Characteristics and Chemical Compositions of Rice Milk Kefir and Process Optimization to Obtain High Antioxidant Kefir

Stephen Moses John

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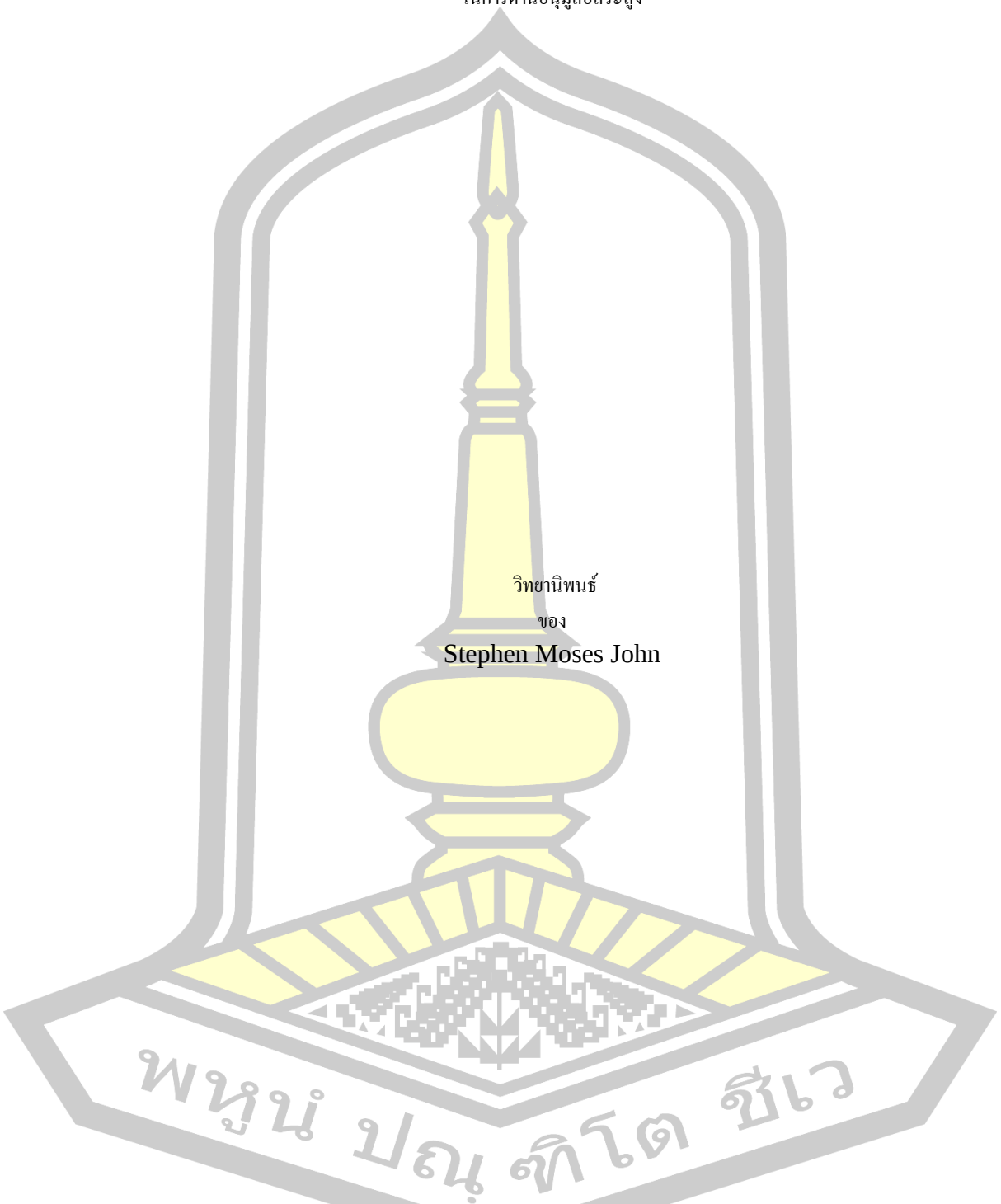
คุณลักษณะและองค์ประกอบทางเคมีของคีเฟอร์นมข้าวและสภาวะที่เหมาะสมในการผลิตคีเฟอร์ที่มีคุณสมบัติในการต้านอนุมูลอิสระสูง



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TITLE Characteristics and Chemical Compositions of Rice Milk Kefir and Process Optimization to Obtain High Antioxidant Kefir

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ABSTRACT

Kefir is fermented using different kinds of milk and consumed to boost health benefits. Kefir cultures from Kamphaeng Phet and Nonthaburi Provinces in Thailand were fermented with rice milk and cow milk. The rice cultivars used were white rice (Khao Hom Mali 105), red rice (Khao Dang) and black rice (Khao Nin) collected from Kalasin Province, Thailand. Characteristics and chemical compounds present in Thai rice milk and cow milk kefir were studied. Results indicated that pH ranged between 4.5 and 6 with viscosity ranging between 1.5 and 7 cps. The ultrasonication method was effective for extraction of volatile compounds and determination of antioxidant activities. Rice milk kefir significantly ($p<0.05$) exhibited higher antioxidant activity than cow milk kefir. DPPH scavenging was recorded between 55% and 89%, while FRAP assay results were between 2.5 and 3 µg $FeSO_4$/ml and total phenolic content ranged 0.1 to 0.6 mg GAE/ml.

Microbial analysis showed the presence of acetic acid bacteria and lactic acid bacteria in both rice milk and cow milk kefir from Nonthaburi Province but yeast was absent. No lactic acid bacteria and yeast were recorded in rice milk and cow milk kefir from Kamphaeng Phet Province. GC-MS analyses showed that amino acids and alcohols were found in variable amounts in both rice milk and cow milk kefir from Kamphaeng Phet and Nonthaburi Provinces, with ethanol and acetic acid found in almost all types of rice milk kefir. Our optimization study revealed that inoculation percentage and incubation temperature modified phenolic contents and acetic acid bacteria population as shown by the response surface model. Optimal conditions were incubation temperature at 27.5 °C and inoculation percentage of 4% v/v. High antioxidant kefir can be considered as a food additive as it contains probiotics or as a cosmetic ingredient.

Keyword : Kefir, Probiotics, Antioxidants, Rice Milk, Rice Milk Kefir

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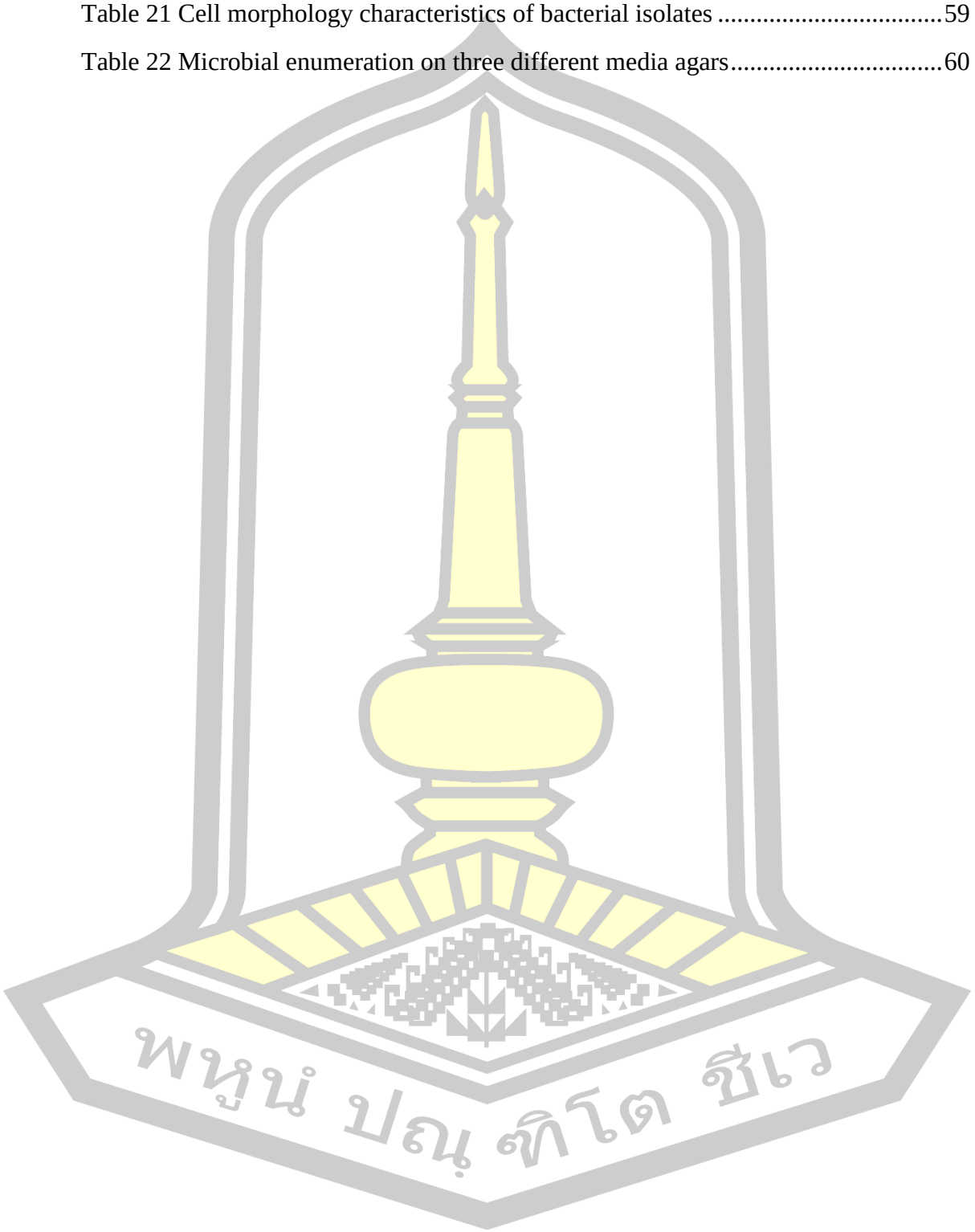

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Chapter 1
Introduction

1.1 Kefir and its background

Kefir is a soured, frothy and mildly alcoholic dairy drink produced by the result of acid and alcohol fermentation. Kefir preparation involves natural fermentation of cow milk with kefir grains (Chandan, 2006). Kefir has frequently been claimed to be effective against a variety of symptoms and diseases. Kefir can be made of any type of milk: cow, goat, sheep, coconut, rice and soy but cow milk is commonly used. Traditionally, kefir is homemade but this product has now been commercialized in many countries (Farnworth, 2005). In Soviet countries, kefir has, anecdotally, been recommended for consumption by healthy people to lower the risk of chronic diseases, and has also been provided to certain patients for clinical treatment of a number of gastrointestinal and metabolic diseases including hypertension, ischemic heart disease (IHD) and allergies (St-Ongeet *et al*., 2002; Farnworth and Mainville, 2003).

Lactic acid bacteria and yeasts are embedded in kefir grains in a slimy polysaccharide matrix named kefiran (La Riviere *et al*., 1967). Various lactic acid bacteria and yeasts have been identified in kefir grains including *Lactobacillus brevis*, *L. helveticus*, *L. kefir*, *Leuconostoc mesenteroides*, *Kluyveromyces lactis*, *K. marxianus*, and *Pichia fermentans* (Angulo *et al*., 1993, Lin *et al*., 1999). Kesenkas *et al.* (2011) determined the antioxidant properties of kefir produced from different cow and soy milk mixtures. Antioxidative activities such as the inhibition of ascorbate autoxidation, reducing activity, the scavenging effect of superoxide anion radicals and hydrogen peroxide of kefir samples were determined. Kefirs produced from whole soy milk had the highest inhibition rate of ascorbate autoxidation.

Bacterial inhibition and antioxidant activity have been reported by several isolated strains from kefir but no studies of the bioactive properties of kefir from a mixture of pure cultures are available, while only a few consider the activity of kefir produced by rice milk (Deeseenthum and Pejovic, 2010). As kefir contains probiotics,

its properties need to be known. Probiotic properties are important for survival in the gastrointestinal tract, and are also important criteria for the selection of starter cultures used to inoculate milk, with metabolism leading to the probiotic and prebiotic characteristics of the fermented milk product (Santos *et al*., 2003).

Therefore, the objectives of this study were to determine the optimal kefir from three kefir grains based on physical, chemical and biological properties and antioxidant capacity before screening and characterization of lactic acid bacteria and yeast from kefir using genetic techniques. Optimization of rice milk kefir production from the optimal kefir grain selected was also performed.

1.2 Objectives

The study objectives were:

1.2.1 To study the characteristics and chemical compositions of colored rice milk kefir.

1.2.2 Screening and characterization of lactic acid bacteria, acetic acid bacteria and yeast from kefir using genetic techniques.

1.2.3 Optimization of rice milk kefir production to obtain high antioxidant kefir.

1.3 Expected outcomes

1.3.1 Obtain knowledge about the various properties of rice milk and kefir grains.

1.3.2 Obtain knowledge about the genetic techniques used in screening lactic acid bacteria, acetic acid bacteria and yeast.

1.3.3. Obtain knowledge on optimization of rice milk kefir production from kefir grains.

1.4 Hypotheses

1.4.1 Different types of rice milk have different functional properties.

1.4.2 Optimization of rice milk influences physical, chemical and biological properties.

1.4.3 Optimization of rice milk kefir production has different effects on the retrieval of bioactive compounds and their activities.

1.5 Scope of research

1.5.1 To source the best possible kefir grain across Thailand by comparing with standard kefir strain DT 500 I.

1.5.2 Study the optimization of rice milk kefir production.

1.6 Definition of keywords

Rice milk: Rice milk is dairy-free and made from rice. Like soy milk and almond milk, rice milk is safe for those who suffer from lactose intolerance. Rice milk is also low in fat, which makes it a good milk substitute for baking.

Kefir: Kefir is an acidic and mildly alcoholic fermented milk with a complex mixture of bacteria that are confined to a matrix of discrete kefir grains. The bacteria include various *lactobacillus*, *lactococcus*, *leuconostoc* and *acetobacter* species and yeasts (both lactose-fermenting and non-lactose-fermenting).

Probiotics: Probiotics are a dietary fibre that activate the growth of bacteria having positive effects on the intestinal flora and can improve host health. (Roberfroid, 2013; Gibson, G. R., & Roberfroid, M. B, 1995).

Chapter 2

Literature Review

2.1 Kefir

Kefir differs from other fermented dairy products in that it is the product of fermentation of milk in the presence of a mixed group of microflora confined to a matrix of discrete 'kefir grains', which are recovered after fermentation (Marshall and Cole, 1985). The bacteria and yeasts in kefir grains digest proteins and other components from milk during the fermentation process. Different original kefir grains, possessing various species of microorganisms, constitute the key factors affecting the functional properties of kefir, which has long been considered good for health.

Table 1 Codex Alimentarius description of kefir*

Definition

Starter culture prepared from kefir grains, *Lactobacillus kefiri* and species of the genera *Leuconostoc*, *Lactococcus* and *Acetobacter* growing in a strong specific relationship. Kefir grains constitute both lactose-fermenting yeasts (*Kluyveromyces marxianus*) and non-lactose-fermenting yeasts (*Saccharomyces unisporus*, *Saccharomyces cerevisiae* and *Saccharomyces exiguus*).

Composition

Milk protein (% w/w)	min. 2.8
Milk fat (% m/m)	<10
Titratable acidity, expressed as % of lactic acid (% m/m)	min. 0.6
Ethanol (% v/w)	not stated

Sum of specific microorganisms constituting the starter culture (CFU/g, in total)	min. 10^7
Yeasts (CFU/g)	min. 10^4

*From Codex Standard for Fermented Milks CODEX STAN 243-2003

2.1.1 Chemical composition of kefir

Wszolek *et al.* (2001) studied the properties of kefir made in Scotland and Poland using bovine, caprine and ovine milk with different starter cultures. They found that the chemical composition of kefir ranged from 10.6% to 14.9% for total solids, 2.9-6.4% for crude protein, 3.8-4.7% for carbohydrate and 0.7-1.1% for ash.

Moreover, the major products formed during fermentation were lactic acid, CO_2 and alcohol (Otles and Cagindi, 2003). Farnworth (2005) found that L (+)-lactic acid was the most abundant organic acid (i.e. the highest concentration) after fermentation and was derived from approximately 25% of the original lactose in the starter milk. The amounts of ethanol and CO_2 produced during the fermentation of kefir depend on the production conditions.

Sarkar (2008) showed that traditional kefir made from caprine milk had lower viscosity and sensory properties than bovine kefir and contained 0.04-0.3% ethanol, while Tratnik *et al.* (2006) found that the ethanol content in bovine and caprine kefir enriched with whey protein concentrate was 0.32 and 0.35%, respectively. Lactic acid, acetic acid, pyruvic acid, hippuric acid, propionic acid, butyric acid, diacetyl and acetaldehyde were generated during the fermentation process. These compounds impart the taste and aroma to kefir (Ahmed *et al*., 2013).

Kesenkas *et al*. (2011) reported lactic acid, citric acid, pyruvic acid and acetic acid as 107.80-282.40, 1.79-5.08, 0.17-0.45 and 0.38-0.66 mg/kg, respectively after 28 days of storage.

2.1.2 Characteristics of kefir

The flavor, viscosity and microbial/chemical composition of the final kefir product can be affected by the size of the inoculums added to the milk, the occurrence of any agitation during fermentation, and the rate, temperature and duration of the cooling and ripening stages following fermentation (Koroleva, 1988b). Natural kefir has a refreshing, yeasty taste and a 'sparkling' mouth feel (Kemp, 1984). The distinctive taste of kefir results from the presence of several flavor compounds that are produced during fermentation (Beshkova *et al*., 2003).

2.1.3 Kefir grains

Kefir grains resemble small cauliflower florets: they measure 1-3 cm in length, are lobed, irregularly shaped, white to yellow-white, and have a slimy but firm texture (La Rivie`re *et al*., 1967; Kosikowski and Mistry, 1997) (Figure 1).

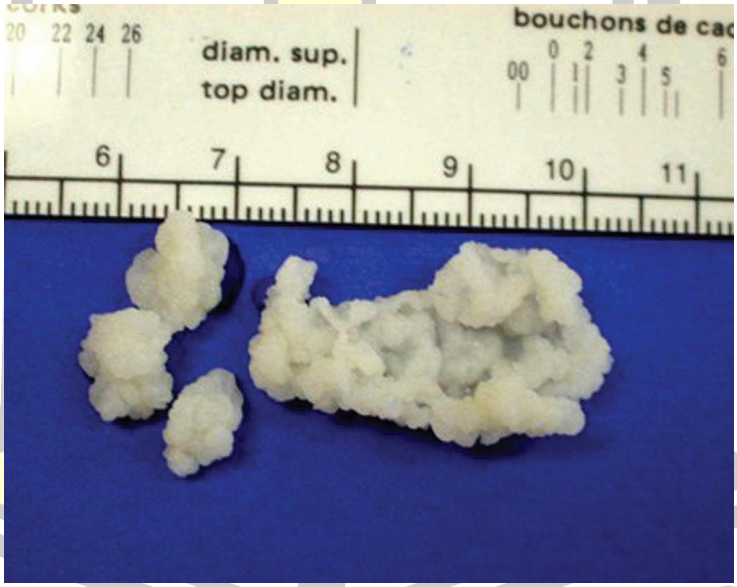


Figure 1 Kefir grains
(Farnworth, 2008)

Grains were kept viable by transferring them daily into fresh milk and allowing them to grow for approximately 20 hr; during this time, the grains increased in mass by 25% (Halle *et al*., 1994). Grains must be treated in this way to retain their viability,

since old and dried kefir grains have little or no ability to replicate (La Rivie`re *et al*., 1967).

Kefir grains replicated in milk ‘at home with daily changes of milk’ and stored for three months either at room temperature or at 48 °C had microbiological profiles that were different to those of fresh grains (Pintado *et al*., 1996).

Washing the grains in water also reduced their viability. In a commercial operation, grains used to produce kefir should be kept viable through daily transfers and should only be replaced if their ability to ferment milk becomes impaired. (Koroleva, 1982). Low temperature storage is the best way to maintain kefir grains for long periods. Garrote *et al*. (1997) showed that storage of kefir grains at -80 °C or -208 °C for 120 days did not change their fermentation properties compared to grains that had not been stored; however, grains stored at -48 °C did not produce acceptable kefir after thawing. Kefir grains replicated in soy milk were smaller in size compared to grains replicated in cow milk Liu *et al*. (2002).

Table 2 Bacteria found in kefir

Bacteria	**References**
Lactobacillus kefir	Koreleva 1991; Pintado *et al*. 1996; Kandler and Kunath 1983; Takizawa et al. 1994; Garrote *et al*. 2001
Lactobacillus delbrueckii	Koreleva 1991; Simova *et al*. 2002; Santos *et al*. 2003
Lactobacillus kefiranofaciens	Fujisawa *et al*. 1988; Takizawa *et al*. 1994; Santos *et al*. 2003
Lactobacillus rhamnosus	Koreleva 1991; Angulo *et al*. 1993
Lactobacillus kefirgranum	Takizawa *et al*. 1994
Lactobacillus casei	Simova *et al*. 2002
Lactobacillus parakefir	Takizawa *et al*. 1994; Garrote *et al*. 2001
Lactobacilli paracasei	Santos *et al*. 2003
Lactobacillus brevis	Ottogalli *et al*. 1973; Simova *et al*. 2002; Santos *et al*. 2003; Angulo *et al*. 1993
Lactobacillus fructivorans	Yoshida and Toyoshima 1994

Lactobacillus plantarum	Garrote *et al*. 2001; Santos *et al*. 2003
Lactobacillus hilgardii	Yoshida and Toyoshima 1994
Lactobacillus helveticus	Koreleva 1991; Lin *et al*. 1999; Simova *et al*. 2002
Lactobacillus fermentum	Angulo *et al*. 1993
Lactobacillus acidophilus	Ottogalli *et al*. 1973; Santos *et al*. 2003; Angulo *et al*. 1993
Lactobacillus viridescens	Angulo *et al*. 1993
Lactococcus lactis subsp.	Koreleva 1991; Pintado *et al*. 1996; Yuksekdag *et al*. 2004; Dousset and Caillet 1993; Ottogalli *et al*. 1973; Simova *et al*. 2002; Yoshida and Toyoshima 1994; Garrote *et al*. 2001; Angulo *et al*. 1993 Luang-In *et al*. 2018
Lactococcus lactis subsp. *Cremoris*	Koreleva 1991; Yuksekdag *et al*. 2004; Dousset and Caillet 1993
Streptococcus thermophilus	Yuksekdag *et al*. 2004; Simova *et al*. 2002
Enterococcus durans	Rosi 1978; Yuksekdag *et al*. 2004
Leuconostoc sp.	Angulo *et al*. 1993
Leuconostoc mesenteroides	Koreleva 1991; Lin *et al*. 1999; Ottogalli *et al*. 1973; Garrote *et al*. 2001
Acetobacter sp.	Garrote *et al*. 2001 Luang-In *et al*. 2018
Acetobacter pasteurianus	Ottogalli *et al*. 1973 Luang-In *et al*. 2018
Acetobacter acetia	Rosi 1978

Bacillus sp.	Angulo *et al*. 1993
Micrococcus sp.	Angulo *et al*. 1993
Bacillus subtilis	Ottogalli *et al*. 1973
Escherichia coli	Angulo *et al*. 1993

2.1.4 Functional properties of kefir

The functional properties of kefir are discussed in detail below and a schematic diagram is presented in Figure 2.

Antimicrobial properties

Kefir has an antibacterial effect against many pathogenic organisms due to the inherent formation of organic acids, hydrogen peroxide, acetaldehyde, carbon dioxide, and bacteriocins (Powell *et al*., 2007). For example, 3.5 kDa bacteriocin was identified from *Lactobacillus plantarum* ST8KF in kefir (Powell *et al*., 2007). The antibacterial effect of kefir produced from a freeze-dried commercial starter culture (PROBAT KC3, Danisco, Denmark) was determined against *Staphylococcus aureus* (ATCC 29213), *Bacillus cereus* (ATCC 11778), *Salmonella enteritidis* (ATCC 13076), *Listeria monocytogenes* (ATCC 7644), and *Escherichia coli* (ATCC 8739) and compared with ampicillin and gentamycin (Colak *et al*., 2007).

The antimicrobial effect was determined after 24 hr and 48 hr fermentations and during 7 days of cold storage. Zones of inhibition formed by the antibiotics and the kefir samples were similar for each pathogen; for example, inhibition zone diameter for *E. coli* was 19.5 mm, 18.6 mm, 20.2 mm, and 20.8 mm for 24 hr fermented kefir, 48 hr fermented kefir, ampicillin, and gentamycin, respectively. Antimicrobial activity of kefir was as effective as ampicillin and gentamycin while neither the length of fermentation nor the duration of cold storage significantly affected the antimicrobial activity (Colak *et al*., 2007).

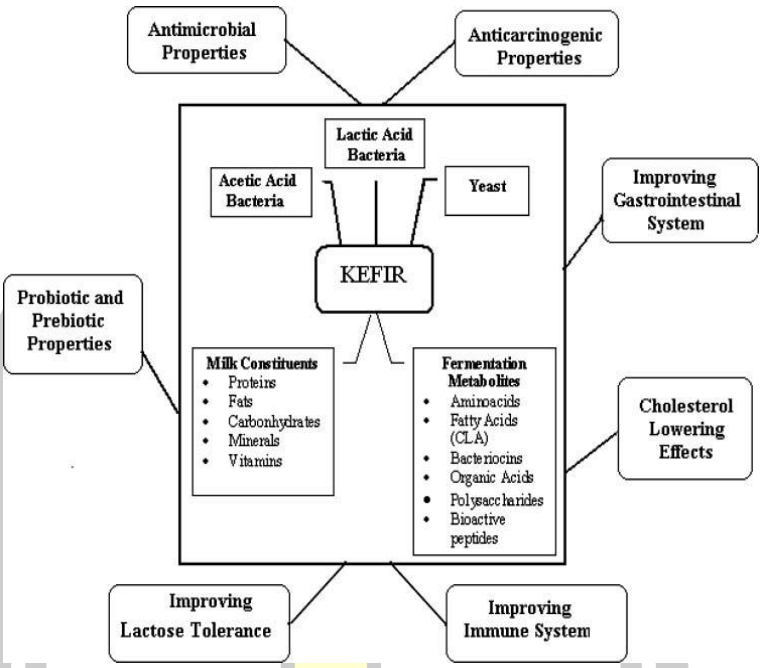


Figure 2 Functional properties of kefir

Adapted from: Guzel-Seydim *et al.* (2011)

2.2 Morphology of lactic acid bacteria, acetic acid bacteria and yeast

Lactic acid bacteria

The *Lactobacillaceae* are represented by the genus *Lactobacillus*, a highly diverse group of gram positive, microaerophilic bacteria that microscopically appear as long to short rods or even coccobacilli (Kandler and Weiss, 1986). Species within this genus are generally catalase-negative, although a few strains decompose peroxide by a non-heme-containing pseudo-catalase (Johnston and Delwiche, 1965). *Lactobacillus* spp. are either homo- or hetero-fermentative with regard to hexose metabolism.

Acetic acid bacteria (AAB)

The Acetobacteraceae family is no exception to this reorganization of species and genera. AAB are considered a lineage within the Acetobacteraceae family, which is characterized by the ability to produce acetic acid, although some of them are very weak producers. Eight new AAB genera have been added to the two traditional genera mentioned above including *Acidomonas*, *Gluconacetobacter*, *Asaia*, *Kozakia*,

Saccharibacter, *Swaminathania*, *Neoasaia* and *Granulibacter* (Guillamon and Albert Mas, 2009).

The most explicitly known and widely applied industrial strains of acetic acid bacteria belong to the genus *Gluconacetobacter*. These bacteria occur in vinegar, sugar cane, flowers and fruits (Brenner *et al*., 2005). Representatives of this genus are gram negative aerobic bacteria whose optimal growth is at 30 °C with pH ranging from 5.4 to 6.3 (Hommel, 2004). Cells of bacteria belonging to the genus *Gluconacetobacter* attain shapes from ellipsoidal to more elongated bacilli, usually straight ones, though slightly bent types also occur. Their sizes range 0.6-1.2 × 1.0-3.0 μm. They occur individually, in pairs or short chains. Only parts of the bacteria are characterized by peri-calcification which provides their motor capacity. They produce catalase, do not produce oxidase, indole nor hydrogen sulfide, and they do not fluidize gelatin (Brenner *et al*., 2005).

Morphology of yeast:

Yeasts are eukaryotic microorganisms classified in the kingdom fungi, with 1,500 species currently described.

Domain: Eucaryota are defined by their enclosed nucleus with a double DNA strand. They have multiple organelles specialized to each species outside of the nucleus, such as ribosomes, endoplasmic reticulum and Golgi apparatus (Campbell, 2009).

Kingdom: Fungi are non-vascular, heterotrophic species. They have cell walls similar to plants but differ from plants because they are made up of chitin. Their reproduction is very diverse and is how the phylum is classified (Campbell, 2009).

Phylum: Ascomycota can reproduce asexually or sexually. They are classified by their internal spores called asci, which is the reason why they are commonly known as sac fungus. Sexual spores are called ascus and asexual spores are called conidia, which means dust in Greek. These asexual spores are found externally. Fungus in this phylum can be either single-celled or multicellular. They also have a wide variety of habitats ranging from marine, to freshwater, to terrestrial (Campbell, 2009).

2.3 Rice

Rice (*Oryza sativa* L.) is the world's most important food crop and responsible for feeding approximately one-third of the Earth's population. It is the dietary staple food in many Asian countries (Shen *et al*., 2009). Rice yields have increased dramatically in China, which contributes 31% of the world's rice production, due to the introduction of hybrid rice varieties (Li, Salas, DeAngelo, & Rose, 2006). Recently, many attempts have been made to develop better rice varieties that are rich in certain functional compounds exhibiting antioxidant activities.

Whole grain rice is the unpolished version of the grains consisting of the germ, bran, and endosperm, and is also called brown rice. Although widely consumed as white rice, many special rice cultivars contain color pigments, such as black rice, red rice and brown rice. Their name refers to the kernel color (black, red or purple) which is formed by deposits of anthocyanins in different layers of the pericarp, seed coat and aleurone (Chaudhary, 2003).

Colored rice varieties have also been reported as viable sources of antioxidants for functional foods (Yawadio, Tanimori, & Morita, 2007). Of these, red rice gained popularity in Japan as a functional food because of its high polyphenols and anthocyanin content (Itani and Ogawa, 2004). Before the health beneficial effects of pigmented rice emerged, Chaudhary (2003) foresaw an upcoming demand of black rice as an organic food coloring agent, made possible due to the increased production of black rice.

Black rice has a number of nutritional advantages over common rice, such as a higher content of protein, vitamins and minerals, although mineral content varies with cultivar and production location (Suzuki *et al*., 2004).

The health benefits of whole grain are mainly contributed by one of its major constituents, the polyphenols. Polyphenols in rice grain can be classified into three subgroups as (1) phenolic acids, which are the most common secondary metabolite in cereal grains, (2) anthocyanins, which only exist in black or dark purple grains, and (3) proanthocyanidins, which mainly consist of catechin and epicatechin block units in red rice and are considered to be the most effective antioxidants in nature (Gunaratne *et al*., 2013; Qiu, Liu, and Beta, 2010).

Anthocyanin pigments have also been reported to be highly effective in reducing cholesterol levels in the human body (Lee *et al*., 2008).

2.3.1 Phenolic acid composition of rice

Goufo *et al*. (2014) determined phenolic acids as substances containing a phenolic ring and an organic carboxylic acid function, with absorption maxima at 280 nm for the C6-C1 skeleton of hydroxyl benzoic acid derivatives (gallic, protocatechuic, p-hydroxybenzoic, vanillic, and syringic acids) and at 320 nm for the C6-C3 skeleton of hydroxycinnamic acid derivatives (p-coumaric, ferulic, caffeic, sinapic, chlorogenic, and cinnamic acids). The phenolic ring can stabilize and delocalize unpaired electrons, conferring an antioxidant property to phenolic acids. The antioxidant property notably depends on the number and position of hydroxyl groups on the phenolic ring (Goffman and Bergman, 2004; Chung and Shin, 2007; Heuberger *et al*., 2010).

Two groups of phenolic acids in rice grain are derivates of hydroxybenzoic acids and hydroxycinnamic acids which can be detected at wavelengths of 260-280 nm and 320-325 nm, respectively (Irakli, Samanidou, Biliaderis, & Papadoyannis, 2012; Jun, Song, Yang, Youn, & Kim, 2012). Hydroxybenzoic acids contain gallic, p-hydroxybenzoic, salicylic, gentisic, protocatechuic, vanillic, and syringic acids. Hydroxycinnamic dehydrodisinapic acid (thomasidioic acid) is not present as a natural product in cereal grains but might be derived from air oxidation during alkaline hydrolysis (Cai, Arntfield, & Charlton, 1999).Twelve phenolic acids are usually identified in rice, with their sum ranging from 7.3 to 8.7 mg/100 g in the endosperm, 177.6 to 319.8 mg/100 g in the bran, 20.8 to 78.3 mg/100 g in the whole grain, and 477.6 mg/100 in the husk depending on the rice color (Goufo and Trindade, 2014).

Min *et al*. (2011) reported phenolics as the major hydrophilic antioxidants in rice, while carotenoids, tocopherol, and gamma-oryzanols formed the principle lipophilic antioxidative constituents. Rice is the most studied cereal in animal and human clinical trials and in food fortification (Fardet *et al*., 2008). This trend is likely to increase in the near future as Europe, South America, and Africa are also becoming interested in the antioxidant potentials of their rice varieties. Wanyo *et al*. (2016)

revealed that Thai pigmented rice had high gamma-oryzanol and alpha-tocopherol content when treated at different temperatures, with also a significant increase in extracted phenolic acids, flavonoids, and antioxidant properties, while Setyaningsih *et al*. (2010) found the ultrasound assisted-extraction method to be effective for extraction of melatonin from rice grain.

2.3.2 Anthocyanin composition of rice

Anthocyanins are a class of flavonoids that exhibit maximum absorbance in the green/blue spectrum at 510 nm. They are water-soluble glycosides of polyhydroxyl and polymethoxyl derivatives of 2-phenylbenzopyrylium or flavylium (2-phenylchromenylium) salt (Zhang *et al*., 2006).

To date, about 18 anthocyanins have been identified in rice, of which only four have been quantified (cyanidin-3-O-glucoside, peonidin-3-O-glucoside, cyanidin-3-O-rutinoside, and cyanidin-3-O-galactoside) as presented in Table 3. Mean value of the sum of the four anthocyanins in pigmented rice varieties was 1,252.7 mg/100 g and 345.8 mg/100 g for the bran and whole grain, respectively, while the anthocyanin content of rice varied more widely than the phenolic acid content (Goufo and Henrique Trindade, 2014).

Table 3 Anthocyanin compounds in rice

Color and rice parts	**Anthocyanin Compounds** (mg/100 g DW)			
	cyanidin-3-O-glucoside	peonidin-3-O-glucoside	3-O-rutinoside	cyanidin-3-O-galactoside
Pigmented rice bran	9.1-2640.4	11.4-534.1	3.17-96.62	2.93-50.00
Pigmented rice whole grain	0.8-784.3	2.9-162.1	13.78-19.90	NA
Non pigmented rice bran	7.36	0.96-2.41	6.19	NA
Non pigmented rice whole grain	NA	NA	NA	NA

Goufo and Henrique (2014)

2.4 Antioxidants

Antioxidants are substances that may protect cells from damage caused by unstable molecules known as free radicals. Free radical damage may lead to cancer. Antioxidants interact with and stabilize free radicals and may prevent some of the damage free radicals may cause. Examples of antioxidants include carotenoids as β-carotene (Em-on Chairote *et al.*, 2008). The antioxidant capacity of fruits, vegetables, and beverages is commonly determined using *in vitro* assay methods. In most fruits and vegetables, the antioxidant capacity of the hydrophilic components is higher than the lipophilic components (Wu *et al*., 2004).

2.4.1 Antioxidant activities of kefir

Kefir is a potent antioxidant that interacts with a wide range of species directly responsible for oxidative damage. The anti-oxidative activity of kefirs may be attributed to their proton-donating ability. Kefirs are potential candidates for the role of useful and natural antioxidant supplements in the human diet (Liu *et al.*, 2005). Significant variations occur among the antioxidant properties of kefir samples produced from different cow/soy milk mixtures in relation to soy milk ratio in kefir milk. The threshold soy milk level for significant antioxidative activities was found to be 50% (Kesenkas et *al*., 2011).

Unfermented soy milk demonstrated a greater DPPH radical-scavenging activity than unfermented milk. Immediately following addition of kefir grains to the milk and soy milk, the DPPH radical-scavenging activity increased, indicating that some components of the antioxidants contained in the kefir grains were transferred to milk and soy milk (Liu *et al*.,2005). The reducing power of both milk and soy milk was increased significantly by kefir fermentation. Some milk-derived proteins and peptides demonstrated levels of antioxidative activity (Ye *et al*., 2000).

2.4.2 Antioxidant compound determination

Different methods have been employed to determine antioxidant compounds. Sreeramulu *et al*. (2009) found that *in vitro* antioxidant activities of rice generally significantly correlated with their antioxidant compound contents.

Studies by Min *et al*., (2011), Chen *et al*. (2012) and Pitija *et al*. (2013) found that phenolic acids possessed higher antioxidant activities than anthocyanins. Phenolic compounds also showed higher reducing power compared with alpha-tocopherol (Laokuldilok *et al*., 2011). These factors determined the choices of method selection as detailed below.

2.4.3 Ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and total phenolic content

A ferric-ferrozine method of antioxidant capacity measurement has been developed for simple, low-cost, and versatile assay of food antioxidants. In the presence of ferrozine (FZ) ligand, ferric ion easily oxidizes antioxidants and is itself reduced to Fe (II)-FZ, yielding a very high molar absorptivity in the order of 2.8×10^4 L $mol^{-1}cm^{-1}$ that enhances sensitivity for most antioxidants (Berker *et al*., 2010).

Electron transfer (ET) based assays generally set a fixed time for the concerned redox reaction and measure thermodynamic conversion (oxidation) during that period. ET-based assays include 2,2'-azino-bis-3 ethylbenzthiazoline-6-sulphonic acid (ABTS)/Trolox equivalent antioxidant capacity (TEAC), DPPH (though the first two assays are considered as mixed HAT/ET-based assays by some researchers), Folin-Ciocalteu reagent (FCR), FRAP, ferricyanide, and CUPRAC (CUPric Reducing Antioxidant Capacity) using different chromogenic redox reagents with different standard potentials. The reducing capacity of a sample is not directly related to its radical scavenging capability but it is a very important parameter of antioxidants. The reaction equations of various ET-based assays can be summarized as follows:

Folin: Mo (VI) (yellow) + e– (from AH) → Mo(V) (blue) (Halliwell & Gutteridge, 1989),

where the oxidizing reagent is a molybdophosphotungstic heteropoly acid comprised of $3H_2O–P_2O_5–13WO_3–5MoO_3–10H_2O$ (heteropoly anion: $P_2Mo_5W_{13}O_{62}^{6-}$), in which the hypothesized active center is Mo (VI) with λmax = 765 nm.

FRAP: $Fe(TPTZ)_2^{3+} + ArOH \rightarrow Fe(TPTZ)_2^{2+} + ArO^{\bullet} + H^+$

(Pandey *et al*., 2010), where TPTZ: 2,4,6-tripyridyl-*s*-triazine ligand with λmax = 595 nm.

DPPH: $DPPH^{\bullet} + ArOH \rightarrow DPPH + ArO^{\bullet} + H^{+}$ (Huang *et al*., 2005),

where DPPH• is the [2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl] stable radical with λmax = 515 nm.

DPPH is a stable radical with a deep purple color whose reaction with other radicals, reducing agents, or compounds capable of hydrogen atom transfer (HAT) leads to loss of color at 515 nm and loss of its electron paramagnetic resonance (EPR) free radical signal (Papariello and Janish, 1966; Blois, 1958). Like ABTS+•, DPPH• reacts with both electron and hydrogen donors (Barclay *et al*., 1999; Litwinienko and Ingold, 2003), though more slowly, and steric accessibility to the radical site is a clear issue (McGowan *et al*., 1959; Hogg *et al*., 1961).

No antioxidant assay is simpler or less expensive to run than the DPPH assay, which accounts for its popularity and extensive use. The only requirements are the reagent, some cuvettes, and a UV-vis spectrophotometer which is found in even the most rudimentary laboratories. DPPH crystals were dissolved in MeOH or EtOH, initial DPPH• absorbance was recorded, an aliquot of the test antioxidant was added, the mixture was incubated for 30 min, and the final absorbance was recorded. The reaction was measured as (A0 − Af) and antioxidant activity was reported either as IC_{50} (the antioxidant concentration required to reduce the DPPH absorbance by half) or % loss or original absorbance or EPR signal (Apak *et al*., 2013).

2.5 Extraction of phytochemicals and optimization of processes for kefir production

Several conventional extraction techniques have been reported for the extraction of polyphenols from rice bran including solvent extraction (Chotimarkon, Benjakul & Silalai, 2008; Iqbal, Bhanger & Anwar, 2005), supercritical fluid extraction (Shen *et al*., 1997) and microwave-assisted extraction (Zigoneanu *et al*., 2008). Disadvantages of conventional solvent extraction include long extraction

times, and large solvent consumption. Disadvantages of supercritical fluid extraction are higher cost of the equipment and blockage in the systems as a result of the presence of water in the sample (Camel, 2000). With the development of the ‘‘Green chemistry’’ concept during the last few years, environment-friendly techniques are becoming increasingly more attractive.

Extraction of bioactive compounds under ultrasound irradiation (20-100 KHz) is one of the upcoming extraction techniques that offers high reproducibility in a shorter time, simplified manipulation, reduced solvent consumption and temperature and lower energy input (Chemat, Tomao & Virot, 2008).

The economic feasibility of an industrial process also requires working in such a way that high extraction efficiency is attained. Many factors have been established to influence extraction efficacy, such as extraction methods, solvent type, solvent concentration, extraction temperature and extraction time (Pinelo *et al*., 2005; Banik & Pandey, 2007; Silva, Rogez & Larondelle, 2007).

Bartnik, Mohler and Houlihan (2006) suggested methanol as a suitable extraction solvent to attain good yields of phenolic compounds. Environmentally benign and non-toxic food grade organic solvents like ethanol, n-butanol and isopropanol are recommended by the US Food and Drug Administration for extraction purposes

Process, optimization can be achieved by either empirical or statistical methods (Liyana-Pathirana and Shahidi, 2005; Juntachote *et al*., 2006). The empirical method is known as the one-factor-at-a-time approach, in which one factor is varied while all other factors are kept constant (Bas and Boyaci, 2007). The major disadvantage of this method is that it does not include interactive effects among the variables studied. As a consequence, this technique does not depict the complete effects of the parameter on the response. Another disadvantage of one-factor optimization is the increase in the number of experiments necessary to conduct the research, which leads to an increase in time and expenses as well as an increase in the consumption of reagents and materials (Bezerra *et al*., 2008).

Response surface methodology (RSM) enables evaluation of variable effects and their interactions on response variables. Thus, RSM as a collection of statistical

and mathematical techniques has been successfully used for developing, improving and optimizing processes (Bartnik, Mohler and Houlihan, 2006). The most common designs as central composite design (CCD) and Box-Behnken design (BBD) of response surface methodology have been widely used in various experiments. Box–Behnken, a spherical and revolving design, has been applied in optimization of chemical and physical processes because of its reasoning design and excellent outcomes (Sun *et al*., 2010).

Both traditional and industrial processes are used for kefir production. Food scientists are currently studying modern techniques to produce kefir with the same characteristics as those found in traditional kefir. Kefir can be made from any type of milk, cow, goat, sheep, coconut, rice or soy. There are many choices for milk such as pasteurized, unpasteurized, whole fat, low fat, skim and no fat (Semih Otles & Ozlem Cagindi, 2003).

Kefir grains are the key ingredient in kefir production. However, the finished product has a different microbiological profile from the grain and therefore cannot be used to inoculate a new batch of milk. The complex microbiological composition of kefir grains explains why it is difficult to obtain a starter with the optimal and constant composition necessary for regular kefir production of standard quality (Mainville *et al*., 2006). Using defined cultures to produce kefir is in progress toward standardizing kefir production (Marshall and Cole, 1985).

Taiwanese researchers have shown that lactic acid bacteria from kefir grains grow more slowly in soy milk compared to cow milk. This may be due in part to the slower production of growth factors at the beginning of fermentation when soy milk is the substrate rather than cow milk. Addition of carbohydrate (e.g. 1% glucose) to soy milk increased yeast numbers, lactic acid production and ethanol production, compared to kefir produced from soy milk alone (Liu and Lin 2000).

