al.*, 2012). This could be due to starch gelatinization during heat treatment. Interestingly after fermentation, some of the starches show

more amorphous extracellular material and breakages within the flour granules (Sotomayor *et al.*, 1999; Yang & Tao, 2008; Peralta-Contreras *et al.*, 2013). Amylose/amylopectin cross linkages are more likely to occur during gelatinization as compared to thermal treatment since all proteins and starches are exposed and toughly mixed. The high sample protein content (Table 2.1) clearly reflects the above protein/starch ratio similar to findings by Syahariza *et al.* (2013).

2.3.7 Fluorescence measurements

A spectrophotometer is an easy and direct instrument that is commonly used to measure the front-face surface fluorescence spectra of cereal flours and cereal-based products. It has been reported that fluorescence spectroscopy is a very sensitive technique able to measure trace substances containing one or more fluorescent chemical groups (Karoui & Blecker, 2011). Fluorescence only due to tryptophan residues can be selectively measured by exciting at 290 nm, because at this wavelength there is no absorption by tyrosine (Karoui *et al.*, 2006). Seemly, three emission bands are present in the range of 280-600 nm spectral (Figure 4).

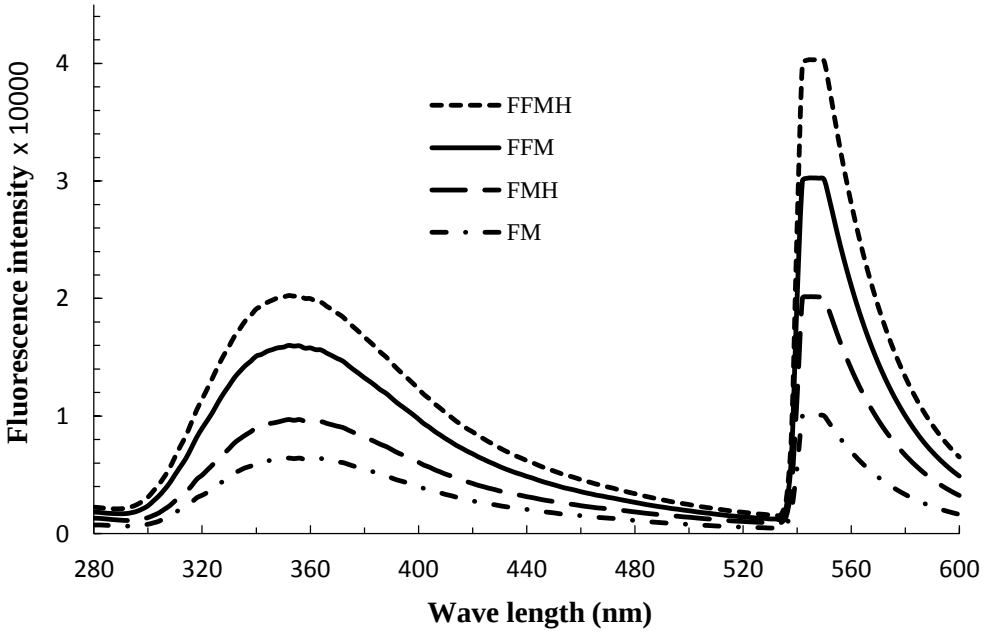


Figure 2.4 Fluorescence spectra of foxtail millet flour (FM), fermented foxtail millet flour (FFM), heat-moisture treated foxtail millet flour (FMH) and heat-moisture treated fermented foxtail millet flour (FFMH) on 280 nm excitation.

The two main fluorescence intensity bands in the range of 280- 420 nm are likely due to aromatic residues present in cereal proteins such as tryptophan (Zandomeneghi, 1999), whereas, the 536 nm fluorescence are reported to be caused by riboflavin (Zandomeneghi *et al.*, 2003) and 420-520 nm region shows low intensity fluorescence suggested to be caused by the presence of ferulic acid (Karoui & Blecker, 2011). However, (Zandomeneghi *et al.*, 2000) attributed the band between 430 and 530 nm to the lutein flour content. Both the fermentation by *L. paracasei* Fn032 and HMT of foxtail millet flour presented significant effect on the fluorescence intensity with the highest being observed in FFMH sample (Figure 2.4). This shows the correlation between the increase in protein content after fermentation (more amino acid residues) as well as the influence of HMT. Furthermore, the constant high fluorescent intensity reflects the heat resistance property of riboflavin (vitamin B2). Huschka *et al.* (2012) reported that moisture is an important factor in inter-protein network formation.

2.4 Conclusions

The results suggested that the effects of *L. paracasei* Fn032 fermentation and heat moisture treatment significantly influence the physicochemical properties of foxtail millet flour. Scanning electron microscopy analysis provided information on differences in the microstructure of the flours as a result of fermentation and heat moisture treatment and on the other side fluorescence spectroscopy showed how changes in chemicals affected the spectra of tryptophan and riboflavin. RVA pasting properties of foxtail millet flour followed the same trends and the crystal structures moderately lost completeness during the fermentation process. The two conditions of fermentation and HMT had significant effects on the fraction content of RDS, SDS, and RS, resulting in higher values after heat moisture treatment. Opportunities may, therefore, exist to explore the potential of foxtail millet meal cooking to enhance its physicochemical and functional properties while improving its nutritional value. There is need for further studies to understand the digestibility of processed foxtail millet flour as a potential gluten free diet formulation.

2.5 References

Adekunle AA (2012) *Agricultural innovation in sub-saharan africa: experiences from multiple-stakeholder approaches*. Ghana.

Amadou I, Gbadamosi OS & Le GW (2011) Millet-based traditional processed foods and beverages—A review. *Cereal Food World* 56: 115-121.

Amadou I, Gounga ME & Le GW (2013) Millets: Nutritional composition, some health benefits and processing — A review. *Emirates Journal of Food and Agriculture* 25: 501-508.

Amadou I, Le GW, Amza T, Sun J & Shi YH (2013) Purification and characterization of foxtail millet-derived peptides with antioxidant and antimicrobial activities. *Food Research International* 51: 422-428.

AOAC (2000) *Official Methods of Analysis of the Association of Official Analytical Chemists International* Suite 400, 2200 Wilson Boulevard, Arlington, Virginia, USA.

Brennan CS (2005) Dietary fibre, glycaemic response, and diabetes. *Molecular Nutrition & Food Research* 49: 560-570.

Buleon A, Colonna P, Planchot V & Ball S (1998) Starch granules: Structure and biosynthesis. *International Journal of Biological Macromolecules* 23: 85-112.

Chung H-J, Cho A & Lim S-T (2012) Effect of heat-moisture treatment for utilization of germinated brown rice in wheat noodle. *LWT - Food Science and Technology* 47: 342-347.

Correia P & Beirão-da-Costa ML (2012) Effect of drying temperatures on starch-related functional and thermal properties of chestnut flours. *Food and Bioproducts Processing* 90: 284-294.

Elyas SHA, El Tinay AH, Yousif NE & Elsheikh EAE (2002) Effect of natural fermentation on nutritive value and in vitro protein digestibility of pearl millet. *Food Chemistry* 78: 75-79.

Englyst HN, Kingman SM & Cummings JH (1992) Classification and measurement of nutritionally important starch fractions. *European Journal of Clinical Nutrition* 46: S33-50.

Fasasi OS, Eleyinmi AF & Oyarekua MA (2007) Effect of some traditional processing operations on the functional properties of African breadfruit seed (*Treculia africana*) flour. *LWT - Food Science and Technology* 40: 513-519.

Gunaratne A & Hoover R (2002) Effect of heat-moisture treatment on the structure and physicochemical properties of tuber and root starches. *Carbohydrate Polymers* 49: 425-437.

Huschka B, Bonomi F, Marengo M, Miriani M & Seetharaman K (2012) Comparison of lipid effects on structural features of hard and soft wheat flour proteins assessed by front-face fluorescence. *Food Chemistry* 133: 1011-1016.

Ibrahim FS, Babiker EE, Yousif NE & El Tinay AH (2005) Effect of fermentation on biochemical and sensory characteristics of sorghum flour supplemented with whey protein. *Food Chemistry* 92: 285-292.

Karoui R & Blecker C (2011) Fluorescence spectroscopy measurement for quality assessment of food systems—a Review. *Food and Bioprocess Technology* 4: 364-386.

Karoui R, Cartaud G & Dufour E (2006) Front-face fluorescence spectroscopy as a rapid and nondestructive tool for differentiating various cereal products: A preliminary investigation. *Journal of Agricultural and Food Chemistry* 54: 2027-2034.

Krishnakumari S & Thayumanavan B (1995) Content of starch and sugars and in vitro digestion of starch by α-amylase in five minor millets. *Plant Foods for Human Nutrition* 48: 327-333.

Léder I (2004) *Sorghum and millets, in Cultivated Plants, Primarily as Food Sources*. Eolss Publishers, Oxford ,UK.

Lin J, Singh H, Wen C & Chang Y (2011) Partial-degradation and heat-moisture dual modification on the enzymatic resistance and boiling-stable resistant starch content of corn starches. *Journal of Cereal Science* 54: 83-89.

Lu Z-H, Li L-T, Min W-H, Wang F & Tatsumi E (2005) The effects of natural fermentation on the physical properties of rice flour and the rheological characteristics of rice noodles. *International Journal of Food Science & Technology* 40: 985-992.

Ma Z, Boye JI, Simpson BK, Prasher SO, Monpetit D & Malcolmson L (2011) Thermal processing effects on the functional properties and microstructure of lentil, chickpea, and pea flours. *Food Research International* 44: 2534-2544.

Natarajan K & Rajendran A (2012) Evaluation and optimization of food-grade tannin acyl hydrolase production by a probiotic Lactobacillus plantarum strain in submerged and solid state fermentation. *Food and Bioproducts Processing* 90: 780-792.

Niba LL (2003) Effect of storage period and temperature on resistant starch and β-glucan content in cornbread. *Food Chemistry* 83: 493-498.

Osman MA (2011) Effect of traditional fermentation process on the nutrient and antinutrient contents of pearl millet during preparation of Lohoh. *Journal of the Saudi Society of Agricultural Sciences* 10: 1-6.

Peralta-Contreras M, Chuck-Hernandez C, Perez-Carrillo E, Bando-Carranza G, Vera-Garcia M, Gaxiola-Cuevas N, Tamayo-Limon R, Cardenas-Torres F & Serna-Saldivar SO (2013) Fate of free amino nitrogen during liquefaction and yeast fermentation of maize and sorghums differing in endosperm texture. *Food and Bioproducts Processing* 91: 46-53.

Puncha-arnon S & Uttapap D (2013) Rice starch vs. rice flour: Differences in their properties when modified by heat–moisture treatment. *Carbohydrate Polymers* 91: 85-91.

Rodríguez-Damian AR, De La Rosa-Millán J, Agama-Acevedo E, Osorio-díaz P & Bello-Pérez LA (2013) Effect of different thermal treatments and storage on starch digestibility and physicochemical characteristics of unripe banana flour. *Journal of Food Processing and Preservation* 37(5): 987–998.

Shih F, King J, Daigle K, An HJ & Ali R (2007) Physicochemical properties of pice ptarch podified by pydrothermal treatments. *Cereal Chemistry* 84: 527-531.

Singh J, Dartois A & Kaur L (2010) Starch digestibility in food matrix: a review. *Trends in Food Science & Technology* 21: 168-180.

Sotomayor C, Frias J, Fornal J, Sadowska J, Urbano G & Vidal-Valverde C (1999) Lentil starch content and its microscopical structure as influenced by natural fermentation. *Starch/Stärke* 51: 152−156.

Syahariza ZA, Sar S, Hasjim J, Tizzotti MJ & Gilbert RG (2013) The importance of amylose and amylopectin fine structures for starch digestibility in cooked rice grains. *Food Chemistry* 136: 742-749.

Wongsagonsup R, Varavinit S & BeMiller JN (2008) Increasing slowly digestible starch content of normal and waxy maize starches and properties of starch products. *Cereal Chemistry* 85: 738-745.

Yang X, Wan Z, Perry L, *et al.* (2012) Early millet use in northern China. *Proceedings of the National Academy of Sciences of the United States of America* 109: 3726–3730.

Yang Y & Tao W-Y (2008) Effects of lactic acid fermentation on FT-IR and pasting properties of rice flour. *Food Research International* 41: 937-940.

Zandomeneghi M (1999) Fluorescence of cereal flours. *Journal of Agricultural and Food Chemistry* 47: 878-882.

Zandomeneghi M, Festa C, Carbonaro L, Galleschi L, Lenzi A & Calucci L (2000) Front-surface absorbance spectra of wheat flour: Determination of carotenoids. *Journal of Agricultural and Food Chemistry* 48: 2216-2221.

Zandomeneghi M, Carbonaro L, Calucci L, Pinzino C, Galleschi L & Ghiringhelli S (2003) Direct fluorometric determination of fluorescent substances in powders: The case of riboflavin in cereal flours. *Journal of Agricultural and Food Chemistry* 51: 2888-2895.

CHAPTER 3

Evaluation of Antimicrobial, Antioxidant Activities, and Nutritional Values of Fermented Foxtail Millet Extracts by *Lactobacillus paracasei* Fn032

3.1 Introduction

Millet is a general term for a wide range of cereals. Foxtail millet (*Setaria italica*) is one of the most important food crops of the semi-arid tropics; it originated from China, and is now planted all over the world. It plays a very important role in the agriculture and food of many developing countries because of its ability to grow under adverse heat and limited rainfall conditions. It was reported that foxtail millet has many nutritious and medical functions (Hegde *et al.*, 2005; Xue *et al.*, 2008). The millet bran is used extensively as animal feed in China (En *et al.*, 2008). Millet is an important cereal and nutritious food in traditional diets, especially for people in the continents of Asia and Africa. However, cereals such as millet are also deficient in some of the basic components (e.g., essential amino acids); fermentation may be the most simple and economical way of improving their nutritional value, sensory properties, and functional qualities (Motarjemi 2002; Dharmaraj *et al.*, 2011). Thus, fermented foods have become very important to human beings all over the world. About 20 to 40% of our food supply is from fermented foods (Campbell-Platt, 1994).

Production of antimicrobial peptides is found as a widespread strategy used by plants, animals, and microorganisms to combat pathogenic microorganisms. Besides the variable structural characteristics, these peptides are mostly cationic, showing an amphipathic nature, containing about 30 to 100 amino acid residues in a linear or cyclic arrangement (Hwang & Vogel, 1998). *Lactobacillus* spp. are gram-positive, non-motile, non-sporulating, facultative anaerobes. *Lactobacillus paracasei* isolated from healthy humans showed antibacterial and anticandidal activities against oral pathogens, such as *S. mutans, S. salivarius, Streptococcus sanguis, Staphylococcus aureus, Actinomyces viscosus, P. gingivalis, Candida albican, Candida tropicalis,* and *Candida grabata* (Sookkhee *et al.*, 2001).

There is a wide range of oxygen-free radicals and other reactive oxygen species (ROS), which include free radicals, such as superoxide anion radicals ($O^{2-\bullet}$); hydroxyl radicals ($HO^{\bullet}$); and

non-free-radical species, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($|O_2$), which may form in the human body and in the food system. These radicals induce not only lipid peroxidation that causes deterioration of foods, but also cause oxidative damage by oxidizing biomolecules leading to cell death and tissue damage, such as atherosclerosis, cancer, emphysema, cirrhosis, and arthritis (Kehrer, 1993). Antioxidant agents can act against free radicals either by retarding their formation (preventive antioxidants) or by inactivating radicals in reaction medium (chain-breaking antioxidants) (Amadou *et al.*, 2011). Currently, the natural antioxidant *α*-tocopherol and some synthetic antioxidants, such as butylated hydroxytoluene, butylated hydroxyanisole, and propyl gallate, are commonly used to act against free radicals in food and biological systems. However, the use of synthetic antioxidants in food products is under strict regulation owing to their potential health hazards (Lee *et al.*, 2008).

Antioxidative property of Lactobacilli would be useful in the food manufacturing industry. They could beneficially affect the consumer by providing another dietary source of antioxidants or by providing probiotic bacteria with the potential of producing antioxidants during their growth in the intestinal tract. It was found that superoxide dismutase "SOD" produced by the engineered *Lactobacillus gasseri* reduces the levels of superoxide radicals in the gut imposed by specific members of the gut microbiota and the immune system (Carroll *et al.*, 2007), demonstrating the potential use of the antioxidative *Lactobacilli*. They also could modulate a redox state in the colonic fermentation system, which is related to their free radical scavenging ability or antibacterial effect. Sun *et al.* (2010) have previously demonstrated that antioxidative *Lactobacillus paracasei* Fn032 is a probiotic with the ability to scavenge or prevent the production of hydroxyl radical in the system mimicking the colon environment with Fe2+ addition. Nutritional, proximate analysis, and functionality of foxtail millet were widely studied (Kamara *et al.*, 2009; Durojaiye *et al.*, 2010; Kamara *et al.*, 2010; Liang *et al.*, 2010; Dharmaraj *et al.*, 2011). However, information on the aqueous extract's antimicrobial effect, antioxidant activity of fermented foxtail millet by *Lactobacillus paracasei* Fn032 is yet to be studied. Therefore, the present chapter was aimed to test the antibacterial effect and antioxidative properties including reducing power of aqueous extracts of fermented foxtail millet by *L. paracasei* Fn032, and also to evaluate their nutritional values using amino acid profile.

