Bioactivity of food-grade curcuminoids and their incorporation into coconut yogurt

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ABSTRACT

Curcuminoids are the bioactive components of turmeric, which comprises pf of 77% curcumin, 17% demethoxycurcumin (DMC) and 3% bisdemethoxycurcumin (BDMC). The application of curcuminoids is limited by its low oral bioavailability due to poor aqueous solubility, low absorption from the gut, rapid metabolism and rapid systemic elimination, which can be improved by incorporating curcuminoids into a suitable food matrix. Thus, the present study aimed at developing a coconut yogurt as a potential vehicle for the delivery of bioactive curcuminoids.

This research project was carried out in three phases. Phase I involved the screening of 10 different commercial food-grade curcuminoid products in three types of yogurt. Each of the 10 food-grade curcuminoid products, were added (0.4% w/w) to three types of commercial yogurt: cow's milk yogurt, coconut cream yogurt and a goat's milk yogurt and subjected to pH measurement and sensory evaluation with a view to selecting the most promising curcuminoid and delivery medium. Results showed that coconut yogurt with added curcuminoids (C7 and C9) were the most acceptable to the sensory panellists.

In phase 2, the two selected curcuminoid products (C7 and C9) were subjected to a cell-based, *in vitro* analysis to measure their anti-inflammatory activity and cytotoxicity using THP-1 macrophages stimulated with lipopolysaccharide (LPS). The anti-inflammatory activity of the two curcuminoid products was compared to analytical grade curcumin (Pure C) as positive control and a dimethyl sulfoxide (DMSO) vehicle control. C7 and C9, as well as pure curcumin presented a varied degree of toxicity towards LPS stimulated macrophages, as measured by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) colorimetric assay. All curcuminoid samples were found to be non-toxic to THP-1 cells at 10 μM. At this concentration, the test products and the control down-regulated the expression of TNF-α by 2.5-fold in the differentiated THP-1 cells stimulated with LPS. Concentrations of DMSO as high as 0.5% were well tolerated by the macrophages. As there were no significant differences ($p>0.05$) in the anti-inflammatory activity of the food-grade curcuminoid samples, the both the test products in coconut cream yogurt were tested in phase III.

Samples of coconut cream were fortified with food-grade curcuminoids (C7 and C9) at 400 mg/150g, prior to yogurt fermentation; a negative control without curcumin was also included. The physico-chemical, microbiological and sensory properties of the fermented coconut cream yogurts were compared to the control coconut yogurt. Results showed curcuminoids did not have any effect on fermentation of coconut cream. During storage (4°C) for 15 days, acidity, yogurt microflora and syneresis of the curcuminoid enriched yogurts were not significantly different from the control yogurts. However, addition of curcuminoids resulted in formation of a weaker gel compared to the control yogurt, and the viscosity of the gels varied during storage. The concentrations of curcuminoids in the coconut cream yogurt during storage of the fermented products were measured by reversed phase HPLC. HPLC analysis showed that 70-75% of the bioactives were retained in the yogurt at the end of the 15-day storage period. The two fermented coconut yogurts fortified with curcuminoids (C7 and C9) were well-accepted by a consumer sensory panel (n=180). Based on the pH, acidity, sensory, texture, microbiological and HPLC results, it can be inferred that coconut yogurt may serve as a suitable delivery medium for bioactive curcuminoids.

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LIST OF ABBREVIATIONS

˚C	Degree Celsius
BDMC	Bisdemethoxycurcumin
CCY	Coconut cream yogurt
cfu	Colony-forming unit
CMY	Cow's milk yogurt
CUR	Curcumin
DMC	Demethoxycurcumin
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme-linked immunosorbent assay
GMY	Goat's milk yogurt
h	Hours
HPLC-DAD	High performance liquid chromatography with diode-array detection
HRP	Horseradish peroxidase
IL-1β	Interleukin-1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8
L. bulgaricus	*Lactobacillus bulgaricus*
LPS	Lipopolysaccharide
M	Molarity
mg	Milligrams
µ	micro
mol	mole
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NaOH	Sodium Hydroxide
NF-κB	Nuclear factor-kappaB
nm	Nanometre
o	ortho
Pure C	Analytical grade curcumin

ROS	Reactive oxygen species
S. thermophilus	*Streptococcus thermophilus*
TNF-α	Tumour necrosis factor alpha
USD	United States dollar
w/w	Weight by weight

CHAPTER 1
INTRODUCTION

1.1 Background

At present, one of the most topical research areas in the Food Sciences is the development of functional foods by incorporating novel, natural bioactive ingredients (Silva, Barreira & Oliveira, 2016). Functional foods are natural or processed foods containing defined and effective levels of bioactives with clinically proven therapeutic roles in the prevention and management of chronic diseases (Martirosyan & Singh, 2015). The term 'functional food' was first coined in Japan in 1984 (Bigliardi & Galati, 2013), and is attributed to the development of the term FOSHU (Food for specified health uses). Since then, a continuous and significant growth in the functional food market has been observed worldwide (Küster-Boluda & Vidal-Capilla, 2017). This has mainly been triggered by awareness of the benefits of consuming functional foods by health-conscious consumers (Siegrist, Stampfli & Kastenholz, 2008; Bigliardi & Galadi, 2013).

The world's functional foods market was estimated to be worth nearly USD 200 billion in 2013, and it is expected to surpass USD 300 billion by 2020 (Santeramo et al., 2018). Whole foods including fruits and vegetables are the simplest forms of functional foods (Day, Seymour, Pitts, Konczak & Lundin, 2009). Other commercially available functional foods include margarine enriched with cholesterol-lowering phytosterols, cereals fortified with soluble fibre, probiotic yogurts, fruit juices fortified with vitamins (C, E and folic acid) and minerals (zinc and calcium), eggs and pasta enriched with Omega-3 (Eussen et al., 2011; Bigliardi & Galati, 2013).

Diet and health are intrinsically linked, with numerous epidemiological studies demonstrating the influential role of a healthy diet in the prevention, treatment or lowering the risk of incidence of lifestyle related chronic diseases (Kyrø et al., 2013; Prasad & Aggarwal, 2014; Vella et al., 2014; Dietz et al., 2015; Zhang et al., 2015 Kimokoti & Millen, 2016). Chronic diseases such as obesity, diabetes, cancer, cardiovascular and neurodegenerative diseases is a result of chronic or uncontrolled inflammation (He et al., 2015). Chronic inflammation involves a complex mechanism

mediated by various biomarkers (Esposito, Chen, Grace, Komarnytsky & Lila, 2014), and the alteration of signalling pathways, resulting in increased levels of inflammatory biomarkers, lipid peroxides and free radicals (Lin, & Karim, 2007; Mueller, Hobiger, & Jungbauer, 2010; Franceschi, & Campisi, 2014; He et al., 2015). Globally, chronic diseases are the leading cause of death, being responsible for about 40 million (68%) of the world's 56 million deaths reported in 2012 (World Health Organization, 2014).

A low-grade chronic, systemic inflammation which occurs in the absence of overt infection ("sterile" inflammation) during the aging process, is known as "inflammaging"; and this is a significant risk factor for both morbidity and mortality in elderly people (Franceschi, & Campisi, 2014). Inflammaging is a result of increased levels of pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), Interleukin-8 (IL-8), Interleukin-1beta (IL-1β) and Interleukin-6 (IL-6) and oxidative stress, which leads to the onset of chronic diseases (Goldberg & Dixit, 2015; Xia et al., 2016). Aging is associated with a weakened immune system, reduced metabolism, limited physical activity and other biomedical and psychosocial factors (Franceschi & Campisi, 2014). Inflammaging combined with these age associated factors make the older adults most susceptible to chronic diseases (Figure 1.1).

According to Statistics New Zealand (Ministry of Health- Manatū Hauora, 2016), the older adult population segment is growing at a much faster rate than the overall population. For those aged 55-64 and 65-74, an incremental growth of 13% and 31% respectively is projected to occur between 2015/16 to 2035/36. The growth in aging population and the elevation in incidence of chronic diseases is posing a significant burden on the world economy and healthcare systems (Vella et al, 2014). According to the World Health Organization (2014), the cumulative economic losses due to chronic diseases in low- and middle-income countries have been estimated at USD 7 trillion during the years 2011-2025.

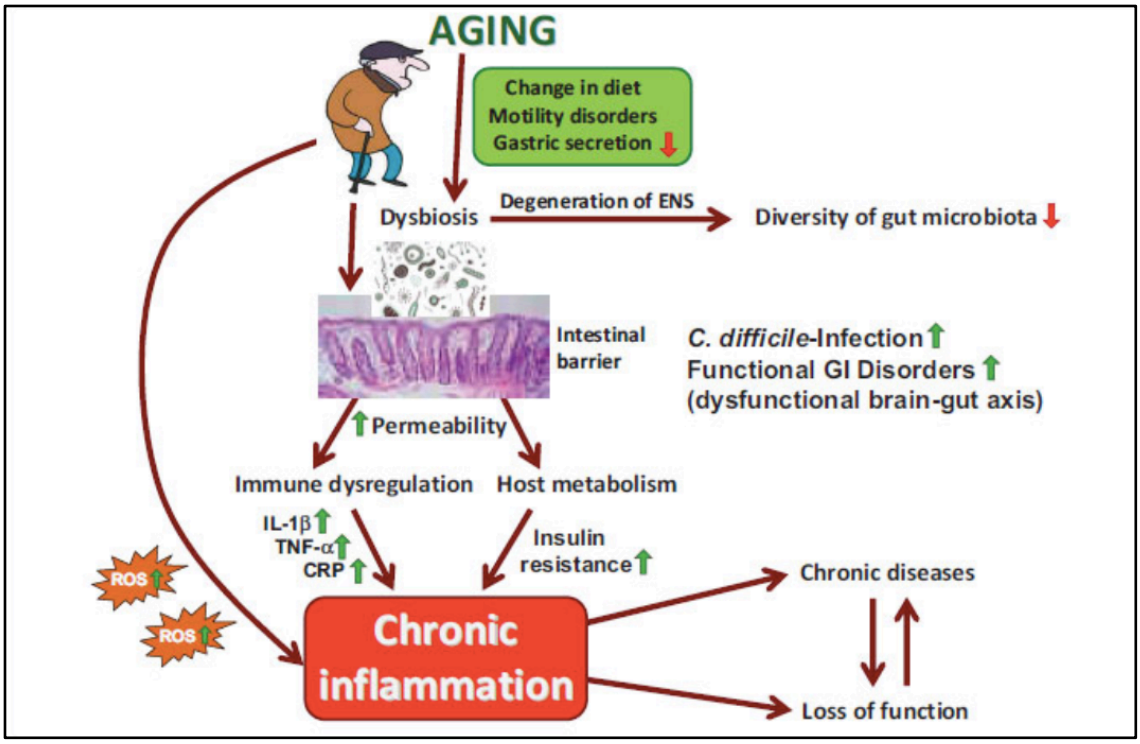


Figure 1.1 Mechanism of inflammaging in older adults

Source: Konturek et al. 2015

Abbreviations: *C. difficile*, *Clostridium difficile*; ENS, enteric nervous system; TNF-α, tumour necrosis factor-alpha; IL-1β, Interleukin-1 beta; CRP, C-reactive protein; ROS, reactive oxygen species.

Since ancient times, inflammation and related chronic diseases have been treated with traditional plants or plant-derived formulations (Muller et al., 2010). These plant-based foods are rich in bioactives compounds such as polyphenols and carotenoids, which protect the body against oxidative damage and hence the development of chronic diseases (Day et al., 2009; Herrero, Plaza, Cifuentes & Ibáñtez, 2010). Polyphenols are a major class of phytochemicals present in fruits, vegetables, olive oil, red wine and tea (Manach, Scalbert, Morand, Rémésy & Jiménez, 2004) and more than 8000 polyphenols have been identified (Seiad & Derriche, 2013). Two main types of polyphenols are flavonoids and phenolic acids. Flavonoids are aromatic compounds and include compounds such as flavonols, flavones, isoflavone and anthocyanidins (Manach et al., 2004). These are commonly found in onion, tea, apple, citrus fruits and berries (Scalbert et al., 2005). Phenolic acids include caffeic acid, chlorogenic acid, ferulic acid and tannins and are commonly

found in coffee, tea and fruits such as strawberries, raspberries and blackberries (Manach et al., 2004; Scalbert et al., 2005).

Curcuminoids are non-flavonoid polyphenols obtained from turmeric. Curcuminoids consist of 77% pure curcumin (CUR), 17% demethoxycurcumin (DMC) and 3% bisdemethoxycurcumin (BDMC) (Wulandari, Urraca, Descalzo, Amran & Moreno-Bondi, 2015). These lipophilic molecules exhibit a wide range of pharmacological activities such as anti-inflammatory, antioxidant, cardioprotective (Zeng et al., 2015), antimicrobial (Magalhães et al., 2009; Reddy, Vatsala, Keshamouni, Padmanaban & Rangarajan, 2005) and potent anti-cancer effects (Ramirez et al., 2018). However, the application of curcuminoids are limited due to its poor solubility and reduced bioavailability, because of low absorption from the gut, rapid metabolism and systemic elimination (Chopra, Jain, Dewangan, Varkey & Mazumder, 2016; Lu, Inbaraj & Chen, 2017). To mitigate some of these problems, researchers have developed numerous pharmaceutical and food-grade curcuminoid formulations with improved aqueous solubility, stability and bioavailability (Cuomo et al., 2011; Chopra et al, 2016).

Improved bioavailability and aqueous solubility of curcuminoids were reported in food matrices such as oil-in-water emulsions (Ahmed, Li, McClements & Xiao, 2012; Zou, Liu, Liu, Xiao & McClements, 2015), proteins (Aditya, Yang, Kim & Ko, 2015; Sneharani, Kakakkat, Singh & Rao, 2010; Tapal & Tiku, 2012), lipids (Jannin et al., 2015; Kumar, Ahuja, Ali & Baboota, 2016) and liposomes (Jin, Lu & Jiang, 2016; Gómez-Mascaraque, Sipoli, Torre & López, 2017;). Most of these food-grade bioavailable formulations involve the interaction of curcuminoids with milk constituents. In fact, administration of turmeric in foods with a high fat content (coconut milk or chocolate) enhances the absorption of the minor curcuminoids- demethoxycurcumin and bisdemethoxycurcumin (Cuomo et al., 2011). Considering the interaction of curcuminoids with milk proteins and lipids, and its stability at acidic pH (Rege & Momin, 2017), it is proposed that yogurt may be a suitable medium for delivery of curcuminoids.

Yogurt is amongst the most popular fermented milk products in the world. A wide range of yogurt products are available in the market such as flavoured yogurts (fruit, vanilla, chocolate), low-fat yogurts, organic yogurts and functional yogurts supplemented with prebiotics, probiotics, vitamins

and minerals (Carlucci, Stasi, Nardone & Seccia, 2013). This continuous evaluation and modification of yogurts to meet consumer expectations is the key to growth of market sales of yogurt (Noh et al., 2013). Furthermore, yogurt's popularity and success is attributed to its intrinsic beneficial properties such as high digestibility and balanced nutritional content (Carlucci et al., 2013). Considering the popularity of functional yogurts, the yogurt fortified with bioactives will most likely be acceptable to consumers (Betoret, Betoret, Vidal & Fito, 2011; Granato, Nunes & Barba, 2017). Consumer acceptance of new products is also based on aspects such as stability, texture and the sensory properties of the product (Day et al., 2009). Thus, the major challenges in product development is to maintain the bioactivity and prevent degradation of the functional ingredient without compromising the quality and sensory attributes of the product (Day et al., 2009; Betoret et al., 2011).

1.2 Aims and objectives

The current study was undertaken to develop a functional curcuminoid yogurt and demonstrate the potential of yogurt as a delivery vehicle for commercial food-grade curcuminoids. The research was carried out in three phases and the principal objectives of each phase were:

1. Phase I (Screening of food-grade curcuminoids and commercial yogurt products)
    - Incorporation of 10 different curcuminoid ingredients into commercial coconut cream yogurt (CCY), cow's milk yogurt (CMY) and goat's milk yogurt (GMY).
    - To determine the sensory properties and pH of the prepared yogurt samples, and, select the most suitable yogurt type for incorporation of curcuminoids.
    - To select two suitable curcuminoid powders for testing of anti-inflammatory activity in phase 2.

2. Phase II (*In vitro* analysis for determination of bioactivity of curcuminoids)
    - To optimize concentration and time of incubation of lipopolysaccharide (LPS) with differentiated THP-1 monocytes.
    - To determine the cytotoxicity of curcuminoids on LPS-stimulated macrophages by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

- To determine and compare anti-inflammatory activity of analytical and food-grade curcumin/curcuminoids by TNF-α Enzyme-linked immunosorbent assay (ELISA).
- Selection of a food-grade curcuminoid ingredient with highest anti-inflammatory activity for incorporation into coconut milk/cream yogurt.

3. Phase III (Laboratory scale production of coconut yogurt supplemented with curcuminoids)
    - To select an optimum commercial coconut milk/cream for preparation of yogurt.
    - To optimize the concentration of curcuminoids in yogurt.
    - Investigation of physico-chemical, rheological, microbiological and sensory properties of yogurt containing curcuminoids during both fermentation and 15-day shelf life period.
    - To quantitate the levels of residual curcuminoids in yogurt after 1, 5, 10 and 15 days of storage using HPLC-DAD (High-performance Liquid Chromatography with Diode-Array Detection).

CHAPTER 2
LITERATURE REVIEW

2.1 Turmeric

Turmeric is an orange-yellow spice obtained from the rhizome of *Curcuma longa*, a member of the ginger family *Zingeberacea*. It originated from South-East Asia (Rathaur, Raja, Ramteke & John, 2012) and is commonly used as condiment, food preservative and colouring agent (Gul & Bakht, 2015). Until the 20th century, European and American herbalists had little interest in turmeric (Lal, 2012), however, the herb has been used in Indian Ayurveda and traditional Chinese medicines for centuries for preventing and curing several diseases (Lal, 2012; Deogade & Ghate, 2015).

Turmeric contains more than 100 components, out of which the main components are turmeric powder, essential oils, oleoresins and curcuminoids (Raina, Srivastava & Syamsundar; 2005; Li et al., 2011; Prasad & Aggarwal, 2011). Volatile oils are responsible for the characteristic aroma of turmeric, whereas curcuminoids impart the bright-yellow colour to this spice. Nutritionally, turmeric contains protein (6.3%), fat (5.1%), minerals (3.5%), carbohydrates (69.4%), and moisture (13.1%) (Prasad, Gupta, Tyagi & Aggarwal, 2014). It is also a source of omega-3 and contains about 2.5% α-linolenic acid (Prasad & Aggarwal, 2011).

Essential oils and curcuminoids are the major bioactives of turmeric that exhibit a wide range of pharmacological activities such as anti-inflammatory, hepatoprotective, antimicrobial, wound-healing, anti-cancer, anti-tumour and anti-viral properties (Raina et al., 2005). The turmeric plant contains approximately 9% volatile oils (Lal, 2012) which are comprised of monoterpenes and sesquiterpenes, along with their oxygenated derivatives (Carvalho, Osorio-Tobón, Rostagno, Petenate & Meireles, 2015). A study by Tyagi et al. (2015) suggested that β-sequiphellanderne (SQP), a constituent of turmeric oil has a potent anti-cancer activity comparable to that of curcumin.

Curcuminoids are isolated from the rhizomes of turmeric in amounts varying from 0.58 to 6.5% (on a dry weight basis) (Li et al., 2011). Commercially available curcuminoids are a mixture of 77% curcumin (CUR), 17% demethoxycurcumin (DMC) and 3% bisdemethoxycurcumin (BDMC) (Wulandari, Urraca, Descalzo, Amran & Moreno-Bondi, 2015). Curcumin exhibits significantly higher bioactivity as compared to DMC and BDMC (Guo et al., 2008). There is an extensive *in vivo* and *in vitro* evidence indicating that curcuminoids possess anti-inflammatory, antioxidant, cardioprotective (Zeng et al., 2015), antimicrobial (Reddy, Vatsala, Keshamouni, Padmanaban & Rangarajan, 2005; Magalhães et al., 2009), antiulcer (Mahattanadul et al., 2009) and potent anti-cancer (Ramirez et al., 2018) properties.

2.2 Traditional uses of turmeric

Traditionally, turmeric is used as a condiment, curry spice, preservative, insect repellent, dye and cosmetic ingredient (Akbar et al., 2018). It is considered auspicious in India and is extensively used during wedding and religious ceremonies (Gupta et al., 2013). Furthermore, it has been used as a remedy for treatment of various ailments such as coryzoa, hepatic disorders, rheumatism, stomach disorders, wounds, cold, cough and sore throats in Asian countries since ancient times ((Ng et al., 2006; Krishnaswamy, 2008; Kim, Lee & Shin, 2015). In Unani culture, topical administration of turmeric has been found to aid ulcers and inflammation (Bhowmilk, Kumar, Chandira & Jayakar, 2009).

Turmeric is widely consumed through foods in the Indian sub-continent, South Asia and Japan (Deogade & Ghate, 2015), although less common in Western cuisine where it is considered a minor ingredient in spice mixtures and sauces (Ng et al., 2006). The average intake of turmeric in Indian populations can be as high as 2- 2.5 g per day (approximately 100 mg of curcuminoids) (Basnet & Skalko-Basnet, 2011). Daily consumption of 200 mg curcuminoids can reduce the incidence of cardiovascular diseases by lowering the level of blood lipid peroxides, high density and low density lipid peroxidation (Ng et al., 2006). A study related to consumption of turmeric reported a significantly lower incidence of Alzheimer's disease in elderly Indians compared to the same age group in United States (Kim, Lee & Shin, 2015).

2.3 Extraction of curcuminoids from turmeric

Curcuminoids are naturally synthesized by turmeric plant and are commercially extracted from the turmeric oleoresin (Jayaprakasha, Rao & Sakariah, 2006). The quality, efficacy and the extraction efficiency of curcuminoids depends on factors such as the extraction solvents used, composition of turmeric, extraction method and processing conditions (Zhan, Zeng, Zhang & Li, 2011; Paulucci, Couto, Teixeira & Freitas, 2013). Turmeric powder containing higher content of volatile oils solubilize the curcuminoids and interferes with the recrystallization process (Pawar, Gavasane & Chaoudhary, 2018), thus, altering the extraction efficiency and quality of the final product.

The extraction procedure has a major impact on the structural characteristics, as well as chemical and biological properties of curcuminoids (Sahne, Mohammadi, Najafpour & Moghadmnia, 2016). Extraction of curcuminoids by Soxhlet extraction is one of the oldest and most common method employed in the industry (Sahne et al., 2016). It involves dissolving curcuminoids in organic solvents and heating the solution, which may take up to 12 h (Mandal, Mohan Hemalatha, 2008). This approach involves a higher risk of curcumin decomposition (Mandal et al., 2008), which may lead to loss of bioactivity and lower extraction yield. To overcome these problems associated with conventional method, researchers have developed modern techniques such as ultra-sound assisted extraction, microwave-assisted extraction and enzymatic-assisted extraction (Sahne et al., 2016; Liang et al., 2017; Sahne et al., 2017).