Gao *et al*. (2012) suggested optimal culture conditions as skim milk concentration 41.6%, temperature 30.05 °C, inoculation amount 1.86%, time, 20 hr and shaker rotating speed 0 r/min, with efficient growth rate. Skim milk concentration, temperature and inoculation amount are also significant factors for biomass production. They used response surface methodology to optimize biomass production.

A series of experiments conducted by Zajšek, Goršek, & Kolar (2013) determined that fermentation temperature, agitation rate, and choices of carbon sources, nitrogen sources, vitamins and minerals were factors that most affected production of kefiran by kefir grains lactic acid bacteria. The 24 hr kefiran production from kefir grains was maximized at 25 °C and agitation rate of 80 rpm. Addition of lactose, thiamine, and $FeCl_3$ sustained a high degree of kefiran production. Their results suggested that the production of exopolysaccharide kefiran from kefir grains can be enhanced dramatically by controlling culture conditions and modifying the composition of the milk medium.

Chapter 3
Materials and Methods

3.1 Materials

3.1.1 Rice

Thai rice cultivars used in this study were unpolished waxy colored rice varieties including black jasmine rice (Khao Nin), red jasmine rice (Khao Dang) and white jasmine rice 105 (Khao Hom Mali 105) from Kalasin, Thailand. The whole grain of the rice was used for the study.

Black jasmine rice Red jasmine rice White jasmine rice 105

Figure 3 Rice Samples

3.1.2 Kefir

Kefir Culture DT 500 I was purchased from Danisco, Poland. Thai kefir cultures were purchased from Kamphaeng Phet and Nonthaburi Provinces, Thailand as homemade milk kefir products. The starter cultures were grown in pasteurized milk (Dutch mill) and incubated at room temperature for 24 hr before cooling at 4 °C until required for use.

Figure 4 Thai kefir culture

3.1.3 Reagents and chemicals

Standard materials, chemical reagents and all solvents of the highest commercial grade were purchased from Merck Millipore. The major materials, chemicals and reagents used in this study are listed below.

Folin-Ciocalteau reagent, 2,2,-diphenyl-1-pricrylhydrozyl (DPPH), gallic acid, methanol, NaOH- phosphate buffer (pH 7), sodium carbonate, MRS, Bromocresol purple, GYC agar, YPD agar, kanamycin, cybersafe, PCR kit, PCR purification kit markers, lactic acid, acetic acid, ethanol and sulfuric acid. These chemicals were purchased from Sigma Aldrich.

3.2 Kefir and rice milk preparation

3.2.1 Kefir preparation and production

Kefir cultures were sourced from Kamphaeng Phet and Nonthaburi Provinces, Thailand. They were sub cultured and incubated in pasteurized cow milk (Dutch mill selected) at room temperature for 24 hr and then kept at 4 °C until required for use. The kefir cultures were inoculated with inoculum at 3% w/v in rice milk. Following this, the kefir cultures were incubated at room temperature. Fermentation was carried out for 24 hr and 48 hr until attainment of pH 4.5. Milk kefir was the inoculum and rice milk was the substrate, using a combination of aerobic and anerobic fermentation. The samples were freeze-dried before using for further analysis.

3.2.2 Preparation of rice milk

Rice milk was prepared using black rice, white rice and red rice cultivars. The ratio used in the preparation of rice milk was 1:5 w/v with soaking for 24 hr. Rice milk was prepared using a blender and ultrasonicated using a Sonics Vibra Cell Ultrasonicator (20 KHz) with tip diameter (25 mm), intensity (low), volume (500-1,000 ml), amplitude (70%) and time (5 min).

Following this, the rice milk was pasteurized at 75° C for 15 min and filtered through cheesecloth. After filtration, the rice milk was analyzed for physical, chemical and biological properties.

3.3 Properties of kefir produced from the three rice milk samples

3.3.1 Methods to determine the physical properties

The pH values of the samples were determined using a digital pH meter (Ezdo PL-600), while viscosities were measured using a viscometer (Syncherd-Lectric, Brookfield) and reported in centipoises (cps).

3.3.2 Methods used in determining chemical properties

Volatile and non-volatile compounds were analyzed using the following methods.

3.3.2.1 Total phenolic content

Chemical preparation: Folin-Ciocalteau reagent and deionized water (1:10), sodium carbonate with a concentration of 7%, gallic acid with a concentration of 10 mg/ml. The chemicals were prepared as follows. Sodium carbonate anhydrous 7% was prepared by adding 7 g of sodium carbonate in 100 ml water and gallic acid of concentration 1,000 µg/ml.

Method: Total phenolic content of rice milk kefir was determined by the modified method of Singleton and Ross (1965) using Folin-Ciocalteu reagent. Briefly, 12.5 µl of rice milk kefir was added in a 96-well microplate reader and 12.5 µl of Folin-

Ciocalteu reagent (1:10) was added with 12.5 µl of water. The mixture was allowed to stand for 6 min at room temperature and then 7% sodium carbonate (125 µl) and 100 µl deionized water were added and the mixture was allowed to stand for 90 min. Absorbance was measured at 765 nm using a microplate reader. De-ionized water was used as the blank. The amount of total phenolic was calculated using the Gallic Acid Calibration Curve.

3.3.2.2 DPPH free radical scavenging

Chemical preparation: To prepare stock solution of DPPH with a concentration of 10 µg/ml, 1 mg of DPPH was added to 10 ml of methanol and the volume was made to 100 ml by adding methanol before covering with aluminum foil and storing at -20 °C. For DPPH with concentration of 0.1 µg/ml, 1 ml of stock solution was pipetted and added with 100 ml of methanol.

Method: Antioxidant activity of rice milk kefir was evaluated through the free radical scavenging effect on 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical. The determination was based on the method followed by Akowvah *et al.* (2010). Briefly, 100 µl of DPPH solution was added to 50 µl of sample using methanol as the control. The mixture was incubated for 30 min in a dark room at room temperature. Absorbance was measured at 517 nm using a microplate reader. Methanol was used as a blank.

The standard of DPPH was prepared as 10, 20, 30, 40, 50, 60, 70, 80 and 90 µg/ml. Absorbance was measured at 517 nm using a microplate reader and a graph was plotted.

3.3.2.3 Determination of Ferric Reducing/Antioxidant Power Assay (FRAP)

Chemicals preparation: The FRAP assay was performed following Benzie and Strain (1999) with slight modifications. FRAP reagent was prepared as follows; 0.0270 g of ferric chloride was added to 5 ml of distilled water and mixed. Then, acetate buffer 300 mM was prepared by adding 2.4609 g of sodium acetate in water and the pH was adjusted to 3.6. HCl 40 mM was prepared in the ratio 1:1 with, water and then 0.66 ml was pipetted and added with 99.44 ml water. An aliquot of 10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ) solution was prepared by adding 2, 4, 6-tripyridyl-

s-triazine 0.0156 g in 5 ml of 40 mM HCl, 300 mM of acetate buffer, 10 mM TPTZ, and 20 mM iron (III) chloride solution.

Method: The prepared FRAP reagent was used for the experiment as follows. An aliquot of 20 µl of sample was added to 1.50 µl of FRAP reagent. The mixture was mixed thoroughly and was incubated in the dark for 30 min. Absorbance was measured at 595 nm using a microplate reader. The standard curve ($r^2 = 0.9995$) for FRAP was plotted with the absorbance at 595 nm. The standard concentration was prepared as 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 µg/ml. The calibration curve was drawn with concentration of $FeSO_4.7H_2O$ along the X axis and OD as the y axis. Values obtained were expressed in µg/ml of ferrous equivalent Fe (II) per µg of sample.

3.3.2.4 Instrument and chromatographic conditions (GC-MS)

Volatile compounds present were determined by gas chromatography mass spectrometry using a Shimadzu GCMS-QP2010NC Instrument. The conditions were as follows:

Column: CP wax 52 CB, Column Oven Temp: 50.0 °C, Injection Temp: 230.00 °C, Injection Mode: Split, Injection volume: 20 µl, Flow Control Mode: Linear Velocity, Pressure: 53.6 kPa, Total Flow: 14.0 ml/min, Column Flow: 1.00 ml/min, Linear Velocity: 36.3 cm/s, Purge Flow: 3.0 ml/min, Split Ratio: 10.0, High Pressure Injection: OFF, Carrier Gas Saver: OFF Splitter Hold: OFF

Oven Temp

Rate: 10 °C/min, Temperature: (°C) 50.0-220 and Hold Time: (min) - 5.0-10.00 min.

Working standard was prepared by mixing the primary standard (250 µl) and methanol (750 µl) in a 1-ml vial. Then, certain portions of WS-I (10, 20, 40 and 80 µl) were withdrawn and added with methanol (990, 980, 960 and 920 µl) in a 1-ml vial. This mixing step yielded working standard concentrations of 5, 10, 20 and 40 ng/µl.

3.3.2.5 Instrument and chromatographic conditions (HPLC)

Non-volatile compounds present were determined using a Shimadzu HPLC CT0-10AS Instrument. The conditions were as follows.

Injection volume: 1 µl, Column: Aminex HPX-87H, Column size: 300 × 7.8 mm in the control, Column temperature: 50 °C, Mobile phase: 0.005 M sulfuric acid, Flow rate: 0.60 ml/min, Run time: 40 min, Wavelength: 210 nm and Detector: UV detector.

3.3.2.6 Determination of gamma-aminobutyric acid (GABA) content

GABA content was determined using high-performance liquid chromatography (HPLC) as described previously. Briefly, 0.5 g of sample was suspended in 12 ml distilled water. The suspension was stirred at 4 °C for 16 hr. Independent extractions were performed for each replicate. Samples were centrifuged at 15,000 rpm at 10 °C for 20 min. The supernatant was vacuum-dried and then dissolved in 500 µl of distilled water. The samples were then vacuum-dried, reconstituted in 500 µl of 0.1 M ammonium acetate pH 6.5 (mobile phase A), and centrifuged at 13,000 rpm at 10 °C for 5 min. The supernatants were passed through a 0.22 µm nylon filter. HPLC analyses were performed using an Alliance Separation Module 2695 (Waters, Milford, USA), equipped with a Photodiode Array Detector 2996 (Waters). Samples (20 µl) were injected onto a C18 Altima (250 × 4.6 mm i.d., 5 µm particle size) column equipped with a guard column, both thermostated at 40 °C. The chromatograms were developed at a flow rate of 0.7 ml/min by eluting the sample in mobile phase A (0.1 M ammonium acetate pH 6.5), and mobile phase B (0.1 M ammonium acetate, acetonitrile, methanol, 44/46/10, v/v/v, pH 6.5) as follows: isocratic flow 100% A for 15 min, gradient flow from 100% A to 100% B for 27 min, isocratic flow 100% B for 8 min, and finally equilibrated with 100% A for 5 min. Data acquisition and integration were performed using Empower II software (Waters). GABA was identified by retention time and spiking the sample with a standard solution. GABA content was quantified by using an external GABA standard calibration curve with a linear range over 0-240 µg/µl. All analyses were carried out

in duplicate. Results were expressed in mg of GABA/100 g of sample on a dry matter basis (d.m.) (Chunchom, Talubmook & Deeseenthum, 2017).

3.3.2.7 Determination of alpha-tocopherol content

Alpha-tocopherol analysis was determined using reversed-phase high-performance liquid chromatography (RP-HPLC) method as described previously. The Shimadzu HPLC system (model L-6200A) equipped with a photodiode array detector and a computer system was applied. Briefly, detection was operated at 292 nm, simultaneously. Spectra from 250 to 600 nm were recorded for all peaks. The samples were injected through a guard column and separated on a C18 column (4.60 × 150 mm, 4 μm). Gradient elution at ambient temperature was used. Mobile phase A was methanol, mobile phase B was water, and mobile phase C was butanol. The gradient used was 0-12 min 92% A, 4% B, and 4% C, 12-25 min linear gradient from 4% B to 3% B, and 4% C to 5% C with flow rate of 1.5 ml/min and injection volume of 20 μl. The tocopherol was detected at 292 nm. Chromatograms were recorded, and peak areas were used to calculate the content of alpha-tocopherol compared with the standard solutions. The results were expressed in mg of alpha-tocopherol/100 g of sample on a dry matter basis (d.m.) (Chunchom, Talubmook & Deeseenthum, 2017).

3.3.3 Methods to determine the biological properties

3.3.3.1 Microbial population

Microbiological analyses were carried out to determine kefir microflora in all kefir samples fermented from three types of rice milk. Samples were serially diluted and plated on MRS agar + 0.05% Bromocresol purple (BCP) plates to isolate lactic acid bacteria (LAB), containing 2 g/l meat extract, 4 g/l yeast extract, 10 g/l peptone from casein, 1 ml Tween 80, 2.5 g/l K_2HPO_4, 5 g/l sodium acetate, 2 g/l diammonium hydrogen citrate, 0.2 g/l magnesium sulfate heptahydrate, 0.038 g/l manganese sulfate monohydrate, 20 g/l glucose and 15 g/l agar at pH 6.5. To isolate acetic acid bacteria (AAB), GYC agar was used (10 g/l yeast extract, 50 g/l D-glucose, 30 g/l calcium carbonate and 15 g/l agar at pH 6.8). To isolate yeasts the serial dilutions were plated on YPD agar plates (10 g/l peptone from casein, 5 g/l

yeast extract and 15 g/l agar, and 20 g/l dextrose at pH 6.5). For single colony identification, the streak plate technique was carried out five times. Colonies of bacteria and yeast were studied based on gram staining and then observed under the microscope. Colonies were reported as CFU/ml.

3.4 Optimization of rice milk kefir production

The best kefir grain and best rice variety selected from the previous methods were used for the optimization of rice milk kefir production.

Two types of variation were carried out:

1. Variation of incubation temperature: 25-30 °C.
2. Variation of inoculation rate: 2.5-5 % (w/v).

3.4.1 Experimental design

Central composite rotatable design (CCRD) was used with two factors to generate. Two factors were varied. Inoculation rate was varied at 2.5-5 %w/v and temperature for fermentation was varied between 23 °C and 30 °C.

After fermentation, the physical, chemical and biological properties were studied.

Table 4 Experimental design of rice milk kefir production with code values and actual values

Run	Factor 1 Inoculation rate (%)	Factor 2 Incubation temperature (°C)
1	2.5	27.5
2	3	25
3	3	30
4	4	23.96
5	4	27.5
6	4	27.5
7	4	27.5
8	4	31.0
9	5.4	27.5
10	5	25.0
11	5	30

3.5 Biodiversity of microorganisms in kefir grains using genetic technique

In this study, isolates of lactic acid bacteria, acetic acid bacteria and yeast were selected based on their morphology. Based on the results, isolates were characterized as bacterial or yeast isolates. Following this, DNA isolation was performed following the procedure below.

3.5.1 Genomic DNA isolation

For DNA isolation, bacteria overnight cultures (1 ml) were centrifuged at 8,000 g for 5 min. The pellet was washed with 1 ml TE buffer containing 1 mM

EDTA and 10 mM Tris at pH 8 and centrifuged again. The pellets were stored at 20 °C. Total DNA isolation was performed from bacterial pellets using the Bacterial Genomic DNA isolation kit (Vivantis, Malaysia) according to the manufacturer's instructions. Isolated DNA was amplified using the restriction fragment length polymorphism (RFLP) genetic technique following the procedure below.

3.5.2 RAPD-PCR

Markers used were random application of polymorphic DNA (RAPD) fragments with single primers of arbitrary sequences. The primer used was M13V (5´-GTTTTC-CCA-GTC-ACG-AC-3´). The PCR reaction (25 µl) contained v25 pmol of primer M13V, 0.2 mM each deoxynucleoside triphosphate, 3.5 mM $MgCl_2$, reaction buffer, 0.75 U Taq polymerase, and 1 µl of DNA solution. Approximately the same amount of DNA (50-100 ng) was used. PCR kit used was purchased from Vivantis, Malaysia. The amplification program was 94 °C for 45 s, 3 cycles of 94 °C for 3 min, 40 °C for 5 min, 72 °C for 5 min, and 32 cycles 94 °C for 1 min, 60 °C for 2 min, 72 °C for 3 min. All PCR products were mixed with 5 µl 6X DNA loading dye (Fermentas) and then electrophoretically separated in 1.3% (w/v) agarose gel (0.5X Tris-borate-EDTA buffer [45 mM Tris-borate, 1 mM EDTA]) (Anna *et al*., 2011).

3.5.3 Sequence analysis and phylogenetic tree construction

Sequence similarity values between the isolate and related taxa were retrieved from GenBank using BLAST (Basic Local Alignment Search Tool). The phylogenetic tree was evaluated by bootstrap analysis (1,000 copies) using the software package MEGA 5.0 (Molecular Evolutionary Genetics Analysis, Version 5.0), and the neighbor-joining method at 1,000 bootstrap replications (Kumar, Tamura, & Nei, 2004).

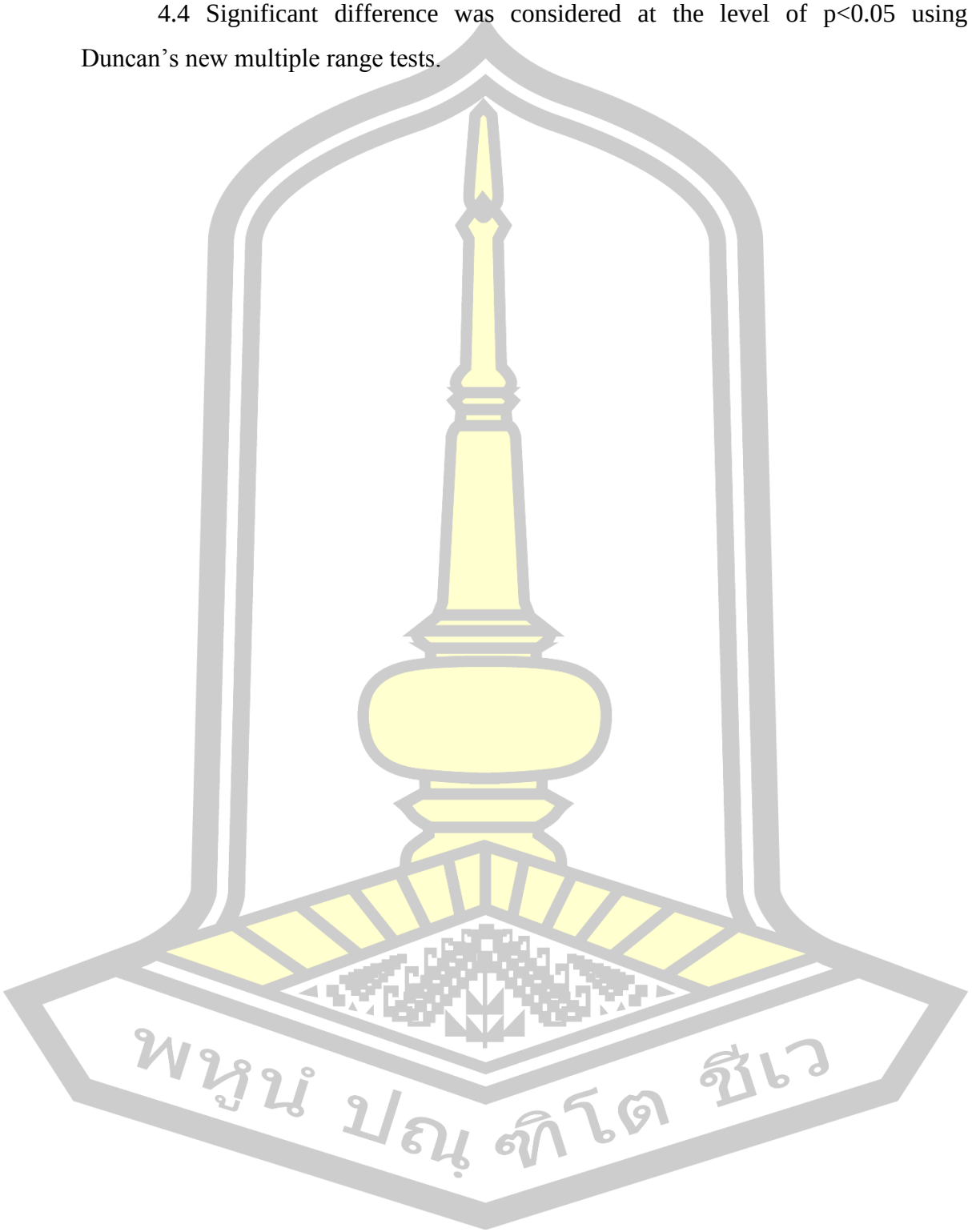
3.6 Data analysis

4.1 All experiments were conducted with three replications.

4.2 Mean and standard deviations were reported from triplicate determinations for each sample.

4.3 Data were analyzed by one-way ANOVA using SPSS.

4.4 Significant difference was considered at the level of $p<0.05$ using Duncan's new multiple range tests.



Chapter 4

Results and Discussions

4.1 Rice milk preparation analysis

Rice milk was prepared by ultrasonic and blender extraction methods based on the conditions mentioned in Chapter 3. The ultrasonic extraction method was determined as the most efficient for extraction of volatile compounds by breaking the cells. The results are given below.

4.1.1 Gamma-oryzanol and alpha-tocopherol content in rice milk varieties

Gamma-oryzanol and alpha-tocopherol content in black jasmine rice, red jasmine rice and white jasmine rice 105 from Kalasin, Thailand were analyzed using two methods. Table 5 shows the amounts of gamma-oryzanol and alpha-tocopherol.

Table 5 Estimation of gamma-oryzanol and alpha-tocopherol

Sample	**Gamma-oryzanol (mg/ml)**		**Alpha-tocopherol (mg/ml)**	
	Blender extraction	**Ultrasonic extraction**	**Blender extraction**	**Ultrasonic extraction**
White rice	0.09 ± 0.005^{BC}	0.33 ± 0.017^{AB}	0.04 ± 0.000^{BC}	0.10 ± 0.0112^{AB}
Red rice	0.08 ± 0.057^{BD}	0.11 ± 0.005^{AD}	0.05 ± 0.0017^{AA}	0.05 ± 0.0020^{AD}
Black rice	0.13 ± 0.001^{BB}	0.17 ± 0.004^{AC}	0.06 ± 0.0109^{BB}	0.11 ± 0.0064^{AC}

Means in horizontal lines with different letters are significantly different ($p<0.05$)

The ultrasonic extraction method was found to be effective for extraction of gamma-oryzanol and alpha-tocopherol contents. Extraction yield was improved using the ultrasonic extraction method.

4.1.2 Volatile compound identification using GC-MS

Based on the results from GC-MS, ethanol, tris(dimethylamino) methane, benzeneethanamine, cystine, propanoic acid, acetic acid, 1-H-purin-6-amine, methoxyacetic acid, ethylene oxide hexamer, methyl ester and dimethyl ester were found in variable amounts among Thai rice milk and cow milk kefir samples. Ethanol was found in all types of milk kefir in a greater amount compared with other compounds. The results are given below.

Table 6 Volatile compounds present in white rice milk at 0 to 24hr fermentation using ultrasonic extraction and blender extraction methods.

Volatile compounds	Formula 1		Formula 2		Formula 3		Formula 4		Formula 5	
	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr
Amino acids										
Tris(dimethylamino)methane	20.94	nd.	nd.	17.42	9.70	nd.	99.75	14.60	nd.	nd.
Cystine	nd.	nd.	nd.	nd.	nd.	73.97	nd.	nd.	nd.	8.30
Alcohols										
Ethanol	nd.	nd.	nd.	82.49	nd.	nd.	nd.	85.33	nd.	91.66
Glycerin	nd.	2.32	2.85	nd.	nd.	nd.	nd.	nd.	nd.	nd.
Benzenemethanol	nd.	nd.	0.01	nd.	nd.	nd.	nd.	nd.	nd.	nd.
Hexadecamethyl	nd.	nd.	0.02	nd.	nd.	nd.	nd.	nd.	nd.	nd.
Hexane	nd.	nd.	nd.	0.01	nd.	nd.	nd.	nd.	nd.	nd.
Dodecamethyl	nd.	nd.	0.03	nd.	nd.	nd.	nd.	nd.	nd.	nd.
1-(5-Bicyclo[2.2.1]heptyl)ethylamine	nd.	91.08	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.
Benzeneethanamine	nd.	nd.	nd.	nd.	nd.	nd.	0.20	nd.	85.52	nd.
Butane-2,3-diol	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	4.56	nd.
(3R)-Heptanol	nd.	nd.	nd.	nd.	nd.	nd.	0.05	nd.	nd.	nd.
1H-Purin-6-amine	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	0.07	nd.

Butane	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	1.99	nd.
Glycyl alcohol	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	2.65	nd.
Tetradecamethyl	nd.	nd.	0.03	nd.	nd.	nd.	nd.	nd.	nd.	nd.
Acids										
Propanoic acid	nd.	6.46	nd.	nd.	nd.	20.01	nd.	nd.	nd.	nd.
3-Nonenoic acid	0.01	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.
Undecanoic acid	nd.	nd.	nd.	0.01	nd.	nd.	nd.	nd.	nd.	nd.
dl-3-Aminobutyric acid	nd.	nd.	0.01	nd.	nd.	nd.	nd.	nd.	nd.	nd.
Cyclopropanetetradecanoic acid	nd.	0.05	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.
9,12,15-Octadecatrienoic acid	0.01	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.
3-Nonenoic acid	nd.	0.02	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.
Acetic acid	nd.	nd.	0.01	nd.	nd.	nd.	nd.	nd.	nd.	nd.
Palmitinic acid	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	5.14	nd.
8-Methoxyoctanoic acid	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	0.01

Formula 1 (White rice milk): Kamphaeng Phet (Ultrasonicator)

Formula 2 (White rice milk): Nonthaburi (Blender)

Formula 3 (White rice milk): Nonthaburi (Ultrasonicator)

Formula 4 (White rice milk): DT 500 I (Blender)

Formula 5 (White rice milk): DT 500 I (Ultrasonicator)

Table 7 Volatile compounds present in black rice milk kefir at 0 to 24 hr fermentation using ultrasonic extraction and blender extraction methods.

Volatile compounds	Formula 1		Formula 2		Formula 3		Formula 4		Formula 5	
	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr
Amino acids										
Tris(dimethylamino)methane	nd.	nd.	81.01	nd.	11.85	nd.	nd.	8.97	nd.	nd.
Cystine	nd.	16.78	nd.	nd.	nd.	nd.	16.81	nd.	nd.	15.31
Esters										
Octaethylene glycol monododecyl ether	32.51	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.
dimethyl ester	nd.	nd.	0.03	nd.	nd.	nd.	nd.	nd.	nd.	nd.
Alcohols										
Ethanol	nd.	83.13	nd.	99.98	nd.	99.92	nd.	90.97	nd.	84.65
2,4-decadienal	0.81	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.
Cyclohexane	0.46	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.
1-Hexen-4-ol	nd.	nd.	0.02	nd.	nd.	nd.	nd.	nd.	nd.	nd.
Hexadecamethyl	nd.	nd.	nd.	nd.	nd.	0.02	nd.	nd.	nd.	nd.
Butane	0.09	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.
1H-Purin-6-amine	0.26	0.02	nd.	nd.	nd.	nd.	0.01	nd.	0.01	nd.
Benzeneethanamine	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	0.02	0.02
Hexadecanoic acid	1.19	nd.	nd.	nd.	nd.	nd	nd.	nd.	nd.	nd.

Volatile compounds	Formula 1		Formula 2		Formula 3		Formula 4		Formula 5	
	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr
Acids										
Methoxyacetic acid,	nd.	nd.	nd.	nd.	nd.	0.01	nd.	nd.	nd.	nd.
3-Deoxyhexonic acid	nd.	nd.	nd.	nd.	nd.	0.01	nd.	nd.	nd.	nd.
Hexanoic acid	0.06	0.02	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.
Butanoic acid	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	0.01	nd.

Formula 1(Black rice milk): Kamphaeng Phet (Ultrasonicator)

Formula 2 (Black rice milk): Nonthaburi (Blender)

Formula 3 (Black rice milk): Nonthaburi (Ultrasonicator)

Formula 4 (Black rice milk): DT 500 I (Blender)

Formula 5 (Black rice milk): DT 500 I (Ultrasonicator

Table 8 Volatile compounds present in red rice milk kefir at 0 to 24 hr fermentation using ultrasonic extraction and blender extraction methods.

Volatile compounds	Formula 1		Formula 2		Formula 3		Formula 4		Formula 5	
	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr
Amino acids										
Tris(dimethylamino)methane	4.81	18.63	nd.	16.9	24.64	11.18	10.83	nd.	19.91	nd.
Arginine	0.24	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.
Cystine	nd.	nd.	nd.	nd.	nd.	nd.	nd.	6.14	nd.	nd.
Ethers										
Heptaethylene glycol monododecyl ether	nd.	nd.	nd.	nd.	nd.	0.02	nd.	nd.	nd.	nd.
Alcohols and Amines										
Ethanol	nd.	81.33	nd.	83.01	nd.	88.75	nd.	93.85	nd.	95.57
2,3-Butanediol	nd.	nd.	nd.	nd.	nd.	3.11	nd.	nd.	nd.	nd.
Benzeneethanamine	0.27	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	4.41
1H-Purin-6-amine	0.28	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.
2,5-Dimethyl-1-hepten-4-ol	nd.	0.01	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.
Phenethylamine	0.24	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.
Butanoic acid	0.24	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.
Hexadecanoic acid	nd.	nd.	nd.	nd.	nd.	0.02	nd.	nd.	nd.	nd.
Tetracosanoic acid	nd.	nd.	nd.	nd.	nd.	0.01	nd.	nd.	nd.	nd.

Volatile compounds	Formula 1		Formula 2		Formula 3		Formula 4		Formula 5	
	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr
5-Aminohexanoic acid	nd.	nd.	nd.	nd.	nd.	0.03	nd.	nd.	nd.	nd.
Pentanoic acid	0.17	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.
3,5-Dihydroxydecanoic acid	nd.	nd.	nd.	nd.	nd.	0.01	nd.	nd.	nd.	nd.
beta-Ethoxypropionic acid	0.17	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.
Thiosulfuric acid	0.19	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.

Formula 1 (Red rice milk): Kamphaeng Phet (Ultrasonicator)

Formula 2 (Red rice milk): Nonthaburi (Blender)

Formula 3 (Red rice milk): Nonthaburi (Ultrasonicator)

Formula 4 (Red rice milk): DT 500 I (Blender)

Formula 5 (Red rice milk): DT 500 I (Ultrasonicator)

Table 9 Volatile compounds present in cow milk kefir at 0 to 24 hr fermentation using ultrasonication and blender methods.

Volatile compounds	Formula 1		Formula 2		Formula 3		Formula 4		Formula 5	
	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr
Aldehydes										
Heptenyl acrolein	nd.	nd.	nd.	nd.	0.21	nd.	nd.	nd.	nd.	nd.
Ethylene oxide cyclic tetramer	nd.	nd.	nd.	nd.	nd.	3.80	nd.	nd.	nd.	nd.
Ethylene oxide hexamer	nd.	nd.	nd.	nd.	nd.	8.98	nd.	nd.	nd.	nd.
1-Methoxy-3-(2-trimethylsilyloxyethyl)octane	nd.	nd.	nd.	nd.	0.04	nd.	nd.	nd.	nd.	nd.
methyl ester	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	0.11
12-Crown-4	0.01	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.
Alcohols										
Ethanol	nd.	91.59	nd.	nd.	nd.	nd.	nd.	nd.	nd.	69.27
3-Hexanol	nd.	nd.	nd.	nd.	0.02	nd.	nd.	nd.	nd.	nd.
4,5-Octanediol	0.01	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.
1H-Purin-6-amine	nd.	0.01	nd.	nd.	nd.	nd.	nd.	nd.	0.01	nd.
1-Propanol	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	0.14
2-Formylhistamine	nd.	8.36	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.

Volatile compounds	Formula 1		Formula 2		Formula 3		Formula 4		Formula 5	
	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr
Organic Acid										
Acetic acid	nd.	nd.	nd.	nd.	0.08	nd.	nd.	nd.	nd.	nd.
Propanoic acid	nd.	0.01	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.
Octadecanoic acid	nd.	0.04	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.
Cyclohexanecarboxylic acid	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	0.03
Carbazic acid	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.	0.11
Tris(dimethylamino)methane	nd.	nd.	nd.	nd.	35.92	70.58	nd.	nd.	nd.	29.56
Benzoic acid	nd.	0.01	nd.	nd.	nd.	nd.	nd.	nd.	nd.	nd.

Formula 1(Cow milk): Kamphaeng Phet (Ultrasonicator)

Formula 2 (Cow milk): Nonthaburi (Blender)

Formula 3 (Cow milk): Nonthaburi (Ultrasonicator)

Formula 4(Cow milk): DT 500 I (Blender)

Formula 5 (Cow milk): DT 500 I (Ultrasonicator)

Based on the results obtained from various methods, kefir from Nonthaburi showed higher antioxidant content with more volatile compounds than kefir from Kamphaeng Phet. Hence kefir from Nonthaburi was selected for further studies.

4.2 Study of characteristics of cow and rice milk kefir prepared by ultrasonication method fermented with Nonthaburi kefir grain

4.2.1 Physical properties

Physical property analyses were performed using a pH meter and a viscometer. The pH of cow milk kefir from Nonthaburi was highest after 24 hr of fermentation. Cow milk kefir from Nonthaburi also had the highest viscosity of 7 cps. Higher pH in cow milk kefir may also explain why LAB can grow better in rice milk. The results are given below.

Table 10 pH and viscosity of milk kefir fermented with Nonthaburi grain

Milk type	**pH**			**Viscosity (Cp)**		
	0 hr	**24 hr**	**48 h r**	**0 hr**	**24 hr**	**48 hr**
Cow	6.29	5.74	5.60	6.8	7.0	6.9
White rice	4.74	4.79	4.56	1.8	1.6	1.5
Red rice	4.90	4.80	4.74	1.6	1.6	1.6
Black rice	5.04	4.65	4.05	2.2	1.3	1.2

Magalhães *et al.* (2011) studied whey-based beverages. They observed a sharp decrease in pH during the first 28 hr, from an initial value of 6.1 to 4.3 at 28 hr, for all the substrates. Moreover, Motaghi *et al*. (1997) reported that kefir manufactured by adding 5% Iranian kefir grains and incubation times of 12, 24, 36, 48, 60 and 72 hr

had pH values in the range 2.98 to 4.00. Also, a study by Sarkar (2008) showed that traditional kefir made from caprine milk had low viscosity and sensory properties, unlike those of bovine kefir.

4.2.2 Chemical properties

- **Total phenolic content analysis**

Total phenolic content of kefir with different fermentation times in three varieties of rice milk was performed. Highest content was found in black rice with the lowest content in cow milk. Results revealed that kefir fermented for 24 hr showed significant antioxidant activity compared to 0 hr and 48 hr. Results are presented in Table 4.7

Table 11 Total phenolic content of milk kefir fermented with Nonthaburi grain during 48 hr of fermentation

Milk type	**(mg GAE/ml)**		
	0 hr	**24 hr**	**48 hr**
Cow	0.15±0.006	0.47±0.027	0.17±0.017
White rice	0.32±0.006	0.46±0.014	0.18±0.021
Red rice	0.36±0.024	0.52±0.025	0.52±0.057
Black rice	0.32±0.020	0.63±0.058	0.69±0.058

Significant difference ($p<0.05$)

Satir and Guzel-Zeydim (2015) reported that feeding regimes and breed type are significant parameters that determine the functional properties of goat milk and kefir. They found total antioxidant capacity and phenolic substances in goat hair samples were noticeably higher than in Saanen breed samples and cow milk. They concluded that kefir made from goat milk had higher bacterial populations including probiotics and more bioactive compounds (total antioxidant capacity and phenolic substances) than kefir produced from cow milk due to the genetic features and botanical differences in feeding regimes.

4.2.3 Biological properties

- **Microbial population**

Microbiological analysis were carried out to determine kefir microflora in two types of kefir grains, fermented with three types of rice milk and cow milk. The results of lactic acid bacteria, acetic acid bacteria and yeast population in kefir from Nonthaburi, Thailand fermented for 24 hr and 48 hr with three varieties of rice milk and cow milk are given below.

Table 12 Microbial population of milk kefir fermented with Nonthaburi grain during 48 hr of fermentation

Milk type	**Yeast** (CFU/ml)		**Lactic acid bacteria** (CFU/ml)		**Acetic acid bacteria** (CFU/ml)	
	24 hr	**48 hr**	**24 hr**	**48 hr**	**24 hr**	**48 hr**
Cow	-	-	2.2×10^{7}	2.5×10^{7}	5.0×10^{9}	1.0×10^{10}

White rice	-	-	6.1×10^{8}	1.3×10^{10}	7.1×10^{9}	1.4×10^{10}
Red rice	-	-	4.9×10^{7}	1.4×10^{11}	5.0×10^{8}	1.4×10^{11}
Black rice	-	-	3.2×10^{7}	2.7×10^{11}	3.6×10^{7}	2.9×10^{7}

Tamang & Thapa (2006) revealed that lactic acid bacteria, yeasts and other bacteria play a dominant role in fermenting cereal-based foodstuff by secreting different hydrolytic enzymes and producing sugars, organic acids, vitamins and other bioactive substances.

Furthermore, Angulo, Lopez, & Lema (1993) and Ottogalli, Galli, Resmini, & Volonterio (1973) found *Bacillus* sp. along with *Lactobacilli*, acetic acid bacteria or yeasts in milk kefirs from Spain, while Chen, Wang, & Chen (2008), Zhou *et al*. (2009) and Magalhaes *et al.* (2010) found *Lactobacillus* species and yeasts prevalent in kefirs. Interestingly, Cruz *et al*. (2000) found that *B. subtilis* produced lactate, acetate, acetoin, ethanol, succinate and 2,3-butanediol from substrates of glucose and pyruvate during anaerobic metabolism. Moreover, a study by Vijitra Luang-In and Sirirat Deeseenthum revealed that only *Bacillus* spp. was identified in Thai milk kefir from Kamphaeng Phet Province, Thailand.

- **Antioxidant activity by DPPH scavenging and Ferric Reducing Antioxidant Power assay**

DPPH scavenging analysis of kefir from Nonthaburi with different fermentation times of three varieties of rice milk was performed. Highest activity was found in red rice and lowest was recorded for cow milk. The data are shown in Table 4.9.

Also, FRAP analysis was performed for kefir at different fermentation times of 0-48 hr. Results in Table 4.9 show that black rice had the highest activity.

Table 13 DPPH of milk kefir fermented with Nonthaburi grain during 48 hr of fermentation

Milk type	DPPH (% scavenging)		
	0 hr	24 hr	48 hr
Cow	55.19±0.01	56.83±0.02	67.21±0.02
White rice	87.97±0.00	87.97±0.00	85.79±0.00
Red rice	89.07±0.01	86.33±0.01	85.24±0.01
Black rice	87.97±0.01	84.15±0.01	80.87±0.01

Significant difference ($p<0.05$)

Table 14 FRAP of milk kefir fermented with Nonthaburi grain during 48 hr of fermentation

Milk type	FRAP (µg $FeSO_4$/ml)		
	0 hr	24 hr	48 hr
Cow	2.55±0.044	2.92±0.043	2.59±0.063
White rice	2.73±0.023	2.61±0.073	2.83±0.093
Red rice	2.75±0.0378	2.90±0.059	2.92±0.049
Black rice	2.66±0.059	2.51±1.225	3.19±0.39

Significant difference ($p<0.05$)

4.3 Modeling by response surface methodology

Based on the results of antioxidant activities, volatile compounds and microbial population analyses, ultrasonication was determined as the most suitable method. Kefir from Nonthaburi and black rice milk optimization were performed using two factors of inoculum size (3-5%) and incubation temperature (25-30 °C). For optimization, central composite design was used with 11 runs.

The responses (total phenolic content and antioxidant activities) of each experimental design run are presented below. Coded and decoded values of independent variables for each experiment are also presented. Total phenolic content of black rice milk kefir extracts varied from 0.4-0.6 (mg GAE/ml). FRAP and scavenging of DPPH radical assays were used to determine the antioxidant activity of the extracts. As shown in Tables 23 and 24, activity values varied from 1.4-1.7 µg $FeSO_4$/ml, 81-86% for FRAP and DPPH assays, respectively. The pH ranged from 4.8 to 5.2, while viscosity ranged from 1.8-2.3 cps.

4.3.1 Physical property analyses after optimization

Physical property analyses after optimization were performed using a pH meter and a viscometer. The highest pH was found in inoculum 5% and 30 °C. Highest viscosity was also found with the same condition. Results are given below.

Table 15 pH and viscosity of optimized kefir

Run (Inoculum, Temp)	**pH**	**Viscosity (cps)**
2.5%, 27.5 °C	4.95	2.26
3%, 25 °C	5.24	2.32
3%, 30 °C	4.86	1.84
4%, 23.9 °C	4.92	2.27

4%, 27.5 °C	4.95	2.28
4%, 27.5 °C	4.93	2.23
4%, 27.5 °C	4.97	2.26
4%, 31 °C	4.90	1.92
5.4%, 27.5 °C	4.97	2.24
5%, 25 °C	5.23	2.36
5%, 30 °C	5.25	2.38

4.3.2 Chemical property analyses after optimization

Chemical properties of optimized kefir were analyzed using GC-MS and total phenolic content. The results are given below.

4.3.2.1 Effect of process variables on volatile compounds

Black rice milk kefir from Nonthaburi was selected and optimization was performed using RSM. Eleven runs were conducted by varying the inoculum rate (2.5%-5%w/v) and incubation temperature (25 °C-30 °C). Compounds were identified using a GC-MS Shimadzu GCMS- QP2010NC Instrument, with results shown below.

Table 16 GC-MS profile of optimized kefir

Run (Inoculum, Temp)	**Compound name**	**Retention time (min)**	**Area%**
2.5%, 27.5 °C	1. Propiolic acid 2. Ethanol 3. Cyclobutanol 4. 2-Butanone, 3-hydroxy- 5. Acetic acid 6. 2,3-Butanediol 7. 2-Undecanol 8. 1,2,3-Propanetriol 9. 3,3Bis(carbamino)diaziridine	1.90 2.71 7.18 9.69 12.12 13.90 20.75 21.63 24.66	0.60 2.36 49.68 3.70 8.28 2.11 0.01 2.53 1.60
3%, 25 °C	1. Ethanol 2. Tris(dimethylamino)methane	2.67 7.21	1.12 53.04

	3. 2-Butanone, 3-hydroxy-	9.68	2.06
	4. Acetic acid	12.12	4.07
	5. 2,3-Butanediol	13.89	2.64
	6. 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	21.25 21.63	2.78
	7. 1,2,3-Propanetriol	26.34	7.20
	8. Octadecanoic acid	27.77	5.41
	9. Heptadecene-(8)-Carbonic Acid		
3%, 30 °C	1. Nitrogen oxide	1.88	0.65
	2. Ethanol	2.69	1.71
	3. Benzeneethanamine, 2,5-difluoro-beta,3,4-trihydroxy-N-methyl	7.09	47.59
	4. 2-Butanone, 3-hydroxy-	9.66	2.98
	5. Acetic acid	12.12	7.07
	6. 2,3-Butanediol	13.89	3.56
	7. 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	21.27	0.47
	8. 1,2,3-Propanetriol	21.63	3.15
4%, 23.9 °C	1. Ethanol	2.70	2.24
	2. Cyclobutanol	7.16	40.98
	3. 2-Butanone, 3-hydroxy-	9.69	3.40
	4. Acetic acid	12.11	9.37
	5. 2,3-Butanediol	13.8	2.93
	6. 2-Hydroxypropanoic acid	20.3	35.50
	7. 1,2,3-Propanetriol	21.64	3.13
	8. 3,3-Bis(carbamino)diaziridine	24.62	1.75
4%, 27.5 °C	1. Ethanol	2.67	5.88
	2. Benzeneethanamine, 2,5-difluoro-beta,3,4-trihydroxy-N-methyl	7.12	65.20
	3. 2-Butanone, 3-hydroxy	9.67	21.8
	4. Acetic acid	12.13	6.78
4%, 27.5 °C	1. Propiolic acid	1.88	1.08
	2. Ethanol	2.69	3.81
	3. (S)-(+)-1-Cyclohexylethylamine	7.10	33.18
	4. 2-Butanone, 3-hydroxy	9.66	4.63
	5. Acetic acid	12.11	9.48
	6. 2,3-Butanediol	13.90	3.83
	7. 1,2,3-Propanetriol	21.62	5.47
	8. Benzene, 1,1'-(1,1,2,2-tetramethyl-1,ethanediyl)bis	22.01	2.87

Run (Inoculum, Temp)	Compound name	Retention time (min)	Area%
4%, 31 °C	1. Ethanol	2.71	1.66
	2. Benzeneethanamine, 2,5-difluoro-beta,3,4-trihydroxy-N-methyl	7.10	45.15
	3. 2-Butanone, 3-hydroxy	9.69	2.89
	4. Acetic acid	12.12	6.35
	5. 2,3-Butanediol	13.87	3.52
	6. (S)-2-Hydroxypropanoic acid	20.34	36.35
	7. 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	21.26	0.40
	8. 1,2,3-Propanetriol	21.63	3.68
5.4%, 27.5 °C	1. Ethanol	2.71	3.32
	2. 1-(5 Bicyclo[2.2.1]heptyl)ethylamine	7.12	44.23
	3. 2-Butanone, 3-hydroxy-	9.68	11.37
	4. Acetic acid	12.12	5.74
	5. 2,3-Butanediol	13.88	3.10
	6. 2-Furanmethanol	14.85	1.60
	7. (S)-2-Hydroxypropanoic acid	20.34	26.98
	8. Glycerin	21.63	1.26
	9. Propyl-1-D1 Hexyl Ether	24.61	2.40
5% 25 °C	1. Docosanoic acid	2.18	0.91
	2. Ethanol	2.71	0.80
	3. Tris(dimethylamino)methane	6.99	70.54
	4. 2-Butanone, 3-hydroxy-	9.63	1.51
	5. Acetic acid	12.12	2.91
	6. 2,3-Butanediol	13.89	1.22
	7. Propanoic acid, 2-hydroxy-	20.34	10.66
	8. Octane	21.23	0.46
	9. 1,2,3-Propanetriol	21.61	2.07
	10. 2-Furancarboxaldehyde, 5-(hydroxymethyl)-	23.39	8.44
	11. Hexadecanoic acid	30.62	8.44

4.3.2.2 Effect of process variables on total phenolic content

Total phenolic content of black rice milk kefir extracts obtained after optimization are shown in Table 17.

