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Editorial

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Heat Stress and Gut Health in Broilers: Role of Tight Junction Proteins

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INTRODUCTION

Sixteen of the seventeen hottest years ever recorded have occurred since 2001 and climate trends are predicted to continue in an upward trend.[1] As this upward trend continues, it will serve as a severe environmental stress factor on all forms of the life.[2,3] One of the most affected industries will be the livestock industry. Poultry, in particular, appears to be very heat sensitive animals, due to lack of sweat glands and high metabolic activity.[4,5] It is estimated that heat stress alone costs the U.S. poultry industry more than 100 million dollars a year and this number is expected to rise.[6] For broiler (meat-type) chickens, the external temperature for optimal performance is 18 to 22 ºC.[7] Under these conditions, the internal body temperature of a broiler is between 40.6 °C-41.7 °C. Nevertheless, when chickens are placed under heat stress conditions, their body temperature may be well above that; up to 45 °C-47.2 °C, which is the lethal limit.[8] Heat stress (HS) or hyperthermia results from failed thermoregulation that occurs when animals produce or absorbs more temperature than it dissipates.[9] The adverse effects of HS can range from discomfort to multiple organ damage and, under severe stress, to death by spiraling hyperthermia. The Gut plays a vital role in nutrient absorption, digestion and transport, yet it is very responsive and susceptible to HS. In this editorial we will review the effect of heat stress on tight junction (TJ) proteins and gut health.

HEAT STRESS AND GUT HEALTH

Under thermoneutral conditions, the gut is able to efficiently digest and absorb most nutrients through cell plasma membranes (transcellular transport) that involves specific receptors. Epithelial cells in the intestine provides a barrier isolating the external environment from the internal body, yet, providing tolerance to water and digested nutrients.[10-12] Intestinal epithelial cells adhere to each other through three distinct intercellular junctional complex known as desmosomes, adheren junctions (AJ), and TJ (Figure 1). Desmosomes are localized dense plaques that are connected to keratin filaments while AJ and TJ both consist of transcellular proteins.[13,14] These proteins are connected intracellularly through adaptor proteins to the actin cytoskeleton.[3,15] In contrast to transcellular transport, the transfer of molecules through the space between the cells across an epithelium (paracellular transport) is passive down a concentration gradient, and this transport is regulated by the TJ.[16] As multi-protein complexes, TJ not only hold cells together, but they form channels allowing the transport of substances across the epithelium.[17] Interestingly, the molecular composition, ultrastructure, and function of TJ is regulated by intracellular proteins through a series of intracellular signaling pathways that includes myosin light kinase (MLCK), mitogen-activated protein kinases (MAPK), protein kinase C (PKC) among others.[18] Occludin phosphorylation on Tyr, Ser and Thr is associated with disruption of TJ, hence, phosphorylation of occludin is involved in TJ permeability.[19] Any factors that affect the balance between protein kinases and protein phosphatases, such as heat stress or inflammation can affect gut permeability due to disruption of TJ.[20,21] In contrast, glycosylation of the Junctional adhesion molecule-A (JAM-A) decreases gut permeability.[22,23] TJ regulate epithelial permeability and paracellular diffusion *via* two pathways, leak and pore.[24] The leak pathway allows transport of large noncharged solutes while the pore pathway allows the transfer of large charged molecules.[25] As transmembrane barrier proteins, TJ also function as a fence between the lumen and host.[26] There are roughly 50 TJ proteins, which include the

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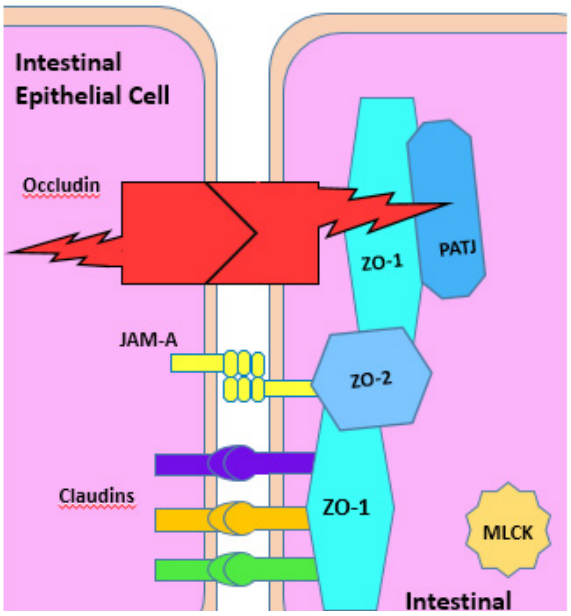
Figure 1: Functions of Tight Junctions. Tight Junctions are the Primary Mechanisms that Control the Epithelium (Tight or Leaky). Several Classes of Tight Junction Proteins including Claudins, JAM-A, and ZO-1/2 Play an Important in Cell Permeability.



claudins, occludin, tricellulin, JAM's, and scaffolding proteins. For instance, tricellulin (also known as MARVELD2) and angulin family proteins, including angulin-1 (also known as LSR), angulin-2 (also known as ILDR1) and angulin-3 (also known as ILDR2), have been identified as molecular constituents of tricellular contacts. Both types of proteins are involved in TJ formation as well as the full barrier function of epithelial cellular sheets. The primary role of scaffolding proteins is to regulate stand formation and placement of transmembrane proteins.[4,27] Under thermal neutral conditions, paracellular junction are rigorously regulated.[14] However, under heat stress conditions, the TJ barrier becomes compromised and luminal substances leak into the blood stream, hence the term leaky gut,[28] a condition that induce chronic systemic inflammation which requires high resources of energy that impact negatively the performance of the animals. Alterations in gut permeability are associated with bacterial translocation (BT) in the portal and/or systemic circulation in several types of leaky gut syndromes leading to systemic bacterial infections.[29] Similarly, FITC-dextrin is a large molecule (3-5 kDa) which does not usually leak through the intact gastrointestinal tract barrier.[4,6] However, when there are conditions which disrupt the tight junctions between epithelial cells, the molecule can enter circulation demonstrated by an increase in trans-mucosal permeability associated with chemically induced disruption of tight junctions by elevated serum levels of FITC-d after oral administration.[30,31] Although studies are very limited, it has been reported that cyclic heat stress up regulated claudin and ZO-1 expression in the chicken jejunum.[32] This indicates that heat stress dysregulates intestinal barrier function and induces leaky gut *via* alteration of tight junction proteins which merit further in depth investigations.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Editorial

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The Regulation of Dietary Supplement in the U.S. and Major Change in the Guidance for New Dietary Ingredient Notifications

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Dietary supplement has become a conservative item on daily menu of Americans. The National Health and Nutrition Examination Survey (NHANES) indicated that one in two Americans take dietary supplement on daily basis.[1] Subsequently, the need of dietary supplement causes the booming of dietary supplement products on U.S. market. According to the Dietary Supplement Label Database (DSLD) of the National Institute of Health (NIH), approximately 50,000 dietary supplement products are currently available on U.S. market.

Here, we want to briefly introduce the dietary supplement related regulations in the U.S., especially the recently proposed dietary ingredient guidance for industry, to help the audience to understand how this type of product is regulated in the U.S. and the important changes in the new guidance.

The U.S. Food and Drug Administration (FDA) regulate both dietary supplement products and dietary ingredients. Dietary supplements are regulated under the Dietary Supplement Health and Education Act of 1994 (DSHEA), which is an amendment to Federal Food, Drug, and Cosmetic Act (FD&C). In another word, dietary supplements are regulated under the general umbrella of "food" in the U.S. DSHEA defines dietary supplement as a vitamin, mineral, herbal or other botanical, amino acid, dietary substances, or concentrate, metabolite, constitute, extraction or combination of substances as aforementioned. To help industry better comply with DSHEA, FDA requires dietary supplement manufactures to comply with current good manufacturing practice in manufacturing, packaging, and labeling set forth in Code of Federal Regulation Title 21, Part 111.[2] However, the law does not require the companies to submit the safety evidence of products to FDA, nor obtain FDA's satisfaction that the claim is accurate or truthful before it appears on the product. FDA begins to play a role after the products enter the market. This role is mainly performed by monitoring the serious adverse event report through Safety Reporting Portal, as well as reviewing product labels and other product information. The reporting of adverse events by industry is mandatory, and it is voluntary for consumers and health professionals. FDA take actions to remove products from market only when there are evidences demonstrating adulteration of the product (e.g., containing unsafe ingredients) or/and misbranded (e.g., false labeling), or causing serious adverse effects to human. In the past decade, several fatal events related to dietary supplement (e.g., ephedra) consumption were reported by social media, which raised strong public concerns to the safety of dietary supplements on U.S. market. People criticize the lack of regulation from FDA on dietary supplement products, which is focused on no premarket approval needed to produce and sell this type of products in the U.S.

Compared to the U.S., Canada has relatively more strict regulation to dietary supplements. In Canada, not only the companies are required to obtain license to sell the products on market, but also the products are required to get licensed from Health Canada.[3] And the com-

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panies are required to demonstrate the evidence of safe and efficacy of the products to the government before marketing.

To improve the public understanding and the rate of compliance with the regulation requirements, FDA is making efforts to enhance the safety of dietary supplement products. One of the recent moves of FDA is to revise the outdated draft guidance of new dietary ingredient (NDI) for industry in 2011.

According to DSHEA, the dietary supplement manufactures do not need to get FDA's approval before producing or selling the products unless the product contains a NDI. NDI is defined as a dietary ingredient that was not marketed in the U.S. before October 15, 1994. If the product contains NDI, the industry must submit NDI premarket safety notification to FDA at least 75 days before marketing. However, it is the manufacture itself determines the presence of NDI in their products.

According to FDA's estimate, more than 55,600 dietary supplements exist on the market, and ~5,000 more enter the market each year. However, the agency has received fewer than 1,000 NDI notifications since DSHEA acted in 1994. These numbers, coupled with the concerns about the presence of undeclared active ingredients in the dietary supplements, made FDA to draft a new dietary ingredient guidance for industry in August 2016.[4]

The new NDI guidance aims to help improving public's understanding of NDI notification requirements and improve the quality of NDI notification submitted by companies. For example, it further explains the term "dietary ingredient" in the NDI definition. Dietary ingredient means the ingredient markets in or as a dietary supplement, or for use in a dietary supplement. In other words, the NDI exemption only applies to the ingredients intended to be used in or as a dietary supplement marketed in the U.S. before October 15, 1994. So, if an ingredient used in the food supply chain of a conventional food but not for dietary supplement purpose, it is still considered an NDI even if the product was marketed in the U.S. before October 15, 1994. This new guidance draft is currently under revision, and will be finalized with the considerations of public comments.

Though the new NDI guidance intends to improve the safety of dietary supplements by restricting the definition of NDI and expanding the scope of NDI requires notification to FDA. The arguments will continue because the submission of safety notification is still on a voluntary basis. In the U.S., the law put dietary supplements under the umbrella of "food" regulation, which determines the regulation of dietary supplements is prone to food rather than drug. With more understanding the situation of dietary supplement regulation, the public can read the information of the products better.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Research

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Fatty Acid Compositions of Olive Oils from Six Cultivars from East and South-Western Algeria

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ABSTRACT

The aim of this work is to characterize olive oils from six algerian cultivars (*Azeradj*, n=4); *Blanquette*, n=7; *Bouricha*, n=2; *Chemlal*, n=5; *Limli*, n=3; *Sigoise*, n=1) by determining their fatty acid compositions. The fatty acid composition of oils is determined using gas chromatography of methyl esters obtained by transesterification of triacylglycerols with 2M KOH/MeOH. Fourteen fatty acids and squalene are identified in all the samples. Oleic acid (18:1ω9), palmitic acid (16:0), linoleic acid (18:2ω6) and stearic acid (18:0) are the major fatty acids commonly found in olive oils. Palmitoleic acid (16:1ω7), hypogeic acid (16:1ω9), oleic acid (18:1ω9) and cis-vaccenic acid (18:1ω7) are considered as separate entities by the European regulation, unlike the Codex Alimentarius which identifies them as a single component. Six minor fatty acids namely margaric acid (17:0), margaroleic acid (17:1ω8), arachidic acid (20:0), gondoïque acid (20:1ω9), behenic acid (22:0) and lignoceric acid (24:0) are identified. These acids, although minor, are important for the characterization of cultivars. Oils of different cultivars are characterized by different fatty acid compositions. All the values of fatty acid compositions are in compliance with the regulations of the International Olive Oil Council and Codex Alimentarius. A radial plot enables the analysis and characterization of each variety as a "morphotype" by creating a "morphogramme". The "morphogramme" is designed as a radial representation of each fatty acid (n=14) using an Excel® spreadsheet, and each axis represents the change in the variable with respect to the mean. The "morphotypes" are real fingerprints of different oil cultivars. Thus, the oils of *Azeradj*, *Blanquette*, *Chemlal*, *Limli* and *Sigoise* cultivars have specific "morphotype". The "morphotype" of the *Bourricha* cultivar is very similar to the *Chemlal* cultivar. In addition, the *Blanquette* "morphotype" is identical to that of the tunisian cultivar *Chetoui*. This mode of representation is particularly effective for the rapid visual identification of characteristics of olive oils.

KEY WORDS: Algerian cultivar; Fatty acids; Olive oil; "Morphotype".

ABBREVIATIONS: PDO: Protected Designation of Origin; NADP: National Plan for Agricultural Development; FAME: Fatty Acid Methyl Esters; FID: Flame Ionization Detector; VOOs: Virgin Olive Oils.

INTRODUCTION

The characterization of olive oils in terms of cultivars and geographical origin is a matter of current debate. The quality and the peculiarity of olive oil is, in fact, influenced by several factors one of them being the associated cultivar.[1]

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Algeria currently has almost 32×10^6 olive trees covering an area of 310,000 ha. Reports of NADP (National Plan for Agricultural Development) indicate that Algeria will achieve an overall coverage area of 420,000 ha in some years.[2] The Algerian olive grove is rich in varieties, despite which only a few systematic studies have been conducted on the local cultivars to evaluate the quality of oil which is a priority relative to the requirements of the international market and international olive-growing council (COI). The authentication of virgin olive oils (VOOs) has always been a problem of topical interest. The determination of the varietal origin of oils is important for quality control in the food industry and to assure fair trading. We have shown that the fatty acid composition of monovarietal olive oils or French protected designation of origin (PDO) oils were distinct characteristics of the cultivar or the geographical origin of olive oils.[3,4]

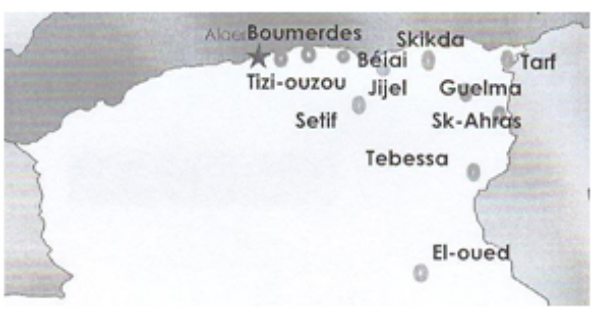
So, the aim of the present study is to investigate the varietal characterization of olive oils from six Algerian cultivars (*Azeradj*, *Blanquette*, *Bouricha*, *Chemlal*, *Limli*, *Sigoise*) by determining their fatty acid compositions and to create a "morphotype" for each cultivar. This data will be particularly effective in ensuring a possible protected designation of origin, their protection from related fraudulence activities,[5] and in improving the quality of our oils by utilizing potential varieties taking the chemical composition of oil into account, to fulfill the requirements of the international market with respect to the commercial standards.

MATERIALS AND METHODS

Oil Samples

The samples came from the east and north-east of Algeria. Figure 1 represents the Algerian olive growing areas. Industrial virgin olive oil samples (n=22) were collected during two crop harvesting seasons (2009 and 2010). Oils came from six Algerian cultivars: *Azeradj* (n=4; Bejaia, Skikda), *Blanquette* (n=7; Guelma, Soouk-Ahras), *Bourricha* (n=2; Skikda), *Chemlal* (n=5; Bourmerdes, Setif, Tarf, Tebessa, Tizi-ouzou), *Limli* (n=3; Jijel) and *Sigoise* (n=1; El-Oued).

Figure 1: Geographical Origins of Algerian Olive Oils.



Fatty Acid Determination

Olive oil in *n*-heptane (0.12 g/2 mL) were transmethylated with a cold solution of KOH (2M) (200 µl) according to the norms of the European Standard NF EN ISO 5509 (2000).[6] Fatty acid methyl esters (FAME) were analyzed in compliance with the European Standard NF EN ISO 5508 Norms (1995). The experimental analysis were performed using an Agilent Technology gas chromatograph 7890 A (GC) equipped with a split/split-less injector (t=250 °C) and a flame ionization detector (FID) (t=250 °C). A silica capillary column (60 m×0.25 mm i.d., 0.25 µm film thickness) coated with Sulpelcowax (Supelco) were used. The operating conditions of separation were such that the gas carriers (hydrogen) were used as the output. The inlet pressure of the hydrogen as carrier gases were 178 κPa with the ratio 1:70. The oven temperature conditions were as follows: 20 min at 210 °C, from 210 °C to 245 °C at 6 °C/min, 20 min at 245 °C and the flow rate were 13 ml/mn. The peaks were identified on the basis of the retention of known methyl esters.

Morphogramme

A radial plot helps create a "morphogramme" which is used in the characterization of each variety as per a "morphotype". The "morphogramme" is designed as a radial representation using an Excel® spreadsheet, and on each axis the change in the variable with respect to the mean is represented. For each variable, centered mean gives the origin of the graphics (0%) that comes from a data base built from more than 2000 samples from various sources, grouped on the basis of variety and origin. The limits of variation (-100% to +100%) is equal to twice the standard deviation of the variable for fatty acids. The solid lines are the median values of individual fatty acids with respect to the dotted lines of first and second quartiles. Thus, on each axis, the value represented gives the percentage change compared to two times the standard deviation.

Nomenclature

The nomenclature of the fatty acids that were found in our study has been given as follows: Fatty acids 14:0, myristic acid (tetradecanoic acid); 16:0, palmitic acid (hexadecanoic acid); hypogeic acid (7-hexadecenoic acid); palmitoleic acid (9-hexadecenoic acid); 17:0, margaric acid (heptadecanoic acid); margaroleic acid (9-heptadecenoic acid); oleic acid (9-octadecenoic acid); cis vaccenic acid (11-octadecenoic acid); linoleic acid (9,12-octadecadienoic acid); linolenic acid (9,12,15-octadecatrienoic acid); 20:0, arachidic acid (eicosanoic acid); gondoic acid (11-eicosenoic acid)[2]; 22:0, behenic acid (docosanoic acid); 24:0, lignoceric acid (tetracosanoic acid).

RESULTS AND DISCUSSION

The fatty acid composition of oils were determined by gas chromatography of methyl esters. Fourteen fatty acids and squalene were identified in all samples. Oleic acid (18:1ω9), palmitic acid

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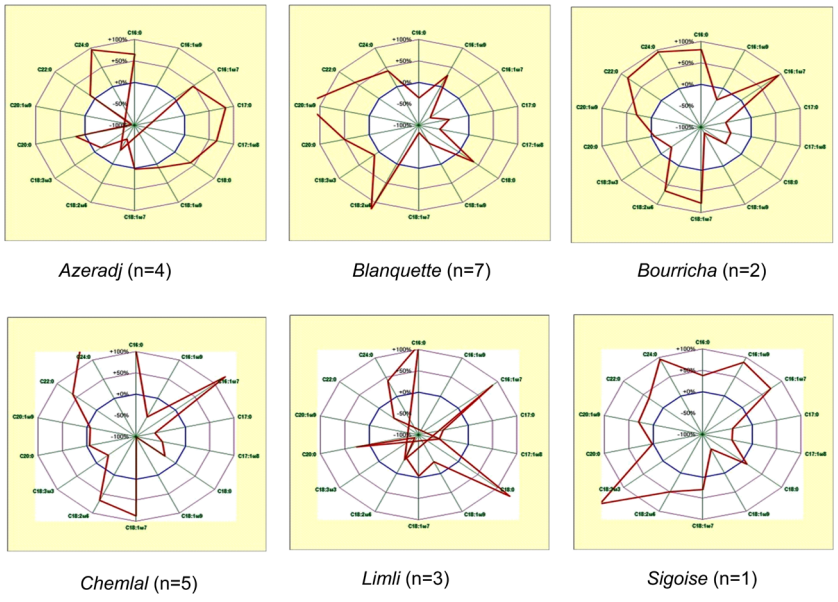
(16:0), linoleic acid (18:2ω6) and stearic acid (18:0) are the major fatty acids commonly found in olive oils. Palmitoleic acid (16:1ω7), hypogeic acid (16:1ω9), oleic acid (18:1ω9) and cis-vaccenic acid (18:1ω7) are considered as separate entities by the European regulation, unlike the Codex Alimentarius which identifies them as a single component. Six minor fatty acids including margaric acid (17:0), margaroleic acid (17:1ω8), arachidic acid (20:0), gondoïque acid (20:1ω9),[2] behenic acid (22:0) and lignoceric acid (24:0) were also determined. The fatty acids which leave slight traces (<0.01%) are not taken into account. Table 1 gives the results of the qualitative analysis of fatty acids of olive oils extracted from six cultivars. The "morphogrammes" are built from average values of fatty acids (Figure 2).