The use of many extraction solvents is limited due to the regulations set by national and international food laws on residual limits in final product (Uematsu et al., 2008; Revathy, Elumalai, Benny & Antony, 2011). Organic solvents such as hexane, ethanol, methanol, acetone, isopropanol and ethyl acetate are commonly used in extraction of curcuminoids from turmeric oleoresins (Revathy et al., 2011; Kulkarni, Maske, Budre & Mahajan, 2012; Osorio-Tobón et al., 2014; Pawar et al., 2018). Out of these solvents, acetone has been reported slightly superior to alcohol and ethyl acetate, resulting in higher and selective extraction of curcuminoids (Pawar et al., 2018). Apart from this, researchers have made efforts to isolate curcuminoids using food grade solvents such as polyglycolized glycerides (Gilda, Kanitkar, Bhonde & Paradkar, 2010) and medium-chain triacylglycerols (Takenaka et al., 2013).

2.4 Detection of curcuminoids

High performance liquid chromatography (HPLC) is the most common technique used for the quantitative analysis of curcuminoids (Priyadarsini, 2014). For detection of curcuminoids, wavelengths of 350 to 450 nm in visible region or 250 to 270 nm in the ultraviolet region are commonly used (Priyadarsini, 2014). Over the years, researchers have modified the traditional HPLC method to improve detection limit and reduce analysis time. An HPLC system based on C_{18} mobile phase with less than 10% water content has been reported to yield better separation of curcumin, demethoxycurcumin and bisdemethoxycurcumin (Jayaprakasha, Rao & Sakariah, 2002). This method has been successfully employed by Fu et al. (2016) to quantitate the curcuminoids extracted from curcuminoids-buttermilk yogurt. Thus, this HPLC method can be used to detect curcuminoids in food products.

Other methods used for estimation of curcuminoids include fluorescence (Nugraha, Batubara, Rahmat & Alatas, 2017), near-infrared spectroscopy (Tanaka, Kuba, Sasaki, Hiwatashi & Komatsu, 2008), column chromatography (Revathy et al., 2011) and high-performance thin layer chromatography (Paramasivam, Poi, Banerjee & Bandyopadhyay, 2009). These methods are often used in conjugation with HPLC, such as HPLC-fluorescence (Zhang et al., 2009). However, limited studies have reported the use of these methods for detection of curcuminoids.

2.5 Chemistry of curcuminoids

2.5.1 Structural characteristics of curcuminoids

The structure of curcumin was first elucidated by Milobedzka, von Kostanecki and Lampe in 1910 (Wanninger, Lorenz, Subhan & Edelmann, 2015), as shown in Figure 2.1. Curcuminoids are naturally occurring β-diketone ligand (Wanninger et al., 2015) with three reactive entities: two aromatic rings with *ortho (o)*-methoxy phenolic group, diketone moiety and a seven-carbon linker chain (Priyadarsini, 2013). Structurally, CUR, DMC and BDMC differ due to presence or absence of *o*-methoxy phenolic group (Figure 2.1). Regardless, all these compounds exist as trans-trans keto-enol tautomer (Amalraj, Pius, Gopi & Gopi, 2017).

Figure 2.1 Structure and molecular weight of curcuminoids
Source- Esatbeyoglu et al. (2012); Ali, Haque & Saleem (2014)

The carbon—carbon (C—C) and carbon—phenyl (C—Ph) units (Figure 2.1) can give rise to a number of conformational isomers (Kolev, Velcheva, Stamboliyska & Spiteller, 2005). Among these isomeric forms, the enolic form (Figure 2.2) is the most stable in gaseous and aqueous phases, and in some organic polar solvents (alcohol and dimethyl sulfoxide) (Kawano, Inohana, Hashi & Lin, 2013; Priyadarsini, 2013; Mahran, Hagras, Sun & Brenner, 2017). Using density functional theory (DFT) and time-dependent density functional theory (TD-DFT) calculations, Shen and Ji (2007) reported that the enol form is more stable than the di-keto form, their energy difference being 7.75 kcal/mol. X-ray diffraction analysis shows that enol form is stabilized by electron delocalization and intramolecular hydrogen bonding in the crystal form (Figure 2.3) (Kolev et al., 2005). Another reason is the extension of conjugated chain to seven carbon atoms in the enol form in contrast to three carbon atom chain in the di-keto form (Wright, 2002). However, the pH of the solvent plays a crucial role in determining the dominant tautomer of curcumin and hence its structure. In acidic medium (pH≤7), curcumin exists in the keto form and acts as a hydrogen atom donor while the enol form predominates under alkaline conditions (pH>7) (Esatbeyoglu et al., 2012).

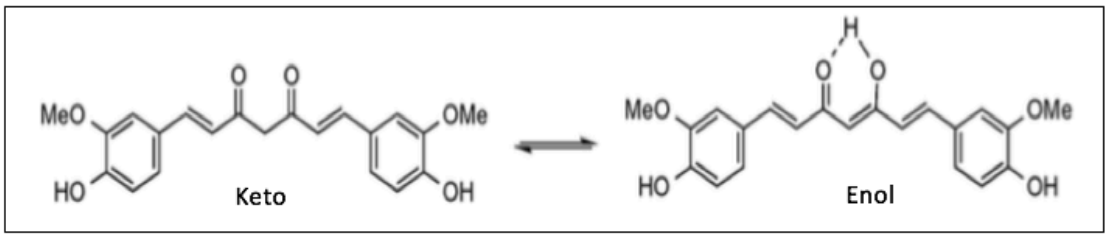


Figure 2.2 Keto-enol tautomerism of curcumin

Source: Wanninger et al. (2015)

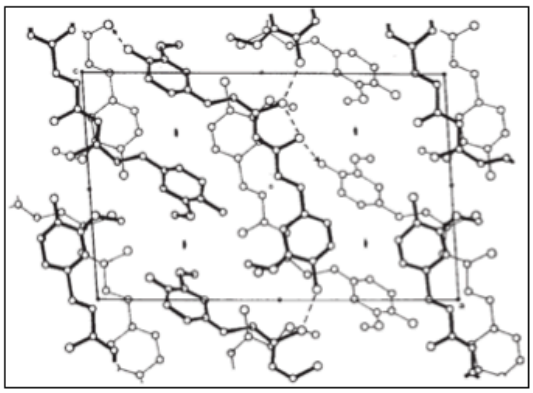


Figure 2.3 Crystal structure of curcumin

Source: Kolev et al. (2005)

The structural properties of curcuminoids are strongly related to its anti-inflammatory, antioxidant and antiamyloid activities. The α, β-unsaturated β-diketone moiety plays an important role in suppressing the nuclear factor-kappaB (NF-kB) and scavenging reactive oxygen species (ROS) (Nabavi et al., 2015). Furthermore, CUR has higher ability to suppress activation of NF-kB while BDMC shows least activity, which may be attributed to the presence of phenyl methoxy groups (Sandur et al., 2007). The interaction between *o*-methoxy oxygen and phenolic hydrogen significantly affects the planarity, conformation and ability to undergo oxidation (Sandur et al., 2007).

2.5.2 Solubility of curcuminoids

Curcuminoids are lipophilic molecules which solubilize to variable degrees in polar solvents such as Dimethyl Sulfoxide (DMSO), methanol, ethanol, acetonitrile, chloroform, acetone and ethyl acetate (Priyadarsini, 2014). Furthermore, the solubility of curcuminoids in organic solvents such as DMSO can be enhanced by pre-treatment with 5% Bovine Serum Albumin or 10% Fetal Calf Serum (Quitschke, 2008). A combination of serum and DMSO can be used to dissolve curcumin for *in vitro* studies (Klawitter et al., 2012).

The aqueous solubility of curcuminoids is approximately 3 x 10^{-8} M, indicating that they are almost insoluble in water (Kharat, Du, Zhang & McClements, 2017). However, the solubility of curcuminoids increases at alkaline pH due to salt formation and rapid hydrolytic degradation (Rao & Rao, 2011), which is characterized by change in colour from yellow to red in alkaline solutions (Bernabé-Pineda, Ramírez-Silva, Romero-Romo, González-Vergara & Rojas-Hernández, 2004). Analysis of the pH (above 7) profiles of curcuminoids indicate that solubility increases with increasing pH up to a maximum at about pH 10.2. Further increase in pH to 11.95 leads to a decline in the solubility of the compound (Price & Buescher, 1997).

2.5.3 Stability of curcuminoids

Curcuminoids are unstable molecules and are susceptible to degradation in aqueous solutions and in the presence of oxygen, light, high temperature and high relative humidity (Jin, Lu & Jiang, 2016; Peram et al., 2017) (Figure 2.4). The individual curcuminoids exhibit variable degrees of degradation towards these factors. BDMC is most stable followed by DMC and CUR (Peram et al., 2017).

Stability of curcuminoids in aqueous solutions

Curcuminoids experience pH-dependent degradation by hydrolysis in aqueous solutions (phosphate, citrate-phosphate, carbonate buffers and serum-free cell culture medium) (Mondal, Ghosh & Moulik, 2016; Zhu et al., 2017). Degradation occurs faster in alkaline environment compared to acidic solutions (Zhu et al., 2017). From chromatographic analysis, it can be interpreted that 44.39% curcuminoids degraded in pH ranging from 3-7, however, only 21.19% curcuminoids remained in the 1M NaOH solution after 2 h of incubation (Peram et al., 2017).

The poor stability of curcuminoids leads to hydrolytic cleavage at physiological pH, resulting in formation of various molecular fragments (Shen & Ji, 2012) as shown in Figure 2.4. *Trans*-6-(40-hydroxy-30-methoxyphenyl)-2,4-dioxo-5-hexanal is the major product of this hydroxyl mediated reaction (Figure 2.4) (Zhu et al., 2017). Ferulic acid is easily metabolised and absorbed in the human body (Ou & Kwok, 2004). Like the parent molecule curcumin, the medicinal properties of ferulic acid and vanillin including anti-inflammatory, immunostimulatory, antioxidant, antimicrobial and potent anti-carcinogenic and neuroprotective activities are well-documented (Kanski, Aksenova, Stoyanova & Butterfield, 2002; Ou & Kwok, 2004; Mourtzinos, Konteles, Kalogeropoulos &karathanos, 2009; Kumar & Pruthi, 2014; Sripanidkulchai & Junlatat, 2014).

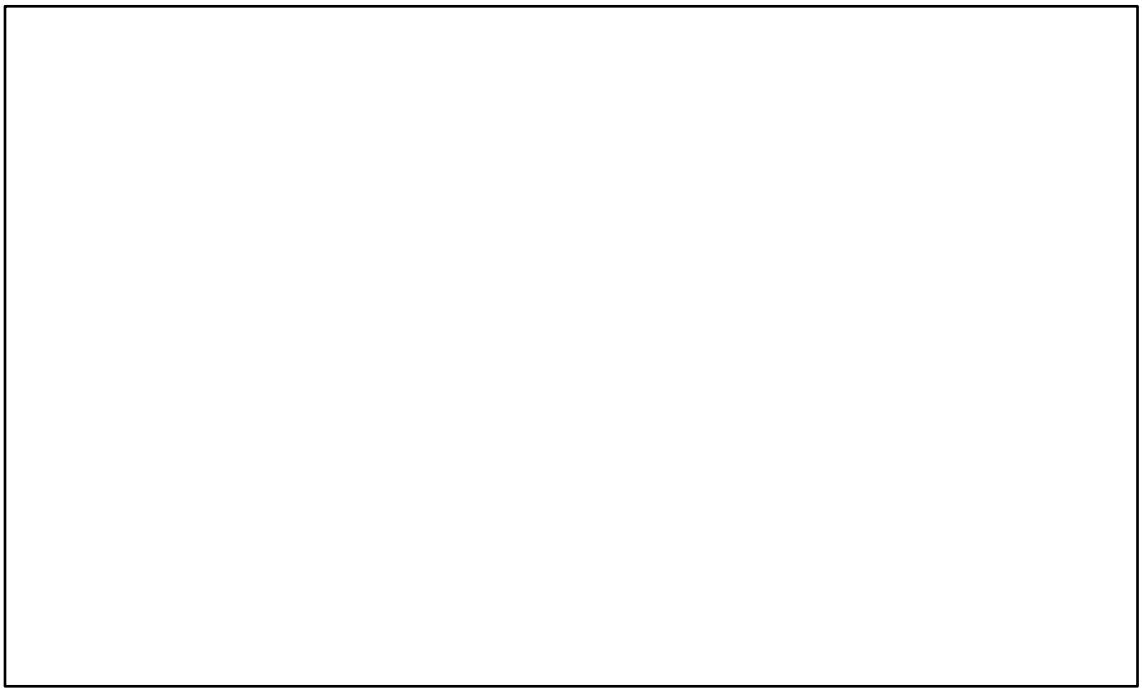


Figure 2.4 Alkaline hydrolysis of curcumin
Source: Mirzaee, Kooshk, Rezaei-Tavirani & Khodarahmi (2014)

Oxidative degradation of curcuminoids

Curcuminoids experience rapid oxidative degradation in aqueous solutions at physiological pH (Zhu et al., 2017). This oxygen-mediated reaction is the main degradation pathway of curcuminoids, resulting in formation of a stable cyclic compound end-product (Gordon et al., 2015) (Figure 2.5 & 2.6). Autoxidation of curcumin, DMC and BDMC yield different end-

products due to their different structures and varying electron density of their phenolic rings (Gordon et al., 2015). Curcumin is most vulnerable to oxidation, while BDMC undergoes oxidation only in the presence of catalysts such as horseradish peroxidase (HRP) or hydrogen peroxide (Gordon et al., 2015; Peram et al., 2017). DMC undergoes combined degradation by autoxidation and enzyme-catalysed reactions (Gordon et al., 2015). Despite their differences, the degradation of all three components follow a similar mechanism, as shown in Figures 2.5 and 2.6 (Gordon et al., 2015; Schneider et al., 2015).

The initial products of the autoxidation are unstable and are formed by abstraction of hydrogen from one of the phenolic hydroxyl groups (Griesser et al., 2011). A series of reactions follow which result in formation of a new C—C bond and oxygen-linked ether bridges (Gordon et al., 2012). The end-products of decomposition of curcumin, DMC and BDMC are bicyclopentadione, demethoxy bicyclopentadione and bisdemethoxy spiroepoxide respectively (Figures 2.5 and 2.6) (Gordon et al., 2015). Formation of these compounds can be confirmed by monitoring the absorbance spectra of curcuminoids at 430 nm (Griesser et al., 2011).

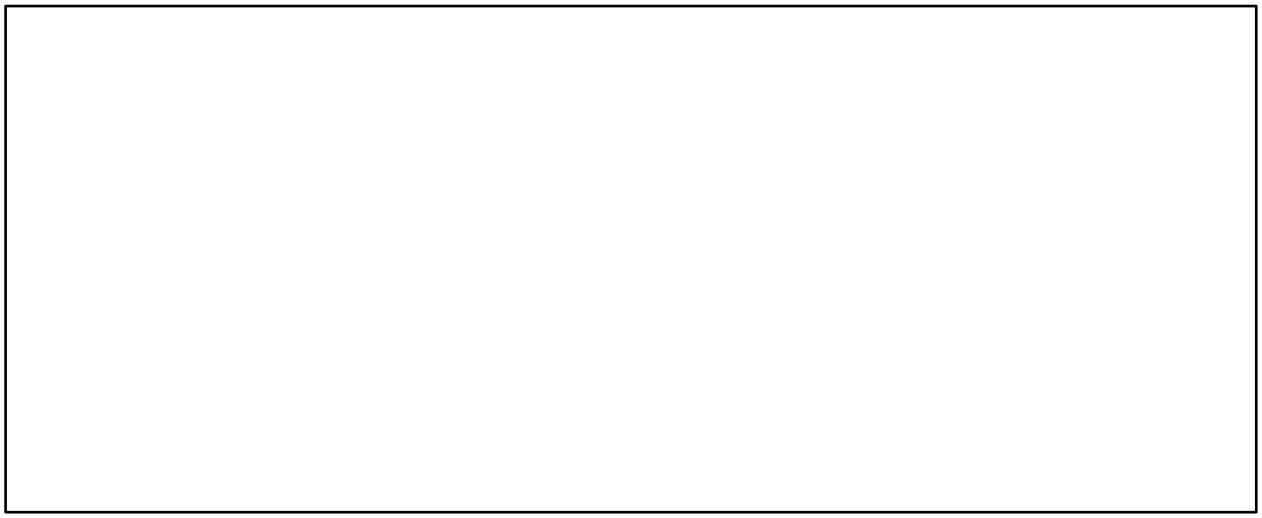


Figure 2.5 Auto-oxidation of curcumin

Source: Schneider, Gordon, Edwards & Luis (2015)

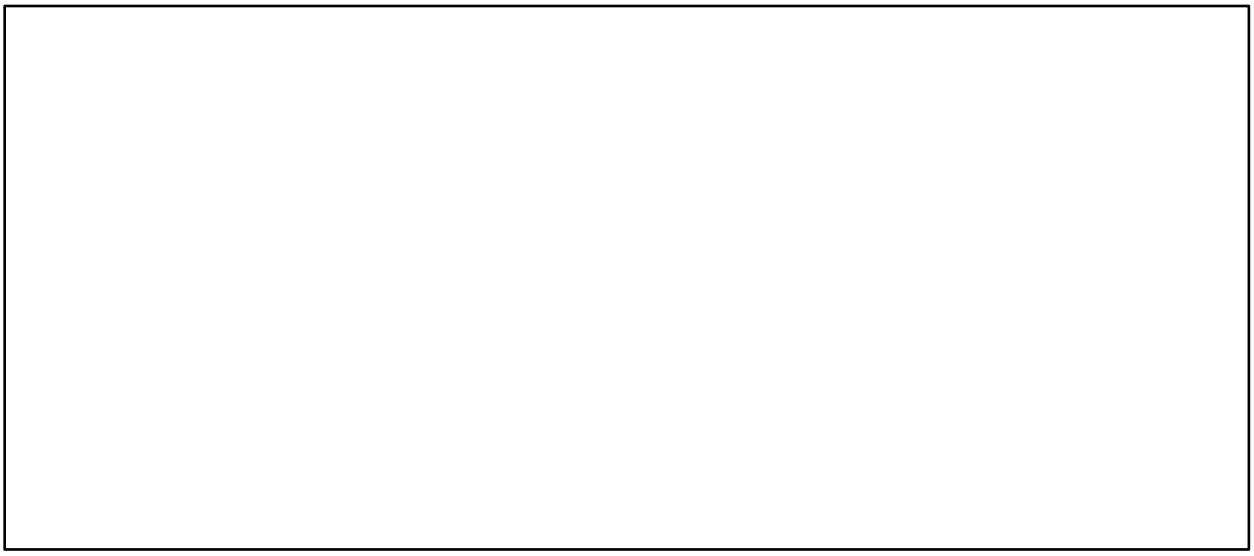


Figure 2.6 Oxidative decomposition of DMC and BDMC
Source: Gordon et al. (2015)

The potential of bicyclopentadione and other unstable intermediates in the treatment of chronic diseases is unknown (Zhu et al., 2017). Sanidad et al. (2017) compared the *in vitro* pharmacological activities of bicyclopentadione (BCP) and a mixture of stable total degradation products (TDP) with curcumin in MC38 cells, and demonstrated that BCP and TDP failed to suppress cell proliferation, cell cycle progression and apoptosis in MC38 cells (Sanidad et al., 2017). However, these compounds weakly promoted the inhibition of inflammatory markers and NF-kB signalling pathway (Sanidad et al., 2017). In fact, Gordon et al. (2015) reported the ability of the intermediates to inactivate topoisomerase IIα, thereby killing cancerous cells.

Light-induced decomposition of curcuminoids

Curcumin, DMC and BDMC manifest similar susceptibility towards exposure to light (Peram et al., 2017). However, curcuminoids dissolved in acid brine exhibit the following order of stability towards photodegradation: curcumin> DMC> BDMC (Price & Buescher, 1996). The stability of curcuminoids depends on factors such as their physical state, type of solvent and presence of oxygen (Price & Buescher, 1996). Curcuminoids were reported to be most stable in dry powder under anaerobic conditions (Price & Buescher, 1996). Furthermore, curcuminoids dissolved in ethyl acetate, chloroform or acetonitrile are more vulnerable to light degradation than in methanol

(Price & Buescher, 1996). The mechanism of photo-degradation follows cleavage of the seven-carbon atom conjugated chain (Griesser et al., 2011). The end-products of this reaction are vanillin, ferulic aldehyde, ferulic acid, feruloylmethane, *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde (Schieffer, 2002; Griesser et al., 2011).

Thermal degradation

Curcuminoids are thermally stable compounds and retain their original composition up to 80˚C (Peram et al., 2017). It was reported that even the most unstable form (pH 8), showed only a low increase in degradation when the temperature was increased from 37˚C to 60˚C (Naksuriya, Steenbergen, Torano, Okonogi & Hennink, 2016). Previous studies have demonstrated losses of up to 2% curcuminoids at 90˚C (Kurien, Singh, Matsumoto & Scofield, 2007) and 23-57% at 121˚C (Suresh, Manjunatha & Srinivasan, 2007). Temperatures above 100˚C promote cleavage of the β-diketone linkage, thus leading to degradation of the curcuminoid molecule (Peram et al., 2017).

2.5.4 Reactivity of curcuminoids

Curcuminoids are weak Brönsted acids (Priyadarsini, 2014) with different chemical properties. Bonab et al. (2017) reported that enolic hydroxyl group and phenolic hydroxyl groups are mainly responsible for the reactivity of these compounds. In curcumin, the acidic hydrogen of hydroxyl group retains a slightly higher charge due to the presence of the methoxy groups (Patil & Gaikar, 2011). However, at least one or both methoxy groups are absent in DMC and BDMC, respectively (Peram, Jalalpure, Palkar & Diwan, 2017). This accounts for the difference in chemical reactivity of the curcuminoids (Peram et al., 2017), with curcumin being more reactive compared to DMC and BDMC.

The chemical properties of curcuminoids contribute significantly towards their biological activity. Of the three reactive groups in curcuminoids, the di-ketone moiety acts as Michael acceptor, the β-dicarbonyl as a metal chelator and phenolic $^{-}$OH as H-donor (Gordon et al., 2015). Curcuminoids have three ionisable protons- enolic O^{-} ($pKa < 8.5$) and two phenolic O^{-} ($8.5 \leq pKa \leq 10.7$) (Figure 2.7) (Priyadarsini, 2013). Thus, they can exist in different forms such as protonated (H_4A^{+}) (at pH<1), deprotonated form (A^{3-}) (pH>9) or neutral form (H_3A) (pH 1-7) (Peram et al., 2017). The

ionic state of curcuminoids plays a crucial role in determining the nature of bioactivity. For example, curcuminoids act as electron donors at alkaline pH but also undergo degradation, resulting in loss of ability to act as antioxidants (Rege & Momin, 2017). However, below pH 7, delocalization followed by resonance stabilization in the diketone and heptadiene moiety takes place, respectively. This results in formation of a stable radical which prevents degradation of curcuminoids (Rege & Momin, 2017). This molecule solely acts as a hydrogen donor with high reactivity in anti-oxidation reactions (Rege & Momin, 2017). The anti-oxidant activity of curcuminoids contributes towards suppressing chronic inflammation in the body (He et al., 2015). The mechanism of the radical scavenging activity of curcuminoids is shown in Figure 2.7.