Final equation in terms of actual factors

Total phenolic content = -2.42704 + 0.135591× Incubation temperature

= -2.42704 + 0.75572 × Inoculation percentage

= -2.42704 + 0.003 × Incubation temperature × Inoculation percentage

= -2.42704 - 0.00313 × Incubation temperature^2

= -2.42704 - 0.10208 × Inoculation percentage^2

Table 17 Total phenolic content of optimized kefir

Run (Inoculum, Temp)	**Total phenolic content (mg GAE/ml)**
2.5%, 27.5 °C	0.47±0.17
3%, 25 °C	057±0.15
3%, 30 °C	0.35±0.85
4%, 23.9 °C	0.68±0.95
4%, 27.5 °C	0.69±0.05
4%, 27.5 °C	0.66±0.07
4%, 27.5 °C	0.61±0.05
4%, 31 °C	0.62±0.04

Run (Inoculum, Temp)	Total phenolic content (mg GAE/ml)
5.4%, 27.5 °C	0.50±0.07
5%, 25 °C	0.62±0.15
5%, 30 °C	0.43±0.03

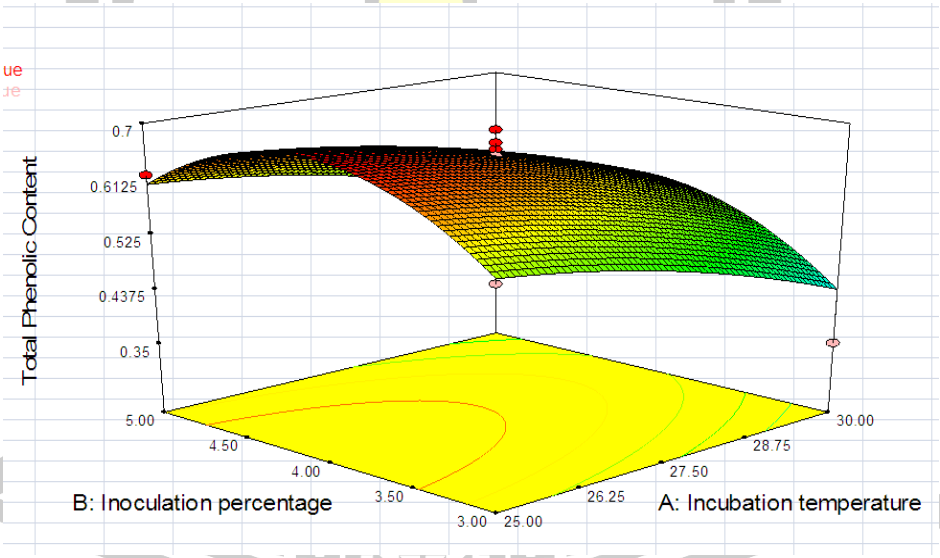


Figure 5 Response surface plots for the effect of (a) Incubation temperature, and (b) Inoculation percentage on the total phenolic content (TPC).

4.3.3 Biological property analyses after optimization

4.3.3.1 Effect of process variables on microbial population

The populations of acetic acid bacteria, lactic acid bacteria and yeast were studied. There was no growth of yeast. Typically, yeast can grow on kanamycin but bacteria cannot. Our results showed growth of lactic and acetic acid bacteria. Population results are given below.

Final equation in terms of actual factors

Acetic acid population = 45.11886 - 3.22812 × Incubation temperature

= 45.11886 + 3.358234 × Inoculation percentage

= 45.11886 - 0.20921 × Incubation temperature × Inoculation percentage

= 45.11886+ 0.072723 × Incubation temperature^2

= 45.11886 + 0.300796 × Inoculation percentage^2

Final equation in terms of actual factors

Lactic acid population = 30.51368 - 1.98589 × Incubation temperature

= 30.51368 +1.802981× Inoculation percentage

= 30.51368 - 0.11035 ×Incubation temperature × Inoculation percentage

= 30.51368 + 0.044003 × Incubation temperature^2

= 30.51368 + 0.169132 × Inoculation percentage^2

Table 18 Microbial population of optimized kefir

Sample **Inoculum (w/v), Temp °C**	**Yeast** CFU/ml	**Lactic acid bacteria** (CFU/ml)	**Acetic acid bacteria** (CFU/ml)
2.5%, 27.5	-	$5.6x10^6$	$6.6\ x10^6$
3%, 25	-	$2.66x10^7$	$2.23x10^7$
3%, 30	-	$9\ x10^7$	$8.9\ x10^7$
4%, 23.9	-	$1.22x10^7$	2.2110^7
4%, 27.5	-	$9.8x10^6$	$1.x10^6$
4%, 27.5	-	$8.8x10^6$	$5.6x10^6$
4%, 27.5	-	$8.7x10^6$	$6.6x10^6$
4%, 31	-	$9.90x10^6$	$1.12x10^7$
5.4%, 27.5	-	$8x10^6$	8.910^6
5%, 25	-	$2.23x10^8$	$2.21x10^8$
5%, 30	-	$6.x10^7$	$7.2x10^6$

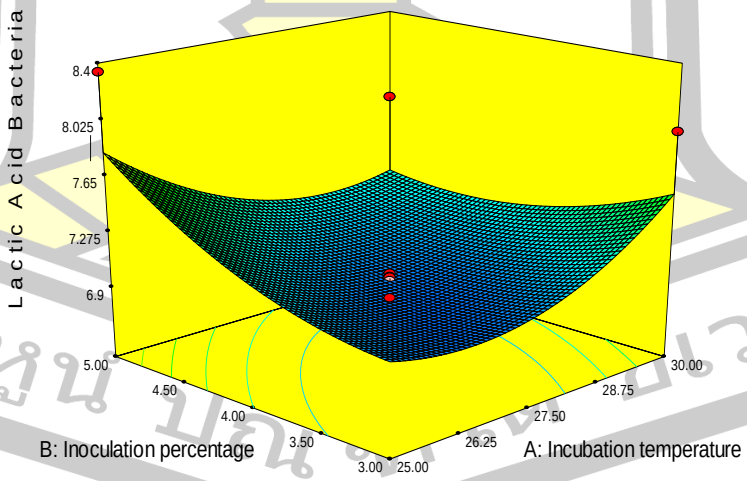


Figure 6 Response surface plots for the effect of (a) Incubation temperature, and (b) Inoculation percentage on population of lactic acid bacteria**.**

4.3.3.2 Effect of process variables on ferric reducing antioxidant power assay

Ferric reducing power assay was performed to optimize kefir. The condition 4%, 31 had the highest activity. Results are given below.

Final equation in terms of actual factors

FRAP = -1.44745 + 0.087783 × Incubation temperature

= -1.44745 + 0.971844 × Inoculation percentage

= -1.44745 - 0.016 × Incubation temperature × Inoculation percentage

= -1.44745 - 0.00063 × Incubation temperature^2

= 1.44745 -0.06146 × Inoculation percentage^2

Table 19 FRAP assay analysis of optimized kefir

Sample **Inoculum (w/v), Temp °C**	**µg $FeSO_4$/ml**
2.5%, 27.5	1.50±0.04
3%, 25	1.52±0.07
3%, 30	1.44±0.12
4%, 23.9	1.63±0.31
4%, 27.5	1.65±0.26
4%, 27.5	1.67±0.03
4%, 27.5	1.58±0.11
4%, 31	1.70±0.26
5.4%, 27.5	1.60±0.21
5%, 25	1.69±0.09
5%, 30	1.45±0.13

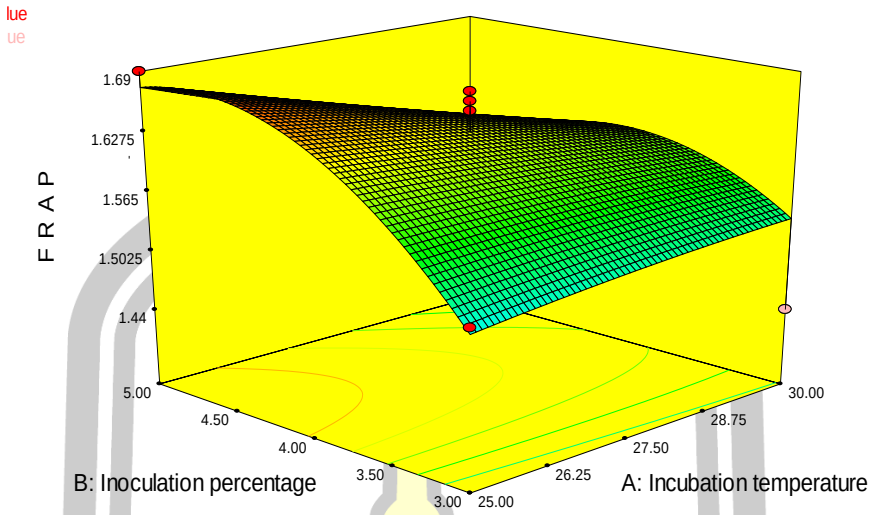


Figure 7 Response surface plots for the effect of (a) Incubation temperature, and (b) Inoculation percentage on FRAP assay.

4.3.3.3 Effect of process variables on antioxidant activities

- **Effect of process variables on DPPH analysis**

DPPH analyses were performed for optimized kefir. Highest activity was found in condition 5%, 30, with results shown below.

Final equation in terms of actual factors

DPPH = 173.7313 - 3.36638 × Incubation temperature

= 173.7313 - 24.1559 × Inoculation percentage

= 173.7313 + 0.461 × Incubation temperature × Inoculation percentage

= 173.7313 + 0.0332331 × Incubation temperature^2

= 173.7313 + 1.357708 × Inoculation percentage^2

Table 20 DPPH analysis of optimized kefir

Sample Inoculum (w/v), Temp ° C	(% Scavenging)
2.5%, 27.5	85.203±0.17
3%, 25	84.888±0.05
3%, 30	84.888±0.32
4%, 23.9	81.110±0.04
4%, 27.5	80.519±0.07
4%, 27.5	82.999±0.31
4%, 27.5	82.802±0.06
4%, 31	82.172±0.53
5.4%, 27.5	82.684±0.39
5%, 25	81.897±0.12
5%, 30	86.501±1.64

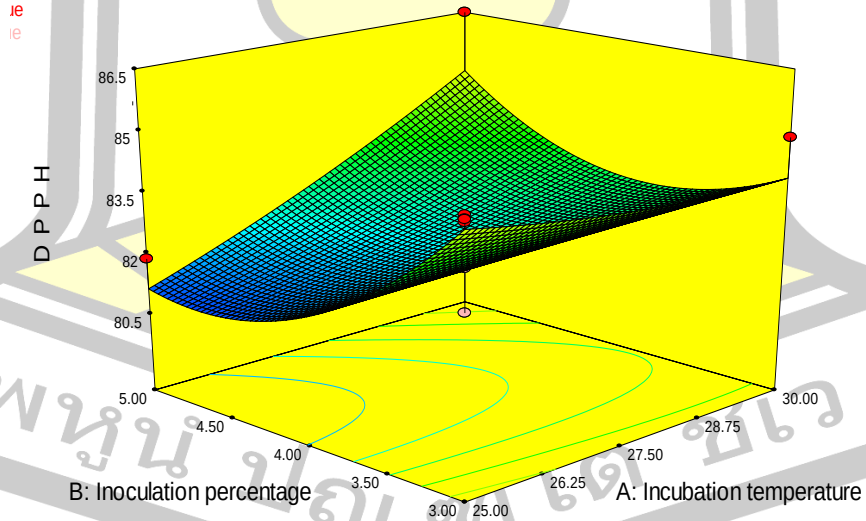


Figure 8 Response surface plots for the effect of (a) Incubation temperature, and (b) Inoculation percentage on DPPH scavenging activity.

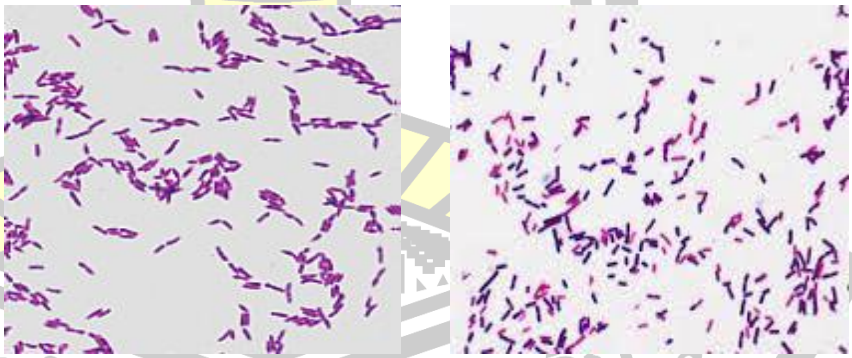
Gao *et al*. (2012) demonstrated that response surface methodology was effective in increasing kefir biomass production. They reported that optimal culture conditions were skim milk concentration 41.6%, temperature 30.05 °C; inoculation amount, 1.86%; time, 20 hr and shaker rotating speed 0 r/min, giving a growth rate of 14.33%, 39.4% more than initial. They also revealed that concentration, temperature and inoculation amount were significant factors for biomass production.

Moreover, Tabaraki and Nateghi (2011) studied ultrasonic technology for extraction of polyphenols and antioxidants from rice bran. They used response surface methodology (RSM) to optimize the experimental variables such as ethanol concentration (%v/v), extraction temperature (C) and extraction time (min). Results indicated that RSM was successful in optimizing the extraction conditions.

4.4 Biodiversity of microorganisms in kefir grains

4.4.1 Cell morphology

There was growth on all three media. On MRS + 0.05% Bromocresol purple there was growth of gram positive bacteria. On YPD there was growth of gram negative bacteria and on GYC there was growth of gram negative bacteria.



(Gram positive bacteria)

Figure 9 Morphology of bacteria

To confirm the presence of yeast, kanamycin 150 µg/150 ml was added to YPD agar plates. After the addition of kanamycin, there was no growth, confirming no presence of yeast.

Table 21 Cell morphology characteristics of bacterial isolates

Isolate	Gram stain	Morphology
A1	Gram positive(+)	Spherical cells
A2	Gram positive(+)	Rod shaped cells
A3	Gram positive(+)	Rod shaped cells
A4	Gram positive(+)	Round shaped cells
A5	Gram positive(+)	Spherical cells
A6	Gram positive(+)	Round shaped cells
A7	Gram positive(+)	Spherical cells
A9	Gram positive(+)	Rod shaped cells
A10	Gram positive(+)	Rod shaped cells
A11	Gram positive(+)	Round shaped cells
A12	Gram positive(+)	Spherical cells, short chains
L3	Gram positive(+)	Spherical cells, short chains
L4	Gram positive(+)	Spherical cells, short chains
L6	Gram positive(+)	Spherical cells, short chains
L7	Gram positive(+)	Spherical cells, short chains
L9	Gram positive(+)	Spherical cells, short chains
L10	Gram positive(+)	Spherical cells, short chains
L11	Gram positive(+)	Spherical cells, short chains
L12	Gram positive(+)	Spherical cells, short chains

L13	Gram positive(+)	Spherical cells, short chains
L14	Gram positive(+)	Spherical cells, short chains
L15	Gram positive(+)	Spherical cells, short chains

A = Acetic acid, L = Lactic acid

Lactococci are typically spherical or ovoid cells of size 1.2 µm by 1.5 µm, occurring in pairs and short chains. They are gram positive, non-motile and do not form spores.

Table 22 Microbial enumeration on three different media agars

Medium agar	**Microbial enumeration**	**Selected colonies/Total colonies**
MRS + BCP	3.6×10^5	27/36
GYC	3.6×10^5	12/36
YPD	nd.	0

4.4.2 Detection and identification of bacterial isolates

The selected PCR products were purified and sequenced. All 36 isolates were successfully sequenced, aligned with BLAST, and bacteria were identified to species level. Based on the PCR and gel electrophoresis results, out of the 36 isolates, 23 pure isolates were detected at 1,500 bp. Bacterial DNA were identified based on the size of the bp.

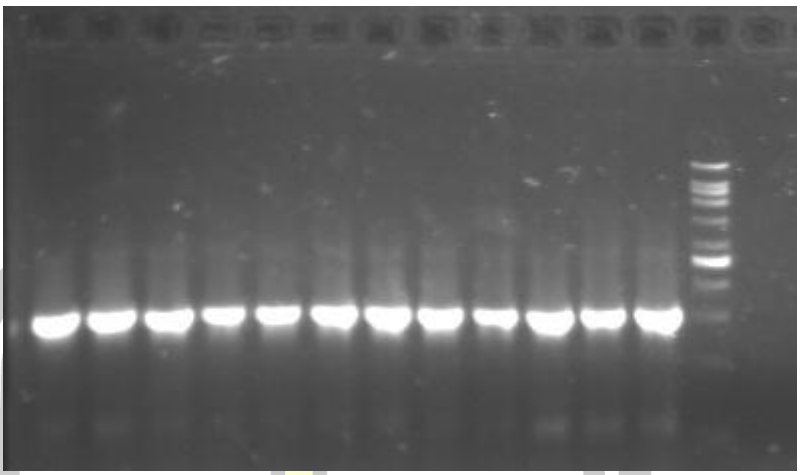


Figure 10 Bands generated from gel electrophoresis detection: 1,500 bp.

4.4.3 Identification of microorganisms

The microflora of kefir from Nonthaburi were analyzed based on the procedure of Anna *et al*. (2011). A total of 36 isolates were obtained and bacteria showing differences in their RAPD patterns were identified by 16 rDNA. The 23 bacterial isolates showing different RAPD patterns and 12 different bacterial species were identified as *Lactococcus lactis* strain Unkn111, *Lactococcus lactis* strain CAU: 2674, *Lactococcus lactis* strain RPWL3, *Lactococcus lactis* subsp. *lactis* strain NM146-2, *Bacillus* sp. strain abc48, *Bacterium* MRG-IF-3, *Lactococcus lactis* strain AF13, *Lactococcus lactis* strain PON37, *Lactococcus lactis* strain RCB476, *Lactococcus lactis* strain KLDS4.0602, *Lactococcus lactis* strain HadRami9 and *Lactococcus lactis* subsp. *lactis* strain UC77. Out of these 12 species, 10 species were *Lactococcus* and 1 *Bacillus*. These species showed differences in their RAPD pattern, as shown below. The identity and possible origin of 10 different species are shown in the appendices.

4.4.4 Microbial diversity of kefir (Nonthaburi, Thailand)

Based on the neighbor-joining method using the Tamura-Nei model (Tamura & Nei, 1993) with 1,000 bootstrap replications, a phylogenetic tree of 10 *Lactococcus* spp. was generated from the PCR-amplified bacterial 16S rDNA genes (ca. 900 bp). The scale bar represents 0.05% estimated distance in the figure below.

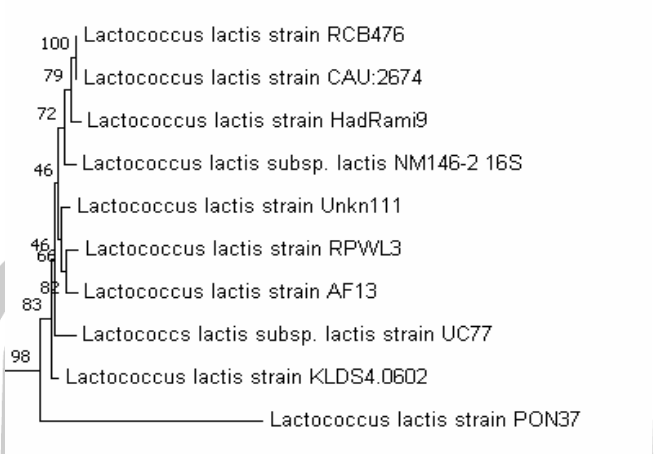


Figure 11 Phylogenetic tree of *Lactococcus sp.*

Camu *et al*. (2008) and Gulitz *et al*. (2011) stated that the use of different media can be justified because cultivation and enumeration of AAB from natural environments is sometimes considered problematic.

Pintado *et al*. (1996) and Witthuhn *et al*. (2004) reported that the use of a single medium gave very dissimilar results (from 0 to 10^8 CFU/ g). Therefore, the combined use of some selective culture media was suggested to provide a more complete picture of the culturable portion of AAB in kefir grains (Camu *et al*., 2008; Papalexandratou *et al*., 2011). Furthermore, Leite *et al*. (2013) reported that diversity in the macroscopic and microscopic views of kefir grains may be due to the origin of the grains sharing the kefir grain ecosystem.

Garofalo *et al*. (2015) found that *Lb. kefiranofaciens* was the main bacterial species found in Italian kefir grains and *Dekkera anomala* was the predominant yeast. They revealed the presence of the sub-dominant species ascribed to *St. thermophilus*, *Lc. lactis* and Acetobacter genera. In addition, they also identified *Lc. lactis*, *Enterococcus* sp., *Bacillus* sp., *A. fabarum*, *A. lovaniensis* and *A. orientalis* as part of the cultivable community. This confirmed the importance of the combination of culture-independent and culture-dependent approaches when studying microbial diversity in food, and how the combination of multiple 16S rRNA gene targets strengthens taxonomic identification by sequence-based identification approaches.

Chapter 5

Conclusions

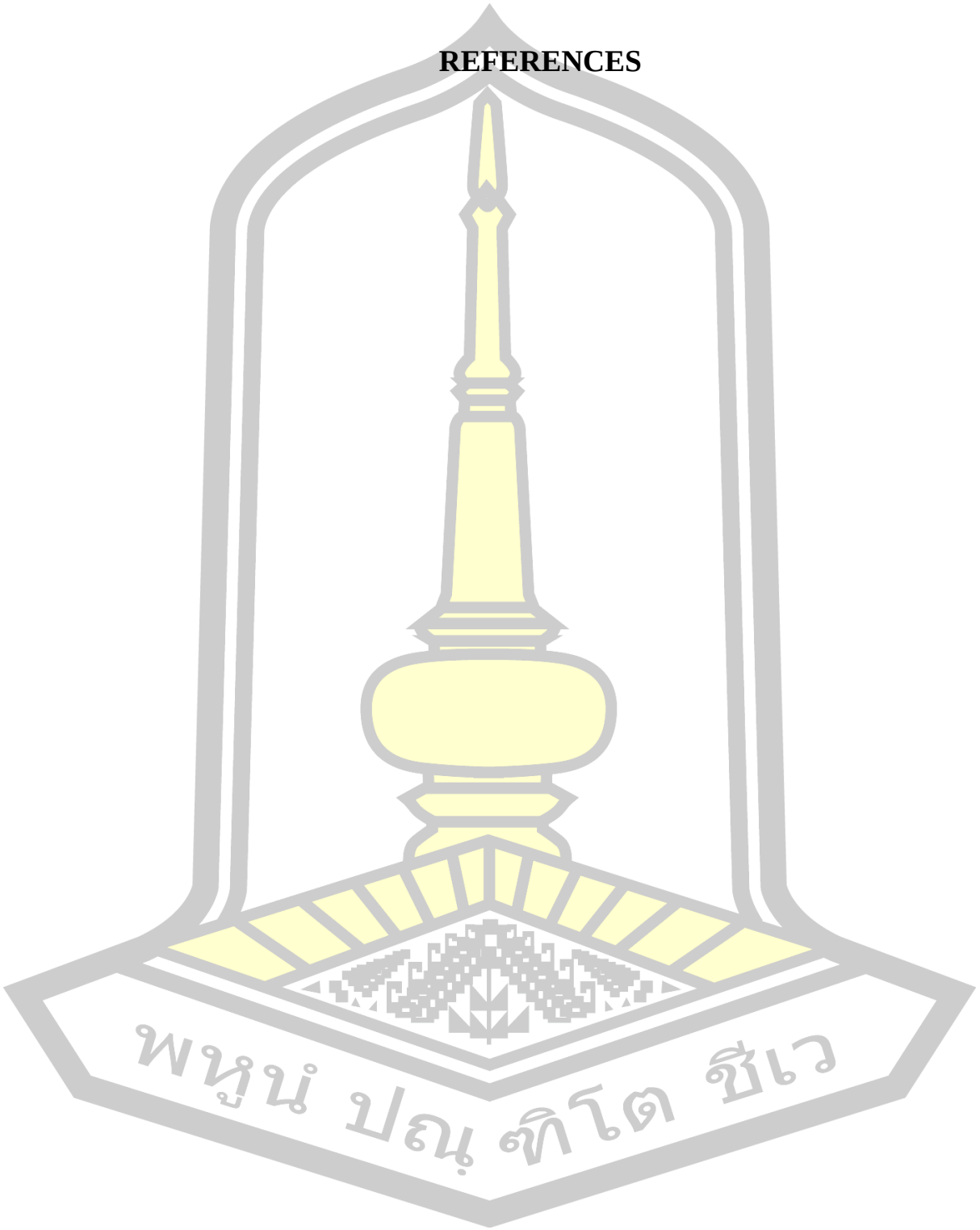
The characteristics and chemical compounds present in Thai rice milk and cow milk kefir were studied. Our results indicated that the pH ranged between 4.5 and 6, while viscosity ranged from 1.5 to 7 cps. The ultrasonication method was the most effective for extraction of volatile compounds and antioxidant activities.

It was found that rice milk kefir ($p \leq 0.05$) exhibited significantly higher antioxidant activity than cow milk kefir. DPPH scavenging was recorded between 55% and 89%, while results from FRAP assay were found between 2.5 and 3 (µg $FeSO_4$/ml) and total phenolic content ranged at 0.1 to 0.6 (mg GAE/ml).

Microbial analysis showed the presence of acetic acid bacteria and lactic acid bacteria in both rice milk and cow milk kefir from Nonthaburi but no presence of yeast. No lactic acid bacteria and yeast were found in rice milk and cow milk kefir from Kamphaeng Phet. Results from GC-MS analysis showed the presence of acids, amino acids and alcohols in variable amounts in both rice milk and cow milk kefir from Kamphaeng Phet and Nonthaburi, Thailand. Ethanol and acetic acid were found in almost all types of rice milk kefir. Our optimization of antioxidant activity by response surface methodology revealed that two factors as inoculation percentage and incubation temperature can modify phenolic contents and acetic acid bacteria population. Optimal conditions were incubation temperature 27.5 °C and inoculation percentage 4%.

Our findings revealed that volatile compounds in Thai rice milk kefir were potential antioxidants. Antioxidant rice milk kefir produced by RSM optimization can also be considered as a nutritional food additive containing probiotics or as a cosmetic ingredient.

REFERENCES



Ahmed, Z., Wang, Y., Ahmad, A., Khan, S. T., Nisa, M., Ahmad, H., & Afreen, A. (2013). Kefir and health: a contemporary perspective. *Critical Reviews in Food Science and Nutrition*, 53(5), 422–434.

Akowuah, G. A., Ismail, Z., Norhayati, I., & Sadikun, A. (2005). The effects of different extraction solvents of varying polarities on polyphenols of *Orthosiphon stamineus* and evaluation of the free radical-scavenging activity. *Food Chemistry*, 93(2), 311–317.

Angulo, L., Lopez, E., & Lema, C. (1993). Microflora present in kefir grains of the Galician region (North-West of Spain). *Journal of Dairy Research*, 60(2), 263–267.

Apak, R., Gorinstein, S., Bohm, V., Schaich, K. M., Ozyurek, M., & Guçlu, K. (2013). Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report). *Pure and Applied Chemistry*, 85(5), 957–998.

Arthitaya Kawee-ai. (2008). Exopolysaccharide production by *Lactobacillus confusus* CMU 198 using coconut water as substituted nitrogen and carbon sources. *Abstract. Chiang Mai University*, Available from: http://archive.lib.cmu.ac.th/full/T/2008/biot1008ak_abs.pdf

Banik, R. M., & Pandey, D. K. (2008). Optimizing conditions for oleanolic acid extraction from *Lantana camara* roots using response surface methodology. *Industrial Crops and Products*, 27(3), 241–248.

Barclay, L. R. C., Edwards, C. E., & Vinqvist, M. R. (1999). Media effects on antioxidant activities of phenols and catechols. *Journal of the American Chemical Society*, 121(26), 6226–6231.

Baş, D., & Boyacı, İ. H. (2007). Modeling and optimization I: Usability of response surface methodology. *Journal of Food Engineering*, 78(3), 836–845.

Berker, K. I., Guclu, K., Demirata, B., & Apak, R. (2010). A novel antioxidant assay of ferric reducing capacity measurement using ferrozine as the colour forming complexation reagent. *Analytical Methods*, 2(11), 1770.

Benzie, I. F. F., & Strain, J. J. (1999). Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods in Enzymology*, 299, 15–27.

Beshkova, D. M., Simova, E. D., Frengova, G. I., Simov, Z. I., & Dimitrov, Z. P. (2003). Production of volatile aroma compounds by kefir starter cultures. *International Dairy Journal*, 13(7), 529–535.

Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S., & Escaleira, L. A. (2008). Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta*, 76(5), 965–977.

Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature*, 181(4617), 1199–1200.

Brenner, D. J., Krieg, N. R., & Staley, J. T. (2005). *Bergey's Manual of Systematic Bacteriology*, the proteobacteria, part b: the gammaproteobacteria, (2)121-131.

Campbell, N., Reece, J., Urry, L., Cain, M., Wasserman, S., Minorsky, P., Jackson, R. (2009) Biology. *Pearson education*, USA, 636, 644, & 651.

Camel, V. (2000). Microwave-assisted solvent extraction of environmental samples. *TrAC Trends in Analytical Chemistry*, 19(4), 229–248.

Camu, N., González, A., De Winter, T., Van Schoor, A., De Bruyne, K., Vandamme, P., De Vuyst, L. (2007). Influence of turning and environmental contamination on the dynamics of populations of lactic acid and acetic acid bacteria involved in spontaneous cocoa bean heap fermentation in Ghana. *Applied and Environmental Microbiology*, 74(1), 86–98.

Cai, R., Arntfield, S. D., & Charlton, J. L. (1999). Structural changes of sinapic acid during alkali-induced air oxidation and the development of colored substances. *Journal of the American Oil Chemists' Society*, 76(6), 757–764.

Chandan, R. C. (2013). History and consumption trends. Manufacturing yogurt and fermented milks. *Dairy Food*, (2), 1–20. doi:10.1002/9781118481301.ch1

Chaudhary, R. C. (2003). Specialty rices of the world: Effect of WTO and IPR on its production trend and marketing. *Journal of Food, Agriculture and Environment*, 1(2), 34–41.

Chen, M.-H., Choi, S. H., Kozukue, N., Kim, H.-J., & Friedman, M. (2012). Growth-inhibitory effects of pigmented rice bran extracts and three red bran fractions

against human cancer cells: Relationships with composition and antioxidative activities. *Journal of Agricultural and Food Chemistry*, 60(36), 9151–9161.

Chen, H.-C., Wang, S.-Y., & Chen, M.-J. (2008). Microbiological study of lactic acid bacteria in kefir grains by culture-dependent and culture-independent methods. *Food Microbiology*, 25(3), 492–501.

Chemat, F., Tomao, V., & Virot, M. (2008). Ultrasound-assisted extraction in food analysis. Handbook of food analysis instruments. *CRC Press*, USA, 85-103.

Chotimarkorn, C., Benjakul, S., & Silalai, N. (2008). Antioxidant components and properties of five long-grained rice bran extracts from commercial available cultivars in Thailand. *Food Chemistry*, 111(3), 636–641.

Chung, H., & Shin, J. (2007). Characterization of antioxidant alkaloids and phenolic acids from anthocyanin-pigmented rice (*Oryza sativa* cv. Heugjinjubyeo). *Food Chemistry*, 104(4), 1670–1677.

Chunchom, S., Talubmook, C., & Deeseenthum, S. (2017). Antioxidant activity, biochemical components and sub-chronic toxicity of different brown rice kefir powders. *Pharmacognosy Journal*, 9(3), 388–394.

Colak, H., Hampikyan, H., Ulusoy, B., & Bingol, E. B. (2007). Presence of listeria monocytogenes in Turkish style fermented sausage (sucuk). *Food Control*, 18(1), 30–32.

Cruz Ramos, H., Hoffmann, T., Marino, M., Nedjari, H., Presecan-Siedel, E., Dreesen, O., John, D. (2000). Fermentative metabolism of *Bacillus subtilis*: physiology and regulation of gene expression. *Journal of Bacteriology*, 182(11), 3072–3080.

Chairote, E., Chairote, G., Niamsup, H., & Lumyong, S. (2008). The presence and the content of monacolins in red yeast rice prepared from Thai glutinous rice. *World Journal of Microbiology and Biotechnology*, 24(12), 3039–3047.

Deeseenthum, S. and Pejovic, J. (2010). Bacterial inhibition and antioxidant activity of kefir produced from Thai jasmine rice milk. *Biotechnology*, 9(3), 332-337.

Dousset, X. and Caillet, F. (1993). Aspects microbiologiques et biochimiques de la fermentation du kefir, *Microbiologie Aliments Nutrition* ,11, 463-470.

Fardet, A., Rock, E., & Rémésy, C. (2008). Is the in vitro antioxidant potential of whole-grain cereals and cereal products well reflected in vivo? *Journal of Cereal Science*, 48(2), 258-276.

Farnworth, ER. (2005). Kefir: a complex probiotic. *Food Science and Technology Bulletin*. 2(1), 1-17.

Farnworth, E. R. (2008). The future for fermented foods. *Functional Foods and Nutraceuticals*, (2), 361–378. doi:10.1201/9780203009727.ch15i

Fitday 2013. What is rice milk and is rice milk good for you? [Online]. Available from: http://www.fitday.com/fitness-articles/nutrition/healthy-eating/what-is-rice-milk-and-is-rice-milk-good-foryou.html#b [Accessed 10 Aug 2013].

Fujisawa, T., Adachi, S., Toba, T., Arihara, K. and Mitsuoka, T. (1988). *Lactobacillus*

kefiranofaciens sp. nov. isolated from kefir grains. *International Journal of Systematic Bacteriology*, 38(1), 12–14.

Gao, J., Gu, F., Ruan, H., Chen, Q., He, J., & He, G. (2012). Culture conditions optimization of Tibetan kefir grains by response surface methodology. *Procedia Engineering*, 37, 132–136.

Garofalo, C., Osimani, A., Milanović, V., Aquilanti, L., De Filippis, F., Stellato, G., Clementi, F. (2015). Bacteria and yeast microbiota in milk kefir grains from different Italian regions. *Food Microbiology*, 49, 123–133.

Garrote, G.L., Abraham, A.G. and De Antoni, G.L. (2001). Chemical and microbiological characterization of kefir grains. *Journal of Dairy Research*, 68(4), 639–652.

Garrote, G. L., Abraham, A. G., & De Antoni, G. L. (1997). Preservation of kefir grains, a comparative study. LWT - *Food Science and Technology*, 30(1), 77–84.

Gibson, G. R., & Roberfroid, M. B. (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *The Journal of Nutrition*, 125(6), 1401–1412.

Giraffa G. (1994). Microbial polysaccharides produced by lactic acid bacteria in the dairy industry, *Industrie Alimentari*, 33, 295-298.

Goffman, F., & Bergman, C. (2004). Rice kernel phenolic content and its relationship with antiradical efficiency. *Journal of the Science of Food and Agriculture*, 84(10), 1235– 1240.

Goufo, P., Pereira, J., Moutinho-Pereira, J., Correia, C. M., Figueiredo, N., Carranca, C., Trindade, H. (2014). Rice (*Oryza sativa* L.) phenolic compounds under elevated carbon dioxide (CO_2) concentration. *Environmental and Experimental Botany*, 99, 28–37.

Goufo, P., & Trindade, H. (2014). Rice antioxidants: phenolic acids, flavonoids, anthocyanins, proanthocyanidins, tocopherols, tocotrienols, γ-oryzanol, and phytic acid. *Food Science & Nutrition*, 2(2), 75–104.

Guillamón, J. M., & Mas, A. (Eds.). (2009). Acetic acid bacteria. Biology of microorganisms on grapes, in must and in wine. *Springer*, Berlin, Heidelberg, 2, 31–46.

Gulitz, A., Stadie, J., Wenning, M., Ehrmann, M. A., & Vogel, R. F. (2011). The microbial diversity of water kefir. *International Journal of Food Microbiology*, 151(3), 284–288.

Gunaratne, A., Wu, K., Li, D., Bentota, A., Corke, H., & Cai, Y.-Z. (2013). Antioxidant activity and nutritional quality of traditional red-grained rice varieties containing proanthocyanidins. *Food Chemistry*, 138(2-3), 1153–1161.

Guzel-Seydim, Z. B., Kok-Tas, T., Greene, A. K., & Seydim, A. C. (2011). Review: Functional properties of kefir. *Critical Reviews in Food Science and Nutrition*, 51(3), 261–268.

Halliwell, B., & Gutteridge, J. M. C. (1989). Free radicals in biology and medicine. *Journal of Free Radicals in Biology & Medicine*, 1(4), 331–332.

Harper, S. L., Walling, J. F., Holland, D. M., & Pranger, L. J. (1983). Simplex optimization of multi-element ultrasonic extraction of atmospheric particulates. *Analytical Chemistry*, 55(9), 1553–1557.

Heuberger, A. L., Lewis, M. R., Chen, M.-H., Brick, M. A., Leach, J. E., & Ryan, E. P. (2010). Metabolomics and functional genomic analyses reveal varietal differences in bioactive compounds of cooked rice. *The Public Library of Science*, 5(9), e12915.

Hirawan, R., Diehl-Jones, W., & Beta, T. (2011). Comparative evaluation of the antioxidant potential of infant cereals produced from purple wheat and red rice grains and LC-MS analysis of their anthocyanins. *Journal of Agricultural and Food Chemistry*, 59(23), 12330–12341.

Ho, C. W., Chew, T. K., Ling, T. C., Kamaruddin, S., Tan, W. S., & Tey, B. T. (2006). Efficient mechanical cell disruption of *Escherichia coli* by an ultrasonicator and recovery of intracellular hepatitis B core antigen. *Process Biochemistry*, 41(8), 1829–1834.

Hogg, J. S., Lohmann, D. H., & Russell, K. E. (1961). The kinetics of reaction of 2, 2-Diphenyl-1-Picrylhydrazyl with phenols. *Canadian Journal of Chemistry*, 39(8), 1588–1594.

Hommel R.K., Ahnert P. (2004). Encyclopedia of Food Microbiology. vol. 1 (eds. C. Batt, P.Patel, R. Robinson). *Elsevier*, New York, 1–7.

Huang, D., Ou, B., & Prior, R. L. (2005). The Chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry*, 53(6), 1841–1856.

Itani, T., & Ogawa, M. (2004). History and recent trends of red rice in Japan.

Japanese Journal of Crop Science, 73(2), 137–147.

Iqbal, S., Bhanger, M. I., & Anwar, F. (2005). Antioxidant properties and components of some commercially available varieties of rice bran in Pakistan. *Food Chemistry*, 93(2), 265–272.

Irakli, M. N., Samanidou, V. F., Biliaderis, C. G., & Papadoyannis, I. N. (2012). Simultaneous determination of phenolic acids and flavonoids in rice using solid-phase extraction and RP-HPLC with photodiode array detection. *Journal of Separation Science*, 35(13), 1603–1611.

Johnston, M. A., & Delwiche, E. A. (1965). Distribution and characteristics of the catalases of *Lactobacillaceae*. *Journal of Bacteriology*, 90(2), 347–351.

Jun, H.-I., Song, G.-S., Yang, E.-I., Youn, Y., & Kim, Y.-S. (2012). Antioxidant activities and phenolic compounds of pigmented rice bran extracts. *Journal of Food Science*, 77(7), 759–764.

Juntachote, T., Berghofer, E., Bauer, F., & Siebenhandl, S. (2006). The application of response surface methodology to the production of phenolic extracts of lemon grass, galangal, holy basil and rosemary. *International Journal of Food Science and Technology*, 41(2), 121–133.

Kandler, O., & Kunath, P. (1983). Lactobacillus kefir sp.nov. A component of the microflora of Kefir. *Systematic and Applied Microbiology*, 4(2), 286–294.

Kandler, O., N and Weiss. (1986). In: Bergey's manual of systematic bacteriology, (Eds), Vol. 2, *Baltimore: Williams and Wilkins*, 1209 – 1234.

Kesenkas, H., Dinkci, N., Seckin, K., Kinik, O., Gönc, S., Ergönül, P. G., & Kavas,

G. (2011) “Physicochemical, microbiological and sensory characteristics of soymilk kefir”. *African Journal of Microbiology Research*, 5, 3737–3746.

Kemp, N. (1984). Kefir, the champagne of cultured dairy products. *Cultured Dairy Products Journal*, 29-30.

Korea Food Additives Code Propionic acid. (2005). Food additives code. Seoul, Korea. *The annual Report of KFDA*.

Koroleva, N.S. (1991). Products prepared with lactic acid bacteria and yeasts. In: Robinson, R.K., editor. Therapeutic properties of fermented milks: 159-179.*Elsevier Applied Sciences Publishers*, London, UK.

Koroleva NS. (1998). Starters for fermented milks. Section 4, kefir and kumys starters. Bulletin of the IDF 227, Chapter 2. *International Dairy Federation*, Brussels, Belgium.

Koroleva, N.S. (1982.) Special products (kefir, koumyss, etc.). Proceedings XXI *International Dairy Congress*, Moscow, 2: 146-151.

Kosikowski, F.V. and Mistry, V.V. (1997). Cheese and fermented milk foods.1, origins and principles (3Ed). *F.V. Wesport*, Connecticut, USA, 728.

Kumar, S., Tamura, K., &Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*, 5, 150-163.

La Riviere, J. W. M.; Kooiman, P.; Schmidt, K. Kefiran. (1967). A noval polysaccharide produced in the kefir grain by *Lactobacillus brevis*. *Archives of Microbiology*.59: 269-278.

Lee, J.-C., Kim, J.-D., Hsieh, F., & Eun, J.-B. (2008). Production of black rice cake using ground black rice and medium-grain brown rice. *International Journal of Food Science & Technology*, 43(6), 1078–1082.

Leite, A. M. de O., Miguel, M. A. L., Peixoto, R. S., Rosado, A. S., Silva, J. T., & Paschoalin, V. M. F. (2013). Microbiological, technological and therapeutic properties of kefir: a natural probiotic beverage. Brazilian Journal of Microbiology, 44(2), 341–349.

Li, C., Salas, W., DeAngelo, B., & Rose, S. (2006). Assessing alternatives for mitigating net greenhouse gas emissions and increasing yields from rice production in China over the next twenty years. *Journal of Environmental Quality*. 35: 1554-1565.

Lin, C. W.; Chen, C. L.; Liu, J. R. (1999). Identification and characterization of lactic acid bacteria and yeasts isolated from kefir grains in Taiwan. *The Australian Journal of Dairy Technology*, 54: 14-18.

Liu, J.-R., Lin, Y.-Y., Chen, M.-J., Chen, L.-J., & Lin, C.-W. (2005 a). Antioxidative activities of kefir. *Asian-Australasian Journal of Animal Sciences*, 18(4), 567–573.

Liu, J.-R., Chen, M.-J., & Lin, C.-W. (2005 b). Antimutagenic and antioxidant properties of milk−kefir and soymilk−kefir. *Journal of Agricultural and Food Chemistry*, 53(7), 2467–2474.

Liu, J.-R., Chen, M.-J., & Lin, C.-W. (2002). Characterization of polysaccharide and volatile compounds produced by kefir grains grown in soymilk. *Journal of Food Science*, 67(1), 104–108.