3.2 Materials and Methods

3.2.1 Materials

Foxtail millet bran and foxtail millet meal were purchased from a local producer (Hebei, China) and Da-Run-Fa Market (Wuxi, China), respectively. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), was purchased from Sigma-Aldrich, Inc. (Shanghai, China). Acid protease (Acid protease–537 from Asp.) was purchased from Sunson Industry Group Co.Ltd. (Beijing, China). Gentamycin was purchased from Xuzhou RYEN PHARMA. Co. Ltd. (Xuzhou, China). All other reagents were of analytical grade.

3.2.2 Bacterial strains

Lactobacillus paracasei Fn032 was obtained by the Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University (Wuxi, China). *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8099, and *Salmonella typhi* CMCC 50013 were provided by the microbiology laboratory culture collection of Jiangnan University (Wuxi, China). Bacteria were maintained as glycerol stocks and stored at −70°C. All strains were serially transferred at least three times prior to use in this study. The strains were inoculated into the Mann Rogosa Sharpe (MRS) broth and incubated for 18 h at 37°C in an anaerobic jar. Cells were collected by centrifugation at 3000× *g* for 15 min and the bacterial pellets were washed twice and re-suspended in 0.1 mol/L phosphate buffered saline (PBS; pH 7.3).

3.2.3 Fermentation and preparation of foxtail millet extracts

An amount of 0.025 mL of *L. paracasei* Fn032 with and without protease was mixed aseptically with 25 g of defatted foxtail millet bran (FFMB and FFMBP) and defatted foxtail millet flour (FFM and FFMP) (10^7 CFU/g) in a polyethylene bag (140 mm ×200 mm) and vacuum sealed, and then solid-state fermentation was performed for 48 h at 37°C. Fermented foxtail millet extracts were prepared according to the method described by Ye *et al.* (2003) Five grams of fermented foxtail millet were mixed with 50 mL of distilled water, homogenized for 1 min and incubated at 37°C for 60 min. The incubated mixture was centrifuged at 9600 rpm for 2 min using a D-3756 Osterode AM Harz Model 4515 Centrifuge (Hamburg, Germany) and the residue was washed with 20 mL of distilled water, centrifuged again at the same speed and time, and the combined

supernatant was freeze-dried and stored at –20°C until further use. The freeze-dried extracts were weighed and the yield was calculated based on the weight of foxtail millet bran and flour.

3.2.4 Chemical analysis

Crude protein content (N = 6.25) was determined using the micro-Kjeldahl method (Tkachuk, 1969). For total sugar determination, a 500-mg portion of sample was extracted three times with 20 mL of hot 80% ethanol and the extract was evaporated and made up to 10 mL. An aliquot was assayed for total soluble sugars by the phenol-sulphuric acid method (Du Bois *et al.*, 1956) using glucose as a standard. *In vitro* protein digestibility (IVPD) was carried out according to the method described by Elkhalil *et al.* (2001) with slight modifications. Twenty milligrams of fermented foxtail millet extract (flour and bran) samples were digested in triplicate in 10 mL of trypsin (0.2 mg/mL in 100 mM Tris-HCl buffer, pH 7.6). The suspension was incubated at 37°C for 2 h. Reaction was stopped by adding 5 mL of 50% trichloroacetic acid (TCA). The mixture was allowed to stand for 30–35 min at 4°C and was then centrifuged at 5000 rpm for 5 min using a D-3756 Osterode AM Harz Model 4515 Centrifuge (Hamburg, Germany). The resultant precipitate was dissolved in 5 mL of NaOH and protein concentrate was measured using the Kjeldahl method. Digestibility was calculated as follows:

$$\text{Protein digestibility (\%)} = \frac{(A-B)}{A} \times 100$$

where A is total protein content (mg) in the sample and B is total protein content (mg) in TCA precipitate.

3.2.5 Molecular weight determination

The samples were determined using a Waters TM 600E Advanced Protein Purification System (Waters Corporation, Milford, MA, USA). A TSK gel, 2000SWXL (7.8 × 300 mm) column was used with 10% acetonitrile + 0.1% TFA in HPLC grade water as the mobile phase. The calibration curve was obtained by running bovine carbonic anhydrase (29,000 Da), horse heart cytochrome C (12,400 Da), bovine insulin (5800 kDa), bacitracin (1450 Da), Gly-Gly-Tyr-Arg (451 kDa), and Gly-Gly-Gly (189 Da). The total surface area of the chromatograms was integrated and separated into eight ranges, expressed as a percentage of the total area.

3.2.6 Amino acid analysis and protein quality evaluation

The freeze-dried samples were digested with HCl (6 M) at 110°C for 24 h under nitrogen atmosphere. Reversed phase high performance liquid chromatography (RP-HPLC) analysis was carried out in an Agilent 1100 (Agilent Technologies, Palo Alto, CA, USA) assembly system after precolumn derivatization with o-phthaldialdehyde (OPA). Each sample (1 μL) was injected on a Zorbax 80 A C18 column (i.d. 4.6 × 180 mm; Agilent Technologies, Palo Alto, CA, USA) at 40°C with detection at 338 nm. Mobile phase A was 7.35 mmol/L sodium acetate/triethylamine/tetrahydrofuran (500:0.12:2.5, v/v/v), adjusted to pH 7.2 with acetic acid, while mobile phase B (pH 7.2) was 7.35 mmol/L sodium acetate/methanol/acetonitrile (1:2:2, v/v/v). The amino acid composition was expressed as g of amino acid per 100 g of protein. Total hydrophobic amino acids (THAA) and hydrophobic indices (*H*Φ) was calculated according to (Adler-Nissen, 1986). The estimated predicted protein efficiency ratio (PER) values of fermented foxtail millet extracts were carried out in accordance with Alsmeyer *et al.* (1974) using three regression equations. The FAO/WHO reference pattern of essential amino acid requirements (g/100 g of protein) FAO (2007) was used as the standard.

PER-1 = –0.684 + 0.456 (Leu) –0.047 (Pro)
PER-2 = –0.468 + 0.454 (Leu) –0.105 (Tyr)
PER-3 = –1.816 + 0.435 (Met) + 0.780 (Leu) + 0.211 (His) – 0.944 (Tyr)

3.2.7 DPPH radicals scavenging activity assay

The scavenging effect of fermented foxtail millet extracts on DPPH free radical was measured according to the method of Shimada *et al.* (1992) with little modification. Two milliliters of each sample solution (3, 5, 7, and 10 mg/mL) were added to 2 mL of 0.1 mM DPPH dissolved in 95% ethanol. The mixture was shaken and left for 30 min at room temperature, and the absorbance of resulting solution was read at 517 nm. A lower absorbance represents a higher DPPH scavenging activity. The scavenging effect was expressed as shown in the following equation:

$$\text{DPPH scavenging activity (\%)} = \frac{\left(Blank\ absorbance - Sample\ absorbance\right)}{Blank\ absorbance} \times 100$$

3.2.8 Reducing power

The reducing power of fermented foxtail millet extracts were measured according to Wu *et al.* (2003). The sample (0, 1, 1.5, 2, and 5 mg/mL) was added to 2 mL of 0.2 M phosphate buffer (pH 6.6) and 2 mL of 1% (w/v) potassium ferricyanide. The mixture was incubated at 50°C for 20 min, and then 2 mL of 10% (w/v) trichloroacetic acid (TCA) added. The mixture was centrifuged for 10 min at 3000× *g*, and 2 mL of the supernatant was mixed with 2 mL of distilled water and 0.4 mL of 0.1% (w/v) $FeCl_3$. After reaction for 10 min, the absorbance of the solution was read at 700 nm. Increase in the absorbance of the reaction mixture indicated increased reducing power.

3.2.9 Antimicrobial activity

Antibacterial activity was determined by the disc diffusion method Collin *et al.* (1995). The suitable nutrient agar media was prepared. Pure *S. aureus, E. coli*, and *S. typhi* culture were added separately to sterile petri plates. Sterilized media was poured into the sterile petri plates. After the media had cooled and solidified, wells (8 mm in diameter) were made in the agar and 50 μL of fermented foxtail millet extract was loaded in the wells. Gentamycin was used as a positive control. The plates were incubated for 24 h at 37°C. After incubation, the zone of inhibition was observed and the diameter was measured.

3.2.10 Statistical analysis

All experiments were conducted at least in triplicate. Analysis of variance (ANOVA) was performed and significant difference in mean values were evaluated by a Tukey HSD multiple range test at ($P < 0.05$) using SPSS version 17.0 (SPSS, Chicago, IL, USA).

3.3 Results and Discussion

3.3.1 Chemical analysis

Both fermented foxtail millet flour with and without added protease revealed appreciable amounts of the protein (FFMP: 21.04%; FFM: 20.54%). Fermented foxtail millet bran with and without added protease exhibited an insignificant increase in protein content, likewise with FFM and FFMP; however, these results affected their nutritional values (Table 3.1). A previous study reported influence of fermentation on nutrients composition using *Lactobacillus plantarum* Amadou *et al.* (2011). Little increase was observed in total soluble sugar content during

fermentation after the addition of protease and this implies that foxtail millet flour or bran were utilized as the main energy source for *L. paracasei* Fn032 activity.

Table 3.1 Chemical composition of fermented foxtail millet extracts.

Analysis (%)	FFM	FFMB	FFMP	FFMBP
Protein content	20.54±0.05b	6.75±0.06a	21.04±1.06b	7.86±0.06a
Protein digestibility	90.25±1.10ab	89.16±1.14a	92.16±1.11b	90.54±0.54ab
Soluble sugar content	3.58±0.68b	2.07±0.59a	3.79±0.50b	2.13±0.06a
Extract Yield	17.97±1.97a	30.22±0.15b	19.59±0.50a	28.17±1.13b

Values are means ± standard deviation of three determinations. Rows with different letters indicate statistical differences ($P < 0.05$).

Table 3.1 shows an insignificant increase ($P < 0.05$) between fermented samples (FFM: 3.58%; FFMB: 2.07%) and that of samples with added protease during the fermentation (FFMP: 3.79%; FFMBP: 2.13%). The entire samples showed a significant difference ($P < 0.05$) *in vitro* trypsin digestibility above 89% (Table 3.1). As expected, fermented foxtail millet flour had higher digestibility, sugar solubility, and more protein content than FFMB extracts. However, the yield of fermented foxtail millet bran extract was the higher (30.22%) with significant difference ($P < 0.05$) compared to FFM flour. Our data corroborated with the work reported by Chavan *et al.* (1988) and Dharmaraj *et al.* (2011).

3.3.2 DPPH radical-scavenging activity

The DPPH radicals were widely used to investigate the scavenging activity of some natural compounds. DPPH free radicals are stable in ethanol and show maximum absorbance at 517 nm. When DPPH radicals encounter a proton-donating substance, such as an antioxidant, the radicals would be scavenged and the absorbance is reduced Wu *et al.* (2003). Results shown in Table 3.2 revealed that FFMP exhibited the highest DPPH radical scavenging activity (91.40% at 3 mg/mL concentration). However, fermented foxtail millet bran extracts (FFMB and FFMBP) showed significant ($P < 0.05$) radical scavenging activity inversely proportional with the concentrations, whereas fermented foxtail millet flour extracts (FFM and FFMP) showed direct correlation. Similar observation was reported by Lee *et al.* (2008) and Amadou *et al.* (2011) on the water

extracts from fermented soybeans. Both fermented foxtail millet bran and flour extracts showed significant scavenging abilities on

Table 3.2 Radical scavenging activity of fermented foxtail millet extracts

Samples (mg/mL)	FFM	FFMB	FFMP	FFMBP
3	29.94±0.17a	79.70±1.79c	77.52±2.50c	54.71±2.26b
5	23.98±1.04a	77.31±1.82d	41.48±1.49b	69.15V2.11c
7	88.23±2.11c	66.56±3.02b	91.4±1.51c	38.14±2.01a
10	85.28±2.06c	64.74±3.80b	89.91±1.28c	32.82±1.11a

Values are means ± standard deviation of three determinations. Rows with different letters (a, b, c and d) indicate statistical differences ($P < 0.05$)

DPPH radicals and that may be explained considering that the extracts containing bioactive peptides, which were electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction.

3.3.3 Reducing power

Duh *et al.* (1999) and Zhu *et al.* (2008) reported earlier that antioxidant activity and reducing power were directly related. In this assay, the presence of antioxidants caused the reduction of the Fe3+/ferricyanide complex to the ferrous form, and the yellow color of the test solution changed to various shades of green and blue depending on the reducing power of all compounds. The reducing power of fermented foxtail millet extracts increased with increasing concentrations and it was observed that the addition of protease enhanced the reducing power at 1 to 5 mg/mL (Figure 3.1). The results of this research showed that the addition of protease has an effect on the reducing power, FFMBP and FFMP extracts were higher than that of FFMB and FFM at 1 to 5 mg/mL concentrations, as shown in Figure 3.1.

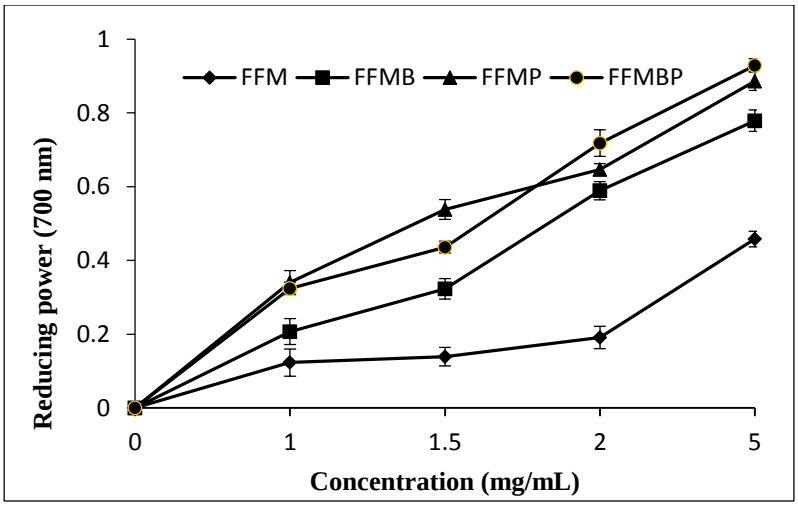


Figure 3.1 Reducing power of fermented foxtail millet extracts

The result indicated that fermented foxtail millet extracts are capable of donating electrons that can react with free radicals to convert them to stable products and strongly inhibiting radical chain reaction (Lee *et al.*, 2008; Rao *et al.*, 2010).

3.3.4 Antimicrobial activity

The foxtail millet extracts obtained from *L. paracasei* Fn032 fermentation were examined for their antimicrobial (Table 3.3) properties. The zones of inhibition for the fermented foxtail millet flour, without and with protease were the bigger (16.27 and 15.10 mm, respectively; see Table 3.3). It is shown in Table 3 that the FFMP extract exhibited significant inhibition ($P < 0.05$) against *S. Aureus*; moreover, the extracts from fermented flour, with and without protease, revealed stronger inhibition than their counterparts from the fermented bran. Since the fermented foxtail millet extract exhibited the highest antioxidant activity, it can be inferred that the peptides derived from *L. paracasei* Fn032 activity under the medium of foxtail millet flour are responsible for the microbial inhibition. It was obvious that all extracts showed lower inhibition than the controlled samples. The fermented foxtail millet flour extracts exhibited higher inhibitory response than the fermented foxtail millet bran due to higher activity of *L. paracasei* Fn032 in the flour. Generally, it is reported that *L. paracasei* Fn032 have the property of inhibiting the proliferation of other microorganisms but the information on the foxtail millet peptides extracts constituents with respect to their inhibitory activity is scanty (Viswanath *et al.*, 2009; Sun *et al.*, 2010; Chuang *et al.*, 2011).

Table 3.3 Antimicrobial activity of fermented foxtail millet extracts

Organism	FFM	FFMB	FFMP	FFMBP	Control
Escherichia coli	16.27±0.34d	12.31±0.47a	15.1±0.37c	13.27±0.41b	18.47±0.39e
Salmonella typhi	13.57±0.47c	11.76±0.21a	12.50±0.38b	12.54±0.33b	17.03±0.07d
Staphyloccocus aureus	14.17±0.42c	12.10±0.41b	15.47±0.44d	11.45±0.42a	17.31±0.24e

Values are means ± standard deviation of five determinations (Zone of inhibition in mm). Rows with different letters (a, b, c, d and e) indicate statistical differences ($P < 0.05$).

3.3.5 Amino acids content and protein quality

The total amino acid composition of the foxtail millet extracts obtained from *L. paracasei* Fn032 fermentation, are shown in Table 3.4, along with the hydrophobic amino acid index and estimated predicted protein efficiency ratio (PER) (FAO, 2007). Table 3.4 showed a significant difference ($P < 0.05$) in their content of essential amino acids as well as nonessential amino acids. FFMP revealed the highest while FFM had the lowest content of hydrophobic and essential amino acids. The solid state fermentation with *L. paracasei* Fn032 in combination with protease was achieved owing to the difference in the content of hydrophobic amino acid, which makes up the peptides. Furthermore, aspartic acid, glutamic acid, leucine, and proline were found to be abundant as expected in most fermented cereals (Sun *et al.*, 2010; Amadou *et al.*, 2011). For both fermented foxtail millet bran and flour, water extracts showed significant balance ($P < 0.05$) in amino acid composition and most of them were at a higher level than FAO/WHO/UNU protein and amino acid requirements in human nutrition (FAO, 2007). Conversely, FFMP with the highest antioxidative activity and relatively higher antimicrobial inhibition ability did correlate well with total hydrophobic amino acids (THAA) and hydrophobic indices ($H\Phi$), respectively, for 51.39 g/100 g and 8.47 Kj/moL AAR. Both the amount and quality of protein provided by a food are important in the human diet.