The profile of the fatty acids of the studied oils were in accordance with that of the International Olive Oil Council's regulation,[7] Codex Alimentarius[8] and European Regulations[9] excepted for *Sigoise* sample (18:3ω3>1%). Oils of different cultivars are characterized by different fatty acid compositions.

Our observations indicate that indeed, all the six varieties are very rich in oleic acid (C18: 1ω9). The flow rate of oleic acid in each studied variety for *Azeradj* is (72.70%) on average, followed by the *Blanquette* (67.18%), *Bouricha* (64.29%),

Figure 2: "Morphogrammes" from Oils of Six Algerian Cultivars.



Azeradj (n=4) *Blanquette* (n=7) *Bourricha* (n=2)

Chemlal (n=5) *Limli* (n=3) *Sigoise* (n=1)

Table 1: Fatty Acid Compositions[1] of Six Algerian Virgin Olive Oils.

Fattyacids	*Azeradj*			*Blanquette*			*Bouricha*			*Chemlal*			*Limli*			*Sigoise*
	n= 4			n=7			n=2			n=5			n=3			n=1
	Min	Max	Moy	Min	Max	Moy	Min	Max	Moy	Min	Max	Moy	Min	Max	Moy	
16:0	13.12	14.54	14.01	9.54	11.66	10.77	14.31	14.72	14.51	14.12	16.29	15.18	15.17	15.84	15.47	13.12
16:1ω9	0.02	0.08	0.04	0.13	0.14	0.14	0.10	0.12	0.11	0.08	0.12	0.10	0.04	0.05	0.04	0.17
16:1 ω7	1.10	1.44	1.26	0.23	0.36	0.33	1.63	1.68	1.65	1.43	2.18	1.90	1.40	1.93	1.59	1.47
17:0	0.10	0.20	0.16	0.05	0.05	0.05	0.04	0.04	0.04	0.03	0.04	0.03	0.04	0.04	0.04	0.05
17:1 ω8	0.17	0.32	0.27	0.06	0.06	0.06	0.08	0.09	0.09	0.07	0.09	0.08	0.06	0.07	0.06	0.09
18:0	2.54	2.95	2.74	2.58	2.80	2.71	1.84	1.93	1.88	1.83	2.15	2.01	3.46	3.97	3.73	2.43
18:1 ω9	71.38	74.52	72.70	64.86	68.79	67.18	64.11	64.47	64.29	60.83	64.52	63.06	68.73	68.98	69.19	66.37
18:1 ω7	2.28	2.58	2.42	1.14	1.48	1.37	3.27	3.40	3.34	3.68	3.20	3.46	2.14	2.73	2.35	2.75
18:2 ω6	4.44	5.44	4.95	13.97	16.85	15.65	12.43	12.70	12.57	11.48	13.80	12.69	6.27	6.33	6.30	11.66
18:3 ω3	0.60	0.67	0.63	0.61	0.78	0.69	0.57	0.65	0.61	0.51	0.69	0.60	0.45	0.55	0.47	1.01
20:0	0.40	0.42	0.41	0.43	0.49	0.45	0.38	0.38	0.38	0.36	0.42	0.38	0.41	0.44	0.42	0.39
20:1 ω9	0.20	0.25	0.21	0.38	0.42	0.39	0.30	0.32	0.31	0.24	0.31	0.28	0.16	0.18	0.17	0.31
22:0	0.11	0.13	0.12	0.13	0.15	0.14	0.15	0.15	0.15	0.12	0.15	0.14	0.10	0.10	0.10	0.13
24:0	0.07	0.08	0.07	0.06	0.07	0.06	0.07	0.07	0.07	0.07	0.09	0.08	0.05	0.08	0.06	0.07

[1]Determined as methyl esters, % area of total fatty acids.

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Chemlal (63.73%), *Limli* (69.19%) and 66.37% for *Sigoise* (Table 1).

The recorded percentage were close to those of the Tunisian varieties *Chemlal*, *Chetoui* and *Zelmati* which are 60.62%, 65.66% and 62.15% respectively[10] like those of the French oil AOC Aix en-provence et Vallée des Baux.[11]

These results show that the six varieties of olive oils contain considerable quantities of essential fatty acids, in particular the *Blanquette* variety which contains 15.65% of linoleic acid followed by oils of the varieties *Chemlal* and *Bouricha* having 12.69% and 12.57% of 18:2ω6 respectively.

In addition, percentage of linolenic acid 18:3ω3 constitution varies between 0.47 to 0. 69% on an average for *Azeradj*, *Blanquette*, *Bouricha*, *Chemlal* and *Limli* except for the *Sigoise* sample.

Azeradj oil is the richest in 18:1ω9 (72.70%), 17:0 (0.16%) and 17:1 and the poorest in 18:2ω6 (4.95%) composition. These results are in agreement with the works of Bakhouche et al,[12] on the olive oils of *Azeradj* variety cultivated in Algeria, where the oleic acid composition ranges between 55 to 83% and low values of linoleic acid. However, to analyse the composition of an essential fatty acid, *Blanquette* is the most promising variety following the *Chemlal* and *Bouricha*.

It should be noted that the average percentages in linoleic and linolenic acid composition in six oils during the 2-seasons prove to be sufficient to prevent a state of deficiency of fatty acids for the people using this as principal source of fat in their food.[13]

The fatty acid composition of *Bouricha* and *Chemlal* oils are similar. The "morphogrammes" allow the identification of "morphotypes" for each variety of oil. The *Bouricha* and *Chemlal* "morphogrammes" (Figure 2) confirm the resemblance between them. Moreover, the *Blanquette* "morphotype" is identical to that of the Tunisian cultivar *Chetoui* (Figure 3). This confirms the hypothesis of Loussert and Brousse[14] on the identity and resemblance between these two cultivars.

This mode of representation is particularly effective to allow rapid visual identification of characteristics of olive oils.

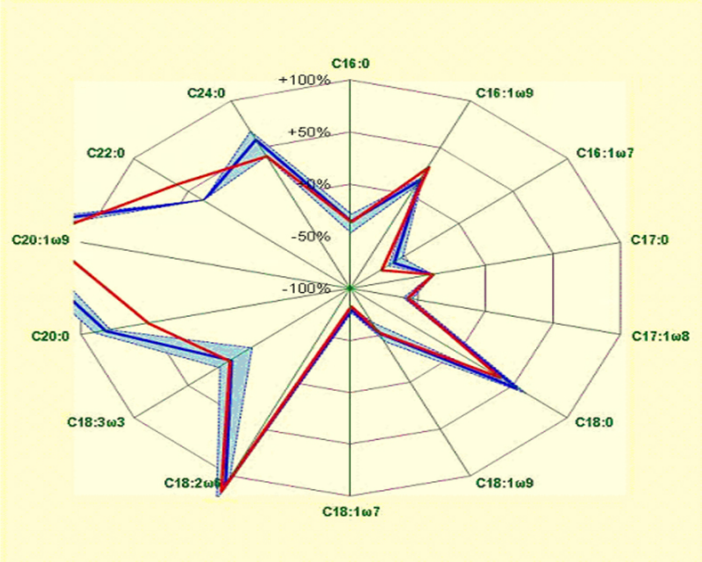
The fatty acids of olive oil play a significant role in contributing to its nutritional and organoleptic quality. Various factors, such as the variety, maturity of the fruit, climatic conditions, storage and extraction process used have an effect on the fatty acid composition of olive oils.[13,15]

CONCLUSION

The analysis of oil from six cultivars from East and South-western Algeria has shown minor differences in their fatty acid profile.

The complete results of this analysis were obtained only with oils belonging to the six varieties produced in the season 2009 and 2010. These varieties were cultivated over several seasons to confirm the real identity of these oils, to guarantee its rank in terms of quality in the world wide market of the olive oils and to select the best varieties amongst them. This will also make it possible to select the best varieties and to spread their cultivation throughout the national territory and confirm the Algerian AOC (designation of origin controlled).

Figure 3: "Morphogramme" Comparaison between Blanquette Oil (red) and Chétoui Oil (blue).



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CONFLICTS OF INTEREST

There are no competing interests related to this article.

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Research

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A Quantitative Enzyme-Linked Immunosorbent Assay for Shiga Toxin 2a Requiring Only Commercially Available Reagents

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ABSTRACT

Aim: This study develops a quantitative ELISA for measuring Shiga toxin 2 (Stx2) produced by Shiga toxin (Stx)-producing *Escherichia coli* (STEC), including foodborne pathogen *E. coli* O157:H7 by all commercially available reagents.
Background: Most foodborne outbreak strains of STEC produce Stx2a, Stx2c or both, which are more frequently associated with human clinical cases, leading to severe gastrointestinal diseases or even death. However, no simple and cheap assays or kits are available for quantitative detection of Stx2a and Stx2c. Therefore, an easy and affordable quantitative method for Stx2 is needed for its pathogenesis study.
Results: We successfully developed a sensitive and specific receptor-based ELISA by using all commercial available agents. Hydroxyl acyl ceramide trihexoside, an analogue of Stx2 receptor globotriaosylceramide (Gb3), was used for antigen capture, and several critical steps were identified that must be adhered to ensure repeatability. No cross reactivity was observed with Stx1, and linear curves could be constructed using either purified Stx2a or a bacterial lysate from an Stx2a-producing *E. coli* O157:H7 strain. We applied this method to quantify Stx2 production by a collection of *E. coli* O157:H7 strains, indicating it can be extended to qualitatively evaluate Stx2c, and providing evidence that toxin production does not necessarily correlate with strain phylogeny.
Conclusion: Our R-ELISA provides a reliable way to quantify Stx2a using commercially available components, and it can also be used for detecting Stx2c. This cost-effective ELISA can be easily performed, suggesting it will be a useful tool for studying pathogenesis of STEC.

KEY WORDS: Shiga toxin-producing *E. coli*; *E. coli* O157:H7; ELISA; Shiga toxin 2.

ABBREVIATIONS: Stx: Shiga toxin; STEC: Stx-producing *Escherichia coli*; Gb3: Globotriaosylceramide; HC: Hemorrhagic Colitis; HUS: Hemolytic Uremic Syndrome; USDA: US Department of Agriculture; LSPA: Lineage Specific Polymorphism Assay.

INTRODUCTION

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) cause gastrointestinal diseases such as Hemorrhagic colitis (HC), and in some cases this develops into the life-threatening hemolytic uremic syndrome (HUS).[1,2] Foodborne pathogen, *E. coli* O157:H7, is a genetically heterogeneous serotype of STEC. Consumption of as low as ten cells of O157:H7 derived from contaminated bovine origin foods such as beef and cheese, people may develop symptoms ranging from asymptomatic carriage, bloody diarrhea, to fatal HUS. It has been estimated to cause 73,000 illnesses and 60 deaths annually in United States (US), with an estimated economic loss of about 400 million dollars. This pathogen continues to cause most of known outbreaks across the globe.[3,4] Therefore, the US Department of Agriculture (USDA) has enforced

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a "zero tolerance policy" to regulate the prevalence of O157:H7 in foods. Genetic methods, most commonly the lineage specific polymorphism assay (LSPA)[5] and clade typing,[6] have been used to separate O157:H7 strains by virulence potential and ecology. For example, human isolates are more commonly classified as lineage I and I/II than lineage II,[7,8] and it has been argued that isolates from clades 6 and 8 are more virulent than isolates from other clades.[6,9,10]

Many virulence factors contribute to the pathogenesis of *E. coli* O157:H7 including Stx.[11,12] This is an AB_5 toxin, comprised of a single A subunit associated with 5 identical B subunits.[13,14] While the pentamer binds to Gb3 located in the host cell membrane,[15] the A subunit functions as a glycosidase, cleaving an adenine nucleotide from 28S rRNA within the 60S subunit. This results in inhibition of protein synthesis, followed by necrosis and cell death.[16] There are 2 immunologically distinct isoforms of the toxin, designated Stx1 and Stx2, which share 56.8% amino acid identity.[17] Stx2 has a lower affinity for Gb3 than Stx1[18]; however, Stx2 is more toxic in animal models[19-21] and associated with more severe clinical cases.[22] Among the seven subtypes of Stx2 (from Stx2a to Stx2g),[23] Stx2a and Stx2c are more often associated with clinical strains causing deadly HUS.[24,25] In this manuscript, we will use "Stx2" when collectively referring to its subtypes.

Stx2 producing strains are correlated with the development of HUS,[26,27] leading to the hypothesis that high toxin-producing strains are more likely to cause severe disease symptoms. Several studies have concluded, for example, that lineage I and I/II isolates produce more Stx2 than lineage II isolates,[28] and that clade 8 isolates are high toxin producers compared to isolates from other clades.[29,30] Most studies measure Stx2 production by semi-quantitative commercial kits,[10] qPCR/microarray,[10,29,30] or by semi-quantitative western blots.[30] Concerning mRNA quantification, several studies have reported that Stx2 transcript levels do not always correlate with Stx2 toxin production.[10,30,31] Thus, immunoassays are preferred methods for quantifying Stx production.

Several immunologic methods were previously described that use either immobilized antibodies specific for Stx,[32,33] or a receptor mimic[34-36] as the antigen capture in the sandwich ELISA. The disadvantage for the former is that it requires two sets of anti-Stx2 antibody from different species for both antigen capture and detection steps, while the latter assay only needs one set of anti-Stx2 antibody for detection. Most commercially available anti-Stx2 antibodies are monoclonal and generated from mice, so we suggest that one drawback preventing most research laboratories from quantifying Stx2 by sandwich ELISA is the limited commercially availability of antibodies from other species. Although, commercial kits such as Premier® Enterohemorrhagic *Escherichia coli* (EHEC) has been developed for detecting Stx, they are not designed to be specifically for Stx2 quantification, because they can not differentiate Stx1 from Stx2. Its high cost also prohibits most laboratories from routinely using it for this purpose. Moreover, previously reported sandwich ELISA by using Gb3 as antigen capture was not developed to be quantitative for Stx2. Therefore, we were motivated to develop a cheap and reliable assay specifically for quantifying Stx2a that required only one set of anti-Stx2a antibody. Our initial attempts to develop a R-ELISA met with several difficulties in consistency and repeatability, an issue echoed in a previous publication.[36] We suggest here that development of a robust assay would require identifying and standardizing critical steps of this assay, so it can be successfully applied to quantify Stx2a.

MATERIALS AND METHODS

Strains and Culture Conditions

The fourteen *E. coli* O157:H7 strains used in this study were obtained from the Pennsylvania Department of Health and previously characterized by our lab.[7] They were stored in 10% glycerol at -80 °C.

Ciprofloxacin Induction and Bacteria Lysate Collection

Each strain was propagated in Luria Broth (LB) at 37 °C with shaking overnight. Cells were diluted to A_{600} of 0.05 in fresh LB broth. To induce toxin expression, ciprofloxacin was added to a sub-leathal concentration of 45 ng/mL. After an eight-hour incubation at 37 °C with shaking, the culture was centrifuged at 4,000 g for 10 min, and supernatants were filtered through 0.2 µM cellulose acetate filters (VWR, Radnor, PA, USA).

Stx2 Specific R-ELISA

A 0.5 mg/ml stock solution of hydroxyl acyl ceramide trihexoside (Matreya Biosciences, Pleasant Gap, PA, USA) was prepared in chloroform: methanol (2:1, v/v). This stock solution was further diluted to 25 µg/mL in methanol, and was either used immediately or stored at -20 °C. Frozen stocks were heated in a 55 °C water bath to re-dissolve hydroxyl acyl CTH before use. To coat wells with antigen capture, a volume of 100 µL hydroxyl acyl CTH working solution was added to wells of an eight-well polystyrene strip plate (Thermo Scientific, Waltham, MA, USA) in a chemical fume hood. Strip plates were rotated manually every 15 minutes until the methanol was fully evaporated. Next, 200 µL blocking buffer [4% bovine serum albumin (BSA) in 0.01 M phosphate buffer saline (PBS) with 0.05% Tween20] was added to each well and incubated at 4 °C for 16 hours. After removing blocking buffer, 200 µL washing buffer (0.01M PBS with 0.05% Tween20) was added to each well and incubated for one minute on a shaking platform. This step was repeated for a total of 5 washes.

The R-ELISA assay was initiated by adding 100 µL sample into each well, followed by shaking at room temperature for one hour. Wells were washed five times in PBS/Tween20 as described above. Next, 100 µL mouse anti-Stx2 antibody (Santa

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Cruz Biotech, Santa Cruz, CA, USA) was diluted in blocking buffer to 1 µg/mL, added to each well, and incubated with shaking at room temperature for one hour. Wells were again washed five times with PBS/Tween20. Goat anti-mouse HRP conjugated secondary antibody was diluted in the blocking buffer (1 µg/mL, 100 µL) and added to each well, and incubated with shaking at room temperature for one hour. Five washes in PBS/Tween20 followed; however, this time each wash was performed for five minutes. Detection was accomplished using 1-Step Ultra TMB (Thermo-Fischer, Waltham, MA, USA), which was equilibrated to room temperature in a foil-wrapped tube for at least 30 minutes prior to use. TMB substrate (100 µL) was added to each well and incubated with shaking for 10 minutes to allow for color development. Finally, 100 µL of stop solution (2 M H_2SO_4) was added to each well, followed by shaking for 30 seconds. The reading values of A_{450} were obtained using a DU®730 spectrophotometer (Beckman Coulter, Atlanta, Georgia, USA).

For all assays, supernatants from *E. coli* O157:H7 strain PA24, which produces only Stx1, were used for the negative control, and a lysate from a *E. coli* O157:H7 strain, PA11, served as the positive control. The Stx2a concentration for PA11 stock was quantified by known concentration of pure Stx2 (BEI Resources, Manassas, VA, USA). The standard curves in R-ELISA were generated using two-fold serially diluted PA11 lysate in PBS. Any A_{450} above 0.1 was considered as positive. Total protein in each unknown sample was measured by the Bradford assay (VMR Life Science, Philadelphia, PA, USA), following the manufacturer's recommended protocol. Stx2 quantities were reported as µg Stx2/mg total protein.

Statistic Analysis

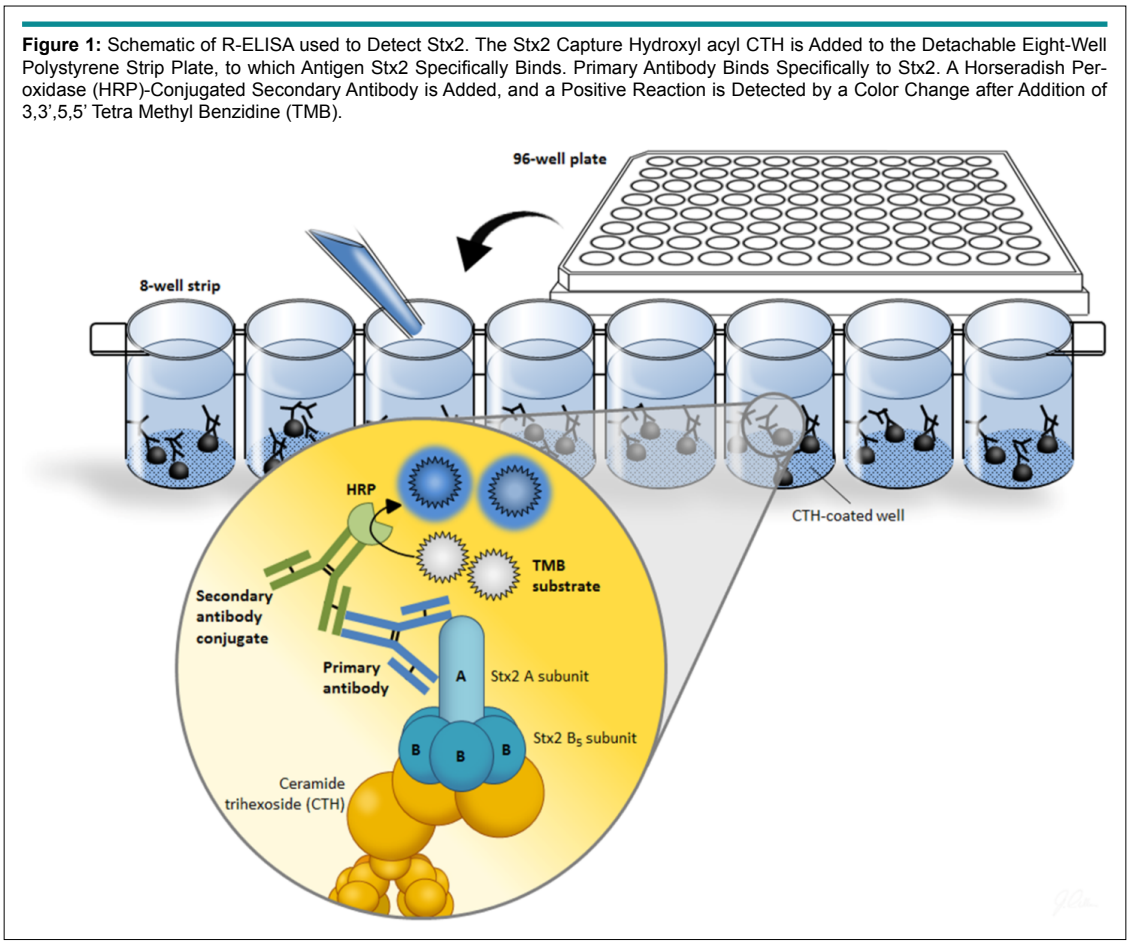
All data were reported as the mean from three biological replicates and each sample had three technical repeats. The mean and standard error were calculated in MS Excel®. Data were analyzed using linear regression and one-way analysis of variance (ANOVA) in Minitab version 17 (Minitab Inc., State College, PA, USA).