Liu, J.-R., & Lin, C.-W. (2000). Production of kefir from soymilk with or without added glucose, lactose, or sucrose. *Journal of Food Science*, 65(4), 716–719.

Luang-In, V., Saengha, W., Yotchaisarn, M., Halaslova, M., Udomwong, P., & Deeseenthum, S. (2018). Microbial strains and bioactive exopolysaccharide producers from Thai water kefir. *Microbiology and Biotechnology Letters*, 46(4), 403–415.

Litwinienko, G and Ingold K. U. (2003). Abnormal solvent effects on hydrogen atom abstractions. 1, the reactions of phenols with 2, 2-diphenyl-1-picrylhydrazyl (dpph) in alcohols. *The Journal of Organic Chemistry*, 68, 3433.

Liyana-Pathirana, C, Shahidi, F. (2005). Optimization of extraction of phenolic compounds from wheat using response surface methodology. *Food Chemistry*, 93(1), 47–56.

Laokuldilok, T., Shoemaker, C. F., Jongkaewwattana, S., & Tulyathan, V. (2011). Antioxidants and antioxidant activity of several pigmented rice brans. *Journal of Agricultural and Food Chemistry*, 59(1), 193–199.

Magalhães, K. T., Pereira, M. A., Nicolau, A., Dragone, G., Domingues, L., Teixeira, J. A., Schwan, R. F. (2010). Production of fermented cheese whey-based beverage using kefir grains as starter culture: Evaluation of morphological and microbial variations. *Bio resource Technology*, 101(22), 8843–8850.

Mainville, I., Robert, N., Lee, B., & Farnworth, E. R. (2006). Polyphasic characterization of the lactic acid bacteria in kefir. *Systematic and Applied Microbiology*, 29(1), 59–68.

Margulis L. (1996). From kefir to death. In: How things are. J. Brockman and K. Matson, Eds. *William Morrow and co*, New York, 69-78.

Marshall, V. M., & Cole, W. M. (1985). Methods for making kefir and fermented milks based on kefir. *Journal of Dairy Research*, 52(3), 451–456.

McGowan, J. C. Powell, T. Raw, R. (1959). The rates of reaction of αα- diphenyl-β – picrylhydrazyl with certain amines and phenols. *Journal of the Chemical Society*. 3103.

Min, B., McClung, A. M., & Chen, M.-H. (2010). Phytochemicals and antioxidant capacities in rice brans of different color. *Journal of Food Science*, 76(1), 117–126.

Motaghi, M., Mazaheri, M., Moazami, N., Farkhondeh, A., Fooladi, M.H., and Goltapeh, E.M. (1997). Short communication: kefir production in Iran. *World Journal of Microbiology and Biotechnology* 13: 579- 581.

Neve H. (1992). Analysis of kefir grain starter cultures by scanning electron microscopy. *Milchwissenschaft*, 47: 275-278.

Ottogalli, G., Galli, A., Resmini, P. and Volonterio, G. (1973). Composizione microbiologia, chimica ed ultrastruttura dei ganuli di kefir. *Annali di microbiologia ed enzimologia*, 23: 109-121.

Pandey, K. B., Mehdi, M. M., Maurya, P. K., & Rizvi, S. I. (2010). Plasma protein oxidation and its correlation with antioxidant potential during human aging. *Disease Markers*, 29(1), 31–36.

Papariello, G. J., & Janish, M. A. M. (1966). Diphenylpicrylhydrazyl as an organic analytical reagent in the spectrophotometric analysis of phenols. *Analytical Chemistry*, 38(2), 211–214.

Papalexandratou, Z., Falony, G., Romanens, E., Jimenez, J.C., Amores, F., Daniel, H.-M., De Vuyst, L. (2011). Species diversity, community dynamics, and metabolite kinetics of the microbiota associated with traditional Ecuadorian spontaneous cocoa bean fermentations. *Applied Environmental Microbiology*. 77 (21), 7698-7714,

Pinelo, M., Rubilar, M., Jerez, M., Sineiro, J., & Núñez, M. J. (2005). Effect of solvent, temperature, and solvent-to-solid ratio on the total phenolic content and antiradical activity of extracts from different components of grape pomace. *Journal of Agricultural and Food Chemistry*, 53(6), 2111–2117.

Pintado, M. E., Da Silva, J. A. L., Fernandes, P. B., Malcata, F. X., & Hogg, T. A. (1996). Microbiological and rheological studies on Portuguese kefir grains. *International Journal of Food Science and Technology*, 31(1), 15–26.

Pitija, K., Nakornriab, M., Sriseadka, T., Vanavichit, A., & Wongpornchai, S. (2012). Anthocyanin content and antioxidant capacity in bran extracts of some Thai black rice varieties. *International Journal of Food Science & Technology*, 48(2), 300–308.

Powell, J. E., Witthuhn, R. C., Todorov, S. D., & Dicks, L. M. T. (2007). Charact -erization of bacteriocin ST8KF produced by a kefir isolate *Lactobacillus plantarum* ST8KF. *International Dairy Journal*, 17(3), 190–198.

Qiu, Y., Liu, Q., & Beta, T. (2010). Antioxidant properties of commercial wild rice and analysis of soluble and insoluble phenolic acids. *Food Chemistry*, 121(1), 140–147.

Roberfroid, M.B. (2013). Prebiotic fibre. [Online]. Available from: http://www.prebiotic.ca/prebiotic_fibre.html. [Accessed 12 Aug, 2013]

Rosi, J. and Rossi, J. (1978). Microrganismi del kefir: I fermenti lattice. *Sciezae Tecnica Lattiero-Casearia*, 29: 291-305.

Srivastava, R., Mukerjee, A., & Verma, A. (2015). GC-MS analysis of phytocomponents in pet ether fraction of *Wrightia tinctoria* seed. *Pharmacognosy Journal*, 7(4), 249–253.

Santos, A., San Mauro, M., Sanchez, A., Torres, J. M., & Marquina, D. (2003). The antimicrobial properties of different strains of *Lactobacillus* spp. isolated from kefir. *Systematic and Applied Microbiology*, 26(3), 434–437.

Sarkar, S. (2008). Biotechnological innovations in kefir production: a review. *British Food Journal*, 110(3), 283–295.

Schoevers, A., & Britz, T. J. (2003). Influence of different culturing conditions on kefir grain increase. *International Journal of Dairy Technology*, 56(3), 183–187.

Semih Ot-es and Oz-em Cagindi C. (2003). Kefir: A probiotic dairy-composition, nutritional and therapeutic aspects. *Pakistan Journal of Nutrition*, 2(2), 54–59.

Setyaningsih, W., Duros, E., Palma, M., & Barroso, C. G. (2016). Optimization of the ultrasound-assisted extraction of melatonin from red rice (*Oryza sativa*) grains through a response surface methodology. *Applied Acoustics*, 103, 129–135.

Shen, Y., Jin, L., Xiao, P., Lu, Y., & Bao, J. (2009). Total phenolics, flavonoids, antioxidant capacity in rice grain and their relations to grain color, size and weight. *Journal of Cereal Science*, 49(1), 106–111.

Shen, Z., Palmer, M. V., Ting, S. S. T., & Fairclough, R. J. (1997). Pilot Scale extraction and fractionation of rice bran oil using supercritical carbon dioxide. *Journal of Agricultural and Food Chemistry*, 45(12), 4540–4544.

Singleton, VL and Ross, JA. (1965). Colorimetry of Total phenolics with phosphomolybdic phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, 16: 144-158.

Silva, E.M, Rogez, H, Larondelle, Y. (2007). Optimization of extraction of phenolics from *Inga edulis* leaves using response surface methodology. *Separation and Purification Technology*, 55(3), 381–387.

Simova, E., Beshkova, D., Angelov, A., Hristozova, T., Frengova, G., & Spasov, Z. (2002). Lactic acid bacteria and yeasts in kefir grains and kefir made from them. Journal of Industrial *Microbiology & Biotechnology*, 28(1), 1–6.

St-Onge, M.-P., Farnworth, E. R., Savard, T., Chabot, D., Mafu, A., & Jones, P. J. (2002). Kefir consumption does not alter plasma lipid levels or cholesterol fractional synthesis rates relative to milk in hyperlipidemic men: a randomized controlled trial [ISRCTN10820810]. *BMC Complementary and Alternative Medicine*, 2(1).

Steffen, Chr. (1971). Methods for the determination of total lactic acid and the lactate configuration in cheese and milk, Switzerland. *Milk Newspaper* 97, 1073-1078.

Sun, Y.-X., Liu, J.-C., & Kennedy, J. F. (2010). Extraction optimization of antioxidant polysaccharides from the fruiting bodies of *Chroogomphis rutilus* (Schaeff. Fr.) O.K. Miller by box-behnken statistical design. *Carbohydrate Polymers*, 82(1), 209–214.

Suzuki, M., Kimura, T., Yamagishi, K., Shinmoto, H., & Yamaki, K. (2004). Comparison of mineral contents in 8 cultivars of pigmented brown rice. *Nippon Shokhhin Kagaku Kogaku Kaishi*, 51(8), 424–427.

Tabaraki, R., & Nateghi, A. (2011). Optimization of ultrasonic-assisted extraction of natural antioxidants from rice bran using response surface methodology. *Ultrasonics Sonochemistry*, 18(6), 1279–1286.

Takizawa, S., Kojima, S., Tamura, S., Fujinaga, S., Benno, Y. and Nakase, T. (1994). *Lactobacillus kefirgranum sp*. nov and *Lactobacillus parakefir sp.* nov., two new species from kefir grains. *International Journal of Systematic Bacteriology*, 44(3), 435–439.

Toma, M., Vinatoru, M., Paniwnyk, L., & Mason, T. (2001). Investigation of the effects of ultrasound on vegetal tissues during solvent extraction. *Ultrasonics Sonochemistry*, 8(2), 137–142.

Thoreux, K., & Schmucker, D. L. (2001). Kefir milk enhances intestinal immunity in young but not old rats. *The Journal of Nutrition*, 131(3), 807–812.

Wanyo, P., Kaewseejan, N., Meeso, N., & Siriamornpun, S. (2016). Bioactive compounds and antioxidant properties of different solvent extracts derived from Thai rice by-products. *Applied Biological Chemistry*, 59(3), 373–384.

Ye, X. Y., & Ng, T. B. (2000). Purification and characterization of glycolactin, a novel glycoprotein from bovine milk. *Life Sciences*, 66(13), 1177–1186.

Yoshida, T., & Toyoshima, K. (1994). Lactic acid bacteria and yeast from kefir. *Nippon Eiyo Shokuryo Gakkaishi*, 47(1), 55–59.

Yuksekgag, Z. N., Beyath, Y., & Aslım, B. (2004). Metabolic activities of *Lactobacillus* spp. strains isolated from kefir. *Nahrung/Food*, 48(3), 218–220.

Zajšek, K., Goršek, A., & Kolar, M. (2013). Cultivating conditions effects on kefiran production by the mixed culture of lactic acid bacteria imbedded within kefir grains. *Food Chemistry*, 139(1-4), 970–977.

Zhou, J., Liu, X., Jiang, H., & Dong, M. (2009). Analysis of the microflora in Tibetan kefir grains using denaturing gradient gel electrophoresis. *Food Microbiology*, 26(8), 770–775.

Zheng, W., & Wang, S. Y. (2001). Antioxidant activity and phenolic compounds in selected herbs. *Journal of Agricultural and Food Chemistry*, 49(11), 5165–5170.

Zigoneanu, I. G., Williams, L., Xu, Z., & Sabliov, C. M. (2008). Determination of antioxidant components in rice bran oil extracted by microwave-assisted method. *Bioresource Technology*, 99(11), 4910–4918.

APPENDICES

APPENDIX A

Solution preparation

1. Preparation of reagents for Total Phenolic content

a. Preparation of 10% Folin-Ciocalteu reagent: A 10% Folin-Ciocalteu reagent was prepared by diluting 10 mL of Folin-Ciocalteu reagent in 900 mL of deionized water.

b. Preparation of 10% Sodium carbonate: A 10% sodium carbonate solution was prepared by dissolving 10.0502 g of 99.5% Na2CO3 in 100 mL of deionized water.

c.Preparation of standard Stock (2 mg mL-1) gallic acid: Standard stock solution (2 mg mL-1) of gallic acid was prepared by dissolving 0.051g of 98% gallic acid and made up to ending volume with deionized water in 25 mL volumetric flask.

2. Preparation of reagents for Free- radical scavenging activity (DPPH) assay

a. Preparation of 0.1 mM DPPH (MW= 394.33): A 0.1 mM DPPH was prepared by dissolving 0.0232 g of 85% DPPH in 500 mL and made up to volume with methanol in 500 mL volumetric flask.

b. Preparation of Stock standard 2 mg ml-1 BHA: Stock standard solution (2 mg mL-1) of BHA was prepared by dissolving 0.0521 g of 96% BHA and made up to volume with methanol in 25 mL volumetric flask.

3. Preparation of reagents for Ferric reducing antioxidant power (FRAP) assay

a. Preparation of 300 mM Sodium acetate buffer, pH 3.6: A 0.025 M sodium acetate buffer (pH 3.6) solution was prepared by dissolving 24.624 g of CH3COONa•3H2O in 500 mL of deionized water. The pH value of 0.3 M of the solution was adjusted by using CH3COOH and made up to volume with deionized water in a 1000 mL volumetric flask.

b. Preparation of 10 mM TPTZ (MW= 312.32): A 10 mM TPTZ solution was prepared by dissolving 0.0789 g of 99% TPTZ in 25 mL and made up to volume with 40 mM HCl in 25 mL volumetric flask.

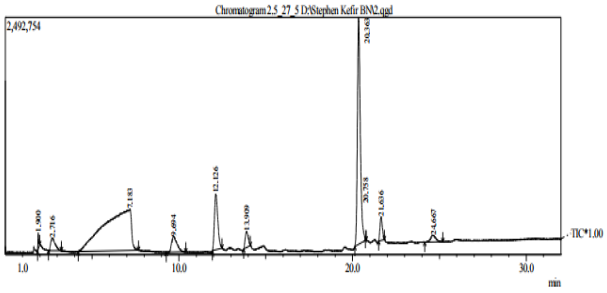
c. Preparation of 20 mM Ferric chloride (MW= 162.21): A 20 mM ferric chloride solution was prepared by dissolving 0.1655 g of 98% FeCl3 in 50 mL and made up to ending volume with deionized water in 50 mL volumetric flask.

d. Preparation of 40 mM Hydrochloric acid (MW= 36.441; 37%; d= 1.19): A 40 mM hydrochloric acid was prepared by dilute 3.30 mL of 37% HCl in 1000 mL and made up to volume with deionized water in 1000 mL volumetric flask.

e. Preparation of 10 mM Ferrous sulphate: Standard stock solution of 10 mM FeSO4 was prepared by dissolving 0.0140 g of 99% FeSO4•7H2O in 5 mL and made up to ending volume with methanol in 5 mL volumetric flask.

APPENDIX B

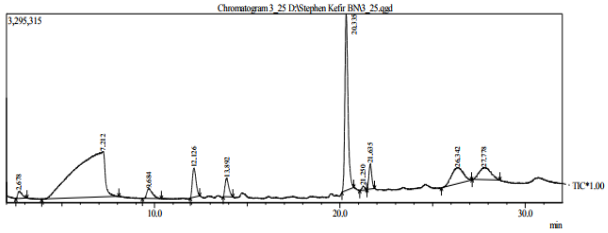
GC-MS output of Volatile compounds after optimization



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	1.900	1.858	1.983	577184	0.60	170342	3.95	3.39		
2	2.716	2.492	3.233	2255260	2.36	132382	3.07	17.04	MI	
3	7.183	4.200	7.675	47539351	49.68	431109	9.99	110.27	MI	
4	9.694	9.275	10.400	3544079	3.70	164817	3.82	21.50	MI	
5	12.126	11.933	12.475	7924900	8.28	586473	13.59	13.51		
6	13.909	13.733	14.117	2020014	2.11	167806	3.89	12.04		
7	20.363	20.133	20.775	27863899	29.12	2340130	54.21	11.91	S	
8	20.758	20.742	20.775	5948	0.01	7681	0.18	0.77	T	
9	21.636	21.500	21.842	2424028	2.53	245001	5.68	9.89		
10	24.667	24.167	25.192	1531089	1.60	71094	1.65	21.43	MI	
				95685752	100.00	4316835	100.00			

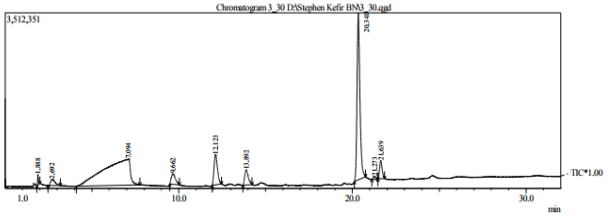
Figure A: GC-MS Output of Run 1



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	2.678	2.517	3.108	1914395	1.12	125547	2.08	15.23	MI	
2	7.212	3.908	8.067	90949720	53.04	789240	13.11	115.22	MI	
3	9.684	9.342	10.367	3533585	2.06	169026	2.81	20.91	MI	
4	12.126	11.925	12.442	6985721	4.07	510393	8.48	13.69		
5	13.892	13.708	14.225	4522411	2.64	324605	5.39	13.93		
6	20.335	20.117	20.750	36519404	21.30	3079658	51.14	11.86		
7	21.250	21.067	21.442	677719	0.40	75313	1.25	8.99	MI	
8	21.635	21.467	21.867	4760003	2.78	445784	7.40	10.68		
9	26.342	25.483	27.100	12347689	7.20	285275	4.74	42.93	MI	
10	27.778	27.142	28.625	9272907	5.41	216860	3.60	42.77	MI	
				171483554	100.00	6021701	100.00			

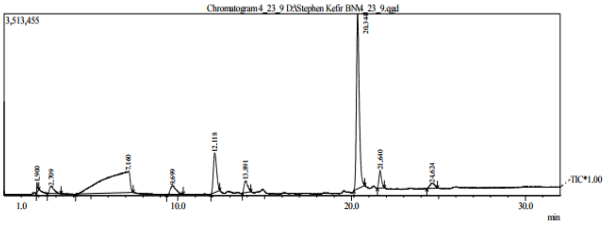
Figure B: GC-MS Output of Run 2



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	1.888	1.833	2.000	765518	0.65	211102	3.67	3.63		
2	2.692	2.475	3.175	2106715	1.79	129405	2.25	16.28	MI	
3	7.094	4.108	7.758	55956369	47.59	528471	9.19	105.87	MI	
4	9.662	9.475	10.042	3498988	2.98	211865	3.69	16.52		
5	12.123	11.925	12.467	8312284	7.07	607722	10.57	13.68		
6	13.892	13.692	14.217	4180437	3.56	301069	5.24	13.89		
7	20.341	20.117	20.750	38494357	32.74	3339249	58.08	11.53		
8	21.273	21.117	21.467	556842	0.47	61092	1.06	9.12	MI	
9	21.639	21.475	21.850	3698721	3.15	359136	6.25	10.30		
				117570231	100.00	5749111	100.00			

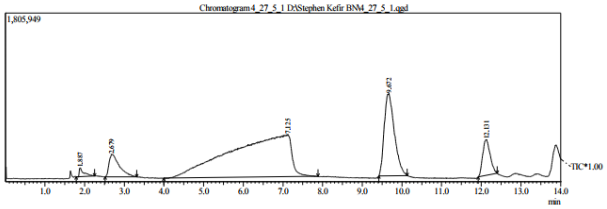
Figure C: GC-MS Output of Run 3



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	1.900	1.858	2.008	743318	0.69	191122	3.34	3.89		
2	2.709	2.492	3.283	2430927	2.24	139033	2.43	17.48	MI	
3	7.160	4.117	7.442	44412249	40.98	412166	7.20	107.74	MI	
4	9.699	9.358	10.317	3689818	3.40	170726	2.98	21.61	MI	
5	12.118	11.917	12.417	10149227	9.37	763673	13.34	13.29		
6	13.891	13.717	14.208	3173547	2.93	229007	4.00	13.86		
7	20.344	20.133	20.750	38473509	35.50	3375933	58.96	11.40		
8	21.640	21.483	21.892	3396288	3.13	341108	5.96	9.96		
9	24.624	24.333	24.933	1897213	1.75	102653	1.79	18.48	MI	
				108366096	100.00	5725421	100.00			

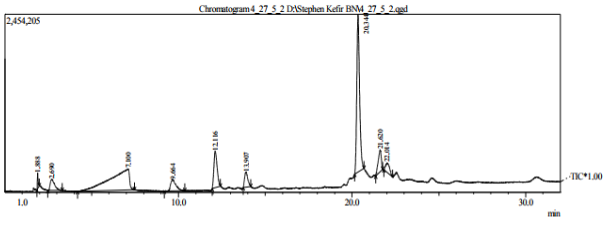
Figure D: GC-MS Output of Run 4



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	1.887	1.792	2.250	718795	0.97	95105	4.62	7.55	MI	
2	2.679	2.517	3.317	4344758	5.88	242072	11.77	17.95	MI	
3	7.125	4.000	7.883	48205698	65.20	454397	22.09	106.08	MI	
4	9.672	9.417	10.133	15656809	21.18	881400	42.86	17.76		
5	12.131	11.925	12.400	5010326	6.78	383680	18.66	13.06		
				73936386	100.00	2056654	100.00			

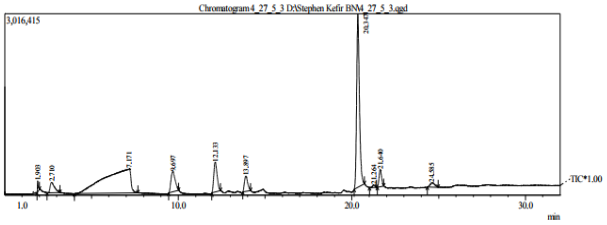
Figure E: GC-MS Output of Run 5



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	1.888	1.850	1.983	744947	1.08	218876	5.28	3.40		
2	2.690	2.467	3.300	2619068	3.81	152992	3.69	17.12	MI	
3	7.100	4.183	7.475	22801114	33.18	295568	7.13	77.14	MI	
4	9.664	9.192	10.367	3183366	4.63	152267	3.67	20.91	MI	
5	12.116	11.942	12.408	6512798	9.48	509160	12.29	12.79		
6	13.907	13.725	14.158	2634025	3.83	208375	5.03	12.64		
7	20.344	20.150	20.683	24495281	35.64	2172601	52.42	11.27		
8	21.620	21.358	21.758	3762160	5.47	303545	7.32	12.39		
9	22.014	21.842	22.342	1970754	2.87	130934	3.16	15.09	MI	
				68723513	100.00	4144318	100.00			

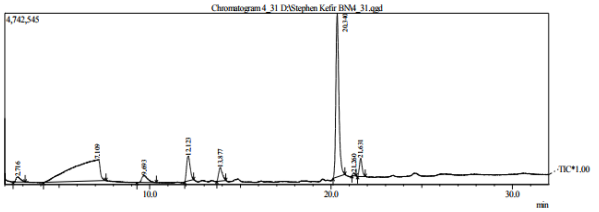
Figure F: GC-MS Output of Run 6



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	1.903	1.858	2.000	663989	0.67	181094	3.52	3.67		
2	2.710	2.433	3.192	2973360	2.98	176573	3.43	16.84	MI	
3	7.171	4.017	7.675	43504148	43.61	405176	7.88	107.37	MI	
4	9.697	9.450	10.017	5712462	5.73	335817	6.53	17.01		
5	12.133	11.933	12.433	6697512	6.71	499806	9.72	13.40		
6	13.897	13.717	14.158	3229185	3.24	252889	4.92	12.77		
7	20.347	20.133	20.717	32250094	32.33	2882926	56.08	11.19		
8	21.264	21.017	21.425	388520	0.39	42302	0.82	9.18	MI	
9	21.640	21.467	21.842	2998036	3.01	292883	5.70	10.24		
10	24.585	24.350	24.967	1329392	1.33	71464	1.39	18.61	MI	
				99746698	100.00	5140930	100.00			

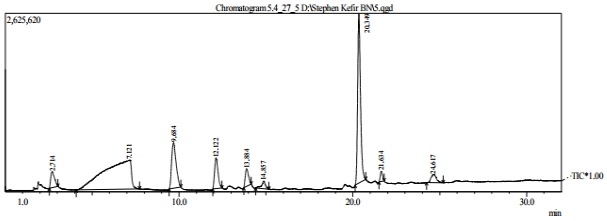
Figure G: GC-MS Output of Run 7



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	2.716	2.458	3.125	2397741	1.66	138525	1.96	17.31	MI	
2	7.109	4.142	7.583	65406873	45.15	588464	8.31	111.13	MI	
3	9.693	9.300	10.367	4189257	2.89	197523	2.79	21.21	MI	
4	12.123	11.925	12.417	9198745	6.35	683431	9.65	13.46		
5	13.877	13.700	14.183	5100388	3.52	370626	5.23	13.76		
6	20.340	20.108	20.758	52661492	36.35	4527000	63.89	11.63		
7	21.260	21.158	21.375	576687	0.40	76355	1.08	7.55		
8	21.631	21.458	21.883	5335244	3.68	503591	7.11	10.59	S	
				14486427	100.00	7085515	100.00			

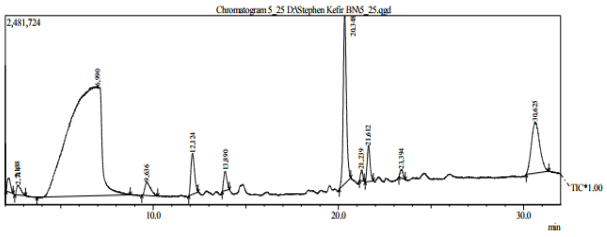
Figure H: GC-MS Output of Run 8



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	2.714	2.542	3.025	3532222	3.32	234579	4.76	15.06		
2	7.121	4.092	7.733	47106539	44.23	426372	8.66	110.48	MI	
3	9.684	9.433	10.108	12112029	11.37	673192	13.67	17.99		
4	12.122	11.942	12.467	6115869	5.74	448488	9.11	13.64		
5	13.884	13.725	14.142	3297929	3.10	258824	5.26	12.74		
6	14.857	14.408	15.175	1707647	1.60	112218	2.28	15.23	MI	
7	20.349	20.133	20.733	28736985	26.98	2493328	50.63	11.53		
8	21.634	21.508	21.792	1344332	1.26	152468	3.10	8.82		
9	24.617	24.242	25.200	2559193	2.40	125456	2.55	20.40	MI	
				106512745	100.00	4924925	100.00			

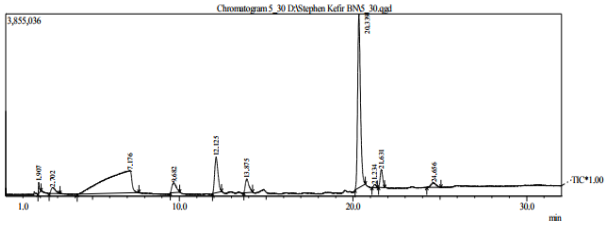
Figure I: GC-MS Output of Run 9



Peak Report TIC

Peak#	R.Time	LTime	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	2.188	2.042	2.425	2284721	0.91	178651	2.83	12.79		
2	2.714	2.508	3.100	2001609	0.80	125864	2.00	15.91	MI	
3	6.990	3.708	8.750	176801573	70.54	1430875	22.69	123.53	MI	
4	9.636	9.375	10.242	3780581	1.51	178331	2.83	21.21	MI	
5	12.124	11.917	12.417	7280982	2.91	537808	8.53	13.54		
6	13.890	13.725	14.108	3052587	1.22	258045	4.09	11.83		
7	20.348	20.050	20.667	26724255	10.66	2191923	34.76	12.19		
8	21.239	21.142	21.400	1150580	0.46	142551	2.26	8.07		
9	21.612	21.450	21.883	5185168	2.07	470451	7.46	11.02	S	
10	23.394	23.258	23.608	1224978	0.49	113374	1.80	10.80		
11	30.625	30.142	31.367	21142133	8.44	676767	10.76	31.14	MI	
				250629167	100.00	6306640	100.00			

Figure J: GC-MS Output of Run 10



Peak Report TIC

Peak#	R.Time	LTime	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	1.907	1.858	2.033	892463	0.74	235754	3.73	3.79		
2	2.702	2.508	3.125	2053962	1.71	122930	1.95	16.70	MI	
3	7.176	4.125	7.675	51049644	42.48	472126	7.47	108.11	MI	
4	9.682	9.475	10.025	3538175	2.94	210321	3.33	16.82		
5	12.125	11.917	12.433	10137637	8.44	762362	12.07	13.30		
6	13.875	13.708	14.225	4113928	3.42	292901	4.64	14.05		
7	20.339	20.108	20.700	41949266	34.91	3671307	58.11	11.43		
8	21.234	21.083	21.408	655306	0.55	73299	1.16	8.96	MI	
9	21.631	21.475	21.808	3903780	3.25	379634	6.01	10.28		
10	24.656	24.242	25.067	1880945	1.57	97397	1.54	19.31	MI	
				120175106	100.00	6318031	100.00			

Figure K: GC-MS Output of Run 11

APPENDIX C

BLAST output of microbial isolates from Thai rice milk kefir (Nonthaburi)

Isolate and accession ID	Full Nucleotide sequence	Blasted sequence	Identity	Origin
A2 Bacterium MRG-IF-3 16S ribosomal RNA gene Accession Id: KF803553.1	GGCGGGCGTCCTA CTCTCACAAGTGA GAGTTGAGCGCTC GAAGGTTGGTA CTTGTACCGACTGG ATAGAGCAGCGAA CGGGTGAGTAACG CGTGGGGAAT CTGCCTTTGAGCGG GGGACAACATTTG GAAACGAATGCTA ATACCGCATA AAAACTTTAAACA CAAGTTTTAAGTTT GAAAGATGCAATT GCATCACTCA AAGATGATCCCGC GTTGTATTAGCTAG TTGGTGAGGTAAA GGCTCACCAA GGCGATGATACAT AGCCGACCTGAGA GGGTGATCGGCCA CATTGGGACTG AGACACGGCCCAA ACTCCTACGGGAG GCAGCAGTAGGGA ATCTTCGGCAA TGGACGAAAGTCT GACCGAGCAACGC CGCGTGAGTGAAG AAGGTTTTCGG ATCGTAAAACTCTG TTGGTAGAGAAGA ACGTTGGTGAGAG TGGAAAGCTC ATCAAGTGACGGT AACTACCCAGAAA GGGACGGCTAACT ACGTGCCAGCA GCCGCGGTAATAC GTATGTCCCGAGC GTTGTCCGGATTTA TTGGGCGTAA AGCGAGCGCAGGT GGTTTATTAAGTCT GGTGTAAAAGGCA GTGGCTCAAC CATTGTATGCATTG GAAACTGGTAGAC TTGAGTGCAGGAG AGGAGAGTGG	GAACGGGTGAGTAAC GCGTGGGGAATCTGC CTTTGAGCGGGGGAC AACATTTGGAAACGA ATGCTAATAC CGCATAACAACTTTAA ACACAAGTTTTAAGTT TGAAAGATGCAATTG CATCACTCAAAGATG ATCCCGCG TTGTATTAGCTAGTTG GTGAGGTAAAGGCTC ACCAAGGCGATGATA CATAGCCGACCTGAG AGGGTGATC GGCCACATTGGGACT GAGACACGGCCCAAA CTCCTACGGGAGGCA GCAGTAGGGAATCTTC GGCAATGGA CGAAAGTCTGACCGA GCAACGCCGCGTGAG TGAAGAAGGTTTTCGG ATCGTAAAACTCTGTT GGTAGAGA AGAACGTTGGTGAGA GTGGAAAGCTCATCA AGTGACGGTAACTAC CCAGAAAGGGACGGC TAACTACGTG CCAGCAGCCGCGGTA ATACGTAGGTCCCGA GCGTTGTCCGGATTTA TTGGGCGTAAAGCGA GCGCAGGTG GTTTATTAAGTCTGGT GTAAAAGGCAGTGGC TCAACCATTGTATGCA TTGGAAACTGGTAGA CTTGAGTG CAGGAGAGGAGAGTG GAATTCCATGTGTAGC GGTGAAATGCGTAGA TATATGGAGGAACAC CGGTGGCGA AAGCGGCTCTCTGGCC TGTAACTGACACTGAG GCTCGAAAGCGTGGG GAGCAAACAGGATTA GATACCCT GGTAGTCCACGCCGTA	95%	Deglycosylation of Isoflavones by Human Intestinal Bacterium, Korea

	AATTCCATGTGTAG CGGTGAAATGCGT AGATATATGGAGG AACACCGGTG GCGAAAGCGGCTC TCTGGCCTGTAACT GACACTGAGGCTC GAAAGCGTGG GGAGCAAACAGGA TTAGATACCCTGGT AGTCCACGCCGTA AACGATGAGT GCTAGATGTAGGG AGCTATAAGTTCTC TGTATCGCAGCTAA CGCAATAAG CACTCCGCCTGGG GGAGTACGACCGC AAGGTTGAAACTC AAAGGAATTGA CGGGGGCCCGCAC AAGCGGTGGAGCA TGTGGTTTAATTCG AAGCAACGCG AAGAACCTTACCA GGTCTTGACATACT CGTGCTATTCCTAG AGATAGGAA GTTCCTTCGGGACA CGGGATACAGGTG GTGCATGGTTGTCG TCAGCTCGT GTCGTGAGATGTTG GGTTAAGTCCCGC AACGAGCGCAACC CCTATTGTTA ATTGCCATCATTAA GTTGGGCACTCTAA CGAGACTGCCGGT GATAAACCG GAGGAAAGGTGGG GAAGAAGTCCAAT CCTCCTGGCCCCTT ATGACCTGGG GCTACCACCCTGCC TACAATGGAAGGG TACAACCAATTCCC CGAAAAAGG GAGGTTTTAGCCA ATCCCTTAAAACCA TTCCCCATTTCCGA ATTTAGAGG GGGCAACCCCCCC CACTTAAATTCGGG AATCCCTTTTAATT CCGGAAAAA ACAACCCCCCGGT	AACGATGAGTGCTAG ATGTAGGGAGCTATA AGTTCTCTGTATCGCA GCTAACGC AATAAGCACTCCGCCT GGGGAGTACGACCGC AAGGTTGAAACTCAA AGGAATTGACGGGGG CCCGCACAA GCGGTGGAGCATGTG GTTTAATTCGAAGCAA CGCGAAGAACCTTAC CAGGTCTTGACATACT CGTGCTAT TCCTAGAGATAGGAA GTTCCTTCGGGACACG GGATACAGGTGGTGC ATGGTTGTCGTCAGCT CGTGTCGT GAGATGTTGGGTTAA GTCCCGCAACGAGCG CAACCCCTATTGTTAG TTGCCATCATTAAGTT GGGCACTC TAACGAGACTGCCGG TGATAAACCGGAGGA AAGTGGGGGATGACG TCAAATCATCATGGCC CCTTATGAC CTGGGGCTACACACGT GCTTACAATGGGAGG GGACAACCAAGTCCC CGAACAAGGGAAGTT TAACTAAAC TCCTTAAAACCATTTT CCAGTTTCCGATTTGA AGGCTGCAACTCCGCT TAATTGAGATCCGGA ATCCCCT TTTAATCCGGGAAACA ACACCCCCCGGTGGAT AATTTCCCCGGGCCTG TTTAACACCGCCGGTC ACCCAC ACGGGGGATTGGGAA GACCCCAAAAAAGTT GGCTAACCCCAGGAG GGGCGTTCTAAT		

	GAAAAAATTTCCC GGCCCTTTGAAACC CCCCCCTCCC CCCCCGGCGGGTT GGGGAAACCCCAA AAAATTGTCCTATT CCCCAAAG			
A7 Lactococcus lactis strain Unkn111 16S ribosomal RNA gene Accession ID: KX881768.1	GGCCGGGGCACCT CACTAATCGTGAG AGTTGAGCGCTGA AGGTTGGTACTTGT ACCGACCGGAAGA GCAGCGAACGGGT GAGTAACGCGTGG GGAATCTGCCTTTG AGCGGGGGACAAC ATTTGGAAACGAA TGCTAATACCGCAT AAAAACTTTAAAC ACAAGTTTTAAGTT TGAAAGATGCAAT TGCATCACTCAAA GATGATCCCGCGTT GTATTAGCTAGTTG GTGAGGTAAAGGC TCACCAAGGCGAT GATACATAGCCGA CCTGAGAGGGTGA TCGGCCACATTGG GACTGAGACACGG CCCAAACTCCTACG GGAGGCAGCAGTA GGGAATCTTCGGC AATGGACGAAAGT CTGACCGAGCAAC GCCGCGTGAGTGA AGAAGGTTTTCGG ATCGTAAAACTCTG TTGGTAGAGAAGA ACGTTGGTGAGAG TGGAAAGCTCATC AAGTGACGGTAAC TACCCAGAAAGGG ACGGCTAACTACG TGCCAGCAGCCGC GGTAATACGTAGG TCCCGAGCGTTGTC CGGATTTATTGGGC GTAAAGCGAGCGC AGGTGGTTTATTAA GTCTGGTGTAAAA GGCAGTGGCTCAA CCAT TGTATGCATTGGAA ACTGGTAGACTTG AGTGCAGGAGAGG	CGGCGGTGGCGGCGT GCTATACATGCAAGTT GAGCGCTGAAGGTTG GTACTTGTACCAACTG GATGAGCAGCGAACG GGTGAGTAACGCGTG GGGAATCTGCCTTTGA GCGGGGGACAACATT TGGAAACGAATGCTA ATACCGCATAAAAAC TTTAAACACAAGTTTT AAGTTTGAAAGATGC AATTGCATCACTCAAA GATGATCCCG CGTTGTATTAGCTAGT TGGTGAGGTAAAGGC TCACCAAGGCGATGA TACATAGCCGACCTGA GAGGGTGATCGGCCA CATTGGGACTGAGAC ACGGCCCAAACTCCTA CGGGAGGCAGCAGTA GGGAATCTTCGGCAAT GGACGAAAGTCTGAC CGAGCAACGCCGCGT GAGTGAAGAAGGTTT TCGGATCGTAAAACTC TGTTGGTAGA GAAGAACGTTGGTGA GAGTGGAAAGCTCAT CAAGTGACGGTAACT ACCCAGAAAGGGACG GCTAACTACGTGCCAG CAGCCGCGGTAATAC GTAGGTCCCGAGCGTT GTCCGGATTTATTGGG CGTAAAGCGAGCGCA GGTGGTTTATTAAGTC TGGTGTAAAAGGCAG TGGCTCAACCATTGTA TGCATTGGAAACTGGT AGACTTGAGTGCAGG AGAGGAGAGTGGAAT TCCATGTGTAGCGGTG AAATGCGTAGATATAT GGAGGAACACCGGTG GCGAAAGCGGCTCTCT GGCCTGTAACTGACAC TGAGGCTCGAAAGCG	96%	Probiotic characteristics of lactic acid bacteria isolated from camel milk, United Arab Emirates.

	AGAGTGGAATTCC ATGTGTAGCGGTG AAATGCGTAGATA TATGGAGGAACAC CGGTGGCGAAAGC GGCTCTCTGGCCTG TAACTGACACTGA GGCTCGAAAGCGT GGGGAGCAAACAG GATTAGATACCCTG GTAGTCCACGCCGT AAACGATGAGTGC TAGATGTAGGGAG CTATAAGTTCTCTG TATCGCAGCTAAC GCAATAAGCAC TCCGCCTGGGGAG TACGACCGCAAGG TTGAAACTCAAAG GAATTGACGGG GGCCCGCACAAGC GGTGGAGCATGTG GTTTAATTCGAAGC AACGCGAAGA ACCTTACCAGGTCT TGACATACTCGTGC TATTCCTAGAGATA GGAAGTTCCTTCGG GACACGGGATACA GGTGGTGCATGGTT GTCGTCAGCTCGTG TCCTGAAATGTTGG GTTAAGTCCCGCA ACGAGCGCAACCC CTATTGTTAGTTGC CATCATTAAGTTGG GCACTCTAACGAA ACTGCCGGTGATA AACCGGAGG AAAGGGGGGGGAT AAAGTCCAAACAT CCTGGCCCCTTTTA ACCGGGGGTAAAA CCTGGTTAAAAGG GAGGGGGCAACCA ATCCCCGAAAAAA GGAGGGTTTACCA AATCCTTTAAAACA TTTTCCCTTTCGGA TTGTAGGGGGGCA ACCCCCCTCACTGA AAGCGGAAACCCT TTTAATTCCGGAAA AAACCCCCCCCGG GGAAAAATTTTCCC GC	TGGGGAGCAAACAGG ATTAGATACC CTGGTAGTCCACGCCG TAAACGATGAGTGCT AGATGTAGGGAGCTA TAAGTTCTCTGTATCG CAGCTAACGCAATAA GCACTCCGCCTGGGG AGTACGACCGCAAGG TTGAAACTCAAAGGA ATTGACGGGGGCCCG CACAAGCGGTGGAAC ATGTGGTTTAATTCGA AGCAACGCGAAGAAC CTTACCAGGTCTTGAC ATACTCGTGCT ATTCCTAGAGATAGG AAGTTCCTTCGGGACA CGGGATACAGGTGGT GCATGGTTGTCGTCAG CTCGTGTCCTGAAATG TTGGGTTAAGTCCCGC AACGAGCGCAACCCC TATTGTTAGTTGCCAT CATTAATTTGGGCACT CTAACGAGACTGCCG GTGATAAACCGGAGA AAAGTTGGGGATGAA GTCCAATCATCAGGCC CCTAAAAA CCGGGGCACCACCTTG GTACAAGGAAGGGGT CACCAATCCGCGGAC GAGAGATTGTTACCCA ACCCCTTAAAAACATT CTCCGGTTCGAATGTA AGG		

A9 Lactococcus lactis strain RPWL3 16S ribosomal RNA gene Accession ID: MF185375.1	CGGCGTGGTGACC TTCACTAACCATGC AGTTGAGCGCTGA GGTTGGTACTTGTA CCGACTGGATAGA GCAGCGAACGGGT GAGTAACGCGTGG GGAATCTGCCTTTG AGCGGGGGACAAC ATTTGGAAACGAA TGCTAATACCGCAT AAAAACTTTAAAC ACAAGTTTTAAGTT TGAAAGATGCAAT TGCATCACTCAAA GATGATCCCGCGTT GTATTAGCTAGTTG GTGAGGTAAAGGC TCACCAAGGCGAT GATACATAGCCGA CCTGAGAGGGTGA TCGGCCACATTGG GACTGAGACACGG CCCAAACTCCTACG GGAGGCAGCAGTA GGGAATCTTCGGC AATGGACGAAAGT CTGACCGAGCAAC GCCGCGTGAGTGA AGAAGGTTTTCGG AT CGTAAAACTCTGTT GGTAGAGAAGAAC GTTGGTGAGAGTG GAAAGCTCATCAA GTGACGGTAACTA CCCAGAAAGGGAC GGCTAACTACGTG CCAGCAGCCGCGG TAATACGTAGGTCC CGAGCGTTGTCCG GATTTATTGGGCGT AAAGCGAGCGCAG GTGGTTTATTAAGT CTGGTGTAAAAGG CAGTGGCTCAACC A TTGTATGCATTGGA AACTGGTAGACTT GAGTGCAGGAGAG GAGAGTGGAATTC CATGTGTAGCGGT GAAATGCGTAGAT ATATGGAGGAACA CCGGTGGCGAAAG CGGCTCTCTGGCCT	CTATACATGCAGTTGA GCGCTGAGGTTGGTAC TTGTACCGACTGGATG AGCAGCGAACGGGTG AGTAACGCGTGGGGA ATCTGCCTTTGAGCGG GGGACAACATTTGGA AACGAATGCTAATAC CGCATAAAAACTTTAA ACACAAGTTTTAAGTT TGAAAGATGCAATTG CATCACTCAAAGATG ATCCCGCGTTGTATTA GCTAGTTG GTGAGGTAAAGGCTC ACCAAGGCGATGATA CATAGCCGACCTGAG AGGGTGATCGGCCAC ATTGGGACTGAGACA CGGCCCAAACTCCTAC GGGAGGCAGCAGTAG GGAATCTTCGGCAATG GACGAAAGTCTGACC GAGCAACGCCGCGTG AGTGAAGAAGGTTTTC GGATCGTAAAACTCTG TTGGTAGAGAAGAAC GTTGGTGAGAGTGGA AAGCTCATCAAGTGA CGGTAACTACCCAGA AAGGGACGGCTAACT ACGTGCCAGCAGCCG CGGTAATACGTAGGTC CCGAGCGTTGTCCGGA TTTATTGGGCGTAAAG CGAGCGCAGGTGGTTT ATTAAGTCTGGTGTAA AAGGCAGTGGCTCAA CCATTGTATGCATTGG AAACTGGTAGACTTG AGTGCAGGAGAGGAG AGTGGAATTCCATGTG TAGCGGTGAAATGCG TAGATATATGGAGGA ACACCGGTGGCGAAA GCGGCTCTCTGGCCTG TAACTGACACTGAGG CTCGAAAGCGTGGGG AGCAAACAGGATTAG ATACCCTGGTAGTCCA CGCCGTAAACGATGA GTGCTAGATGTAGGG AGCTATAAGTTCTCTG TATCGCAGCTAACGCA ATAAGCACTCCGCCTG GGGAGTACGACCGCA	94%	Fujian Agriculture and Forestry University, Fujian, China.