Table 3.4 Amino acid composition (g/100g protein), hydrophobic index and estimated predicted protein efficiency ratio

Amino acid	Total amino acid				FAO/WHO/UNU, 2007	
	FFM	FFMB	FFMP	FFMBP	Child	Adult
Essential Amino Acid						
Histidine	3.19±0.02c	2.13±0.01a	3.17±0.01c	2.18±0.02b	1.6	1.5
Threonine	4.84±0.01c	4.32±0.01a	4.27±0.02a	4.57±0.04b	2.5	2.3
Valine	6.91±0.01c	6.26±0.03b	6.01±0.02a	6.22±0.03b	2.9	3.9
Methionine	2.92±0.01c	1.08±0.01a	2.73±0.02b	3.31±0.03d		
Met + Cys*	3.77±0.03b	3.96±0.03c	3.55±0.05a	6.01±0.02d	2.3*	1.6*
Phenylalanine	4.02±0.02c	3.34±0.01b	4.16±0.01d	3.21±0.02a		
Phe + Tyr**	6.56±0.02d	5.36±0.03a	6.45±0.09c	5.64±0.03b	4.1**	3.8**
Isoleucine	4.09±0.02c	3.57±0.01a	4.27±0.01d	3.86±0.02b	3.0	3.0
Leucine	8.30±0.02c	5.41±0.01a	11.98±0.01d	6.55±0.02b	6.0	5.9
Lysine	5.06±0.01c	2.94±0.00b	2.87±0.01a	2.93±0.01b	4.8	4.5
Tryptophan	1.19±0.02d	0.97±0.01b	1.09±0.01c	0.92±0.01a	0.66	0.6
Non-Essential Amino Acid						
Aspartic acid	10.09±0.09b	12.10±0.02d	7.82±0.05a	11.37±0.03c		
Glutamic acid	14.98±0.01c	13.47±0.01a	19.27±0.08d	14.32±0.01b		
Serine	4.94±0.01b	5.12±0.01c	4.76±0.03a	5.79±0.01d		
Glycine	6.47±0.02b	14.30±0.08d	4.41±0.03a	9.65±0.04c		
Arginine	4.57±0.01c	1.64±0.06a	1.93±0.02b	1.69±0.02a		
Alanine	9.78±0.01c	8.79±0.02b	10.57±0.03d	8.43±0.01a		
Tyrosine	2.54±0.01d	2.02±0.01a	2.29±0.02b	2.43±0.01c		
Cysteine-s	0.85±0.01a	2.88±0.00c	0.82±0.02a	2.70±0.02b		
Proline	5.38±0.01a	10.08±0.01c	7.53±0.02b	10.07±0.03c		
Hydrophobicity						
THAA (g/100g)	41.47±0.42a	44.30±0.03b	51.39±0.03d	47.65±0.05c		
*H*Φ (Kj/mol AAR)	8.10±0.01a	8.36±0.01b	8.47±0.02c	8.37±0.02b		
Estimated predicted protein efficiency ratio (PER)						
PER-1	2.48±0.01c	1.32±0.01a	4.40±0.04d	1.82±0.02b		
PER-2	3.02±0.02b	2.20±0.02a	4.70±0.03c	2.25±0.02a		
PER-3	4.25±0.10c	1.41±0.02a	7.16±0.12d	2.87±0.06b		

THAA: Total Hydrophobic Amino Acid, HΦ: Hydrophobic index (Kilojoule/ mole Amino Acid Residue); *Requirements for methionine + cysteine, ** Requirements for phenylalanine + tyrosine. Raw with different letters indicate statistical differences ($P < 0.05$).

Many benefits are attributed to fermentation. It preserves and enriches food, improves digestibility, and enhances the taste and flavor of foods (Motarjemi, 2002; Amadou *et al.*, 2011). The protein quality, also known as the nutritional or nutritive value of a food, depends on its amino acid content and on the physiological utilization of specific amino acids after digestion, absorption, assimilation, and minimal obligatory rates of oxidation. Because direct assessment of protein nutritional value in human subjects is impractical for regulatory purposes, methods based on *in vitro* (chemical) and animal bioassays for assessment of protein quality have been developed (Motarjemi, 2002). Good quality protein should exceed 2.00 predicted PER values (Friedman, 1996). FFMP has the highest PER value compared to FFM, FFMB, and FFMBP (Table 3.4). Furthermore, the PER values of FFMP surpassed significantly the required level of 2.00 PER values and that of standard casein PER of 2.5 (Friedman, 1996). Solid state fermentation of foxtail millet using *L. paracasei* Fn032, not only improved the physiochemical quality but also enhanced the nutritional values.

3.3.6 Molecular weight distributions

The molecular weight distributions of the various extracts were determined by SEHPLC. The molecular weights for all samples were calculated according to the standard equation below: Log Mol 6.77–0.217 T (R^2 = 0.992). The size of peptides is known to be a significant factor in the overall antioxidant activity of hydrolyzed proteins.

Table 3.5 Molecular weight distribution of fermented foxtail millet extracts

Molecular weight (Da)	Area (%)			
	FFM	**FFMB**	**FFMP**	**FFMBP**
> 5000	1.24	0.05	0.14	0.02
5000 – 3000	3.05	0.26	0.38	0.18
3000 – 2000	4.06	0.39	0.63	0.37
2000 – 1000	9.29	1.18	2.72	1.50
1000 – 500	11.71	2.69	6.70	4.06
500 – 180	34.60	16.88	45.25	21.76
< 180	36.05	78.55	44.19	72.11

FFM: Fermented foxtail millet; FFMB: Fermented foxtail millet bran; FFMP: Fermented foxtail millet in combination with protease; FFMBP: Fermented foxtail millet bran in combination with protease.

Results in Table 3.5 showed that the molecular weight distribution of different extracts (FFM, FFMB, FFMP, and FFMBP), have different molecular weight (MW) distributions. Fermentation with *L. paracasei* Fn032 have the ability to produce low molecular weight distributions peptides with bioactivity, such as antimicrobial and antioxidative properties, compared with a previous investigation by Kamara *et al.* (2009) There was significant ($P < 0.05$) influence of the protease in combination with *L. paracasei* Fn032 on the extracts' characteristics (Sun *et al.*, 2010; Amadou *et al.*, 2011). The MW distributions ranged below 180 Da to 5000 Da above (Table 3.5). These findings are in agreement with observations from other studies and support the fact that antioxidative, antimicrobial peptides properties are highly influenced by their molecular mass (Chavan *et al.*, 1988; Lee *et al.*, 2008; Amadou *et al.*, 2011).

3.4 Conclusion

From the results obtained, it may be inferred that the fermented foxtail millet is a good source of bioactive peptides with signifilcantly higher antioxidant and antimicrobial activities. Thus, solid state fermentation of foxtail millet using *L. paracasei* Fn032, in combination with protease, not only improves the physiochemical quality but also enhanced the nutritional values of the meal. Remarkable antioxidant activity and protein quality exhibited by all the extracts could be associated with the potential activity of *L. paracasei* Fn032 during the bioprocessing. Further investigation in this direction may be helpful in the purification and utilization of the specific peptides constituents of the fermented foxtail millet bran or flour extracts as food preservatives.

3.5 References

Adler-Nissen J (1986) *Enzymic Hydrolysis of Food Proteins*. New York: Elsevier Applied Science Publishers, New York.

Alsmeyer RH, Cunigham AE & Happieh ML (1974) Equations predicted (PER) from amino acid analysis. *Food Technology* 28: 34-40.

Amadou I, Le GW, Shi YH & Jin S (2011) Reducing, radical scavenging, and chelation properties of fermented soy protein meal hydrolysate by *Lactobacillus plantarum* Lp6. *International Journal of Food Properties* 14: 654-665.

Amadou I, Le GW, Shi YH, Gbadamosi OS, Kamara MT & Jin S (2011) Optimized *Lactobacillus plantarum* Lp6 solid-state fermentation and proteolytic hydrolysis improve some nutritional attributes of soybean protein meal. *Journal of Food Biochemistry* 35: 1686-1694.

Campbell-Platt G (1994) Fermented foods — a world perspective. *Food Research International* 27: 253-257.

Carroll IM, Andrus JM, Bruno-Barcena JM, Klaenhammer TR, Hassan HM & Threadgill DS (2007) Anti-inflammatory properties of *Lactobacillus gasseri* expressing manganese superoxide dismutase using the interleukin 10-deficient mouse model of colitis. *American Journal of Physiology Gastrointestinal and Liver Physiology* 293: G729-738.

Chavan UD, Chavan JK & Kadam SS (1988) Effect of fermentation on soluble proteins and in vitro protein digestibility of sorghum, green gram and sorghum-green gram blends. *Journal of Food Science* 53: 1574-1575.

Chuang LC, Huang CS, Ou-Yang LW & Lin SY (2011) Probiotic Lactobacillus paracasei effect on cariogenic bacterial flora. *Clinical Oral Investigations* 15: 471-476.

Collin CH, Lyne DM & Grange JM (1995) *Microbiological Methods, 7th*. Oxford Publishers, London.

Dharmaraj U, Ravi R & Malleshi NG (2011) Physicochemical and textural characteristics of expanded finger millet. *International Journal of Food Properties* 15: 336-349.

DuBois M, Gilles KA, Hamilton JK, Rebers PA & Smith F (1956) Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* 28: 350-356.

Duh PD, Tu YY & Yen GC (1999) Antioxidant Activity of Water Extract of Harng Jyur (*Chrysanthemum morifolium* Ramat). *LWT - Food Science and Technology* 32: 269-277.

Durojaiye FA, Falade KO & Akingbala JO (2010) Chemical composition and storage properties of fura from pearl millet (*Pennisetum americanum*). *Journal of Food Processing and Preservation* 34: 820-830.

Elkhalil EAI, El Tinay AH, Mohamed BE & Elsheikh EAE (2001) Effect of malt pretreatment on phytic acid and in vitro protein digestibility of sorghum flour. *Food Chemistry* 72: 29-32.

En H, Pang ZH & Xiong BH (2008) Comparative analysis of composition and nutritive value of millet bran feed. *China Feed* 18: 39-41.

FAO (2007) Protein and amino acid requirements in human nutrition. pp. Geneva, Switzerland.

Friedman M (1996) Nutritional value of proteins from different food sources: A review. *Journal of Agriculture and Food Chemistry* 44: 26-29.

Hegde PS, Rajasekaran NS & Chandra TS (2005) Effects of the antioxidant properties of millet species on oxidative stress and glycemic status in alloxan-induced rats. *Nutrition Research* 25: 1109-1120.

Hwang PM & Vogel HJ (1998) Structure-function relationships of antimicrobial peptides. *Biochemistry and Cell Biology* 76: 235-246.

Kamara MT, Kexue Z, Amadou I, Tarawalie F & Zhou H (2009) Functionality, in Vitro Digestibility and Physicochemical Properties of Two Varieties of Defatted Foxtail Millet Protein Concentrates. *International Journal of Molecular Sciences* 10: 5224–5238.

Kamara MT, Amadou I, Tarawalie F & Huiming Z (2010) Effect of enzymatic hydrolysis on the functional properties of foxtail millet (*Setaria italica* L.) proteins. *International Journal of Food Science & Technology* 45: 1175-1183.

Kehrer JP (1993) Free radicals as mediators of tissue injury and disease. *Critical Reviews in Toxicology* 23: 21-48.

Lee YL, Yang JH & Mau JL (2008) Antioxidant properties of water extracts from Monascus fermented soybeans. *Food Chemistry* 106: 1128-1137.

Liang S, Yang G & Ma Y (2010) Chemical characteristics and fatty acid profile of foxtail millet bran oil. *Journal of the American Oil Chemists' Society* 87: 63-67.

Motarjemi Y (2002) Impact of small scale fermentation technology on food safety in developing countries. *International Journal of Food Microbiology* 75: 213-229.

Rao AS, Reddy SG, Babu PP & Reddy AR (2010) The antioxidant and antiproliferative activities of methanolic extracts from Njavara rice bran. *BMC Complementary and Alternative Medicine* 10: 4.

Shimada K, Fujikawa K, Yahara K & Nakamura T (1992) Antioxidative properties of xanthan on the antioxidation of soybean oil in cyclodextrin emulsion. *Journal of Agricultural and Food Chemistry* 40: 945-948.

Sookkhee S, Chulasiri M & Prachyabrued W (2001) Lactic acid bacteria from healthy oral cavity of Thai volunteers: inhibition of oral pathogens. *Journal of Applied Microbiology* 90: 172-179.

Sun J, Hu XL, Le GW & Shi YH (2010) Lactobacilli prevent hydroxy radical production and inhibit *Escherichia coli* and Enterococcus growth in system mimicking colon fermentation. *Letter Applied Microbiology* 50: 264-269.

Tkachuk R (1969) Nitrogen-to-protein conversion factors for cereals and oilseed meals. *Cereal Chemistry* 46: 419-424.

Viswanath V, Urooj A & Malleshi NG (2009) Evaluation of antioxidant and antimicrobial properties of finger millet polyphenols (*Eleusine coracana*). *Food Chemistry* 114: 340-346.

Wu H-C, Chen H-M & Shiau C-Y (2003) Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). *Food Research International* 36: 949-957.

Xue YY, Li P & Lin QB (2008) Research evolution on chemical component and physical character of foxtail millet. *Journal of Chinese Cereals Oils Association* 22: 51-56.

Ye YT, Xue M, Lin SM, Wang YH, Luo L & Tian JS (2003) Enzymolysis kinetics of digestive enzyme from intestine and hepatopancreas in grass carp to four kinds of raw feed materials. *Journal of Fishery Science China* 10: 470-473.

Zhu YP, Cheng YQ, Wang LJ, Fan JF & Li LT (2008) Enhanced Antioxidative Activity of Chinese Traditionally Fermented Okara (*Meitauza*) Prepared with Various Microorganism. *International Journal of Food Properties* 11: 519-529.

CHAPTER 4

Purification and Characterization of Foxtail Millet-Derived Peptides with Antioxidant and Antimicrobial Activities

4.1 Introduction

Cereals are good source of natural antioxidants and serve as a major staple food for many populations around the globe, millets are placed sixth among other cereals, accounting for about 1.3% of total cereal production in 2009 (Devi *et al.*, 2011; Yang *et al.*, 2012). Millets are highly nutritious among cereals, and as they do not contain gluten, there is a good prospect for their use in the development of gluten-free foods and beverages for patients with gluten sensitivity (Taylor & Emmambux, 2008; Amadou *et al.*, 2011). Little studies have demonstrated that millets are not only rich sources of phenolic compounds but are also beneficial proteins (Xu *et al.*, 2011; Kamara *et al.*, 2012). Foxtail millet (*Setaria italica*) is one of the most important food crops of the semi-arid tropics, originated from China, and is now planted all over the world (Yang *et al.*, 2012).

Most of probiotic foods generate fatty acids, vitamins and other vital nutrients such as bioactive peptides that improve the body's resistance against pathogenic microorganisms (Castillo *et al.*, 2012). Fermented foods are important to human beings and account for 20 to 40% of food supply (Anukam & Reid, 2009). Lactic acid bacteria and their fermented food products are thought to confer a variety of important nutritional and therapeutic benefits to consumers, including antioxidant and antimicrobial activities (Chandrasekara & Shahidi, 2011; Amadou *et al.*, 2013). Many microorganisms possess enzymatic and nonenzymatic antioxidative mechanisms and minimize generation of reactive oxygen species (ROS) to levels that are not harmful to the cells. Antioxidative Lactobacillus could modulate redox state in colonic fermentation system, which is related to their free radical- scavenging ability or antibacterial effect (Li *et al.*, 2012). It has been previously demonstrated that antioxidative *Lactobacillus paracasei* Fn032 is a probiotic with an ability to scavenge or prevent the production of hydroxyl radical in the systemmimicking colon fermentation and prevent hydroxyl radical production (Sun *et al.*, 2010).

Production of antimicrobial and antioxidative peptides is found as a widespread strategy used by plants, animals and microorganisms to combat pathogenic microorganism and stress. It is

generally believed that a protein, which is resistant to proteolytic digestion in the digestive tract retains sufficient structural integrity to have an increased probability of stimulating natural defense of the organism against invading pathogens (Guani-Guerra *et al.*, 2010; Ntwasa *et al.*, 2012) though, it may cause allergenic effect when ingested by humans as foods (Toda *et al.*, 2011).

The detection of foodborne pathogens is an important issue for ensuring food safety and security. Moreover, it is also important to explore selective antimicrobial peptides in detail, in particular with regard to their characteristics (Guani-Guerra *et al.*, 2010; Nan *et al.*, 2012). We previously evaluated the antibacterial and antioxidant activities of fermented foxtail millet meal extracts by *L. paracasei* Fn032 (Amadou *et al.*, 2013) and demonstrated the ability of *L. paracasei* Fn032 to inhibit Escherichia coli growth (Sun *et al.*, 2010). Therefore, in the present study, we have characterized peptides generated from the fermentation of foxtail millet meal by *L. paracasei* Fn032; and furthermore, determine the peptide resistance to trypsin digestion and their implication to inhibit E. coli ATCC 8099 growth.

4.2 Materials and Methods

4.2.1 Materials

Foxtail millet meal was obtained from Anhui Yanzhifang Food Co., Ltd (Hefei, China). Trypsin (E.C. 3.4.21.4, trypsin>250 N.F.units/mg) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich, Inc. (Shanghai, China). All other chemicals and reagents (from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) were of analytical grade.