RESULTS

Assay Optimization

We started to develop our R-ELISA based on the traditional sandwich ELISA. Although, polyclonal anti-Stx2 antibody from rabbit (BEI resource, Manassas, VA, USA) was tried as the antigen capture in our initial attempt, it gave very high background noise. Therefore, we replaced the capture antibody with a Gb3 chemical analogue, hydroxyl acyl ceramide trihexoside (CTH) (Figure 1). Mouse anti-Stx2 A subunit monoclonal antibody (Santa Cruz Biotech, Santa Cruz, CA, USA) was selected as the

Figure 1: Schematic of R-ELISA used to Detect Stx2. The Stx2 Capture Hydroxyl acyl CTH is Added to the Detachable Eight-Well Polystyrene Strip Plate, to which Antigen Stx2 Specifically Binds. Primary Antibody Binds Specifically to Stx2. A Horseradish Peroxidase (HRP)-Conjugated Secondary Antibody is Added, and a Positive Reaction is Detected by a Color Change after Addition of 3,3',5,5' Tetra Methyl Benzidine (TMB).



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primary antibody. As a result, we were able to reduce the background noise, by giving optical density at 450 nm (A_{450}) less than 0.1 when measuring the negative control.

Using previous publications[34,36] as guidelines, several critical steps which were not explicitly stated in those manuscripts were identified and further improved to make our assay robust and quantitative. First, although several Gb3 receptor mimics are available (CTH is available as hydroxyl acyl, non-hydroxyl acyl and lyso-derivatives), we found that hydroxyl acyl CTH was best at capturing Stx2a at room temperature (Table S1). Second, although the supplier recommended methanol as the solvent, we found that dissolving hydroxyl acyl CTH first in chloroform:methanol (2:1), and further diluting in methanol to the working concentration gave the best results. Commercial hydroxyl acyl CTH better dissolved in the former without leaving any solid residual, and the signal got improved by average five times higher (Table S2). Third, we used detachable eight-well polystyrene strip plates instead of traditional 96-well plates. This minimized edge effects, a common observation with ELISA where peripheral wells give a higher absorbance than central wells. Fourth, the coating of R-ELISA plate wells with hydroxyl acyl CTH needs to be performed as rapidly as possible, so evaporation of methanol occurs evenly across the wells. Last, each of the washing steps used to remove the secondary antibody was extended to five minutes to minimize background noise.

Specificity and Sensitivity of Stx2 Specific R-ELISA

The specificity of this assay was first tested by using bacteria lysate of Stx1-producing strain previously designated PA24.[7] The absorbance at 450 nm (A_{450}) for bacteria lysate from PA24 is routinely less than 0.1, indicating that no cross-reactivity was observed in this assay with Stx1. As pure Stx2a from biodefense and emerging infectious (BEI) resource is available in quite limited quantity, we decided to use lysates from a high Stx2a-producing strain previously designated PA11[7] as the toxin source for standard curves. The Stx2a concentration in PA11 supernatants after ciprofloxacin induction was first quantified by using known concentration of pure Stx2a and around 23.2 µg/mL. We routinely obtained a linear range between an A_{450} of 0.3 to 3.2, corresponding to a Stx2a concentration from 23 ng/mL to 363 ng/mL (Figure 2).

Application of Stx2 Specific R-ELISA to Quantify Toxin in Bacteria Lysates

This assay was designed specifically for Stx2a. Although, Stx2a and Stx2c were reported to vary in their affinities to Gb3 *in vitro*, we expected this assay to detect Stx2c because the primary antibody used targets at the A subunit of Stx2, which is conserved between the 2 subtypes.[23,37] Therefore, we next evaluated whether this assay could be applied to measure the Stx2 levels in bacteria lysates from phylogenetically distinct strains. Thirteen strains from 6 of the known nine clades (Table 1) were chosen. These strains consisted of four defined Stx-allelic types: Stx2a only, Stx2c only, Stx1 & 2c and Stx2a & 2c previously.[7] One strain (PA7) was previously reported to be Stx1 and Stx2 negative by polymerase chain reaction (PCR), although it was toxin-positive using Premier® EHEC that simultaneously detected both isoforms; and another strain (PA48) could not be categorized by lineage subtype method. We quantified Stx2 in eleven of the thirteen strains (Figure 3), with strains PA7 and PA48 under the detection limit. Therefore, this assay could be applied to detect Stx2c levels in bacteria lysates of strains harboring either Stx2c (PA38, 40) or both Stx2c and Stx1 (PA22, 41).

Among them, the highest producer (PA39) had approximately five times more Stx2 production than the lowest strain (PA5). Strains sharing the same lineage, clade or Stx allelic type showed varied Stx2 levels as well. For example, strains PA32, 49 from lineage I produced similar amounts of Stx2 as PA31, but significantly more than lineage I strain PA5. Likewise, among

Figure 2: Linear Curves Generated Using Bacteria Lysate Supernatant from *E. coli* O157:H7 Strain PA11 in R-ELISA. Two-fold Dilutions were Assayed using R-ELISA, Revealing the Linear Ranges of the Assay. The Linear Equation of the Best Fit Line and R-square are Shown by using Raw Data from Three Replicates.

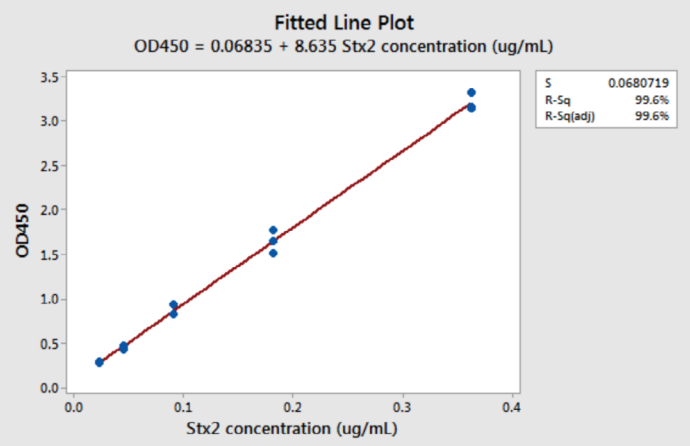

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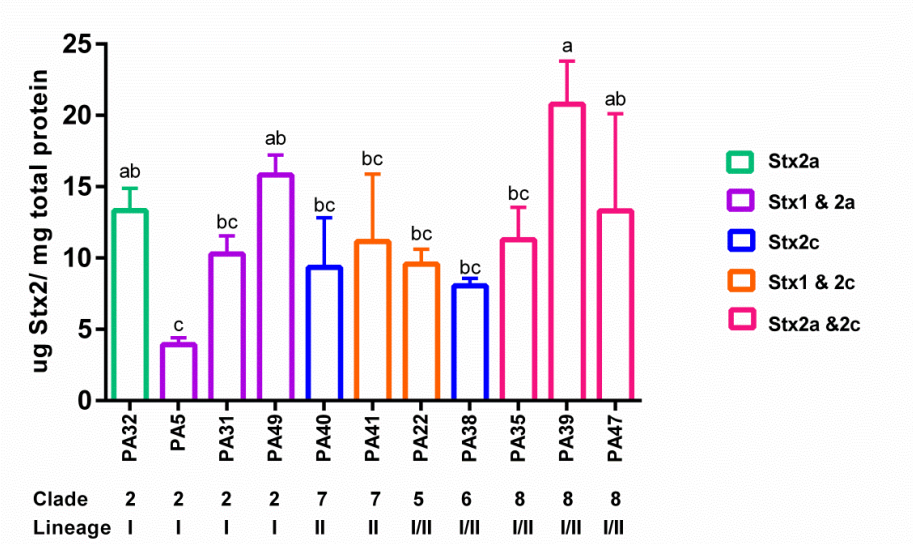
Table 1: Characteristics Summary of STEC Strains Used in this Study.

Clade type	Toxin profile	Strain name	Lineage type*
Clade 2	Stx2a	PA 32	lineage I
Clade 2	Stx1 & 2a	PA 5, 31, 49	lineage I
Clade 5	Stx1 & 2c	PA 22	lineage I/II
Clade 6	Stx2c	PA 38	lineage I/II
Clade 7	Stx2c	PA 40	lineage II
Clade 7	Stx1 & 2c	PA 41	lineage II
Clade 8	Stx1	PA 24	lineage I/II
Clade 8	Stx2a & 2c	PA 35, 39, 47	lineage I/II
Clade 9	Stx1 & 2c	PA 48	Undefined
Unclassified	Unknown	PA 7	lineage I

*The lineage and Stx profiles of all strains were previously reported.[7]

Figure 3: Quantification of Stx2 Production by *E. coli* O157:H7 Strains from Various Lineages, Clades, and Toxin Profiles. The Lineage or Clade Type is Shown below the Strain Designation, while the Stx Profile is Differentiated by Color. Data is Reported as the Average Concentration±One Standard Error. Means that do not Share a Common Letter (a, b, c) are Significantly Different (Turkey's test, $p<0.05$).



the three strains in clade 8, the Stx2 level in strain PA39 was similar to PA47; however, significantly higher than PA35. Lastly, among strains producing both Stx1 and Stx2a, strain PA49 produced significantly higher amounts of toxin than strain PA5, but was not different from PA31.

DISCUSSION

Stx2a and Stx2c are responsible for severe clinical complications such as life-threatening HUS caused by foodborne pathogen *E. coli* O157:H7. Thus, the accurate measurement of Stx2 production is important to study its pathogenesis. A globotetraosylceramide (Gb4)-based ELISA was reported for quantitative detection of Stx2e[38]; however, it can not be applied to measure other Stx2 subtypes because the latters preferentially recognize different receptor, Gb3. Therefore, no quantitative receptor-based ELISA was available for measuring Stx2a before our study by using all commercial available agents. In this study, we have developed a robust assay for the quantification for Stx2a, and it is also expected to detect Stx2c. The cost for this quantitative assay is one third of qualitative Premier® EHEC kit.

Although, Gb3 have been used previously in immunoassays to capture Stx,[35,36] we identified several steps that were critical to follow in order to achieve repeatable results. In addition, there are three other modifications from the literature worth highlighting. First, it was reported that the lysoform of Gb3 provided more reliable Stx1 detection, presumably because it is less hydrophobic.[36] However, during assay development we found that lyso-CTH was less effective at capturing Stx2a than hydroxyl acyl CTH. When testing the same bacteria lysate from O157:H7 strain with a high Stx2a concentration, the hydroxyl

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acyl CTH gave saturated reading while the A_{450} for lyso-CTH is as low as negative control (Table S1). This could simply reflect differences in affinity of Stx1 and Stx2a for CTH derivatives. Secondly, another group previously reported that they could improve R-ELISA sensitivity by increasing the amount of receptor used to coat wells.[36] While we saw similar results in initial trials, we decided that 25 µg/ml hydroxyl acyl CTH was the optimal concentration for detecting Stx2a. Under this concentration, we were able to detect Stx2a as low as 23 ng/mL which met our need. In Gb4-based quantitative ELISA for Stx2e, the limit of detection is 20 ng/mL,[38] as comparable as ours. Moreover, two 96 well R-ELISA plates can be prepared from one vial of commercial available CTH (0.5 mg), instead of one 96 well plate if a higher concentration was used. Lastly, it was reported that the presence of cholesterol alone or along with lecithin caused statistically significant increases in the binding of Gb3 to Stx2.[39] However, no enhancement of Stx2a capture was found when combining these with hydroxyl acyl CTH during the coating step.

Using R-ELISA, we were able to quantify toxin levels from various *E. coli* O157:H7 strains, and obtained results that were both confirmed and contrasted with those previously reported. First, we did not notice a correlation between Stx2 production and lineage. It was previously reported that *E. coli* O157:H7 isolates from lineage II produced less Stx2 than those from lineage I and I/II[28]; however, our findings show that the Stx2 levels in strains from lineage II (PA40, 41) were not significantly different from strains belonging to lineage I (PA5, 31, 32, 49) or lineage I/II (PA22, 35, 38, 47). Second, although strains from clade 8 were suggested to produce higher Stx2 levels than isolates from other clades,[29,30] our data indicates this is not universal. In our study, clade 8 strain PA35 produced similar amounts of Stx2 as others clades, including clade 2 (PA31), clade 5 (22), clade 6 (PA38) and clade 7 (PA40, 41). Lastly, it was previously suggested that Stx2-only producing strains synthesized more toxin than Stx1/Stx2-producers, due to crosstalk between phage-encoded repressors encoded in different Shiga toxin-converting phages.[40] However, the Stx2a-only strain PA32 produced toxin level that was similar to most Stx1/Stx2-producing strains we tested. It appears likely that phylogeny or toxin profile alone does not explain differences in toxin levels and thus virulence differences observed among strains. More factors such as colonization, adherence capabilities and other virulence genes, need to be considered as well.

Two strains, PA7 and PA48, produced Stx2 levels that were below the detectable limit using our R-ELISA. Strain PA48 is classified as clade 9, and was previously shown to be LSPA type 311111.[7] This strain is related to *E. coli* O157:H7 strain G5101,[41] which is also LSPA 311111[42] and is atypical among pathogens of this serotype in that it is β-glucuronidase-positive. Little is known about the toxin production levels of these strains. PA7 is a strain previously characterized[7] and identified as PCR-negative for both Stx1 and Stx2; however, the commercial kit that simultaneously detects both subtypes identified this strain as Shiga toxin-positive. Our results here suggest the commercial assay was detecting Stx1, although it is possible that PA7 produces Stx2 level below the limit of detection of our R-ELISA.

While this assay is useful for quantifying Stx2a in the bacterial lysates, we have observed limitations when applying it to environmental samples. In a previous publication,[43] we were unsuccessful using this method to quantify toxin levels in cattle mucus; however, we could accomplish this using the Premier EHEC kit sold by Meridian Biosciences, Cincinnati, OH, USA. We also found that our assay was one tenth less sensitive than the commercial kit for mouse feces. The sensitivity of the ELISA may be improved by using more hydroxyl acyl CTH as the receptor, or optimizing sample preparation steps to remove inhibitory components from these samples. Therefore, this R-ELISA may not be practical at the moment for detecting toxin in complex biological matrixes. However, it is advantageous when one needs to quantify toxin from laboratory samples while it is impractical to generate or obtain antibodies needed for a traditional sandwich ELISA.

CONCLUSION

Our R-ELISA provides a reliable way to quantify Stx2a using commercially available components, and it can also be used for detecting Stx2c. The application of this assay can be expanded to other Stx2 subtype once corresponding pure toxin is available. It is a readily easy and cheap way to quantify toxin levels especially for groups that currently assess toxin production by qPCR or other transcription-based methods. We predict a similar quantitative assay can be developed to detect Stx1, although as stated above this may require selecting a different receptor mimic.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interesrt.

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SUPPLEMENTARY DATA

Table S1: The A450 for CTH Derivatives as Receptors in ELISA. The Data was Showed in Mean±Standard Deviation and Derived from Three Prelicates.

Stx2a concentration (ng/mL)	Hydroxyl acyl CTH	Non hydroxyl acyl CTH	Lyso CTH
240.0	2.8639±0.0132	0.0909±0.0122	0.1011±0.0097
120.0	1.8045±0.0115	0.0809±0.0106	0.0878±0.0072
60.0	0.9532±0.0107	0.0838±0.0094	0.0987±0.0080
30.0	0.5854±0.0091	0.0946±0.1010	0.0891± 0.0090
0	0.1012±0.0041 (negative control)		

Table S2: The A450 for Using Different Solvents for Dissolving CTH. The Data was Showed in Mean±Standard Deviation and Derived from Three Prelicates.

Stx2a Concentration (ng/mL)	Chloroform: Methanol	Methanol
240.0	2.8639±0.0132	0.5788±0.114
120.0	1.8045±0.0115	0.3224±0.090
60.0	0.9532±0.0107	0.1632±0.120
30.0	0.5854±0.0091	0.1232±0.098

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Preparation of Thymo-Rutin Green Tea and its Active Ingredients Evaluation

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ABSTRACT

Introduction: Buckwheat (*Fagopyrum esculentum*) and wild thyme (*Thymus serpyllum*) leaves and flowers were used to develop thymo-rutin green tea. The process involved cultivation of buckwheat collection of flowers and leaves after flowering and collection of wild thymus leaves and flowers.
Materials and Methods: The leaves and flowers washed and pass through steam to fix color and kept in solar house for dehydration. Dehydrated leaves, flowers and some other additives mixed and developed mixture of green tea. All these parameters used in this process have been standardized. Chemical and mineral analyses of thymo-rutin green tea have been carried out.
Results: The results of green tea showed the highest content of rutin content (1.99%) along other constituent i.e., thymol (0.21%) extracting value of ethanol (18.93%) and caffeine (0.35%). Thymol (2-isopropyl-5-methylphenol) is the main compounds have shown anti-inflammatory, immunomodulatory, antioxidant, antibacterial and antifungal properties.[1,2] Rutin is one of the flavonoid, flavonoids belongs to the plant phenolics.
Conclusion: The increasing interest in powerful biological activity of plant rutin outlined the necessity of determining rutin content in plant leaves and flowers. The highest content of rutin was found in the developed green tea. The developed green tea contained rutin, thymol and caffeine. The developed green tea is nutritious, delicious and health promoting.

KEY WORDS: Thymol; Rutin; Green tea; Buckwheat; Thyme.

ABBREVIATION: PCSIR: Pakistan Council of Scientific & Industrial Research; MARC: Mountain Areas Agricultural Research Center; GB: Gilgit-Baltistan.

INTRODUCTION

The Thymo-Rutin Green Tea has been developed by the utilization off buckwheat and thymus leaves flowers from the stand point of its nutritional importance. The Thymo-Rutin Green Tea can be used by common people especially for mountain expediters, soldier performing their duties in high altitudes i.e., siachen glacier, kargil, and other common peoples of high altitudes who feels headache due to deficiency of Oxygen, players of football, hockey and athletes who have chances to rupture capillary walls. The product also provides appreciable amount of different nutrients like caffeine, thymol and rutin.

Wild Thyme

Wild thyme belongs to genus *Thymus* known as thyme, it has 215 species that has commercial importance.

In Pakistan (Gilgit-Baltistan), the genus is represented by the species *Thymus serpy-*

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llum wild thyme. Thyme is most widely used culinary herbs. The dried leaves and flowers are used for food flavoring and the source of essential oil (EO) in pharmaceutical and food industries. A number of benefits in human and animal wellbeing have been associated with the use of thyme EO by the industry.[3] At this point, this plant can be considered as a potential impulse of new trends in food, pharmaceutical and cosmetic industries.[4] The thyme essential oil antioxidant potential has shown the uses of this product in food industry and effectiveness of oil demands for development of dietetic supplement.[5] Recent studies have showed that thyme have strong antibacterial, antifungal, antiviral, antiparasitic and antioxidant activities.[6-10]

The antiseptic, antioxidative, insecticidal, preservative, and anesthetic properties of thyme EO are owed mainly to the presence of thymol, carvacrol, geraniol and other volatile components.[11] Thymol helps in blood vessel treatment and purification of blood that helps in the easy dissolution of more oxygen in blood that is an important remedy for high altitudes like siachen glacier and other hits where there problems of deficiency of oxygen cause headache and other problems.