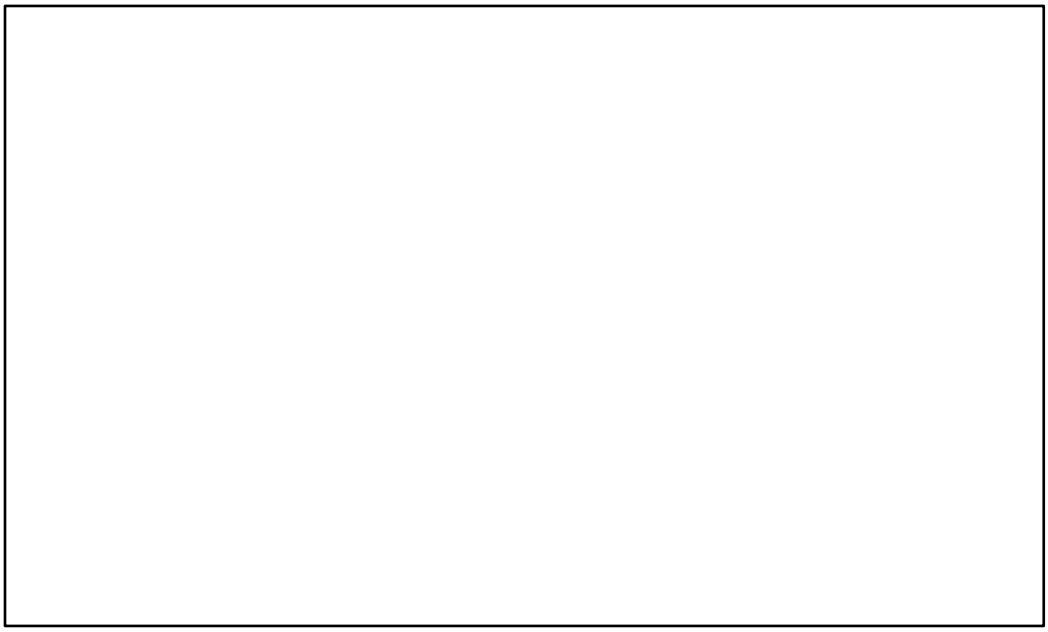


Figure 2.7 Dissociation equilibrium of curcumin

Source: Priyadarsini (2014)

Antioxidation reactions

Curcuminoids are natural antioxidants due to their ability to inhibit generation of reactive oxygen species (ROS), hydroxyl free radical and nitrogen oxide free radicals (Chen, Xue & Mu, 2014). This radical trapping and chain-breaking activity of curcuminoids is attributed to its reactive moieties (Itokawa, Shi, Akiyama, Morris-Natschke & Lee, 2008). Using the phosphomolybdenum method, Jayaprakasha et al. (2006) reported that CUR (3099 ± 66 μmol/g) exhibits higher

antioxidant activity than DMC (2833 ± 25 μmol/g) which is greater than BDMC (2677 ± 30 μmol/g). The higher antioxidant activity of CUR is due to presence of *o*-methoxy groups in curcumin, which increases the charge on the phenolic OH groups (Elsayed, 2016).

The active site and mechanism by which curcumin is able to neutralize reactive free radicals and molecular oxidants is controversial (Barzegar, 2012). A study conducted by Barzegar (2012) indicated that the phenolic groups are mainly responsible for the antioxidant activity of curcumin. Further, the reaction can either follow hydrogen atom transfer (HAT) or sequential electron and proton transfer (SET) mechanisms (Priyadarsini, 2013). The phenolic OH-groups can undergo oxidation to generate phenoxyl radicals (1) (Priyadarsini, 2014). These radicals react with ROS to form a table compound (2). The anti-oxidation reaction generally occurs in two steps (Masuda et al., 2001):

$$S—OO\bullet + AH \leftrightarrow SOOH + A\bullet \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

$$A\bullet + X\bullet \rightarrow \text{non-radical materials} \ldots\ldots\ldots\ldots\ldots \quad (2)$$

where, S is the substance oxidized, AH is the phenolic antioxidant (curcumin), A• is the anti-oxidant radical, and X• is another radical species (e.g. ROS, nitrogen oxide free radicals or peroxy radicals) (Masuda et al., 2001).

2.6 Medical applications of curcuminoids

Curcuminoids are renowned for their pharmaceutical properties and are thus described as “multi-anti spice” and “curecumin” (Goel, Kunnumakkara & Aggarwal, 2008). Various *in vitro* and *in vivo* studies have validated the therapeutic potential of curcuminoids in the treatment of chronic diseases caused by chronic inflammation and oxidative stress (Choi & Kim, 2007; Jin, Lee, Park, Choi & Kim, 2007; Dhillon et al., 2008; He et al., 2011; Kant et al., 2011; Rong et al, 2012; Panahi et al., 2015; Kunnumakkara et al., 2016). In addition, these bioactives have potent anti-microbial properties (Reddy et al., 2005; Magalhães et al., 2009). This section of the literature review will focus on the *in vitro* and *in vivo* anti-inflammatory effects of curcuminoids, along with its

signalling pathways. Some of the diseases that may be treated by curcuminoids are summarised in Table 2.1.

Table 2.1: Potential use of curcuminoids in treatment of diseases

CONDITION OF HUMAN BODY	TYPE OF DISEASES CAUSED
Chronic Inflammation (leading to chronic disease)	Acquired immune deficiency syndrome (AIDS), Alzheimer's, Arthritis, Atherosclerosis, Cancer, Cystic fibrosis, Diabetes, Epilepsy, Gastric ulcer, Hypothyroidism, Lewy body disease, Liver diseases, Lung diseases, Multiple sclerosis, Myocardial infarction, Osteoporosis, Parkinson's, Psoriasis, Renal diseases, Scleroderma.
Infection	Leishmaniasis, Malaria, Sexually transmitted disease.
Inflammation	Allergy, Contraceptive, Fever, Inflammatory bowel disease, Pancreatitis.
Other	Antidepressant, Antihelminthic, Antispasmodic, Antivenomic, Cataract, Fanconi anemia, Gall stone, Hyaline membrane disease, Hypolopidemia, Wounds.

Source: Mullaicharam & Maheswaran, 2012.

2.6.1 Curcuminoids and anti-inflammation

Inflammation is the hallmark of various chronic diseases (Ameruoso et al., 2017). Inflammation is defined as the body's innate response to traumatic, infectious, post ichaemic, toxic or autoimmune disorders (Xu et al., 2014). An initial and short-term response is termed as acute inflammation (Buckley, Gilroy & Serhan, 2014) and is beneficial for the host. However, the endurance of inflammation for longer periods may lead to chronic inflammation (Horadagoda et al., 1999; Weiss et al., 2015). Various factors such as bacterial, viral and parasitic infections, chemical irritants (phorbolmyristate acetate) and non-digestible particles (asbestos and silica) are known to induce chronic inflammatory responses in the body (Prasad & Aggarwal, 2014). As a result of these inflammatory responses, oxidative stress accumulates in body and is indicated by increased levels of pro-inflammatory cytokines (IL-6, IL-1 and TNF-α) and genes encoded by activation of NF-κB (He et al., 2015).

NF-κB is a key regulator of more than 500 gene products involved in inflammation, tumour cell transformation, cell proliferation, invasion, angiogenesis, metastasis and chemoresistance (Buhrmann et al., 2011). It is normally found in an inactive form in the cytoplasm, where it exists as a heterodimer complex, consisting of two sub-units: RelA (p65) and NF-κB1 (p50) with an additional inhibitory unit, IκBα. Exposure of cells to mitogens, inflammatory cytokines, ultraviolet radiation, viral proteins, bacterial lipopolysaccharides (LPS) and reactive oxygen species (ROS) activates the enzyme NF-κB-inducing kinase (NIK), which further activates IκBα Kinase (IKK) (Jobin et al., 1999; Surh et al., 2001). The activated IKK kinase leads to phosphorylation of IκBα at Ser-32 and Ser-36 residues and subsequent degradation of IκBα. The activated NF-κB subunits translocate to nucleus, where it binds to the promoter region and intervene the transcription of various inflammatory agents such as TNF-α, IL-6, IL-8, and other chemokines; MHC class II, ICAM-1; inducible nitric oxide synthase, and COX-2, which promotes inflammation (Jobin et al., 1999; Buhrmann et al., 201; Li et al., 2017). The cytokine mediated NF-κB signalling pathway is illustrated in Figure 2.8.

The MAPK signalling pathway is another mechanism that leads to the activation of NF-κB (Surh et al., 2001; Hua et al., 2010) (Figure 2.8). The family of serine/ threonine kinases includes three major members- extracellular-regulated protein kinase (ERK 1, 2 and 5), c-Jun NH_2-protein kinase (JNK)/ stress activated protein kinase (SAPK) and p38 (Surh et al., 2001; Liang et al., 2015). Following external stimuli, the MAP kinase is activated, leading to phosphorylation of the transcription factors located in the cytoplasm or nucleus (Kaminska, 2005). A series of interactions in the MAPK cascade leads to the activation of NF-κB (Kaminska, 2005). This leads to enhanced expression of COX-2 and iNOS (Surh et al., 2001). COX-2 production is also stimulated by IL-1β, a pro-inflammatory cytokine activated through the phosphatidylinositol 3-kinase (PK-I3)/Akt signalling pathway (Buhrmann et al., 2011).

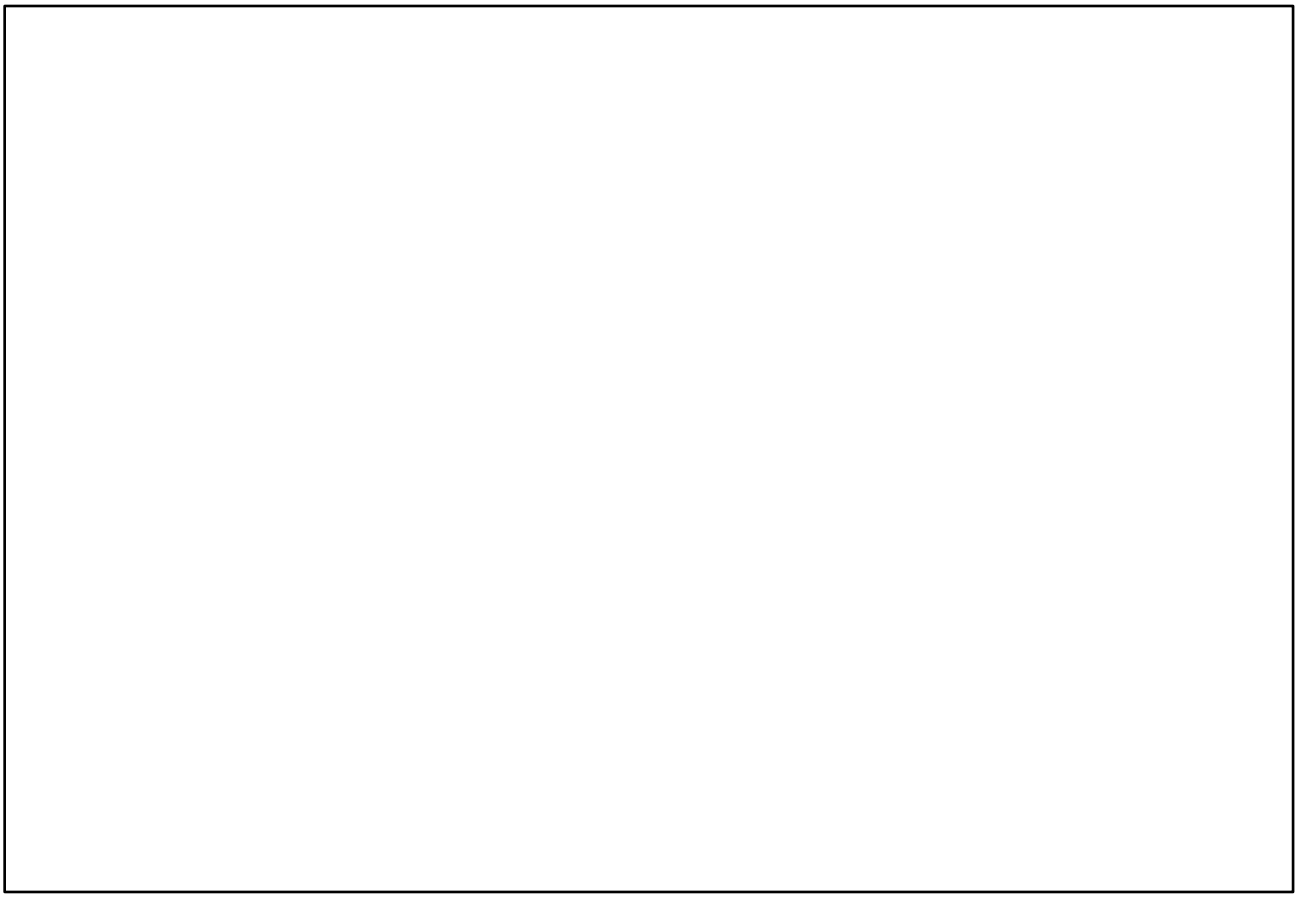


Figure 2.8 Role of curcuminoids in NF-κB inactivation

Source: Lin & Lin (2008)

Abbreviations: IκBα**,** nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; IKKα/β, IκBα Kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; TNF, tumour necrosis factor

Curcuminoids are multi-target agents with the ability to modulate the effect of gene expression (Buhrmann et al., 2011; Lozada-García et al., 2017). They can bind to as many as 33 different proteins, including transcription factors, growth factors, pro-inflammatory cytokines, enzymes, cell surface adhesion molecules and genes regulating cell proliferation and apoptosis (Buhrmann et al., 2011; Lozada-García et al., 2017). The anti-inflammatory activity of curcuminoids is due to their ability to block the activation of several transcription factors (e.g. NF-κB) and enzymes such as p38, mitogen activated protein kinase (MAPK) and c-jun N-terminal kinase (JNK), suppress the expression and release of pro-inflammatory cytokines and acute phase reactants, scavenge

ROS, down-regulate lipid peroxidation and amplify the activity of anti-oxidant enzymes (Panahi, et al., 2015). Curcuminoids are known to block the signals induced by pro-inflammatory cytokines and external stimuli, thus resulting in degradation of IκB (Jobin et al., 1999) (Figure 2.8). This prevents the activation of NF-κB, and thereby down-regulates inflammation. The molecular targets of curcuminoids are shown in Figure 2.9.

Figure 2.9 Molecular targets of curcuminoids

Source: Anand, Sundaram, Jhurani, Kunnumakkara & Aggarwal (2008)

Abbreviations: 5-LOX, 5-lipoxygenase; AAPK, autophosphorylation-activated protein kinase; AATF-1, arylamine N-acetyltransferases-1; AHR, aryl hydrocarbon receptor; AP-1, activating protein-1; AR, androgen receptor; Bcl-2, beta-cell lymphoma protein; Bcl-xL, beta-cell lymphoma extra-large; Ca^{2+}PK,

Ca^{2+}-dependent protein kinase; COX-2, cyclooxygenase-2, CREB-BP, CREB- binding protein; CTGF, connective tissue growth factor; CXCR4, alpha-chemokine receptor; DNA pol, DNA polymerase; DFF-40, DNA fragmentation factor, 40-kd subunit; DR-5, death receptor-5; EGF, epidermal growth factor; EGFR, EGF-receptor; EGFR-K, EGF receptor-kinase; EGR-1, early growth response gene-1; ELAM-1, endothelial leukocyte adhesion molecule-1; EPCR, endothelial protein C-receptor; ER-α, estrogen receptor- alpha; ERE, electrophile response element; ERK, extracellular receptor kinase; FAK, focal adhesion kinase; FAS R, Fas receptor; FGF, fibroblast growth factor; FPT, farnesyl protein transferase; GCL, glutamate cysteine ligase; GST, glutathione-s-transferase; H2R, histamine (2)-receptor; HER-2, human epidermal growth factor-2; HGF, hepatocyte growth factor; HIF-1, hypoxia inducible factor-1; IAP-1, inhibitory apoptosis protein-1; ICAM-1, intracellular adhesion molecule-1; IL, interleukin; IL-1, receptor associated kinase; IL-8R, interleukin-8 receptor; IR, integrin receptor; iNOS, inducible nitric oxide oxidase; InsP3-R, inositol 1,4,5-triphosphate receptor; JAK, janus kinase; JNK, c-jun N-terminal kinase; LDLR, low density lipoprotein receptor; MaIP, macrophage inflammatory protein; MAPK, mitogen activated protein kinase; MCP, monocyte chemoattractant protein; MDRP, multi-drug resistance protein; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; NF-κB, nuclear factor-kappaB; NGF, nerve growth factor; Notch-1, highly conserved cell signalling system; NQO-1, NAD(P)H:quinoneoxidoreductase-1; Nrf-2, nuclear factor-2 related factor; ODC, ornithine decarboxylase; PAK, protamine kinase; PhpD, phospholipase D; PDGF, platelet derived growth factor; PhK, phosphorylase kinase; PKA, protein kinase A; PKB, protein kinase B; Pp60c-tk, a non-receptor protein tyrosine kinase c-Src, cellular src kinase; PPAR-γ, peroxisome proliferator-activated receptor; PTK, protein tyrosine kinase; Src-2, src homology 2 domain containing tyrosine phosphatase; STAT, signal transducers and activators of transcription; TF, tissue factor; TGF-1, transforming growth factor-1; TMMP-3, tissue inhibitor of metalloproteinase-3; TNF-α tumour necrosis factor- alpha; uPA, urokinase-type plasminogen activator; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; WT-1 Wilms' tumour gene 1.

2.6.2 *In vitro* anti-inflammatory benefits of curcuminoids

The anti-inflammatory activity of curcuminoids has been studied both human and animal cell lines. Treatment of IL-1β stimulated human tenocytes with curcumin (5 μM) down-regulated the phosphorylation, degradation and kinase activity of IκBα. Simultaneously, the expression of cyclooxygenase-2 (COX-2), suppressed the levels of Bax and active capsase-3, leading to inhibition of NF-κB through the phosphatidylinositol 3-kinase/Akt pathway (Buhrmann et al., 2011). In contrast, Jobin et al. (1999) argued that inactivation of NF-κB occurs due to blocking of secretion of cytokines such as (interleukin) IL-8, IL-1β and TNF-α in human (Caco-2 and HT-29)

cells and rat intestine epithelial IEC-6 cells. Another study (Jin, Lee, Park, Choi & Kim, 2007) reported the down-regulation of pro-inflammatory cytokines accompanied by inactivation of NF-κB, nitric oxide (NO) and prostaglandin E_2 in LPS-stimulated BV2 microglial cells, when treated with curcumin. Similar results, demonstrating inhibition of TNF-α and NO followed by NF-κB inactivation by curcumin were observed in RAW 264.7 macrophages (Woo et al., 2007). Another study (Abe, Hashimoto and Horie,1999) demonstrated the ability of curcumin to inhibit the secretion of pro-inflammatory cytokines IL-8, (macrophage inflammatory protein) MPI-1α, (monocyte chemoattractant protein) MCP-1, IL-1β and TNF-α in human peripheral blood monocytes and macrophages stimulated with LPS or Phorbol 12-myristate 13-acetate (PMA). While curcumin has been suggested to possess the greatest anti-inflammatory activity, demethoxycurcumin was found to have stronger inhibitory response towards NF-κB activation as compared to bisdemethoxycurcumin (Guo et al., 2008). Based on the results from these studies, it may be speculated that NF-κB and the pro-inflammatory cytokines (TNF-α and interleukins 1β, 6 and 8) are the primary biomarkers of inflammation.

2.6.3 Anti-inflammatory effects of curcuminoids in animal models

The pharmacological efficacy of curcuminoids has been demonstrated through various pre-clinical trials. Li, Suwanwela & Patumraj (2017) studied the effect of curcumin in a transient ischemia/ reperfusion rat model. Immunohistochemistric analysis showed a decline in NF-κB expression in the MCAO (Middle Cerebral Arterial Occlusion) cells treated with curcumin compared to control MCAO cells. Injection of curcumin-carboxymethyl cellulose acetate butyrate nanoparticles (10 mg/kg or 30 mg/kg) in Freund's complete adjuvant-induced arthritic rat demonstrated a reduction in inflammation thereby reducing the pain (Dewangan et al., 2017). Similar anti-inflammatory results were observed using the rat model treated with 3 mg/kg emu-oil containing curcumin (Jeengar, Shrivastava, Veeravalli, Naidu & Sistla, 2016). A study by Kant et al. (2014) showed application of curcumin to wounds increased its contraction and suppressed the release of inflammatory cytokines such as TNF-α and IL-1β in diabetic rats. Moreover, curcumin enhanced IL-10 (an anti-inflammatory cytokine) and anti-oxidant enzymes (sodium oxide dismutase, catalase and glutathione peroxidase). Similar anti-inflammatory effects were observed in cardiopulmonary bypass (CPB) and cardiac global ischemia and reperfused rabbits, in which curcumin significantly suppressed the levels of IL-6, IL-8, IL-10, TNF-α along with inhibition of

NF-κB, cardiac troponin I, myocardial matrix metallopeptidase (MMP-9) and myleoperoxidase (MPO) activities (Yeh, Chen, Wu, Lin & Lin, 2005).

Several studies have been conducted to demonstrate and compare the bioactivity of curcuminoids. Most of these studies have demonstrated the superiority in bioactivity of curcumin when compared to the minor constituents (Ramsewak et al., 2000; Jayaprakasha et al., 2006; Sandur et al., 2007; Ahmed & Gilani, 2009). Anti-inflammatory effects of a demethoxycurcumin and curcuminoid mixture were analysed in an infused rat model (infused with amyloid-β and ibotenic acid) (Ahmed & Gilani, 2011). The results demonstrated a significant decline in the level of IL-1β by curcuminoids (from 373.99 ± 15.28% to 188.59 ± 10.07%) and demethoxycurcumin (from 373.99 ± 15.28% to 136.67 ± 31.96%) at a dose of 30 mg/kg (Ahmed & Gilani, 2011). This study indicated that demethoxycurcumin had a greater neuroprotective activity compared to the other two constituents. The bioactivity of the minor compound bisdemethoxycurcumin was studied in a pylorus-ligated rat model by Mahattanadul et al. (2009), with BDMC being demonstrated to be a superior inhibitor of inducible nitric oxide oxidase (iNOS) compared to curcumin. Furthermore, BDMC has been shown to be effective as a healing agent for acetic acid-induced chronic gastric ulcers (Mahattanadul et al., 2009). The various experiments indicate that all three curcuminoids possess different levels of bioactivity.

2.6.4 Anti-inflammatory effects of curcuminoids in humans

Clinical trials are the most appropriate way to test the efficacy and toxicity of a bioactive (Pincus, Bergman & Yazici, 2015). However, these studies suffer from several drawbacks such as such as participant withdrawal and high cost of trials (Pincus et al., 2015). Thus, there are only a limited number of reported clinical trials related to the anti-inflammatory activity of curcuminoids (as reviewed by Schaffer, Schaffer, Zidan & Sella, 2011).