4.2.2 Bacterial strains

L. paracasei Fn032 was obtained by the Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University (Wuxi, China). Escherichia coli ATCC 8099 was provided by the microbiology laboratory culture collection of Jiangnan University (Wuxi, China). Bacteria were maintained as glycerol stocks and stored at −70°C. Stock cultures of all microorganisms were grown in nutrient agar broth at 37°C for 18 h. Few colonies of single bacteria were transferred into a test tube containing sterilized distilled water. The cells were collected by centrifugation at 3000×g for 15 min using an Anker TGL-16C centrifuge (Shanghai, China) and the bacterial pellets

were washed twice. The E. coli ATCC 8099 (OD600≈0.2) was re-suspended in 0.1 moL/L phosphate buffered saline (PBS pH 7.3) prior to antimicrobial assay.

4.2.3 Fermentation and sample preparation

A 25 μL of *L. paracasei* Fn032 was mixed aseptically with 25 g defatted foxtail millet meal (10^7 CFU/g) in polyethylene bag (140 mm×200 mm) and vacuum sealed and then solid-state fermentation was performed for 48 h at 37°C. Fermented foxtail millet extract was prepared according to the method described by Ye *et al.* (2003). Five grams of fermented foxtail millet was mixed with 50 mL of distilled water, homogenized for 1 min and incubated at 37°C for 60 min. The incubated mixture was centrifuged at 9000×g for 2 min using a D-3756 Osterode AM Harz Model 4515 Centrifuge (Hamburg, Germany) and the residue (pellet) was washed with 20 mL distilled water, centrifuged under the same conditions, and the combined supernatant was filtered twice (filter paper Whatman) prior to freeze-drying and stored (as crude peptides) at −20°C until use. Then the collected supernatant in which 8.32% of the peptides were 1 to 2 kDa and 78.40% were ≤1 kDa (data not shown), was subjected to high-performance liquid chromatography (HPLC) analysis.

4.2.4 Peptide purification and identification

The crude peptides were purified by reverse-phaseHPLC (Lab Alliance, USA) on a Sepax Bio C-18 column (10.0×250 mm) operated at ambient temperature, using a gradient of ater/acetonitrile (solvent system was the following: 0.1% TFA in water and 0.1% TFA, 80% acetonitrile in water, the gradient was: 0→40% B in 60 min, flow 3 mL/min and detection completed at 280 nm). Each fraction was collected and concentrated using a rotary evaporator before the antioxidant activity of the peptides was analyzed. The reversed-phase HPLC fractions that exhibited strong radical scavenging activity were subjected to HPLC-MS analysis using a Waters Platform ZMD 4000 system consisting of a Micromass ZMD mass spectrometer (Micromass, Beverly, MA) and a Waters 2690 HPLC (Waters, Milford, MA) equipped with a photodiode detector. Data were collected and processed with MassLynx software version 3.1 (Micromass, a diversion of Waters Corp., Beverly, MA). The samples were injected by an autosampler and subsequently separated by a C18 column (Atlantis, 2.1 mm i.d. 150 mm). The HPLC gradient was linear from 5 to 100% mobile phase B in 10 min using mobile phase A (H_2O, 0.1% formic acid) and mobile phase B

(acetonitrile). The Waters Micromass ZMD mass spectrometer was operated using the electrospray ionization (ESI) source. All measurements were carried out using the positive ESI. A source temperature of 100°C and a desolvation temperature of 250°C were optimal. The capillary voltage was 3.88 kV, and the cone voltage was 60 V.

4.2.5 Determination of radical scavenging activity (RSA)

The RSA of fractionated foxtail millet peptides (FFMp) by RP-HPLC was tested in two systems: DPPH scavenging activity was tested by using the method of Brand-Williams *et al.* (1995); and the superoxide anion ($O^{2\bullet-}$) scavenging activity was measured according to Marklund & Marklund (1974). FFMp samples were mixed 1:1 (v/v) with 0.1 mM DPPH in anhydrous ethanol. The mixture was shaken and left to stand at room temperature for 30 min. The absorbance of the mixture was measured at 517 nm. The DPPH• radical scavenging effect was expressed as the percentage inhibition of DPPH•. Briefly, 1.0 mL of FFMp samples was mixed with 1.8 mL of 50 mM Tris–HCl buffer (pH 8.2). The mixture was incubated at 25°C for 10 min, and then 0.1 mL of 10 mM pyrogallol (dissolved in 10 mM HCl) was added. The absorbance of the solution at 320 nm was measured up to 4 min. The oxidation rate of pyrogallol for samples was calculated as the slope of the absorbance line (ΔA1). The autoxidation rate of pyrogallol for control was measured with 1.0 mL of double-distilled water (ΔA0). The $O^{2\bullet}$ scavenging activity was calculated as $[(\Delta A0-\Delta A1)/\Delta A0]\times100\%$.

4.2.6 Proteolytic digestion

Prior to the subsequent experiments the identified fractionated foxtail millet peptides (FFMp4 FFMp6 and FFMp10) were chemically synthesized and then analyzed for purity (95%) by Shanghai Biotech Bioscience & Technology Co., Ltd (Shanghai, China). Digestion of FFMp4 FFMp6 and FFMp10 by trypsin was carried out using 50 μg/mL peptide and 0.2 μg/mL trypsin in PBS at room temperature (26°C). The progress of peptide cleavage after two time reaction 10 and 30 min was monitored chromatographically using a HPLC.

4.2.7 Antimicrobial activities of synthesized peptides

Antimicrobial activity was determined by the well diffusion assay method (Collin *et al.*, 1995). The suitable nutrient agar media was prepared. E. coli ATCC 8099 culture was added to the sterile

petri plates. Sterilized media were poured into the sterile petri plates. After the media had cooled and solidified, wells (8 mm in diameter) were made in the agar and 50 μL of the synthesized peptides (FFMp4, FFMp6 and FFMp10) was loaded in the wells at concentrations (30, 40, 60 and 80 μg/mL). Following incubation (37°C, 5% CO_2, 24 h), Gentamicin (60 μg/mL) was used as a positive control. After incubation, the zone of inhibition was observed and the diameter was measured.

4.2.8 Statistical analysis

All experiments were conducted at least in triplicate. Analysis of variance (ANOVA) was performed and significant differences in mean values were evaluated by Tukey HSD multiple range test at ($P < 0.05$) using SPSS version 19.0 (SPSS, Chicago, IL, USA).

4.3 Results and Discussions

4.3.1 Radical scavenging activity

Radicals produced by different sources which include free radicals such as superoxide anion radicals ($O^{2\bullet-}$), hydroxyl radicals ($HO^{\bullet}$) and non-free-radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($|O_2$) have different reactivity, multiple methods are generally recommended for assessing the impact of the specific oxygen radicals in the human body and in the food system (Marklund & Marklund, 1974; Suetsuna *et al.*, 2000; Chandrasekara & Shahidi, 2011). Foxtail millet meal was fermented by *L. paracasei* Fn032 and its ability to scavenge superoxide and $DPPH^{\bullet}$ was determined. Fractionated fermented foxtail millet peptides by RP-HPLC exerted different scavenging potencies on the tested radicals (Figure 4.1a). Chromatography with Sepax Bio C-18 column produced 13 fractions collected (Figure 4.1b).

As shown in Figure 4.1a fractions 4 (FFMp4) and 6 (FFMp6) exhibited relatively stronger $DPPH^{\bullet}$ and $O^{2\bullet-}$ scavenging activities, whereas, fraction 10 (FFMp10) which eluted later displayed the maximum antioxidant activity with nearly 47% $DPPH^{\bullet}$ and 69% $O^{2\bullet-}$ at the concentration of 8 mg/mL. Our results further corroborate with the findings that short peptides with 2–10 amino acids exhibit significant ($P < 0.05$) antioxidant activity and other bioactive properties than their parent native proteins or large polypeptides (Tang *et al.*, 2010; You *et al.*, 2010; Nan *et al.*, 2012).

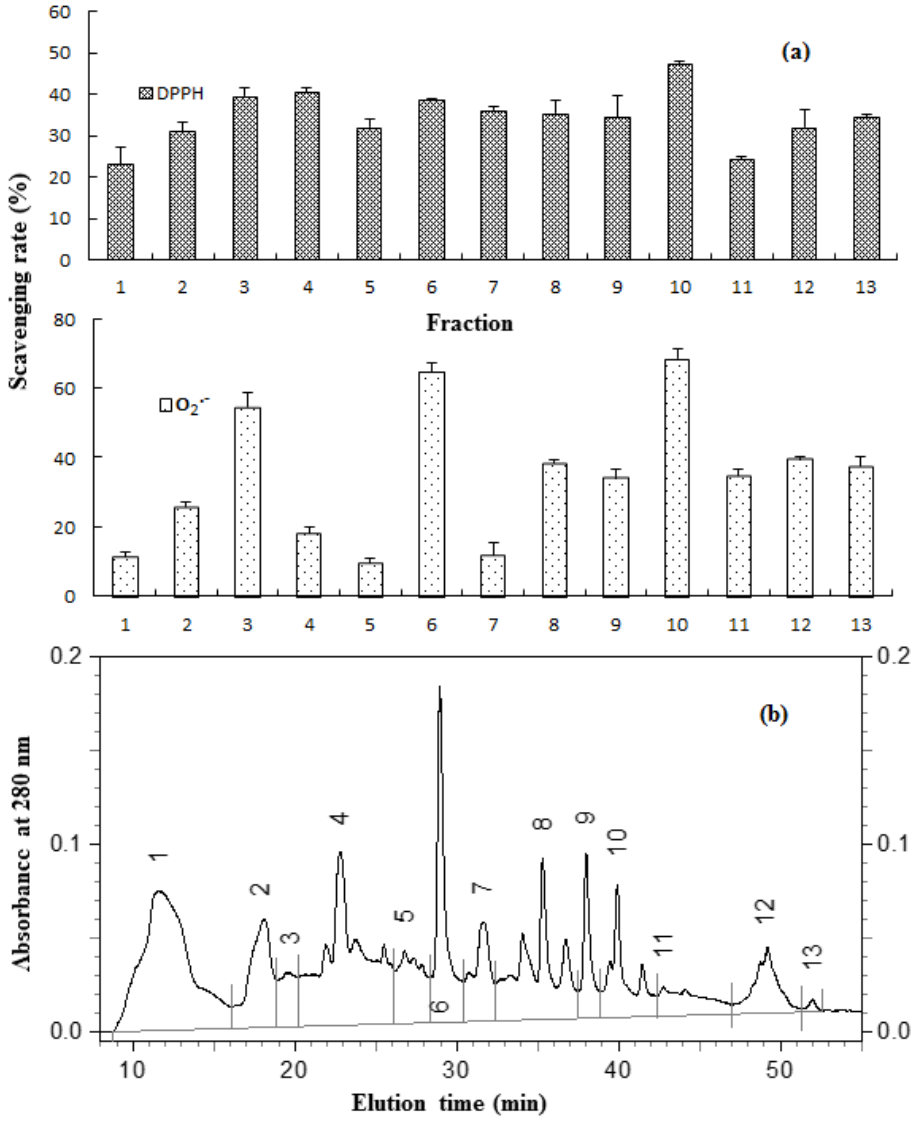


Figure 4.1 Scavenging activity of reversed- phase HPLC fractions of the fermented foxtail millet against DPPH• (1,1-diphenyl-2-picrylhydrazyl) and $O^{2\bullet}$ (a) and HPLC chromatogram (b). The results are plotted as means and standard error from triple determinations. The assay peptide concentrations were 8 mg/mL of DPPH• and $O^{2\bullet}$

It was reported that antioxidant peptides possess some metal chelation or hydrogen electron donating activity, which could allow them to interact with free radicals and terminate the radical chain reaction or prevent their formation (Amadou *et al.*, 2010; Tang *et al.*, 2010). Moreover, peptides containing Tyr, Pro and His, showed strong antioxidant (Ren *et al.*, 2008; You *et al.*, 2010). The antioxidant activity of synthesized peptides was tested as described above; 5 mg/mL

of synthesized peptide sample concentration was used. The scavenging activities of the synthesized peptides are shown in Figure 4.2 where FFMp10 performed better activity than the FFMp4 and FFMp6, this might have linked with the FFMp10 sequence residue and its higher hydrophobicity (Table 4.1). Chen *et al.* (1996) reported the implied role of Leu, His, Gly and Pro in the antioxidant activity.

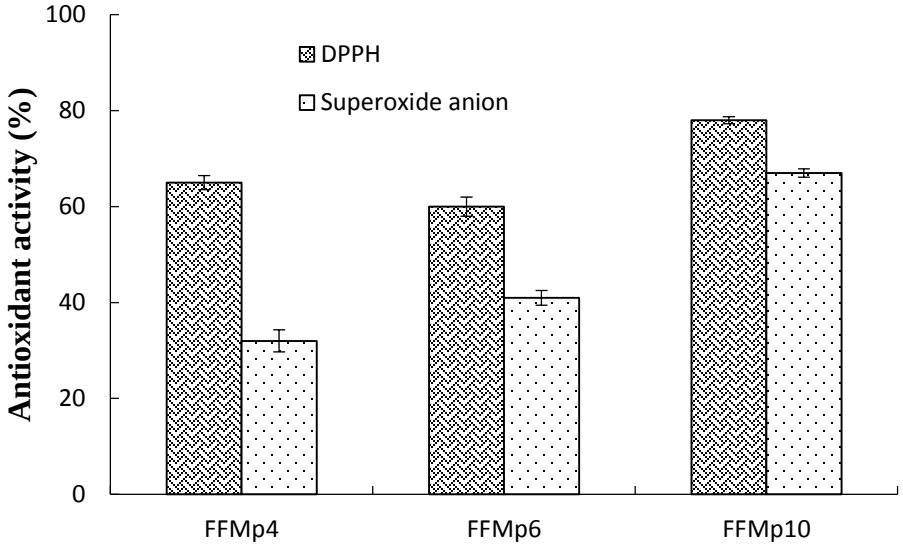


Figure 4.2 Antioxidant activity of synthesized peptides (FFMp4, FFMp6 and FFMp10) identified from fermented foxtail millet

4.3.2 Identification of potent radical scavenging peptide sequences

The molecular mass of peptide fractions purified by RP-HPLC, which had relatively strong radical scavenging activity as shown above was subsequently subjected to HPLC MALDI–TOF–TOF MS analysis for peptide sequence identification. The MassLynx software was used to identify with 100% certainty the prominent peptides in FFMp4 (756.84), FFMp6 (678.74) and FFMp10 (678.87 Da): Ser-Gly-Tyr-Tyr-Met-His, Leu-Gly-Thr-Phe-Gln-Asn and Leu-His-Ala-Leu-Leu-Leu, respectively. The MS and the MS/MS spectra of the most active identified fractions are shown in Figure 4.3.