The chemical polymorphism of thyme has been reviewed by Stahl-Biskup. The most important component found in this genus is thymol and carvacol followed by linalool, p-cymene, α-terpinene, borneol, terpinene-4-ol and 1, 8-cineole.[12] In Pakistan the composition of this genus and detailed research work has not been undertaken so far.

The chemical composition of aromatic plants is significantly influenced by the plant part, season and plant ontogeny[14-16] location of growing[17] and drying.[18,19]

Buckwheat

Buckwheat (*Fagopyrum esculentum*) belongs to family Polygonaceae considered as pseudo cereals and used like wheat. Buckwheat is native to temperate East Asia and was grown in China before 1000 AD; it is now adapted in many areas of the world. Although buckwheat production is concentrated in China, Japan and North America, it is also produced in Europe, India, Tibet, Tasmania, Australia, Argentina, Bhutan and numerous other countries. The name "buckwheat" comes from the Anglo-Saxon words boc (beech) and whoet (wheat) because the seed resembles a small beech nut. The presence of rutin in buckwheat plants is one of the main reasons for the production of different kinds of buckwheat foods. Buckwheat is a very short season crop so that it will bloom in cooler weather i.e. high altitude or alpine zone as crop seeds, flowers and leaves are used as health food. Buckwheat occupies 948 hectares during 2014 in Gilgit Baltistan of Pakistan. The leaves and flowers of buck wheat contain rutin content that strengthens capillary walls. Food produced from buckwheat is gluten free and good source of nutrients including protein starch and essential minerals thus buckwheat have beneficial effect on human health,[20-22] increasing attention to buckwheat as a functional food has been currently paid,[23,24] there are two cultivated species common buckwheat (sweet) and tartary buckwheat sweet buckwheat is commonly used.

Globally the major deaths caused by cardiovascular disease. Although, typically considered a disease of developed countries, its incidence is increasing in the developing world. There may be more than 20 million people will die with cardiovascular disease per year mainly due to heart attacks and strokes up to 2025, if immediate action is not taken.[25]

In recent times, especially in all industrially developed countries, consumers are becoming more interested in foods which offer an added value in terms of health benefits. Functional foods have disease preventing and health promoting properties along with its basic nutritional functions, these foods may be processed or fresh. The term was first used in Japan in the 1980s, where there is a government approval process for functional foods, called Foods for Specified Health Use (FOSHU).[26] The consumption of functional foods with an increased content of rutin may be a way of reducing the risk of cardiovascular diseases in the population.

Rutin

Rutin is responsible for blood vessel elasticity, strengthening of capillary walls, circulatory disorder treatment due to its therapeutic ability. It belongs to bioflavonoids group of phenolic metabolites. It has antioxidant activity, reduce blood pressure and utilize vitamin C.[27-30]

Rutin abundantly found in plants, that is flavonoid glycoside, but unfortunately industrial extraction is possible from only a small number of plants[31] Schunck in 19th century first discovered rutin content in buckwheat; he isolated only 240 gram from 30 pounds fresh buckwheat leaves.[32]

Rutin in Buckwheat

Approximately 50 years ago, buckwheat was cultivated as a rich source of rutin for herbal drug production in the USA. At present, buckwheat rutin is the well-known dietary source until know. Common Buckwheat (pseudocereal) (*Fagopyrum esculentum Moench*) belonging to family polgonaceae is a dicotyledonous plant (and Tartary buckwheat (*Fagopyrum tataricum Gaertn*) is the only two of the many buckwheat species known that are cultivated for human consumption. Buckwheat is a natural functional food, because it positively affects the human organism biologically (for example low glycemic index and reduced capillary fragility) without the necessity of adding any other components. Buckwheat is uniquely rich in proteins (12-15%) and essential amino acids, such as lysine (5-7%), that are deficient in major cereal crops, but also contains an abundance of lipids, fibers, minerals (zinc, manganese, selenium, iron, phosphorus, and copper), and vitamins (B1 and B2).[33-38]

Buckwheat Usage

The leaves and flowers are used to develop green tea due to its

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rutin content. The dehulled seed, or groat, is used in breakfast cereals and milled into grits. Buckwheat gluten free flour can be used as noodles, pancake mix, biscuits, etc. buckwheat gluten free flour also blended with wheat flour for development of bread, and other breakfast product prepration. roasted groats, may be steamed, roasted, boiled. In Eastern Europe, buckwheat flour is used in cooking similar to wheat flour. Bread, cakes and dumplings are made with the addition of wheat flour. Buckwheat gluten free flour also blended 30-40% for development of baby foods. Buckwheat flour also use in manufacturing of ice-cream, dietetic foods, canned meat products. Pasta produced from a mixture of wheat and buckwheat flour has been characterized to possess shorter cooking time. Extruded and snack products can also be developed from buckwheat flour. Extruded buckwheat products are of very high nutritional quality when compared with products extruded from wheat.[39] Buckwheat extruded products developed for special nutritional needs due to it specific protein character. As buckwheat does not contain gluten, it is a common supplement for patients with celiac disease. Buckwheat intolerance is rare among patients with gluten intolerance alone, but more common in those with celiac disease combined with other food allergies. Allergic reactions are caused by ingestion of allergenic buckwheat proteins.[40] Other products made from buckwheat are green buckwheat tea, buckwheat beer and vinegar, spirit, buckwheat floral honey, buckwheat sprouts and fresh green plant parts used as a vegetable. Rutin content in buckwheat depends on species, harvest conditions, and the environmental conditions under which it is produced.[41,42]

Easily dehulled and large size of seeds and sweet taste of common buckwheat increased its consumption.

Rutin and other components are higher in Tartary buckwheat as compared to common or sweet buckwheat so the demand of Tartary buckwheat use is also increased in food companies. Consumption of tartary buckwheat is very less because the dehulling of tartary buckwheat is very difficult due to firm adherences of testa, seed small in size and bitter taste.

Different parts of buckwheat has different amount of rutin content i.e. flour, leaves, flower and stem.

The highest content of rutin can be found in buckwheat leaves, but buckwheat tops are much easier to use as a source of Rutin, considering the costs connected with the harvesting technology. Rutin content is higher in dry leaves, flowers as compared to flour and stem.

MATERIALS AND METHODS

The process of preparation for thymo-rutin green tea involves collection of leaves and flowers, washing, cleaning, steaming, dehydration, addition of additives, cutting and packaging.

Green Tea Preparation Method

Leaves and flowers of buckwheat (*Fagopyrum esculentum*) and wild thyme (*Thymus serpyllum*) were collected for the development of thymo-rutin green tea. The thyme leaves collected from devosi plane (dehydrated thyme leaves and flowers also available in Skardu market) and buckwheat cultivated in Pakistan Council of Scientific and Industrial Research (PCSIR) orchard the leaves and flowers were collected after flowering before seed formation. The harvested leaves and flowers washed to remove dust and cleaned for removal of weeds and other particles. The cleaned, washed leaves and flowers passed through steam to fix green colour and sterilization. The cleaned and washed leaves, flowers of buckwheat and wild thyme dehydrated in solar dehydrator. The dehydrated leaves and flowers chopped with chopper up to desired size. Wild thyme removed the pungent smell of buckwheat leaves and flowers. The developed thymo-rutin green tea packed in polyethylene zip bags and packets (Figures 1 and 2).

Sensory/Organoleptic Evaluation of Thymo-Rutin Green Tea

The organoleptic/sensory evaluation for taste, color, flavour, mouth feel and overall acceptability conducted using nine point hedonic scale in accordance with the method described by Larmond.[43] The panel members were selected on the basis of their ability to discriminate and scale abroad range of different attributes of Green tea. An orientation program was organized for the panel members to brief them the objective of the study. The

Figure 1: Pictorial View of Buckwheat Crop, Dehydrated wild Thyme Leaves and Developed Product Thymo-Rutin Green Tea.

Mr. Faizullah Khan collecting Buckwheat Leaves and Flowers for Thymo-Rutin Green Tea

Thymo-Rutin Green Tea

Dehydrated wild thyme leaves and flowers

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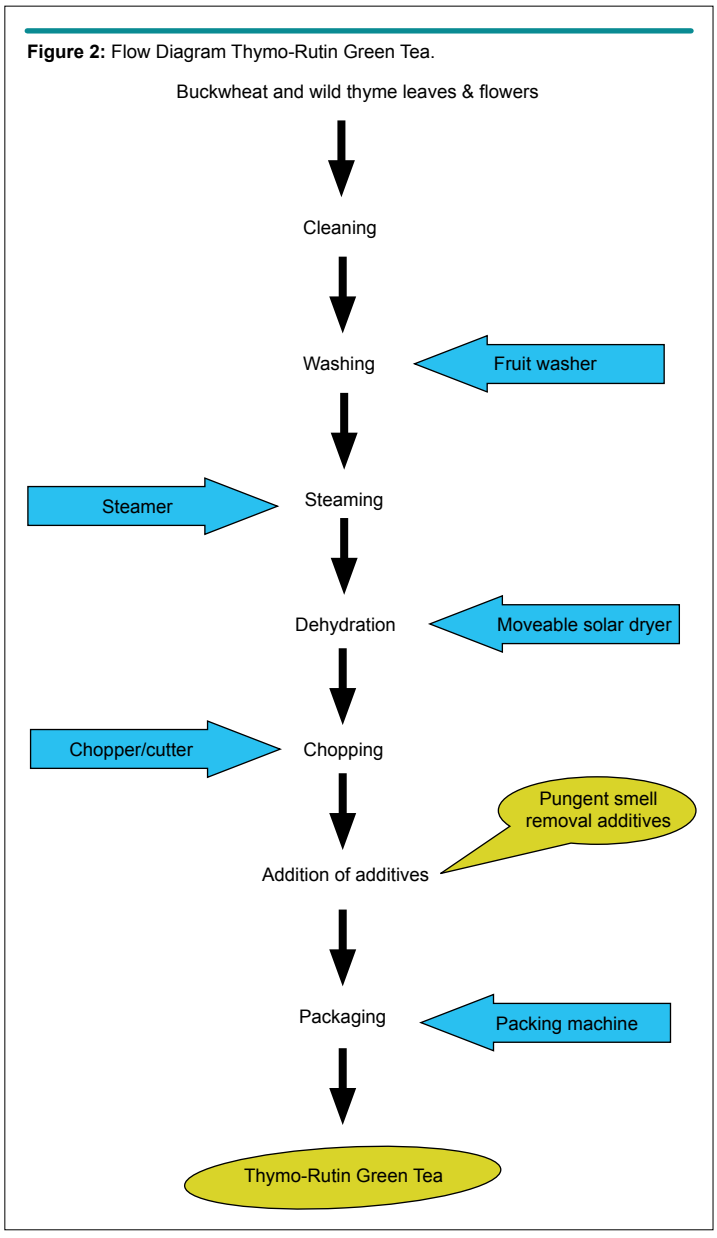

Figure 2: Flow Diagram Thymo-Rutin Green Tea.

sample was served members of organoleptic/sensory analysis. The members were advised to record their observation as per questionnaires. Larmond nine point hedonic scale used to develop Performa i.e. 1=Disliked extremely; 2=Disliked very much; 3=Disliked moderately; 4=Disliked slightly; 5=Neither liked nor disliked; 6=Liked slightly; 7=Liked moderately; 8=Liked very much; 9=Liked extremely; The members of sensory evaluation expectorated the samples and rinsed mouth using distilled water between samples. The experiment was repeated twice and the values are presented as means.

Chemical Analysis

The thymo-rutin green tea (final product) prepared were chemically analyzed for their moisture, total ash, Extracting value (ethanol), rutin, caffeine and thymol by standard method (AOAC-2000) respectively. Moisture was determined by oven drying at 105 ºC, up to constant weight.

Rutin: The analysis of rutin content in dry leaves and flowers were performed according to the Association of Official Agricultural Chemists (AOAC) official method.[44] This method has been modified using high-performance liquid chromatography (HPLC) 80% methanol. 0.5 g of the sample is dissolved into 50 ml HPLC 80% methanol. Then 2 ml of the extract thus obtained is transferred into 50 ml volumetric flask. Two ml dabble distilled water and 5 ml ammonium molybdate are added. Then the mixture is diluted to 50 ml. The standard solution is prepared through dissolving of 0.02 g rutin in 50 ml HPLC 80% methanol. Then 1 ml of this solution is used. The absorbance of the sample against dabble distilled water as a blank sample was determined at 360 nm with an ultraviolet and visible (UV-Vis)

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Spectrophotometer. Samples were analyzed in duplicate. Thymol extraction was carried out in a solvent extraction unit and determination of thymol was conducted by gas chromatograph (GC) mass spectrometer. Ash count was determined in a muffle furnace at 550 ºC for 6 hours. For all these determinations dry green tea samples used in duplicate in accordance with standard procedures.[45]

RESULTS AND DISCUSSION

For the development of thymo-rutin green tea an optimal recipe was explored and required ingredients was considered carefully. Some food grade additives were selected for the study. The ingredients were selected after conducting exploratory and subsidiary trails. Physical trails were employed during above trails using a 9-point hedonic scale.

The sensory evaluation of the product was carried out to check its color, taste, flavour, mouth feel and overall acceptability as shown in Table 1. On the basis of sensory evaluation of the developed product the thymo-rutin green tea was found acceptable. The organoleptic result of the product shows that the mix product of thyme and buckwheat gives better results in term of taste, colour, flavour, mouth feel and over all acceptability. The final product was further analyzed for rutin, thymol, and caffeine contents to check its suitability for nutritional purpose.

Table 2 depicts the ratio tried for acceptable buckwheat leaves, flowers and thyme leaves, flowers. Optimum ratio was found to be 4:1 of buckwheat and thyme. The leaves and flowers after cleaning and washing passed through steam and dehydrated in solar dryer and immediately packed in polyethylene bags for maximum availability of thymol and rutin.

Table 3 depicts the nutritional composition of the developed green tea. The moisture, ash, caffeine, ethanol, rutin and thymol contents observed were moisture 5.01%, total ash 11.91%, caffeine 0.35%, ethanol 18.93%, rutin content 1.99% and thymol content was observed 0.21%. The data showed that the developed product is a good source of thymol, rutin and caffeine. Thymol (2-isopropyl-5-methylphenol) is the main compounds have shown anti-inflammatory, immunomodulatory, antioxidant, antibacterial and antifungal properties.[43,44] Rutin is one of the flavonoid, flavonoids belongs to the plant phenolics.

The importance of rutin content due to its strong biological activity attracts researchers for its evaluation in different parts of buckwheat i.e., leaves flowers and flour, etc. The highest content of rutin was found in the developed green tea. The developed product is a mixed product of wild thyme leaves and buckwheat leaves and flowers.

Nutritional data of product also shows that it is an im-

Table 1: Sensory/Organoleptic Evaluation of Thymo-Rutin Green Tea.

Parameter	Panel member 1	Panel member 2	Panel member 3
Color	8	8.4	8.5
Taste	9	8	8.5
Flavour	8	8.5	8.7
Mouth feel	8.3	8.8	8
Overall acceptability	8	8.5	8.6

Table 2: Ratios of ingredients Tried for Acceptable Green Tea Production.

Ingredients	Level used							Optimum level
	1	2	3	4	5	6	7	
Buckwheat	1	2	3	4	5	4	3	4
Thyme	4	3	3	3	0	1	2	1

Table 3: Chemical Analysis of Thymo-Rutin Green Tea.

S. No	Parameters	Thymo-Rutin Green Tea %
1	Moisture	5.01±0.26
2	Ash	11.91±0.09
3	Caffeine	0.35±0.09
4	Extractive value (ethanol)	18.93±0.17
5	Rutin	1.99±0.07
6	Thymol	0.21±0.02

Values are mean of duplicate determinations±SD

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portant therapeutic product that contains ethanol and caffeine active ingredient with rutin and thymol that is helpful for reduction of cardiovascular disease, blood vessel elasticity, treatment of circulatory disorders, blood vessel treatment, purification of blood, increase oxygen dissolubility, atherosclerosis, reduction of blood pressure, stimulates of Vitamin C utilization and antioxidant activity.

CONCLUSION

It is concluded from the study that the developed product green tea is acceptable in buckwheat and thyme leaves and flowers combination. The develop product is a good source of rutin, thymol and caffeine which are beneficial to health and wellbeing of all individuals. The rutin and thymol makes the green tea an essential nutritional supplement especially for athletes, soldiers, mountain climbers and common peoples of above 8000 feet altitude. Rutin strengthen capillary walls, increase elasticity of veins, and thymol blood vessel treatment and increase dissolubility of more oxygen (O_2). The product green tea can be used by all age group.

CONFLICTS OF INTEREST

The authors have not declared any conflicts of interest.

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Review

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Microbial Safety of Foods in the Supply Chain and Food Security

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ABSTRACT

Most foodborne outbreaks in recent years have been linked to microbial contamination of food products. These food outbreaks can cause considerable food losses, and hence can play a role in global food insecurity. We discuss the importance of microbial food safety in the supply chain to reduce the potential for contamination. Microbial contamination may take place at pre-farming, farming or post-farming stages of the food supply chain. *Campylobacter, Salmonella, Listeria monocytogenes, Escherichia coli* O157:H7 and non-O157:H7 STEC *E. coli* are the most common pathogenic bacteria associated with food safety issues in the food supply chain. Efficient process controls and effective food safety management systems are vital elements to reduce microbial contamination and improve food security.

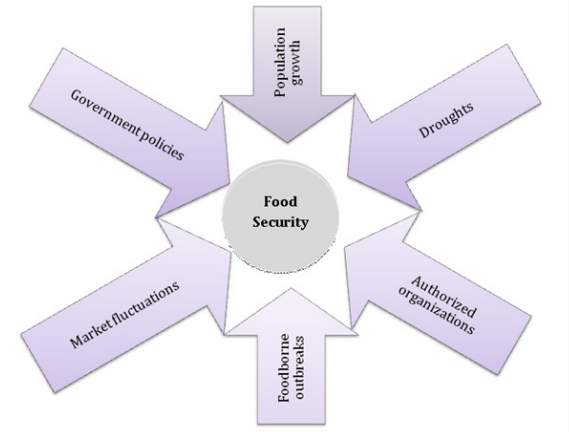
KEY WORDS: Food security; Food safety; Food supply chain; Microbial contamination.

ABBREVIATIONS: FSC: Food Supply Chain; FAO: Food and Agriculture Organization; STEC: Shiga toxin-producing *E. coli*; CDC: Centers for Disease Control and Prevention; CARMA: *Campylobacter* Risk Management and Assessment; COI: Cost-of-Illness.

INTRODUCTION

The difference between the terms "food safety" and "food security" is commonly misunderstood. These are separate issues but are, nevertheless, closely interrelated. The definition of food safety is the inverse of food risk and is the probability of a specific food not causing health problems after consumption.[1] Food security is defined as ensuring that all people at all times have both physical and economic access to the basic food they need.[2] Food security, a complex issue, is affected by multiple factors including microbial contamination of food, government policies, drought, global and national market fluctuations, and population growth (Figure 1). The importance of microbiological food safety is paramount because of the potential for harm-

Figure 1: Factors that Influence Food Security either Directly or Indirectly.



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ful microorganisms to grow and multiply in food commodities.[3] Entry of possible contaminants such as microbiological agents into food is a threat to the safety of food products. This can result in food poisoning, increase in foodborne outbreaks and a decrease in food availability because of discarding the contaminated food products.[4] Drought also can significantly affect the availability of irrigation water used in agricultural production and cause crop failures.[5,6] The aforementioned factors affect food availability and food access and can result in an increase in global commodity prices and food market fluctuations.[7]

Globalization of the food trade is one of the factors responsible for the increased number of foodborne outbreaks caused by microbes.[8,9] Bacterial pathogens are the most prevalent contaminants in food products followed by viruses, pesticide residues and mycotoxins.[10] The presence of harmful bacteria on food surfaces can increase the risk of cross-contamination, causing food poisoning and/or food losses. Food safety in the manufacturing and production of foods is important to protect the consumer from potential health risks and to reduce food losses. Food safety and quality in the supply chains are crucial to achieving food security and allows food to flow from areas of surplus to areas of deficit in local, national and global markets.[11,12] Emphasis as a priority is on microbiological quality throughout the food production chain, to minimize the risk of foodborne illnesses and, consequently to improve food security.