The effectiveness of curcuminoids in treatment of inflammation have been demonstrated via phase II clinical trials. Based on published data, approximately 120 human studies have been successfully completed including some which are ongoing (Kunnumakkara et al., 2016). Panahi et al. (2015) conducted a randomized double-blind clinical trial on individuals diagnosed with metabolic syndrome. Oral administration of curcuminoid-piperine supplementation (80 mg- 6 g per day; for

8 weeks) led to a significant decrease in the levels of serum CRP due to the down-regulation of pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α. In a phase II clinical trial, curcuminoids (8 g/day for 8 weeks) effectively suppressed NF-κB and pSTAT3 (signal transducers and activators of transcription) activation in patients with advanced pancreatic cancer (Dhillon et al., 2008). Another human trial conducted to study the anti-inflammatory effect of curcuminoids in patients with colorectal cancer (He et al., 2011), demonstrated a significant decrease in TNF-α serum concentration and increase in cancer cell death. The anti-inflammatory effects of curcuminoids has also been observed in obese patients. Obese patients administered with NCB-02 (two capsules containing 150 mg curcumin) twice a day for 8 weeks (randomized, parallel-group and placebo-controlled trials), had significant decreases in the levels of the anti-inflammatory cytokines IL-6 and TNF-α (Usharani, Mateen, Naidu, Raju & Chandra, 2008).

2.7 Cytotoxicity of curcuminoids

2.7.1 Cytotoxicity of curcuminoids towards cell lines

Curcuminoids show variable toxicity towards different cell lines (Chang, Sun & Webster, 2014). In all cell lines, the toxicity increases with increased concentration and uptake of curcuminoids (Kunwar et al., 2008). Curcuminoids exhibit a selective toxicity against tumour and cancer cells (Romero-Hernández et al., 2013). In these cells, curcuminoids produce a cell death tumour and an antineoplastic effect resulting in inhibition of tumour growth. In addition, curcumin arrests the cell cycle, exercises anti-metastatic action and induces cell death by apoptosis, autophagy, mitotic catastrophe and endoplasmic reticulum stress (Romero-Hernández et al., 2013). Curcuminoids show different cytotoxicity to healthy and infected cells (Chang, Sun & Webster, 2014). This was demonstrated following the incubation of 10 μM curcumin with MG-63 osteosarcoma cells and healthy osteoblast cells for 24 h, where the cell viability of the osteosarcoma cells was less than 50% that of the control. In contrast, healthy osteoblasts showed a cell viability of 75% compared to control cells at curcumin doses of up to 25 μM (Chang, Sun & Webster, 2014). Furthermore, the cell death mechanism was different between healthy and tumour or cancerous cells (Romero-Hernández et al., 2013). Cell death of human astrocytoma cells incubated with 100 μM curcumin for 24 hours occurred due to accumulation of lucent vesicles in the cytoplasm, a process called methuosis (Romero-Hernández et al., 2013). Further, it was reported that incubation of curcumin

(15 μM for 24 hours) with ovarian cancer cells (SKOV3, MDAH2774 and PA1) significantly reduced cell viability by apoptosis. However, the same treatment did not affect the cell viability of normal cells (ovary surface epithelial cells and peripheral blood mononuclear cells) (Seo, Kim, Dhanasekaran, Tsang & Song, 2016).

2.7.2 Dose-dependent effects of curcuminoids in humans and animal models

Curcuminoids have already been subjected to extensive preclinical and clinical toxicology testing and have a favourable safety profile (Park, Amin, Chen & Shin, 2013). Humans can tolerate curcumin doses as high as 8-12 g/day with no adverse side effects (Buhrmann et al., 2011; Li et al., 2017). However, regular consumption of a higher dosage (above 2 g) over three months can occasionally cause diarrhoea or nausea (Li et al., 2017). In a phase 1 dose-escalation trial, 15 patients with advanced colorectal cancer were administered a daily dosage of 450 mg to 3.6 g curcuminoids for 4 months. Results showed that curcuminoids had no toxic effects on any patients except mild diarrhoea experienced by two patients at a high dose (3.6 g) (Sharma et al., 2004). In another study, 24 healthy volunteers were tested for tolerance to curcuminoids at 12 g (Lao et al., 2006). Each volunteer was administered a single oral dosage of 500 mg to 12 g daily. Results showed good tolerance to curcuminoids at high dosages of 12 g. The peak serum concentration after 4 g, 6 g and 8 g of curcuminoid oral dosage was found to be 0.51 ± 0.11 μM, 0.64 ± 0.06 μM and 1.77 ± 1.87 μM, respectively (Lao et al., 2006). The safety and tolerance of curcuminoids was also tested with 13 women suffering from cervical dysplasia (Gattoc et al., 2016). Intravaginal administration of 2 g curcuminoid gelatin capsules for 14 days was well-tolerated by patients, with no dose-limiting toxicities reported. However, some grade 1 adverse events such as genital pruritus, vaginal discharge, vaginal dryness, abnormal prothrombin and hypokalaemia occurred in some patients (Gattoc et al., 2017). Another study showed no toxic effects in the human body after administration of 8 g curcuminoids per day for 3 months (Cheng et al., 2001). Results of six healthy subjects tested for toxicity of formulated curcumin (theracurmin) at 210 mg showed significant increases in plasma curcuminoid levels (Kanai et al., 2012).

Several clinical trials have shown that a minimum dosage of 3.6g/ day is required in humans to achieve measurable plasma levels, with respect to the limit of detection of the assays used (Sharma, Gescher, & Steward, 2005; Anand, Kunnumakkara, Newman & Aggarwal 2007; Cui et al., 2009;).

However, there is a lack of substantial data that favour a dose-response relationship for any biomarker and the activity of curcumin (Sharma et al., 2005). A typical therapeutic curcumin dosage is 400-600 mg (three times) a day. However, some clinical studies on have reported side effects at curcumin and curcuminoids doses ranging from 180 mg to 12 g, when consumed for more than 3 months (Asher & Spelman, 2013).

2.8 Pharmacokinetics of curcuminoids

2.8.1 Metabolic pathway of curcuminoids in humans

Upon oral administration, curcumin undergoes degradation to form curcumin glucuronide and curcumin sulphate in the liver and intestinal tract (Anand et al., 2007) in two phases (Figure 2.10). Phase I involves reduction of four double bonds of curcumin to form dihydrocurcumin. Further bio-reduction leads to formation of tetrahydrocurcumin, hexahydrocurcumin, octahydrocurcumin and hexahydrocurcuminol. The reaction converting curcumin to tetrahydrocurcumin is catalysed by nicotinamide adenine dinucleotide phosphate (NADPH)-dependent curcumin/ dihydrocurcumin reductase (CurA) (Hassaninasab, Hashimoto, Tomita-Yokotani & Kobayashi, 2011). During phase II, curcumin and its metabolites are conjugated with a monoglucuronide, a monosulfate and a mixed sulphate/ glucuronide leading to formation of conjugated metabolites (Hassaninasab et al., 2011; Ghosh, Banerjee & Sil, 2015). (Figure 2.10). Phase II metabolites are usually detected in the plasma (Cuomo et al., 2011). The enzyme involved in formation of hexahydrocurcumin from sulphate metabolites is alcohol dehydrogenase (Ireson et al., 2001). Tetrahyrdrocurcumin and hexahydrocurcumin are major products of the metabolic pathway (Hassaninasab et al., 2011) (Figure 2.10). The two minor constituents demethoxycurcumin and bisdemethoxycurcumin undergo reductive metabolism similar to that of curcumin with major metabolites as hexahydrocurcumin glucuronide and hexahydrocurcumin sulphate (Metzler, Pfeiffer, Schulz & Dempe, 2013).

Figure 2.10 Metabolism of curcumin in human body

Source: Mahran, Hagras, Sun & Brenner (2017)

Approximately 75% of ingested curcuminoids that are not metabolised is excreted directly in faeces (Yadav, Sah, Jha, Sah & Shah, 2013). The rapid metabolism of curcuminoids in gastrointestinal tract followed by rapid systemic elimination is one of the main reasons for its poor bioavailability in humans (Prasad, Tyagi & Aggarwal, 2014; Ghosh et al., 2015). Transport proteins in the luminal membrane of enterocytes act on the conjugated metabolites and transfer them back to the intestinal lumen (Kocher, Schiborr, Behnam & Frank, 2015), thereby affecting the bioavailability of curcuminoids. Only a small amount of curcumin reaches target organs, while that remaining is metabolised in the liver and intestine (Prasad et al., 2014).

2.8.2 Absorption of curcuminoids

Curcuminoids are poorly absorbed in both humans and animals. An early study by Wahlström & Blennow (1978) demonstrated that about 75% of 1 g/kg orally administered curcumin was excreted in the rat faeces due to poor absorption in the gut. However, Ravindranath & Chandrasekhara (1980) reported that 40% of orally delivered curcumin (in arachis oil suspension) was excreted in faeces of male albino rats within 5 days. The small amount of curcumin absorbed within the intestine undergoes rapid degradation in the liver and plasma (Liu et al., 2016). This absorbed curcumin is in the form of glucuronide or sulphate conjugates (Vareed et al., 2008).

2.8.3 Improving bioavailability of curcuminoids

Therapeutic use of curcuminoids is limited due to its poor aqueous solubility, inherently low absorption from the gut, rapid metabolism, rapid systemic elimination and short biological half-life (Zhongfa et al., 2012; Jäger et al., 2014; He et al., 2015). These factors all contribute to the low bioavailability of curcuminoids. Upon oral administration, only 1% of curcuminoids are bioavailable in rats (Zhang, Tang, Xu & Li, 2013). Comparison amongst the curcuminoid constituents have indicated a higher bioavailability of bisdemethoxycurcumin compared to demethoxycurcumin and curcumin (Zhongfa et al., 2012). Over the years, researchers have developed several delivery systems to improve the bioavailability of curcuminoids (Figure 2.11). The delivery systems work by accelerating the rate and capacity of drug solubilisation into aqueous intestinal fluids (Maiti, Mukherjee, Ganitat, Saha & Mukherjee, 2007). Some of these approaches are discussed below.

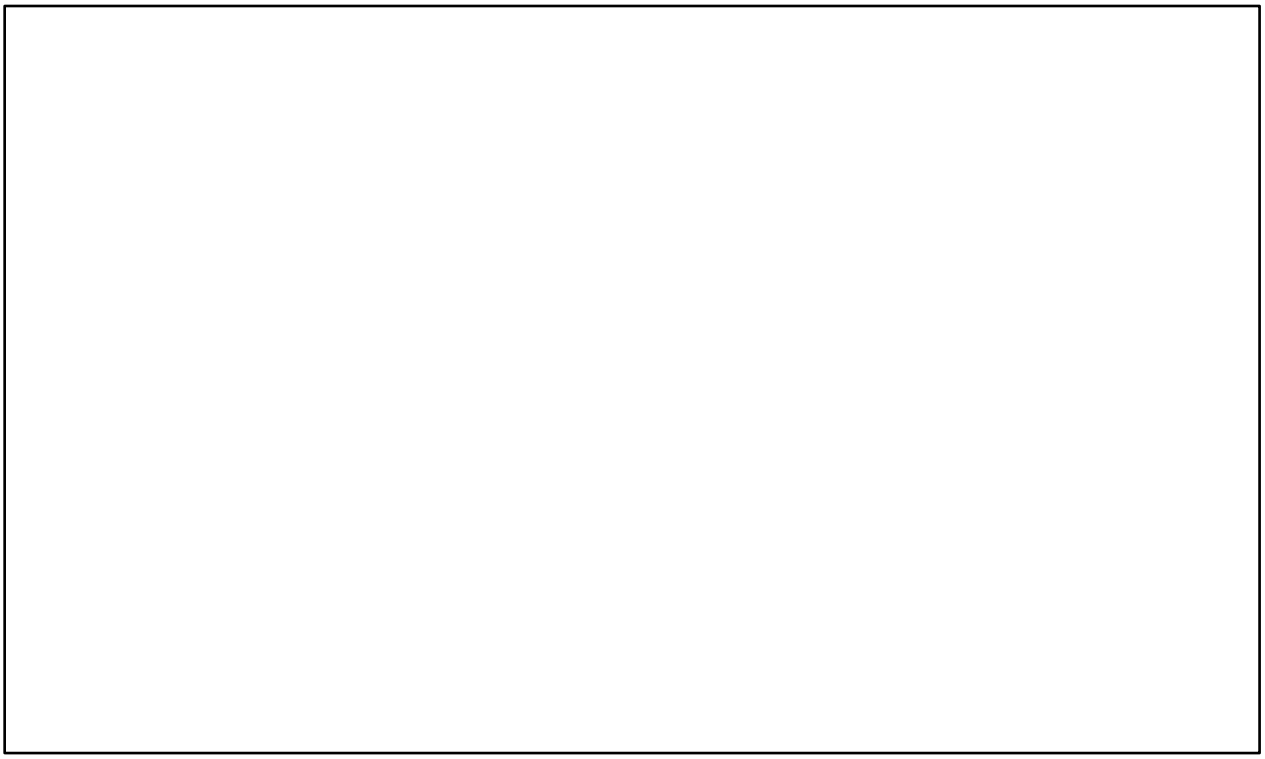


Figure 2.11 Potential delivery systems for improved bioavailability of curcumin

Source: Prasad, Tyagi & Aggarwal (2014)

Abbreviations: GMO, glyceryl monoleate; PGLA, polylactic-co-glycolic acid; PHEMA, poly (2-hydroxymethyl methacrylate); PEG-PEI, polyethylene glycol-poly (ethylene imine).

Curcuminoids combined with adjuvants

Naturally occurring compounds such as piperine, quercetin, resveratrol, fibre, dextrins, phospholipids and turmeric oils have been used to enhance the bioavailability of curcumin (Prasad et al., 2014; Mahran et al., 2017). Most of these compounds act synergistically with curcumin to enhance the overall therapeutic effects (Mahran et al., 2017). Use of adjuvants such as piperine (Moorthi, Krishnan, Manavalan & Kathiresan, 2012; Sehgal, Kumar & Dhawan, 2012; Shoba et al., 1998) and phospholipids (Maiti, Mukherjee, Ganitat, Saha & Mukherjee, 2007; Lin, Lin, Chen, Yu & Lee, 2009; Zhang et al., 2013) are a common strategy to improve the bioavailability of curcuminoids.

Piperine, a bioactive compound from black pepper can enhance serum concentrations and curcumin absorption in humans and rats with no adverse effects (Shoba et al., 1998). Concomitant administration of 20 mg/kg piperine with 2g/kg curcumin can increase the bioavailability by 154% in rats and by 2000% in humans (Shoba et al., 1998). Another compound, lecithin acts in synergy with curcumin-piperine to enhance the overall absorption and bioavailability of curcumin by 126-fold (Antony et al, 2008; Jäger et al., 2014). Various pharmaceutical grade encapsulated curcumin-piperine nanoparticles have been developed for sustained and efficient delivery of curcumin in humans (Moorthi et al., 2002). Piperine enhances the absorption of curcumin by suppressing the activity of glucuronidase enzymes, both at the site of the intestinal border and the liver. Further, an increase in intestinal perfusion and enterocyte permeability contribute to improved bioavailability of curcumin (Panahi et al., 2015). However, piperine is known to inhibit phase-1 and phase-2 xenobiotic enzymes, while curcumin inactivates various classes of cytochromes (including CPYP3A4) along with P-glycoprotein activity (Cuomo et al., 2011). In this scenario, the bioavailability of curcuminoids is improved at a cost of potential interaction of these bioactives with mainstream drugs (Cuomo et al., 2011).

Curcuminoids can form complexes with **phospholipids** via hydrogen bonding and dipole interactions with the polar heads of phosphatidylcholine (Cuomo et al., 2011). Phospholipids are small lipids with a hydrophobic fatty acid tail and a polar phosphate group (Semalty et al., 2010). Due to their amphipathic nature, phospholipids can enhance the water and lipid solubility of curcumin, thereby increasing its absorption and bioavailability (Liu et al., 2016). A dose of 200 mg/kg curcumin-phospholipid in rats was able to restore the toxic effects caused by carbon tetrachloride by two-fold compared to unformulated curcumin (Maiti et al., 2007). In another study, male wistar rats were administered a 340 mg/kg dose of either curcumin or curcumin-phosphatidylcholine (Mervia). The peak plasma level concentration was reported to be five-fold higher for rats administered the Mervia complex compared to curcumin (Marczylo et al., 2007). The Mervia formulation resulted in a 20-fold increase in absorption of curcumin (Marczylo et al., 2007). Curcumin-phospholipid complex has been reported to increase the absorption of curcumin 50% more efficiently compared to the curcumin-piperine combination (Jäger et al., 2014).

Nano-curcumin formulations

In the era of nanotechnology, scientists have developed various nanoformulations of curcumin. These include polymer nanoparticles, polymeric micelles, liposomes, micro-/ nanoemulsions, nanogels, polymer conjugates and self-assemblies (Yallapu et al., 2012; Prasad et al., 2014; Liu et al., 2016; Mahran et al., 2017). The main target of these formulations is to increase solubility and bioavailability of curcuminoids with a controlled release of the bioactives in the gastrointestinal tract (Naksuriya, Okonogi, Schiffelers & Hennink, 2014).

Liposomes are spherical bilayer vesicles which are amphipathic in nature, consisting of an external lipophilic phospholipid bilayer and an internal aqueous core (Jin et al., 2016). They are commonly used in the food industry to encapsulate nutraceuticals, antimicrobials, flavours and colorants (Jin, et al., 2016). Curcuminoids encapsulated in phosphatidylcholine vesicles down-regulated the activity of inflammatory biomarkers in RAW 264.7 cells by two- to six-fold compared to unformulated curcuminoids (Basnet, Hussain, Tho & Skalko-Basnet, 2011). Liposomal curcumin prepared using milk fat globule membrane (MGFM) phospholipids are more stable with a higher encapsulation capacity as compared to traditional soybean lecithin liposomes (Jin et al., 2016).

Nanoemulsions are highly stable systems with droplet sizes ranging from 1-100 μm (Wang et al., 2008). Due to their small particle size, curcumin-loaded nanoemulsions can resist phase I degradation in the liver (Zhongfa et al., 2012), resulting in increased absorption of the curcuminoids. A curcuminoid-loaded nanoemulsion in polyethylene glycol increased absorption (10-fold) and serum peak concentrations (40-fold) in mice compared to unformulated curcuminoids (Zhongfa et al., 2012). In female CD-1 mice, curcumin organogel in oil phase as a nanoemulsion increased the bioavailability of curcumin by 9-fold compared to unformulated curcumin (Yu & Huang, 2012). Combinations of two or more delivery systems, such as microemulsions and piperine combined with curcumin has resulted in higher bioavailability, increased absorption and higher solubility of curcuminoids (Zhang et al., 2013). A curcumin nanoemulsion with a droplet size of 30 nm was prepared using phosphatidylcholine enriched with medium chain fatty acids and glycerol as surfactants in the oil and water phases (Ochoa-Flores et al., 2017). Delivery of curcuminoids in this nanosystem reported a significantly higher curcumin

concentration in the liver and lungs compared to an unmodified phosphatidylcholine nanoemulsion or unformulated curcumin (Ochoa-Flores et al., 2017).

A novel formulation, developed by coating curcumin nanoparticles (SLCNs) on a cationic polysaccharide, *N*-trimethyl chitosan (TMC) was tested for bioavailability and drug release capability (Ramalingam & Ko, 2015). Evaluation of these nanoparticles under simulated gastrointestinal conditions showed a resistance to an acidic environment and controlled release of curcumin in the intestinal tract. Further, an increase in bioavailability by 23-foldwas reported in formulated curcumin as compared to native curcumin (Ramalingam & Ko, 2015). The mechanism by which the formulated system increases the bioavailability of curcumin is illustrated in Figure 2.12. Comparison of three food-grade curcuminoid containing nanoparticles in an *in vitro* gastrointestinal model indicated a higher bioaccessibility in lipid nanoparticles (92%) compared to phospholipid (74%) and protein (52%) nanoparticles (Zou et al., 2016).

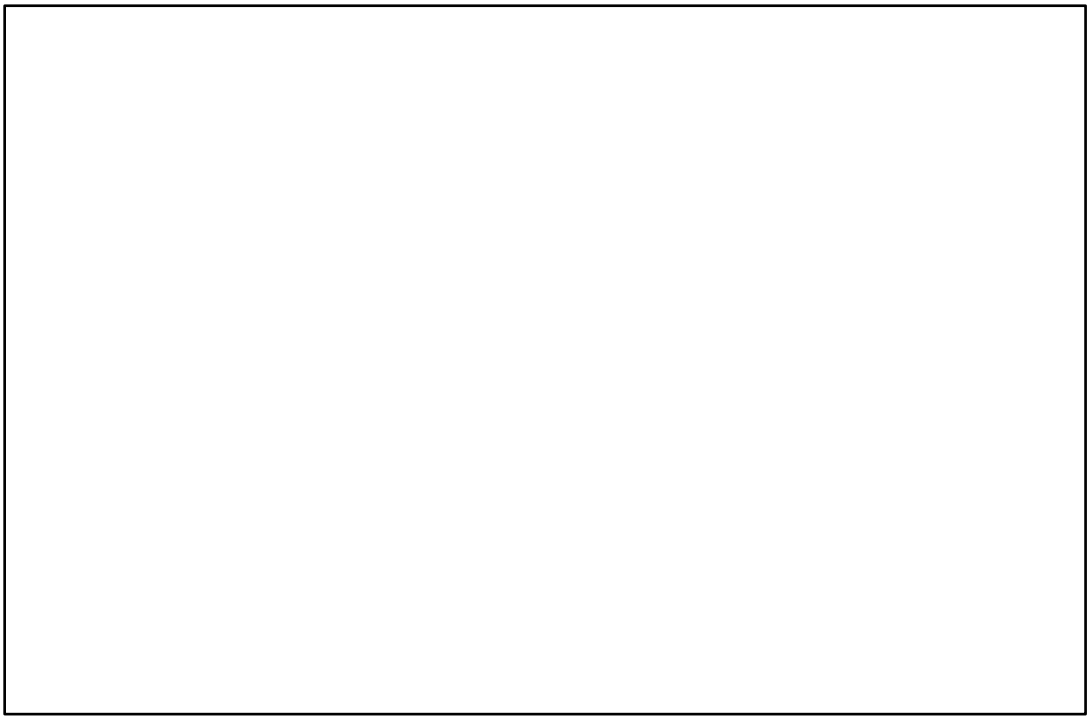


Figure 2.12 Sustained drug delivery of curcuminoids loaded chitosan nanoparticles
Source: Mohammed, Syeda, Wasan & Wasan (2017)

Microemulsions are stable solutions comprising of surfactant(s), oil and water (Yallapu, Jaggi & Chauhan, 2012). Emulsification is a simple process that can be produced by blending or homogenising various food-based ingredients (Pinheiro, Coimbra & Vicente, 2016). This delivery system protects curcuminoids from degradation, thereby increasing the bioavailability of these bioactives (Pinheiro et al., 2016). Microemulsions prepared using curcumin and food-grade ingredients such as soybean lecithin as a surfactant and soybean oil in the oil phase were effective in causing apoptosis of HepG2 cancerous cell lines (Lin et al., 2014). Further modification of the microsystem has led to development of a **self-microemulsifying drug delivery system (SEMDDS)**, composed of a mixture of curcuminoids, oil, surfactant and co-surfactant, produced following gentle mixing of these ingredients in aqueous media (Wu, Xu, Huang & Wen, 2011). With a mean droplet size of less than 100 nm, the capsules are able to protect curcumin against mucus interactions and allow high permeability into the membranes (Köllner et al., 2017).