	GTAACTGACACTG AGGCTCGAAAGCG TGGGGAGCAAACA GGATTAGATACCCT GGTAGTCCACGCC GTAAACGATGAGT GC TAGATGTAGGGAG CTATAAGTTCTCTG TATCGCAGCTAAC GCAATAAGCACTC CGCCTGGGGGAGT ACGACCGCAAGGT TGAAACTCAAAGG AATTGACGGGGGC CCGCACAAGCGGT GGAACCAGGTGGT TTAATTCGAAGCA ACGCGAAGAACCT TACCAGGTCTTGAC ATACTCGTGCTATT CCAGAAAAATAGG AA GTTCCTTCGGGACA CGGGAAACAGGTG GGGGGAAGGGTTG TCGTCAGCTCGTGT CCTGAAAAGGTTG GGGTTAAGTCCCG CAACGAGCGCAAC CCCTATTGTTAGTT GCCATCATTAAGTT GGGCACTCTAACG AGACTGCCGGTGA TAAACCGGAGGAA AGGGGGGGGGAAG AAGTTCACATAATC CTGCCCCCTAATG ACCTGGGGCTACC ACCCTGGTTACAAT GGGAGGGGACAAC CAATCCCGGAAA AGTGAGTGTTTTGC TAACTCCTTAAAAC AATTCTCCCCTTCC GAATGGAAGGGGG CAACTCGCCCCACT GAAGATCGGAAAC CCCTGTTAATCCCG GATAAACAACCCC CGCGGGAAAAAAT TTCCCGCGCCTTGT AAACCCGCCCGGT TCCACCCCGGGGG GTTGGGGAAAACC CCAAAAAAAATC	AGGTTGAAACTCAAA GGAATTGACGGGGGC CCGCACAAGCGGTGG AGCATGTGGTTTAATT CGAAGCAACGCGAAG AACCTTACCAGGTCTT GACATACTCGTGCTAT TCCTAGAGATAGGAA G TTCCTTCGGGACACGG GATACAGGTGGTGCA TGGTTGTCGTCAGCTC GTGTCGTGAGATGTTG GGTTAAGTCCCGCAAC GAGCGCAACCCCTATT GTTAGTTGCCATCATT AAGTTGGGCACTCTAA CGAGACTGCCGGTGA TAAACCGGAGGAAGG TGGGGATGACGTCAA ATCATCATGCCCCTTA TGACCTGGGCTACACA CGTGCTACAATGGATG GTACAACGAGTCGCG AGACAGTGATGTTTAG CTAATCTCTTAAAACC ATTCTCAGTTCGGATT GTAGGCTGCAACTCGC CTACATGAAGTCGGA ATCGCTAGTAATCGCG GATCAGCACGCCGCG GTGAATACGTTCCCGG GCCTTGTACACACCGC CCGTCACACCACGGG AGTTGGGAGTACCCG AAGTAGGTTGCCTAAC CGCAAGGAGGGCGCT CCTAAG		

A10 Lactococcus lactis subsp. lactis strain NM146-2 16S ribosomal RNA gene Accession ID: HM218576.1	GGCGTGTGACGTA CTAGTCGTGCAGTT GAGCGCTGAAGGT TGGTACTTGT ACCGACTGGATAG AGCAGCGAACGGG TGAGTAACGCGTG GGGAATCTGCC TTTGAGCGGGGGA CAACATTTGGAAA CGAATGCTAATAC CGCATAAAAAC TTTAAACACAAGTT TTAAGTTTGAAAG ATGCAATTGCATCA CTCAAAGAT GATCCCGCGTTGTA TTAGCTAGTTGGTG AGGTAAAGGCTCA CCAAGGCGA TGATACATAGCCG ACCTGAGAGGGTG ATCGGCCACATTG GGACTGAGACA CGGCCCAAACTCCT ACGGGAGGCAGCA GTAGGGAATCTTC GGCAATGGAC GAAAGTCTGACCG AGCAACGCCGCGT GAGTGAAGAAGGT TTTCGGATCGT AAAACTCTGTTGGT AGAGAAGAACGTT GGTGAGAGTGGAA AGCTCATCAA GTGACGGTAACTA CCCAGAAAGGGAC GGCTAACTACGTG CCAGCAGCCGC GGTAATACGTAGG TCCCGAGCGTTGTC CGGATTTATTGGGC GTAAAGCGA GCGCAGGTGGTTT ATTAAGTCTGGTGT AAAAGGCAGTGGC TCAACCATTG TATGCATTGGAAA CTGGTAGACTTGA GTGCAGGAGAGGA GAGTGGAATTC CATGTGTAGCGGT GAAATGCGTAGAT ATATGGAGGAACA CCGGTGGCGAA	GTGCCTAATACATGCA AGTTGAGCGCTGAAG GTTGGTACTTGTACCG ACTGGATGAGCAGCG AACGGGTG AGTAACGCGTGGGGA ATCTGCCTTTGAGCGG GGGACAACATTTGGA AACGAATGCTAATAC CGCATAAAA ACTTTAAACACAAGTT TTAAGTTTGAAAGATG CAATTGCATCACTCAA AGATGATCCCGCGTTG TATTAG CTAGTTGGTGAGGTAA AGGCTCACCAAGGCG ATGATACATAGCCGA CCTGAGAGGGTGATC GGCCACATT GGGACTGAGACACGG CCCAAACTCCTACGGG AGGCAGCAGTAGGGA ATCTTCGGCAATGGAC GAAAGTCT GACCGAGCAACGCCG CGTGAGTGAAGAAGG TTTTCGGATCGTAAAA CTCTGTTGGTAGAGAA GAACGTTG GTGAGAGTGGAAAGC TCATCAAGTGACGGTA ACTACCCAGAAAGGG ACGGCTAACTACGTGC CAGCAGCC GCGGTAATACGTAGG TCCCGAGCGTTGTCCG GATTTATTGGGCGTAA AGCGAGCGCAGGTGG TTTATTAA GTCTGGTGTAAAAGG CAGTGGCTCAACCATT GTATGCATTGGAAACT GGTAGACTTGAGTGC AGGAGAGG AGAGTGGAATTCCAT GTGTAGCGGTGAAAT GCGTAGATATATGGA GGAACACCGGTGGCG AAAGCGGCTC TCTGGCCTGTAACTGA CACTGAGGCTCGAAA GCGTGGGGAGCAAAC AGGATTAGATACCCTG GTAGTCCA CGCCGTAAACGATGA	96%	Isolation and identification of lactic acid bacteria from naturally fermented dairy products in Inner Mongolia, China

	AGCGGCTCTCTGGC CTGTAACTGACACT GAGGCTCGAAAGC GTGGGGAGC AAACAGGATTAGA TACCCTGGTAGTCC ACGCCGTAAACGA TGAGTGCTAG ATGTAGGGAGCTA TAAGTTCTCTGTAT CGCAGCTAACGCA ATAAGCACTC CGCCCGGGGGAGT ACGACCGCAAGGT TGAAACTCAAAGG AATTGACGGGG GCCCGCACAAGCG GTGGAACCAGGTG GTTTAATTCGAAGC AACGCGAAGA ACCTTACCAGGTCT TGACATACTCGTGC TATTCCTAGAAAAT AGGAAGTT CCTTCGGGACACG GGATACAGGTGGG TGCATGGTTGTCGT CAGCTCGTGT CGTGAGATGTTGG GTTAAGTCCCGCA ACGAGCGCAACCC CTATTGTTAGT TGCCATCATTAAGT TGGGCACTCTAAC GAGACTGCCGGTG ATAAACCGGA GGAAAGGTGGGGA TGAAGTCAAATCA TCAAGGCCCCTTAT GACCGGGGCT ACACCCTGCTACA ATGGAGGTACAAC CAATCTCCGAACA ATGATGTTTAG CTAATCTCTTAAAA CCATCCTCATTTCC GAATGTAAGGCCG CAACTCCGC CCACTGGAAGTCG GAAACCCTATTATA TCCGGAATAACAC CCCCCCGGTG AAGAGTTTCCCGG CTTGGTACACCGCC CCTCCCCCCCCGGG ATTTGGGAA CCCCTACCCCAGG	GTGCTAGATGTAGGG AGCTATAAGTTCTCTG TATCGCAGCTAACGCA ATAAGCAC TCCGCCTGGGGAGTAC GACCGCAAGGTTGAA ACTCAAAGGAATTGA CGGGGGCCCGCACAA GCGGTGGAG CATGTGGTTTAATTCG AAGCAACGCGAAGAA CCTTACCAGGTCTTGA CATACTCGTGCTATTC CTAGAGA TAGGAAGTTCCTTCGG GACACGGGATACAGG TGGTGCATGGTTGTCG TCAGCTCGTGTCGTGA GATGTTG GGTTAAGTCCCGCAAC GAGCGCAACCCCTATT GTTAGTTGCCATCATT AAGTTGGGCACTCTAA CGAGAC TGCCGGTGATAAACC GGAGGAAGGTGGGGA TGACGTCAAATCATCA TGCCCCTTATGACCTG GGCTACAC ACGTGCTACAATGGAT GGTACAACGAGTCGC GAGACAGTGATGTTTA GCTAATCTCTTAAAAC CATTCTC AGTTCGGATTGTAGGC TGCAACTCGCCTACAT GAAGTCGGAATCGCT AGTAATCGCGGATCA GCACGCCG CGGTGAATACGTTCCC GGGCCTTGTACACACC GCCCCTCACCCCACGG GAGTTGGGAGTACCC GAAGTAG GTTGCCTAACCGCAAG GAGGGCGCTTCCTAA GGTAAGA		

A11 Bacillus sp. strain abc48 16S ribosomal RNA gene Accession ID: KX426042.1	GGGTTGGGGTCAG TCTATACTGCTAGT CGAGCGCGACAGA TGCGGAGCTT GCTCCCTGATGTTA GCGGCGGACGGGT GAGTAACACGTGG GTAACCTGCC TGTAAGACTGGGA TAACTCCGGGAAA CCGGGGCTAATAC CGGATGGTTGT TTGAACCGCATGGT TCAAACATAAAAG GTGGCTTCTGCTAC CACTTACAG ATGGACCCGCGGC GCATTAGCTAGTTG GTGAGGTAATGGC TCACCAAGGC AACGATGCGTAGC CGACCTGAGAGGG TGATCGGCCACACT GGGACTGAGA CACGGCCCAGACT CCTACGGGAGGCA GCAGTAGGGAATC TTCCGCAATGG ACGAAAGTCTGAC GGAGCAACGCCGC GTGAGTGATGAAG GTTTTCGGATC GTAAAGCTCTGTTG TTAGGGAAGAACA AGTACCGTTCGAAT AGGGCGGTA CCTTGACGGTACCT AACCAGAAAGCCA CGGCTAACTACGT GCCAGCAGCC GCGGTAATACGTA GGTGGCAAGCGTT GTCCGGAATTATTG GGCGTAAAGG GCTCGCAGGCGGT TTCTTAAGTCTGAT GTGAAAGCCCCCG GCTCAACCGG GGAGGGTCATTGG AAACTGGGGAACT TGAGTGCAGAAAA GGAGAGTGGAA TTCCACGTGTACCG GTGAAATGCCTAA AGATGTGGAGGAA CACCATTGGC CAAAGGCAACTCT	GGGGGGGTCTGCCTAT ACTGCAGTCGAGCGG ACAGATGGGAGCTTG CTCCCTGATGTTAGCG GCGGACGG GTGAGTAACACGTGG GTAACCTGCCTGTAAG ACTGGGATAACTCCG GGAAACCGGGGCTAA TACCGGATG GTTGTTTGAACCGCAT GGTTCAAACATAAAA GGTGGCTTCGGCTACC ACTTACAGATGGACCC GCGGCGC ATTAGCTAGTTGGTGA GGTAACGGCTCACCA AGGCAACGATGCGTA GCCGACCTGAGAGGG TGATCGGCC ACACTGGGACTGAGA CACGGCCCAGACTCCT ACGGGAGGCAGCAGT AGGGAATCTTCCGCA ATGGACGAA AGTCTGACGGAGCAA CGCCGCGTGAGTGAT GAAGGTTTTCGGATCG TAAAGCTCTGTTGTTA GGGAAGAA CAAGTACCGTTCGAAT AGGGCGGTACCTTGA CGGTACCTAACCAGA AAGCCACGGCTAACT ACGTGCCAG CAGCCGCGGTAATAC GTAGGTGGCAAGCGT TGTCCGGAATTATTGG GCGTAAAGGGCTCGC AGGCGGTTT CTTAAGTCTGATGTGA AAGCCCCCGGCTCAA CCGGGGAGGGTCATT GGAAACTGGGGAACT TGAGTGCAG AAGAGGAGAGTGGAA TTCCACGTGTAGCGGT GAAATGCGTAGAGAT GTGGAGGAACACCAG TGGCGAAGG CGACTCTCTGGTCTGT AACTGACGCTGAGGA GCGAAAGCGTGGGGA GCGAACAGGATTAGA TACCCTGGT AGTCCACGCCGTAAA CGATGAGTGCTAAGT	94%	Molecular and Microbial Studies of infant food to Detect Microbial Contamination, Saudi Arabia

	CTGATCTGTAACTG ACGCTGAGGAACT AAATCCTGGG GATGCAACACGAT TAGATCACCTGGAT ATCAAAGCGCTAA ACAAGAATGC GTAGTGCTACGGG GTTTCCTCCCCTTA ATGCTGGCGACTA CAACTTTAAG CTCCCCCCCGGGG GAATGACGTCCAC AAACTGAAAATCC ACCTAATTTTA AGGCGCCGCCCCC TTCCACGGACGAA GGAATCTTCCTCGA CCACCCTTAA CGACTTAACTTGCT TGGAACCTTCCCCC CGTTCTAAAATACG GGGGGGCC GGCTCCGGTTTAAA CGGAAAGTTCCTTG GGGTGGACCCCGC CTTGGGCCC CCAAATTAGGGAA ATTTTGTTTACCCC GAAACCCAGTTTTT TTTTTGGAA AAAAATTTTTAAG GCCCGGGGGGGGT TCCCCAAGGAACC CTTCCGGGAAA GGGAAAAACCCCG GGGGGGCCCCCCA AAAAAAGGGGGGG GTTTTAACCCT GGGGGGGGGGAAA ACCCTTTTTGGGGG GGAAAAGGGGCCG GGATTTTTGG GGGATAATTCCAA AACCCCAAAAGGG TGTGGGGCCGGG	GTTAGGGGGTTTCCGC CCCTTAGTGCTGCAGC TAACGCAT TAAGCACTCCGCCTGG GGAGTACGGTCGCAA GACTGAAACTCAAAG GAATTGACGGGGGCC CGCACAAGC GGTGGAGCATGTGGTT TAATTCGAAGCAACG CGAAGAACCTTACCA GGTCTTGACATCCTCT GAAAATCC TAGAGATAGGACGTC CCCTTCGGGGGCAAA ATGACAGGTGGTGCA TGGTTGTCGTCACTCC GGGCCGGGA AAAGTTGGGTTAATTC CCGCAAAGAGGGAAC CTTTGATCTAATTGCC CGCCTTCAGTGGGCCC TCTAAGG GACTGCCGGTGACAA ACCGGAGAAAGTGGG GGAAGACGCCAAATC AAAAGGCCCCTTAAG ACGGGGGACA CACAGTGTCCAAATG GACAAAAAAAGGGGC GCCCAACCGCCGGGTT AGGCCATCCCCAAATC TTTTCTTT TTTGGGAGGCGGGTC GGCCTCTTGGCGGGG AAGGGGAAACCCTTT ATATTCGGAAACACA GCCCGGGGAA AAATTTTTCGGGTCTG TTCACCCCGCCTCTCA CCCAAGATGG		
A13 Lactococcus lactis strain AF13 16S ribosomal RNA gene	GGCGTGGCGGTAT GTATATACATGCA GTTGAGCGCTGAA GGTTGGTACTT GTACCGACTGGAT AGAGCAGCGAACG GGTGAGTAACGCG TGGGGAATCTG	CGCAGTGCGGGGAGC TATACATGCAGTTGAG CGCTGAAGGTTGGTAC TTGTACCGACTGGATG AGCAGCG AACGGGTGAGTAACG CGTGGGGAATCTGCCT TTGAGCGGGGGACAA	97%	Fujian Academy of Agricultural Sciences, China.

Accession ID: KY438201.1	CCTTTGAGCGGGG GACAACATTTGGA AACGAATGCTAAT ACCGCATAAAA ACTTTAAACACAA GTTTTAAGTTTGAA AGATGCAATTGCA TCACTCAAAG ATGATCCCGCGTTG TATTAGCTAGTTGG TGAGGTAAAGGCT CACCAAGGC GATGATACATAGC CGACCTGAGAGGG TGATCGGCCACATT GGGACTGAGA CACGGCCCAAACT CCTACGGGAGGCA GCAGTAGGGAATC TTCGGCAATGG ACGAAAGTCTGAC CGAGCAACGCCGC GTGAGTGAAGAAG GTTTTCGGATC GTAAAACTCTGTTG GTAGAGAAGAACG TTGGTGAGAGTGG AAAGCTCATC AAGTGACGGTAAC TACCCAGAAAGGG ACGGCTAACTACG TGCCAGCAGCC GCGGTAATACGTA GGTCCCGAGCGTT GTCCGGATTTATTG GGCGTAAAGC GAGCGCAGGTGGT TTATTAAGTCTGGT GTAAAAGGCAGTG GCTCAACCAT TGTATGCATTGGAA ACTGGTAGACTTG AGTGCAGGAGAGG AGAGTGGAAT TCCATGTGTAGCGG TGAAATGCGTAGA TATATGGAGGAAC ACCGGTGGCG AAAGCGGCTCTCT GGCCTGTAACTGA CACTGAGGCTCGA AAGCGTGGGGA GCAAACAGGATTA GATACCCTGGTAGT CCACGCCGTAAAC GATGAGTGCT AGATGTAGGGAGC	CATTTGGAAACGAAT GCTAATACC GCATAAAAACTTTAA ACACAAGTTTTAAGTT TGAAAGATGCAATTG CATCACTCAAAGATG ATCCCGCGT TGTATTAGCTAGTTGG TGAGGTAAAGGCTCA CCAAGGCGATGATAC ATAGCCGACCTGAGA GGGTGATCG GCCACATTGGGACTG AGACACGGCCCAAAC TCCTACGGGAGGCAG CAGTAGGGAATCTTCG GCAATGGAC GAAAGTCTGACCGAG CAACGCCGCGTGAGT GAAGAAGGTTTTCGG ATCGTAAAACTCTGTT GGTAGAGAA GAACGTTGGTGAGAG TGGAAAGCTCATCAA GTGACGGTAACTACCC AGAAAGGGACGGCTA ACTACGTGC CAGCAGCCGCGGTAA TACGTAGGTCCCGAGC GTTGTCCGGATTTATT GGGCGTAAAGCGAGC GCAGGTGG TTTATTAAGTCTGGTG TAAAAGGCAGTGGCT CAACCATTGTATGCAT TGGAAACTGGTAGAC TTGAGTGC AGGAGAGGAGAGTGG AATTCCATGTGTAGCG GTGAAATGCGTAGAT ATATGGAGGAACACC GGTGGCGAA AGCGGCTCTCTGGCCT GTAACTGACACTGAG GCTCGAAAGCGTGGG GAGCAAACAGGATTA GATACCCTG GTAGTCCACGCCGTAA ACGATGAGTGCTAGA TGTAGGGAGCTATAA GTTCTCTGTATCGCAG CTAACGCA ATAAGCACTCCGCCTG GGGAGTACGACCGCA AGGTTGAAACTCAAA GGAATTGACGGGGGC CCGCACAAG		

	TATAAGTTCTCTGT ATCGCAGCTAACG CAATAAGCAC TCCGCCTGGGGAG TACGACCGCAAGG TTGAAACTCAAAG GAATTGACGGG GGCCCGCACAAGC GGTGGAGCATGTG GTTTAATTCGAAGC AACGCGAAGA ACCTTACCAGGTCT TGACATACTCGTGC TATTCCTAGAGATA GGAAGTTC CTTCGGGACACGG GATACAGGTGGTG CATGGTTGTCGTCA GCTCGTGTCG TGAGATGTTGGGG TTAAGTCCCGCAAC GAGCGCAACCCCT ATTGTTAGTT GCCATCATTAAGTT GGGCACTCTAACG AGACTGCCGGTGA TAAACCGGAG GAAAGAGGGGGGA AGAACGTCAAAAC ATCCTGGCCCCTAT TAACCGGGGG TACAACCTGGTTAC AAGGGAGGGGTCA ACCAATCCGCCAG ACAAGGAGTG TTTTGCCAATCTCT TAAACCATTCTCCT CTCGGAAAGGAGG GCGGGAACG CCCCCCCTGAAAG GCGGAAACCGCTG TTAATCCGGAAAC ACACACCCCGC CGGGAAAATAATT CCCGCGCCG	CGGTGGAGCATGTGG TTTAATTCGAAGCAAC GCGAAGAACCTTACC AGGTCTTGACATACTC GTGCTATT CCTAGAGATAGGAAG TTCCTTCGGGACACGG GATACAGGTGGTGCA TGGTTGTCGTCAGCTC GTGTCGTG AGATGTTGGGTTAAGT CCCGCAACGAGCGCA ACCCCTATTGTTAGTT GCCATCATTAAGTTGG GCACTCT AACGAGACTGCCGGT GATAAACCGGAGGAA GGTGGGGATGACGTC AAATCATCATGCCCCT TATGACCTG GGCTACACACGTGCTA CAATGGATGGTACAA CGAGTCGCGAGACAG TGATGTTTAGCTAATC TCTTAAAA CCATTCTCAGTTCGGA TTGTAGGCTGCAACTC GCCTACATGAAGTCG GAATCGCTAGTAATCG CGGATCA GCACGCCGCGGTGAA TACGTTCCCGGGCCTT GTACACACCGCCCGTC ACACCACGGGAGTTG GGAGTACC CGAAGTAGGTTGCCTA ACCGCAAGGAGGGCG CTCCTAAGTAGACCCA TGCC		
A14 Lactococcus lactis strain PON37 16S ribosomal RNA gene Accession ID: KC545887.1	GGCCGGGGCAACG TATATTCGGAGAG AGTTGAGCGCTCG CATCGTTGGTG ACTTGTACCGCACC GTGATGAGCAGCG AACGGGTGAGTAA CGCGTGGGGA ATCTGCCTTTGAGC GGGGGACAACATT TGGAAACGAATGC TAATACCGCA	GCTTCAATCCGACCTT ACGTCCGTAAGTTGAG CGCTGTCGTTGGTACT TGCTACCGCACTGAGA TGAGCA GCGAACGGGTGAGTA ACGCGTGGGGAATCT GCCTTTGAGCGGGGG ACAACATTTGGAAAC GAATGCTAAT ACCGCATAAAAACTTT AAACACAAGTTTTAA	97%	Ecology and technological aptitudes of lactic acid bacteria isolated from PDO Vastedda della Valle del Belice cheese Italy.

	TAAAAACTTTAAA	GTTTGAAAGATGCAAT		
	CACAAGTTTTAAGT	TGCATCACTCAAAGAT		
	TTGAAAGATGCAA	GATCCCG		
	TTGCATCACT	CGTTGTATTAGCTAGT		
	CAAAGATGATCCC	TGGTGAGGTAAAGGC		
	GCGTTGTATTAGCT	TCACCAAGGCGATGA		
	AGTTGGTGAGGTA	TACATAGCCGACCTGA		
	AAGGCTCACC	GAGGGTGA		
	AAGGCGATGATAC	TCGGCCACATTGGGAC		
	ATAGCCGACCTGA	TGAGACACGGCCCAA		
	GAGGGTGATCGGC	ACTCCTACGGGAGGC		
	CACATTGGGAC	AGCAGTAGGGAATCT		
	TGAGACACGGCCC	TCGGCAATG		
	AAACTCCTACGGG	GACGAAAGTCTGACC		
	AGGCAGCAGTAGG	GAGCAACGCCGCGTG		
	GAATCTTCGGC	AGTGAAGAAGGTTTTC		
	AATGGACGAAAGT	GGATCGTAAAACTCTG		
	CTGACCGAGCAAC	TTGGTAGA		
	GCCGCGTGAGTGA	GAAGAACGTTGGTGA		
	AGAAGGTTTTC	GAGTGGAAAGCTCAT		
	GGATCGTAAAACT	CAAGTGACGGTAACT		
	CTGTTGGTAGAGA	ACCCAGAAAGGGACG		
	AGAACGTTGGTGA	GCTAACTACG		
	GAGTGGAAAGC	TGCCAGCAGCCGCGG		
	TCATCAAGTGACG	TAATACGTAGGTCCCG		
	GTAACTACCCAGA	AGCGTTGTCCGGATTT		
	AAGGGACGGCTAA	ATTGGGCGTAAAGCG		
	CTACGTGCCAG	AGCGCAGG		
	CAGCCGCGGTAAT	TGGTTTATTAAGTCTG		
	ACGTAGGTCCCGA	GTGTAAAAGGCAGTG		
	GCGTTGTCCGGATT	GCTCAACCATTGTATG		
	TATTGGGCGT	CATTGGAAACTGGTA		
	AAAGCGAGCGCAG	GACTTGAG		
	GTGGTTTATTAAGT	TGCAGGAGAGGAGAG		
	CTGGTGTAAAAGG	TGGAATTCCATGTGTA		
	CAGTGGCTCA	GCGGTGAAATGCGTA		
	ACCATTGTATGCAT	GATATATGGAGGAAC		
	TGTAAACTGGTAG	ACCGGTGGC		
	ACTTGAGTGCAGG	GAAAGCGGCTCTCTG		
	AGAGGAGAGT	GCCTGTAACTGACACT		
	GTAATTCCCTGTGT	GAGGCTCGAAAGCGT		
	AGCGGGGAAATAC	GGGGAGCAACAGGAT		
	GTATATATATGCAG	TAGATACCC		
	GAACACCGA	TGGTAGTCCACGCCGT		
	TGGCGAAATCGAC	AAACGATGAGTGCTA		
	TCTCTGACCTGTAA	GATGTAGGGAGCTAT		
	CGGAGACTGAGGC	AAGTTCTCTGTATCGC		
	TGGAAGCCCA	AGCTAACG		
	GCGGACGAAACAG	CAATAAGCACTTCGCT		
	AATGTATATACACT	GGGGAGTACGACCGC		
	GCGCCGTACACGA	AAGGTTGAAACTCAA		
	CGAGCGATTA	AGGAATTGACGGGGG		
	TGAGCAGCGAGTA	CCCGCACAA		
	TATAGGGCAGTAC	GCGGTGGAGCATGTG		
	ATACATTTCTTCTG	GTTTAATTCGAAGCAA		
	CTCTCGGCAA	CGCGAAGAACCTTAC		
	ACTCACTGCGATCA	CAGGTCTTGACATACT		

	TGTCACTCCTGCCC TCGGGGAGGACGA AACTACACA GGTTTTTAGTCGGC AGCACAATGGGGC GGGGCCCGCGTCC TATTCATGTG AGCGTAGTGAGAA AGCAGCTCTGAGC ATCCGTTACTAAAA CCTTAAAAAT CTTCTTGAGAATAC CTCGGGGACTATTC CTTCATAATTCAGG GAGGTTCC TGGCAGGGTACTTT GGAAGACACCGCT GCTTTCCCGGGGGT GGTCGTCCC GGCTCCGGTTGAG AAGCCCTTTTTCCG GGCTCCAAAATCCT TTGGGTTTT TATTTCCCCCAACC AAAATAAGTTTGCT TACCCCCCAAAAG GGAAAATCC TTTGGCTAACCCTT ACAAAGGGGGGGG GAGTCGGGCCGAA AGGAAAAAAA ACCTTGGCGGGGG CAAGGGAGCCAAA AAAAAAAACCTCC CAAGGGCCCGC CTACGGCCCCCCCT ATAAAGAAACAAG GGGGCGGGGCCCC CTTTTTTTCT TACCCCCCTGGCCG GTGGGGAAGGGGG CCTCCCCCCAAAAC CCCCCCGGG TTTGGGAGGGGGT TGAAAAAATATTA TTTAAGGCCCCCCC CAAATTCCGC CCGGTGATGGGGG GTTTTTTTTGCGCC CTCCCCACCCCATC CTTTAAAAA AAACAAAGGGGAA ACTTTTTTTAAACC ACCACCCGCGCCC GGGGGGAGAA AAAAAATTATTAA TCCCCCCTGGGTGG	CGTGCTAT TCCTAGAGATAGGAA GTTCCTTCGGGACACG GGATACAGGTGGTGC ATGGTTGTCGTCAGCT CGTGTCGT GAGATGTTGGGTTAA GTCCCGCAACGAGCG CAACCCCTATTGTTAG TTGCCATCATTAAGTT GGGCACTC TAACGAGACTGCCGG TGATAAACCGGAGGA AGGTGGGGATGACGT CAAATCATCATGCCCC TTATGACCT GGGCTACACACGTGCT ACAATGGATGGTACA ACGAGTCGCGAGACA GTGATGTTTAGCTAAT CTCTTAAA ACCATTCTCAGTTCGG ATTGTAGGCTGCAACT CGCCTACATGAAGTCG GAATCGCTAGTAATCG CGGATC AGCACGCCGCGGTGA ATACGTTCCCGGGCCT TGTACACACCGCCCGT CACACCACGGGAGTT GGGAGTAC CCGAAGTAGGTTGCCT AACCGCAAGGTAGGG CGCTTCCTAAGGTAAG ACTCGATGACTGGAG GTAGACGC AACCAGTAGACAACG CT		

	GACCCCCTGTTTTT GCCCCCACA TCTTCCCCCCGCCG GGTACACCCATTCC CCCCCACCAAAAC CCCTAATAT AAAAATATATAAT AAACCCTTTTTTTA AAAAA			
A15 Lactococcus lactis strain RCB476 16S ribosomal RNA gene Accession ID: KT260688.1	GGCCGGGCCCACT GACGCGTAGTGAG AGTTGAGCGCTCG AAGGTTGGTAC TTGTACCGACCGG AAGAGCAGCGAAC GGGTGAGTAACGC GTGGGGAATCT GCCTTTGAGCGGG GGACAACATTTGG AAACGAATGCTAA TACCGCATAAA AACTTTAAACACA AGTTTTAAGTTTGA AAGATGCAATTGC ATCACTCAAA GATGATCCCGCGTT GTATTAGCTAGTTG GTGAGGTAAAGGC TCACCAAGG CGATGATACATAG CCGACCTGAGAGG GTGATCGGCCACA TTGGGACTGAG ACACGGCCCAAAC TCCTACGGGAGGC AGCAGTAGGGAAT CTTCGGCAATG GACGAAAGTCTGA CCGAGCAACGCCG CGTGAGTGAAGAA GGTTTTCGGAT CGTAAAACTCTGTT GGTAGAGAAGAAC GTTGGTGAGAGTG GAAAGCTCAT CAAGTGACGGTAA CTACCCAGAAAGG GACGGCTAACTAC GTGCCAGCAGC CGCGGTAATACGT AGGTCCCGAGCGT TGTCCGGATTTATT GGGCGTAAAG CGAGCGCAGGTGG TTTATTAAGTCTGG TGTAAAAGGCAGT GGCTCAACCA	GACGCTGCGGCGTGCT AATACATGGCAAGTT GAAGCGCTGAAGGTT GGTACTTGTACCGACT GGATGAGC AGCGAACGGGTGAGT AACGCGTGGGGAATC TGCCTTTGAGCGGGGG ACAACATTTGGAAAC GAATGCTAA TACCGCATAAAAACTT TAAACACAAGTTTTAA GTTTGAAAGATGCAAT TGCATCACTCAAAGAT GATCCC GCGTTGTATTAGCTAG TTGGTGAGGTAAAGG CTCACCAAGGCGATG ATACATAGCCGACCTG AGAGGGTG ATCGGCCACATTGGG ACTGAGACACGGCCC AAACTCCTACGGGAG GCAGCAGTAGGGAAT CTTCGGCAAT GGACGAAAGTCTGAC CGAGCAACGCCGCGT GAGTGAAGAAGGTTT TCGGATCGTAAAACTC TGTTGGTAG AGAAGAACGTTGGTG AGAGTGGAAAGCTCA TCAAGTGACGGTAACT ACCCAGAAAGGGACG GCTAACTAC GTGCCAGCAGCCGCG GTAATACGTAGGTCCC GAGCGTTGTCCGGATT TATTGGGCGTAAAGC GAGCGCAG GTGGTTTATTAAGTCT GGTGTAAAAGGCAGT GGCTCAACCATTGTAT GCATTGGAAACTGGT AGACTTGA GTGCAGGAGAGGAGA GTGGAATTCCATGTGT AGCGGTGAAATGCGT	97%	Microbial Culture Collection, National Centre for Cell Science, Maharashtra, India

	TTGTATGCATTGGA AACTGGTAGACTT GAGTGCAGGAGAG GAGAGTGGAA TTCCATGTGTAGCG GTGAAATGCGTAG ATATATGGAGGAA CACCGGTGGC GAAAGCGGCTCTC TGGCCTGTAACTGA CACTGAGGCTCGA AAGCGTGGGG AGCAAACAGGATT AGATACCCTGGTA GTCCACGCCGTAA ACGATGAGTGC TAGATGTAGGGAG CTATAAGTTCTCTG TATCGCAGCTAAC GCAATAAGCA CTCCGCCCGGGGG AGTACGACCGCAA GGTTGAAACTCAA AGGAATTGACG GGGGCCCGCACAA GCGGTGGAACCAG GTGGTTTAATTCGA AGCAACGCGA AGAACCTTACCAG GTCTTGACATACTC GTGCTATTCCTAGA AGATTAGGA AGTTCCTTCGGGAC ACGGGATACAGGT GGGTGCATGGTTGT CGTCAGCTC GTGTCGTGAAAAT GTTTGGGTTAAGTC CCGCAACGAGCGC AACCCCTATT GTTAGTTGCCATCA TTAAGTTGGGCACT CTAACGAAACTGC CGGTGATAA ACCGGAGGAAAGG TGGGGGATGAACG TCAAATCATCCTGC CCCCTTATGA ACCTGGGGCTACA CACCTGCCTACAA AGGGAAGGTTACA ACCAATTCCCC GAGACAAGTGATG TTTAACCAAACCCT TTAAACA	AGATATATGGAGGAA CACCGGTGG CGAAAGCGGCTCTCTG GCCTGTAACTGACACT GAGGCTCGAAAGCGT GGGGAGCAAACAGGA TTAGATAC CCTGGTAGTCCACGCC GTAAACGATGAGTGC TAGATGTAGGGAGCT ATAAGTTCTCTGTATC GCAGCTAA CGCAATAAGCACTCC GCCTGGGGAGTACGA CCGCAAGGTTGAAAC TCAAAGGAATTGACG GGGGCCCGCA CAAGCGGTGGAGCAT GTGGTTTAATTCGAAG CAACGCGAAGAACCT TACCAGGTCTTGACAT ACTCGTGC TATTCCTAGAGATAGG AAGTTCCTTCGGGACA CGGGATACAGGTGGT GCATGGTTGTCGTCAG CTCGTGT CGTGAGATGTTGGGTT AAGTCCCGCAACGAG CGCAACCCCTATTGTT AGTTGCCATCATTAAG TTGGGCA CTCTAACGAGACTGCC GGTGATAAACCGGAG GAAGGTGGGGGATGA CGTCAAATCATCATGC CCCTTATG ACCTGGGCTACACAC GTGCTACAATGGATG GTACAACGAGTCGCG AGACAGTGATGTTTAG CTAATCTCT TAAAACCATTCTCAGT TCGGATTGTAGGCTGC AACTCGCCTACATGAA GTCGGAATCGCTAGTA ATCGCG GATCAGCACGCCGCG GTGAATACGTTCCCGG GCCTTGTACACACCGC CCG		

L2 Lactococcus lactis strain CAU:2674 16S ribosomal RNA gene Accession ID: MF354497.1	GGGGGTCAGTATA TTACTGCAGTTGAG CGCTGAAGGTTGG TACTTGTACC GACTGGATGAGCA GCGAACGGGTGAG TAACGCGTGGGGA ATCTGCCTTTG AGCGGGGGACAAC ATTTGGAAACGAA TGCTAATACCGCAT AAAAACTTTA AACACAAGTTTTA AGTTTGAAAGATG CAATTGCATCACTC AAAGATGATC CCGCGTTGTATTAG CTAGTTGGTGAGGT AAAGGCTCACCAA GGCGATGAT ACATAGCCGACCT GAGAGGGTGATCG GCCACATTGGGAC TGAGACACGGC CCAAACTCCTACG GGAGGCAGCAGTA GGGAATCTTCGGC AATGGACGAAA GTCTGACCGAGCA ACGCCGCGTGAGT GAAGAAGGTTTTC GGATCGTAAAA CTCTGTTGGTAGAG AAGAACGTTGGTG AGAGTGGAAAGCT CATCAAGTGA CGGTAACTACCCA GAAAGGGACGGCT AACTACGTGCCAG CAGCCGCGGTA ATACGTAGGTCCC GAGCGTTGTCCGG ATTTATTGGGCGTA AAGCGAGCGC AGGTGGTTTATTAA GTCTGGTGTAAAA GGCAGTGGCTCAA CCATTGTATG CATTGGAAACTGG TAGACTTGAGTGC AGGAGAGGAGAGT GGAATTCCATG TGTAGCGGTGAAA TGCGTAGATATATG GAGGAACACCGGT	ACTGCAGTTGAGCGCT GAAGGTTGGTACTTGT ACCGACTGGATGAGC AGCGAACGGGTGAGT AACGCGTG GGGAATCTGCCTTTGA GCGGGGGACAACATT TGGAAACGAATGCTA ATACCGCATAAAAAC TTTAAACAC AAGTTTTAAGTTTGAA AGATGCAATTGCATCA CTCAAAGATGATCCCG CGTTGTATTAGCTAGT TGGTGA GGTAAAGGCTCACCA AGGCGATGATACATA GCCGACCTGAGAGGG TGATCGGCCACATTGG GACTGAGAC ACGGCCCAAACTCCTA CGGGAGGCAGCAGTA GGGAATCTTCGGCAAT GGACGAAAGTCTGAC CGAGCAAC GCCGCGTGAGTGAAG AAGGTTTTCGGATCGT AAAACTCTGTTGGTAG AGAAGAACGTTGGTG AGAGTGGA AAGCTCATCAAGTGA CGGTAACTACCCAGA AAGGGACGGCTAACT ACGTGCCAGCAGCCG CGGTAATACG TAGGTCCCGAGCGTTG TCCGGATTTATTGGGC GTAAAGCGAGCGCAG GTGGTTTATTAAGTCT GGTGTAA AAGGCAGTGGCTCAA CCATTGTATGCATTGG AAACTGGTAGACTTG AGTGCAGGAGAGGAG AGTGGAATT CCATGTGTAGCGGTGA AATGCGTAGATATATG GAGGAACACCGGTGG CGAAAGCGGCTCTCTG GCCTGTA ACTGACACTGAGGCTC GAAAGCGTGGGGAGC AAACAGGATTAGATA CCCTGGTAGTCCACGC CGTAAACG	98%	College of Food Science & Nutritional Engineering, Beijing, China

	GGCGAAAGCG GCTCTCTGGCCTGT AACTGACACTGAG GCTCGAAAGCGTG GGGAGCAAAC AGGATTAGATACC CTGGTAGTCCACGC CGTAAACGATGAG TGCTAGATGT AGGGAGCTATAAG TTCTCTGTATCGCA GCTAACGCAATAA GCACTCCGCC CGGGGGAGTACGA CCGCAAGGTTGAA ACTCAAAGGAATT GACGGGGGCCC GCACAAGCGGTGG AACCAGGTGGTTT AATTCGAAGCAAC GCGAAGAACCT TACCAGGTCTTGAC ATACTCGTGCTATT CCTAGAAAATAGG AAGTTCCTT CGGGACACGGGAT ACAGGTGGTTGCA TGGTTGTCGTCAGC TCGTGTCGTG AGATGTTGGGGTT AAGTCCCGCAACG AGCGCAACCCCTA TTGTTAGTTGC CATCATTAAGTTGG GCACTCTAACGAA ACTGCCGGTGATA AACCGGAGGA AAGGTGGGGGAAG AAGTCCAATCAAT CATGCCCCCTTATA ACCTGGGGCT ACACCCTGCTTAAA AGGAAGGGTACAA CCAAGTCCCCGAA AAAATGGTGG TTTTTCTTAATTTCT TTAAAACATTTTCC TTTTCGGA	ATGAGTGCTAGATGTA GGGAGCTATAAGTTCT CTGTATCGCAGCTAAC GCAATAAGCACTCCG CCTGGGG AGTACGACCGCAAGG TTGAAACTCAAAGGA ATTGACGGGGGCCCG CACAAGCGGTGGAGC ATGTGGTTTA ATTCGAAGCAACGCG AAGAACCTTACCAGG TCTTGACATACTCGTG CTATTCCTAGAGATAG GAAGTTCC TTCGGGACACGGGAT ACAGGTGGTGCATGG TTGTCGTCAGCTCGTG TCGTGAGATGTTGGGG TTAAGTCC CGCAACGAGCGCAAC CCCTATTGTTAGTTGC CATCATTAAGTTGGGC ACTCTAACGAGACTGC CGGTGAT AAACCGGAGGAAGGT GGGGATGACGTCAAA TCATCATGCCCCTTAT GACCTGGGCTACACA CGTGCTACA ATGGATGGTACAACG AGTCGCGAGACAGTG ATGTTTAGCTAATCTC TTAAAACCATTCTCAG TTCGGATT GTAGGCTGCAACTCGC CTACATGAAGTCGGA ATCGCTAGTAATCGCG GATCAGCACGCCGCG GTGAATAC GTTCCCGGGCCTTGTA CACACCGCCCGTCACA CCACGGGAGTTGGGA GTACCCGAAGTAGG		
L3 Lactococcus lactis strain KLDS4.0602	GGGGGGGTGGGCG ATCTTTATATACAT GCAGTTGAGCGCT GAAGGTTGGT	GCAGGGGGTGGGCGG CTTCCTTAATACATGC AAGTTGAGCGCTGAA GGTTGGTACTTGTACC	98%	Key Lab of Dairy Science, Northeast Agricultural University, Heilongjiang, China.