The gap between the current global population and food production, as well as the difference between food supply and demand in many countries is widening.[13,14] Understanding the relationships between microbial contamination, food safety and food security, and how these affect the global food supply, will help highlight areas in the food supply chain (FSC) that require more attention to improve food security. Governmental organisations have therefore introduced more rigorous policies to reduce the risk of food contamination and, thereby, ensure the supply of safe food.[15]

This paper discusses how microbiological food safety in the FSC could be useful in improving global food security. For the purpose of explanation in this report, the term "food safety" will refer to foodborne microbial infections and outbreaks and "food insecurity" as food losses and microbial spoilage of food during and post-food production.

FOOD LOSSES AND WASTE IN THE FOOD SUPPLY CHAIN

According to the Food and Agriculture Organisation (FAO), the world produces enough food to feed everyone living on earth. However, each year, almost one out of every four calories produced to feed people is not consumed and about one-third of total food production (1.3 billion tonnes) was either lost or discarded during production in the FSC. The FSC has been defined as "the total supply process from agricultural production, harvest or slaughter, through primary production and/or manufacturing to storage and distribution to retail sale or use in catering and by consumers".[3] It is designed to positively influence quality, safety, sustainability, logistics and efficiency of food production and processing from the farm to the fork.[16] Food losses and waste can take place at any stage of the FSC, e.g., agricultural production, post-harvest, processing, distribution and consumption (Figure 2). The term food loss most commonly refers to food products that are intended for human consumption but have instead been lost in production, storage, transport and processing, mostly due to microbial contamination and/or spoilage.[17-19] Food waste occurs at the end of the FSC within the retail and final consumption stages and it refers to edible food products that have been discarded, degraded and not consumed by humans.[20]

For most countries, food losses and waste of fruit and vegetables are higher than for other products, such as dairy and meat.[21,22] Liu et al[23] reported that fruit and vegetables suffered losses of up to 20-30% compared to meat and aquatic products (>15% loss) in the FSC in China. According to FAO,[24] the losses related to fruits and vegetables in the FSC in Latin America were over 70% while the dairy and meat product losses were only 22% and 25%, respectively (Figure 3). Insufficient refrigeration facilities in the FSC, particularly during transportation are noted as a major factor in these losses. In developed countries better FSC management has led to a reduction of food losses compared to less developed nations. It is widely accepted that an improvement in food safety reduces potential microbial risks and opera-

Figure 2: Major Stages in the Food Supply Chain where Microbial Contamination is Likely to Occur.

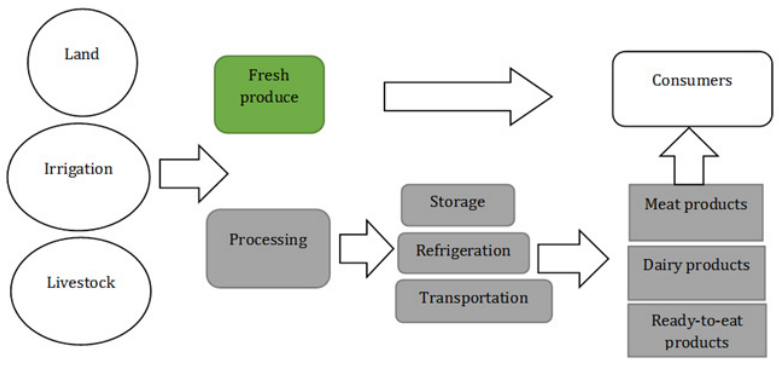

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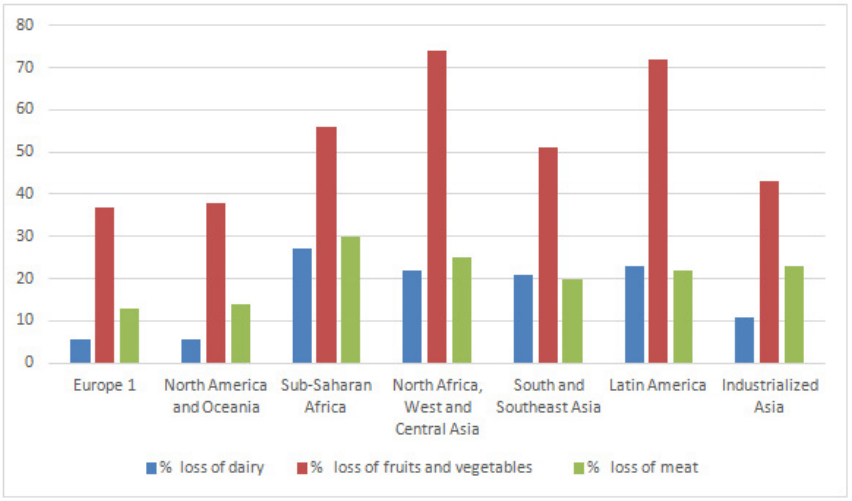
Figure 3: Total Supply Chain Loss of Dairy, Meat, and Fruit and Vegetable Products in Different Regions of the World.[24]



tional costs, both of which are vital in reducing food losses and foodborne outbreaks and thereby enhance food security.

Food may become contaminated with a range of microorganisms during harvesting, processing and handling operations as a result of the behaviour of farmers, retailers and consumers.[17,19,25] It is not well-documented how much food loss or wastage is caused by microbial contamination each year. However, the cost of foodborne illnesses caused by microbial pathogens can be used as an indicator to evaluate the extent of the contamination problem. For example, organisations such as the Centers for Disease Control and Prevention (CDC) in the USA provide details of the 48 million Americans annually who suffer from foodborne illnesses associated with identified microbial contaminants.[26] Buzby and Roberts[27] estimated that 70% of diarrhoeal diseases are foodborne. Thus it is clear that microbial pathogens are associated with a large number of foodborne outbreaks, which results in food losses. Hence, it is important to understand the common microbiological hazards in foods.

COMMON MICROBIOLOGICAL HAZARDS IN FOODS

Food security, as we have seen above, not only means paying attention to the reasons behind shortages in food supply, but also addressing issues like food contamination and foodborne outbreaks that indirectly contribute to food losses. A foodborne outbreak is generally defined as an incident when two or more people become sick as a result of consuming a common food or meal.[28] The symptoms and severity of food poisoning vary, depending on the nature of the hazard (i.e., biological, chemical or physical agents) and its ability to cause adverse health effects. Pathogenic bacteria are the most common cause of foodborne outbreaks and food scares around the world. More than 50% of foodborne outbreaks in the USA have been linked to bacterial infections.[29,30] In addition, microbial food contamination in the FSC that causes food losses and foodborne illnesses can result in heavy economic losses. Many studies have used cost-of-illness (COI) to estimate the economic burden of an illness on a society.[26,27,31] Such studies were also useful for making sound policy decisions about food safety interventions.[32,33]

Lake et al[31] used COI to estimate the burden of disease for certain potentially foodborne diseases (e.g., campylobacteriosis, salmonellosis, listeriosis) and their sequelae in New Zealand. The cost of foodborne infections in New Zealand is considerable and has been estimated at $86 million per year, with approximately 90% due to campylobacteriosis. In Sweden, the estimated cost of foodborne illnesses is about $171 million per year.[34] The Economic Research Service of the United States Department of Agriculture (USDA) reported that, in the USA, five foodborne pathogens (*Campylobacter, Salmonella*, *L. monocytogenes*, *E. coli* O157:H7 and *E.coli* non-O157:H7 STEC) cost $6.9 billion each year.[35] Examples of a number of bacterial foodborne outbreaks are given in Table 1. International organisations such as the FAO and the World Health Organization (WHO) have accepted the challenge to work together in order to initiate risk assessment studies of a number of pathogens in food commodities to improve consumer health and indirectly the economy.[36] Some of the most prevalent foodborne pathogens are discussed below.

Salmonella spp., one of the leading causes of foodborne outbreaks and foodborne illnesses, are a serious threat to public health worldwide. In the USA, the annual estimated economic loss was $2.4 billion in 2014 due to foodborne *Salmonella* infections.[37] *Salmonella* spp. are commonly associated with foods of animal origin (e.g., red meat, chicken and pork). The most common symptoms of salmonellosis are abdominal cramps, diarrhoea and fever.[38] Contamination by *Salmonella* can occur during production or due to inappropriate food handling during manufacturing. Data from foodborne outbreaks in the USA indicate that *Salmonella* infections were responsible for 18% of foodborne diseases in 2006,[39] but increased to 35% in 2011[40] and 38% in 2013.[41] In New Zealand, *Salmonella* spp. were respon-

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Table 1: Selected Examples of Foodborne Outbreaks and Recalls Caused by Pathogenic Bacteria

Foodborne outbreak	Pathogenic bacteria	Region/Country	Initial announcement	Casualties	Estimated economic losses	Reference
Bean sprouts	*Salmonella*	USA	November 2014	115 people infected, 25 % of ill persons were hospitalized	Contaminated bean sprouts and any remaining products were destroyed	CDC[a] 2014[51]
Nut mix	*Salmonella*	New Zealand	December 2014	No illnesses were reported	Contaminated nut mixes batches were recalled	MPI[b] 2014[95]
Fresh cream	*E. coli*	New Zealand	January 2014	No illnesses were reported	8,700 bottles of fresh cream distributed to retail and foodservice outlets were recalled	MPI 2014[95]
Cheese	*L. monocytogenes*	Australia and New Zealand	March 2014	No illnesses were reported	Cheese was recalled from Australian and New Zealand supermarkets	MPI 2014[95]
Chicken	*Salmonella*	Puerto Rico and USA	March 2013	No illnesses were reported	More than 23,000 units (approximately 102,000 pounds) of contaminated chicken were recalled	CDC 2014[51]
Cheeses	*L. monocytogenes*	USA	September 2013	Six ill persons hospitalized. One death in Minnesota	All cheese products made on a specified date or earlier were recalled and destroyed	CDC 2014[51]
Sprouts	*E. coli* O104:H4	Europe and North America	May 2011	More than 4,000 persons were infected	Ban by the EU on the importation of fenugreek seeds and various other seeds, beans, and sprouts from Egypt that were the source of the sprouts responsible for the outbreaks in Germany and France	WHO[c] 2011[96]
Spinach	*E. coli* O157:H7	USA	2006	Not specified	Spinach was recalled and banned to sell	CDC 2007[97]

[a] Centers for Disease Control and Prevention; [b] Ministry for Primary Industries; [c] World Health Organization

sible for 6.6% of foodborne outbreaks in 2011 (Institute of Environmental Science and Research (ESR))[42] and increased to 10 % in 2012.[43] Despite the improvements to food safety standards in the FSC, *Salmonella* infections have continued to increase and cause considerable losses to global food safety through productivity/production losses and recalls.

Campylobacter spp. cause serious bacterial food poisoning. In the USA, *Campylobacter* is responsible for more than 600,000 illnesses that cost over $1.3 billion each year.[44] Approximately, 50% of these illnesses were attributed to poultry products such as chicken burgers and nuggets.[45] In the Netherlands, the *Campylobacter* Risk Management and Assessment (CARMA) estimated the cost of campylobacteriosis at 21 million euros annually, with 20-40% of cases attributed to contaminated poultry.[46] These bacteria infect about 1% of the population of Western Europe, and most of the infections are caused by inappropriate handling of contaminated food.[17,47] In New Zealand, campylobacteriosis has been a notifiable disease since 1980.[48] ESR reported that *Campylobacter* spp. were the most commonly identified agents in poultry and dairy outbreaks in 2013 and caused 13.3 % of the total reported foodborne disease outbreaks.[49] The economic loss due to a *Campylobacter* outbreak in August 2012 was estimated at $1.184 million.[50]

Some *E. coli* strains are pathogenic and flourish in the gut of many host species. *E. coli* O157:H7 was identified in 1982 and is now recognized as a dangerous foodborne pathogen. This, and the foodborne Shiga toxin-producing *E. coli* (STEC), have been implicated in many outbreaks around the world and the illnesses cost about $280 million annually in the USA.[35] The annual foodborne outbreaks reported by CDC[51] attributed 29 confirmed outbreaks to STEC in the USA in 2013, mostly caused by fresh produce (raw fruits and vegetables). These strains were also responsible for an outbreak in the UK in 2007 that caused 157 hospitalized cases and one death.[52] In Germany, in 2011, approximately 941 people were infected with *E. coli* O104:H4 from food.[53] In New Zealand, STEC caused two outbreaks, and 11 illnesses were reported by ESR.[54] In the case of *E. coli* O157:H7, most of the infections were from beef and minced meat.[29]

L. monocytogenes is a pathogenic bacterium that has caused a number of food outbreaks over the last decade with dairy products being the main vehicle associated with foodborne illnesses, with the capacity to affect infants and the elderly. Unlike other bacteria, infection by this bacterium has a high fatality rate of 20-30%,[55] and the annual estimated economic loss is $2.6 billion in the USA.[35] *L. monocytogenes* has been the most infamous foodborne pathogen in Australia because of its high numbers of fatal cases and substantial economic losses of $1.2 billion per year.[56] One of the most serious listeriosis outbreaks was reported in Europe in 2009-2010. The reported data showed a total of 26 people were infected, with eight fatalities in three different regions.[57] In Australia, five outbreaks caused by *L. monocytogenes* infections between the years 2001 and 2010 led to 57 cases and 14 deaths.[58] The severity of listeriosis and the difficulty of avoiding *L. monocytogenes* in the environment have highlighted the importance of improving the food safety system against *Listeria*. However, listeriosis outbreaks have recently been connected to the ability of a food to harbour viable *L. monocytogenes* throughout the manufacturing process[59] and

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have often been associated with inappropriate storage processes. As outlined above, the number of outbreaks caused by each pathogenic bacterium, number of reported illnesses and the associated food vehicles are well-documented in many Western countries, including New Zealand. This information has been used to estimate economic losses and highlight food safety issues in the FSC[9,60] but losses are probably underestimated because many foodborne illnesses are not reported unless they are severe. It is suggested that the pathogenic bacteria associated with foodborne outbreaks are a major cause of foodborne illnesses and will have a considerable influence on future efforts to enhance food security.

FOOD SAFETY AND FOOD SECURITY

Compromised food safety can disrupt the supply of food at any time and create the condition of food insecurity. A supply chain strategy emphasizes the management of all food safety issues that can arise due to improper transferring, handling and distribution of the product.[61,62] In fact, when managing food safety, it is essential to implement proactive strategies to minimize the probability of delivering an unsafe product. Ensuring this will reduce food scares and food losses.

Many studies have investigated different stages in the FSC where strategies for food safety have failed. A recent study investigated the occurrence of *L. monocytogenes* in 12 meat and dairy products from small-scale direct marketers in Europe.[63] The study categorized these food business operators into uncontaminated and contaminated sectors according to existing data on the occurrence of *L. monocytogenes* in each food business, which showed that *L. monocytogenes* was a common colonizer of processing environments in European food processing factories. The study revealed how environmental factors can cause cross-contamination during food production if poor hygiene practices are in place, effects that can lead to significant food losses. The influence of environmental factors on foodborne outbreaks and food security is therefore a widely debated and investigated issue.

Foodborne illnesses associated with the consumption of specific food products, such as fresh produce, cause serious issues for public health.[6,64] According to international organizations (FAO/WHO), agro-food products present the greatest concern in terms of microbiological hazards that influence public health.[65,66] In rapidly developing China, agro-food products account for more than 70% of the total food consumption.[67] This has prompted the Chinese government to establish efficient food control systems to reduce foodborne illnesses and outbreaks caused by agro-food products. The establishment of food control systems in China was delayed; however, as the various difficulties and problems in the FSC were investigated.[74] The many studies on the subject agreed; however, that lack of agro-food legislation and food safety structures were major obstacles to food security, and that in the absence of an effective food safety system, numbers of foodborne illnesses and foodborne outbreaks increase, thereby leading to food insecurity.

The production and consumption of foods, especially fresh produce and agro-food products, involves growing, transferring and handling food under conditions that vary considerably.[68] At any stage in the FSC if food safety is compromised, it can lead to food spoilage and microbial food contamination.[65,69] A research report from Canada investigated the relationship between the incidence of *Salmonella*, pathogenic *E. coli* and *Campylobacter* infections between 1992 and 2000 in two Canadian provinces, using weekly reports of confirmed cases of these three pathogens.[70] The results showed a strong association between ambient temperature and the occurrence of all three enteric pathogens. Pathogenic bacteria are present in the food processing environment because of their saprophytic lifestyles.[71] An inadequate hygiene system, poor hygiene practices and unhygienic design of equipment may cause pathogenic contamination of the food manufacturing plants.[72] This contamination can be the initial step in the transmission of pathogenic bacteria from their original source in the food crop to food processing elements[60] and, ultimately to the consumer, which can result in outbreaks of foodborne illnesses.

In order to protect consumers from microbiological and also chemical hazards, many countries have evaluated their current food control systems.[73,74] For example, two national studies were conducted, in Kuwait and the Sultanate of Oman, on the effectiveness of their current food management systems and elements of these, including the food control systems used by food producers, implementation of food legislation, food inspection protocols and the use of accredited food testing laboratories.[9,75] Both reports highlighted deficiencies in the development of standards relating to food safety and quality, and the weak food and food products inspection system at different stages during production and processing. Without addressing these problems, food losses and food insecurity are unavoidable. The impact of unclear information and knowledge among stakeholders and food handlers about the importance of food safety control can result in an ineffective food safety system.

Uncertainty in the food service sectors in the FSC and limited knowledge of food safety strategies can negatively affect food safety control during production and handling.[24,76] An inadequate system for training food handlers in food safety practices contributes to increasing incidences of microbial contamination of food.[77] With the recent increase in global food production, some international food manufacturers are relying on third parties to produce and export thousands of tonnes of food ingredients. Inadequate food safety training for these third parties handling food in the early stages of food manufacturing can lead to an increase in the number of foodborne outbreaks. Consequently, this will cause a significant loss of food products, damage the reputation of the international food manufacturing companies as food producers and, ultimately, influence food insecurity. Thus, provision of information and education about food safety and quality issues for food handlers across the FSC is important and a significant first step towards reducing food safety problems and improving food security.

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It is now clearly evident that a national food safety management system is important for any country and, if compromised, can significantly increase food losses and foodborne incidents with consequent food insecurity.

IMPROVED MICROBIAL FOOD SAFETY IN THE FSC IS VITAL TO ENHANCE FOOD SECURITY

A food safety management system, therefore, is vital for ensuring the safety and quality of foods prepared for consumers. An improvement in food safety control systems can significantly reduce microbial contamination of foods throughout the FSC.[16] Therefore, it is necessary to understand how best to manage the FSC to improve microbiological food safety. Currently, different food industries apply different food safety controls in their food safety management systems, and the functioning of such systems is also variable. To ensure sustainable control of product quality it is important to have a well-managed operation at each step within the FSC. With the world facing a challenge to reduce the large amount of food produced globally that is currently lost (Figure 3), managing the different aspects of the FSC is essential.

Implicated as sources of infection that may cause microbial contamination, food outbreaks and economic losses are the primary materials for food manufacturing, such as milk, meat, fruits and vegetables (Table 1). Farmers are responsible for supplying consumers and manufacturers with the raw products for use in food manufacturing and processing. Fresh produce and raw products receive special attention because they are more likely to contain pathogenic bacteria, such as *Salmonell*a spp. and *E. coli* O157:H7.[78-80] This concern has demanded the use of Good Agricultural Practices (GAP) in both crop and animal production on farms, in order to reduce the risk of microbial contamination.[81,82] Practising GAP includes all activities before and during production and at harvest. GAP also requires product inspection reports from suppliers, and these are essential records to ensure that the products are not contaminated by pathogens or toxins. Thus, applying GAP and more hygienic procedures at the farming stage is important to ensure maximum safety of the products.

However, food safety control systems are also important in other stages of the FSC to ensure the production of safe food. An ever-increasing number of food outbreaks around the world each year are linked to processed foods and cross-contamination.[83-85] Microbial contamination during food processing can result from poor application of Hazard Analysis and Critical Control Point (HACCP) principles.[86,87] In many countries, including Australia, processed and ready-to-eat meats are, potentially, a vehicle for foodborne illnesses associated with *Clostridium perfringens, Salmonella, E. coli* (EHEC) and *L. monocytogenes*.[88,89] An increase during manufacturing in the incidence of foodborne outbreaks by these bacteria[74] has resulted in the identification of specific risk profiles for these bacteria in different foods to provide the industry with risk ratings for hazardous meat and meat product combinations.[90] Such development of risk management systems and the implementation of HACCP-based food safety strategies across the FSC for different food products are essential for all countries to reduce food poisoning outbreaks and improve global food security.