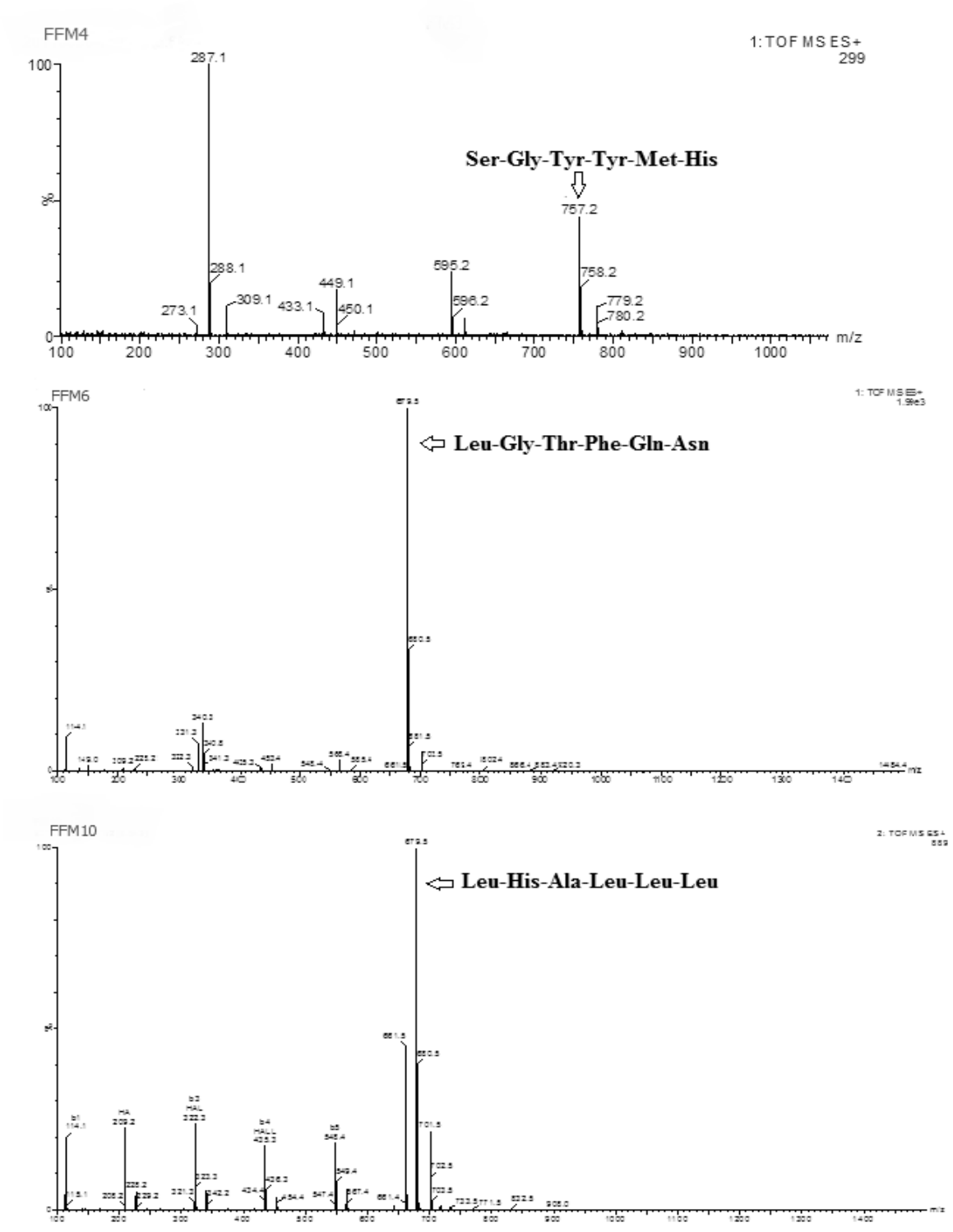
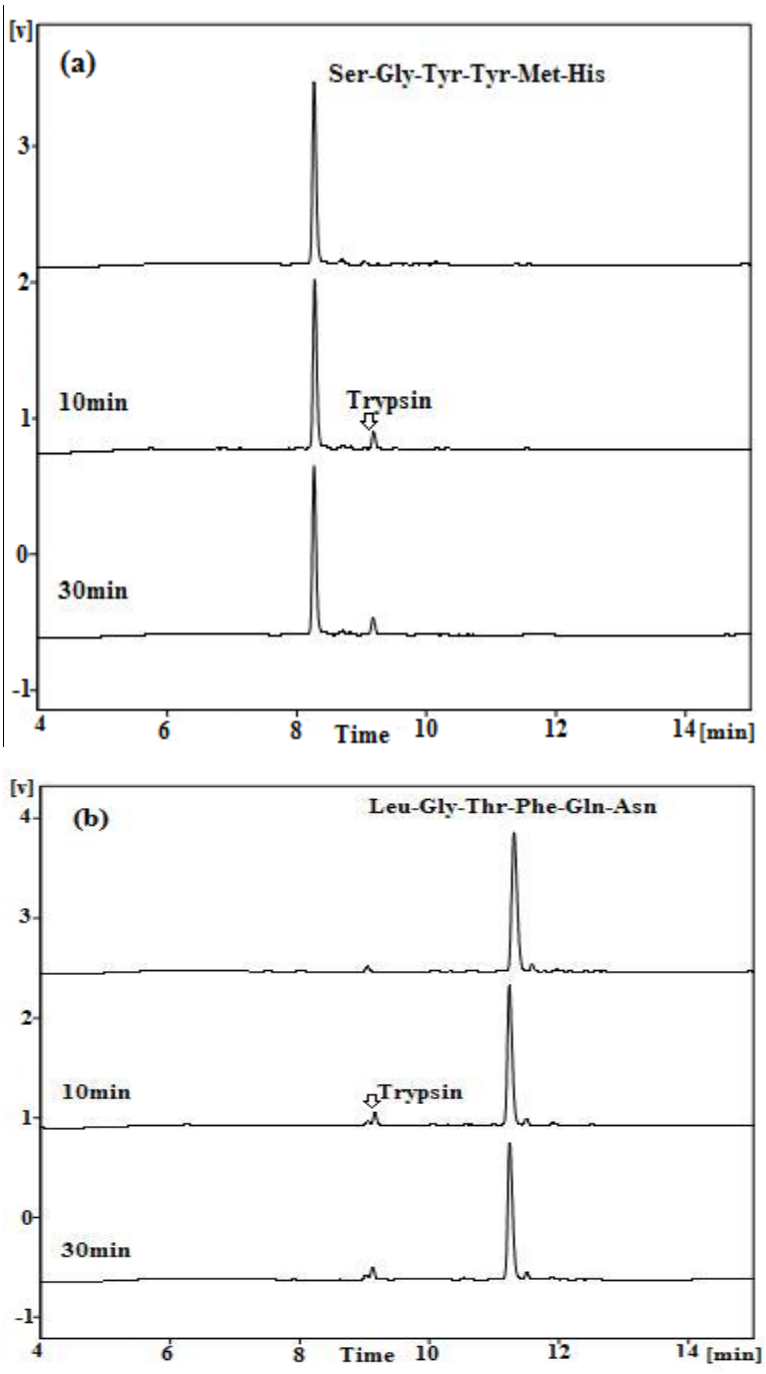


Figure 4.3 Elution profile (LC-MS spectrum of reversed-phase HPLC fractions FFMp4, FFMp6 and FFMp10) of the fermented foxtail millet: (FFMp4=756.84), (FFMp6=678.74) and (FFMp10=678.87 Da).

Previous research has shown that short peptides and amino acids are the most efficient antioxidants because their accessibility to the oxidant/antioxidant test systems is greater than that of large peptides and proteins (Ren *et al.*, 2008; Tang *et al.*, 2010; Li *et al.*, 2012). You *et al.* (2010) isolated an antioxidative peptide from loach (*Misgurnus anguillicaudatus*) protein hydrolysate with Pro-Ser-Tyr-Val. Suetsuna *et al.* (2000) isolated a radical scavenging hexapeptide from casein hydrolysate. The Glu-Leu sequence in the identified peptide, Tyr-Phe-Tyr-Pro-Glu-Leu, was thought to be critical to the antimicrobial activity (Sousa *et al.*, 2009). However, the presence of amino acid such as His, Leu, Gly and Pro was suggested to play an important role in radical scavenging activity (Tessier *et al.*, 2005).

4.3.3 Proteolytic digestion

The susceptibilities to proteolytic degradation of FFMp4, FFMp6 and FFMp10 peptides were tested in the presence of trypsin. Figure 4.3 shows the HPLC elution profiles of three peptides before and after 10 and 30 min trypsin treatments. The results clearly indicate (Figure 4.4a and b) that the peptides FFMp4 and FFMp6 performed resistant shield from trypsin degradation. Whereas, FFMp10's behavior makes it susceptible to proteolytic digestion (Figure 4.4c) where peaks 1, 2 and 3 were eluted before the FFMp10 elution time thus, this is a sign that the peptide was partially cleaved after 10 and 30 min trypsin digestion. The structural dimension of peptides has played a role in their susceptibilities to proteolytic degradation (De Zotti *et al.*, 2009). Bracci *et al.* (2003) work made mentioned of short peptides from being proteolytically digested.


[v]
(a)
Ser-Gly-Tyr-Tyr-Met-His
3
2
10min
Trypsin
1
0
30min
-1
4
6
8
Time
10
12
14
[min]
[v]
(b)
Leu-Gly-Thr-Phe-Gln-Asn
4
3
2
10min
Trypsin
1
0
30min
-1
4
6
8
Time
10
12
14
[min]

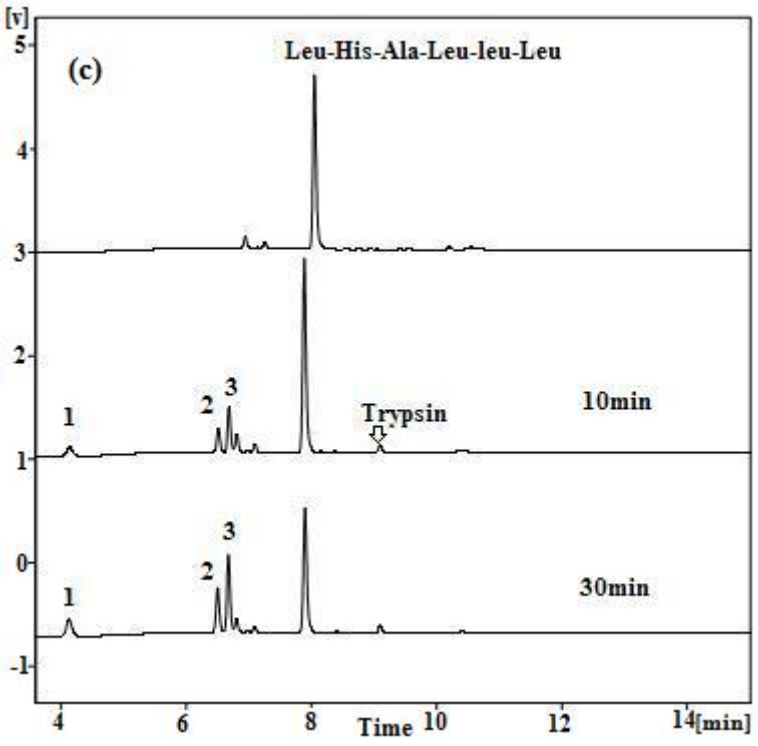


Figure 4.4 Trypsin treatment analytical HPLC elution profiles (retention times) of: (a) FFMp4, (b) FFMp6 and (c) FFMp10 peptide sequences. Experimental conditions: Galaksil EF-C18 (H), 5um 4.6*250 reverse-phase column; gradient from 2 to 90% B in 20 min A= 0.1% TFA in a 95:5(v/v) mixture of H_2O/CH_3CN; B=0.1% TFA in a 5:95 (v/v) mixture of H_2O/CH_3CN]. The absorption was monitored at 220 nm.

4.3.4 Antimicrobial activities of synthesized peptides

The theoretical estimation of the physiochemical properties of identified peptides from fermented foxtail millet (FFMp4, FFMp6 and FFMp10) presented in Table 4.1 shows that the hydrophobicity based on the amino acid contributions gives the Leu rich peptide FFMp10 higher value (2.62) compared to FFMp4 and FFMp6 with 0.63 and 0.5 respectively. It has been reported that the net positive charge and the hydrophobicity are involved in their antimicrobial and antioxidant activities (Chen *et al.*, 1996; Rosenfeld *et al.*, 2010).

Table 4.1 Estimations on physiochemical properties of identified peptides from fermented foxtail millet (FFMp4, FFMp6 and FFMp10).

Peptides	Molecular weights (Da)	Hydrophobicity contributions**	Isoelectric points*	Net charges at neutral pH*	Sequences
FFMp4	756.84	0.63	pH 7.69	0.1	[Ser - Gly - Tyr - Tyr - Met – His]
FFMp6	678.74	0.5	pH 6.01	0	[Leu - Gly - Thr - Phe - Gln – Asn]
FFMp10	678.87	2.62	pH 7.85	0.1	[Leu - His - Ala - Leu - Leu – Leu]

* innovagen's peptide property calculator (http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp); ** Hydrophobicity base on amino acids contributions (partition coefficient log *P*).

Antimicrobial activity test of the synthesized peptides were determined by well diffusion assay method (Collin *et al.*, 1995). The zones of inhibition against *E. coli* ATCC 8099 exhibited by the synthesized peptides initially identified from fermented foxtail meal are shown in Table 4.2. The FFMp10 peptide revealed a relatively larger inhibition zone (13.4±0.8) than their counterparts FFMp4 and FFMp6 (12.5±0.6, 11.8±0.9 mm) at 60 μg/mL. The present study demonstrated that FFMp4, FFMp6 and FFMp10 displayed some activity against the *E. coli* ATCC 8099 growth at a minimum inhibitory concentration of 60 μg/mL for FFMp4, and 40 μg/mL for both FFMp6 and FFMp10. It was obvious that the three synthesized peptides showed lower inhibition against *E. coli* ATCC 8099 than the control sample (Gentamicin).

Table 4.2 Inhibitory activity of three chemically synthesized peptides (FFMp4, FFMp6 and FFMp10) against Escherichia coli ATCC 8099, as determined by well diffusion assay method (Collin *et al.*, 1995).

Synthesized Peptides concentrations (μg/mL)	30	40	60	80
FMp4	-	-	12.5±0.6	12.5±0.5
FFMp6	-	9.7±1.2	11.8±0.9	12.0±0.5
FFMp10	-	10.8±0.2	13.4±0.8	14.0±1.0
Gentamycin	NA	NA	17.6±0.4	NA

Values are means ± standard deviation of two determinations (Zone of inhibition plus diameter of well (8 mm)); NA: not available.

This tells us that shorter peptides (4–7 amino acid residues) are likely to have lower inhibition capacity due to their length to make secondary structure, the absence of Arg residue in the sequence or insufficient hydrophobicity to transcend bacteria cell walls (Lee *et al.*, 2011; Nan *et al.*, 2012). However, the FFMp10 activity could be due to the Leu rich content in its sequence (Table 4.1). Sousa *et al.* (2009) reported Glycine/Leucine-rich antimicrobial peptide with the ability to inhibit the growth of Gram-negative bacteria such as *E. coli*. Whereas, antimicrobial peptide with a short size and a simple amino acid composition would more favorably lead the molecule to reduce production costs and to facilitate pharmaceutical optimization (Lee *et al.*, 2011; Nan *et al.*, 2012).

4.4 Conclusion

Antioxidative antimicrobial peptides generated from fermented food present the great advantage to be derived from harmless substances, therefore, the major findings of this study are that peptide fractions obtained by RP-HPLC purification of the fermented foxtail millet of Tyrosine/Leucine-rich exhibit significant radical scavenging activity and antibacterial fairly activity against *E. coli* ATCC 8099 and FFMp4 and FFMp6 were completely resistant to trypsin proteolysis. Thus, food derived peptides can be regarded not only for their nutritive value, safety but also as a possible resource to increase the natural defense of the organism against invading pathogens. Results indicated that it is feasible to derived natural antioxidants along with antimicrobial activity from foxtail millet meal by *L. paracasei* Fn032 fermentation.

4.5 References

Amadou I, Gbadamosi OS & Le GW (2011) Millet-based traditional processed foods and beverages—A review. *Cereal Foods World* 53: 115-121.

Amadou I, Le G-W & Shi Y-H (2013) Evaluation of antimicrobial, antioxidant activities, and nutritional values of fermented foxtail millet extracts by *Lactobacillus paracasei* Fn032. *International Journal of Food Properties* 16: 1179-1190.

Amadou I, Gbadamosi OS, Shi YH, Kamara MT, Sun J & Le GW (2010) Identification of antioxidative peptides from *Lactobacillus plantarum* Lp6 fermented soybean protein meal. *Research Journal of Microbiology* 5: 372-380.

Anukam KC & Reid G (2009) African traditional fermented foods and probiotics. *Journal of Medicinal Food* 12: 1177-1184.

Bracci L, Falciani C, Lelli B, Lozzi L, Runci Y, Pini A, De Montis MG, Tagliamonte A & Neri P (2003) Synthetic Peptides in the Form of Dendrimers Become Resistant to Protease Activity. *Journal of Biological Chemistry* 278: 46590-46595.

Brand-Williams W, Cuvelier ME & Berset C (1995) Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology* 28: 25-30.

Castillo NA, de Moreno de LeBlanc A, Galdeano CM & Perdigón G (2012) Probiotics: An alternative strategy for combating salmonellosis: Immune mechanisms involved. *Food Research International* 45: 831-841.

Chandrasekara A & Shahidi F (2011) Bioactivities and antiradical properties of millet grains and hulls. *Journal of Agricultural and Food Chemistry* 59: 9563-9571.

Chen H-M, Muramoto K, Yamauchi F & Nokihara K (1996) Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein. *Journal of Agricultural and Food Chemistry* 44: 2619-2623.

Collin CH, Lyne DM & Grange JM (1995) *Microbiological methods. (7th ed.)*. Oxford Publishers.

De Zotti M, Biondi B, Formaggio F, Toniolo C, Stella L, Park Y & Hahm KS (2009) Trichogin GA IV: an antibacterial and protease-resistant peptide. *Journal of Peptide Science* 15: 615-619.

Devi P, Vijayabharathi R, Sathyabama S, Malleshi N & Priyadarisini V (2011) Health benefits of finger millet (*Eleusine coracana* L.) polyphenols and dietary fiber: a review. *Journal of Food Science and Technology* 1-20.

Guani-Guerra E, Santos-Mendoza T, Lugo-Reyes SO & Teran LM (2010) Antimicrobial peptides: general overview and clinical implications in human health and disease. *Clinical Immunology* 135: 1-11.

Kamara MT, Amadou I & Zhou HM (2012) Antioxidant activity of fractionated foxtail millet protein hydrolysate. *International Food Research Journal* 19: 59-66.

Lee SH, Kim SJ, Lee YS, Song MD, Kim IH & Won HS (2011) De novo generation of short antimicrobial peptides with simple amino acid composition. *Regulatory Peptides* 166: 36-41.

Li S, Zhao Y, Zhang L, Zhang X, Huang L, Li D, Niu C, Yang Z & Wang Q (2012) Antioxidant activity of Lactobacillus plantarum strains isolated from traditional Chinese fermented foods. *Food Chemistry* 135: 1914-1919.

Marklund S & Marklund G (1974) Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *European Journal of Biochemistry* 47: 469-474.

Nan YH, Bang JK, Jacob B, Park IS & Shin SY (2012) Prokaryotic selectivity and LPS-neutralizing activity of short antimicrobial peptides designed from the human antimicrobial peptide LL-37. *Peptides* 35: 239-247.

Ntwasa M, Goto A & Kurata S (2012) Coleopteran antimicrobial peptides: Prospects for clinical applications. *International Journal of Microbiology* doi.org/10.1155/2012/101989.

Ren J, Zhao M, Shi J, Wang J, Jiang Y, Cui C, Kakuda Y & Xue SJ (2008) Purification and identification of antioxidant peptides from grass carp muscle hydrolysates by consecutive chromatography and electrospray ionization-mass spectrometry. *Food Chemistry* 108: 727-736.