More than 100 different types of systems for the delivery of curcuminoids have been developed by scientists (Mahran et al., 2017). Experimental trials conducted using these formulations involve the comparison formulated curcumin with the unformulated curcumin (Liu et al., 2016). Some of the parameters tested showed increase in bioavailability are increase in absorption (area under plasma concentration-time curve) (Schiborr et al., 2014), measurement of peak plasma and serum concentration (Marczylo et al., 2007; Zhongfa et al., 2012), increased solubility (Shaikh, Ankola, Beniwal, Singh & Kumar, 2009) and increased half-life of curcuminoids (Wan, Sun, Qi & Tan, 2012). Most of the formulations are pharmaceutical grade and it is not feasible to use encapsulated curcuminoids in food systems. Thus, there is a need to develop a cost-effective food matrix for delivery of curcuminoids.

2.9 Availability of curcuminoids to consumers

Turmeric as a traditional medicine is accessible to modern consumers due to its usage into modern medicines and others commercial products (Corson & Crews, 2007). Various commercial products containing curcuminoids/ turmeric include dietary supplements, colouring agents, beauty and medicated creams, dairy, oils, drinks, extracts, gels and nasal sprays (Yallapu, Jaggi & Chauhan, 2012; He et al., 2015).

Dietary supplements

According to Cassandra (2016), turmeric/curcumin dietary supplements were one of the top 10 best-selling supplements in the United States worth over $ 100 million in 2012 The sales reached nearly USD 200 million in 2015 and an accelerated growth in revenue is projected in the future (Kumar, 2016). A wide range of dietary supplements are available on the market which can be categorised as turmeric supplements, curcuminoid supplements and special curcuminoid formulations (*"Choosing"*, n.d.). Several *in vitro* and *in vivo* studies have been conducted to analyse the bioactivity of the commercial products (Table 2.2).

Table 2.2 Commercially available curcumin dietary supplements

Category of supplements	Formulation name	Composition	Applications	Scientific studies	Commercially available products
Turmeric	No name	Approximately 5% curcuminoids	Maintain overall health and reduce inflammation in body	Local inactivation of NF-κB and inhibition of chemokines and COX-2 in female Lewis rats (Funk et al., 2006).	Botanic Choice™ capsules, Starwest Botanicals™ turmeric root powder and Simply Organic™ powder
Curcuminoids	Sabsina C[3] complex	Approximately 95% curcuminoids (curcumin, 75-81%; DMC, 15-19%; BDMC, 2.2- 6.5%)	Maintain overall health and reduce inflammation in body	Inhibition of NF-κB and STAT-3 activation in humans with advanced pancreatic cancer (Dhillon et al., 2008)	Micro Ingredients™ Organic curcumin, Jarrow Formulas™ Curcumin 95
Special curcumin formulations	Mervia®	Curcumin (20%), hydroxypropyl methylcellulose and calcium citrate laurate, hypromellose capsule, leucine, silicon dioxide, microcrystalline cellulose and phytosome	Treatment of inflammatory diseases such as osteoarthritis, uveitis, rheumatoid arthritis, inflammatory bowel diseases	In a randomized, placebo-controlled, single blind pilot trial, Mervia® supplementation reduced inflammation in muscles (Drobnic et al., 2014).	Source Naturals™ Turmeric with Mervia®, Throne Research™ Mervia-SF®, GO® Healthy Mervia® Curcumin
	Longvida®	Curcumin (20%), Plant derived cellulose, soy lecithin, stearic acid, dextrin, ascorbyl palmitate, silicon dioxide and microcrystalline cellulose	Potentially in brain diseases such as Alzheimer's	Down-regulation of IL-6, NO and PGE_2; Inactivation of NF-κB in LPS-stimulated RAW 264.7 cultured murine macrophages (Nahar, Slitt & Seeram, 2015).	Nutrivene Longvida™, Blackmores™ Brain active, Vbyotics™ Longvida curcuma capsules, Now Foods™ Curubrain capsules
	BCM 95®	Curcuminoids (86%) and essential oils (7-9%)	Inflammatory conditions, gastroprotective, beneficial for type-II diabetic patients	An increased bioavailability of curcumin in BCM-95® by 6.3-fold as compared to pure curcumin as tested in blood samples of 11 healthy volunteers (Antony et al., 2008)	Life Extension™ Super Biocurcumin, Terry™ Naturally CuraMed, Progressive labs™ BCM-95® and Curcu Gel Ultra
	Theracurcumin®	Curcumin (10%)	solubilizing properties	A dose of 210 mg/ day administered to six healthy volunteers resulted in increase in peak plasma concentrations without any adverse effects (kanai et al., 2012)	Swanson™ High Absorption Theracumin, Source Naturals™ capsules, Natural Factors™ Curmin Rich
	Curcumin C3 complex® with BioPerine®	Curcuminoids plus black pepper standardized extract *(Piper nigrum)*	Improve bioavailability and reduce inflammation in body	Concomitant administration of 20 mg/kg piperine with 2g/kg curcumin can increase in bioavailability by 154% in rats and by 2000% in humans (Shoba et al., 1998).	Doctor Danille™ curcumin with Bioperine®, Schwartz Bioresearch™, VitaBreeze™ capsules

Source: *Choosing* , *n.d.*; Ullah et al., 2017

Food products

Food companies have made considerable efforts to incorporate turmeric into beverages, semi-solid and solid foods. Turmeric has been added to beverages such as juices, milk, raw vinegars, cider, probiotic drinks, sparkling water, and latte. Some foods containing added turmeric include cheese, bread, yogurt and chewing gums.

Temple Turmeric™, a U.S.-based company invented a range of beverages using Hawaiian Oana turmeric as the core ingredient (Temple Turmeric, n.d.). The company states that every bottle of Temple turmeric contains 5 mg to 13 mg of whole root, organic Hawaiian Oana turmeric, turmeric juice, and adaptogenic ingredients such as black pepper, ginger and plant-based fats. Some of their more popular products include Turmeric Original Elixir, Turmeric- Ginger- Aid Elixir, Pure Fire Tonic apple cider drink and detox shots (Temple Turmeric, n.d.). Another company, Arya™ has developed various products using curcumin, including curcumin-infused sparkling water, energy drinks and chewable gummies. Arya™ chewable gums, commercially available as 'Curcumin on the go', contains 10 mg of curcumin per serving (*"curcumin,"* 2016). NutraOrganics™ golden latte and turmeric beef bone-broth have added diversity in the market to the already available turmeric products. Another interesting product in the food range is Organic Traditions™ probiotic fibre blend containing turmeric. Foods such as bread and cheese have also been fortified with turmeric and are sold by Paleo™. Raglan™ has a wide range of yogurt products which include products containing mango, turmeric and coconut milk. Most of these food products are fortified with turmeric, which contains only 0.58 to 6.5% curcuminoids (Li et al., 2011). However, for many of these products the amount of curcuminoids remaining following food processing has not been specified.

2.10 Delivery of curcuminoids in food products

Multi-functional curcuminoids have recently gained tremendous attention in the food industry (Jitoe-Masuda, Fujimoto & Masuda, 2012). Thus, food companies are developing new techniques to incorporate this bioactive into food products. Development of suitable delivery systems depends on factors such as the ability of the food matrix to disperse bioactives, prevent degradation of

bioactives and minimising the effect of added curcuminoids on sensory, rheological or textural properties on food system (Sagalowicz & Leser, 2010).

2.10.1 Effect of curcuminoids on the functional and sensory properties of food products

In recent years, researchers have demonstrated the application of curcuminoids in various food products such as cheese, bread and yogurt. "Karishcum" is an Egyptian product prepared by adding 0.3% (w/v) curcuminoids into karish cheese (Hosny, El Kholy, Murad & El Dairouty, 2011), to investigate the anti-microbial activity of curcuminoids. Karish cheese without curcuminoids was found to be contaminated with coliform, *Escherichia coli, Pseudomonas, Staphylococcus aureus* and *Listeria monocytes,* with the counts as high as 10^3 cfu/g of each pathogen (Hosny et al., 2011). In contrast, cheese supplemented with curcuminoids had not growth of pathogens. A novel cottage cheese was formulated with milk and turmeric powder (Kishor & David, 2016). A high overall sensory acceptability score (7.80 using a 9-point hedonic scale) was reported for the cottage cheese made using 80% skim milk, 20% chick pea milk, 0.5% turmeric powder and 0.2% black pepper (Kishor & David, 2016). Similar consumer acceptance results were obtained in Ras cheese formulated with 0.5% turmeric (El-Shazly, El-Tawil & Hammam, 2011). However, the stability of curcuminoids in cheese and the effect of curcuminoids on functional properties of cheese is unknown.

The addition of free or encapsulated curcuminoids (1%) in staple foods such as bread has been reported (Vitaglione et al., 2012). Consumption of bread containing encapsulated curcuminoids increased the bioavailability of curcuminoids significantly compared to bread enriched with unformulated curcuminoids (Vitaglione et al., 2012). In a novel formulation, wheat flour was replaced by turmeric in concentrations ranging from 0-8% (Lim, Park, Ghaffor, Hwang & Park, 2011). Sensory evaluation of the bread indicated that substitution of turmeric up to 4% was acceptable by the consumers (Lim et al., 2011). In another study, bread dough was enriched with 0.10-0.30% turmeric powder, essential oils and turmeric residues (Sikkhamondhol, Teanpook, Boonbumrung & Chittrepol, 2009). Proximate analysis of that bread showed it contained 28.52% moisture, 56.91% carbohydrate, 10.53% protein, 1.79% fat, 0.47% fibre and 1.74% ash

(Sikkhamondhol et al., 2009). However, the stability of curcuminoids in bread and the effect of curcuminoids on functional properties of bread is unknown.

Curcuminoids can be incorporated into food emulsions such as milk, yogurt drinks, sauces or mayonnaise (Sagalowica & Leser, 2010). Fortification of yogurt with turmeric has significant effects on the chemical, rheological and sensory properties of the yoghurt (Foda, El-Aziz & Awad, 2007). Foda et al. (2007) reported that addition of turmeric (0.1-1% w/w) in yogurt does not have any significant effect on the acidity of yogurt. Further, addition of turmeric increases the gel firmness and decreases the rate of syneresis in yogurt in concentration dependent manner (Foda et al., 2007). Similar findings were reported by Fu et al. (2016), where addition of curcuminoids (0.3% w/w) increased the viscosity of yogurt during storage. However, addition of curcuminoids have been reported to impact the yogurt microflora. Addition of curcuminoids (0.3% w/w) to yogurt mix resulted in lower counts of total lactic acid bacteria count by <1 log (Fu et al., 2016) which may be due to the effect of curcuminoids on the protease activity of *L. bulgaricus* (Sánchez-Vega, 2013). In terms of consumer acceptance, yogurt containing 0.1% turmeric was highly favoured by the panellists (Foda et al., 2007). Delivery of curcuminoids in buttermilk yogurt increased the bioaccessibility of curcuminoids by 15-fold compared to aqueous curcuminoids (Fu et al., 2016). A study by Gutierres et al (2012) reported that yogurt supplemented with curcumin exhibited anti-diabetic activity in rats. This provided an evidence that the bioactivity of curcumin is not lost on treatment with yogurt. From previous studies, it can be hypothesised that yogurt may be a potential matrix for the delivery of curcuminoids.

2.10.2 Delivery of curcuminoids in yogurt

Yogurt is among one of the most popular fermented milk products in the world (Weerathilake, Rasika, Ruwanmali & Munasinghe, 2014). Yogurt has been known as a carrier for probiotics such as *Lactobacillus acidophilus* and *Bifidobacterium* (Ejtahed et al., 2011), prebiotic fibres (Allgeyer, Miller & Lee, 2010) and bioactive polyphenols (Petrotos et al., 2012). Fermentation of milk occurs due to conversion of milk sugars into lactic acid by the action of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Brabandere & Baerdemaekaer, 1999). The acidification process reduces the pH of milk to 4.6-4.5, which causes the agglomeration of casein micelles (Brabandere &

Baerdemaeker, 1999; Haque, Richardson & Morris, 2001). The acidic environment of yogurt is favourable for curcuminoids as they undergo minimum degradation at acidic and neutral pH (Peram et al., 2017).

The principal constituents of milk are water, milk sugar, proteins, fat and minerals (Huppertz, Kelly & Fox, 2002). Curcuminoids interact with proteins, fat and sugars through hydrophobic interactions and hydrogen bonding (Jakobek, 2015). The interaction of curcuminoids with milk proteins and lipids has been widely investigated. Bovine milk proteins (80% casein and 20% whey) can serve as natural carriers of hydrophobic bioactives with an ability to release these substances in the human body (Kimpel & Schmitt, 2015). Curcuminoids bind to caseins, while whey increases their binding affinity (Yazdi & Corredig, 2012). Structural analysis demonstrated that curcuminoids bind casein via hydrophobic interactions mostly with overall binding constant of 2.8 ($\pm$0.8) x 10^4 M^{-1} and 3.1 ($\pm$0.5) x 10^4 M^{-1} for α- and β-caseins respectively (Bourassa, Bariyanga & Tajmir-Riahi, 2013). The binding capacity of caseins can be further increased by heat treatment (80 ºC for 10 minutes) (Yazdi & Corredig, 2012) or high hydrostatic pressure treatment of milk (Yazdi et al., 2013). Curcuminoids can also interact with β-lactoglobulin, the major constituent of whey protein (Sneharani, Karakkat, Singh & Rao, 2010), with the resulting complex having a higher aqueous solubility (approximately 625 μM), higher stability (half-life of 206 minutes) and delayed degradation compared to aqueous curcumin (Sneharani et al., 2010). Curcumin loaded in β-lactoglobulin is resistant to pepsin, which prevents the release of curcumin in stomach. However, the complex is sensitive to trypsin, and this leads to higher permeability and higher absorption in the intestinal tract (Li, Cui, Ngadi & Ma, 2015). Furthermore, this interaction enhances the antioxidant capability of curcuminoids (Li, Ma & Ngadi, 2013).

Bovine milk constitutes approximately 3.5-5% total lipid, existing in globules as an oil-in-water emulsion (Jensen, Ferris & Lammi-Keefe, 1991). These lipid globules are 2-4 μm in diameter and are coated with a novel biophysical membrane called milk fat globule membrane (MGFM) (Jensen et al., 1991; Singh, 2006). MGFM consists of 70% enzymes, 25% phospholipids, 3% cerebrosides and 2% cholesterol (Månsson, 2008). The interaction of curcuminoids with lipids has been discussed in Section 2.6.3. Lipids have the ability to capture bioactives (Jakobek, 2015), thereby enhancing the absorption and bioavailability of curcuminoids (Fricker et al., 2010). Thus,

curcuminoids may show positive interaction with milk lipids and proteins, thereby resulting in increased stability of the curcuminoids. Overall, yogurt may be a potential carrier for delivery of curcuminoids.

CHAPTER 3
MATERIALS AND METHODS

3.1 Introduction

The main aim of the current study was to demonstrate the application of yogurt as a potential carrier of curcuminoids. The experimental research was divided into three integrated phases, with each phase serving definitive objectives. **Phase I** involved screening of commercial food-grade curcuminoid samples. This phase was designed as a two-factor experimental design aimed at finding the most suitable curcuminoid ingredient and a suitable yogurt base for future experiments. **Phase II** comprised of *in vitro* analysis aimed at determining the anti-inflammatory activity of two food-grade curcuminoids, C7 and C9 (selected from phase I) and compare their bioactivity with analytical grade curcumin (≥98%). The food-grade curcuminoids possessing the highest level of anti-inflammatory activity would be selected for incorporation into yogurt to be prepared in phase III. **Phase III** aimed to develop a new product: a coconut yogurt supplemented with curcumin/ curcuminoid ingredients (C7 and C9). In this phase, the effect of curcuminoids on fermentation of coconut cream and properties of yogurt such as acidity, microflora, texture, rheology and sensory characteristics during 15 days' shelf life period was determined. Furthermore, the stability of curcuminoids in coconut yogurt during refrigerated storage for 15 days was determined by HPLC-DAD.

3.2 Phase I: Screening of curcuminoids and yogurt products

Ten curcuminoid powders were obtained from different commercial suppliers and each of these were added to commercial CMY, CCY or GMY at a concentration of 0.4% (w/w). pH was measured (in duplicate) just after mixing the curcuminoids with yogurt and thereafter at 2 h, 6 h and 24 h storage at 4˚C. The samples were also subjected to round table sensory evaluation after 24 h of storage. The results of this phase to determine the suitable curcuminoid ingredient and yogurt were based on chemical (pH) and sensory characteristics.

3.2.1 Raw materials

Curcuminoid ingredients used in this study were obtained from different suppliers as shown in Table 3.1.

Table 3.1 List of food-grade curcuminoid raw ingredients used in this study and their suppliers

Sample code	Sample composition	Brand name	Batch no./ Product code	Supplier
C1	> 95% curcumin	Sanat Products Ltd.	186314	RMF Nutraceuticals
C2	> 95% curcumin	Apollo Ingredients India Pvt. Ltd.	CLRE9510110514	RMF Nutraceuticals
C3	> 95% curcuminoids (75-81% curcumin, 15-19% demethoxycurcumin, 2.2- 6.5% bisdemethoxycurcumin)	Sami Labs Ltd.	0330AU	RMF Nutraceuticals
C4	Approximately 95% curcumin	Changsha Vigorous-Tech Co. Ltd.	Not specified	RMF Nutraceuticals
C5	> 95% curcuminoids	Xi'an Lukee Bio-Tech. Co. Ltd.	LK20170108	RMF Nutraceuticals
C6	> 95% curcumin	Xiamen Boten Biological Technology Co. Ltd.	BTTRE161101	RMF Nutraceuticals
C7	> 95% curcumin	Xi'an Guanyu Bio-technology Co. Ltd.	CU170117	RMF Nutraceuticals
C8	> 12.5% curcuminoids, more than or equal to 10% tumerosaccharides, 0-2% silica colloidal anhydrous, 1% rhizome essential oil	Network Nutrition™	NNCL1812.5	IMCD New Zealand Ltd.
C9	65% curcuminoids, 20% maltodextrin	Medipro botanical™	CUR150190	RMF Nutraceuticals
C10	Approximately 18% curcuminoids, approximately 80% γ-cyclodextrin	Wacker Chemie AG™	N49020417	IMCD New Zealand Ltd.

Commercial yogurts (Cathedral Cove Naturals™ coconut cream yogurt, Naturalea™ cow's milk unsweetened yogurt and Naturally organic™ bio goat's milk yogurt) used in this experimental phase were obtained from local supermarkets (Naturally Organic and Pak n Save, Albany, Auckland, NZ).

3.2.2 Preparation of curcuminoid-yogurt samples

200 g of commercial yogurt was weighed out into a kitchen mixer bowl and 800 mg of curcumin was added. The contents were mixed using a bowl-lift stand mixer (KitchenAid®, 5K5SSAWH, USA) for 1 minute at minimum speed (illustrated in Figure 3.1). Excessive or high-speed mixing results in churning of yogurt and thereby affecting the intrinsic textural properties (Vélez-Ruiz, Cánovas & Peleg, 1997). Thereafter, the contents were transferred into 120 mL sterile plastic containers with screwtop lids (LabServ, NZ) and stored at 4 ˚C.

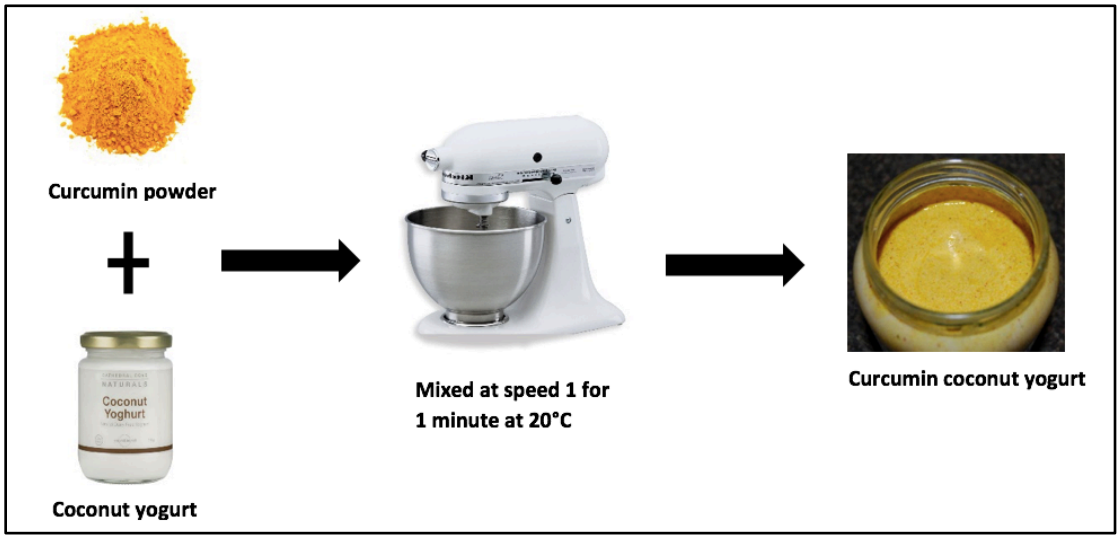


Figure 3.1. Mixing of curcumin (0.4%, w/w) ingredients with coconut yogurt in Phase I

3.2.3 Measurement of pH

The pH of curcuminoid yogurt samples was measured in duplicate, following the AOAC method 981.12 (AOAC, 2005). A bench-top pH meter (Sartorius® PB-11, Germany) equipped with a glass electrode was calibrated using Cetripur® standard buffers at pH 4 and 7 (Sigma-Aldrich®, NZ). pH was measured by dipping the electrode into 30 g of curcuminoid yogurt sample until a stable reading was obtained. After each measurement, the pH electrode was rinsed with distilled water.

3.2.4 Sensory evaluation

A round-table sensory evaluation method was used to evaluate attributes (appearance, colour, taste and overall acceptability) of 30 different yogurt samples (Salvador & Fiszman, 2004; Allgeyer, Miller & Lee, 2010; Galán-Soldevilla, Pérez-Cacho & Campuzano, 2013). A panel of 5 experienced panellists who consumed yogurt on regular basis were recruited for this study. Each panellist was served 10 g of chilled yogurt (4 ˚C) in a 25 mL transparent plastic cup (coded with 3-digit random number) and were asked to describe the product in terms of appearance, colour, taste and overall acceptability. Prior to tasting of each sample, the panellists were required to rinse their mouth with reverse osmosis (RO) water to remove the after taste of the previous sample. The views of each panellist and rating of products were manually recorded.

3.2.5 Data analysis

All values were expressed as mean ± SD (standard deviation) as calculated in Microsoft Excel 2016. Data was statistically analysed by using procedures of Minitab 18 software (Minitab Inc., State College, PA, USA). The data was tested for normality using general linear model and the data with normally distributed residues was further analyzed using ANOVA. One-way ANOVA (Analysis of Variance) was used to determine the effect of curcuminoids on the pH of yogurts at α=0.05.