Applying HACCP or a similar system of hazard control in food manufacturing business is important to control hazards and, thereby, improve the safety of food.[91,92] Table 2 lists common microbial contamination issues and the possible solutions using a food safety management system. Nevertheless, many recent studies have suggested that a combination of two or more

Table 2: Most Common Microbial Contamination Issues and the Possible Solutions Using a Food Safety Management System.

Microbial contamination issue	Frequency/impact	How to improve	References
Contamination of raw materials	• Fresh vegetable products implicated as sources of infection • Inappropriate suppliers' processes or wrong storage may cause microbial contamination	• Apply good agricultural systems and more hygienic performance in both crops and on farms • Establish effective cleaning/sanitizing programmes • Request a product inspection report from the suppliers that contains a self-evaluation document to ensure the materials were not contaminated by pathogens or toxins	Lehto et al[82]; Gustavsson et al[22]; Wu et al[98]
Risk in food packaging	• Air combined with packaging in the food supply chain is one of the major potential sources of pathogenic microorganisms	• Use appropriate air filters combined with production processes at all times that can control high-risk microbial aerosol generation • Use good hygienic practices in food processing and supply a product inspection report form to ensure the products are not contaminated by pathogens or toxins	Harris et al[99]
Risk in food refrigeration	• Inappropriate refrigeration systems can cause microbial contamination that can result in a big loss of food products	• Refrigerate food products as that should produce a safe product by reducing the temperature of the meat and vegetables to a point where the rate of growth of spoilage microorganisms is slowed down and the growth of most pathogenic microorganisms is prevented • Supply a refrigeration inspection report	James et al[100]; Sampers et al[101]; Carpentier et al[102]
Risk in transportation and food service operations	• Unsuitable and untimely transportation services can cause microbial contamination • Product handling at the end destination and/or at wrong temperatures can cause microbial contamination and food loss	• Employ time and temperature control in all stages of transportation • Use good personal hygiene • Establish effective cleaning/sanitizing programmes • Supply product inspection report forms at the products destination for all products from the initial process to the ultimate end-products	Gitahi[103]; Hassouneh et al[104]; Linton and McSwane[105]

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safety control programmes, such as the International Organization for Standardization (ISO) quality management system, good manufacturing practices (GMP) and HACCP, markedly improves microbial food safety management[93,94] and, thereby reduce food contamination. Implementing one or more of these systems during food production is therefore widely recommended for food manufacturing businesses to achieve more effective food safety management to improve food security.

CONCLUSION

The threat of food insecurity is real. Many approaches have been suggested to tackle this challenge. One-third of total food produced globally never makes its way to consumers' tables due to several factors, microbial contamination being the important one. A wide variety of pathogens (e.g., *Salmonella,* pathogenic *E. coli, L. monocytogenes and Campylobacter*) are associated with foodborne illnesses and outbreaks and the consequent food and economic losses. Reducing microbial contamination and other food safety issues will significantly improve the food supply. Efficient food process controls and effective food safety systems (e.g., GAP, HACCP, ISO, GMP) are helpful in controlling microbial contaminations. Such improvements in microbial food safety practices will definitely bring additional food to the table and thereby improve food security.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Low-Protein Rice (LPR) Product: Processing Method and Product Safety

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ABSTRACT

Background: Dietary therapy through a low-protein diet (LPD) has long been used for preserving the renal function of patients with chronic kidney disease (CKD). Reducing the amount of protein ingested from rice would make it possible to allocate the difference to side dishes, thereby improving the quality of meals and facilitating adherence to LPD.
Methods: Forica Foods Co., Ltd., Uonuma City, Niigata, Japan developed exclusive technology in 1994 for obtaining low-protein rice (LPR), using proteolytic enzymes to digest and remove the protein in white rice. In 2004, we developed processing technology to enhance the palatability of said LPR. By combining these technologies, we established a manufacturing method for producing delicious, high quality processed rice products which can serve as a daily staple.
Results: For more than two decades since being launched in 1995, nearly 100 million Protein Low Content (PLC) rice products have been sold, and to date, there have been zero complaints of health problems attributable to them. This demonstrates their high safety even when eaten repeatedly over a long period.
Discussion: The PLC Rice series of products, which compare favorably with, and can be substituted for, regular rice, are delicious (high quality) enough to be eaten everyday. They enhance the quality of mealtimes for CKD patients by increasing their range of food choices.
Conclusion: PLC Rice product offers high added value, as they not only facilitate adhesion to LPD but also add satisfaction and contentment to daily meals, helping to enhance the quality of life (QoL) of patients with CKD.

KEY WORDS: Chronic kidney disease (CKD); Low-protein diet (LPD); Low-protein rice (LPR); Dietary therapy.

ABBREVIATIONS: CKD; Chronic Kidney Disease, WR: White Rice; LPD; Low-Protein Diet, LPR; Low-Protein Rice; PLC: Rice Series of Products; GDL: Glucono Delta-Lactone; EM: Enzyme Mix.

BACKGROUND

Dietary therapy through a low-protein diet (LPD) has long been used to preserve the renal function of patients with chronic kidney disease (CKD).[1-3] LPD restricts the amount of protein in the total dietary intake and it lowers the production of urea nitrogen, an end product of protein metabolism.[4] It is considered that the level of protein intake modulates the filtration load on the kidneys and tempers the decline of renal function.[5,6] Over the past 50 years, LPD have been successfully used to treat chronic renal failure in Japan.[7] Since the easiest course of action is to cut down on foods which are high in protein, the main dishes, such as meat and fish, are usually reduced to smaller servings. However, such frugality compromises the quality of meal times. Patients may have difficulty adhering to the LPD, and as a result, be unable to reap the benefits of LPD.

Removing protein from rice would minimize the need to cut down on main dishes.

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Mealtime quality would be maintained, making it easier for patients to follow the LPD.

To address this issue, in 1992 the Niigata Agricultural Research Institute Food Research Center developed a technology (JP2706888B) using lactobacillus fermentation to reduce the protein, phosphate, and potassium contained in white rice (WR).[8] However, their method posed technical challenges such as extremely long processing times and the difficulty of microorganism control hampering reliable production. In 1994, Forica Foods Co., Ltd., Uonuma City, Niigata, Japan built on this technology to develop an exclusive proteolysis technology (JP3156902B) utilizing an enzyme product which can break down protein in WR more quickly and reliably than lactobacilli fermentation.[9] The following year, the manufacture and sales of "Cooked PLC Rice 1/3 (PLC: Protein Low Content)" began.

PLC Rice is processed, protein reduced WR. The palatability (quality) of this low-protein rice (LPR) is such that it can be eaten day after day. The PLC Rice products were developed to facilitate adhesion to the LPD, thereby improving the quality of life (QoL) of patients with CKD. In this article, the manufacturing process for the PLC Rice series will be described.

METHODS

Raw Materials and Protein Digestion

Raw materials are WR, pH adjuster, and protease.

There is no particular restriction on the type of WR. For example, either *Japonica* rice (short grain) or *Indica* rice (long grain) can be used. Highly clean raw material is used to reduce the risk of microbiological contamination.

In the protein digesting step, the temperature and pH must be maintained for a certain amount of time at levels conducive to enzyme activity. To prevent in-process microbiological proliferation and consequent product contamination, it is desirable to keep the reaction solution pH acidic and at or above 50 °C. To control pH, a suitable quantity of organic acid is added. Suitable organic acids include citric acid, lactic acid, fumaric acid, gluconic acid, and glucono delta-lactone (GDL). Out of these, citric acid is chosen because of its high buffering property, ability to maintain pH within the enzyme solution stably over a long period, and for having the smallest negative effect on the taste and aroma of cooked rice.

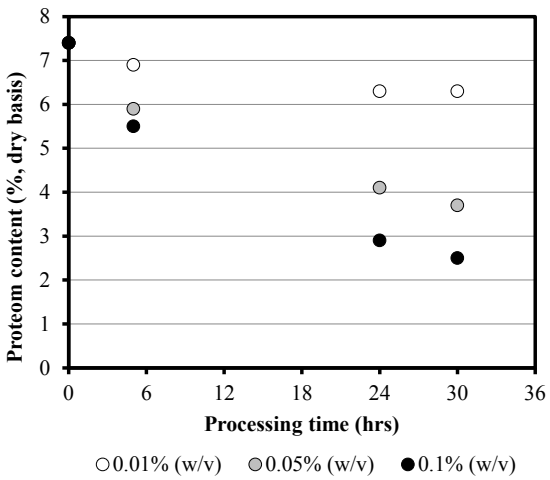
As proteolytic enzyme, acidic protease approved by the Ministry of Health, Labour and Welfare (MHLW) as a food additive and available on the market is used. In our method, we use an enzyme mix (EM) consisting of an exclusive formulation of at least 3 kinds of enzyme products, comprising at least 1 each derived from *Aspergillus oryzae, Rhizopus niveus,* and *Aspergillus niger* respectively. Each enzyme product is a crude enzyme product extracted from the source microorganisms, and the main component is protease in the aspartic protease family (EC 3.4.23).

Protein Digesting Process

The protein digesting process is broadly divided into enzyme digestion and washing. In the first step, surface bran residue and germs were washed away with water. The washed WR is placed in a reaction solution containing dissolved EM and citric acid, and allowed to soak for a certain period of time (up to 24 hours) in a temperature and pH conducive to enzyme activity. This breaks down the protein in the WR. Subsequently, thorough washing removes any unnecessary matter: rice protein decomposition product, EM, and excess citric acid. These steps yield LPR (Raw LPR), which is raw, protein reduced WR.

Figure 1 shows the time lapsed changes of protein mass in rice treated with enzyme with a single protease derived from

Figure 1. The Effect of Processing Time and Enzyme Concentration on Protein Removal.



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Aspergillus oryzae. Protein mass in rice is highly dependent on enzyme concentration and reaction time. By adjusting these factors, the rice protein mass can easily be reduced to target levels. In the actual process of protein enzyme treatment, EM is used instead of a single enzyme, resulting in better removal rates and production efficiency compared to a single enzyme. This is likely to be because the combination of multiple enzymes each with different protein cleavage sites reduces the molecular weight of the decomposition product. The smaller the molecular weight of rice protein, the easier it is to elute from the rice grain.

Manufacturing Method

The manufacturing flow of these products is outlined in Figure 2. Regardless of product form, low protein rice products need to have their rice protein content broken down and removed. The method employed by Forica Foods is based on the invention by Nakajo et al (JP3156902B), and consists of utilizing protease to process protein.[9] The very simple production process is shared across product forms.

Cooking Process

The Raw LPR obtained by said protein digestion process requires special techniques and know-how to produce rice grains which maintain their shape while offering palatability. This is because Raw LPR grains are extremely fragile, and when subjected to steaming or cooking like ordinary WR, they dissolve into a gruel-like mass which is far from delicious. In order to solve this problem, we developed a method (WO 2017037799 A1) for processing LPR into a palatable, high quality product.[10]

Characteristics of the Manufacturing Method for "Cooked PLC Rice"

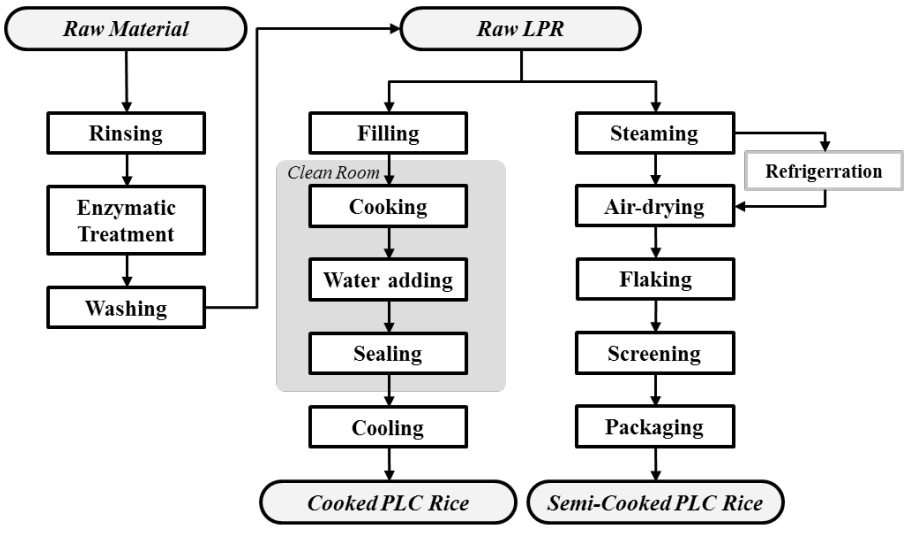
The manufacturing method for "Cooked PLC Rice" can be broadly categorized into cooking, hot water injection, sealing, and cooling. In the first step, each serving of the Raw LPR is filled into heat resistant containers. Without adding water, the containers are transported to our exclusive rice steamers installed in clean rooms. The rice steamer chamber is sealed and the interior de-aired to create a vacuum. Immediately, steam is injected powerfully to instantly raise the temperature within the chamber to 130 °C to 140 °C. This state maintained for 30 to 120 seconds during which the rice is steamed rapidly. Immediately after steaming the rice, sterile hot water of at least 90 °C is added. The steamed LPR is promptly sealed by heat resistant film with oxygen absorber affixed to it. The temperature is maintained for an additional 5 to 10 minutes to ensure thorough steaming of the content. Subsequently, the packaged rice is allowed to air cool gradually for 20 to 30 minutes until its temperature reaches 30 to 35 °C, then subjected to X-ray inspection to check for foreign matter and weighed. Articles which pass the inspections become product.

Using the steaming method described above, in which rice is steamed by elevating the temperature in the chamber instantaneously without adding water, the starch can be prevented from eluting from the Raw LPR, thereby avoiding loss of the flavor of white rice and making it possible to obtain low protein rice with appropriate viscosity and elasticity that is suited to Japanese preferences. In addition, the hot water injection process makes it possible to change the amount of added water at will. Thus, the moisture content of the product can be adjusted easily to obtain the desired hardness/softness of the cooked rice. As has been described, this technique is an extremely unique method of cooking rice which offers both practicality and flexibility.

Characteristics of the Manufacturing Method for "Semi-Cooked PLC Rice"

The manufacturing method for "Semi-Cooked PLC Rice" can be

Figure 2. Production Flow Chart of PLC Rice.



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broadly categorized into steaming, drying, and flaking to separate into individual grains. The most basic manufacturing method consists of spreading out Raw LPR into a uniform thickness, steaming with steam, then loosely separating the grains, drying uniformly overall, then flaking to separate the rice grains into individual grains. By gelatinizing and then drying the raw starch contained in Raw LPR, it is possible to obtain "Semi-Cooked LPR" whose rice grains are strong enough for long-term storage and which can be cooked similarly to WR.

However, simply steaming the rice with saturated steam results in highly viscous starch eluting from the surface of the LPR grains. This causes the LPR grains to stick strongly to each other, making it extremely difficult to separate the grains into uniform size. Variations in the size of LPR clusters can cause uneven drying. Trying to separate unevenly dried LPR clusters generates large quantities of cracked and crushed rice, resulting in significant loss of productivity. Furthermore, when LPR thus obtained is cooked, it is stickier than necessary, compromising the quality of the product.

To solve the issues of productivity and reliable product quality, we invented the 2 processing methods shown below.

Refrigeration to retrograde the starch: Raw LPR is spread out evenly into a thickness of 30 to 50 mm, then steamed for 5 to 10 minutes with saturated steam. This is transferred to a refrigerator and kept at 0 to 5 °C for 24 to 72 hours. This causes the starch, gelatinized by steaming, to retrograde, resulting in weakened adhesion between rice grains, thereby facilitating the flaking process. After the grains are separated, the rice is uniformly dried to bring the moisture content of the grains to 15 to 26%. The separated LPR grains are screened for grain shape and color, and the grains with uniform shape and color are weighed and sealed in packaging material together with oxygen absorber to obtain product.

Superheated steam: Raw LPR is spread out evenly into a thickness of 30 to 50 mm, then steamed for 4 to 10 minutes using superheated steam of at least 105 °C. The superheated steam, obtained by heating saturated steam, makes it possible to heat efficiently using higher temperatures than saturated steam. Furthermore, superheated steam is drier than saturated steam, thereby keeping down excess moisture in the rice during steaming. Therefore, the steamed rice is less viscous and more easily separated.

The subsequent steps are similar to method described above. Immediately after steaming, the Steamed LPR is separated loosely and dried by warm air so that the moisture level is uniform throughout, at 16% to 20%. The rice is then separated into individual grains using a cracking device, etc. The separated LPR grains are screened for grain shape and color, and the grains with uniform shape and color are weighed and sealed in packaging material together with oxygen absorber to obtain product.

Compared to method using retrogradation, in which the process is interrupted for the refrigeration step, this method consists of fewer steps and is amenable to continuous production conveyor-style. Therefore, it is possible to manufacture "Semi-Cooked PLC Rice" efficiently.

RESULTS

Nutrition

Nutrition information on PLC Rice products is shown in Table 1. Test samples were taken from at least 3 separate production days. As shown in the Table 2, there is minimal interlot variability of nutritional values, indicating extremely high reproducibility. Particularly noteworthy is the protein content, which demonstrates reliability in meeting the respective target values.

Product Overview

PLC Rice is LPR which is processed, protein reduced WR (Fig-

Table 1: Nutrient Content in 100 Grams of "Cooked PLC Rice" Products.

Content	Unit	WR	Mean (±SD)				
			"1/3"	"1/5"	"1/10"	"1/20"	"1/25"
Calories	kcal	168	160 (3.1)	162 (6.8)	162 (2.6)	160 (7.1)	160 (1.7)
Moisture	%	60.0	60.4 (0.7)	60.1 (1.7)	60.1 (0.8)	60.5 (1.6)	60.6 (0.5)
Total Protein	%	2.5	0.87 (0.1)	0.44 (0.0)	0.24 (0.1)	0.13 (0.0)	0.10 (0.0)
Total Fat	%	0.3	0.5 (0.1)	0.5 (0.1)	0.6 (0.1)	0.3 (0.1)	0.5 (0.2)
Total Carbohydrate	%	37.1	38.1 (0.7)	38.9 (1.7)	39.0 (0.7)	39.1 (1.4)	38.8 (0.7)
Ash	%	0.1	0.1 (0.0)	0.1 (0.1)	0.1 (0.1)	0 (0)	0 (0.1)
Sodium	mg	1	2 (0.3)	2 (0.0)	2 (0.2)	2 (0.1)	2 (0.1)
Potassium	mg	29	0.6 (0.3)	0.5 (0.2)	0.9 (0.2)	0.6 (0.1)	0.7 (0.2)
Calcium	mg	3	5 (0.0)	5 (0.3)	5 (1.2)	5 (0.7)	5 (0.6)
Phosphorus	mg	34	15 (1.0)	15 (1.5)	14 (1.0)	13 (0.7)	13 (1.2)

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Table 2: The Effect of Processing Time and Enzyme Concentration on Protein Removal.

Protein content (%, dry basis)			
Processing time (hrs)	Enzyme concentration (%, w/v)		
	0.01	0.05	0.1
0	7.4	7.4	7.4
5	6.9	5.9	5.5
24	6.3	4.1	2.9

Figure 3. The List of PLC Rice Products.

Products				
Protein content (in 100 g of product)	0.83 g	0.5 g	0.25 g	0.125 g
Net weight	160 g	180 g	180 g	180 g
Products			 (Uonuma Koshihikari)	 (Semi-Cooked)
Protein content (in 100 g of product)	0.1 g	0.125 g	0.1 g	0.24 g
Net weight	180 g	150 g	180 g	1 kg

ure 3). Some of the products in the series have been approved as "Food for special uses" by the Ministry of Health, Labour and Welfare, and for bearing claims on the label that they are suitable foods for kidney disease patients. In 1995, when we launched the first product, it contained 1/3 of the protein in WR, that is, 2/3 of the WR protein had been removed. With continued technological development, we achieved a reduction rate of 1/25 by 2007. As of 2017, 22 years from its development in 1994, the PLC Rice series consists of 10 products to accommodate consumer needs in terms of product form, reduction rate (1/3 to 1/25), and serving size (140 to 180 grams).

PLC Rice comes in 2 product forms: "Cooked PLC Rice" which can be microwaved or boiled in the pouch, and "Semi-Cooked PLC Rice" which requires cooking in a rice cooker. In "Cooked PLC Rice," LPR is aseptically packaged. In "Semi-Cooked PLC Rice," LPR is steamed, dried, flaked, and sieved, and requires a finishing step of cooking such as boiling, steaming, or microwaving before serving. Both types of products can be stored and distributed at room temperature. The best-before date of "Cooked PLC Rice" is 7 months and "Semi-Cooked PLC Rice" is 12 months.