Rosenfeld Y, Lev N & Shai Y (2010) Effect of the hydrophobicity to net positive charge ratio on antibacterial and anti-endotoxin activities of structurally similar antimicrobial peptides. *Biochemistry* 49: 853-861.

Sousa JC, Berto RF, Gois EA, *et al.* (2009) Leptoglycin: a new Glycine/Leucine-rich antimicrobial peptide isolated from the skin secretion of the South American frog Leptodactylus pentadactylus (Leptodactylidae). *Toxicon* 54: 23-32.

Suetsuna K, Ukeda H & Ochi H (2000) Isolation and characterization of free radical scavenging activities peptides derived from casein. *The Journal of Nutritional Biochemistry* 11: 128-131.

Sun J, Hu XL, Le GW & Shi YH (2010) Lactobacilli prevent hydroxy radical production and inhibit Escherichia coli and Enterococcus growth in system mimicking colon fermentation. *Letters in Applied Microbiology* 50: 264-269.

Tang X, He Z, Dai Y, Xiong YL, Xie M & Chen J (2010) Peptide fractionation and free radical scavenging activity of zein hydrolysate. *Journal of Agricultural and Food Chemistry* 58: 587-593.

Taylor JRN & Emmambux MN (2008) *Gluten-free foods and beverages from millets*. Elsevier, Academic Press., San Diego, CA.

Tessier B, Schweizer M, Fournier F, Framboisier X, Chevalot I, Vanderesse R, Harscoat C & Marc I (2005) Prediction of the amino acid composition of small peptides contained in a plant protein hydrolysate by LC–MS and CE–MS. *Food Research International* 38: 577-584.

Toda M, Reese G, Gadermaier G, *et al.* (2011) Protein unfolding strongly modulates the allergenicity and immunogenicity of Pru p 3, the major peach allergen. *Journal of Allergy and Clinical Immunology* 128: 1022-1030.e1027.

Xu W, Wei L, Qu W, Liang Z, Wang J, Peng X, Zhang Y & Huang K (2011) A novel antifungal peptide from foxtail millet seeds. *Journal of the Science of Food and Agriculture* 91: 1630-1637.

Yang X, Wan Z, Perry L, *et al.* (2012) Early millet use in northern China. *Proc Natl Acad Sci U S A* 109: 3726–3730.

Ye YT, Xue M, Lin SM, Wang YH, Luo L & Lian JS (2003) Enzymolysis kinetics of digestive enzyme from intestine and hepatopancreas in grass carp to four kinds of raw feed materials. *Journal of Fishery Science China* 10: 470-473

You L, Zhao M, Regenstein JM & Ren J (2010) Purification and identification of antioxidative peptides from loach (Misgurnus anguillicaudatus) protein hydrolysate by consecutive chromatography and electrospray ionization-mass spectrometry. *Food Research International* 43: 1167-1173.

CHAPTER 5

Antimicrobial Effects of Modified FFMp10 Chemically Synthesized Short Peptides on *Escherichia coli* ATCC 8099 Hydrophobicity

5.1 Introduction

Antimicrobial peptide with a short (4-7 amino acid residues) size and a simple amino acid composition would be a more favorable lead molecule to reduce production costs and to facilitate pharmaceutical optimization (Nan *et al.,* 2012). In this line, potent antimicrobials have been produced based on natural peptides and the active domains of larger antimicrobial proteins (Ntwasa *et al.,* 2012). Short, positively charged antimicrobial peptides (AMPs), have been found in plants and animals as part of the innate immune system that helps to defend against invading microorganisms (Huo *et al.,* 2011). Hydrophobic amino acid stretches can be used to enhance bactericidal potency of ultra-short AMPs with limited toxicity (Pasupuleti *et al.,* 2009). Enzymatic instability of a peptide could limit its in vivo use. Studies indicated that in the gastrointestinal tract, amino acid peptides are more readily absorbed than the amino acid themselves (Kodera *et al.,* 2006; Gilbert *et al.,* 2008). Digestive enzymes such as pepsin, trypsin are suggested to exert a synergistic effect on protein digestion in the body (Getz *et al.,* 2011; Dickey & Potter 2011).

Plants, animals and microorganisms commonly use the release of antimicrobial peptides as a defensive strategy against pathogenic microorganism and stress (Ntwasa *et al.,* 2012; Banerjee *et al.,* 2013; Ruthu *et al.,* 2013). It is generally believed that a protein, that resist proteolytic digestion in the digestive tract, retains sufficient structural integrity and that increases the probability of stimulating natural defense of the organism against invading pathogens (Ntwasa *et al.,* 2012; Guani-Guerra *et al.,* 2010). Detecting foodborne pathogenic bacteria such as *Escherichia coli* is an important factor ensuring food safety (Gregory & Mello, 2005). *E. coli* is commonly found in the gut of humans and warm-blooded animals. Findings from structural activity analysis of AMPs revealed that the cell penetrating efficiency and affinity to DNA are critical factors in determining the potency of antimicrobial peptides; and binding of ultra-short peptides to bacterial DNA or DNA fragments could be a mechanism to show the antimicrobial efficacy of these compounds (Huo *et al.,* 2011; Hao *et al.,* 2013).

Hydrophobicity appears to be imparted by different chemical components of the cell wall in different bacteria; these components include lipoteichoic acids and proteins (Thwaite *et al.,* 2009; Van der Mei & Busscher 2012). The cell surface hydrophobicity (CSH) of bacteria is been recognized as a physical measurable macroscopic characteristic which, reflects the ratio of hydrophobic to hydrophilic cell envelope constituents (Jones *et al.,* 1996). CSH responds to a wide variety of environmental factors and, it's suggested to be involved in several activities like cell-to-cell interaction, adherence of bacteria to solid surfaces and host tissue, partitioning at liquid–liquid, solid–liquid or liquid–air interfaces, resistance of cells to specific treatments like organic solvents or antibiotics, which activities are essential in technologies and natural processes (Jones *et al.,* 1996; Choi *et al.,* 2013; Zikmanis *et al.,* 2007).

A characterized and identified FFMp10 peptide fraction from foxtail meal fermentation using *Lactobacillus paracasei* Fn032 was suggested to inhibit the growth of *E. coli* ATCC 8099 (Amadou *et al.,* 2013; Amadou *et al.,* 2013); however there is little information about the effect of FFMp10 peptide on the cell surface hydrophobicity, DNA binding ability of *E. coli* ATCC 8099 and its proteolytic response. Therefore, we set to assess the effect of chemically synthesized FFMp10 peptide replicate residues on the cell surface hydrophobicity, biomass growth and DNA retardation ability of *E. coli* ATCC 8099. Furthermore, these peptides responses to trypsin and pepsin proteolysis were also tested.

5.2 Materials and Methods

5.2.1 Materials

The chemically synthesized peptides (FFMp10, Mp1, Mp2, Mp3 and Mp4) with 99% purity tested by the company were purchased from Shanghai Biotech Bioscience & Technology Co., Ltd (Shanghai, China). All other chemicals and reagents (from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) were of analytical grade.

5.2.2 Proteolytic digestion

Digestion of FFMp10, Mp1, Mp2, Mp3 and Mp4 peptides by trypsin was carried out using 50 μg/mL peptide and 0.2 μg/mL trypsin in phosphate buffer saline (0.01 M PBS) at room temperature (26^{o}C). The progress of peptides cleavage after two times reaction 5 and 15 min were

monitored chromatographically using a HPLC (Shimadzu LC-20A, Japan). We also digested the chemically synthesized peptides residues (FFMp10, Mp1, Mp2, Mp3 and Mp4) using 0.2 µg/mL pepsin in PBS at 50 µg/mL peptide and followed by incubation at 37ºC for 1 h. The peptide cleavage was also analyzed using a HPLC (Shimadzu LC-20A, Japan).

5.2.3 Determination of bacterial cell surface hydrophobicity

Bacteria (*E. coli* ATCC 8099) were maintained as glycerol stocks and stored at -70°C. Stock cultures of all microorganisms were grown in nutrient agar broth at 37ºC for 18 h. Aliquots of single bacteria colonies were transferred into a test tube containing sterilized distilled water, centrifuged at 10000 rpm for 1 min using an Eppendorf centrifuge 5430 (Hamburg, Germany) and the deposited bacterial cells pellets were washed three times. The *E. coli* ATCC 8099 (OD_{600}= 0.4 ± 0.02) pellets were sampled into 2 parts of which one part was directly re-suspended in sterile phosphate buffered saline (0.1 mM PBS, pH 7.2) and the other part was treated with FFMp10, Mp1, Mp2, Mp3 and Mp4 peptides (1 mg/mL) for 2 h prior to bacterial cell surface hydrophobicity (CSH) assay as described by Jones *et al.* (1996) with little modification testing the bacterial adherence to hydrocarbons (BATH).

After 2 h of incubation the *E-coli* ATCC 8099 cells were washed twice with sterilized PBS and then re-suspended in PBS. Then 2.4 mL of bacterial-sterilized PBS suspension were added to 0.2 mL xylene and vortex mixed at constant speed for 3 min. After phase separation, the lower aqueous layer was carefully removed and the absorbance was measured at 600 nm (SPM-UV2300 spectrophotometer, Shanghai, China). The hydrophobicity was expressed as a percentage of the applied cell suspension absorbance which had been excluded from the aqueous phase. The 2 h treated *E-coli* ATCC 8099 cells with peptides and cells suspended in sterilized PBS were then grown in sterilized liquid broth Mann Rogosa Sharpe (MRS) to test their biomass growth at different times (4, 8, 12, 18 and 24 h).

5.2.4 DNA binding assay

Gel-retardation experiments were performed using 2 µL of *E. coli* ATCC 8099 genomic DNA (100 ng) for each reaction, 0.8 µL each of FFMp10, Mp1, Mp2, Mp3 and Mp4 peptide concentrations (0.5, 0.8 and 1.0 mg/mL) were mixed with 2 µL of binding buffer (10% Ficoll 400, 10 mM Tris-HCl; pH 7.5, 50 mM EDTA, 0.25% bromophenol blue). The reaction mixtures were

incubated at room temperature for 10 min and loaded to 0.8% agarose gel with 2 μg/mL ethidium bromide in 0.5× Tris borate-EDTA buffer then subjected to electrophoresis (Zhang *et al.,* 1999).

5.2.5 Statistical analysis

Analysis of variance (ANOVA) was performed and significant differences in mean values were evaluated at $P < 0.05$ confidence test, using SPSS version 19.0 (SPSS, Chicago, IL, USA).

5.3 Results and Discussion

5.3.1 Estimated physicochemical properties of the peptides

The Arg and Lys residues within a given peptide are known for their role in DNA binding (Huo *et al.,* 2011; Deber *et al.,* 2001; De Rouchey *et al.,* 2013). The theoretical estimated physiochemical properties of identified Leu rich peptide (FFMp10 fraction) from fermented foxtail millet meal and its replicates (modified peptides: Mp1, Mp2, Mp3 and Mp4) are presented in Table 5.1. In our previous report we demonstrated that the Leu rich peptide had significant contribution to the high hydrophobicity of peptide sequences (Amadou *et al.,* 2013). Substitution of His by Lys at second amino acid sharply increased the hydrophobicity contributions value of Mp2 to 3.1, higher than its counterparts. Moreover, substitution of Arg and Lys in the sequence FFMp10 increased both the net charge at neutral pH and respective isoelectric points of the replicate peptides (Table 5.1). The net positive charge and the hydrophobicity are critical factors in determining the antimicrobial potency of the peptides (Zikmanis *et al.,* 2007; Rosenfeld *et al.,* 2010; Sousa *et al.,* 2009).

Table 5.1 Estimated physiochemical properties of identified Leu rich peptide (FFMp10 fraction) from fermented foxtail millet and its replicates (Mp1, Mp2, Mp3 and Mp4 peptides)

Peptides	Molecular weights (Da)	Hydrophobicity contributions**	Isoelectric points*	Net charges at neutral pH*	Sequences
FFMp10	678.88 g/moL	2.62	pH 7.85	0.1	[Leu - His - Ala - Leu - Leu – Leu]

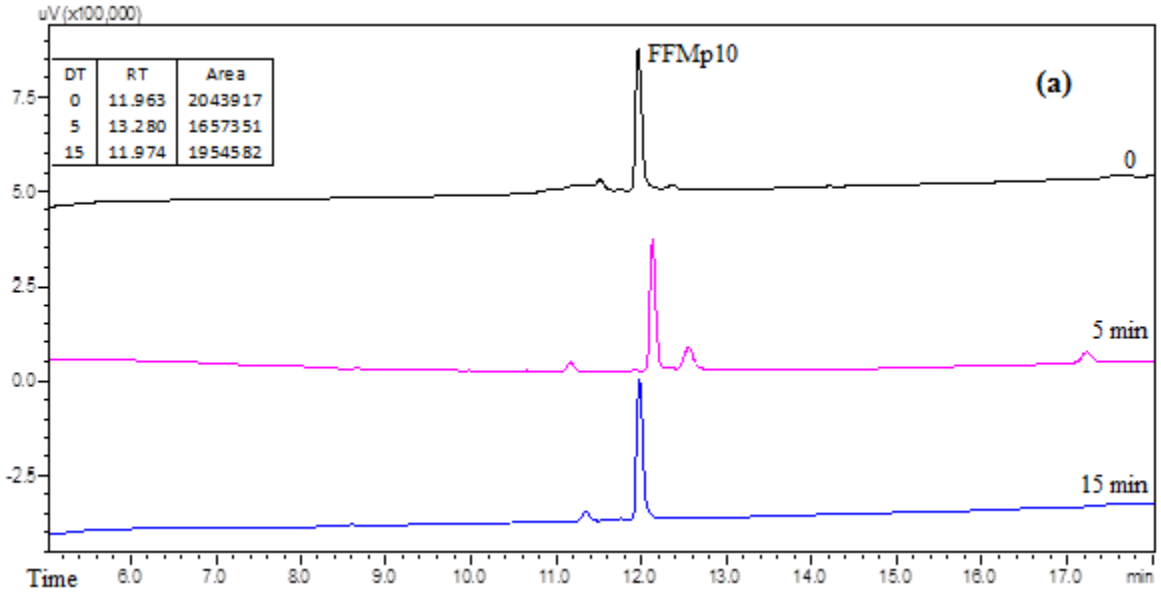
Mp1	697.92 g/moL	2.14	pH 11.04	1	[Leu - Arg - Ala - Leu - Leu- Leu]
Mp2	735.97 g/moL	3.1	pH 10.1	1.1	[Leu - Lys - Ala - Leu - Leu – Leu]
Mp3	721.9 g/moL	1.03	pH 11.04	1.1	[Arg - His - Ala - Leu - Leu – Leu]
Mp4	721.9 g/moL	1.03	pH 11.04	1.1	[Leu - His - Ala - Leu - Leu – Arg]

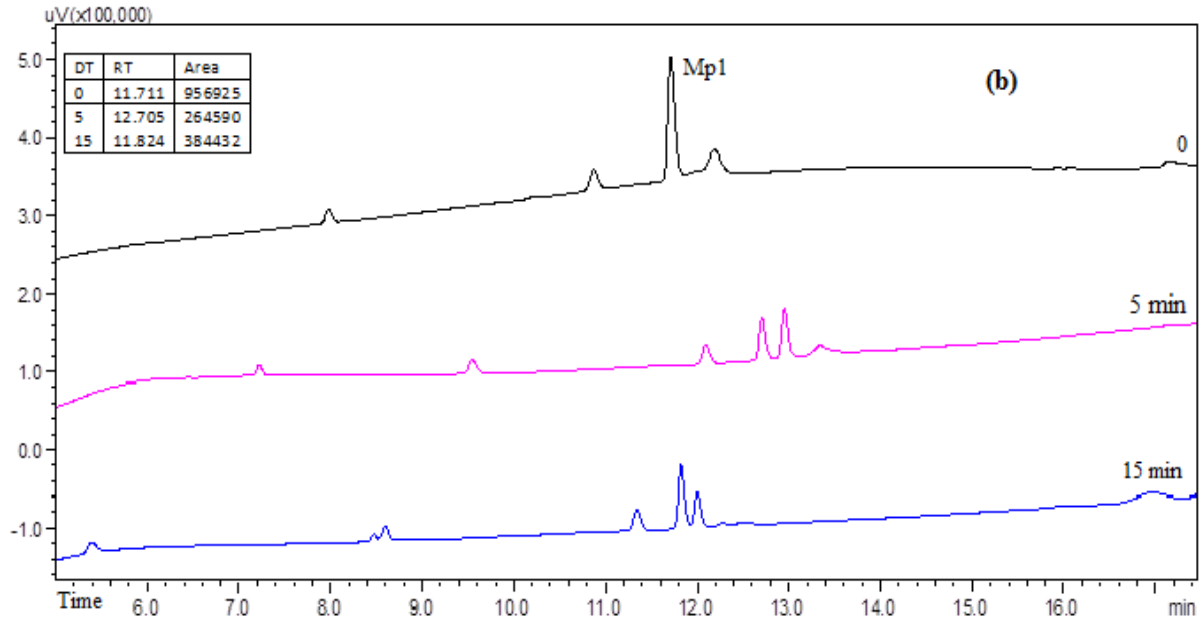
* Innovagen's peptide property calculator (http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp); ** Hydrophobicity base on amino acids contributions (partition coefficient log *P*).