3.3. Phase II: *In vitro analysis* for determination of bioactivity of curcuminoids

A human monocytic acute leukaemia cell line, THP-1 was used in this study to test the bioactivity of curcuminoids. THP-1 cells were cultured in complete RPMI 1640 medium and differentiated into macrophages by treating the cells with 200 nM Phorbol 12-myristate 13-acetate (PMA) for 72 hours. Cells were maintained at 37 ˚C in 5% CO_2 humidified incubator at all times. Differentiated macrophages were stimulated with an endotoxin called LPS at doses varying from 0-1000 ng/mL. Dosage and incubation period of macrophages with LPS was optimized by TNF-α ELISA. Further, different curcumin (0-50 μM) dissolved in DMSO were tested for their ability to supress the levels of TNF-α produced by stimulated macrophages, as measured in cell supernatant using ELISA. Simultaneously, the cytotoxicity of curcumin on differentiated macrophages was assessed by MTT colorimetric assay. Prior to testing the effect of curcumin on cell viability, various experimental trials were performed to optimize the cell number per well and incubation time period for MTT

assay. A standard procedure, as demonstrated in section 3.3.2.4 was followed. Lastly, this study investigated the significant of DMSO on the cell viability and TNF-α production. All the experiments were performed thrice in triplicates and the data was statistically analysed using one-way ANOVA and Tukey's multiple comparison test in Minitab 18 software (Minitab Inc., State College, PA, USA).

3.3.1 Materials

3.3.1.1 Chemicals and reagents

All the chemicals and reagents used in this study were of analytical grade (Table 3.2) except the curcuminoid samples which were food-grade. Samples coded as C7 and C9 in phase I (Table 3.1) were used in phase II experiments.

3.2 List of chemicals and reagents used in phase II experiments

Product	**Catalogue #**	**Supplier**
Curcumin (≥98%)	08511	Sigma-Aldrich®, NZ
Gibco™ RPMI 1640 (GlutaMAX™ supplement, HEPES)	72400120	ThermoFisher Scientific™, NZ
Gibco™ Fetal Bovine Serum (FBS), NZ origin	10091148	ThermoFisher Scientific™, NZ
Gibco™ Penicillin/ Streptomycin (5000 U/mL)	15070063	ThermoFisher Scientific™, NZ
Phorbol 12-myristate 13-acetate (PMA) (≥99%)	P1585	Sigma-Aldrich®, NZ
Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 (1 mg/mL; 0.2 µm filtered)	L5293	Sigma-Aldrich®, NZ
Invitrogen™ MTT (3-(4,5- Dimethylthiazol-2-yl)-2,5- Dipehnyltetrazolium Bromide)	M6494	ThermoFisher Scientific™, NZ
Gibco™ RPMI 1640 medium, no phenol red	11835030	ThermoFisher Scientific™, NZ
Fluka® Analytical 0.4% trypan blue solution	93595	Sigma-Aldrich®, NZ

3.3.1.2 Cell line

THP-1 cells (ATCC®; American Tissue Culture Collection, TIB-202, isolated from the peripheral blood of a 1-year-old human male infant with acute monocytic leukemia) were purchased from Sigma-Aldrich®, NZ (Catalogue # 88081201, passage # 21).

3.3.1.3 Solutions and media

Complete RPMI cell culture medium

Complete RPMI cell culture medium was aseptically prepared by supplementing Gibco™ RPMI 1640 (GlutaMAX™ supplement, HEPES) with 10% Gibco™ Fetal Bovine Serum (NZ origin) and 1% antibiotics (Gibco™ Penicillin/ Streptomycin). Prior to mixing, all reagents were warmed to room temperature (20 ˚C) and complete RPMI medium was stored at 4 ˚C.

Phorbol 12-myristate 13 acetate (PMA) stock solution

A stock solution at 1 mM was prepared by dissolving 1 mg of PMA into 1.62 mL of DMSO. The solution was aliquoted into 500 µL Eppendorf tubes and stored at -20 ˚C.

Lipopolysaccharide (LPS) stock solution

A stock solution of LPS at 0.1 mg/mL was prepared by diluting 1 mg/mL LPS by ten-fold in complete RPMI medium. The solution was aliquoted into 500 µL Eppendorf tubes and stored at -20 ˚C until use.

Curcumin stock solution

A stock solution of curcumin (analytical as well as food-grade) at 10 mM was prepared by dissolving 10 mg powdered curcumin in 2.72 mL DMSO. The solution was stored at -20 ˚C until use.

Phosphate buffered saline (PBS)

To prepare PBS, 80 g sodium chloride, 2 g potassium chloride, 14.4 g disodium hydrogen phosphate and 2.4 g potassium dihydrogen phosphate were weighed out and added to milli-Q water to a final volume up to ten litres. pH of the PBS buffer was 7.4; measured using a bench-top pH

meter (Sartorius® PB-11, Germany) (as described in Section 3.2.3). The buffer was stored at room temperature.

Coating buffer (1x)

Coating buffer for ELISA was prepared according to manufacturer's instructions (BioLegend®, USA). For coating one plate (96-wells), 2.4 mL of coating buffer (5x) was diluted with 9.6 mL of milli-Q water.

Capture antibody solution

As per manufacturer's instructions (BioLegend®, USA), 60 µL of capture antibody (200x) was diluted with 12 mL of 1x coating buffer.

Assay diluent A (1x)

To prepare 1x assay diluent, 12 mL of 5x assay diluent A was dissolved in 48 mL PBS buffer (BioLegend®, USA).

Detection antibody solution

As per manufacturer's instructions (BioLegend®, USA), 60 µL of detection antibody (200x) was pipetted out and diluted with 12 mL of 1x assay diluent A.

Avidin-HRP solution

As per manufacturer's instructions (BioLegend®, USA), 12 µL of avidin-HRP antibody (1000x) was diluted with 12 mL of 1x assay diluent A.

ELISA standard reconstitution

According to manufacturer's protocol (BioLegend®, USA), a standard stock solution (55 ng/mL) was prepared by dissolving 11 ng lyophilized human TNF-α standard in 0.2 mL of 1x assay diluent A. Ten microliters of this stock solution was diluted with 990 µL of 1x assay diluent A to obtain a top standard at 500 pg/mL. A range of standards (7.8125- 500 pg/mL) were prepared by performing six two-fold serial dilutions (in 1x assay diluent A) using the top standard using. The 1x assay diluent A was used as the blank.

3,3',5,5'- Tetramethylbenzidine (TMB) substrate solution

TMB substrate solution was prepared by mixing substrate A and substrate B in equimolar volumes. For 1 plate, 5.5 mL of substrate A and 5.5 mL of substrate B were mixed in a 15 mL falcon™ tube (Fisher Scientific, NZ) covered with aluminium foil (BioLegend®, USA).

MTT stock solution

Five milligrams of MTT was weighed and dissolved in 1 mL of Gibco™ RPMI 1640 medium without phenol red. The solution was filtered through a 0.2 µm Whatman® disposable syringe filter (Sigma-Aldrich®, NZ) and stored at 4˚C.

MTT working solution

MTT working solution was prepared by diluting the MTT stock solution in Gibco™ RPMI 1640 medium without phenol red by 1:10.

Acidified isopropanol

Acidified isopropanol is a solution of 0.04 M HCl in absolute isopropanol (≥99.9%) (Sigma-Aldrich®, NZ). To prepare 200 mL of acidified isopropanol, 787 µL of 32% HCl (w/w) was added to 199 mL absolute isopropanol. The solution was stored at room temperature.

3.3.2 Methods

3.3.2.1 Culturing THP-1 cells

Upon arrival, the cryovial containing THP-1 cells was stored at -80 ˚C. To thaw cells, the cryovial was warmed to room temperature by gentle shaking; until no frozen material was visible. The contents of the cryovial were transferred to a centrifuge tube containing 20 mL of pre-warmed complete RPMI cell culture medium (Hirsch, 2016). Cells were then centrifuged for 6 minutes at 300 x g at 20 ˚C. The supernatant was discarded and the cells were re-suspended into fresh cell culture medium in a T75 flask (ThermoFisher Scientific™, NZ). These suspension cells were maintained at 37 ˚C in 5% carbon dioxide (CO_2) in a humidified incubator (Mitre 4000 series, Contherm™, NZ). Cells were sub-cultured every third or fourth day with the dilution ratio depending on the level of confluence reached (Theus, Cave & Eisenach, 2004; Wang et al., 2013)

The growth of cells was monitored by viewing under an inverted phase contrast microscope (CKX31, Olympus™, Japan).

3.3.2.2 Cell differentiation

Prior to differentiating THP-1 cells, cell viability was determined using a 0.4% trypan blue solution. Viable cells are resistant to trypan blue and thus appear unstained under the microscope (Belloni, Pepe & Palitti, 2010). Cell suspensions and trypan blue were mixed in a 1:1 ratio and 10 µL of solution was pipetted onto a haemocytometer. Total number of viable cells were counted in the haemocytometer chambers (Hirsch, 2016).

$$\text{Number of cells/mL} = \text{average cell count} \times 10^4 \times \text{dilution factor} \quad \ldots\ldots(1)$$

Cells were seeded in 6-well plates at a density of 1 x 10^6 cells/mL. To induce differentiation in THP-1 cells, filtered PMA was added to each well to a final concentration of 200 nM. The plates were incubated at 37 ˚C in a 5% CO_2 humidified incubator for 3 days (Takashiba et al., 1999; Zhou et al., 2004; Daigneault, Preston, Marriott, Whyte & Dockrell, 2010; Hirsch, 2016). At the end of the three-day stimulation period, medium containing PMA was discarded and cells were washed with cell culture medium. The cells were then rested in 1 mL of fresh cell culture medium for 2-3 hours prior to further experiments.

3.3.2.3 Treatment of LPS stimulated THP-1 macrophages with curcuminoids

Appropriately diluted curcumin stock solution (in complete RPMI medium) was added to cells at a final concentration varying from 0.1 µM to 50 µM. Corresponding to this, diluted DMSO (1:100 in complete RPMI medium) was added to cells at a final concentration of 0.001% to 0.5%. DMSO concentration of 0.001% is equivalent to the concentration of DMSO in the 0.1 µM curcumin working solution. Both curcumin and DMSO working solutions were filtered through a 0.2 µm Whatman® disposable syringe filter (Sigma-Aldrich®, NZ) prior to use. Cells were stimulated with curcumin for 1 hour at 37 ˚C in a 5% CO_2 humidified incubator. The plate was removed from the incubator and the cells were treated with LPS. A stock solution of LPS (0.1 mg/mL) was appropriately diluted in pre-warmed complete RPMI medium and added to differentiated macrophages (stored in 1 mL fresh complete RPMI medium) to achieve a final concentration of

200 ng/mL in 6-well plate. The plate was incubated at 37 ˚C in 5% CO_2 humified incubator for 4 hours. At the end of the incubation period, the supernatant was carefully removed and aliquoted into 1.5 mL Eppendorf tubes and stored at -80 ˚C until ELISAs (Section 3.3.2.5) were carried out. The remaining cell pellet was utilised for the MTT assay (Section 3.3.2.4) to assess the cytotoxicity of curcumin on differentiated macrophages (Figure 3.2).

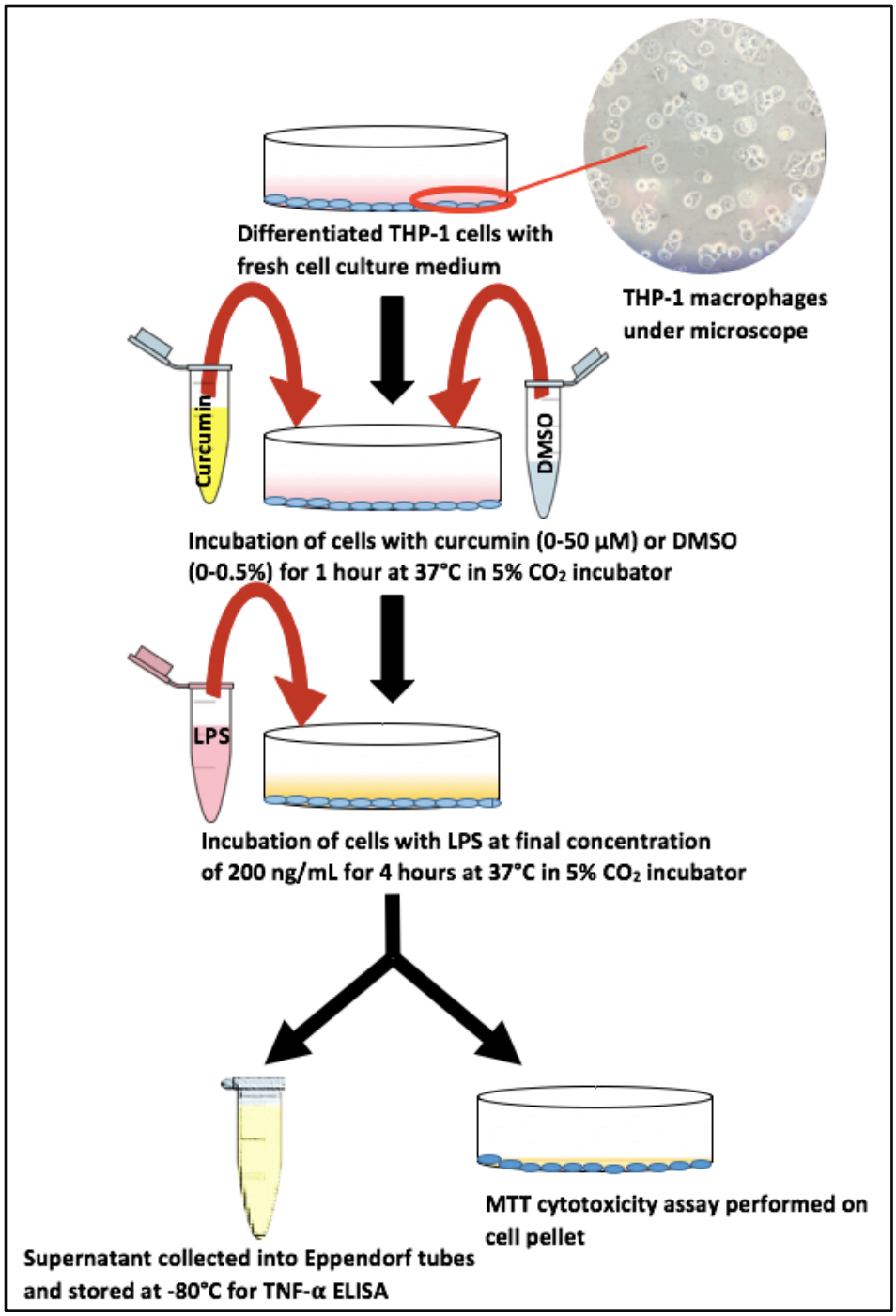


Figure 3.2 Systematic procedure for the treatment of macrophages with LPS, curcumin and DMSO

3.3.2.4 MTT cytotoxicity assay

Cultured cells seeded at a density of 0.5 x 10^6 cells/ mL in 6-well plates were washed with warm RPMI-1640 without phenol red to remove PMA. The well without cells was treated as the blank, while the cells treated with LPS (without curcumin) represent a negative control. 1 mL of MTT working solution was added to cells in each well of 6-well plate and incubated for 30 minutes to 3 hours at 37˚C in 5% CO_2 Contherm™ (MITRE 4000 series, New Zealand) humidified incubator. At the end of the incubation period, violet crystals were formed in the wells due to reduction of MTT to formazan by viable cells (Popovich & Kitts, 2002). The cell medium was removed and the converted dye was solubilized with 1 mL of acidified isopropanol. The plates were wrapped in aluminium foil and placed on orbital shaker for 20 minutes to ensure complete dissolution of the dye. The contents of each well were transferred to Eppendorf tubes and centrifuged (My SPIN™ mini centrifuge, ThermoFisher Scientific™, NZ) at 12,500 rpm for 2 minutes. 200 µL of sample solution was pipetted in wells of a 96-well plates (in triplicate) and the absorbance was recorded at 595 nm using a FLUOstar® Optima microplate reader (BMG Labtech™, Germany). Cell viability % was calculated using the formula:

$$\% \text{ cell viability} = \frac{(\text{Abs. of sample} - \text{Abs. of blank})}{(\text{Abs. of negative control} - \text{Abs. of blank})} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

3.3.2.5 TNF-α ELISA

ELISA was performed using TNF-α human ELISA MAX™ deluxe kits (BioLegend®, United States), with a sensitivity of 2 pg/mL. All the reagents from the kit were stored at 4 ˚C prior to use. A sample containing only assay diluent was treated as blank and the absorbance obtained was subtracted from blank to minimize the background noise. The assay procedure was carried out according to the manufacturer's instructions, as illustrated in Figure 3.3. Absorbance of standards and test samples at 450 nm was displayed as optical density (OD) in BMG OPTIMA Windows™ based software and transferred to Microsoft® Excel (2016). Standard curves were generated and the resulting regression equation was used to calculate the concentration of TNF-α (ng/mL) in the supernatant samples stimulated with LPS (with or without curcumin and DMSO).

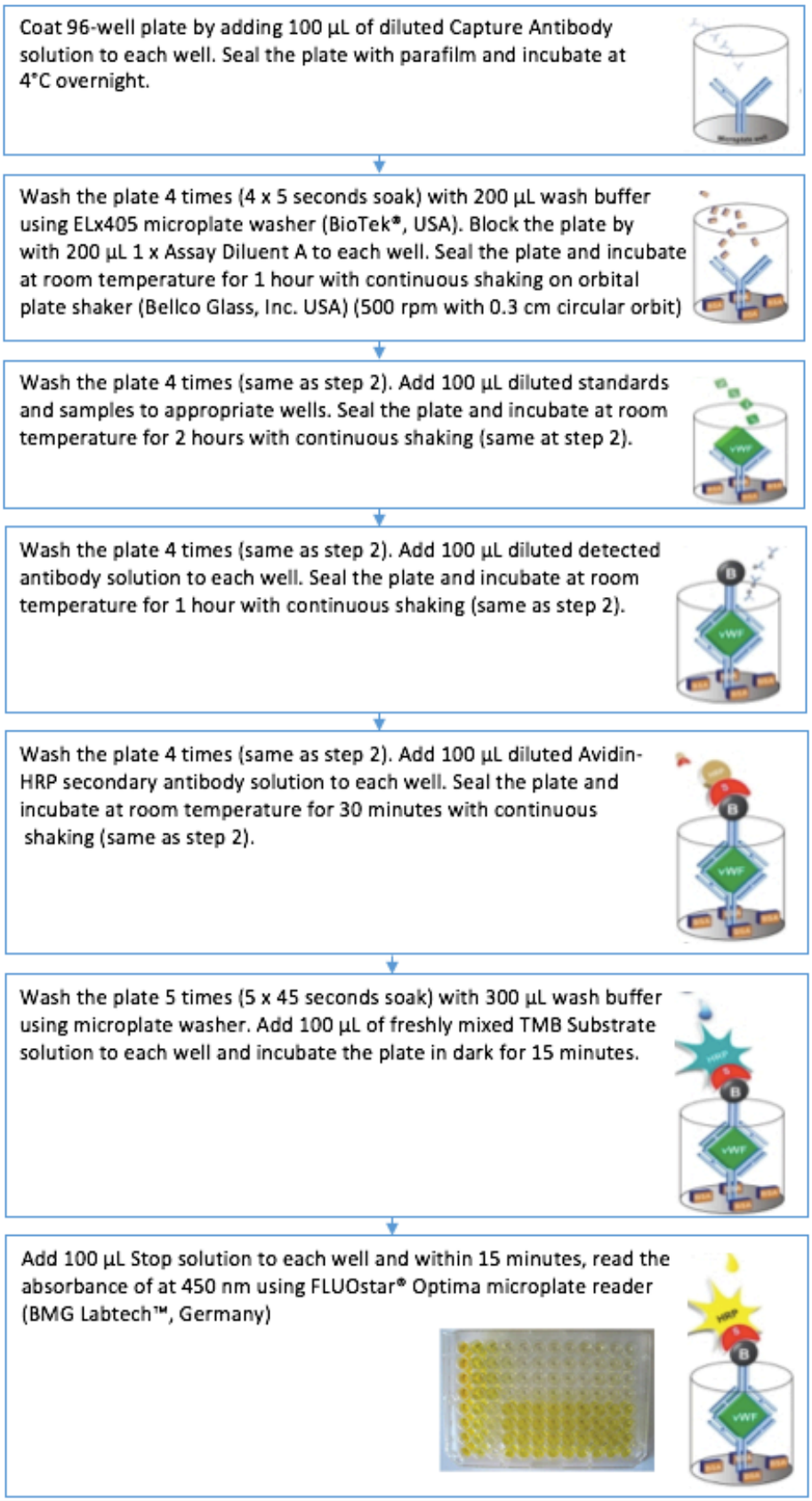


Figure 3.3 Systematic procedure for TNF-α ELISA (BioLegend®, USA)

3.3.2.6 Data analysis

The experiments were repeated three times and all measurements were conducted in triplicate. Results were presented as mean ± SD (standard deviation) in average of three replicate values. Graphs were generated using Prism 7 (GraphPad Software Inc., California, USA) and Microsoft® Excel 2016 (Microsoft Corporation, WA, USA) and error bars in the graphs represent standard deviation of three replicate values. Statistical analysis of data was carried out using procedures of Minitab 18 software (Minitab Inc., State College, PA, USA). One-way ANOVA (analysis of variance) was performed to determine significant differences amongst the data at a confidence level of 95%. Tukey's test performed to compare the statistically significant differences ($P < 0.05$) between analytical grade curcumin (Pure C), C7, C9 and DMSO.

3.4 Phase III: Laboratory scale production of coconut yogurt enriched with curcuminoids

Yogurt was prepared by following the process illustrated in Figure 3.5. Kara™ coconut cream was considered as an optimum raw material for preparation of coconut yogurt fortified with curcumin (0.27%, w/w). Fermentation of coconut cream using 2% YO MIX® 726 LYO 50 DCU starter culture was carried out until the pH reached 4.5± 0.1. Three different types of yogurts were prepared- coconut yogurt without curcumin and coconut yogurts containing C7 and C9 (400mg/150g) respectively. During fermentation, yogurt samples were subjected to pH, titratable acidity and the microbiological analysis (*L. bulgaricus* and *S. thermophilus* counts) every two hours. Furthermore, time taken by the starter culture to ferment coconut cream to form yogurt was recorded. Yogurt was stored for 15 days at 4 ˚C and the effect of curcumin on properties of yogurt was investigated at Day 1, 5, 10 and 15 (Figure 3.4). Chemical, rheological, microbiological and sensory properties of fortified yogurts were measured and compared with coconut yogurt without curcumin (negative control). Further, amount of curcuminoids remaining in coconut yogurt at day 1, 5, 10 and 15 of storage period was quantitatively measured using HPLC-DAD (Section 3.4.6). All experiments were repeated thrice and with duplicate measurements within each experiment were recorded. Data was presented in Microsoft excel 2016 spreadsheet and statistically analysed using various procedures in Minitab 18 (as described in Section 3.4.8).