Residual Enzyme in Product

Figure 4 shows the SDS-PAGE profile (CBB stain) of Raw LPR extract. Compared to extract from non-treated WR (L1), Raw LPR extract (L2) no longer shows any protein-derived bands except for some in the low molecular weight side. There were no bands common to Raw LPR (L2) and EM (L3), indicating that enzyme derived protein is absent (or under the CPB stain detection limit). These results show that rice protein decomposition product and EM are washed away from Raw LPR, leaving no residue in the product.

Microbiological Safety of Product

To store the packaged "Cooked PCR Rice" safely in room temperature, the contents must be sufficiently sterilized and sealed in the sterile state. Raw LPR is steamed with high temperature, high pressure steam in a steaming device installed in a clean room. At the same time, it undergoes thorough sterilization un-

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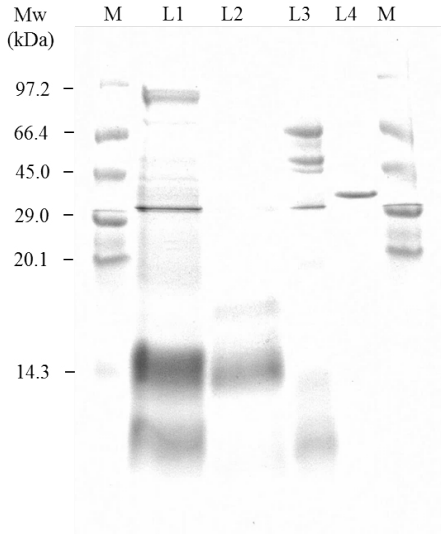
Figure 4: CBB-Stained SDS-PAGE Patterns of the Raw LPR Extract.



M; Marker (AE-1440, ATTO, USA), L1; 5-times concentrated extract from non-treated rice, L2; 5-times concentrated extract from Raw LPR, L3; EM, L4; Thermo-treated EM (50 °C, 24 hrs).

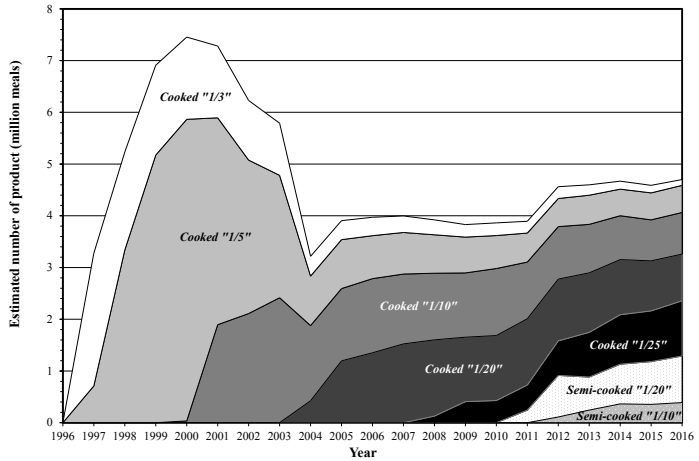
der conditions which are equivalent to the 120 °C for 4 minutes (F_0=3.1) stipulated in the Japanese Food Sanitation Act for retort pouch food. Subsequently, the contents are kept sterile while sealing together with oxygen absorber, thereby securing sterility of the product. In fact, since its adoption in 2004, there have been no incidents of product spoilage or deterioration attributable to insufficient sterilization, proving the efficacy and safety of this technique.

According to our findings relating to "Pre-Cooked PLC Rice," drying steamed LPR to a moisture content of 15 to 26% yields desirable quality (particularly mouthfeel) when it is cooked. By sealing this together with oxygen absorber and maintaining an anaerobic state inside the package, it becomes possible to store it safely at room temperature. However, once the package is opened, new oxygen flows into the package. The anaerobic state cannot be maintained and there is risk of mold and other microbiological growth. A product with moisture level higher than 20% requires refrigeration or freezing. However, a product dried to a moisture level of 16% to 20% can be stored safely at room temperature. This is due to the reduced water activity of the product, which prevents mold etc. from proliferating after the package is opened.

Product Safety and Quantities Manufactured

Quantities of PLC Rice produced from launch to the present day are shown in Figure 5. In more than 20 years since its launch,

Figure 5: The Production Amount of PLC Rice.



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nearly 100 million PLC Rice products have been provided to CKD patients through many hospitals throughout Japan. Although sales have declined since the peak of 7.5 million servings in 2000, the quantities have been growing gradually since 2004 when the above-mentioned high temperature steaming method was adopted. As of 2016, we are producing the equivalent of 4.7 million servings annually.

In the 22 years of producing and selling "Cooked PLC Rice" and the 7 years of "Semi-Cooked PLC Rice," there have been zero reports of health problems attributable to these products. Given the high frequency of LPD Rice ingestion by CKD patients who eat it every day as part of their LPD, and the fact that PLC Rice products are composed almost entirely of natural ingredients derived from WR, it is reasonable to say that PLC Rice, regardless of product form, is safe to eat and has no negative health effects, even for CKD patients who ingest them repeatedly over the long-term.

DISCUSSION

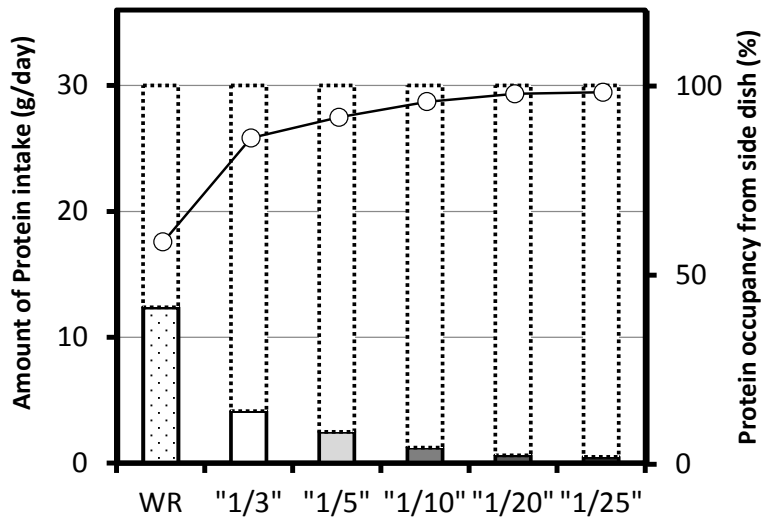
From clinical findings, the recommended protein intake for Japanese CKD patients is under 0.5 g/kg body weight (BW)/day.[11] The cooked rice that is the staple for Japanese people contains 2.5% protein. If a person eats a bowl (approximately 165 g) of cooked rice 3 times a day, he or she will ingest 12.5 g of protein from the rice. A patient weighing 60 kg should ideally ingest less than 30 g of protein per day. If the patient eats regular rice, it would account for 40% of the protein allowance. To keep with the allowance, the entire meal plan needs to be adjusted.

Figure 6 shows the how different proportions of a hypothetical patient's daily protein allowance of 30 g is taken up by eating the same 165 g (1 serving) of WR or PLC as the staple food 3 times a day. Unprocessed WR translates to roughly 40% of the daily protein allowance, thereby leaving only about 60% to be allocated to side dishes (Figure 2). By contrast, LPR, even at the reduction rate of 1/3, only accounts for 8.4% of the daily allowance, leaving more than 90% to be occupied by other dishes. The smaller the reduction rate, the less protein from the staple, thereby permitting the rest of the allowance to be filled by other foods. This greatly expands the patient's food choices.

Another dietary consideration for CKD patients is the desirability of reducing phosphorus and potassium intake in addition to protein. At the same time, there is a need to ensure the patient takes in enough nutrients, particularly energy. With PLC Rice products, not only can protein intake be reduced to the desired level, but the products contain 50% less phosphorus and 95% less potassium compared to regular cooked rice while offering the same energy as WR. They meet all of the requirements as a staple food for CKD patients (Figure 7).

We developed exclusive technology for digesting and removing protein from WR using microorganism-derived enzyme product, and technology for processing the Raw LPR thus

Figure 6: Relationship between Total Protein Allowance and Protein Intake from Staple Out of Total Protein Allowance.



Bars with solid outline: Protein intake when ingesting 165 g (1 serving) of regular rice or LPR 3 times a day
Bars with dotted outline: Daily protein allowance (30 g/day)
White circle and solid line: Difference and percentage - Total protein allowance and protein intake from staple out of total protein allowance
WR; White Rice, "1/3"; PLC Rice 1/3 (0.83 % protein), "1/5"; PLC Rice 1/5 (0.5 % protein), "1/10"; PLC Rice 1/10 (0.25 % protein), "1/20"; PLC Rice 1/20 (0.125 % protein), "1/25"; PLC Rice 1/25 (0.1 % protein)

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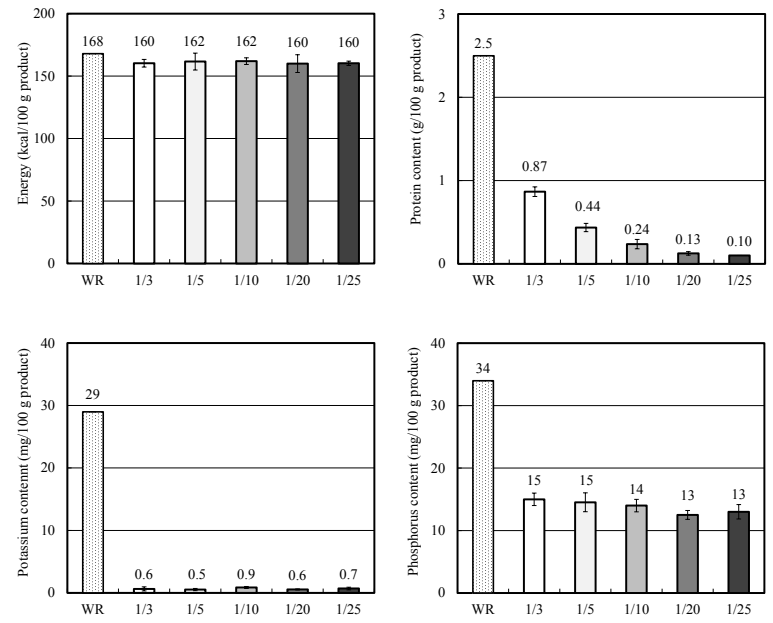
Figure 7: Nutrient Content in "Cooked PLC Rice" Series.



obtained into a palatable food product. By combining these technologies, we can reliably manufacture delicious, high quality, low protein rice product, which can be eaten repeatedly as staple. In the 20 years from its launch, nearly 100 million PLC Rice products have been sold, with zero complaints relating to health problems arising from their consumption to date. This demonstrates their safety even when eaten repeatedly over a long period.

The PLC Rice series of products, which compare favorably with, and can be substituted for, regular rice, are delicious (high quality) enough to be eaten everyday.

CONCLUSION

LPR enhances the quality of mealtimes for CKD patients by increasing their range of food choices. It is therefore, reasonable to say that PLC Rice products offer high added value, as it not only facilitates adhesion to LPD but also add satisfaction and contentment to daily meals, helping to enhance the QoL of patients with CKD.

CONFLICTS OF INTEREST

All authors are employee of Forica Foods Co., Ltd., Uonuma City, Niigata, Japan. All of this study and work has been performed at the Forica Food Co., Ltd., Uonuma City, Niigata, Japan.

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Short Communication

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Effects of Glyphosate or "Organic Herbicide" on Rumen Bacterial Isolates *In Vitro*

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ABSTRACT

Glyphosate (*N*-(phosphonomethyl) glycine) is widely used by agriculture and has been claimed to potentially influence microorganisms; for example, *Lactobacillus* spp., which forms part of the normal microbiota of humans and animals. Comparing a commercial glyphosate containing herbicide against a recipe widely distributed on the internet, Kniss (2014) described as an organic herbicide the growth of five lactobacilli isolated from the bovine rumen and found little influence on rumen microbe isolates growth on agar plates. The growth of 5 isolates from the bovine rumen was not significantly influenced by glyphosate, a commercial glyphosate herbicide, and an organic herbicide and its components suggested that glyphosate influence is at most minor and likely to be of no consequence *in vivo*.

KEY WORDS: Glyphosate; *Lactobacillus*; Rumen isolate; Antibiotic; Organic.

INTRODUCTION

Glyphosate (*N*-(phosphonomethyl) glycine) is widely used by agriculture for control of weeds. Genetically modified soy, corn, canola, alfalfa, cotton, and sorghum have been developed to be resistant to glyphosate, allowing farmers to control weeds without damage to crops. The use of glyphosate for weed control is controversial.[1-3] Glyphosate is a broad-spectrum herbicide which interferes with the synthesis of aromatic amino acids in plants and microorganisms by inhibiting the activity of the 5-enolpyruvyl shikimate 3-phosphate synthase (EPSPS) enzyme.[4] It is assumed that glyphosate does not directly affect vertebrates as they lack this enzyme.[5,6] It has been claimed that it may be possible for glyphosate to influence microorganisms (for example, *Lactobacillus* spp., which forms part of the normal microbiota of humans and animals).[7] *Lactobacillus* spp. are beneficial bacteria, which promote general well-being in the host by balancing the gut. The response of lactobacilli to different stress conditions such as low pH, bile salt stress, high or low temperature stress, nutrient stress, etc., has been widely studied, due to their applications in healthy food and complementary medicines.[8-11] It has been suggested that impact on beneficial microbes by glyphosate may interfere with human and animal health,[2,7,12] although this is not necessarily a widely-held opinion. A number of recipes for so called "organic roundup" or "organic herbicide" have become widely distributed on the internet[13] as a way to address the perceived problems with glyphosate. It is claimed that unlike glyphosate containing products, these organic herbicides do not impact microbes, be they be in the soil or gut of animals.

To our knowledge, the reports investigating possible glyphosate effects on bacteria from the bovine rumen are limited.

MATERIALS AND METHODS

We compared a commercial glyphosate herbicide (Zero Weedkiller- Super Concentrate; Yates

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Ltd, Auckland, New Zealand) containing glyphosate (3.92 mg/ml once diluted 8 ml/l for application) against a recipe widely distributed on the internet[13] described as an organic herbicide on the growth of five lactobacilli isolated from the bovine rumen. Variations in the amounts of each component of the organic herbicide are common; for this study, we used the following recipe: 1.89 l white vinegar (Pams Products Ltd; Auckland, New Zealand), 120 g salt (Cerebos Ltd; Dunedin, New Zealand) and 30 ml dish soap (Ecostore Ltd; Auckland, New Zealand). The isolates used were genetically identified as, RC 2 (*Lactobacillus plantarum* 16), RC 5 (*L. plantarum* 16), RC 13 (*L. plantarum* subsp. *plantarum* ST- III), RC 25 (*L. plantarum* 16) and RC 30 (*L. plantarum* subsp. *plantarum* ST- III). The microbiological and probiotic characteristics of these *Lactobacillus* have been previously reported.[11]

The disc diffusion method was used to assess the antimicrobial activity of commercial glyphosate, glyphosate (N-phosphonomethyl glycine, Sigma-Aldrich New Zealand; Auckland, New Zealand), organic herbicide and the organic herbicide individual ingredients. One hundred microliter of overnight lactobacilli cultures were evenly spread onto the surface of MRS agar plates (Oxoid, Basingstoke, UK) and 7 mm diameter blank sterile paper discs (Oxoid, Basingstoke, UK), antibiotic (ampicillin) discs or papers discs dipped in commercial glyphosate, organic herbicide, white vinegar, glyphosate (3.92 mg/ml), salt (62.5 mg/ml) or detergent (15.6 µl/ml) were transferred aseptically onto the surface of the agar. Replicate plates (n=3) were prepared for this study. All test solutions were sterilised by filtration through a 0.10 µm filter. The plates were then incubated for 24 h at 37 ºC under anaerobic conditions. Following incubation, the plates were then inspected for zones of inhibition around the paper discs. The sizes of zones were measured from the outer edge of the disc to the outer edge of the zone.

RESULTS

Following incubation, the plates were then inspected for zones of inhibition around the paper discs, see Figure 1 for typical examples.

Small inhibition zones of up to approximately 1 mm were observed around commercial herbicide, organic herbicide, glyphosate and the individual components of the organic herbicide (Figure 2). The inhibition zones for ampicillin ranged from about 5 to 10 mm depending on the isolate. The inhibition zone produced by ampicillin was significantly greater ($p<0.01$) compared to the commercial, organic herbicide and blank discs. Differences between the commercial, organic herbicide and glyphosate and the individual components of the organic herbicide were not significant ($p>0.05$).

DISCUSSION AND CONCLUSIONS

In a similar study that compared the effects of glyphosate on potential pathogens and beneficial members from poultry,[14] it was reported that most pathogenic bacteria were highly resistant towards the glyphosate, although *Campylobacter* spp. were susceptible. However, the beneficial bacteria had a moderate to high degree of susceptibility to glyphosate.[14] It has also been reported that commercial glyphosate products, such as Roundup® are more toxic towards food microorganisms than glyphosate alone at similar concentrations.[12] Many substances have either positive or negative influence on the growth of microbes. Some studies of glyphosate and bacteria growth have drawn conclusions about the toxicity of glyphosate towards microbes without suitable controls or specifically testing individual components such as glyphosate from a mixture as having an influence without examining it alone or in comparison with positive and nega-

Figure 1: Inhibition of Rumen Isolates RC 2 (left) and RC 25 (right) by the Agar Disc Diffusion Assay. RC 2 (left) Plate Shows Zone of Inhibition Around Discs Containing Clockwise from Top Left; Antibiotic, Blank (Control), Organic Herbicide or Commercial Glyphosate. The RC 25 (right) Plate Shows Zone of Inhibition Around Discs Containing Clockwise from Top Left; Vinegar, Salt, Detergent or Glyphosate.

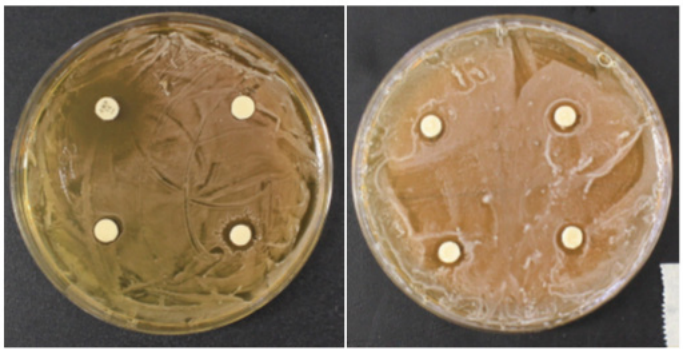
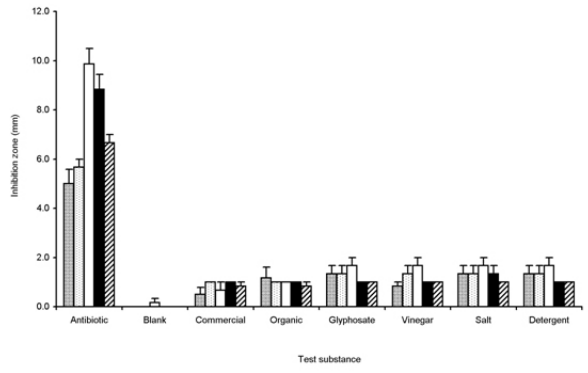


Figure 2: Inhibition Zones (mm) for Rumen Isolates RC2 ( ), RC5 ( ), RC13 (□), RC25 (■) and RC30 ( ) after Incubation with Discs Containing; Antibiotic, Blank, Commercial Herbicide, Organic Herbicide, Glyphosate and the Individual Components of the Organic Herbicide. Error Bars are Standard Error Means (n=3).



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tive control. Kurenbach et al[15] have reported that glyphosate can reduce antibiotic susceptibility in *Escherichia coli* and *Salmonella enterica serovar Typhimurium*. While these researchers did not investigate glyphosate alone (they evaluated a commercial glyphosate containing product), the claim glyphosate reduced antibiotic sensitivity is not clearly supported by their data; furthermore, many of the test combinations they evaluated appeared to increase antibiotic sensitivity.