16S ribosomal RNA gen Accession ID: GQ337894.1	ACTTGTACCGACTG GATGAGCAGCGAA CGGGTGAGTAACG CGTGGGGAAT CTGCCTTTGAGCGG GGGACAACATTTG GAAACGAATGCTA ATACCGCATA AAAACTTTAAACA CAAGTTTTAAGTTT GAAAGATGCAATT GCATCACTCA AAGATGATCCCGC GTTGTATTAGCTAG TTGGTGAGGTAAA GGCTCACCAA GGCGATGATACAT AGCCGACCTGAGA GGGTGATCGGCCA CATTGGGACTG AGACACGGCCCAA ACTCCTACGGGAG GCAGCAGTAGGGA ATCTTCGGCAA TGGACGAAAGTCT GACCGAGCAACGC CGCGTGAGTGAAG AAGGTTTTCGG ATCGTAAAACTCTG TTGGTAGAGAAGA ACGTTGGTGAGAG TGGAAAGCTC ATCAAGTGACGGT AACTACCCAGAAA GGGACGGCTAACT ACGTGCCAGCA GCCGCGGTAATAC GTATATCCCGAGC GTTGTCCGGATTTA TTGGGCGTAA AGCGAGCGCAGGT GGTTTATTAAGTCT GGTGTAAAAGGCA GTGGCTCAAC CATTGTATGCATTG GAAACTGGTAGAC TTGAGTGCAGGAG AGGAGAGTGG AATTCCATGTGTAG CGGTGAAATGCGT AGATATATGGAGG AACACCGGTG GCGAAAGCGGCTC TCTGGCCTGTAACT GACACTGAGGCTC GAAAGCGTGG GGAGCAAACAGGA	GACTGGAT GAGCAGCGAACGGGT GAGTAACGCGTGGGG AATCTGCCTTTGAGCG GGGGACAACATTTGG AAACGAATG CTAATACCGCATAAA AACTTTAAACACAAGT TTTAAGTTTGAAAGAT GCAATTGCATCACTCA AAGATGA TCCCGCGTTGTATTAG CTAGTTGGTGAGGTAA AGGCTCACCAAGGCG ATGATACATAGCCGA CCTGAGAG GGTGATCGGCCACATT GGGACTGAGACACGG CCCAAACTCCTACGGG AGGCAGCAGTAGGGA ATCTTCGG CAATGGACGAAAGTC TGACCGAGCAACGCC GCGTGAGTGAAGAAG GTTTTCGGATCGTAAA ACTCTGTTG GTAGAGAAGAACGTT GGTGAGAGTGGAAAG CTCATCAAGTGACGGT AACTACCCAGAAAGG GACGGCTAA CTACGTGCCAGCAGCC GCGGTAATACGTAGG TCCCGAGCGTTGTCCG GATTTATTGGGCGTAA AGCGAGC GCAGGTGGTTTATTAA GTCTGGTGTAAAAGG CAGTGGCTCAACCATT GTATGCATTGGAAACT GGTAGAC TTGAGTGCAGGAGAG GAGAGTGGAATTCCA TGTGTAGCGGTGAAAT GCGTAGATATATGGA GGAACACCG GTGGCGAAAGCGGCT CTCTGGCCTGTAACTG ACACTGAGGCTCGAA AGCGTGGGGAGCAAA CAGGATTAG ATACCCTGGTAGTCCA CGCCGTAAACGATGA GTGCTAGATGTAGGG AGCTATAAGTTCTCTG TATCGCAG CTAACGCAATAAGCA		

	TTAGATACCCTGGT AGTCCACGCCGTA AACGATGAGT GCTAGATGTAGGG AGCTATAAGTTCTC TGTATCGCAGCTAA CGCAATAAG CACTCCGCCTGGG GAGTACGACCGCA AGGTTGAAACTCA AAGGAATTGAC GGGGGCCCGCACA AGCGGTGGAGCAT GTGGTTTAATTCGA AGCAACGCGA AGAACCTTACCAG GTCTTGACATACTC GTGCTATTCCTAGA GATAGGAAG TTCCTTCGGGACAC GGGATACAGGTGG TGCATGGTTGTCGT CAGCTCGTG TCGTGAGATGTTGG GTTAAGTCCCGCA ACGAGCGCAACCC CTATTGTTAG TTGCCATCATTAAG TTGGGCACTCTAAC GAGACTGCCGGTG ATAAACCGG AAGGAAGGTGGGG ATGACGTCAAATC ATCATGCCCCTTAT GACCTGGGGC TACCCCCGTGCTAC AATGGGATGGGAC AACCAATCCCCGA AAAAGGGAAG TTTAACCTAATCCC TTTAAAACCATTTT CCAGTTCGGAATTG AAG	CTCCGCCTGGGGAGTA CGACCGCAAGGTTGA AACTCAAAGGAATTG ACGGGGGCC CGCACAAGCGGTGGA GCATGTGGTTTAATTC GAAGCAACGCGAAGA ACCTTACCAGGTCTTG ACATACTC GTGCTATTCCTAGAGA TAGGAAGTTCCTTCGG GACACGGGATACAGG TGGTGCATGGTTGTCG TCAGCTC GTGTCGTGAGATGTTG GGTTAAGTCCCGCAAC GAGCGCAACCCCTATT GTTAGTTGCCATCATT AAGTTG GGCACTCTAACGAGA CTGCCGGTGATAAACC GGAGGAAGGTGGGGA TGACGTCAAATCATCA TGCCCCTT ATGACCTGGGCTACAC ACGTGCTACAATGGAT GGTACAACGAGTCGC GAGACAGTGATGTTTA GCTAATC TCTTAAAACCATTCTC AGTTCGGATTGTAGGC TGCAACTCGCCTACAT GAAGTCGGAATCGCT AGTAATC GCGGATCAGCACGCC GCGGTGAATACGTTCC CGGGCCTTGTACACAC CGCCCGTCACACCACG GGAGTTG GGAGTACCCGAAGTA GGTTGCCTAACCGCAA GGAGGGCGCTTCCTA AGGTAAGACCGATGA CTGGGGTGA AGTCGTAACAAGTAG CCGGAGGG		
L7 Lactococcs lactis subsp. lactis strain	GCGGGGTGCCTCG TATTTTCGTGCAGT TGAGCGCTGAAGG TTGGTACTTG	GGCCACATTGGGACT GAGACACGGCCCAAA CTCCTACGGGAGGCA GCAGTAGGGAATCTTC	97%	School of Microbiology, University College Cork, Munster, Ireland.

UC77 Accession ID: CP015906.1	TACCGACTGGATA GAGCAGCGAACGG GTGAGTAACGCGT GGGGAATCTGC CTTTGAGCGGGGG ACAACATTTGGAA ACGAATGCTAATA CCGCATAAAAA CTTTAAACACAAGT TTTAAGTTTGAAAG ATGCAATTGCATCA CTCAAAGA TGATCCCGCGTTGT ATTAGCTAGTTGGT GAGGTAAAGGCTC ACCAAGGCG ATGATACATAGCC GACCTGAGAGGGT GATCGGCCACATT GGGACTGAGAC ACGGCCCAAACTC CTACGGGAGGCAG CAGTAGGGAATCT TCGGCAATGGA CGAAAGTCTGACC GAGCAACGCCGCG TGAGTGAAGAAGG TTTTCGGATCG TAAAACTCTGTTGG TAGAGAAGAACGT TGGTGAGAGTGGA AAGCTCATCA AGTGACGGTAACT ACCCAGAAAGGGA CGGCTAACTACGT GCCAGCAGCCG CGGTAATACGTAG GTCCCGAGCGTTGT CCGGATTTATTGGG CGTAAAGCG AGCGCAGGTGGTT TATTAAGTCTGGTG TAAAAGGCAGTGG CTCAACCATT GTATGCATTGGAA ACTGGTAGACTTG AGTGCAGGAGAGG AGAGTGGAATT CCATGTGTAGCGGT GAAATGCGTAGAT ATATGGAGGAACA CCGGTGGCGA AAGCGGCTCTCTG GCCTGTAACTGAC ACTGAGGCTCGAA AGCGTGGGGAG CAAACAGGATTAG	GGCAATGGA CGAAAGTCTGACCGA GCAACGCCGCGTGAG TGAAGAAGGTTTTCGG ATCGTAAAACTCTGTT GGTAGAGA AGAACGTTGGTGAGA GTGGAAAGCTCATCA AGTGACGGTAACTAC CCAGAAAGGGACGGC TAACTACGTG CCAGCAGCCGCGGTA ATACGTAGGTCCCGA GCGTTGTCCGGATTTA TTGGGCGTAAAGCGA GCGCAGGTG GTTTATTAAGTCTGGT GTAAAAGGCAGTGGC TCAACCATTGTATGCA TTGGAAACTGGTAGA CTTGAGTG CAGGAGAGGAGAGTG GAATTCCATGTGTAGC GGTGAAATGCGTAGA TATATGGAGGAACAC CGGTGGCGA AAGCGGCTCTCTGGCC TGTAACTGACACTGAG GCTCGAAAGCGTGGG GAGCAAACAGGATTA GATACCCT GGTAGTCCACGCCGTA AACGATGAGTGCTAG ATGTAGGGAGCTATA AGTTCTCTGTATCGCA GCTAACGC AATAAGCACTCCGCCT GGGGAGTACGACCGC AAGGTTGAAACTCAA AGGAATTGACGGGGG CCCGCACAA GCGGTGGAGCATGTG GTTTAATTCGAAGCAA CGCGAAGAACCTTAC CAGGTCTTGACATACT CGTGCTAT TCCTAGAGATAGGAA GTTCCTTCGGGACACG GGATACAGGTGGTGC ATGGTTGTCGTCAGCT CGTGTCGT GAGATGTTGGGTTAA GTCCCGCAACGAGCG CAACCCCTATTGTTAG TTGCCATCATTAAGTT GGGCACTC TAACGAGACTGCCGG		

	ATACCCTGGTAGTC CACGCCGTAAACG ATGAGTGCTA GATGTAGGGAGCT ATAAGTTCTCTGTA TCGCAGCTAACGC AATAAGCACT CCGCCTGGGGAGT ACGACCGCAAGGT TGAAACTCAAAGG AATTGACGGGG GCCCGCACAAGCG GTGGAGCATGTGG TTTAATTCGAAGCA ACGCGAAGAA CCTTACCAGGTCTT GACATACTCGTGCT ATTCCTAGAGATA GGAAGTTCC TTCGGGACACGGG ATACAGGTGGTGC ATGGTTGTCGTCAG CTCGTGTCGT GAGATGTTGGGTT AAGTCCCGCAACG ACCGCAACACCTA TTGTTAGTTGC CATCATTACCTTGG GCACTCTACGGAG ACTGCCGGTGATA AACCGGCAGG AAGATGAGGAATG ACGTCCAACCGTCC AGCCCCTTTATGAC CTGGGGTCA CCACCTGCCAGAA AGGAAGGGTCAAA CAAGCTCGGGAGG AAGGGAATTTT TACCCAAGCTCTTG ATAACCTTCTTCCA ATCCGAAAAG	TGATAAACCGGAGGA AAGTGGGGGATGACG TCAAATCATCATGGCC CCTTATGAC CTGGGGCTACACACGT GCTTACAATGGGAGG GGACAACCAAGTCCC CGAACAAGGGAAGTT TAACTAAAC TCCTTAAAACCATTTT CCAGTTTCCGATTTGA AGGCTGCAACTCCGCT TAATTGAGATCCGGA ATCCCCT TTTAATCCGGGAAACA ACACCCCCCGGTGGAT AATTTCCCCGGGCCTG TTTAACACCGCCGGTC ACCCAC ACGGGGGATTGGGAA GACCCCAAAAAAGTT GGCTAACCCCAGGAG GGGCGTTCTAAT		
L9 Lactococcus lactis strain HadRami9 16S ribosomal RNA gene Accession ID: KU324909.1	CGGCGGTGTCGAC GTATATTCGTGCAG TTGAGCGCTGAAG GTTGGTACTT AGTACCGACTGGA TAGAGCAGCGAAC GGGTGAGTAACGC GTGGGGAATCT GCCTTTGAGCGGG GGACAACATTTGG AAACGAATGCTAA TACCGCATAAA AACTTTAAACACA	GGACATGGCGGCGTT GCTATACATGCAGTTG AGCGCTGAAGGTTGG TACTTGTACCAACTGG ATGAGCAG CGAACGGGTGAGTAA CGCGTGGGGAATCTG CCTTTGAGCGGGGGA CAACATTTGGAAACG AATGCTAATA CCGCATAAAAACTTTA AACACAAGTTTTAAGT TTGAAAGATGCAATTG	98%	Biological Sciences, Faculty of Sciences, King Abdulaziz University, Jeddah, Saudi Arabia

	AGTTTTAAGTTTGA	CATCACTCAAAGATG		
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	CGATGATACATAG	CGGCCACATTGGGACT		
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	GTGATCGGCCACA	CTCCTACGGGAGGCA		
	TTGGGACTGAG	GCAGTAGGGAATCTTC		
	ACACGGCCCAAAC	GGCAATGG		
	TCCTACGGGAGGC	ACGAAAGTCTGACCG		
	AGCAGTAGGGAAT	AGCAACGCCGCGTGA		
	CTTCGGCAATG	GTGAAGAAGGTTTTCG		
	GACGAAAGTCTGA	GATCGTAAAACTCTGT		
	CCGAGCAACGCCG	TGGTAGAG		
	CGTGAGTGAAGAA	AAGAACGTTGGTGAG		
	GGTTTTCGGAT	AGTGGAAAGCTCATC		
	CGTAAAACTCTGTT	AAGTGACGGTAACTA		
	GGTAGAGAAGAAC	CCCAGAAAGGGACGG		
	GTTGGTGAGAGTG	CTAACTACGT		
	GAAAGCTCAT	GCCAGCAGCCGCGGT		
	CAAGTGACGGTAA	AATACGTAGGTCCCG		
	CTACCCAGAAAGG	AGCGTTGTCCGGATTT		
	GACGGCTAACTAC	ATTGGGCGTAAAGCG		
	GTGCCAGCAGC	AGCGCAGGT		
	CGCGGTAATACGT	GGTTTATTAAGTCTGG		
	AGGTCCCGAGCGT	TGTAAAAGGCAGTGG		
	TGTCCGGATTTATT	CTCAACCATTGTATGC		
	GGGCGTAAAG	ATTGGAAACTGGTAG		
	CGAGCGCAGGTGG	ACTTGAGT		
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	GGCTCAACCA	CGGTGAAATGCGTAG		
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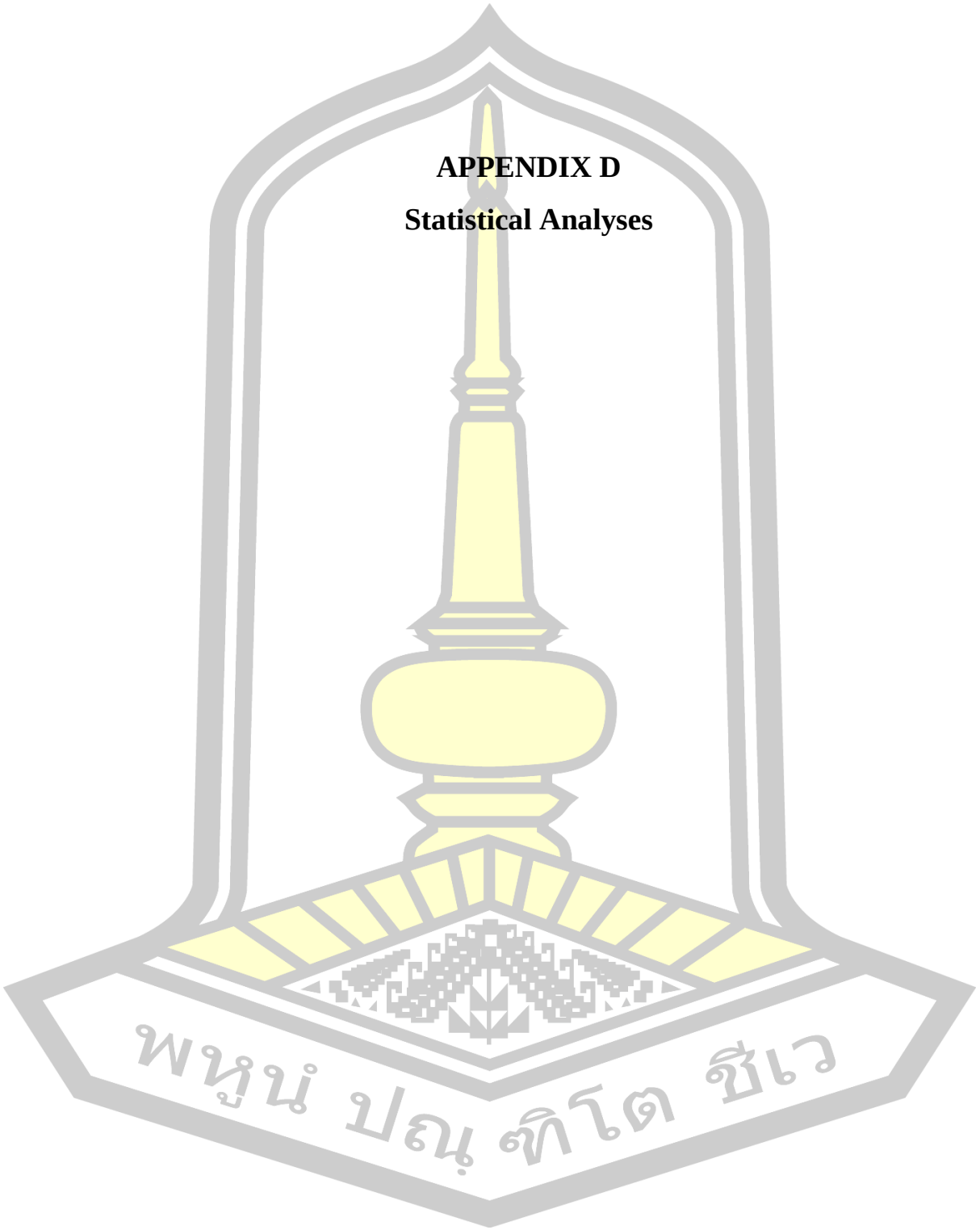
	CACCGGTGGC	TGGTAGTCCACGCCGT		
	GAAAGCGGCTCTC	AAACGATGAGTGCTA		
	TGGCCTGTAACTGA	GATGTAGGGAGCTAT		
	CACTGAGGCTCGA	AAGTTCTCTGTATCGC		
	AAGCGTGGGG	AGCTAACG		
	AGCAAACAGGATT	CAATAAGCACTCCGCC		
	AGATACCCTGGTA	TGGGGAGTACGACCG		
	GTCCACGCCGTAA	CAAGGTTGAAACTCA		
	ACGATGAGTGC	AAGGAATTGACGGGG		
	TAGATGTAGGGAG	GCCCGCACA		
	CTATAAGTTCTCTG	AGCGGTGGAGCATGT		
	TATCGCAGCTAAC	GGTTTAATTCGAAGCA		
	GCAATAAGCA	ACGCGAAGAACCTTA		
	CTCCGCCTGGGGA	CCAGGTCTTGACATAC		
	GTACGACCGCAAG	TCGTGCTA		

	GTTGAAACTCAAA GGAATTGACGG GGGCCCGCACAAG CGGTGGAGCATGT GGTTTAATTCGAAG CAACGCGAAG AACCTTACCAGGTC TTGACATACTCGTG CTATTCCTAGAAGA TAGGAAGT TCCTTCGGGACACG GGATACAGGTGGT GCATGGTTGTCGTC AGCTCGTGT CGTGAGATGTTTGG GTTAAGTCCCGCA ACGAGCGCAACCC CTATTGTTAG TTGCCATCATTTAA GTTGGGCACTCTAA CGAGACTGCCGGT GATAAACCG AAGAAAAAGGTGG GGGAAGAAAAACC AAAACATCAAGGC CCCCTTAGAAC CGGGGGGTACAAC CGGGTTCCAAAGG AAGTGTGCCACCA AATCCCCCAAC AAAAGGAGGTGTT TCCCAAACCTCTTA AAACAATTCTCCTT TTCTGGAAA TTAAGAGGGGGAA ACTTT	TTCCTAGAAGATAGG AAGTTCCTTCGGGACA CGGGATACAGGTGGG TGCATGGTTGTCGTCA GCTCGTGT CGTGAGATGTTTGGGT TAAGTCCCGCAACGA GCGCAACCCCCTATTG TTAGTTGCCATCATTT AATTTGG GCACTCTAACCAAAA CTGCCGGTGATAAACC CGAAGAAAGGGTGGG GGATGAACTCCAAAT CCTCCATGG CCCCTTATGACCTGGG GGTACCACCCT		

APPENDIX D

Statistical Analyses



Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
DPPH	4% 27.5 C	3	85.6767	.13279	.07667	85.3468	86.0065	85.60	85.83
	5.4 % 27.5 C	3	84.2967	1.81555	1.04821	79.7866	88.8068	82.76	86.30
	2.5 % 27.5	3	83.6700	.13856	.08000	83.3258	84.0142	83.59	83.83
	3% 30	3	86.1067	1.61290	.93121	82.1000	90.1133	84.65	87.84
	4% 31.04	3	82.8800	2.26442	1.30736	77.2549	88.5051	80.28	84.42
	4% 27.5	3	80.0867	2.79438	1.61333	73.1451	87.0283	76.86	81.70
	4% 23.9	3	81.8567	1.25125	.72241	78.7484	84.9650	80.52	83.00
	3% 25	3	85.2433	1.63830	.94587	81.1736	89.3131	84.18	87.13
	4% 27.5	3	82.0900	2.64059	1.52454	75.5304	88.6496	80.28	85.12
	50% 30 C	3	81.5800	1.12743	.65092	78.7793	84.3807	80.87	82.88
	5% 25C	3	82.1700	2.09716	1.21080	76.9604	87.3796	80.52	84.53
	Total	33	83.2415	2.38198	.41465	82.3969	84.0861	76.86	87.84
FRAP	4% 27.5 C	3	155.4600	6.09753	3.52041	140.3129	170.6071	149.01	161.13
	5.4 % 27.5 C	3	164.2133	1.17577	.67883	161.2926	167.1341	163.14	165.47
	2.5 % 27.5	3	164.2133	4.56107	2.63333	152.8830	175.5437	161.58	169.48
	3% 30	3	154.2767	9.85527	5.68995	129.7948	178.7585	144.12	163.80
	4% 31.04	3	152.8300	8.14643	4.70334	132.5931	173.0669	147.01	162.14
	4% 27.5	3	161.9900	6.36342	3.67392	146.1824	177.7976	155.13	167.70
	4% 23.9	3	165.3600	5.03852	2.90899	152.8436	177.8764	160.02	170.03
	3% 25	3	153.4600	7.57151	4.37141	134.6513	172.2687	145.23	160.13
	4% 27.5	3	158.9833	8.64472	4.99103	137.5087	180.4580	149.23	165.70
	50% 30 C	3	167.4767	8.06782	4.65796	147.4351	187.5182	158.91	174.93
	5% 25C	3	160.8767	14.31424	8.26433	125.3181	196.4352	147.01	175.60
	Total	33	159.9218	8.29476	1.44393	156.9806	162.8630	144.12	175.60
Phenolic	4% 27.5 C	3	45.1300	8.52293	4.92071	23.9579	66.3021	35.99	52.86
	5.4 % 27.5 C	3	67.4200	2.72820	1.57513	60.6428	74.1972	64.41	69.73
	2.5 % 27.5	3	58.5833	7.22121	4.16917	40.6449	76.5218	50.25	63.00
	3% 30	3	48.5067	10.89547	6.29050	21.4408	75.5725	40.91	60.99
	4% 31.04	3	54.1333	17.29863	9.98737	11.1612	97.1055	34.79	68.12
	4% 27.5	3	65.0800	5.08469	2.93565	52.4489	77.7111	59.49	69.43
	4% 23.9	3	58.0500	7.18871	4.15040	40.1923	75.9077	49.75	62.30
	3% 25	3	52.5933	8.14921	4.70495	32.3496	72.8371	43.52	59.29
	4% 27.5	3	58.0467	19.10969	11.03299	10.5756	105.5178	35.99	69.63
	50% 30 C	3	64.2067	3.06270	1.76825	56.5985	71.8148	62.10	67.72
	5% 25C	3	52.6933	9.35391	5.40048	29.4569	75.9297	44.53	62.90
	Total	33	56.7676	10.86816	1.89190	52.9139	60.6213	34.79	69.73

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
DPPH	Between Groups	110.039	10	11.004	3.385	.008
	Within Groups	71.524	22	3.251		
	Total	181.563	32			
FRAP	Between Groups	820.134	10	82.013	1.306	.287
	Within Groups	1381.565	22	62.798		
	Total	2201.699	32			
Phenolic	Between Groups	1467.381	10	146.738	1.396	.246
	Within Groups	2312.361	22	105.107		
	Total	3779.742	32			

FRAP

Duncan[a]

sample	N	Subset for alpha = 0.05
		1
4% 31.04	3	152.8300
3% 25	3	153.4600
3% 30	3	154.2767
4% 27.5 C	3	155.4600
4% 27.5	3	158.9833
5% 25C	3	160.8767
4% 27.5	3	161.9900
5.4 % 27.5 C	3	164.2133
2.5 % 27.5	3	164.2133
4% 23.9	3	165.3600
50% 30 C	3	167.4767
Sig.		.065

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Phenolic

Duncan[a]

sample	N	Subset for alpha = 0.05	
		1	2
4% 27.5 C	3	45.1300	
3% 30	3	48.5067	48.5067
3% 25	3	52.5933	52.5933
5% 25C	3	52.6933	52.6933
4% 31.04	3	54.1333	54.1333
4% 27.5	3	58.0467	58.0467
4% 23.9	3	58.0500	58.0500
2.5 % 27.5	3	58.5833	58.5833
50% 30 C	3	64.2067	64.2067
4% 27.5	3	65.0800	65.0800
5.4 % 27.5 C	3		67.4200
Sig.		.052	.064

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Post Hoc Tests

Homogeneous Subsets

DPPH

Duncan[a]

sample	N	Subset for alpha = 0.05			
		1	2	3	4
4% 27.5	3	80.0867			
50% 30 C	3	81.5800	81.5800		
4% 23.9	3	81.8567	81.8567	81.8567	
4% 27.5	3	82.0900	82.0900	82.0900	
5% 25C	3	82.1700	82.1700	82.1700	
4% 31.04	3	82.8800	82.8800	82.8800	82.8800
2.5 % 27.5	3		83.6700	83.6700	83.6700
5.4 % 27.5 C	3		84.2967	84.2967	84.2967
3% 25	3			85.2433	85.2433
4% 27.5 C	3				85.6767
3% 30	3				86.1067
Sig.		.106	.119	.055	.064

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

APPENDIX E

Conference Presentation (Poster)

FOOD INNOVATION ASIA CONFERENCE 2014
"Science and Innovation for Quality of Life"

12-13 June, 2014
BITEC, Bangkok, Thailand

The conference will provide opportunity to meet and share experiences as well as strengthen networking among international food scientists and scientists in related fields from academia, government and food industries. **"FOOD INNOVATION ASIA 2014"** is to highlight significant developments in research and innovations in food science and technology with an emphasis on food products innovation. The conference will feature a series of presentations and discussions in plenary, concurrent and poster ses-sions, informal gatherings, competitions and exhibitions in areas:

Presentation areas:
- Food Health and Nutrition
- Food Processing and Engineering
- Food Microbiology, Food Safety and Quality
- Food Chemistry and Analysis
- Food Product Development and Ingredient Innovations
- Sensory and Consumer Research
- Food & Agricultural Packaging Technology & Innovations
- Food Supply Chain Management
- Food Security and Sustainability

- ProPak ASIA 2014
- FoSTAT-Nestle Quiz Bowl 2014
- Food Innovation Contest 2014 (final round
- 50th Anniversary of Food Science & Technology,Kasetsart University
- 3rd International Seminar on Food & Agricultural Science (ISFAS) 2014
- International Food & Agricultural Packaging Meeting 2014
- FIFSTA Annual meeting
- AIAC Annual meeting

Important Dates

Period for abstract submission	January 1 – February 28, 2014 March 1-10, 2014 (extended period)*
Notification of abstract acceptance	March 15, 2014 for abstract submitted by February 28, 2014 March 25, 2014 for abstract submitted by March 10, 2014
Deadline for full paper submission	March 31, 2014
Notification of paper acceptance	May 10, 2014

*Participants who submit their abstract(s) during the extended period should be aware that the deadline for full paper submission either for the conference proceedings or kasetsart Journal is March 31, 2014. Such deadline will not be extended any further.

Registration

Delegate	Early bird rate	Regular rate(After May 31,2014)
Participant	200 USD	250 USD
Student	100 USD	120 USD
Accompany / Visitors (Student only-Not included coffee & lunch conference bag and book of abstract)	60 USD	60 USD

(Bank charges must be paid by the delegate)
Deadline for Registration Fee Payment May 31,2014
พิเศษ – อัตราค่าลงทะเบียน (คนไทย)

Organizers

- Faculty of Agro-Industry, Kasetsart University
- Food Science and Technology Association of Thailand (FoSTAT)


Antioxidant activities and microbial studies of Thai red rice milk kefir
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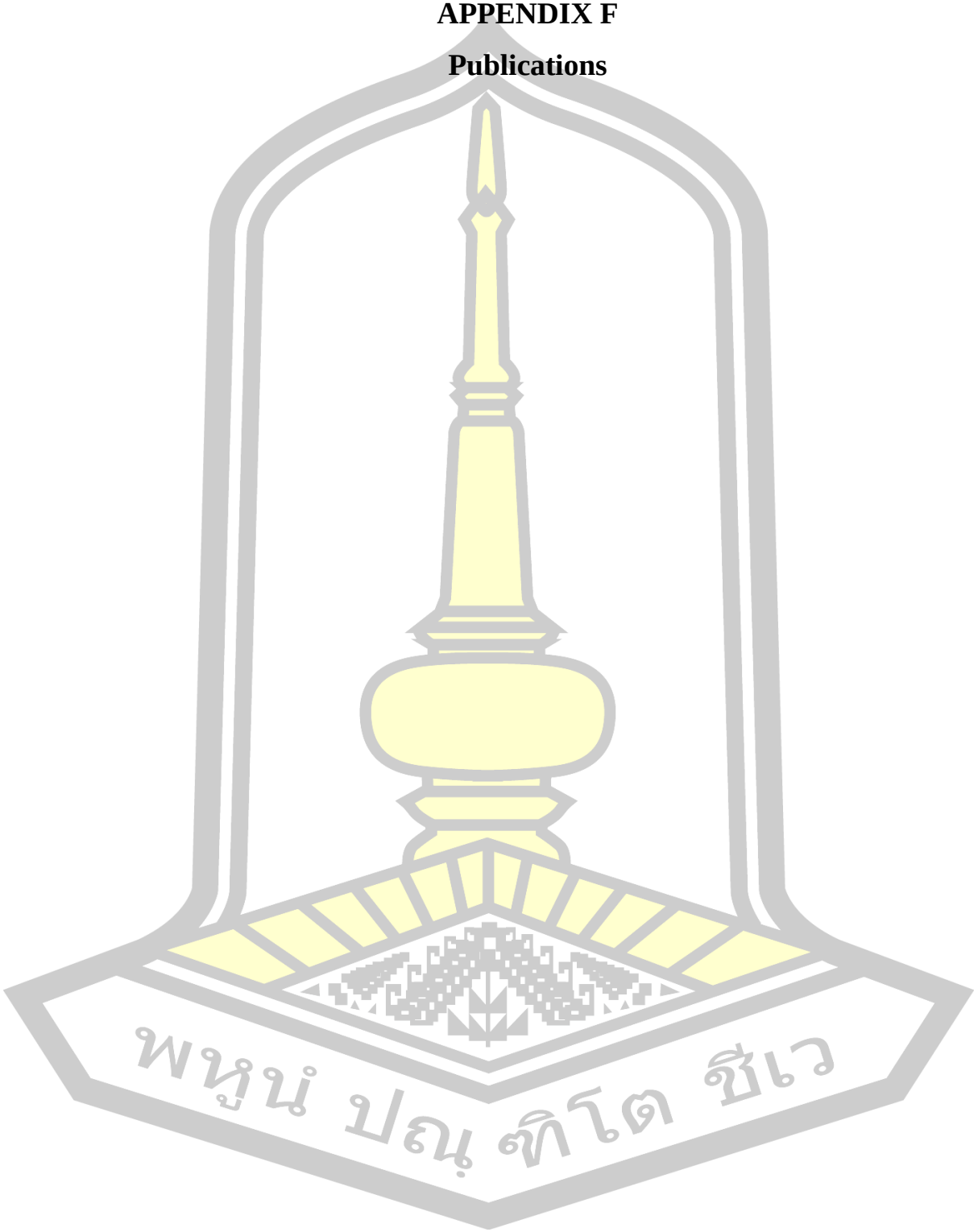
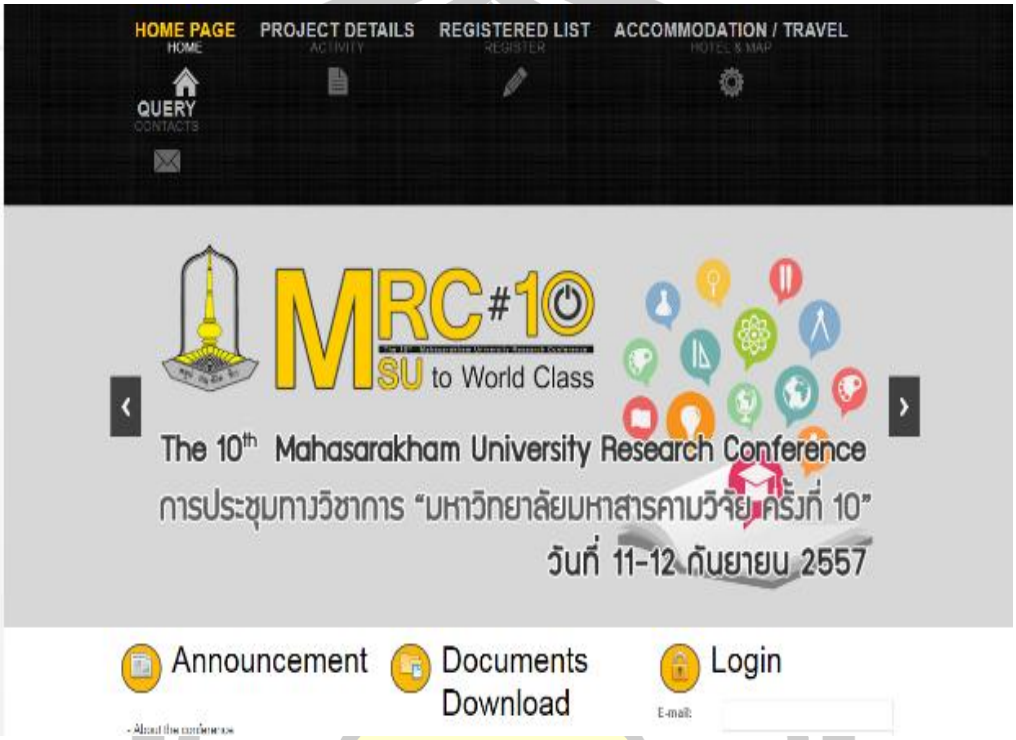
Abstract
Kefir is a popular fermented milk beverage which has its origin from caucasis region. Different varieties of milk can be used for kefir production. In this study, milk kefir grains from Khamphengpet province Thailand and Thai Red rice (Khao Dang) were used. For the rice milk preparation, ultrasonication and blender methods were used. The kefir grains were incubated in rice milk for 24 hrs, 48 hours and until an optimum pH of 4.2 was reached. Physicochemical properties such as pH and viscosity of kefir were calculated. The presence of lactic acid bacteria, acetic acid bacteria and yeast were analyzed. MRS agar, Acetic Acid Medium (AAM) agar and Glucose Yeast (GY) extract agar were used as the medium. Antioxidant studies were done using FRAP, DPPH and Total phenolic methods. From the study, it was found that, as the fermentation time increased the antioxidant activities were higher. With physicochemical properties, ph of rice milk kefir dropped as the fermentation time increased so was with the viscosity. Results from the microbial studies showed that, there was presence of yeast and acetic acid bacteria on rice milk kefir and there was no presence of LAB on red rice milk kefir from Khamphengpet province Thailand. Further studies are needed to identify the different strains of bacteria.
Keywords: Milk kefir, fermented beverage, Lactic acid bacteria, acetic acid bacteria and yeast.
Materials and Methods
Kefir Sample: Kefir grains from Khamphengpet province, Thailand.
Materials
Khao Dang (Red rice)
Kefir grains
1. Preparation of Rice Milk
2. Kefir Culturing
Kefir grains
UHT milk
Incubated 25 °C 24 hr
Filtered
Grains
3. Physicochemical properties and microbial studies
Kefir grains (5%)
PH, Viscosity
4. Antioxidant Activities
TPC
DPPH Free radical scavenging
FRAP assay
Using DPPH solution and Abs at 517 nm
Using FRAP reagent and Abs at 595 nm
Results
Table 1. Physiochemical properties (viscosity and pH) of Rice milk Kefir and UHT milk kefir
Treatments | Viscosity(centipoises) 0 hr, 24 hrs, 48 hrs, pH 4.2 | pH 0 hr, 24 hrs, 48 hrs
Red rice milk kefir | 3.0 | 1.6 | 1.6 | 2.0 | 6.63 | 5.35 | 4.32
UHT Milk kefir | 3.3 | 7.0 | 7.0 | 7.2 | 6.72 | 6.36 | 6.0
Table 2. Microbial population found in Rice milk Kefir and UHT milk kefir
Treatments | Acetic acid bacteria population (CFU/ml) 24 hrs, 48 hrs, pH 4.2 | Yeast population (CFU/ml) 24 hrs, 48 hrs, pH 4.2
Table 3. Antioxidant activities (DPPH, FRAP and Total phenolic content) of Rice milk Kefir and UHT milk kefir
Treatment
DPPH
FRAP
TPC
Red rice milk kefir
UHT Milk kefir
References
Conclusion
In this study, red rice milk was used and kefir grains from Khamphengpet province Thailand were inoculated and was studied on its microbial population and antioxidant activities. The values obtained from physiochemical properties prove that the pH and viscosity values dropped with the increase in fermentation time. Moreover, the highest total phenolic content value was found for red rice milk kefir with pH 4.2.

APPENDIX F

Publications





กิจกรรมการต้านอนุมูลอิสระของคีเฟอร์นมข้าวสี

Antioxidant Activities of Color Rice-Milk Kefir

สตีเฟน มอร์เสส จอห์น[1*], ศิริรัตน์ ดีศีลธรรม[2]
Stephen Moses John[1*], Sirirat Deesenthum[2]

บทคัดย่อ

งานวิจัยนี้นำข้าวสายพันธุ์ไทย 3 สายพันธุ์ได้แก่ ข้าวแดง (ข้าวเจ้ามะลิแดง), ข้าวเหนียวกล่ำเปลือกดำ และข้าวดำ (ข้าวเหนียวดำ มข) มาผลิตเป็นนมข้าวแต่ละสีหมักด้วยหัวเชื้อคีเฟอร์และแปรผันสูตรนมที่มีการผสมและไม่ผสมนมโค หมักเป็นเวลา 72 ชั่วโมง วิเคราะห์หาคุณสมบัติทางกายภาพของผลิตภัณฑ์ ปริมาณฟีนอลิกทั้งหมดโดยวิธี Folin-Ciocalteau วิเคราะห์กิจกรรมการต้านอนุมูลอิสระของคีเฟอร์นมข้าวสีสูตรต่าง ๆ โดยใช้วิธี 2, 2,-diphenyl-1-pricrylhydrozyl (DPPH) scavenging, Ferric reducing antioxidant power (FRAP) และ Hydroxyl radical (OH) scavenging จากผลการทดลองพบว่า คีเฟอร์นมข้าวแดงที่ไม่ผสมนมโคเมื่อหมักเป็นเวลา 48 ชั่วโมงมีสารประกอบฟีนอลทั้งหมดสูงสุดเท่ากับ 1.60 มิลลิกรัมต่อลิตร และกิจกรรมการต้านอนุมูลอิสระของคีเฟอร์นมข้าวแดงที่ผสมนมโคซึ่งวิเคราะห์ด้วยวิธี DPPH ให้ค่ากิจกรรมสูงสุดเท่ากับ 64.37% ในขณะที่คีเฟอร์จากนมข้าวดำที่ผสมนมโคพบว่ามีกิจกรรมการต้านอนุมูลอิสระซึ่งวิเคราะห์โดยวิธี FRAP มีค่าสูงสุดเมื่อหมักเป็นเวลา 24 ชั่วโมง ให้ค่ากิจกรรมการต้านอนุมูลอิสระเท่ากับ 4.00 มิลลิกรัม Fe (II) / ลิตร และคีเฟอร์นมข้าวแดงที่ไม่ผสมนมโคให้ค่ากิจกรรมการต้านอนุมูลอิสระสูงสุด เมื่อวิเคราะห์ด้วยวิธี Hydroxyl radical เท่ากับ 99.37% จากผลการศึกษานี้ชี้ให้เห็นว่าคีเฟอร์จากนมข้าวสีแต่ละชนิดมีคุณสมบัติการต้านอนุมูลอิสระ ซึ่งน่าจะสามารถนำไปพัฒนาเป็นผลิตภัณฑ์สุขภาพในอนาคตได้

Abstract

In this study, different brans of Thai color rice cultivars namely, red rice (*mali dang*), brown rice (*gum pleuak dum*) and black rice (*dum moko*) was used to produce kefir by fermentation of kefir culture for 72 hours. The aim of this study was to find the difference between color rice-milk kefir with and without UHT milk addition on their total phenolic, antioxidant activities and physiochemical properties. The samples were analyzed using the Folin-Ciocalteau method for total phenolic content, 2, 2,-diphenyl-1-pricrylhydrozyl (DPPH) scavenging method, Ferric reducing antioxidant power (FRAP) and Hydroxyl radical (OH) scavenging activity. The results indicated that the highest total phenolic compound was found to be 1.60 mg/l for Kefir produced from 48 hours of fermentation of red rice milk without UHT milk. With the DPPH scavenging method, methanolic extract of kefir

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produced from fermented red rice milk with UHT milk for 0 hour showed the highest scavenging activity of 64.37% while FRAP values were higher for kefir produced from 24 hours fermented black rice milk with UHT milk, which was found to be 4.00 mg Fe (II)/l. Likewise with the hydroxyl scavenging method, Kefir produced from 24 hours fermented red rice milk without UHT milk showed the highest scavenging activity of 99.37%. However, all three color rice milk kefir showed the possibility of presence of polyphenols which have antioxidant properties. These results conclude that Kefir produced from fermented rice milk without UHT milk and rice milk with UHT milk could be a promising source of natural antioxidants with good potential for improving health.

Keywords: Kefir, colored rice, rice-milk, UHT milk, natural antioxidant.

Introduction

The word "Kefir" is derived from the Turkish word "keyif, which means "feeling good" after its ingestion (Lopitz *et al.*, 2006; Tamime, 2006). Kefir is defined as a beverage produced by the action of lactic acid bacteria (LAB) (lactobacilli, lactococci, leuconostocs), yeasts, and acetic acid bacteria (aceterobacteria) on milk (Farnworth and Mainville, 2008; Halle *et al.*, 1994). Kefir is characterized by its distinct flavour, typical of yeast, and an effervescent effect felt in the mouth (Lopitz *et al.*, 2006; Rattray and Connell, 2011). The main products of Kefir fermentation are lactic acid, ethanol and carbon dioxide, which confer upon this beverage viscosity, acidity and low alcohol content. Minor components can also be found, including diacetyl, acetaldehyde, ethyl and amino acids contributing to the flavor composition (Rattray and Connell, 2011). Moreover, Kefir has antimicrobial, antihypertensive, antiinflammatory, anticarci-nogenic, antiallergic, and antioxidant activity; participates in immune-system modulation; reduces cholesterol levels; and alleviates lactose intolerance (Farnworth 2005).

In addition, nitrogen compounds and carotenoids, as well as ascorbic acids, are natural antioxidants which are obtained from plants (Laandrault *et al.*, 2001; Iqbal *et al.*, 2005). Similarly Lin & Yen (1999) showed that natural antioxidants from foods may reduce the oxidative damage on the human body. Moreover, there are many studies relating to antioxidant activity in rice, but few relating to rice milk. Furthermore, colored rice reported as potent sources of antioxidants and encouragements as viable sources of antioxidants for functional foods were made (Yawadio *et al.*, 2007).

Besides, the antioxidant activities of the brown rice extract were higher than BHA (Supasit Chooklin. 2013). But, a study by Wang *et al.*, (2006); Rekha and Vijayalakshmi, (2008) on soy milk showed that there is an increase in antioxidative activities of soymilk after fermentation with lactic acid bacteria, *bifidobacteria* and Probiotic Yeast. Similarly, Sirirat and Jelena, (2010) showed rice milk kefir has high

antioxidant activity compared to BHA. Moreover there are variations among antioxidant properties of Kefir samples produced from different cow/soy milk mixtures related to the soymilk ratio in kefir milk (Kesenkas *et al.*, 2011). Besides this, a study by Je Ruei *et al.*, (2005) proved that Kefirs are potential antioxidants that interact with a wide range of species directly responsible for oxidative damage. There are only few studies relating to the activity of rice milk kefir (Sirirat and Jelena, 2010).

This research has aimed to investigate the antioxidant activities and find the difference between Thai glutinous rice-milk kefir with and without UHT milk addition. Firstly, the total phenolic content (TPC) of rice milk Kefir, rice and cow milk kefir was measured following the Folin-Ciocalteu method, using gallic acid as the standard. Antioxidant activity was determined using Hydroxyl radical (OH) scavenging, the FRAP method and the DPPH method. The physiochemical properties were also determined.

Materials and methods

The experiments were carried out at the Department of Biotechnology, Faculty of Technology, Mahasarakham University, Thailand, during the period December 2012 to September 2013.

Materials

Thai rice cultivars used in this study were unpolished waxy color rice (*mali dang* (brown color), *gum pleuak dum* (red color) and *dum moko* (black color) from (Roi-et, and Udonthani Thailand).

Reagents and Chemicals

Folin-Ciocalteau reagent, 2, 2,-diphenyl-1-pricrylhydrozyl (DPPH), Gallic acid, Methanol, NaOH – phosphate buffer (pH7), Sodium carbonate.

Methods

Preparation of Rice Milk

Firstly, 500 g of (*mali dang*, *gum pleuak dum* and *dum moko*) rice was soaked in 1 L distillated water for 24 hrs. Then it was blended, filtrated with cotton sheet and cooked at 72 °c for 15 min, after which the milk was cooled and ready for use.

Kefir Culturing

Kefir cultures DC 500 I from (Danisco Biolacta, Poland) was used as starter cultures. The starter cultures were grown in MRS medium and were incubated at 25°c for 24 hrs then frozen at 4° C until they were used.