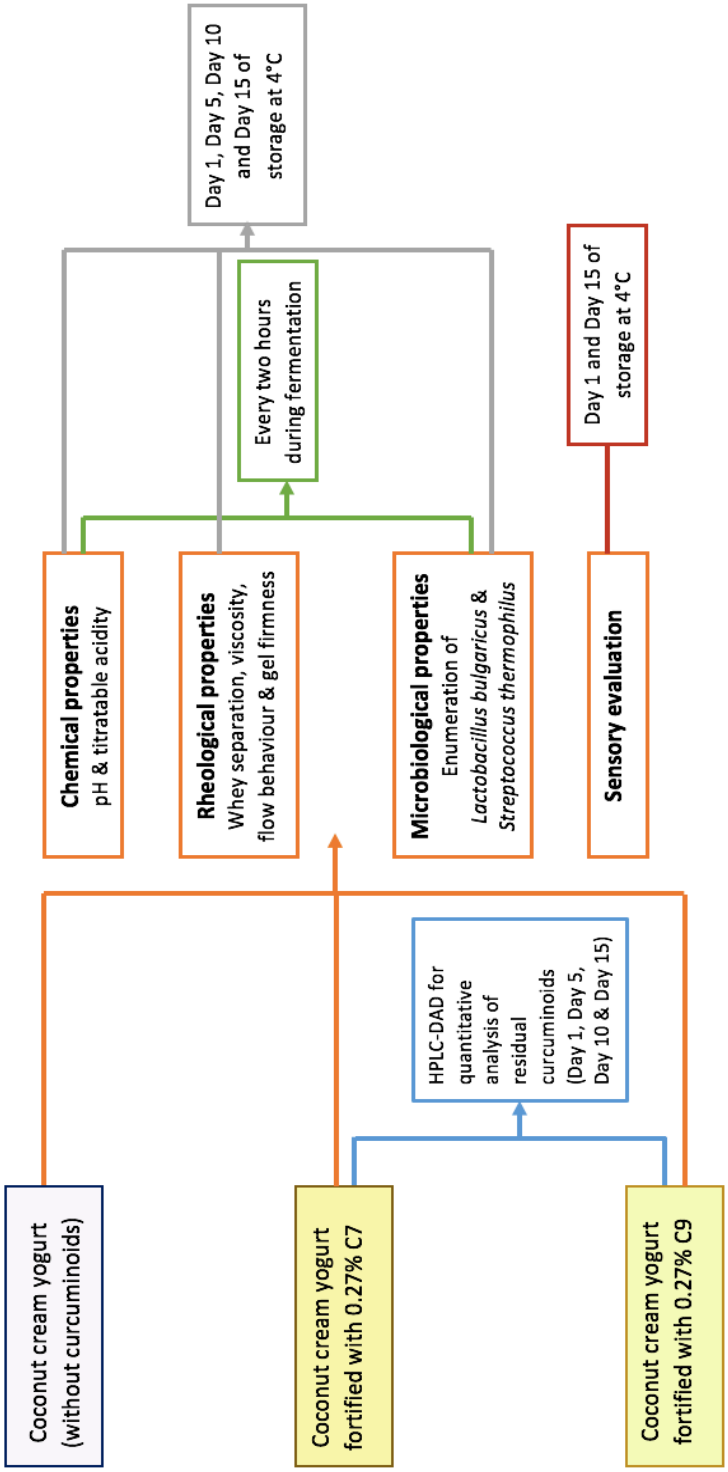


Figure 3.4 Experimental approach for investigating the effect of curcuminoids on the functional and sensory properties of coconut yogurt and determination of curcuminoid stability in phase III

3.4.1 Raw materials

Various commercial coconut milks and creams (Table 3.3) used in the experimental trials of phase III were obtained from local supermarkets (Pak n Save and Street Organics) Auckland, NZ.

Table 3.3 List of coconut milk/ cream used in production of coconut yogurt

Code	Raw material	Ingredients	Sterilization technique	Brand name
R1	So Good™ coconut milk (unsweetened)	Water, coconut cream (9%), chicory inulin, mineral salt (tricalcium phosphate), flavour, emulsifier (sunflower lecithin), salt and vegetable gum (gellan)	Ultra-high temperature pasteurization	Sanitarium™, NZ
R2	Coconut milk organic BPA free	Coconut flesh and water	Retort canning	Roar®, Sri Lanka
R3	Kokonati™ organic coconut milk	Milled and strained coconut kernel	Retort canning	Pure Ceylon Kokonati™, Sri Lanka
R4	Spiral foods™ organic coconut cream	Coconut extract, less than 0.5% guar gum	Retort canning	Spiral foods, Australia
R5	Kara™ coconut cream	Fresh natural coconut cream (99.9%), stabilizers (xanthan gum (E415), guar gum (E412), carrageenan (E407))	Ultra-high temperature pasteurization	Kara Marketing Sdn Bhd.

3.4.2 Process of set-yogurt making

Yogurt was manufactured by following a traditional method, as described by Mohamed, Zayan & Shahein (2014) with minor modifications (Figure 3.5). Prior to processing of yogurt, all equipment was sanitized and the containers were sterilized using hot potable water and air-dried. Three Kilogram Coconut cream was weighed into a KitchenAid mixer bowl and curcuminoids were added at concentration of 0.27% (w/w). The contents were mixed at minimum speed 1 for 1 minute and poured into 1 litre glass jars. The jars were placed in a temperature controlled water bath (Grant, Global Science, NZ) set at 42˚C.

The temperature of the solutions was monitored using a hand-held digital thermocouple thermometer (Fluke 51, ThermoFisher Scientific™, NZ). When the solutions reached a temperature of 40 ± 2 ˚C, 2% (w/v) YO-MIX® 726 (50 DCU) (Danisco™, NZ) was inoculated into coconut cream. Culture was mixed properly and the cream was poured into two 500 mL glass jars (for sensory evaluation) and twelve 120 mL screw cap sterile plastic containers (LabServ, NZ). Containers were carefully placed back in a water bath set at 42 ˚C. pH and titratable acidity were measured every two hours until the pH reached 4.5 ± 0.1, at which point incubation was terminated and the yogurt samples were allowed to cool to room temperature. Simultaneously, 5 g of each type of yogurt was weighed into a stomacher bag (every two hours) during fermentation and subjected to microbiological analysis as discussed in Section 3.4.4. The remaining samples were then stored at 4 ˚C to minimize post-acidification of the acid gels without disturbing the gel until further analysis. Coconut yogurt without curcuminoids was also prepared as described above and treated as a negative control.

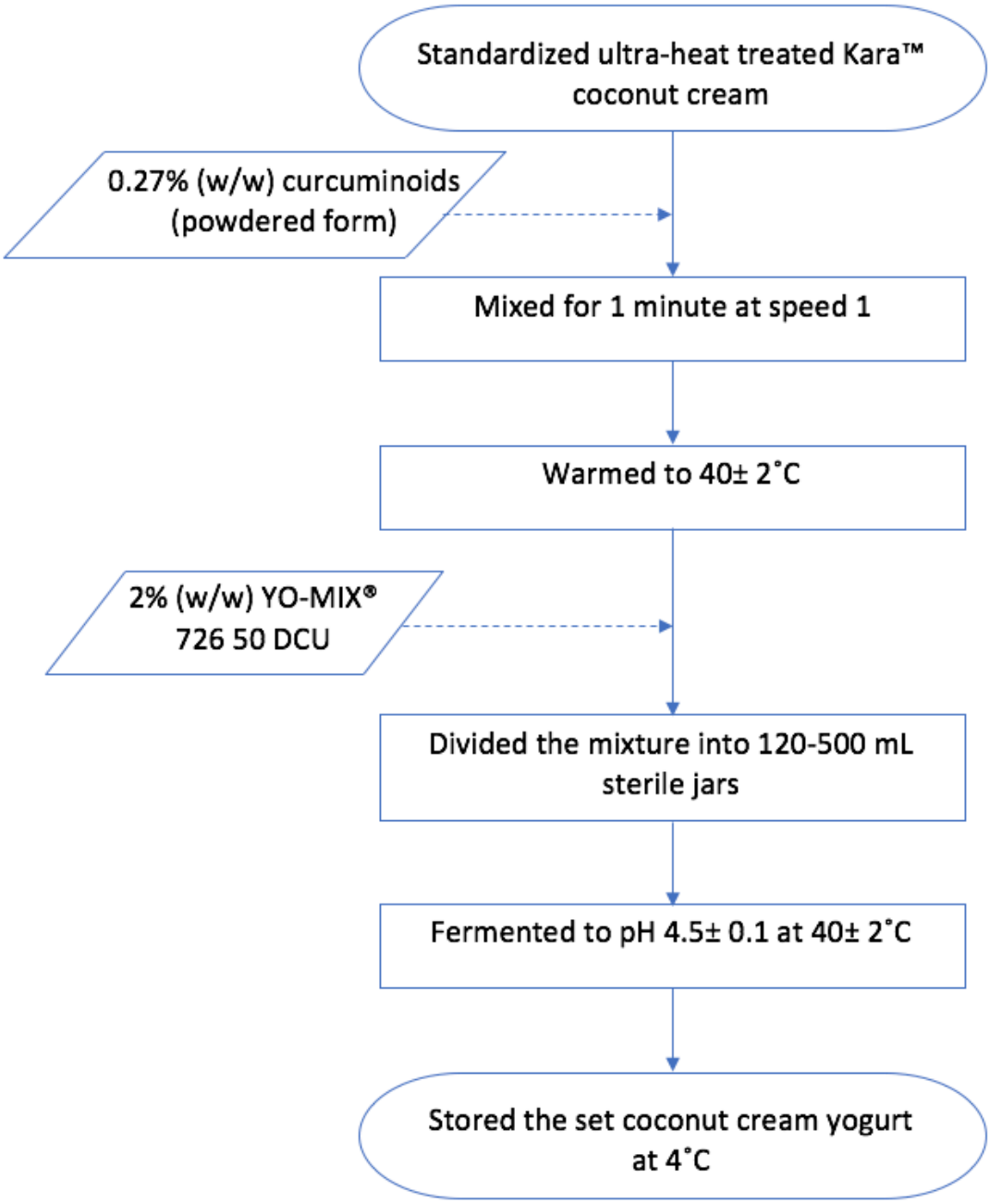


Figure 3.5 Laboratory scale production of set coconut yogurt

3.4.3 Chemical analysis

3.4.3.1 pH

The pH of the set yoghurts was determined as described in Section 3.2.3.

3.4.3.2 Titratable acidity

Standardization of 0.1M sodium hydroxide (NaOH) solution

AOAC 936.16 method (AOAC, 1990) was followed to standardize a 0.1M NaOH solution. Briefly, 20 g of potassium hydrogen phthalate (KHP) (Univar, AjaxFinechem Pty. Ltd., NZ) was dried at 120 ˚C for 2 h, then cooled in a desiccator for 1 h. 200 mg of dried KHP was weighed into 5 x 150 mL Erlenmeyer flasks and dissolved completely in 40 mL of distilled water with continuous stirring at 50 ˚C. The flasks were cooled to room temperature and 3-5 drops of 1% phenolphthalein solution were added to each flask and swirled thoroughly to mix. KHP was titrated against 0.1 M NaOH solution until a faint persistent pink colour appeared. The volume of NaOH used was recorded and the titration was repeated 5 times. Molarity was calculated using the formula:

$$\text{NaOH Molarity (mol/L)} = \frac{\text{mass of potassium hydrogen phthalate (g)}}{\text{NaOH titrant volume } (V_a) \text{ x } 0.204229} \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

Determination of acidity of curcumin coconut yogurt

Titratable acidity of the yogurt samples was measured in duplicate following the AOAC method 947.05 (AOAC, 2005). Ten grams of coconut yogurt samples were weighed into Erlenmeyer flasks and mixed thoroughly with 30 mL of distilled water. 3-5 drops of 1% phenolphthalein indicator was added to each sample solution. Samples were titrated against the standardized 0.1 M NaOH solution until a faint pink, lasting for about 5-10 seconds appeared. The exact volume of NaOH ±0.05 mL was recorded and the titratable acidity was calculated using equation (4).

$$I = \frac{(v \text{ x } 10)}{m} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4)$$

I= titratable acidity; expressed in millimoles of NaOH per 100g of product; v= average volume in millilitres of 0.1M sodium hydroxide solution used for the titration; m= mass (in grams) of the test portion of the yogurt samples

3.4.4 Microbiological analysis

YO MIX® 726 LYO 50 DCU starter culture composed of *S. thermophilus, L. bulgaricus, Biofidobacterium lactis* and *Lactobacillus acidophilus.* Of these, *L. bulgaricus* and *S. thermophilus* play a vital role in fermentation of milk to form yogurt (Ashraf & Shah, 2011).

3.4.4.1 Sample preparation

Five grams of yogurt sample was weighed in a stomacher bag (Global Science, NZ) using an analytical balance (Sartorius® PB-11, Germany). 45 mL of 0.1% peptone water (Merck, Germany) was added to the test sample to obtain a 10^{-1} dilution. The mixture was stomached for 1-2 minutes in a laboratory paddle stomacher blender (Masticator 400 mL, ILU, Spain).

3.4.4.2 Serial dilutions

Ten-fold serial dilutions up to 10^{-9} were performed by successively diluting 1 mL of 10^{-1} sample into 9 mL of 0.1% peptone water (Ben-David & Davidson, 2014). The dilution bottles were vortexed to ensure homogenization of the sample in the peptone water.

3.4.4.3 Enumeration of S. thermophilus

S. thermophilus was isolated on selective media M17 agar (Gorrasi et al, 2016) following the ISO method 7889 (ISO, 2003). M17 agar was prepared by mixing M17 broth (Oxoid™, UK) and 1.5% bacteriological agar (Oxoid™, UK) as per the manufacturer's instructions. Briefly, to prepare 400 mL of M17 agar, 14.9 g M17 broth and 6 g of bacteriological agar was weighed out and dissolved in 380 mL of distilled water. Simultaneously, a 10% lactose solution was prepared by dissolving 10 g of lactose powder (ThermoFisher Scientific™, NZ) in 100 mL of distilled water. Both suspensions were autoclaved (Astell Scientific, UK) at 121 ˚C for 15 minutes. The media were cooled to 45- 48 ˚C by placing the solutions in a water bath set at 45 ˚C (Grant™, UK). Twenty millilitres (20 mL) of 10% of lactose solution was added to 380 mL of molten M17 agar as a nutrient supplement immediately before pour plating the sample. One millilitre (1 mL) of each of the appropriately diluted samples was pipetted into petri dishes and covered with 12-16 mL of M17 agar and gently swirled. Upon solidification, the plates were inverted and placed in an incubator (Heraeus Series 6000, 50042307, Germany) at 37 ˚C for 48 hours. M17 agar plates with

30-300 developed colonies were counted using a colony counter (SC6PLUS, Stuart™, UK) and the results expressed as cfu/g (colony forming unit/ grams of yogurt).

3.4.4.4 Enumeration of L. delbrueckii subsp. *bulgaricus*

L. bulgaricus, L. acidophilus and *Bifidobacterium lactis* are phylogentically closely related species and thus have similar a morphology and biochemical profiles (Süle, Kõrösi, Hucker & Varga, 2014). These species can all be enumerated on MRS (De Man, Rogosa and Sharpe) agar media which makes it difficult to achieve distinct colonies of individual species (Tabsco, Paarup, Janer, Peláez & Requena, 2007; Ashraf & Shah, 2011). Numerous methods have been developed by the researchers for selective and differential enumeration of *L. acidophilus* and *Bifidobacterium lactis* (Lankaputhra, Shah & Britz, 1996; Vinderola & Reinheimer, 1999; Talwalkar & Kailasapathy, 2004; Tharmaraj & Shah, 2003; Casteele et al., 2006; Darukaradhya, Philips & Kailasapathy, 2006). However, there are limited studies on selective/ differential enumeration of *L. bulgaricus* in the presence of the probiotics- *L. acidophilus* and *Bifidobacterium lactis.*

Selective enumeration of *L. bulgaricus* was done on acidified MRS agar (pH 4.58), as described by Tharmaraj & Shah (2003), with minor modifications. MRS agar (CM0361, Oxoid, UK) was prepared according to manufacturer's instructions. Briefly, 24.8 g of dry MRS powder was dissolved in 400 mL RO water. The pH of the media was adjusted to 4.58 using 1.0 M HCl to obtain acidified MRS agar and sterilized by autoclaving at 121 ˚C for 15 minutes. MRS media was cooled to 45- 48 ˚C and applying the pour plate technique, 1 mL of appropriately diluted sample was pipetted into petri dishes and covered with 12-16 mL of pH modified MRS agar and gently swirled. Upon solidification, the plates were overlaid with 5-7 mL of MRS agar to maintain an anaerobic environment for the growth of *L. bulgaricus.* Solidified plates were inverted and placed at 43 ˚C in a 5% CO_2 humidified incubator (Heraeus BBD6220, 51012344, Germany) for 72 hours. Agar plates with 30-300 developed colonies were counted using a colony counter (SC6PLUS, Stuart™, UK) and results expressed as cfu/g (colony forming unit/ grams of yogurt).

3.4.5 Rheological analysis

3.4.5.1 Syneresis

The term 'syneresis' refers to the expulsion of liquid (whey) from the yogurt gel, which causes instability in the gel network and ultimately leads to shrinkage of the yogurt gel (Vareltzis, Adamopoulos, Stavrakakis, Stefanakis & Goula, 2016). The whey separated from the gel appears as a liquid on the surface and can be quantified using the siphon method (Amatayakul, Sherkat & Shah, 2006). Yogurt samples were removed from storage at 4 ˚C and any water droplets formed due to condensation were wiped off before weighing the container on an analytical balance (Sartorius® PB-11, Germany). The yogurt containers were placed at an angle of approximately 45˚ for 10-15 seconds and any whey deposited on the surface of yogurt and against the side of the container was carefully collected using a 5 mL disposable plastic pipette. The collected whey was weighed on the analytical balance and the syneresis was expressed as the percentage weight of the separated whey compared to the initial weight of the yogurt sample (Equation 5).

$$\text{Syneresis (\%)} = \frac{\text{Weight of whey (liquid supernatant) (g)}}{\text{Initial weight of yogurt sample (g)}} \times 100 \quad \text{.....(5)}$$

3.4.5.2 Apparent Viscosity the of broken gel

Analysis of viscosity and flow behaviour of coconut yogurt samples was performed using an AR500 Advanced Rheometer (TA instruments, New Castle, UK) as described by Paseephol, Small & Sherkat (2008), with minor modifications. Prior to rheological measurements, yogurt samples were removed from cold storage (4 ˚C) and stirred manually 20 times with a spatula. The broken yogurt gel was allowed to equilibrate to room temperature (20 ˚C). Meanwhile, the Rheometer was set up according to the manufacturer's instructions. The pressure was maintained at 37.5 psi at all times and the black bearing clamp was removed and replaced with a 40-mm diameter 2˚ angle cone plate geometry. Rotational mapping was performed for correction of residual torque around one completer revolution of the shaft. The geometry was calibrated by performing zero gap, with a 0.105-mm gap setting.

Approximately two grams of yogurt sample was spread uniformly on the lower plate. The geometry was set at 0.105-mm and extra sample was wiped off from the plate, as either over-filling or under-filling of sample can result in inaccurate results. A flow procedure was set up to determine the viscosity of the yogurt samples. At 20 ˚C, the test sample was exposed to a shear rate of 500/s for 60 seconds, followed by a 300 seconds equilibrium period. A flow curve was obtained recording the shear stress with increasing shear rates from 0 to 200 s^{-1} within 200 seconds (upward flow curve). A viscosity curve plot from shear rate and shear stress was generated and used to calculate the apparent viscosity of yogurt samples at a shear rate of 60 s^{-1}.

3.4.5.3 Texture profile analysis

Gel firmness of set coconut yogurt was determined by performing a single compression test using a TA-XT plus Texture Analyzer (Stable Micro Systems, Godalming, UK) equipped with a 5-kg load cell as described by Paseephol et al. (2008). Plastic containers containing approximately 50 grams of yogurt samples were removed from cold storage (4 ˚C) and compressed with a calibrated 20-mm diameter cylindrical probe (P20) at a constant speed of 1 mm/s to a depth of 15 mm. Force-distance curves were obtained and gel firmness was expressed as the maximum force (N) on this force-distance curve. The measurements were done in duplicates and the experiment was repeated thrice.

3.4.6 Degradation analysis of curcuminoids in yogurt

3.4.6.1 Extraction of curcuminoids

Curcuminoids can be extracted from food samples by dissolving in solvents such as ethanol, methanol, acetone and DMSO (Revathy, Elumalai, Benny & Antony, 2011). In this study, acetone was used as the extraction solvent following the method described by Fu et al. (2016). One gram of yogurt sample was weighed and suspended in 5 mL of acetone (≥99.9%, Fisher Scientific, UK). The mixture was ultrasonicated for 10 minutes in an ultrasonic water bath (Sonorex Super RK 501, Bandelin electronic GmbH & Co. KG, Berlin). The objectives of ultrasonication are two fold-extraction of curcuminoids from the yogurt matrix and complete dispersion of curcuminoids in the acetone (Kohn, 2010). The ultrasonicated samples were centrifuged at 2270 x g for 10 minutes at 20 ˚C using a Multifuge 1S-R centrifuge (D-37520, Heraeus, Germany). The supernatant was recovered using 5 mL disposable pipettes and the sediment was re-extracted with aqueous acetone

(5 mL acetone + 1 mL distilled water). The combined extract was analysed using HPLC-DAD analysis as described below. Extraction of curcuminoids from the yoghurt samples was performed in duplicate and the experiment was repeated thrice.

3.4.6.2 High Performance Liquid Chromatography (HPLC)- Diode array detector (DAD)

Quantitative analysis of curcuminoids was performed based on the HPLC protocol described by Fu et al. (2014) with minor modifications. A C_{18} Grace Smart column (250 x 4.6 mm, particle size 5 µm, Grace Davison Discovery Sciences, USA) equipped with reverse phase HPLC system (Prominence UFLC, Shimadzu Corporation, Japan) was used to separate the curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin). The HPLC system consisted of a CBM-20A system controller, LC-20AD solvent delivery unit, DGU-20A3 online degasser, rack changer, SIL-20AHT auto-sampler, CTO-20A column oven and SPD-M20A photodiode array detector. All chemicals used in this experiment were of HPLC grade and were purchased from Fisher Scientific, UK. The mobile phases used in this experiment consisted of

A. 2% (v/v) Acetic acid, 4% Methanol, 35% Acetonitrile and 59% Milli-Q water
B. 2% (v/v) Acetic acid, 10% Methanol and 88% Acetonitrile

10 mM curcumin standards (C7 and C9) prepared in ethanol were diluted 1:100 in milli-Q water into 2 mL HPLC vials (ThermoFisher Scientific™, NZ). 10 µL of each of seven working standards containing 0.005-1.6 µg of diluted standards (C7 and C9) were loaded in the auto-sampler (Jayaprakasha, Rao & Sakariah, 2002). The test samples were diluted by 1:10 in milli-Q water and 10 µL of diluted samples were injected via the auto-sampler. The gradient (0-35 % B) was run for 17 minutes at a flow rate of 1 mL/min. Curcuminoids were detected at 425 nm and the peak height and peak area were obtained by integration using LC solutions software (Prominence UFLC, Shimadzu Corporation, Japan). Standard calibration plots (one for each of C7 and C9) were obtained by plotting mass of compound (g) versus peak area. The concentration of curcumin or curcuminoids in the test samples were quantified by comparing the retention times and peak areas to the standards. Standard curve graphs were generated in Microsoft® Excel 2016 (Santa Rosa, CA, USA) and a regression equation was generated to allow calculation of the concentrations of the curcuminoids. Descriptive statistical analysis was performed to obtain mean and standard

deviation (SD) in the average of test sample values. The concentration of curcuminoids injected was calculated from standard curve and expressed in percentage (%). The concentration of curcuminoids in day 1 sample was set at 100% and concentration of curcuminoids remaining at day 5, day 10 and day 15 was expressed relative to day 1 concentration. This gives a measure of amount of curcuminoids (%) retained in coconut yogurt. Results were presented as mean± SD.