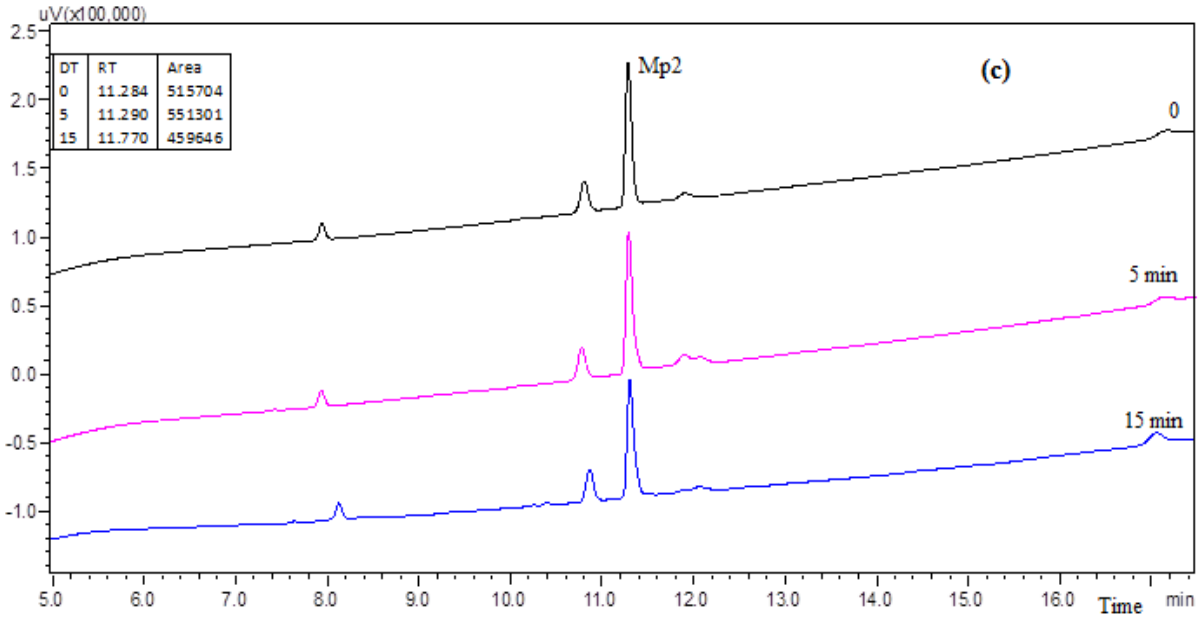
5.3.2 Proteolytic digestion

The susceptibilities of FFMp10, Mp1, Mp2, Mp3 and Mp4 peptides to trypsin and pepsin proteolysis HPLC elution profiles are shown in Figure 5.1. Figure 1a HPLC elution profiles of FFMp10 sequence showed considerable area reduction, an increase in retention time from 11.96 to 13.28 min. Furthermore, FFMp10 responded to trypsin proteolysis right after 5 min digestion. Mp1 showed a similar HPLC elution profile trend as FFMp10, though appeared with more peaks (Figure 5.1b) after trypsin treatment. However, HPLC elution profiles of Mp2, Mp3 and Mp4 peptides showed no peaks modifications after trypsin treatment (Figure 5.1c, d and e). This shows that the respective peptides were resistant to trypsin proteolysis; this might have been due to the sequence modification (Table 5.1). On the other hand, HPLC elution profiles of all the five synthesized peptides showed almost no significant response to 1 h pepsin treatment (Figure 5.1f). However, the fact that Mp2, Mp3 and Mp4 were more resistant to proteolytic digestion than FFMp10 and Mp1 indicates that locations besides the type of amino acid also play a vital role in peptide digestion. Previous studies demonstrated that structural dimension is an important factor for peptides in their susceptibilities to proteolytic degradation (Getz *et al.*, 2011; Bracci *et al.*, 2003; De Zotti *et al.*, 2009). Interestingly, both Arg and Lys residues contributed remarkably to the

proteolytic activities of these synthetized peptides, thus, this may extend interest in their bioactivities.



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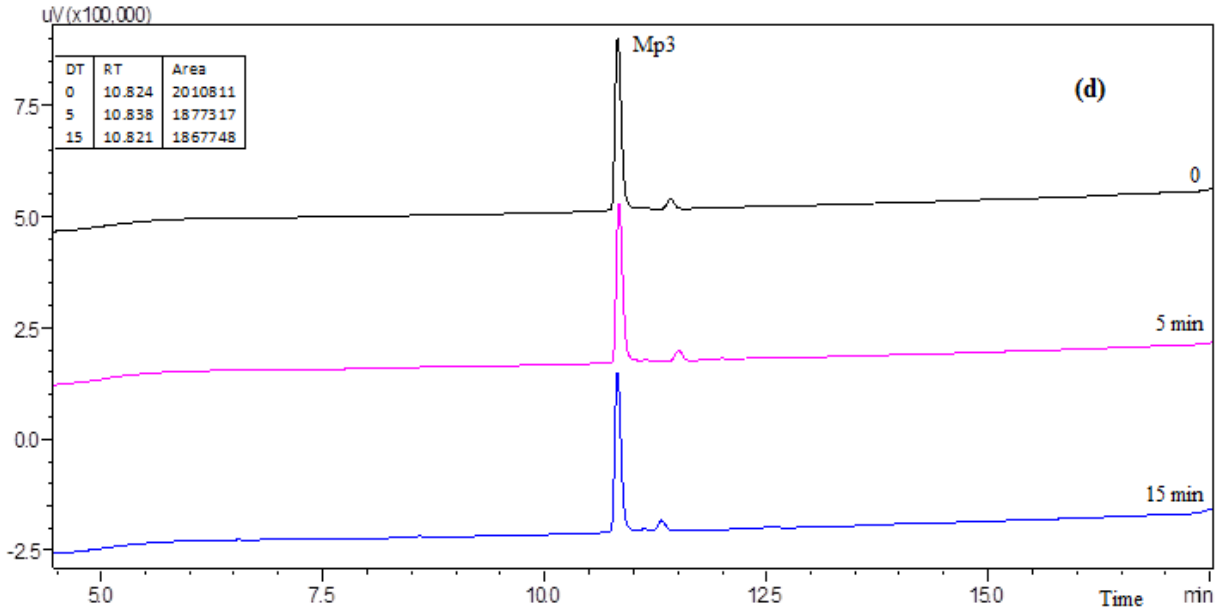
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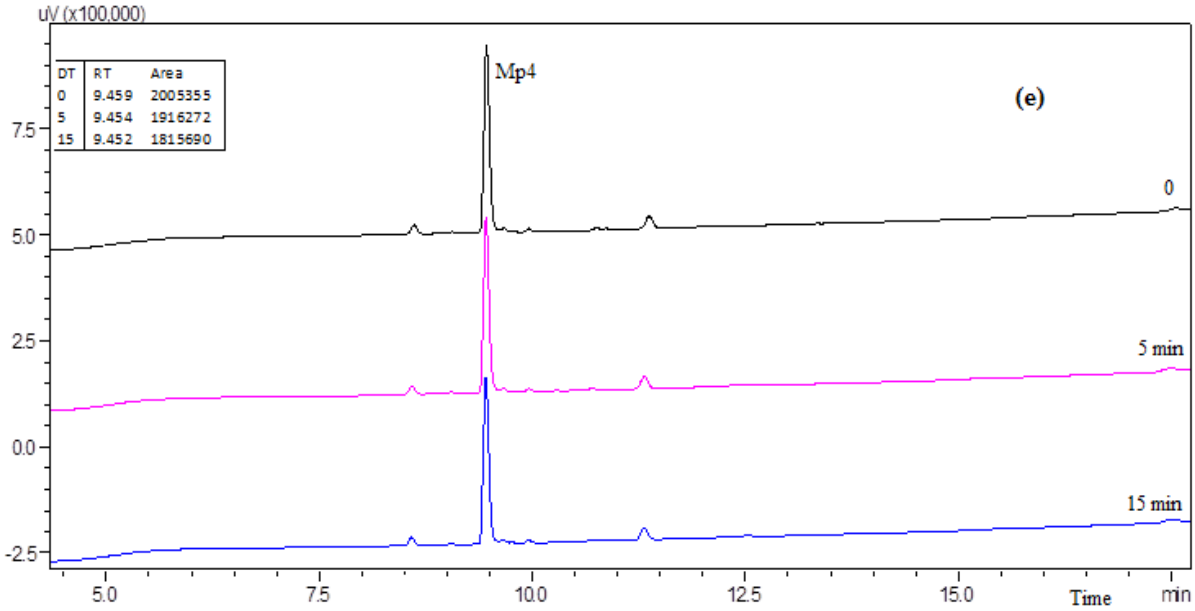

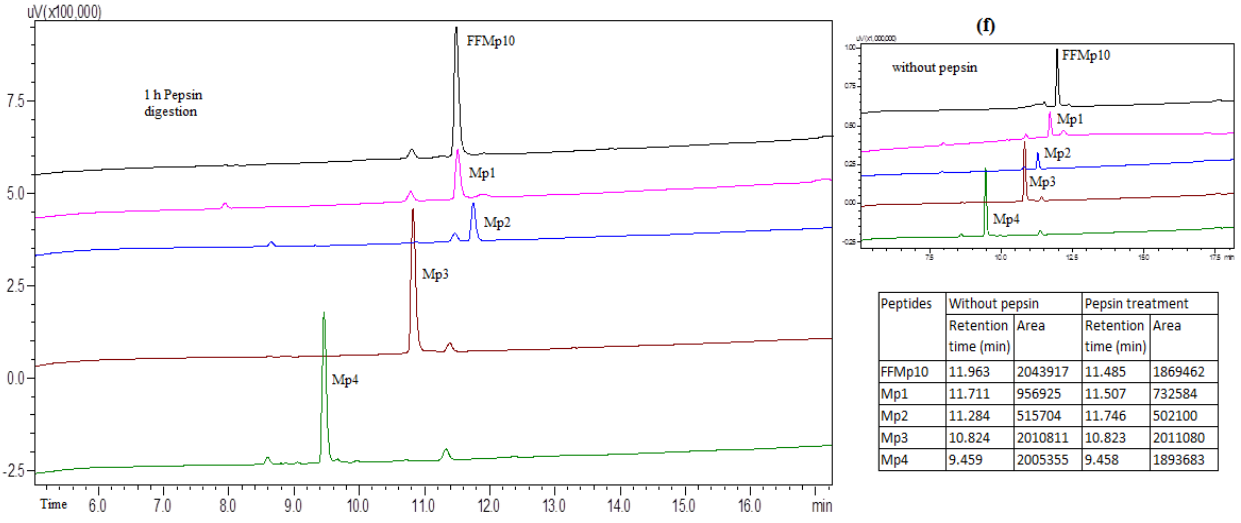

Peptides	Without pepsin		Pepsin treatment	
	Retention time (min)	Area	Retention time (min)	Area
FFMp10	11.963	2043917	11.485	1869462
Mp1	11.711	956925	11.507	732584
Mp2	11.284	515704	11.746	502100
Mp3	10.824	2010811	10.823	2011080
Mp4	9.459	2005355	9.458	1893683

Figure 5.1 HPLC elution profiles (retention times and areas) of: (a) FFMp10, (b) Mp1, (c) Mp2, (d) Mp3, and (e) Mp4 peptides sequences treated with trypsin; and (f) represent (FFMp10, Mp1, Mp2, Mp3 and Mp4) peptides sequences treated with pepsin. Experimental conditions: Galaksil EF-C18 (H), 5µm 4.6*250 reverse-phase column; gradient from 2 to 90% B in 25 min A = 0.1% TFA in a 95:5 (v/v) mixture of H_2O/CH_3CN; B = 0.1% TFA in a 5:95 (v/v) mixture of H_2O/CH_3CN]. The absorption was monitored at 220 nm.

5.3.3 Changes in bacterial cell hydrophobicity

Majority of vital activities in Gram-negative bacteria such as sensory, protective, transport and energy generation functions are dependent on the structural and functional properties of the cell envelope containing the cytoplasmic and the outer membrane as the principal components (Nikaido, 2003). *E coli* ATCC 8099 cells treated with FFMp10, Mp1, Mp2, Mp3 and Mp4 peptides showed significant ($P < 0.05$) changes in cell surface hydrophobicity values (Figure 5.2). The involvement of net positive charge and the hydrophobicity indexes (Table 5.1) showed that FFMp10 had higher impact on the CSH followed by Mp1 and Mp2; whereas Mp3 and Mp4 CSH values were considerably lower when compared to the *E coli* ATCC 8099 cells that were not treated with peptides. Peptides with higher hydrophobicity index contributed to higher CSH percentages (Figure 5.2). Indeed, more profound increases of CSH values were observed after the FFMp10, Mp1 and Mp2 treatment. Study suggested that high CSH values tend to promote the self-aggregation of bacteria and it enhances the possibility of modifying cell surface properties (Zikmanis *et al.,* 2007). The hydrophobicity of the cell surface enhances in vivo bacteria pathogenicity by either increasing the rate of phagocytosis or enhancing the colonization of mucosal surfaces (Thwaite *et al.,* 2009), in this line, Mp3 and Mp4 could provide prominent action against the *E coli* ATCC 8099 activity. Substitutions of Arg at end terminals of Mp3 and Mp4 might have contributed to this activity by reducing the CSH of this strain.

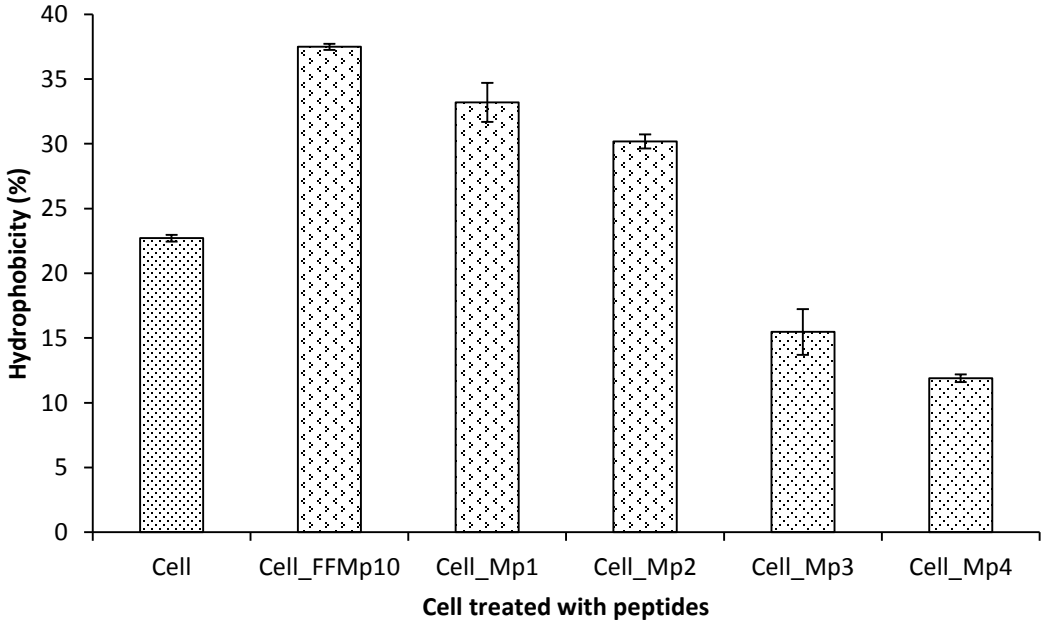

Figure 5.2 Changes in the *E. coli* ATCC 8099 cell surface hydrophobicity by FFMp10, Mp1, Mp2, Mp3 and Mp4 peptide treatment

Previous study has shown that hydrophobic peptides can cause alterations to the surface morphology of bacterial cells (Tagai *et al.*, 2011). The present study demonstrated that disparity in amino acid composition and sequence of FFMp10, Mp1 and Mp2 induced higher CSH in *E. coli* ATCC 8099 cells compared to Mp3 and Mp4 with lesser CSH display, and that might have resulted in morphological changes. The Gly/Leu-rich antimicrobial peptides with ability to inhibit the growth of Gram-negative bacteria such as *E. coli* were reported (Sousa *et al.*, 2009); similarly, this study suggests that the synthesized short peptides replicate (6 residues rich in Leu) from FFMp10 fraction induced changes (Figure 5.2) across the *E coli* ATCC 8099 cell membrane in a similar trend to the Gly/Leu-rich peptides.

5.3.4 Bacterial biomass growth

Antimicrobials, for instance, first have to approach an organism and interact with its cell surface before they can become effective. Bacteria have a natural tendency to adhere to surfaces as a survival mechanism, they can adapt quickly to a new environment triggered by environmental signals to change their phenotypic appearance, but it is of apparent advantage that not all clones in a population follow the same trend (Choi *et al.*, 2013).

When a microorganism is introduced into the fresh medium, it takes some times to adjust with the new environment; therefore, the effect of these peptides (FFMp10, Mp1, Mp2, Mp3 and Mp4) treatment on *E coli* ATCC 8099 followed the same trend. Figure 5.3 shows the exponential or Logarithmic (log) phase of bacterial biomass growth after adaptation time up to 12 h, followed by stationary phase, that all the treated cells have lower growth rate than the control. The survival mechanism by this strain demonstrated that the peptides must have played an important role on the starvation of *E coli* ATCC 8099. Interestingly, FFMp10 with lower net charges at neutral pH exercised more effect on the cell growth retardation and also resulted later in the highest CSH formation (Table 5.1, Figure 5.2). Thus, this could have resulted into the more biofilm formation which helps strain in its adaptation to the new environmental conditions (Van der Mei & Busscher, 2012; Choi *et al.*, 2013).