Kefir and Rice milk Fermentation

Rice milk prepared earlier and UHT milk was used to produce kefir. Three varieties of rice milk (*mali dang*, *gum pleuak dum* and *dum moko*) and UHT milk (total fat 9 % were used. Flask Fermentation was carried out at 24-26°C for 0 (start), 24, 48 and 72 hrs by using 10% (v/v) of Kefir Starter culture. Time 0 hr is considered as the start or initiation time. The experiments were conducted in triplicate.

Physiochemical properties

The pH value was determined using a digital pH meter. The viscosity of the sample was determined using a viscometer. The

titratable acidity (°Th) for the sample was determined by the method according to (Steffen, 1971; Sirirat and Jelena, 2010). Briefly, 20 ml of deionized water was added to 10 ml of milk and then 5 ml of alcoholic solution of phenolphthalein was added then the mixture was titrated with 1 M NAOH to the first persistent pink color. The amount of NAOH required was recorded. This amount was multiplied by 10 and gave the Th per 100 ml of milk.

Total Phenolic content

The total phenolic contents of Kefir produced from colored rice milk with and without UHT cow milk were determined by modified method of Singleton and Ross, (1965) using Folin-Ciocalteu's reagent. Briefly, 1 ml of colored rice milk kefir, color rice milk with and without UHT milk was added to 1 mL of gallic acid. Then 10 ml of water was added to the mixture after that, Folin-Ciocalteu's reagent (1:10) 0.5 ml was added and allowed to stand for 30 mins in room temp. At 20% (w/v) sodium carbonate (3 ml) and deionized water was added to the mixture and the volume was made to 50 ml. After being kept in darkness for 15 min, absorbance was measured at 725 nm using a spectrophotometer. The amount of total phenolic was calculated using the Gallic Acid Calibration Curve.

DPPH Free radical scavenging

The antioxidant activity of Kefir produced from colored rice milk with and without UHT milk was evaluated through a free radical scavenging effect on 2, 2-diphenyl-1- picrylhydrazyl (DPPH) radical. The determination was based on the method followed by Sirirat and Jelena, (2010). Briefly, 3 ml of DPPH solution was added to 0.1 ml of sample or 95% ethanol, which was used as control, mixed well and incubated for 30 min in a dark room at room temperature. Absorbance was measured at 517 nm using a spectrophotometer. The percentage of DPPH scavenging was calculated as:

$$\frac{(control\ absorbance - extract\ absorbance)}{(Control\ absorbance)} \times 100$$

Determination of Ferric Reducing /Antioxidant Power Assay (FRAP)

A FRAP assay was done with slight modification to the method of Benzie and Strain, (1999). FRAP reagent was prepared from acetate buffer (300 mM acetate buffer and adjusted to a pH of 3.6 by acetic acid and made up to 100 ml), 10 mM 2,4,6-Tripyridyl-s-Triazine TPTZ solution in 40 mM HCL and 20 mM iron (III) chloride solution and 300 mM acetate buffer in proportion of 1:1:10 (v/v) respectively. The prepared FRAP reagent was used for the experiment as follows: 300 µl sample or the standard was added to 1.7 ml of FRAP reagent. The mixture was mixed thoroughly and was incubated in the dark for 10 mins. The absorbance was measured at 595 nm using a spectrophotometer. The standard curve (r^2 = 0.9995) for FRAP was plotted with the absorbance at 595 nm and the values obtained were expressed in mM of ferrous equivalent Fe (II) per gram of sample.

$$\frac{(control\ absorbance - extract\ absorbance)}{(Control\ absorbance)} \times 100$$

Determination of Hydroxyl Radical (OH) scavenging activity

Hydroxyl radical (OH) scavenging activity was performed according to the method followed by Sirirat and Jelena, (2010).Briefly, 0.075 ml of sample was mixed with 0.2 M sodium phosphate buffer (pH of 0.7), 0.15 ml 2-Deoxyribose (10 mM), 0.15 ml of EDTA (10 mM), 0.15 mL of $FeSO_4$ (10 mM), 0.15 mL of hydrogen peroxide (10 mM), 0.525 ml of water. Samples were then incubated at 37ºC for 4 hrs. After incubation, 0.75 ml of trichloroactric acid (2.8%) acid and thiobarbituric (0.1%) acid were added. Then, samples were kept in a boiling water bath for 10 mins. The absorbance of each sample was measured at 520 nm and ethanol was used as a control. The percentage of hydroxyl scavenging was calculated as:

$$\frac{(control\ absorbance - extract\ absorbance)}{(Control\ absorbance)} * 100$$

Statistical Analyses

Results obtained were reported as mean+ SD of triplicate measurements. Significant differences for multiple comparisons were determined by a two way analysis of variance (ANOVA) followed by a Duncan test with α = 0.05, significance level of $P < 0.05$ using SPSS (version 19).

Results and Discussion

Physiochemical properties

After the fermentation, a foamy drink was obtained. The viscosity of Kefir produced from 0, 24, 48 and 72 hrs fermented rice milk without UHT milk was slightly higher than the kefir produced from 0, 24, 48 and 72 hrs fermented rice milk with UHT milk. With regard to viscosity, the highest value was found to be 25 cps for kefir produced from 0 hrs fermented black rice milk without UHT milk. In terms of pH, the highest was found to be 4.23 for Kefir produced from fermented black rice milk with UHT milk. The highest acidity value was found to be 1.89 ° Th for Kefir produced from 48 hrs fermented red rice milk without UHT milk. The values are presented in table 1. No significant difference was found in the acidity, pH and viscosity between the Kefir produced from fermented rice milk with and without UHT milk.

The results from physiochemical properties prove that, pH, viscosity (centipoises) and acidity (° Th) values decreased when the fermentation time was increased (Table 1). The decrease in viscosity may be due to the amount of polysaccharide content present in rice milk kefir during the start of fermentation till the end. Toba and group (1987) reported that, the viscosity of the fermented drink was directly proportional to the polysaccharide content. The decrease in acidity may be due to the growth of microorganisms and fermentation time. A study by Shiva *et al.*, (2011) suggests that the acid production in kefir depends on the growth of microorganisms and their ability for fermentation of the carbohydrates in milk and soymilk. Moreover, Sirirat and Jelena, (2010) stated that the decrease in pH may be due to increased lactic acid bacteria population at the beginning of fermentation.

Total Phenolic content

By the folin-Ciocalteu reagent method using gallic acid as standard, the average

quantity of total phenolic compounds (mg/l) was measured for Kefir produced from fermented brown, black and red rice milk with and without UHT milk. The measured values are represented in table 2. Values shown in all tables are means±standard deviation from triplicate experiments. The highest total phenolic content was found to be 1.60 (mg/L) for kefir produced from 48 hrs fermented red rice milk without UHT milk. Muntana and prasong (2010) also reported that red rice showed high total phenolic content and they also suggested that the higher color pigments may also be the main compounds for antioxidant activity. There was no significant difference at ($P<0.05$) between kefir produced from fermented rice milk without UHT milk and rice milk with UHT milk. Besides this, (Patrick and kalidas, 2005) proved soluble phenolic content of soymilk increased during the initial 40 hrs of culture time with active Kefir cultures.

According to Petti and Scully, (2009) phenolic compounds are plant metabolites characterized by the presence of several phenol groups. Some of them are very reactive in neutralizing free radicals by donating a hydrogen atom or an electron, chelating metal ions in aqueous solutions. However, the phenolic compounds possess many biological properties such as antitumor, antimutagenic and antibacterial properties, and these activities might be related to their antioxidant activity (Shui and Leong, 2002). Some researchers have shown that pigmented rice, such as red and black rice are composed of high content of phenolic compounds (Oki *et al.*, 2002).

DPPH Radical Scavenging

The result presented in table 3 was expressed as % scavenging, while the highest % scavenging was found to be 64 % for Kefir produced from 0 hrs fermented brown rice milk and cow milk. On the other hand, kefir produced from 72 hrs fermented black rice milk showed the lowest % scavenging of 23.40 %. So, when the fermentation time was increased, the scavenging % decreased. According to our study the increased scavenging activity may be due to the milk protein or plant protein.

In addition, Liu and team (2005) proved that some antioxidant components were transferred from kefir grains to milk and soy-milk during fermentation. Besides this, Je Ruei Liu and group (2005) found that milks fermented by kefir grains demonstrated an enhanced activity as regards scavenging the DPPH radical as compared to unfermented milks, stating that kefir has got an influence on scavenging the DPPH radicals.

Moreover, (Villano *et al.*, 2007) proved that 1, 1-Diphenyl-2-picrylhydrazyl (DPPH; I) is a stable free radical. On accepting hydrogen from a corresponding donor, its solutions lose the characteristic deep purple (λmax 515–517 nm) colour. DPPH is very popular for the study of natural antioxidants.

Hydroxyl radical scavenging

The result presented in table 4 shows that Kefir produced from 24 hrs fermented red rice milk without UHT milk showed the highest scavenging activity of 99.37%. On the

contrary, when the fermentation time was increased to 72 hrs, the percentage of scavenging decreased to 50.12 for Kefir produced from fermented red rice milk with UHT milk. The higher % of hydroxyl scavenging may be due to the presence of polyphenolic substances in rice or kefir which can scavenge the free radicals. Gulcin and group (2010) stated that hydroxyl radical is the most highly reactive oxygen radical in the presence of transition metal ions and participates in free-radical reaction.

According to Boguslaw Lipinski, (2011) Antioxidant properties of polyphenols are based on their ability to be oxidized, polyphenols can scavenge hydroxyl radical (•OH) by virtue of their addition to double bonds with the formation of a corresponding hydroxyl derivative:

R–CH=CH–R + •OH ⟶ R–CH–(OH)–CH2–R. (Patterson *et al.,* 2000)

Polyphenols are capable candidates for scavenging hydroxyl radicals which also indicates the uncertainity in the presence of polyphenols in rice milk kefir. Moreover, Q.D do and group (2013) found rice paddy herb contains high amount of antioxidants which play an important role in getting rid of hydroxyl radicals considered to be the free radicals causing several serious diseases such as cancer, heart disease, diabetes, etc.

Ferric Reducing antioxidant power

The result presented in table 5 shows that the FRAP values were higher for Kefir produced from 24 hrs fermented black rice milk with UHT milk, which was found to be 4.00 mg Fe (II) /L. The lower value was recorded for kefir produced from 24 hrs fermented brown rice milk without UHT milk, which was found as 1.33 mg Fe (II) /L. The higher FRAP values may be due to the active compounds present in rice, for the reason that , Je Ruei Liu and group (2005) found that milks fermented by kefir grains did not significantly affect their ferrous ion chelating ability However, Schafer and Buettner, (2001) stated that higher FRAP values give higher antioxidant capacity because FRAP value is based on reducing ferric ion, where antioxidants are the reducing agent and are compounds capable of donating a single electron or hydrogen atom for reduction.

Conclusions

The values obtained from physiochemical properties prove that the pH and acidity values increase with the addition of UHT milk. In relation to viscosity, without UHT milk the liquid was found more viscous. Moreover, the highest total phenolic content was found in kefir produced from 48 hours fermented red rice milk without UHT milk, and there were higher levels of phenolic compounds, which may act as antioxidant properties. There was no difference between the treatment and fermentation time. Furthermore, Kefir produced from both fermented rice milk without UHT milk and rice milk with UHT milk showed stronger antioxidant activity, provided that optimum fermentation time was maintained for greater antioxidant activity.

Kefir produced from 24 hrs fermented red rice milk without UHT milk showed the highest Hydroxyl scavenging

activity of 99.37%, which scavenged more hydroxyl radicals. This may be due to the phenolic content in the red rice. Red rice milk with UHT milk had higher DPPH radical scavenging with a percentage of 64.37 % which may be due to the presence or milk or plant protein. Our study proves that rice milk Kefir has greater antioxidant activity individually and also when combined with UHT milk. Study also indicates that the Rice used in this study certainly had a great influence towards antioxidant activity which may be be due to the presence of polyphenols. Colored rice milk Kefir can be a natural antioxidant supplement in the human diet. Further study should be conducted to identify the antioxidant compound and also processing of colored rice milk Kefir to be used as a food product.

Table1 Physiochemical properties (viscosity, pH and acidity) of Kefir produced from 0, 24, 48, 72 hrs fermented rice milk with and without UHT milk.

Treatment	Viscosity(centipoises)				pH				Acidity(°Th)			
	0 hr	24 hrs	48 hrs	72 hrs	0 hr	24 hrs	48 hrs	72 hrs	0 hr	24 hrs	48 hrs	72 hrs
Kefir (Black rice milk without UHT milk)	25	14.58	10.41	8.33	4.09	3.96	3.75	3.70	0.351	0.51	0.387	0.342
Kefir (Black rice milk with UHT milk)	18.75	12.5	8.33	8.33	4.11	4.23	3.99	3.91	0.387	0.54	0.837	0.621
Kefir (Red rice milk without UHT milk)	16.67	8.33	12.5	2.08	4.1	3.69	3.59	3.54	1.71	1.602	1.89	0.603
Kefir (Red rice milk with UHT milk)	14.83	8.33	12.5	2.08	4.11	4.8	3.94	3.91	0.927	1.62	1.35	0.765
Kefir (Brown rice milk without UHT milk)	16.67	10.41	12.5	6.25	3.92	4.03	3.84	3.81	0.351	0.288	0.405	0.333
Kefir (Brown rice milk with UHT milk)	16.67	14.58	14.58	4.16	4.1	4.21	3.94	3.96	0.396	0.675	0.99	0.342

Data represented are mean values from triplicate experiments

Table 2 Total phenolic content of Kefir produced from 0, 24, 48, 72 hrs fermented rice milk with and without UHT milk

Treatment	Total phenolic content (mg/L)		
	0 hr	48 hrs	72 hrs
Kefir (Black rice milk without UHT milk)	0.26 ± 0.01^{bC}	0.63 ± 0.03^{bB}	0.44 ± 0.02^{bA}
Kefir (Black rice milk with UHT milk)	0.28 ± 0.01^{cC}	0.49 ± 0.01^{cB}	0.31 ± 0.01^{cA}
Kefir (Red rice milk without UHT milk)	0.26 ± 0.00^{aC}	1.60 ± 0.02^{aB}	0.55 ± 0.01^{aA}
Kefir (Red rice milk with UHT milk)	0.21 ± 0.01^{dbC}	0.44 ± 0.02^{dbB}	0.50 ± 0.02^{dbA}
Kefir (Brown rice milk without UHT milk)	0.23 ± 0.01^{dC}	0.31 ± 0.03^{dB}	0.28 ± 0.01^{dA}
Kefir (Brown rice milk with UHT milk)	0.23 ± 0.01^{abC}	1.20 ± 0.02^{abB}	0.62 ± 0.01^{abA}

Values with different lowercase letters in the same row and values with different uppercase letters in the same column have significant difference ($p < 0.05$). No significant difference was found.

Table 3 DPPH Free radical scavenging of Kefir produced from 0, 24, 48, 72 hrs fermented rice milk with and without UHT milk

Treatment (1000 ppm)	DPPH (% Scavenging)			
	0 hr	24 hrs	48 hrs	72 hrs
Kefir (Black rice milk without UHT milk)	57.80±0.39eA	22.94±0.16eB	53.57±0.37eA	23.40±0.48eC
Kefir (Black rice milk with UHT milk)	43.24±0.47dA	48.37±0.21dB	55.85±0.36dA	24.38±0.52dC
Kefir (Red rice milk without UHT milk)	58.50±3.59aA	56.65±0.26aB	48.20±0.32aA	34.57±0.33aC
Kefir (Red rice milkwith UHT milk)	64.37±0.67aA	44.21±0.24aB	53.05±0.58aA	45.96±0.47aC
Kefir (Brown rice milk without UHT milk)	52.53±0.42bA	46.07±0.41bB	50.12±0.75bA	45.86±0.74bC
Kefir (Brown rice milk with UHT milk)	42.57±0.52cA	42.82±0.21cB	54.62±0.45cA	44.81±0.47cC

Values with different lowercase letters in the same row and values with different uppercase letters in the same column have significant difference ($p < 0.05$). No significant difference was found.

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References

1. Akowah GA., Ismail Z., Norhayati I., Sadikun A. 2005.The effect of different extraction solvents of varying polarities of polyphenols of orhosiphon. Stamineus and evaluation of the free radical scavenging activity. Food Chem. 93: 311-317.
2. Benzie IFF., strain JJ. 1999. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Meth enzymol. 299: 15-27.
3. Farnworth ER. 2005. Kefir- a complex probiotic. Food Sci Technol Bull Funct food 2:1–17
4. Farnworth, E., Mainville, I. 2008. Kefir -A Fermented Milk Product. In Handbook of Fermented Functional Foods.Second EditionFarnworth Edward R.Broken Sound Parkway NW, Taylor & Francis Group, LLC. Pp. 89-127.
5. Gulcin I, Huyut Z, Elmastas M & Aboul-Enein H Y 2010. Radical scavenging and antioxidant activity of tannic acid. Arabian Journal of Chemistry 3: 43-53.
6. Halle, C., Leroi, F., Dousset, X., Pidoux, M. 1994. Les kéfirs: des associations bactéries lactiques- levures. In: Bactéries Lactiques: Aspects Fondamentaux et Technologiques, pp. 169–182.
7. Iqbal, S., Bhanger, M.I and.Anwar, F. 2005. Antioxidant properties and components of some commercially available varieties of rice bran in Pakistan. Food chem. 93: 265-272.
8. Je Ruei Liu., Yuh-Yih Lin., Ming-Ju Chen., Li-Ju Chen and Chin-Wen Lin. 2005. Antioxidative activities of Kefir. Asian-Aust. J. Anim. Sci. 18(4): 567-573.
9. Kesenkas, H., Dinkci, N., Seckin, K., Kinik, O., Gonc, S. 2011. Antioxidant properties of kefir produced from different cow and soy milk mixtures. Tarım Bilimleri Dergisi. Journal of Agricultural Sciences. 17: 253-259.
10. Laandrault,N ., P. Pouchert, P., Ravel, F., Gase, G., Cros and P.L. Teissedro. 2001. Antioxidant activities and phenolic level of French wines from different varieties and vintages. J. Agric. Food chem. 49: 3341-3343.
11. Liu, J., Chen, M and Lin, C. 2005. Antimutagenic and Antioxidant Properties of Milk-Kefir and Soymilk-Kefir. Journal of Agricalture and Food Chemistry. 53: 2467-2474.
12. Lin M Y & Yen C L.1999. Antioxidative ability of lactic acid bacteria. Journal of Agriculture and Food Chemistry. 47: 1460-1466.

13. Lopitz-Otsoa F., Rementeria A., Elguezabal N and Garaizar J. 2006. Kefir: A symbiotic yeasts bacteria community with alleged healthy capabilities. Rev Iberoam Micol. 23: 67-74.
14. Muntana, N and prasong, S. 2010. Study on total phenolic contents and their antioxidant activities of Thai white, red and black rice bran extracts. Pakistan journal of biological sciences. 13 (4): 170-174.
15. Oki, T., Masuda, M., Kobayashi, M., Nishiba, Y., Furuta, S., Suda, I., Sato, T. 2002. Polymeric procyanidins as radical scavenging components in red- hulled rice. J. Agric .Food chem. 50: 7524-7529.
16. Patterson, c, N. R. Madamanchi, and M. S. Runge. 2000. The oxidative paradox: another piece in the puzzle. Circulation Research. 87(12): 1074–1078.
17. Patrick P. McCue and Kalidas Shetty. 2005. Phenolic antioxidant mobilization during yogurt production from soymilk using Kefir cultures. Process Biochemistry. 40 : 1791–1797.
18. Petti, S., Scully, C. 2009. Polyphenols, oral health and disease: a review. J. Dent. 37: 413-423.
19. Q.D. Do, A.E. Angkawijaya, P.L. Tran-Nguyen, L.H. Huynh, F.E. Soetaredjo, S Ismadji, Y-H Ju. 2013. Effect of extraction solvent on total phenol content, total flavonoids content, and antioxidant activity of *limnophila aromatic*. Journal of food and drug analysis. pp. 1- 7.
20. Rattray FP., O'Connell MJ. 2011. Fermented Milks Kefir. In: Fukay, J. W. (ed.), Encyclopedia of Dairy Sciences (2th ed). Academic Press , San Diego, USA, Pp. 518-524.
21. Rekha, C., Vijayalakshmi, G. 2008. Biomolecules and Nutritional Quality of Soymilk Fermented with Probiotic Yeast and Bacteria. Applied Biochemistry and Biotechnolgy.151: 452–463.
22. Schafer, F. Q and Buettner, G. R. 2001. Redox environment of the cell as viewed through the redox state of the gluthathione disulfide/ gluthathione couple. Free Radical Biology Medicinal 30(11): 1191-1212.
23. Shiva Dadkhah., Rezvan Pourahmad., Mahnaz Mazaheri Assadi and Ali Moghimi. 2011. Kefir production from soymilk. Scholars Research Library. 2 (6): 293-299.
24. Shui, G., Leong, L.P.2002. Separation and determination of organic acids and phenolic compounds in fruit juices and drinks by high-performance liquid chromatography. J. Chromatogr. A. 977: 89-96.
25. Singleton,VL and Ross, JA. 1965. Colorimetry of Total phenolics with phosphomolybdic phosphotungstic Acid reagents. American J Enol Vitic. 16: 144-58.
26. Sirirat, D and Jelena, P. 2010. Bacterial Inhibition and Antioxidant Activity of Kefir Produced From Thai Jasmine Rice Milk. Asian network for scientific information. 9(3): 332-337.
27. Steffen, Chr. (1971) Methoden zur Bestimmung der Gesamtmilchsäure und der Lactatkonfigurationin Käse und Milch, Schweiz.Milchzeitung 97: 1073-1078.

28. Supasit Chooklin. 2013. Ultrasound-assisted extraction of phenolic compounds from brown rice and their antioxidant activities. Kasetsart J. (Nat. Sci.) 47: 864 – 873.

29. Tamime AY. 2006. Production of Kefir, Koumiss and Other Related Products. In: Tamime, AY (ed.),Fermented Milk Blackwell Science Ltd , Oxford, UK.Pp.174-216. Toba, T., Abe, S., and Adachi, S. 1987. Modification of KPL medium for polysaccharide production by *Lactobacillus* sp. isolated from kefir grain, *Jpn.* J. Zootechnol. Sci. 58: 987–990.

30. Villano D., Fernandez-Pachon MS., Moya ML., Troncoso AM., Garcia-Parilla MC. 2007. Radical scavenging ability of phenolic compounds towards DPPH free radical. Talanta 71: 230–235.

31. Wang, Y., Yu, R., Chou, C. 2006. Antioxidative activities of soymilk fermented with lactic acid bacteria and bifidobacteria. Food Microbiology. 23: 128–135.

32. Yawadio, R., Tanimori, S., & Morita, N. 2007. Identification of phenolic compounds *isolated* from pigmented rices and their aldose reductase inhibitory activities. Food Chemistry, 101(4), 1616–1625

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Review Article

Properties and benefits of kefir -A review

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Abstract

Kefir is becoming increasingly popular as a result of new research into its health benefits. It is a fermented milk drink which has its origin in the Caucasus Mountains of Russia. Kefir is prepared by inoculating milk with kefir grains which are a combination of bacteria and yeasts in a symbiotic matrix. The common microorganisms present are non-pathogenic bacteria, especially *Lactobacillus* sp. and yeasts. Kefir has a long history of health benefits in Eastern European countries. It is believed that kefir has therapeutic effects, thus it is important to study the various properties contained in, and exhibited by it. This review includes a critical revision of the antimicrobial, anti-carcinogenic, probiotic and prebiotic properties of kefir. Other health benefits, like reducing cholesterol and improving lactose tolerance are also discussed.

Keywords: kefir, antimicrobial, anti-carcinogenic, cholesterol, probiotic

1. Introduction

In recent years fermented milk and milk products have had a strong influence on health. They are considered to be beneficial with therapeutic effects and various other properties. Researchers have identified yet another fermented milk drink, kefir. The word 'kefir' is derived from the Turkish word 'keif' which means 'good feeling' (Kaufmann, 1997). The drink originated in the Caucasus Mountains of Russia, which are between the Black and the Caspian Seas. Kefir is produced by the fermentation of lactic acid and alcohol by mesophilic bacteria and yeasts, respectively (Ahmed *et al.*, 2013).

Kefir can also be prepared by inoculating milk with kefir grains which are a combination of bacteria and yeasts in a symbiotic matrix. Most microorganisms present in kefir are non-pathogenic bacteria, especially *Lactobacillus* sp. and yeasts. Kefir is enriched with vitamins, amino acids, carbon dioxide, acetoin, alcohol and essential oils which have been shown to have health benefits. Recently, the antibacterial, immunologic and antitumor effects of kefir were studied on human beings (Lin and Change, 2000).

Various properties are exhibited by kefir. Some of the main ones discussed here are antimicrobial, anti-carcinogenic, probiotic and prebiotic. Kefir has long been considered good for health (Liu *et al.*, 2006 a). Guven *et al.* (2003) proposed an alternative suggestion as to how kefir may protect tissues. They found that mice exposed to carbon tetrachloride (a hepatotoxin to induce oxidative damage) and given kefir by gavage showed decreased levels of liver and kidney malondialdehyde, indicating that kefir was acting as an antioxidant.

Their data also indicated that kefir was more effective than vitamin E (which is well known to have antioxidative properties) in combating oxidative damage. Many studies have shown evidence to warrant the use of probiotic foods like kefir in the treatment of gastrointestinal disturbances (Reid *et al.*, 2003). One example is diarrhea, which can be caused by a variety of conditions. Probiotics help in preventing diarrhea and in reducing its duration; they also alleviate conditions such as infant's diarrhea, irritable bowel

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syndrome, colitis, Crohn's disease, gastroenteritis, and traveler's diarrhea (Heyman, 2000). The consumption of kefir has shown good results in mitigating the symptoms of chronic constipation (Maeda *et al*., 2004).

This review outlines the properties and benefits of kefir and its effects regarding health remedies.

2. Origin of Kefir

Kefir is a popular traditional Middle Eastern beverage. Consumption of kefir leads to a 'good-feeling' (Chaitow and Trenev, 2002). It originated in the Caucasus Mountains in the former Soviet Union, in Central Asia and has been consumed for thousands of years (Libudzisz and Piatkiewicz, 1990). Kefir grains were first described by the tribes in the Northern Caucasian mountain region of Russia (Seydim, 2001).

Historically kefir grains were considered as gifts from Allah among the Muslim peoples of the Caucasian Mountains. They were passed down from generation to generation among the tribes of Caucasus and considered a source of family wealth. Traditional authentic kefir can be prepared by culturing fresh or pasteurized milk with kefir grains in homes all over the world (Roberts *et al*., 2000).

2.1 Codex alimentarius description of kefir

According to the Codex Standard for Fermented Milks CODEX STAN 243-2003, kefir contains the following: milk protein minimum (2.7%w/w), milk fat (<10m/m), titratable acidity expressed as percentage of lactic acid minimum (0.6% m/m), ethanol (not stated), sum of specific microorganisms constituting the starter culture minimum (10^7 cfu/g, in total) and yeast minimum (10^4 cfu/g).

3. Kefir Production

There are several methods for kefir production and commonly both traditional and industrial processes are used. Food scientists are currently studying modern techniques to produce kefir with the same characteristics as those found in traditional kefir. Kefir can be made from any type of milk, cow, goat, sheep, coconut, rice or soy. There are many choices for milk; pasteurized, unpasteurized, whole fat, low fat, skim and no fat (Semih, and Cagindi, 2003). Similarly, several processes have been developed to produce a kefir-like beverage in which no grains are used. In Russia, a mother culture is prepared by carrying out traditional kefir fermentation and sieving the grains. About 1 to 3% of this mother culture is added to pasteurized milk and incubated at 19 to 28ºC for 24 hours. (Farnworth and Mainville, 2003).

4. Functional Properties of Kefir

The functional properties of kefir are discussed in detail below and a schematic diagram is presented in Figure 1.

4.1 Antimicrobial properties

Kefir has an antibacterial effect against many pathogenic organisms due to the inherent formation of organic acids, hydrogen peroxide, acetaldehyde, carbon dioxide, and bacteriocins. For example, 3.5 kDa bacteriocin was identified from *Lactobacillus plantarum* ST8KF in kefir (Powell *et al*., 2007). Besides this, hydrogen peroxide is another metabolite produced by some bacteria as an antimicrobial compound. Yuksekdag *et al*. (2004a) showed that all 21 isolates of lactic acid bacteria from Turkish kefir produced hydrogen peroxide (0.04-0.19 µg/ml). In a later paper, they reported that 11 out of 21 strains of kefir *Lactococci* produced hydrogen peroxide (Yuksekdag *et al*., 2004). All *Lactococci* strains were effective in inhibiting the growth of *Streptococcus aureus,* but were less effective against *Escherichia coli* NRLL B-704 and *Pseudomonas aeruginosa.*

Furthermore, Santos *et al*. (2003) stated that the bacteriocin named lacticin 3147, which was produced by *Lactococcus lactis* strain DPC3147 isolated from kefir grains, had antimicrobial activity against *Escherichia Coli*, *Listeria monocytogenes*, *Salmonella typhimurium, S. enteritidis, S. flexneri*, and *Yersinia enterocolitica*. The molecular structure of lactin 3147 is shown in Figure 2. In addition, Ahmed *et al*. (2011) reported that kefir suspension, kefiran (a proposed molecular structure of kefiran is shown in Figure 3), and kefir grains showed antibacterial activity against some unicellular bacterial species and new antifungal activity against filamentous fungal species.

Moreover, many scientists (Diniz *et al*., 2003; Kwon *et al*., 2003; Rodrigues *et al*., 2005; Schneedorf and Anfiteatro, 2004) stated that kefir and its exopolysaccharide, kefiran, had antimicrobial activity. Both were reported to exhibit significant antibiotic activity against Gram-positive and Gram-negative bacteria as well as yeast, *Candida albicans*. Similarly,

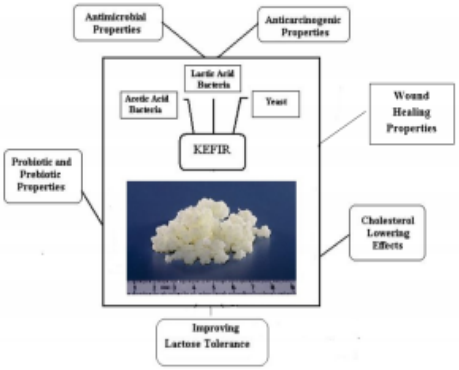

Figure 1. Schematic diagram of the functional properties of kefir (Zeynep, *et al*., 2011; Kniesel, 2005)

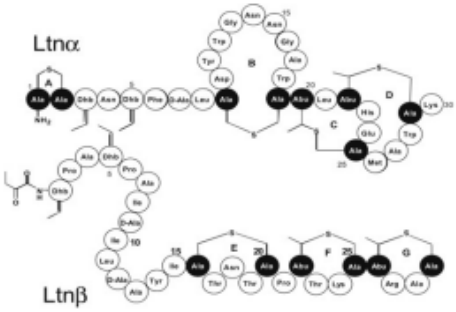

Figure 2. Molecular structure of Lacticin 3147. (Srinivas *et al*., 2010)

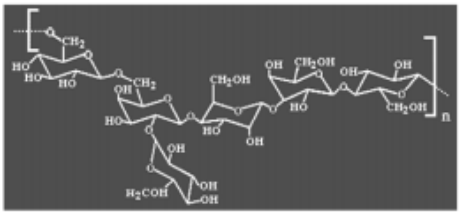

Figure 3. Chair form diagram of the proposed molecular structure of kefiran (Anfiteatro. 2013)

Medrano *et al*. (2008) reported that kefiran, an exopolysaccharide produced from kefir grains, protected against *Bacillus cereus* B 10502 damage to Caco-2 cells when introduced at a concentration range from 300 to 1000 mg/l. Their study also revealed that kefiran was capable of protecting cultured enterocytes significantly from the activity of *B. cereus* B10502 supernatants.

Data presented by Beyza *et al*. (2007) also suggested that kefir may be a good antimicrobial agent in food technology for food safety. More research related to this subject has still to be performed, in order to put the antimicrobial activity of kefir into practice for food technology.

4.2 Anti-carcinogenic properties and inhibition of tumor growth

According to the Merriam Webster medical dictionary, the definition of a anti-carcinogenic is 'tending to inhibit or prevent the activity of a carcinogen or the development of carcinoma'. Tumors are classified as carcinomas or sarcomas. Sarcoma tumors are derived from supportive or connective tissues such as bone, fat, and cartilage (Kuby, 1994). In addition, Liu *et al*. (2002) studied the effects of freeze-dried kefir, produced from soy milk and cows' milk with kefir grains, on the growth of tumors in mice. Mice were injected with Sarcoma 180 cells for one week before the start of the feeding stage of the experiment. Tumor growth (volume) was estimated for up to 30 days. Both soy milk kefir (70.9%) and cows' milk kefir (64.8%) significantly inhibited tumor growth, compared with mice in the positive control group.

In a study on induced breast cancer in mice, De Moreno *et al*. (2006) reported that mice receiving two days cyclical feeding with both kefir and a cell-free fraction of kefir over 27 days had a reduced tumor growth and increase in the IgA(+) cells. They also suggested that the IgA(+) cells might be able to bind the toxic metabolites produced during tumor development and indicate the importance of non-microbial components released during milk fermentation.

In addition, kefir extracts have been shown to suppress the growth of breast cancer cells *in vitro*. Some antitumorigenic abilities of kefir have been associated with the exopolysaccharide kefiran. Kefiran was shown to inhibit Ehrlich carcinoma and Sarcoma 180 in a mouse study, where it was proposed that the polysaccharide stimulated the host immune system via T-cell activity, rather than acting against the cancerous cells directly.

Furthermore, several studies have shown that kefir extracts and kefir bacterial isolates have the potential to reduce the risk or arrest the development of cancerous growths *in vitro* or in animal models (Rattray and Connell, 2011).

In 2008, Topuz and his team conducted the first study on the effect of oral kefir consumption on serum pro-inflammatory cytokines and on CT induced oral mucositis in humans with cancer. Their results showed that oral kefir consumption did not have any effect on the serum pro-inflammatory cytokines or a protective effect on mucositis due to 5-FU (a drug used in the treatment of cancer) based CT in humans. The team also suggested that further studies were needed to understand the effects of oral kefir consumption on the human immune system.

4.3 Cholesterol lowering effect

The evidence that kefir consumption reduces serum cholesterol is limited. Some research results have indicated a decrease in total serum cholesterol and phospholipids, in rats fed with a high cholesterol diet supplemented with kefir. Other biomarkers, such as high density lipoprotein (HDL) and serum triglycerides were unaffected by kefir consumption (Rattray and Connell, 2011). However, Liu *et al*. (2006) reported that milk kefir and soy milk kefir lowered the serum triacylglycerol and total cholesterol concentrations in hamsters. They also showed that the increase in the cholesterol-lowering effect of soy milk kefir, compared with soy milk, might be attributable to hypocholesterolaemic compounds other than genistein present in the kefir but absent from the soy milk.

Furthermore, some scientists (Brashears *et al*., 1998; Tamai Y *et al*., 1996) suggested that reduced serum choles-

terol concentration induced by kefir could be attributed to the deconjugation of bile acids by *Lactobacillus* spp. A study by Reynier and his team (1981) revealed that deconjugation of bile acids reduced serum cholesterol levels by increasing the formation of new bile acids needed to replace those that have escaped the enterohepatic circulation. They also showed that higher cholesterol metabolism lowered the serum cholesterol level.

In 2006, Begley *et al*., studied the mechanisms behind the deconjugation of bile acids by bile salt hydrolase. A detailed drawing is given in Figure 4a. In their study, they showed that the key enzyme bile salt hydrolase from *Lactobacillus* spp was responsible for the conversion of conjugated bile acids to unconjugated bile acids. They also showed that the deconjugation of bile salts could lead to a reduction in serum cholesterol, either by increasing the demand for cholesterol "de novo" synthesis of bile acids, to replace those lost in feces, or by reducing cholesterol solubility and thereby absorption of cholesterol through the intestinal lumen.

In addition, Cenesiz *et al*. (2008) found that there was a decrease in serum cholesterol levels of all kefir-treated chickens in a dose-dependent manner. They also concluded that a decrease in cholesterol levels could be associated with both a reduction in cholesterol biosynthesis in the liver and an increase in degradation of bile acids by *Lactobacillus* species. Similarly, Sanders (2000) suggested that the inhibition of 3HMG-Co A, which is an intermediate of mevalonate, during the synthesis of cholesterol from acetyl-Co A by fermented milk products, was the reason for the reduced level of cholesterol in the serum. A detailed drawing is given in Figure 5.

Moreover Yoon *et al*. (1999) reported that cholesterol assimilation was strain -dependent and *L. acidophilus* CU673 isolated from kefir displayed the highest cholesterol assimilation activity with a 68.8% reduction. According to the report by Kalavathy *et al*. (2009), cholesterol removal from the growth medium by the *Lactobacillus* strains may be strain dependent. However, further studies are required to determine the mechanisms involved in the removal of cholesterol by these *Lactobacillus* strains *in vitro*.

In addition, Maeda *et al*. (2005) found that kefiran-fed rats had a serum cholesterol lowering effect in 2 rat models which were loaded with cholesterol and given orotic acid.

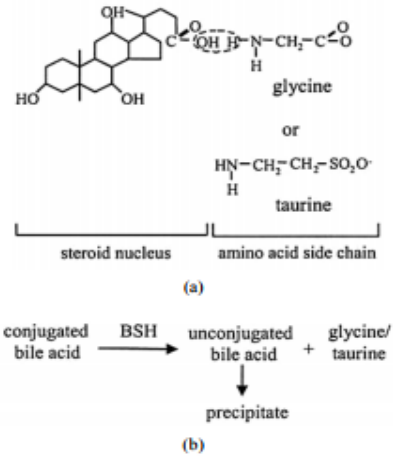

Figure 4. (a) Chemical structure of bile acids. Bile acids are conjugated with either glycine or taurine prior to secretion. (Begley *et al*., 2006). (b) Reaction catalyzed by bile salt hydrolase enzymes. BSHs cleave the peptide linkage of bile acids, which results in the removal of the amino acid group from the steroid core. The resulting unconjugated bile acids precipitate at low pH. (Begley *et al*., 2006).

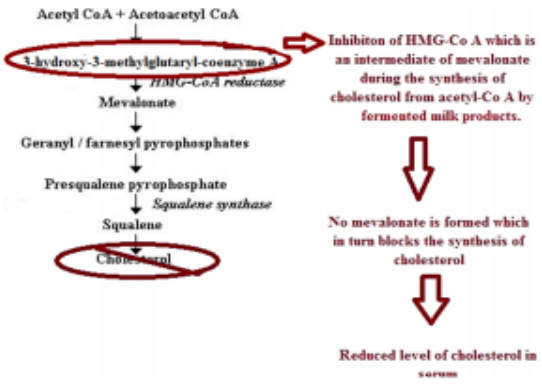

Figure 5. Cholesterol biosynthesis pathway (modified) (Bate *et al*., 2007; Sanders. 2000)

Kefiran accelerated sterol excretion and protected hepatic injuries (glutamate oxaloacetate transaminase [GOT], glutamic pyruvic transaminase [GPT]) in both rat models. The mechanisms for this are not well understood.

4.4 Improving lactose tolerance

Lactose maldigestion is the inability to completely digest lactose, the major carbohydrate in virtually all mammalian milks. Lactose maldigestion affects 75% of adults in the world and occurs most often as the result of a genetically programmed decrease in intestinal lactose activity after the age of 3 to 5 years (Sahi, 1994; Swaggerty *et al.*, 2002).

Hertzler and Clancy (2003) demonstrated that a commercial kefir produced from a starter culture containing six bacteria (but not *L. acidophilus*) and one yeast, was equally as effective as yoghurt in reducing breath hydrogen in adult lactose maldigestors. It has also been shown that fermented milk products have a shorter transit time than milk, which may further improve lactose digestion (Vesa *et al*., 1996; Labayen *et al*., 2001). Furthermore, Rattray and Connell (2011) found that kefir with a diverse microbial population invariably has some degree of β-galactosidase activity, that converts lactose into glucose and galactose, which can then be easily digested.

In addition, Steven *et al*. (2003) reported that although it seems plausible that kefir might improve lactose digestion in a manner similar to yogurt, there is a lack of research to support such a claim. Kefir contains different starter culture microorganisms from yogurt and the bile acid sensitivity, galactosidase activity, or lactose transport of these organisms may be different. This was the first result found, which demonstrated that plain kefir improved lactose digestion just as well as plain yogurt.

4.5 Wound healing properties

As kefir is a probiotic mixture of a diverse spectrum of bacteria and yeasts Witthuhn *et al*., (*2005*), it can stimulate innate immune responses in defense against pathogens (Koutinas *et al*., 2007; Atalan *et al*., 2003). Chena *et al*. (2008) and Kyoung *et al*. (2007) stated that the anti-inflammatory properties of polysaccharide present in kefir extract may also be influential in the process of wound healing. The lactic acid, acetic acid, polysaccharides and other chemicals present in kefir were important factors for wound healing properties observed in a study by Hassan *et al*. (2012).

In 2005, Kamila *et al*. conducted a study on rats, treating them with a simple kefir formulation made from dried grains. The results showed better wound healing properties compared with those treated with the clostebol–neomycin emulsion. Similarly, In 2005 Rodrigues and his team proved that rats treated with 70 % kefir gel made with kefiran, showed a faster reduction of the infected-induced wound compared with clostebol–neomycin emulsion. A study by Hassan *et al*. (2012) also showed that kefir had better wound-healing properties than conventional silver sulfadiazine treatment with regard to thermal injuries.

5. Probiotic and Prebiotic Properties

Kefir is a complex microbial system that has not only been found to be nutritionally beneficial, but has also been proven to inhibit a number of food-borne pathogens and spoilage microorganisms (Paucean and Carmen, 2008). Many probiotic products have been formulated that contain small numbers of different bacteria. The microbiological and chemical compositions of kefir indicate that it is a much more complex probiotic. Since yeasts and bacteria present in kefir grains have undergone a long association, the resultant microbial population exhibits many similar characteristics, making isolation and identification of individual species difficult. Many of these microorganisms are only now being identified by using advanced molecular biological techniques (Edward, 2006).

Kefir can be considered an amazing example of co-evolution of a microbial consortium. It has acquired a strong resistance against several microorganisms, as well as improving the natural immunity of mammals from early times. It is reasonable to think of the consortium as a potential naturally-occurring drug, able to decrease a large variety of illness afflictions (Jose, 2012). In 2003, Santos and his team reported that several strains of *Lactobacillus* spp. isolated from kefir in various countries have good adhesion to Caco-2 cells. These strains were resistant to low pH and bile acid and had antimicrobial activity against common enteropathogenic bacteria, which are popular criteria required by probiotic bacteria.

In addition, prebiotics are considered non-digestible but fermentable oligosaccharides, involving health promotion for the host (Barbosa *et al*., 2011).These compounds are known to provide improvements in nutritional status, in addition to health benefits such as protection against carcinogenesis, mutagenesis, prevention of injuries caused by free radicals, control of intestinal flora, and gastrointestinal resistance. Importantly kefir is able to produce peptide and sugar prebiotics, e.g., lactacin, bacteriocins, and kefiran (Schneedorf and Anfiteatro, 2004).

6. Benefits of Kefir for Pregnant and Nursing Women

According to the National Kefir Association, pregnant and nursing women can safety consume kefir. This promotes the absorption of nutrients, increases immunity, helps the body adjust to hormonal changes and prevents infections such as yeast overgrowth (Sandra, 2013). Also, the consumption of kefir by pregnant women can prevent the overgrowth of a bacterium called group B Beta Streptococcus. Beta streptococcus is a harmful bacterium which can cause infections such as sepsis, pneumonia, and meningitis (Sandra, 2013).

7. Conclusions

Scientific studies indicate kefir to be a complex probiotic, which is a combination of bacteria and yeasts. Kefir has certainly been shown to contain various functional properties such as antimicrobial, anti-carcinogenic, probiotic and others. It provides healthful benefits in the cholesterol lowering effects and improved lactose tolerance in humans. This fermented milk appears to have a great potential and this should inspire researchers to carry out further studies on kefir in order to analyze the hidden therapeutic and functional properties which have not been revealed to date.

References

Ahmed, A., Ismaiel, M., Mohamed, F., Ayman, G.K. and Naggar, E.L. 2011. Milk Kefir: Ultrastructure, Antimicrobial Activity and Efficacy on Aflatoxin B1 Production by *Aspergillus flavus*. Current Microbiology. 62, 1602-1609.

Ahmed, Z., Wang, Y., Ahmad, A., Khan, S.T., Nisa, M., Ahma, H. and Afreen A. 2013. Kefir and health: a contemporary perspective. Critical Reviews in Food Science and Nutrition. 53, 422-434.