We have shown that for 5 isolates from the bovine rumen glyphosate, a commercial glyphosate herbicide, and an organic herbicide and its components appear to all have very little (if any) influence on microbial growth. This very small influence by glyphosate (be it alone or in the form of a commercial product) and organic alternatives when compared to that of an antibiotic that the isolates are known to be sensitive to suggest that this influence is minor and likely to be of no consequence *in vivo*.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Research

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Producing Rinse-Free Rice by the Bran-Grind Method: A Way to Stop Environmental Pollution From Rice Industry Waste Water

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ABSTRACT

Introduction: Removing the bran from the surface of polished rice is important for taste and palatability. However, rice washing waste water contains rich microbial nutrients, such as phosphate and nitrogen, which causes water pollution through the overgrowth of algae and bacteria.
Methods: Rinse-free rice made by the bran grind (BG) method is an effective solution to this problem. The principle is to make bran aggregates by taking advantage of the adhesive properties of "*hadanuka*" present on the surface of white rice. There is no waste water produced in the manufacturing process, and the bran by-product can be used as fertilizer or animal feed.
Results: The BG method therefore contributes to sustainable agriculture and to the prevention of water pollution.
Conclusion: Currently, rinse-free rice made by the BG method accounts for more than 70% of the rinse-free rice market.

KEY WORDS: Rinse-free rice; Prewashed rice; Rice mill technology; Taste, Nutrient.

INTRODUCTION

The taste and palatability of rice are inversely proportional to its nutritional value (Figure 1). The taste of rice depends on natural conditions, for example: the rice species, harvesting methods, or the climate. However, the method of rice processing after harvest is also very important.[1-3] In parallel with industrial development, consumers have sought "more delicious", "easier to

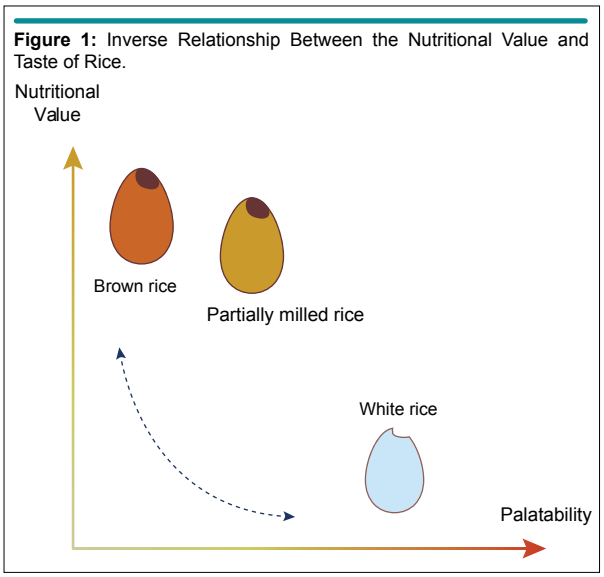

Figure 1: Inverse Relationship Between the Nutritional Value and Taste of Rice.

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eat" and "more appealing" rice. Responding to such demands, the food industry has increasingly processed rice as a market commodity.[4] Novel industrial methods have become popular, using a variety of milling machines, polishing machines, polishing whitening effects, and processing standards.[2]

The health benefits of brown rice are well known, but consumers prefer polished white rice. Brown rice is made up of three concentric layers, respectively: (i) the outer layer of the endosperm (starch storage cell layer), (ii) the aleurone layer and (iii) the bran layer. Removing the bran layer makes the rice tastier (Figure 2).

Proteins, vitamins, and other functional nutrients are mostly present in the bran layer. They are covered by an extremely hard waxy layer, which decreases the palatability of full grains.

Historically, several methods have been employed to remove bran: (i) the washing method (rinse off the skin bran with water and then dry), (ii) the grind method (rub the rice with a brush, nonwoven fabric, or polishing machine, to remove the skin bran), (iii) the neo-tasty method (add a small amount of water to rice, pressurize it, stir it, remove skin bran with hot granulated tapioca, and let it dry).

Until the 1950s' white rice was sold with a large amount of white bran powder covering the surface of each grain. Since 1955, rice mill manufacturers have been increasing the commodity value of rice. They developed the so-called "friction-type jet rice mill", where by the bran powder adhering to the white rice surface is removed by air-flow to produce a glistening appearance.[4,5] This results in the removal of the outer layer of the endosperm. This type of rice mill has continuously been used as the mainstream technique for rice processing from around 1965 until now

Around 1975, the method was improved to further increase the commodity value of rice by using a friction-type jet rice mill to let the rice grains rub against each other, leaving a tiny amount of water attached to the white rice surface. This method scraped the exterior of the softened outer layer of the endosperm and smoothed it to make the rice grain surface glossy.[6]

Since rice grains now appear extremely smooth and shine like a pearl, the commodity value increased a lot compared with former rice (Figure 3).

As small amount of water is added at the time of polishing, the method produced is charge water should bediluted to fit the environmental protection law. Indeed, rice washing waters contain large amounts of phosphate and nitrogen, causing water pollution in rivers and lakes by overgrowth of microorganisms and algae.[7,8]

Rice mills processing paddy rice release huge amounts of wastewaters and other by-products, including toxic inorganic and organic contaminants which cause environmental pollution. Accordingly, cost-effective techniques for removing contaminants are needed.

The "bran grind method" (BG method) was invented by Keiji Saika as an alternative method of rice processing avoiding the use of water.[9,10] To make rinse-free rice, the viscosity of bran itself is used to remove "skin bran," which is normally the basic component of turbid rice-washing water. Preserving the endosperm surface is also important to keep the taste.

METHOD

The Development of BG-Type, Rinse-Free Rice Machine

The purpose of our research was to develop a method and device for manufacturing rinse-free rice that does not require wastewater treatment and can consistently produce high-quality rice. The adhesive properties of fine bran (*hadanuka*) on the surface

Figure 2: Three External Layers of Brown Rice. These Cover Endosperm with Starch.

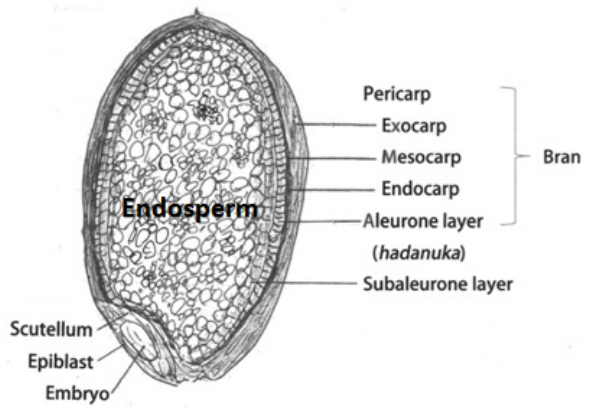
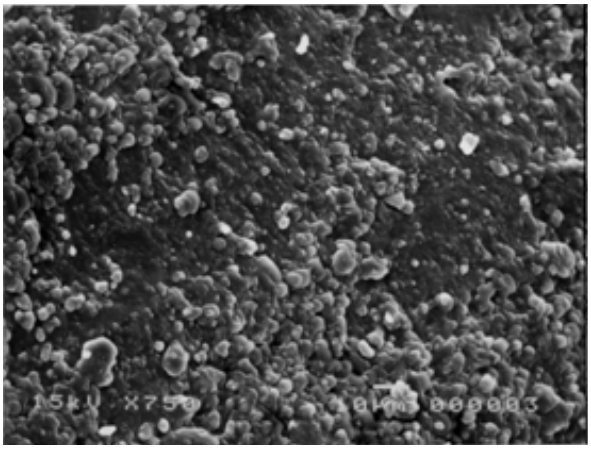


Figure 3: Surface of Polished Rice. Tiny Bran Particles Cover the Surface of Polished Rice. Scanning Electron Microscopic View (x750).



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of polished rice was exploited to aggregate each other from the surface of rice (Figure 3).

A new machine was invented and improved to adjust the best conditions to separate remnant bran from rinse-free rice. Sorted bran was further processed into secondary bran products, such as fertilizer, animal feed or baits for fisheries.

Nutrients

The nutrient contents of BG rinse-free rice, original polished white rice, and hand washed white rice were measured at the Nippon Food Analysis Center, Tokyo, Japan.[11] Energy, major nutrients, vitamins, minerals and number of bacilli and heat-resistant spores were measured according to guidelines of the Ministry of Agriculture, Forest and Fishery.

Taste

The taste of the rinse-free rice made by the BG method or other washing method was compared to that of ordinary polished white rice. The Taste Visualization scale (Aissy Laboratory, Tokyo, Japan) was used.[12] The Taste analysis machine and software computes taste by using statistical inferences from six different taste parameters: sweet, salty, acidic, bitter, *umami* and *koku*. *Koku* means a kind of deepness in the taste. Each rice sample was tested 10-14 days after processing rice from brown rice.

RESULTS

Development of the BG Machine

Separation of *hadanuka* from the surface pits of polished rice is done through the following method (Figure 5), typically with an output of about 50 kg/minute. White rice is introduced into the orifice of a cylinder and pushed forward by a spiral blade.

The inside of the stainless steel cylinder, a thick rotating shaft has 3-5 short blades and large blades alternatively. The short blades stir rice grain to remove remnant fine bran of the rice surface, which adheres on the inner surface of the cylinder. The large blades scratch off the stuck bran on the wall. The mixture of rice and detached tiny bran particles move to the sorting part of the machine, and clear rinse-free rice is obtained (Figure 5a).

In summary, four steps were carried out: (1) Flip fine bran by the short blade toward inner wall of the cylinder. (2) Attached wall bran grows larger by adhesion with other fine bran. (3) The large blade scratched off the wall attached bran layer. (4) Mixture of rinse-free rice and tiny particles of fine bran are sorted.

The removal of *hadanuka* from the surface of rice is shown by scanning electron microscopy (Figure 6).

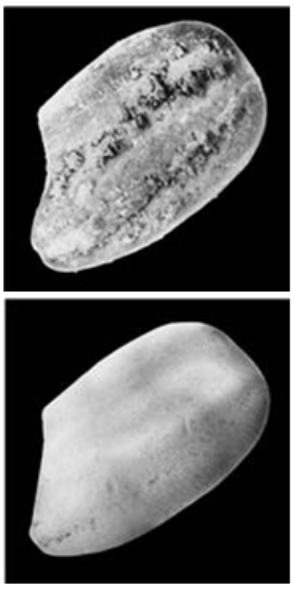

Figure 4: Polished Rice Made by Water Washing Method (Upper) and BG Method (Lower).

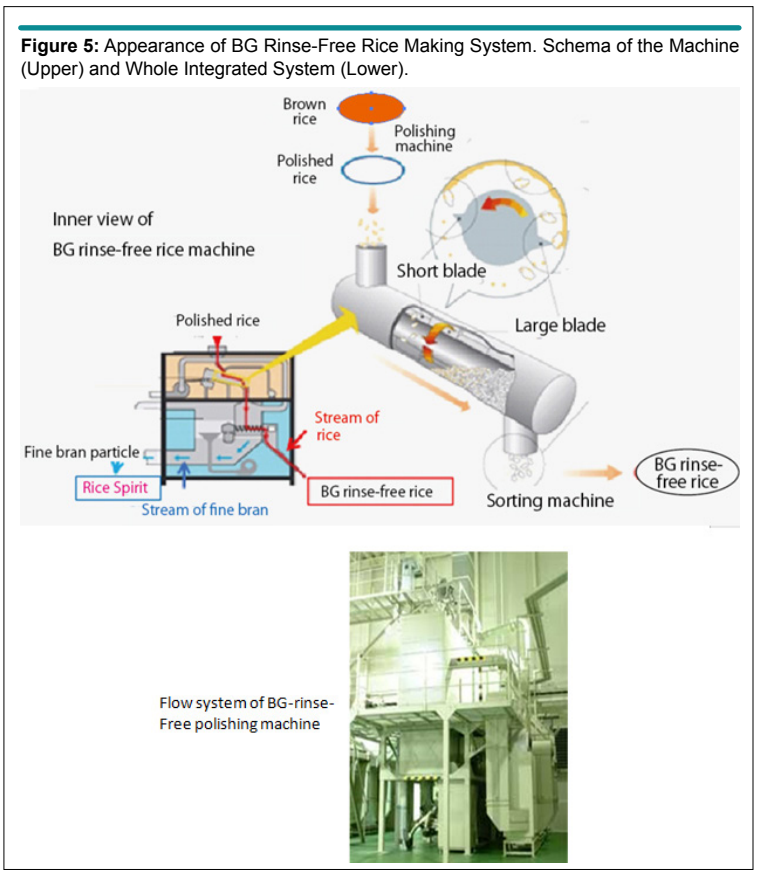

Figure 5: Appearance of BG Rinse-Free Rice Making System. Schema of the Machine (Upper) and Whole Integrated System (Lower).

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Figure 6: Surface of BG Rinse-Free Rice. Fine Bran has been Removed from the Surface. Scanning Microscopic view (x750).

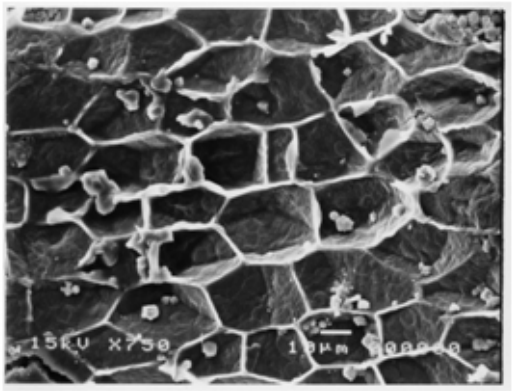

Figure 7: Recycling Agriculture Supported the BG Rinse-Free Rice Factory.

Then, bran and rice are separated by the sorting machine. Bran contains lipase and other enzymes, which are inactivated by heating after adding a small amount of water to make larger ball, about 1 cm in diameter. It is named "*kome-no-sei*" which means bran spirit. This is used as fertilizer and bait for farming fishes in the paddy (Figure 7). This system meets the objectives of the recycling agriculture.

The entire process does not require water treatment at all. The energy expenditure to make 1 kg rinse-free rice is estimated to be 18.5 g CO_2.

Nutrientsin Rinse-Free Rice

Nutrients in the rinse-free rice are similar to hand-washed rice (Table 1). Major nutrients, such as carbohydrate, protein and fat are not significantly different from the original rice. Concentrations of vitamin B_1, B_6, vitamin E, niacinare less than in the original rice, but higher than in hand-washed rice. The concentration of phosphate and potassium is as low as in hand-washed rice.

Table 1: Comparison of Nutrients Among Ordinary Polished Rice, Hand-Wash and Dried Rice, and BG Rinse-Free Rice.

	Unit	Ordinary rice	Hand wash & dry	BG rinse free rice
Water	g/100 g	13.8	16.1	14.1
Protein	g/100 g	5.3	4.9	5.1
Fat	g/100 g	1.2	1.0	1.0
Ash	g/100 g	0.4	0.2	0.2
Carbohydrate	g/100 g	79.3	77.8	79.6
Energy	kcal/100 g	364.0	355.0	363.0
Phosphate	mg/100 g	102.0	56.0	59.7
Iron	g/100 g	0.18	0.15	<0.1
Calcium	g/100 g	5.5	5.9	4.3
Sodium	g/100 g	0.4	0.9	0.4
Potassium	g/100 g	84.6	50.0	56.7
Magnesium	g/100 g	29.3	9.4	10.4
Cupper	ug/100 g	172.0	170.0	163.0
Zinc	mg/100 g	1.51	1.36	1.42
vitamin B_1	mg/100 g	0.1	0.03	0.05
vitamin B_2	mg/100 g	0.01	0.01	0.01
vitamin B_6	mg/100 g	0.11	0.025	0.035
vitamin E	mg/100 g	0.2	<0.1	0.1
Niacin	mg/100 g	1.06	0.24	0.44
Baccilus	n/100 g	$3.3x10^3$	$7.5x10^4$	$1.2x10^3$
Spore*	n/100 g	<300	<300	<300

*number of heat tolerable spore by soaking in boiling water for 10 min

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The number of bacilli is the lowest in rinse-free rice, reflecting a hygienic operation.

In addition, the fatty acid score of rinse-free rice is 5.8 mg/100 g after one month of processing, compared to 11.7 mg/100 g with polished ordinary rice.

Taste of Rinse-Free Rice

BG rinse-free rice ranked the best in comprehensive evaluations of taste (sweetness+umami-bitterness). The higher the point of sweetness and umami, the more taste increased a difference of 0.2 in each factor was easily recognizable by human sensory organ.

Credibility of BG Machine

The core technology is protected by a patent (#P2615314). Land facilities for industrial production were identified by a contractor in various places. The core part of the mill is not visible from the outside. The raw material is introduced through a belt conveyor for rinse-free processing. Because of confidential management, each mill is connected to the headquarters *via* a direct line, and facilities are operated by company engineers through an automatic control system.

DISCUSSION

Comparison with other Rinse-Free Rice Producing Machine

Around 1993, a new rinse-free rice machine called the BG type, was developed and marketed to prevent water pollution caused by turbid rice-washing water.[9] White rice finished with a friction-type jet rice mill was further processed by the BG machine to turn the white rice into rinse-free rice that did not require washing (thus, no rice-washing water was discharged).

Around the time when the BG-type machine appeared on the market, another manufacturer had released a different type of rinsing machine. A simple prewashed-type rubbed hard on the white rice grains in water. It scraped the surface of endosperm, and could not successfully deal with large amounts of turbid rice-washed water generated in rice milling plants, so rice dealers became unable to use it consequently.

The tapioca-type, pre-wash rice machine washed white rice with a small amount of water and a large quantity of tapioca particles to wipe the rice-washed water on the surface of rice. The surface of white rice was grinded with added water to soften the bran layer and mechanically rubbed by tapioca particles heated to 100 °C for wiping off the viscous rice-washing water. As the surface layer of the endosperm is rich in nutrients and delicious, the removal of this part from the pre-washed rice led to poor eating quality.

Market share of BG type rinse-free rice

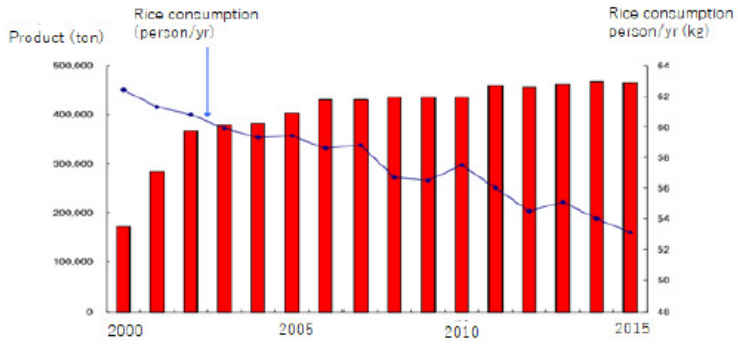
The consumption of rice by Japanese has steadily decreased from 74.6 kg/capita/year in 1985 to 56.9 kg/capita/year in 2013 (Figure 8). In 2014, the production of rinse-free rice was 467,000 tons, i.e., 6.5% of the total national rice consumption (7.22 million tons). However, the total annual rice consumption in Japanese market is estimated to be 4 to 5 million ton, so the rinse-free rice was considered to account for 10%.

Although, brand rice is popular as delicious, delicious

Table 2: Taste of the BG Rinse-Free Rice, Non-BG Rice and Ordinary Rice.

	Evaluation	Sweetness	Salt	Acid	Bitter	*Umami*	*Koku*
BG rinse-free rice	3.71	2.68	1.24	1.15	1.14	2.24	4.00
Non BG type rinse-free rice	3.38	2.37	1.23	1.12	1.13	2.14	3.77
Polished rice	3.45	2.42	1.21	1.13	1.15	2.18	3.83

Figure 8: Rice Consumption in Japan.



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rice cannot be obtained without good rice milling method and cooking method.[13-17] These are all important together with the production method. The volume of waste water for washing polished rice is about ten times more of the weight of rice, and phosphate and nitrogen contained in the waste water cause environmental water pollution, so rinse-free rice is environmentally friendly. The BOD loading amount representing the index of water pollution is 10% or less of discharged water from ordinary milled rice.

Rinse-free rice is actively handled in many company's cafeterias and major restaurants for business use. Recently, Japanese cuisine has been employed for most school lunches now, but for them, rice cooking is cumbersome. Among such circumstances rinse-free rice is employed by about half of school meals. There is a merit in terms of economic reason, such as saving time, unnecessary for washing machine and manpower. There is also an advantage to support rice-making farmers by supplying domestic rinse-free rice for school meals. About 30% of the major rice dealers in Japan have turned to rinse-free rice.

CONCLUSION

While human beings have first improved rice-processing technologies to enhance deliciousness, from now on we should equally aim at preserving the nutritional properties.[18] However, enhancing deliciousness and preserving the nutritional properties are difficult to reconcile. Nevertheless, rice that meets both of these characteristics has been available as early as 2005. Novel techniques exist for converting waste products into energy and value-added products.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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