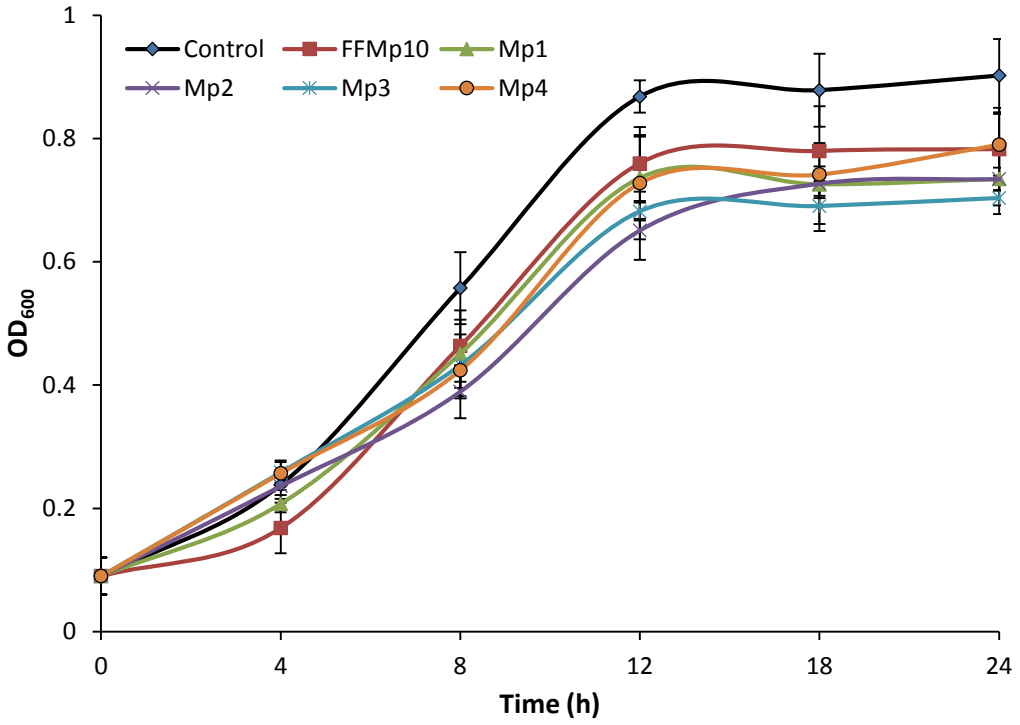

Figure 5.3 Biomass of *E. coli* ATCC 8099 growth following FFMp10, Mp1, Mp2, Mp3 and Mp4 peptide treatment

5.3.5 DNA binding

The CSH of *E. coli* ATCC 8099 appears to be imparted by the effect of the synthetized peptides (Figure 5.2); however, efficiency and the DNA binding ability were critical factors for determining the antimicrobial potency of a peptide (Huo *et al.,* 2011; Hao *et al.,* 2013; DeRouchey *et al.,* 2013; Ulvatne *et al.,* 2004). Therefore, the DNA binding ability of FFMp10, Mp1, Mp2, Mp3 and Mp4 were evaluated using an electrophoresis gel mobility assay (Figure 5.4). The positively charged peptides have ability to bind to phosphate negatively group in the DNA back bone (Deber *et al.,* 2001). The migration of DNA was not retarded in most cases even with increased peptide concentrations. However, a slight attempt to retard DNA migration was observed in Mp1 and Mp2 to DNA ratio in their lanes 2, 3 and lane 3 respectively (Figure 5.4). There was significant *E. coli* ATCC 8099 DNA binding affinity which was similar to the trend observed by Huo *et al.* (2011) that demonstrated that MUC7 12-mer a 12 amino acid peptide did not inhibit DNA electrophoretic mobility at a mass ratio of 500:1. Thus, this suggests that the peptide may have antimicrobial

activity (Amadou *et al.*, 2013) but with no ability to retard bacterial DNA migration. Furthermore, the antimicrobial activity corresponded with the CSH data showed that the *E. coli* ATCC 8099 cell wall surface was affected by changes in surface hydrophobicity instead of the bacteria intracellular DNA binding. This was in agreement with our previous study where we demonstrated that short peptides of such kinds have difficulties in retarding DNA migration (data not shown). In contrary to a study by Ulvatne *et al.* (2004) who demonstrated that high concentration of lactoferrin at mucosal surface and in milk did not bind bacterial DNA.

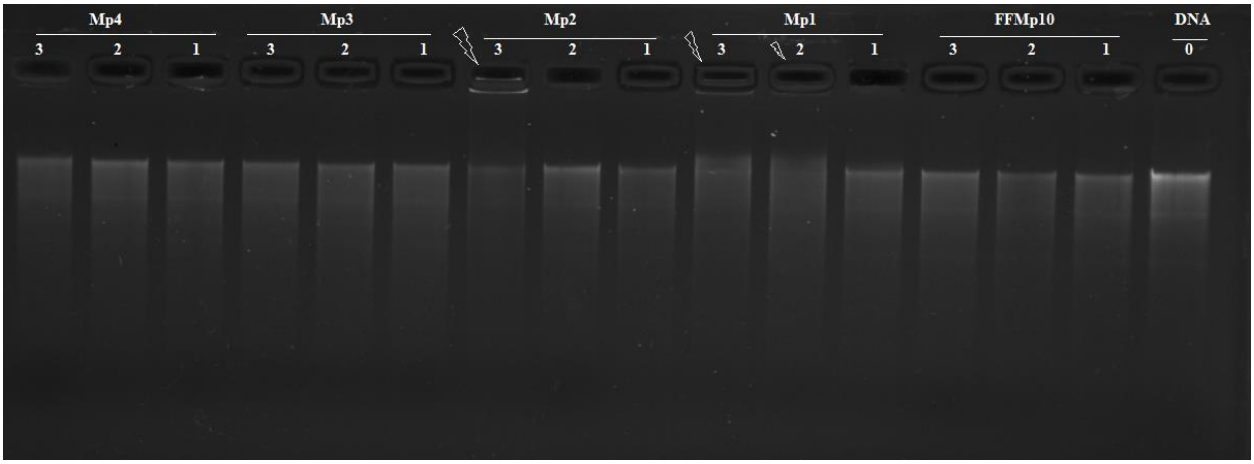

Figure 5.4 Interaction of *E. coli* ATCC 8099 genomic DNA with peptides; (2 µL of 100 ng DNA) was incubated with 8 µL (0.5, 0.8 and 1.0 mg/mL, lanes 1, 2 and 3 respectively) of FFMp10, Mp1, Mp2, Mp3 and Mp4 peptides at room temperature for 10 min. The reaction mixtures mixed with binding buffer (2 µL) were applied to a 0.8% agarose gel electrophoresis.

5.4 Conclusion

In this study, we demonstrated that the synthesized Leu rich short peptides displayed proteolytic resistance to pepsin, and trypsin proteolysis as a result of Arg and Lys residues substitutions in the FFMp10 peptide sequence. The observed multiple relationships between the CSH values and theoretical hydrophobicity indexes to overall biomass growth and DNA binding of *E coli* ATCC 8099 strain were attained. On the basis of these data, short peptides reduced hydrophobicity, but not the viability of *E coli* ATCC 8099; moreover, the Mp3 and Mp4 peptides may provide an attractive function against *E coli* ATCC 8099 CSH activity. Further studies to understand these

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5.5 References

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Banerjee S, Dora K & Chowdhury S (2013) Detection, partial purification and characterization of bacteriocin produced by *Lactobacillus brevis* FPTLB3 isolated from freshwater fish. *Journal of Food Science and Technology* 50: 17-25.

Bracci L, Falciani C, Lelli B, Lozzi L, Runci Y, Pini A, De Montis MG, Tagliamonte A & Neri P (2003) Synthetic peptides in the form of dendrimers become resistant to protease activity. *The Journal of Biological Chemistry* 278: 46590-46595.

Choi NY, Kim BR, Bae YM & Lee SY (2013) Biofilm formation, attachment, and cell hydrophobicity of foodborne pathogens under varied environmental conditions. *Journal of the Korean Society for Applied Biological Chemistry* 56: 207-220.

De Zotti M, Biondi B, Formaggio F, Toniolo C, Stella L, Park Y & Hahm KS (2009) Trichogin GA IV: an antibacterial and protease-resistant peptide. *Journal of Peptide Science* 15: 615-619.

Deber CM, Wang C, Liu LP, Prior AS, Agrawal S, Muskat BL & Cuticchia AJ (2001) TM Finder: a prediction program for transmembrane protein segments using a combination of hydrophobicity and nonpolar phase helicity scales. *Protein Science* 10: 212-219.

DeRouchey J, Hoover B & Rau DC (2013) A comparison of DNA compaction by arginine and lysine peptides: a physical basis for arginine rich protamines. *Biochemistry* 52: 3000-3009.

Dickey DM & Potter LR (2011) Dendroaspis natriuretic peptide and the designer natriuretic peptide, CD-NP, are resistant to proteolytic inactivation. *Journal of Molecular and Cellular Cardiology* 51: 67-71.

Getz JA, Rice JJ & Daugherty PS (2011) Protease-Resistant Peptide Ligands from a Knottin Scaffold Library. *ACS Chemical Biology* 6: 837-844.

Gilbert ER, Wong EA & Webb KE Jr (2008) Board-invited review: Peptide absorption and utilization: Implications for animal nutrition and health. *Journal of Animal Science* 86: 2135-2155.

Gregory K & Mello CM (2005) Immobilization of Escherichia coli cells by use of the antimicrobial peptide cecropin P1. *Applied and Environmental Microbiology* 71: 1130-1134.

Guani-Guerra E, Santos-Mendoza T, Lugo-Reyes SO & Teran LM (2010) Antimicrobial peptides: general overview and clinical implications in human health and disease. *Clinical Immunology* 135: 1-11.

Hao G, Shi YH, Tang YL & Le GW (2013) The intracellular mechanism of action on Escherichia coli of BF2-A/C, two analogues of the antimicrobial peptide Buforin 2. *Journal of Microbiology* 51: 200-206.

Huo L, Zhang K, Ling J, Peng Z, Huang X, Liu H & Gu L (2011) Antimicrobial and DNA-binding activities of the peptide fragments of human lactoferrin and histatin 5 against Streptococcus mutans. *Archives of Oral Biology* 56: 869-876.

Jones DS, Adair CG, Mawhinney WM & Gorman SP (1996) Standardisation and comparison of methods employed for microbial cell surface hydrophobicity and charge determination. *International Journal of Pharmaceutics* 131: 83-89.

Kodera T, Hara H, Nishimori Y & Nio N (2006) Amino Acid Absorption in Portal Blood After Duodenal Infusions of a Soy Protein Hydrolysate Prepared by a Novel Soybean Protease D3. *Journal of Food Science* 71: S517-S525.

Nan YH, Bang JK, Jacob B, Park IS & Shin SY (2012) Prokaryotic selectivity and LPS-neutralizing activity of short antimicrobial peptides designed from the human antimicrobial peptide LL-37. *Peptides* 35: 239-247.

Nikaido H (2003) Molecular basis of bacterial outer membrane permeability revisited. *Microbiology and Molecular Biology Reviews* 67: 593-656.

Ntwasa M, Goto A & Kurata S (2012) Coleopteran antimicrobial peptides: prospects for clinical applications. *International Journal of Microbiology* 101989: 1-8.

Oliveira A, Segovia J, Sousa V, Mata E, Goncalves M, Bezerra R, Junior P & Kanzaki L (2013) Antimicrobial activity of amazonian medicinal plants. *SpringerPlus* 2: 371.

Pasupuleti M, Schmidtchen A, Chalupka A, Ringstad L & Malmsten M (2009) End-Tagging of Ultra-Short Antimicrobial Peptides by W/F Stretches to Facilitate Bacterial Killing. *PLoS ONE* 4: e5285.

Rosenfeld Y, Lev N & Shai Y (2010) Effect of the hydrophobicity to net positive charge ratio on antibacterial and anti-endotoxin activities of structurally similar antimicrobial peptides. *Biochemistry* 49: 853-861.

Ruthu, Murthy P, Rai A & Bhaskar N (2012) Fermentative recovery of lipids and proteins from freshwater fish head waste with reference to antimicrobial and antioxidant properties of protein hydrolysate. *Journal of Food Science and Technology* 1-9.

Seukep J, Fankam A, Djeussi D, Voukeng I, Tankeo S, Noumdem J, Kuete A & Kuete V (2013) Antibacterial activities of the methanol extracts of seven Cameroonian dietary plants against bacteria expressing MDR phenotypes. *SpringerPlus* 2: 363.

Sousa JC, Berto RF, Gois EA, Fontenele-Cardi NC, Honorio JE, Jr. Konno K, Richardson M, Rocha MF, Camargo AA, Pimenta DC, Cardi BA & Carvalho KM (2009) Leptoglycin: a new Glycine/Leucine-rich antimicrobial peptide isolated from the skin secretion of the South American frog *Leptodactylus pentadactylus* (Leptodactylidae). *Toxicon* 54: 23-32.

Tagai C, Morita S, Shiraishi T, Miyaji K & Iwamuro S (2011) Antimicrobial properties of arginine- and lysine-rich histones and involvement of bacterial outer membrane protease T in their differential mode of actions. *Peptides* 32: 2003-2009.

Thwaite JE, Laws TR, Atkins TP & Atkins HS (2009) Differential cell surface properties of vegetative Bacillus. *Letters in Applied Microbiology* 48: 373-378.

Ulvatne H, Samuelsen O, Haukland HH, Kramer M & Vorland LH (2004) Lactoferricin B inhibits bacterial macromolecular synthesis in Escherichia coli and Bacillus subtilis. *FEMS Microbiology Letters* 237: 377-384.

Van der Mei HC & Busscher HJ (2012) Bacterial Cell Surface Heterogeneity: A Pathogen's Disguise. *PLoS Pathogens* 8: e1002821.

Zhang L, Benz R & Hancock RE (1999) Influence of proline residues on the antibacterial and synergistic activities of alpha-helical peptides. *Biochemistry* 38: 8102-8111.

Zikmanis P, Shakirova L, Auzina L & Andersone I (2007) Hydrophobicity of bacteria Zymomonas mobilis under varied environmental conditions. *Process Biochemistry* 42: 745-750.

General Conclusions and Recommendations

6.1 General conclusion

Millets are still the staple food for millions of poor people in Africa and Asia. Like many other cereals, millets are high carbohydrate energy content and nutritious, making them useful components of dietary and nutritional balance in foods. Combination of millets with other sources of protein would compensate the deficiency of certain amino acids such as lysine. Successful improvement of these attributes would be a crucial key to expand the spectrum of applications of millet grains. Therefore, our work has provided a potential platform for the fermentation by *L. paracasei* Fn032 of foxtail millet meal; and it can be deduced from this thesis that foxtail millet meal have diverse potentials when fermented to produce bioactive peptides.

The effects of *L. paracasei* Fn032 fermentation and heat moisture treatment significantly influence the physicochemical properties of foxtail millet flour. Scanning electron microscopy analysis provided information on differences in the microstructure of the flours as a result of fermentation and heat moisture treatment and on the other side fluorescence spectroscopy showed how changes in chemicals affected the spectra of tryptophan and riboflavin. RVA pasting properties of foxtail millet flour followed the same trends and the crystal structures moderately lost complete-ness during the fermentation process. The two conditions of fermentation and HMT had significant effects on the fraction content of RDS, SDS, and RS, resulting in higher values after heat moisture treatment.

It can be inferred that the fermented foxtail millet is a good source of bioactive peptides with significantly higher antioxidant and antimicrobial activities. Thus, solid state fermentation of foxtail millet using *L. paracasei* Fn032, in combination with protease, not only improves the physiochemical quality but also enhanced the nutritional values of the meal. Remarkable antioxidant activity and protein quality exhibited by all the extracts could be associated with the potential activity of *L. paracasei* Fn032 during the bioprocessing. Furthermore, antioxidative antimicrobial peptides were generated by RP-HPLC purification of the fermented foxtail millet. FFMp4 and FFMp6 peptides fractions were completely resistant to trypsin proteolysis.

This study demonstrated that the synthesized Leu rich short peptides displayed proteolytic resistance to pepsin, and trypsin proteolysis as a result of Arg and Lys residues substitutions in the FFMp10 peptide sequence. The observed multiple relationships between the CSH values and

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6.2 Key innovations

- For the first time short peptides with antioxidant and antimicrobial activity were derived from fermented foxtail millet.

- Naturally derived six amino acids sequence peptides from fermented foxtail millet by *L. paracasei* Fn032 resisted to trypsin proteolysis.

- Study have also revealed how short peptides of these kind inhibit the growth of *E. coli* ATCC 8099 by affecting its surface hydrophobicity level.

6.3 Recommendations

- Future trends should focus on the millet consumption in the developed countries that could help its industrial revolution.

- Opportunities may, therefore, exist to explore the potential of foxtail millet meal processing to enhance its physicochemical and functional properties while improving its nutritional value. There is need for further studies to understand the digestibility of processed foxtail millet flour as a potential gluten free diet formulation.

- Further investigation may be helpful in the application or utilization of the purified antioxidative antimicrobial peptides from fermented foxtail millet meal as specific constituents in food preservation.

- Further studies to understand these peptides involvement in *in vivo* activity may be helpful in elucidating the development of enzyme resistant stable short peptide ligands suitable for possible applications.

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Glossary of Local Words

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Braza: fermented millet drinks made in Romania.

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