Anfiteatro, D.N. 2013. Kefir in detail+ health benefits of kefiran, kefir- grains + kefir. Available from: http://users.chariot.net.au/~dna/kefiran.html. [October, 2013].

Atalan, G., Demirkan, I., Yaman, H. and Cina, M. 2003. Effect of topical kefir application on open wound healing on *in vivo* study. Kafkas Universitesi Veteriner Fakultesi Dergisi. 9(1), 43-47.

Barbosa, A.F., Santos, P.G., Lucho, A.S. and Schneedorf, J.M. 2011. Kefiran can disrupt the cell Membrane through induced pore formation. Journal of Electroanalytical Chemistry. 653, 61-66.

Bate, C., Rumbold, L. and Williams, A. 2007. Cholesterol synthesis inhibitors protect against platelet-activating factor-induced neuronal damage. Journal of Neuroinflamation. 4, 5.

Begley, M., Hill, C. and Cormac, G.M. 2006. Bile Salt Hydrolase Activity in Probiotics. Applied and Environmental Microbiology. 72(3), 1729-1738.

Beyza, H.U., Hilal, C., Hamparsun, H. and Mehmet, E. 2007. An *in vitro* study on the antibacterial effect of kefir against some food-borne pathogens. Turkish Microbiological Society. 37 (2), 103-107.

Brashears, M., Gilliland, S.E. and Buck, L.M. 1998. Bile salt deconjugation and cholesterol removal from media by *Lactobacillus casei*. Journal of Dairy Science. 81, 2103-2110.

Cenesiz, S., Yaman, H., Ozcan, A., Kart, A. and Karademir, G. 2008. Effects of kefir as a probiotic on serum cholesterol, total lipid, aspartate amino transferase and alanineamino transferase activities in broiler chicks. Medycyna Weterynaryna. 64, 2.

Chaitow, L. and Trenev, N. 2002. Probiotics. Natasha Trenev Website. Available from: http://www.natren.com. [October, 2013].

Chena, H.C., Wanga, S.Y. and Chena, M.J. 2008. Microbiological study of lactic acid bacteria in kefir grains by culture-dependent and culture-independent methods. Food Microbiology. 25, 492- 501.

De Moreno, A., Matar, A.C., Farnworth, E. and Perdigon, G. 2006. Study of immune cells involved in the antitumor effect of kefir in a murine breast cancer model. Journal of Dairy Science. 90, 1920-1928.

Diniz, R.O., Garla, L.K., Schneedorf, J.M. and Carvalho, J.C.T. 2003. Study of anti-inflammatory activity of Tibetan mushroom, a symbiotic culture of bacteria and fungi encapsulated into a polysaccharide matrix. Pharmacological Research. 47(1), 49-52.

Edward, R.F. 2006. Kefir A Complex Probiotic. Food Science and Technology Bulletin: Functional Foods. 2(1).

Farnworth, E.R. and Mainville, I. 2003. Kefir: A fermented milk product. Hand book of Fermented Functional Foods. E. R. Farnworth, editor. CRC Press, London, U.K., pp. 77-112.

Guven, A., and Gulmez, M. 2003. The effect of kefir on the activities of GSH-Px, GST, CAT, GSH and LPO levels in carbon tetrachloride-induced mice tissues. Journal of Veterinary Medicine B. 50, 412-416.

Hassan, F.H., Golnar, R., Mohammad, R.F., Mitra, M. and Mitra, S. 2012. Evaluation of wound healing activities of kefir products. Elsevier. 38, 719-723.

Hertzler, S.R. and Clancy, S.M. 2003. Kefir improves lactose digestion and tolerance in adults with lactose maldigestion. Journal of the American Dietetic Association. 103, 582 -587.

Heyman, M. 2000. Effect of lactic acid bacteria on diarrheal diseases. Journal of the American College of Nutrition. 19, 137-146.

Kalavathy, R., Norhani, A., Michael, C.V.L.W., Chinna, K. and Yin, W.H. 2009. Bile salt deconjugation and cholesterol removal from media by Lactobacillus strains used as probiotics in chickens. Wiley Inter science. 90(1), 65-69.

Kamila, L.R., Lucelia, R.G.C., Jose, C.T.C., Joao, E. and Jose, M.S. 2005. Antimicrobial and healing activity of kefir and kefiran extract. International Journal of Antimicrobial Agents. 25, 404-408.

Kaufmann, K. 1997. Kefir Rediscovered, ed. Alive Books, Burnaby, Canada, pp.3-17.

Kniesel, A. 2005. Kefirpilze. Available from: http://en.wikipedia.org/wiki/File:Kefirpilze.jpg#file. [June, 2013].

Koutinas, A., Athanasiadis, I., Bekatorou, A., Psarianos, C., Kanellaki, M. and Agouridis, N. 2007. Kefir-yeast technology: industrial scale-up of alcoholic fermentation of whey, promoted by raisin extracts, using kefir-yeast granular biomass. Enzyme and Microbial Technology. 41, 576-582.

Kuby, J. 1994. Immunology. W.H. Freeman and Company. Second Edition, New York, U.S.A., pp.580.

Kwon, CS., Park, MY., Cho, JS., Choi, ST. and Chang, DS. 2003. Identification of effective microorganisms from kefir fermented milk. Food Science and Biotechnology. 12,476-479.

Kyoung, K., Leeb, I.Y., Oha, S.R. and Leea, H.K. 2007. Anti-inflammatory and anti-allergic effects of kefir in a mouse asthma model. Immunobiology. 212, 647-654.

Labayen, I., Forga, L., Gonzalez, A., Wijnkoop, L.I. and Martinez, J.A. 2001. Relationship between lactose digestion, gastrointestinal transit time and symptoms in lactose malabsorbers after dairy consumption. Alimentary Pharmacology and Therapeutics .15, 543-549.

Libudzisz, Z. and Piatkiewicz, A. 1990. Kefir production in Poland. Dairy Industries International. 55, 31-33.

Lin, M.Y. and Change, F.J. 2000. Antioxidative effect of intestinal bacteria *Bifidobacteriumlongum* ATCC 15708 and *Lactobacillus acidophilus* ATCC 4356. Digestive Diseases and sciences. 45, 1617-1622.

Liu, J.R., Wang, S.Y., Lin, Y.Y. and Lin, C.W. 2002. Antitumor activity of milk kefir and soy milk kefir in tumor-bearing mice. Nutrition and Cancer. 44, 182-187.

Liu, J.R., Wang, S.Y., Chen, M.J., Chen, H.L., Yueh, P.Y. and Lin, C.W. 2006. Hypocholesterolaemic effects of milk-kefir and soyamilk-kefir in cholesterol-fed hamsters. British Journal of Nutrition. 95(5), 939-946.

Liu, J.R., Wang, S.Y., Chen, M.J., Yueh, P.Y. and Lin, C.W. 2006 a. The anti-allergenic properties of milk kefir and soy milk kefir and their beneficial effects on the intestinal microflora. Journal of the Science of Food and Agriculture. 86, 2527-2533.

Maeda, H., Zhu, X., Omura, K., Suzuki, S. and Kitamura, S. 2004. Effects of an exopolysaccharide (kefiran) on lipids, blood pressure, blood glucose, and constipation. BioFactors. 22 (1-4), 197-200.

Maeda, H., Mizumoto, M., Suzuki, K. and Tsuji. 2005. Effects of Kefiran-Feeding on Fecal Cholesterol Excretion, Hepatic Injury and Intestinal Histamine Concentration in Rats. Bioscience and Microflora. 24(2), 35-40.

Medrano, M., Perez, P.F. and Abraham, A.G. 2008. Kefiran antagonizes cytopathic effects of *Bacillus cereus* extracellular factors. International Journal of Food Microbiology. 122:1-2.

Paucean, A. and Carmen, S. 2008. Probiotic activity of mixed cultures of kefir's lactobacilli and non-lactose fermenting yeasts Bulletin UASVM, Agriculture. 65(2).

Powell, J.E., Witthuhn, R.C., Todorov, S.D. and Dicks, L.M.T. 2007. Characterization of bacteriocin ST8KF produced by a kefir isolate *Lactobacillus plantarum* ST8KF. International Dairy Journal. 17, 190-198.

Rattray, F.P. and Connell, M.J. 2011. Fermented Milks Kefir. In: Fukay JW, editor. Encyclopedia of Dairy Sciences. 2th ed. Academic Press; San Diego, U.S.A., pp. 518-524.

Reid, G., Jass, J., Sebulsky, M.T. and McCormick, J.K. 2003. Potential uses of probiotics in clinical practice. Clinical Microbiological Review. 16, 658-72.

Reynier, M.O., Montet, J.C., Gerolami, A., Marteau, C., Crotte, C., Montet, A.M. and Mathieu, S. 1981. Comparative effects of cholic, chenodeoxycholic & ursodeoxycholic acids on micellar solubilization and intestinal absorption of cholesterol. Journal of Lipid Research. 22, 467-473.

Roberts, M., Yarunin, S. and Danone. 2000. Moves into Russian kefir market. New Nutrition Business. 6, 22-24.

Rodrigues, K.L., Caputo, L.RG., Carvalho, J.C.T., Evangelista, J. and Schneedorf, J.M. 2005. Antimicrobial and healing activity of kefir and kefiran extract. International Journal of Antimicrobial Agents. 25, 404-408.

Sahi, T. 1994. Genetics and epidemiology of adult-type hypolactasia. Scandinavian Journal of Gastroenterology. 29(202), 7-20.

Sandra. E. 2013. No fear of kefir. Benefits, love stories about kefir. Available from: http://www.benefitsofkefir.com. [January, 2013].

Sanders, E.M. 2000. Considerations for use of probiotic bacteria to modulate human health. Journal of Nutrition. 130, 384-390.

Santos, A., Sanmauro, M., Sanchez, A., Torres, J.M. and Marquina, D. 2003. The antimicrobial properties of different strains of Lactobacillus spp. isolated from kefir. Systematic and Applied Microbiology. 26, 434-437.

Schneedorf, J.M and Anfiteatro, D. 2004. Kefir, A probiotic produced by encapsulated microorganism and inflammation. In Anti-inflammatory Phytotherapics (Portuguese), JCT. Carvalho, editor. Tecmed, Brazil, pp, 443-467.

Schneedorf, J.M. 2012. Kefir D'Aqua and its Probiotic Properties. Intech Open Science, Brazil, pp. 53-76.

Semih, O.E and Cagindi C. 2003. Kefir: A Probiotic Dairy-Composition, Nutritional and Therapeutic Aspects. Pakistan Journal of Nutrition. 2(2), 54-59.

Seydim, Z.B. 2001. Studies on fermentative, microbiological and biochemical properties of kefir and kefir grains. Ph.D. Dissertation, Clemson University, Clemson, South Carolina, U.S.A.

Srinivas, S., Alja, W., Paula, R., Paul, R., Colin H. and Paul, D.C. 2010. Effect of Bioengineering Lacticin 3147 Lanthionine Bridges on Specific Activity and Resistance to Heat and Proteases. Chemistry and Biology. 1151-1160.

Steven, R., Hertzler, R.D., Shannon, M. and Clancy, M.S. 2003. Kefir improves lactose digestion and tolerance in adults with lactose maldigestion. Journal of the American Dietetic association. 103(5), 582-587.

Swaggerty, D.L., Walling, A.D. and Klein, R.M. 2002. Lactose intolerance. American Family Physician. 65, 1845-1850, 1855-1856.

Tamai,Y., Yoshimitsu, N., Watanabe Y., Kuwabara, Y and Nagai, S. 1996. Effects of milk fermented by culturing with varius lactic acid bacteria and a yeast on serum cholesterol level in rats. Journal of Fermentation and Bioenergy. 81, 181-182.

Topuz, E., Derin, D., Can, G., Kurklu, E., Cinar, S. and Aykan, F. 2008. Effect of oral administration of kefir on serum proinflammatory cytokines on 5-FU induced oral mucositis in patients with colorectal cancer. Investigational New Drugs. 6, 567-572.

Vesa, T.H., Marteau, P., Zidi, S., Briet, F., Pochart, P. and Rambaud, J.C. 1996. Digestion and tolerance of lactose from yoghurt and different semi-solid fermented dairy products containing Lactobacillus acidophilus and bifidobacteria in lactose maldigesters-is bacterial lactase important. European Journal of Clinical Nutrition. 50, 730-733.

Witthuhn, R.C., Schoeman, T. and Britz, T.J. 2005. Characterization of the microbial population at different stages of kefir production and kefir grains mass cultivation. International Dairy Journal. 15, 383- 389.

Yoon, Y.H., Cho, J.K., Baek, Y.J. and Huh, C.S. 1999. Antimutagenic activity of *Lactobacillus* spp. isolated from kefir and yoghurt and non-starter strains. Korean Journal of Animal Science. (In Korean, abstract only). 41, 39-44.

Yuksekdag, Z.N., Beyath, Y. and Aslim, B. 2004a . Metabolic activities of *Lactobacillus* spp. strains isolated from kefir. Nahrung / Food. 48, 218-220.

Yuksekdag, Z.N., Beyatli, Y. and Aslim, B. 2004. Determination of some characteristics coccoid forms of lactic acid bacteria isolated from Turkish kefirs with natural probiotic. Lebensmittel-Wissenschaft und-Technologie. 37, 663-667.

Zeynep, B., Guzel, S., Tugokok, T., Annelk, G. and Atifc, S. 2011. Review: Functional properties of kefir. Critical Reviews in Food Science and Nutrition. 51, 261-268.

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Effects of Kefir Fermentation on Antioxidation Activities (*in vitro*) and Antioxidative Stress (*in vivo*) of Three Thai Rice Milk Varieties Prepared by Ultrasonication Technique

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ABSTRACT

Aims: The effects of kefir fermentation were investigated on antioxidation activities (*in vitro*) and antioxidative stress (*in vivo*) for different Thai rice; Hawm Nil rice, Red Hawm rice and Khao Dawk Mali 105 rice. **Methodology:** Antioxidant activity (*in vitro*) was investigated using ferric reducing antioxidant power and 2, 2′-diphenyl-1-picrylhydrazyl assays. In addition, antioxidative stress (*in vivo*) was performed using colitis rat models to study nitric oxide (NO), lipid peroxidation (LPO) and superoxide dismutase (SOD) compared with rats treated with prednisolone and cow's milk kefir. **Results:** Antioxidant activity of rice kefir powder from both assays had higher antioxidant activity than cow's milk kefir powder. NO levels of colitis rats received Hawm Nil rice kefir powder (HNKP) was reduced when compared to phosphate buffered saline (PBS) group. Moreover, colitis rats received HNKP did not differ in NO levels from colitis rats that received prednisolone and non-colitis rats. The result of LPO product malondialdehyde (MDA) indicated that colitis rats treated with HNKP had reduced TBARS compared to PBS group, and did not differ in TBARS levels from rats that received prednisolone and non-colitis rats. Surprisingly, increase in SOD activity was observed in colitis rats that received HNKP compared to PBS, with similar results of increased SOD in rats that received prednisolone and cow's milk kefir powder. **Conclusion:** Hawm Nil rice kefir may offer a protective effect for antioxidative stress resulting from chemical induction; it has potential as a supplementary food with high antioxidant activity and is regarded as safe for consumer health.

Key words: Antioxidant, Antioxidative stress, Lactic acid, Rice kefir, Thai rice

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INTRODUCTION

Kefir is an acidic, fermented milk beverage that originated thousands of years ago in the Caucasus Mountains.[1] Popularity and availability of kefir are increasing globally due to the well-known health benefits and longevity related to daily consumption.[2-10] Kefir beverage is commonly manufactured by fermenting milk with kefir grains. This process supports a complex microbial symbiotic mixture of lactic acid bacteria (e.g., *Lactobacillus, Lactococcus, Leuconostoc* and *Streptococcus*) and yeasts (e.g., *Kluyveromyces* and *Saccharomyces*).[11]

The main products of kefir fermentation are lactic acid, ethanol and carbon dioxide which confer the beverage with viscosity, acidity and low alcohol content. Minor components include diacetyl, acetaldehyde, ethyl and amino acids which contribute to the flavor.[12,13] demonstrated kefirs as potential antioxidants; interacting with a wide range of species that are directly responsible for oxidative damage.[2] stated that kefir could be made from any type of milk: cow, goat, sheep, coconut, soy and rice; however, cow is commonly used.[14] Reported that cereal grains, especially rice, contain special phenolic acids (such as ferulic, p-coumaric and diferulic) that are not present in significant quantities in fruit and vegetables,[15] found that brown rice milk kefir powders had higher α-tocopherol, γ−Aminobutyric acid (GABA) and phenolic contents than cow's milk kefir powder.

The antioxidant activity of plant phenolic is primarily due to their redox properties which allow them to act as reducing agents, hydrogen donors, free radical scavengers and singlet oxygen quenchers.[16,17] showed that rice milk kefir had high antioxidant activity compared to butylated hydroxyanisole (BHA). Moreover,[15] reported rice milk kefir powder as having higher antioxidant activity than that cow's milk kefir. Many authors have examined the antioxidant activity of rice milk *in vitro* but few have considered the antioxidative stress of rice milk kefir *in vivo*. Therefore, this study investigated the unique properties of rice milk kefir and analyzed the effects of different varieties of Thai rice fermentation with kefir grains on the content of antioxidant activity *in vitro* and antioxidative stress *in vivo*.

Cite this article: Deeseenthum S, Luang-In V, John SM, Chottanom P, Chunchom S. Effects of Kefir Fermentation on Antioxidation Activities (*in vitro*) and Antioxidative Stress (*in vivo*) of Different Thai Rice Milk Varieties. Pharmacog J. 2018;10(5):1061-6.

METHODOLOGY

Rice materials and kefir samples

Thai colored rice varieties namely Khao Dawk Mali 105 rice (KDML 105; white rice), Red Hawm rice (KDML105R-PSL-E-14; red rice) and Hawm Nil rice (PSL00288-4-21-5R;dark purple or black rice) were used. The rice materials were obtained from Selaphum Farmer Group, Roi Et Province, Thailand (2014 harvest season), who used grain from Roi-et Rice Seed Center, Rice Seed Division, Rice Department, Thailand. Rice material and kefir sample preparations followed[18] with minor modifications. Each 250 g of rice was left in 500 mL of water for 24 h before ultra-sonication using a Vibra-Cell Ultrasonicator (20 KHz) with tip diameter (25 mm), intensity (low), volume (500-1,000 mL, amplitude (60%) and time (5 min). The rice was blended, and then filtered with a cotton sheet and pasteurized at 75°C for 15 min. The pasteurized rice milks were immediately cooled and stored in dark plastic bags at a cool temperature of 4°C until required for use. The rice and cow milks prepared earlier were used to produce kefir. All the milk samples were maintained at 25°C for 24 h with 10% (v/v) of kefir starter culture. Following this, the milk kefirs were blended, filtered with a cotton sheet and pasteurized at 75°C for 15 min. All experiments were conducted in triplicate. Rice milk cultures were inoculated at 3% w/v and the kefir grains were incubated at 25°C with fermentation at pH 4.2. Samples were freeze-dried and analyzed.

Kefir powder

All milk kefirs at pH 4.2 were freeze-dried using a SJIA-10N Freeze Dryer (Shanghai Beiyi Bioequip Information Co., Ltd., China) at -55°C. The freeze-dried kefirs from the Khao Dawk Mali 105 rice, Red Hawm rice, Hawm Nil rice and cow milks were powdered with a mortar and pestle under aseptic condition and packed into bottles; the caps were tightened, wrapped with foil and the bottles were kept at -20°C until required for use.

Antioxidant activity *in vitro*

2, 2-diphenyl-1-picrylhydrazyl hydrate (DPPH) free radical scavenging assay

2, 2-diphenyl-1-picrylhydrazyl hydrate (DPPH) free radical scavenging assay determination followed the method of[19] with some modifications. Briefly, 100 µL of DPPH solution was added to 50 µL of each kefir sample. Methanol was used as the control, mixed well and incubated for 30 min in the dark at room temperature. Absorbance of each sample was measured at 517 nm using a micro plate reader. Percentage of inhibition was calculated using the following equation:

$$\text{Inhibition (\%)} = [(A_{control}-A_{sample})/ A_{control}] \times 100$$

The standard curve of DPPH was prepared as 10, 20, 30, 40, 50, 60, 70, 80 and 90 µg/mL, absorbance was measured at 517 nm using a micro plate reader and the graph was plotted. The DPPH radical scavenging activity was expressed as the IC_{50} value; this represented the amount of antioxidant in the kefir solution.

Necessary to reduce the initial DPPH concentration by 50%. The IC_{50} value was determined from the standard curve of percent scavenging plotted against the rice kefir powder solution concentration. All experiments were performed in triplicate.

Ferric reducing antioxidant activity (FRAP) assay

A FRAP assay was performed following the method of Benzie and Strain (1999)[20] with slight modifications. FRAP reagent was prepared by adding 0.0270 g of ferric chloride to 5 mL of distilled water and mixing. Then, 300 mM of acetate buffer was prepared by adding 2.4609 g of sodium acetate in water with pH adjusted to 3.6. Next, 40 mM of HCl was prepared in the ratio 1:1 with water and 0.66 mL was pipetted and added with 99.44 mL water. Then, 10 mM of 2,4,6-Tripyridyl-s-triazine (TPTZ) solution was prepared by adding 2,4,6-Tripyridyl-s-triazine 0.0156 g in 5 mL of 40 mM HCl, 300 mM of acetate buffer, 10 mM TPTZ, and 20 mM of iron (III) chloride solution. The prepared FRAP reagent was used as follows: 20 µL of each kefir sample was added to 1.50 µL of FRAP reagent. The mixture was stirred thoroughly and incubated in the dark at room temperature for 30 min.

Absorbance was then measured at 595 nm using a micro plate reader. The standard curve (R_2 = 0.9995) for FRAP was plotted and prepared as 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 µg/mL. A calibration curve was drawn with concentration of $FeSO_4.7H_2O$ on the X-axis and optical density (OD) on the Y-axis. Values obtained were expressed in µg/ml of ferrous equivalent Fe (II) per µg of each kefir sample.

Anti-oxidative stress activity studies

Experimental design

The Hawm Nil rice kefir power is the highest antioxidant activity, biochemical components values such as gamma amino butyric acid (GABA) content, alpha-tocopherol (α-tocopherol) content and total phenolic content. Moreover, this rice kefir power also had not toxicity in the rat model.[15] So that, the experimental design for antioxidative stress activity in this studied chose only Hawm Nil rice kefir power from three rice kefir powder. In addition, the method was followed the originally method described by Deeseenthum *et al.*[18] Each 6 rats were randomly divided into 7 groups; (1): non-colitis rats received phosphate buffered saline (PBS), (2): non-colitis rats received Hawm Nil brown rice kefir powder (150 mg/kg dissolved in PBS), (3): non-colitis rats received cow's milk kefir powder (150 mg/kg dissolved in PBS), (4): colitis rats received PBS, (5): colitis rats received Hawm Nil brown rice kefir powder (150 mg/kg dissolved in PBS), (6): colitis rats received cow's milk kefir powder (150 mg/kg dissolved in PBS), and (7): colitis rats received prednisolone (5 mg/kg).

Rat colitis groups were induced on day 4 by 2,4,6-trinitrobenzene sulfuric acid (TNBS) while groups treated with Hawm Nil brown rice kefir power, cow's milk kefir powder or prednisolone were left for 10 days.

Colitis induction

Rats were colitis induced on day 4 and thereafter. Colitis induction followed the method originally described by Scarminio *et al.*[21] After fasting overnight, the rats were anesthetized with halothane. Under anesthesia, they were given 10 mg of TNBS dissolved in 0.25 mL of 50% (v/v) ethanol by means of a Teflon cannula inserted 8 cm into the anus. During and after TNBS administration, the rats were kept in a head-down position until they recovered from the anesthesia. Rats from the non-colitis group received 0.25 ml of saline.

Serum sample collection

At the end of the experiment, rats were fasted for 24 h, weighed and then euthanized with 50 mL of chloroform. Blood samples were placed in heparinized and non-heparinized tubes and centrifuged at 1,500 g for 10 min to separate serum.

Nitric oxide measurement

Serum samples were treated with Centricon 10 (7,500 rpm, 4°C, 1 h) to remove hemoglobin and proteins. The nitric acid (NO) content was assessed by the Griess reaction method using 23479 Nitrate/Nitrite Assay Colorimetric Kit (Sigma-Aldrich, Inc., USA), that is a commercial kit for NO assay. Briefly, preparation of the nitrite calibration curve was performed by adding sodium nitrite ($NaNO_2$) standard solution and buffer solution to each well Plot the concentration of $NaNO_2$ solution

on the X-axis and the absorbance value on the Y-axis to prepare the calibration curve. Plot the concentration of $NaNO_3$ solution on the X-axis and the absorbance value on the Y-axis to prepare the calibration curve. Determine the concentration of nitrite in the sample solution from the calibration curve. Determine the concentration of nitrate + nitrite in the sample solution using the calibration curve. Then, nitrate concentration can be obtained by the following equation:

$$[\text{Nitrate}] = [\text{Nitrate + Nitrite}] - [\text{Nitrite}]$$

Lipid peroxidation estimation

The lipid peroxide (LPO) product malondialdehyde (MDA) was estimated using a Lipid Peroxidation (MDA) Assay Kit of thiobarbituric acid reactive substances (TBARS) in serum (Sigma-Aldrich, Inc., USA), that is a commercial kit for Lipid Peroxidation (MDA) Assay. The LPO products were expressed in terms of nmole MDA/µL. Concentration of MDA can be obtained by the following equation:

$$(Sa/Sv) \times 4 \times D = C$$

Where,

Sa is the amount of MDA in unknown sample (nmole) from the standard curve

Sv is the sample volume (µL) or amount (µg) added into the wells

C is the concentration of MDA in the sample

D is the dilution factor

4 is the correction factor using 200 µL of 800 µL reaction

Superoxide dismutase (SOD) activity

The SOD activity was estimated using a SOD Assay commercial Kit-WST (19160 SOD determination kit, Sigma-Aldrich, Inc., USA). Read the absorbance at 450 nm using a micro plate reader. Calculate the SOD activity (inhibition rate %) using the following equation:

$$\text{SOD activity} = \{[(A_{\text{blank1}} - A_{\text{blank3}}) - (A_{\text{sample}} - A_{\text{blank2}})]/(A_{\text{blank1}} - A_{\text{blank3}})\} \times 100$$

Statistical analysis

The experiment used a randomized block split-plot design. Plot effect was rice type and the sub-plot was fermentation variable when comparing fermentation effect. Complete block design was used to compare the antioxidant activities of different rice milks and rice kefir samples. All experiments were performed in triplicate. Experimental data were analyzed for divergence using Duncan's multiple range test and SPSS (SPSS 19.0, SPSS Inc. statistical program). Significance was established at $p \leq 0.05$.

RESULTS AND DISCUSSION

DPPH free radical scavenging and FRAP values

Antioxidant activity of Red Hawm rice kefir powder showed the highest % inhibition of DPPH and was significantly different ($p \leq 0.5$) from the other kefirs (85.79±0.34). Cow's milk kefir powder showed the lowest antioxidant activity from DPPH free radical scavenging with FRAP assays at 77.59±0.24and 2.672±0.115, respectively (Table 1).

Anti-oxidative stress in rat models

Nitric oxide

The NO level in the serum was higher in colitis rats that received PBS (control) compared to non-colitis rats (Figure 1, $p \leq 0.05$). However, NO levels compared to negative controls ($p \leq 0.05$). Moreover, colitis rats that received HNKP did not show reduced NO levels compared to rats that received prednisolone and non-colitis rats (Figure 1).

Nitric oxide is a potent, endogenous vasodilator that modulates renal function and plays a key role in endothelial dysfunction.[22] Colitis rats that received PBS (control) had higher NO levels in serum than non-colitis rats. However, colitis rats treated with HNKP showed reduced NO levels compared to the controls. These findings indicated that rice kefir powder may reduce NO excretion in colitis rats compared to controls. Other authors investigated the role of probiotic bacteria in the generation of local NO in the intestinal lumen by nitrate reduction or acid dependent mechanisms. This may be counteracted through rapid NO consumption by other strains or diffused into the surrounding tissues,[23] and explain some of the health promoting effects of this kefir by reducing NO levels in rat models.

Lipid peroxidation

Colitis rats that received PBS showed increased TBARS in serum (20.78±0.58, $p \leq 0.05$). However, colitis rats treated with HNKP gave reduced TBARS compared to controls (10.10±1.06 vs 20.78 ± 0.58, $p \leq 0.05$). Moreover, colitis rats that received HNKP showed similar TBARS levels to rats that received prednisolone and non-colitis rats (Figure 2).

Table 1: Antioxidant activity in cow and pigmented rice's milk fermented with kefir grain at pH 4.2.

Treatments	(%) Inhibition of DPPH	FRAP values
Cow Milk kefir powder	77.59±0.24^{a}	2.672±0.115^{b}
Khaw Dawk Mali 105 rice kefir powder	82.51±0.12^{ab}	2.725±0.107^{a}
Red Hawm Rice kefir powder	85.79±0.34^{b}	2.874±0.197^{a}
Hawm Nil Rice kefir powder	78.14±0.16^{a}	2.876±0.145^{a}

Mean values within each column with different superscripts are significantly different, Duncan's test at $p \leq 0.05$.

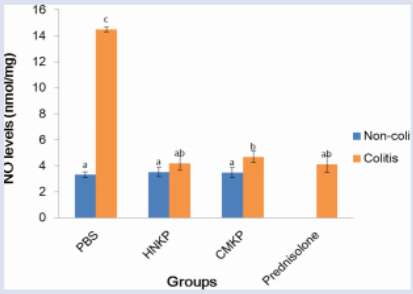

Figure 1: Nitric oxide (NO) levels in the treated rat colitis compared to those in controls. The different lowercase letters are significantly different, Duncan's test at $p \leq 0.05$; PBS = phosphate buffered saline, HNKP = Hawm Nil Rice kefir powder, CMKP = Cow Milk kefir powder.

Lipid peroxidation is the degradation of lipids that occurs because of oxidative damage and a useful marker for oxidative stress. Polyunsaturated lipids are susceptible to oxidative attack, typically by reactive oxygen species, resulting in a well-defined chain reaction with end products such as malondial dehyde (MDA). Lipid peroxidation may contribute to the pathology of many diseases including atherosclerosis, diabetes and Alzheimer's. Here, a significant increase of LPO (TBARS) was recorded in colitis rats, indicating that peroxidative injury involved the reduction of antioxidant defense mechanisms and development of colitis complications.[24] Colitis rats treated with RKP showed significantly decreased LPO. Bioactive peptides released during fermentation e.g., α-tocopherol, γ-amino butyric acid and total phenolic contents, by proteolytic lactic acid bacteria can scavenge reactive oxygen species (ROS) and inhibit LPO, consistent the reported by Pihlanto.[25]

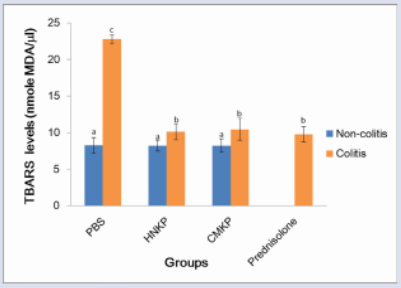

Figure 2: Thiobarbituric acid reactive substances (TBARS) levels in the treated rat colitis compared with controls. The different lowercase letters are significantly different, Duncan's test at $p \leq 0.05$; PBS = phosphate buffered saline, HNKP = Hawm Nil Rice kefir powder, CMKP = Cow Milk kefir powder.

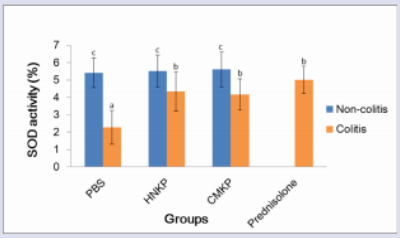

Figure 3: Superoxide dismutase activities (SOD) in the treated rat colitis compared with controls. The different lowercase letters are significantly different, Duncan's test at $p \leq 0.05$; PBS = phosphate

Superoxide dismutase (SOD)

Increase in SOD activity was observed in colitis rats that received HNKP compared to colitis rats that received PBS (controls). This indicated that the antioxidant defense system was functional in colitis rats that received HNKP; similar findings of increased SOD were seen in rats that received prednisolone and cow's milk kefir powder (Figure 3).

Moreover, increase in SOD activity was observed in colitis rats treated with HNKP compared to colitis rats that received PBS (controls). SOD is the primary enzymatic antioxidant defense system in the cell and catalyzes the dismutation of the superoxide anion (O2 -) into hydrogen peroxide and molecular oxygen as one of the most important anti oxidative enzymes. Several direct and indirect methods have been developed to determine SOD activity. A common, convenient and easy indirect method uses nitro blue tetrazolium (NBT). However, there are several disadvantages to the NBT method such as poor water solubility of the formazan dye and interaction with the reduced form of xanthine oxidase. The SOD Assay Kit-WST is very convenient and utilizes Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt) that produces a water-soluble formazan dye on reduction with a superoxide anion.[26] The IC_{50} (50% inhibition activity of SOD or SOD-like materials) can be determined by a colorimetric method. Antioxidant defense systems were functional in colitis rats that received HNKP, with similar findings of increased SOD in rats subjected to prednisolone and CMKP.

CONCLUSION

Thai rice kefir powder included high antioxidant activity. Hawm Nil rice kefir powders had the highest antioxidant activity, followed by Red Hawm rice kefir powder and Khao Dawk Mali 105 rice kefir powder, respectively. Moreover, the Hawm Nil rice kefir powder also may offer protection against chemically induced antioxidative stress such as nitric oxide, lipid peroxidation, while stimulate superoxide dismutase. Thus, this rice milk kefir has potential as a supplementary food with high antioxidant activity and it is regarded as safe for consumer health.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

ABBREVIATIONS

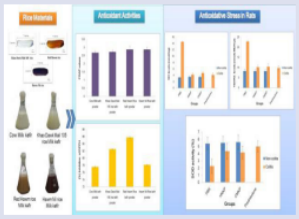
PBS: Phosphate buffered saline; **HNKP:** Hawm Nil Rice kefir powder; **CMKP:** Cow Milk kefir powder; **TNBS:** 2, 4, 6-trinitrobenzene sulfuric acid; **FRAP:** Ferric reducing antioxidant power; **DPPH:** 2, 2´-diphenyl-1-picrylhydrazyl; **NO:** Nitric oxide; **LPO:** Lipid peroxidation; **SOD:** Superoxide dismutase; **MDA:** Malondialdehyde; **TBARS:** Thiobarbituric acid reactive substances.

REFERENCES

1. Angulo L, Lopez E, Lema C. Microflora present in kefir grains of the Galician region (North-West of Spain). J Dairy Res. 1993;60(2):263-7.
2. Farnworth ER. Kefir: A complex probiotic. Food Sci Technol Bull. 2006;2(1):1-17
3. Kabak B, Dobson AD. An introduction to the traditional fermented foods and

10.1080/10408390903579029.
5. Gao J, Gu F, Abdella NH, Ruan H, He G. Investigation on culturable microflora in Tibetan kefir grains from different areas of China. J Food Sci. 2012;77(8):425-33. https://doi: 10.1111/j.1750-3841.2012.02805.x.
6. Leite AM, Mayoa B, Rachid CT, Peixoto RS, Silva JT, Paschoalin VM, *et al.* Assessment of the microbial diversity of Brazilian kefir grains by PCR-DGGE and pyrosequencing analysis. Food Microbiol. 2012;31(2):215-21. https://doi: 10.1016/j.fm.2012.03.011.
7. Leite AM, Miguel MA, Peixoto RS, Rosado AS, Silva JT, Paschoalin VM. Microbiological, technological and therapeutic properties of kefir: A natural probiotic beverage. Braz J Microbiol. 2013;44(2):341-9. https://doi: 10.1590/S1517-83822013000200001.
8. Leite AM, Leite DC, Del Aquila EM, Alvares TS, Peixoto RS, Miguel MA, *et al.* Microbiological and chemical characteristics of Brazilian kefir during fermentation and storage processes. J Dairy Sci. 2013;96(7):4149-59. https://doi: 10.3168/jds.2012-6263.
9. Diosma G, Romanin DE, Rey-Burusco MF, Londero A, Garrote GL. Yeast from kefir grains: Isolation, identification, and probiotic characterization. World J Microbiol Biotechnol. 2014;30(1):43-53. https://doi: 10.1007/s11274-013-1419-9.
10. Pogacic T, Sinko S, Zamberlin S, Samarzija D. Microbiota of kefir grains. Mljekarstvo. 2013;63(1):3-14.
11. Magalhães KT, Dragone G, De Melo Pereira GV, Oliveira JM. Comparative study of the biochemical changes and volatile compound formations during the production of novel whey-based kefir beverages and traditional milk kefir. Food Chem. 2011;126(1):249-53. https://doi: 10.1016/j.foodchem.2010.11.012.
12. Rattray FP, O'Connell MJ. Fermented Milks Kefir. In: Fukay JW (ed) Encyclopedia of Dairy Sciences, 2nd ed n. Academic Press, San Diego, USA. 2011;518-24.
13. Je-Ruei Liu, Yuh-Yih Lin, Ming-Ju Chen, Li-Ju Chen, Chin-Wen Lin. Antioxidative activities of Kefir. Asian-Australas J Anim Sci. 2005;18(4):567-73. https://doi.org/10.5713/ajas.2005.567.
14. Adom KK, Liu RH. Antioxidant activity of grains. J Agric Food Chem. 2002;50(21):6182-7.
15. Chunchom S, Talubmook C, Deeseenthum S. Antioxidant activity, biochemical components and sub-chronic toxicity of different brown rice kefir powders. Pharmacogn J. 2017;9(3):388-94. https://doi: 10.5530/pj.20173.66.
16. Rice-Evans CA, Miller NJ, Bolwell PG, Bramley PM, Pridham JB. The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Radic Res. 1995;22(4):375-83.
17. Deeseenthum S, Pejovic J. Bacterial inhibition and Antioxidant Activity of Kefir Produced from Thai Jasmine Rice Milk. Biotechnol. 2010;9(3):332-7 https://doi: 10.3923/biotech.2010.332.337.
18. Deeseenthum S, Luang-In V, Chunchom S. Characteristics of Thai Pigmented Rice Milk Kefirs with Potential as Antioxidant and Anti-Inflammatory Foods. Pharmacogn J. 2018;10(1):154-61. https://doi: 10.5530/pj.2018.1.26.
19. Akowvah GA, Ismail Z, Norhayati I, Sadikun A. The effect of different extraction solvents of varying polarities of polyphenols of orthosiophonstamineus and evaluation of the free radical scavenging activity. Food Chem. 2005;93(2):311-7. https://doi: 10.1016/j.foodchem.2004.09.028.
20. Benzie IF, Strain JJ. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol. 1999;299:15-27.
21. Scarminio V, Fruet AC, Witaicenis A, Rall VL, Di Stasi LC. Dietary intervention with green dwarf banana flour (Musa sp AAA) prevents intestinal inflammation in a trinitrobenzenesulfonic acid model of rat colitis. Nutr Res. 2012;32(3):202-9. https://doi: 10.1016/j.nutres.2012.01.002.
22. Komers R, Anderson S. Paradoxes of nitric oxide in the diabetic kidney.
23. Am J Physiol Renal Physiol. 2003;284(6):1121-37. https://doi: 10.1152/ajprenal.00265.2002.
24. Sobko T, Huang L, Midtvedt T, Norin E, Gustafsson LE, Norman M, *et al.* Generation of NO by probiotic bacteria in the gastrointestinal tract. Free Radic Biol Med. 2006;41(6):985-91. https://doi:10.1016/j.freeradbiomed.2006.06.020
25. Szkudelski T. The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. Physiol Res. 2001;50(6):537-46.
26. Pihlanto A. Antioxidative peptides derived from milk proteins. Int Dairy J. 2006;16(11):1306-14. https://doi.org/10.1016/j.idairyj.2006.06.005.
27. Gulitz A, Stadie J, Wenning M, Ehrmann MA, Vogel RF. The microbial diversity of water kefir. Int J Food Microbiol. 2011;151(3):284-8. https://doi: 10.1016/j.ijfoodmicro.2011.09.016.

GRAPHICAL ABSTRACT



SUMMARY

- The rice kefir powder had high antioxidant activity.
- Hawm Nil rice kefir powder (HNKP) can reduce NO levels in the colitis rats.
- HNKP can reduce TBARS levels in the colitis rats.
- Surprisingly, this rice kefir takes SOD activity increased in colitis rats.
- The NO, TBARS and SOD levels of colitis rats that received HNKP had no differ from the prednisolone, which is a current medicine.

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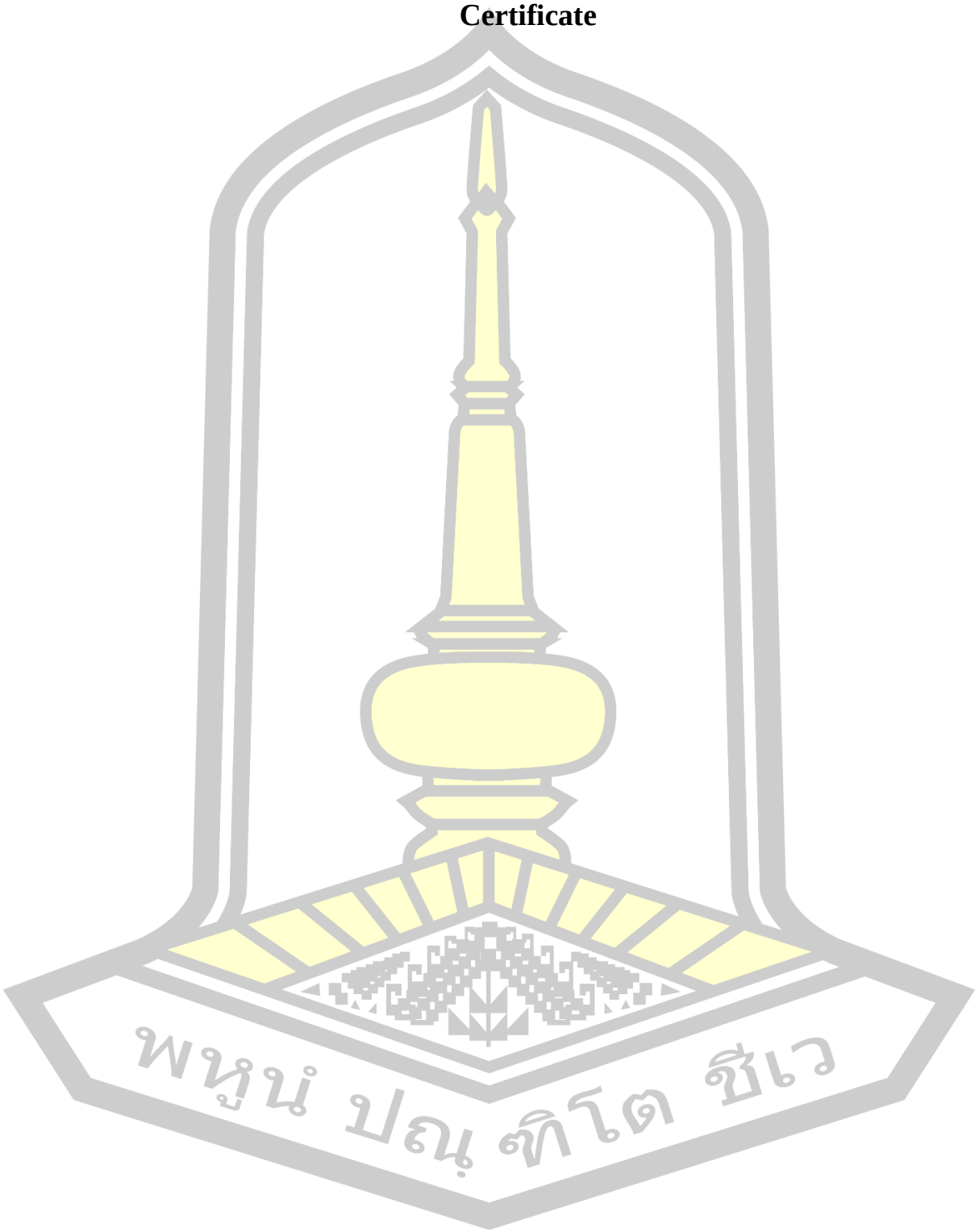
APPENDIX G

Certificate



To Whom It May Concern

This is to certify that the following Thesis has been proofread for grammar and syntax by Mr. Peter Humphrey Charge, an experienced native British editor at PROOFREADING4THAIS.

Characterization and Chemical Compositions of Rice Milk Kefir and Process Optimization to Obtain High Antioxidant Kefir

by

Stephen Moses John

If you have any questions or require further details, please do not hesitate to contact me.

Sincerely,

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พหุน ปณฺ ฑิโต ชีเว

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Research output	Dissertation and Publications