3.4.7 Sensory evaluation

The main objective of sensory evaluation is to obtain measures of sensory characteristics of food such as appearance, flavour, taste, colour and texture; as they would be perceived by humans (Silva et al., 2013). Sensory analysis was conducted based on the method described by Irvine & Hekmat (2011). A total of six sessions were carried out with each session requiring the participation of 30 consumers. The consumer group composed of academic staff, administrative staff, under-graduate and post-graduate students and PhD scholars from Massey University (Albany, New Zealand) were invited to sensory sessions through emails and personal invitations. Participants were recruited based on their familiarity and regular consumption (at least once a week) of any type of yogurt product. Sensory evaluations were conducted in individual sensory booths (Sensory Evaluation and Product Development Laboratory, Building 26, School of Food and Nutrition, Albany, NZ). Prior to sensory evaluation, a brief introduction about the sensory analysis process was provided to panellists, including disclosure of all ingredients to ensure cultural or religious restrictions were not violated. Any panellist allergic to coconut cream or turmeric was excluded from the study. Panellists were requested to complete a consent form (Appendix A.1).

In each sensory session, panellists were required to evaluate three types of yogurt samples by filling in the questionnaire based on the nine-point hedonic scale in which 1= “disliked extremely”, 5= “neither liked nor disliked” and 9= “liked extremely” (Appendix A.2). Panellists were with presented 15 grams of refrigerated (4 ˚C) yogurt samples (one at a time) in a 25 mL plastic cup (coded with a random 3-digit number) and a plastic spoon. Panellists were required to cleanse their mouth with RO water in between the tastings.

3.4.8 Data analysis

Yoghurt samples were prepared and fermented in triplicate and measurements were recorded in duplicates. All values were expressed as mean ± SD (standard deviation) as calculated in Microsoft Excel 2016. Data was statistically analysed by using procedures of Minitab 18 software (Minitab Inc., State College, PA, USA). The data was tested for normality using general linear model and the data with normally distributed residues was further analyzed using ANOVA. One-way ANOVA (Analysis of Variance) was used to determine the effect of curcuminoids on the chemical, rheological, microbiological and sensory properties of yogurt at α=0.05. Further, Tukey's multiple comparison test was employed to separate the significantly different mean values between the three yogurt groups (P <0.05). Graphs were generated using Prism 7 (GraphPad Software Inc., California, USA) and Microsoft® Excel 2016 (Microsoft Corporation, WA, USA) and error bars in the graphs represent standard mean deviation in average of three replicate values.

CHAPTER 4
EFFECT OF CURCUMINOIDS ON THE ACIDITY AND SENSORY PROPERTIES ON ADDITION TO COMMERCIAL YOGURTS

4.1 Introduction

Traditionally, yogurt is prepared by fermentation of milk from the cow, water buffalo, sheep or goat (Weerathilake, Rasika, Ruwanmali & Munasinghe, 2014). Of these, pasteurised cow milk is the most commonly used for yogurt preparation in western countries (Nuñez, 2016). Yogurt can also be produced from non-dairy sources such as legume milk (Jiménez-Martínez, Hernández-Sánchez & Dávila-Oritiz, 2003), soy milk (Lee, Morr & Seo, 1990), coconut milk (Yaakob, Ahmed, Daus, Malek & Rahman, 2012) and sesame milk (Afaneh, 2013).

Mammalian and plant-based milks differ in their nutritional (Table 4.1), physico-chemical and sensory properties (Hajirostamloo, 2009; Kundu, Dhankhar & Sharma, 2018). One of the major differences is the absence of lactose in non-dairy milk (Table 4.1), therefore consumption of plant-based milk products is suitable for people with lactose intolerance (Sousa & Kopf-Bolanz, 2017); a metabolic disturbance arising from insufficient production of lactase, the key enzyme responsible for the digestion of lactose present in dairy products (Granato, Branco, Nazzaro, Cruz & Faria, 2010).

Table 4.1 Nutritional composition of mammalian and plant milks

Milk source	Moisture (%)	Protein (%)	Fat (%)	Carbohydrate (%)	Main sugar	Ash (%)
Cow	87-88.2	3.0-3.9	3.3-5.4	4.4-5.6	Lactose	0.7-0.8
Sheep	80-81.9	4.5-7.0	5.0-9.0	4.1-5.9	Lactose	0.8-1.0
Goat	83.7-88.1	3.0-5.2	3.0-7.2	3.2-5.0	Lactose	0.7-0.9
Coconut	55.1-74.9	2.0-4.0	20.0-35.0	2.7-4.7	Sucrose	0.6-1.0
Soy	89.8-93.0	2.8-3.6	1.9-2.0	1.8-2.9	Sucrose	0.3-0.5

Source: Liu, 1997; Seow & Gwee, 1997; Hou, Yu & Chou, 2000; Tansakul & Chaisawang, 2006; Raynal-Ljutovac, Lagriffoul, Paccard, Guillet & Chilliard, 2008; Ceballos et al., 2009; Madukwe & Eme, 2012; Giri & Mangaraj, 2012; Claeys et al., 2014; Gantner, Mijić, Baban, Škrtić & Turalija, 2015)

Fortification of yogurt with a bioactive can influence certain sensory attributes of the yogurt such as flavour, taste, texture, aroma and colour (Arslan & Bayrakci, 2106). In this preliminary experiment (phase 1) the effect of the addition of 10 different commercially available curcuminoids (coded as C1 to C10) on the acidity and sensory properties of commercial yogurts (set-style CMY, stirred CCY and drinking GMY) was investigated. The acidity of the fortified yogurts was determined by measuring pH.

4.2. Effect of curcuminoids on the pH of yogurts

Yogurt is a fermented product with a pH normally ranging between 4.2- 4.6 (Adhikari, Mustapha, Grün & Fernando (2000). The pH of commercial yogurts measured before addition of curcuminoids are shown in Appendix B.1. These results show that GMY was the most acidic, followed by CMY and CCY ($p<0.05$), with pH of 3.96 ± 0.01, 4.07± 0.01 and 4.25± 0.01 respectively. The pH of the yogurts remained stable throughout storage at 4˚C (Appendix B.1).

Results of addition of curcuminoids into yogurts indicated that addition of C10 increased the pH of CCY while it decreased the pH of the other two yogurt products ($p<0.05$). Yogurt samples containing C6 and C7 presented a significantly ($p<0.05$) higher acidity (lower pH) when compared to the yogurts without curcuminoids (negative control). Variable pH results were reported in yogurts containing C8. In all the three types of yogurts, a decrease in pH was reported after 6 h of addition of C8. However, addition of other commercial curcuminoid samples (C1, C2, C3, C4, C5 and C9) did not have any significant effect on the acidity of the yogurts (Appendix B.1). Overall, the addition of curcuminoid powders had no adverse impact on the pH of the yogurt samples. Thus, pH was not a suitable parameter on which to base the selection of the most suitable curcuminoid samples for further experimentation.

4.3 Sensory properties of yogurts containing curcuminoids

One of the most evident results of informal focus group sensory evaluation was the aversion of the panellists towards the goat milk yogurt (with and without curcuminoids). The yogurt was described as "too acidic" and "sour" with a "goaty odour", which is a typical characteristic of goat milk yogurt (Vargas et al., 2008; Young, Gupta & Sadooghy-Saraby, 2012). The acidic flavour, characterised by the low pH of GMY (Section 4.2) is probably due to the lower level of acetaldehyde production in this yogurt type (Vargas et al., 2008) compared to cow milk yogurt. Comparison of sensory attributes of CCY and CMY without curcuminoids showed that even though panellists were familiar with the "typical flavour" of CMY, the "creamy" coconut cream yogurt was equally acceptable.

Curcuminoid is a yellow pigment with a distinctly earthy, slightly bitter flavour and a mustardy smell (Nampoothiri, Praseetha, Venugopalan & Menon, 2012). These characteristics can impact on the flavour, colour, taste and overall acceptance of the yogurt. The colour of the yogurt samples with added curcuminoids ranged from "light orange-yellow" to "bright orange/yellow". Among these, the light coloured yogurt with C9 was rated the highest by the panellists, followed by samples containing C7 and C6. However, yogurt supplemented with C8 presented a "beige" colour, similar to the colour of the raw C8 powder. Overall, the colour intensity of yogurt containing C8 was comparable to the colour of the powder with no adverse effects observed on the colour of the yogurt.

Based on the panellists' comments, it was reported that the appearance of yogurt varied significantly in different yogurt types. In this study, appearance was referred to the uniform distribution of curcuminoids in yogurt, graininess, liquid (whey) separation and lumpy structure of the yogurt (Karagül-Yüceer & Drake, 2007). Based on visual observations, small visible curcuminoid particles were observed by panellists in samples containing C7, C8, C9 and C10. C1 was not soluble in any of the yogurts resulting in the formation of small lumps in CCY and CMY and on the surface of GMY drinking yogurt. The remaining curcuminoid samples (C2, C3, C4 and C5) produced grainy products in all yogurt types.

The flavour of yogurt is attributed to the taste and aroma of the yogurt (Chen et al., 2017). According to the panellists, the smell of yogurts containing curcuminoids was less intense compared to their respective controls (yogurts without curcuminoids). According to panellists, the addition of C1, C2, C7, C9 and C10 to GMY decreased its "sourness". GMY containing C4 and C5 tasted "similar to the control," while C3 increased the sourness of GMY. In all the types of yogurts, C8 conferred a distinct taste which was expressed as "feels like eating medicine" and "traditional medicinal taste". Two of the five panellists perceived a turmeric flavoured yogurt with C8. One of the possible reasons for this distinct taste of C8 is the presence of the principal flavouring components of turmeric- tumerosaccharides and rhizome essential oil (Silva, Nelson, Drummond, Dufosśe & Glória, 2005). These compounds may be absent in other curcuminoid samples. In general, the addition of curcuminoids to coconut cream yogurt suppressed the intense "coconut flavour" of the yogurt, which was preferred by the panellists. Less coconuty flavour may be due to the coconut flavour being nullified by the slightly bitter flavour of curcuminoids. Interestingly, CCY and CMY containing C7 was perceived to enhance the sweetness of these yogurts. One of the panellists described the sweet taste as being "similar to pudding". Overall, panellists liked the taste of CCY and CMY containing C7, C9 and C10.

The panellists were required to screen the yogurts based on overall acceptability focusing on, "if they would eat the product or not". Samples CMY and CCY containing C10 had the "best texture" and "mild taste" but was rejected due to the intense "bright neon yellow" colour, which was perceived as "artificial" by consumers (Spence, 2015). Sample CCY containing C6 was "acceptable" to the panellists in contrast to CMY enriched with C6 which was rejected by the panellists due to better flavour of the former as compared to the latter.

Based on the views of the panellists, a Pugh decision matrix table (Appendix B.2) was generated to screen for the most promising curcuminoids, in terms of sensory characteristics (Cervone, 2009). The views were represented numerically as:

Yes: (+1)

No: (-1)

Neither yes nor no: (0)

The overall scores were calculated and C7 had the highest score of 9 followed by C9 with a score

of 8. Overall, C7 and C9 in CCY and CMY were the products most liked by the panellists. Further, it was noted that 4 out of 10 curcuminoids (C6, C7, C9 and C10) could be incorporated into coconut yogurt while only C7 and C9 could be used in CMY to develop a product that may be acceptable to the consumers. Thus, CCY was selected as the most suitable matrix for adding curcuminoids.

4.4 Summary of Phase 1

Results from Phase 1 indicated that addition of C7, C8 and C9 into yogurt samples CCY, GMY and CMY increased the acidity of yogurt while the other samples did not have on the pH of the products. However, the addition of curcuminoids markedly affected the colour and flavour of the yogurts. Curcuminoids C2, C3, C4 and C5 largely remained insoluble in the yogurts and therefore imparted a grainy appearance. A Pugh decision-making matrix of the views of informal focus group panellists was generated and results showed that C7 and C9 were the most promising curcuminoids for addition into yogurts. The addition of C7 and C9 in coconut cream yogurt showed the most promising results compared to cow milk yogurt and goat milk yogurt.

CHAPTER 5
DETERMINATION OF THE *IN VITRO* ANTI-INFLAMMATORY ACTIVITY OF FOOD-GRADE CURCUMINOIDS

5.1 Introduction

One of the major stages in the development of a functional food is to investigate the bioactivity of the functional ingredient prior to its addition into the food product (Day et al., 2009; Rein et al., 2013). The current study was undertaken with an aim to develop a novel yogurt, containing food-grade curcuminoids as a functional ingredient with potential health benefits. Several studies have provided an insight into the beneficial effects of curcuminoids which are attributed to its anti-inflammatory activity (Section 2.6). Curcuminoids are able to produce an anti-inflammatory response in the body, and thereby suppress chronic inflammation, a hallmark of diseases such as obesity, cancer, arthritis, pancreatitis, cardiovascular, neurodegenerative and metabolic diseases (He et al., 2015).

In phase II of this research work, the anti-inflammatory activity of food-grade curcuminoids was tested using an *in vitro* cell culture based system. THP-1 human monocytic cells were differentiated into macrophages and further stimulated with LPS to increase the expression of inflammation related biomarkers. One of the key mediators of inflammation is TNF-α (Tang, Marciano, Leeman & Amar, 2005), a proinflammatory cytokine primarily secreted by macrophages, astroglia, Langerhans cells and Kupffer cells (Parameswaran & Patial, 2010). Prolonged overproduction and secretion of TNF-α is a driving force for chronic inflammation, resulting in increased risk of cardiovascular and other inflammatory diseases (as presented in Table 2.1) (Popa, Netea, Riel, Meer & Stalenhoef, 2007; Moreira-Tabaka et al., 2012). Therefore, in the current study, the ability of curcuminoids to down-regulate the expression of TNF-α in activated macrophages stimulated with LPS was investigated and results are discussed in the following sections.

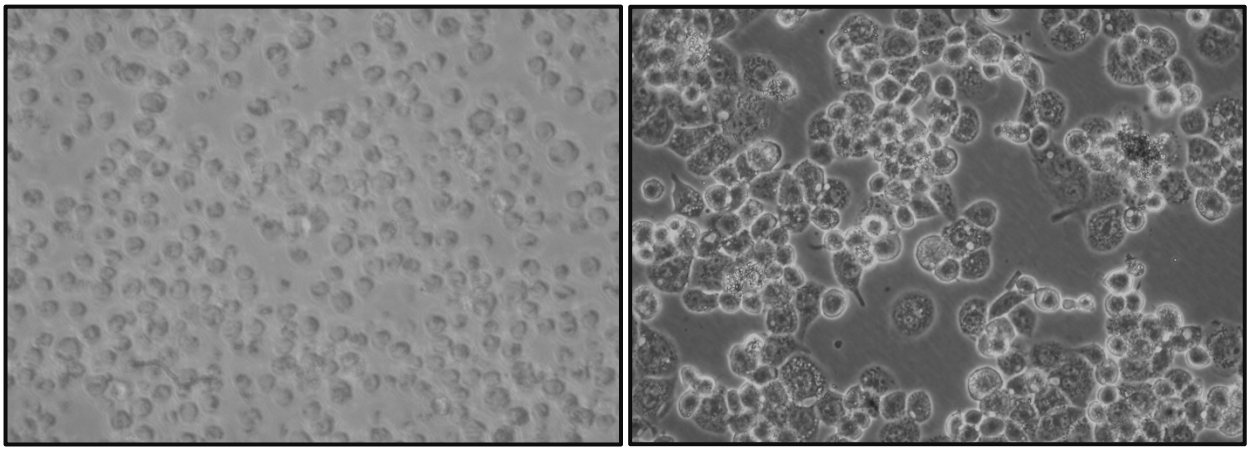
5.2 Advantages of using THP-1 cell line in immunomodulatory studies

The cell line used in this research is a human leukemia monocytic THP-1 cell line, a biologically representative cell line, widely used to study monocyte/macrophage functions, mechanisms, signalling pathways, and nutrient and drug transport (Chanput, Mes & Wichers, 2014). The bioactivity of a number of food compounds including astragalus, resveratrol, tea flavonoids and olive oil phenolics have been tested in using this cell model either in THP-1 monocytes or activated macrophages (Ludwig et al., 2004; Oh et al., 2009; Chanput, 2012; Cicerale, Lucas & Keast, 2012; He et al., 2012). Advantages of using THP-1 cells include its rapid replication time (35 to 50 hours), absence of infectious viruses or toxic products, prolonged storage in liquid nitrogen and homogenous genetic background, which minimizes the variability in cell phenotype and promotes reproducibility of result findings (Chanput et al., 2014).

5.3 PMA-induced differentiation of THP-1 monocytes

Phorbol 12-myristate 13-acetate (PMA), a derivative of croton oil is a potent tumour promoter (Hawrylowicz & Klaus, 1984) which induces differentiation of THP-1 monocytes into a macrophage-like phenotype (Figure 5.1). Treatment of cells with PMA leads to activation of protein kinase C (PKC) resulting in cell adherence and increased expression of macrophage differentiation related surface markers (Daigneault, Preston, Marriott, Whyte & Dockrell, 2010). Upon differentiation, the cell loses their ability to divide and their anti-bacterial properties are markedly elevated, allowing the cell to actively participate in inflammatory and immune responses (Takashiba et al., 1999). The activation level of macrophages depends on the concentration of PMA used. A concentration of PMA of 150-200 nM results in higher levels of expressed genes associated with inflammation and pro-inflammatory cytokines (TNF-α and IL-8) as compared to a concentration of 5-20 nM (Lund et al., 2016). The phenotypic and functional characteristics of these activated macrophages are similar to primary human macrophages (Lund, To, O'Brien & Donnelly, 2016) and thus THP-1 macrophages have been extensively used as a physiological *in vitro* model to study the involvement of macrophages in inflammatory diseases (Park et al., 2007; Chang et al., 2012; Lund et al., 2016). Therefore, all subsequent experiments were performed with THP-1 cells differentiated using 200 nM PMA.

As reported in previous studies (Takashiba et al., 1999; Traore et al., 2005; Starr, Bauler, Malik-Kale & Steele-Mortimer, 2018), monocytes or undifferentiated cells remained unattached in the suspension (A), while differentiated macrophages adhered to the plate surface (B). In addition, a shift in morphology was observed following differentiation; from a small spherical monocyte (A) to large, granulated, irregular shaped differentiated macrophages (B). Post differentiation, the cells were rested in culture media without PMA for at least 3 to 4 hours in order to increase the expression of macrophage markers which have been up-regulated during differentiation (Chanput et al., 2014).



(A) (B)

Figure 5.1 THP-1 monocytes before (A) and after (B) treatment with 200 nM Phorbol-12-myristate 13-acetate (PMA) for 72 h at 37˚C. Image captured by Dino-Eye edge microscope eye-piece camera (AM7025X, Dino-Lite, USA) at 40X magnification.

5.4 Stimulation of THP-1 macrophages with LPS

LPS is an endotoxin derived from the outer cell wall of gram-negative bacteria (Bruggen, Nijenhuis, Raaij, Verhoef & Asbeck, 1999), which plays a vital role in mediation of inflammation (Yang et al., 2016). LPS interacts with the CD14 cell surface protein (Clemons-Miller, Cox, Suttles & Stout, 2000) which results in activation of TLR receptors (Sherry et al., 2007; Ngkelo et al., 2012; Yang et al., 2016). As a result of this binding, a systemic inflammatory response is initiated (Chanput, 2012) and NF-kB, MAPK and AP-1 signalling pathways are activated (Ngkelo et al., 2012; Yang et al., 2016). This stimulation by LPS results in enhanced phagocytic ability, and

increased secretion of pro-inflammatory mediators, such as superoxide and tumour necrosis factor (TNF) in the activated macrophages (Nishio et al., 2013; Chanput et al., 2014; Lund et al., 2016).

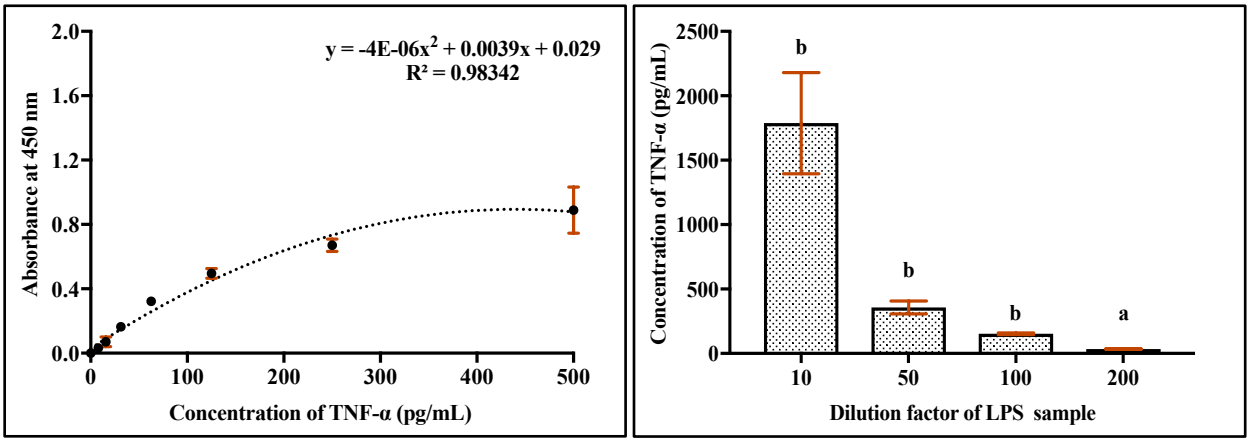
In the present study, differentiated THP-1 cells were exposed to LPS to achieve an up-regulation of the inflammation-related markers mentioned above. These biological alterations generated in LPS stimulated THP-1 macrophages mimic LPS signalling *in vivo* (Chanput et al., 2014) and thus provides an adequate model for understanding the role of LPS in chronic inflammation. THP-1 cells were differentiated into macrophages and treated with varying concentrations of LPS (50-1000 ng/mL) for 0.5-6 hours (Section 3.3.2.3). The stimulatory response was detected by quantitatively measuring the levels of TNF-α, one of the earliest known pro-inflammatory cytokine secreted by activated macrophages (Takashiba et al., 1999). On the basis of TNF-α produced by macrophages, an appropriate LPS dose, incubation time period and dilution were selected, as described in the following sections of this chapter.

5.4.1 Optimization of the sample dilution for TNF-α measurement from LPS stimulated cells

LPS (final concentration 1 µg/mL) was added to 6-plates with differentiated macrophages (1x 10^6 cells/ mL) rested in culture medium without PMA and incubated for 2 hours at 37˚C. The cell supernatant was collected and tested for TNF-α production by ELISA (Section 3.2.3.5). The test samples were diluted in duplicate by 1/10, 1/50, 1/100 and 1/200 in 1x assay diluent A.

The concentration of TNF-α (pg/mL) was calculated from the regression equation generated from the standard curve in Figure 5.2A and the results are presented in Figure 5.2B. The levels of TNF-α produced by the test sample diluted by 1/200 was lower as compared to the other three test samples ($p<0.05$). However, no significant difference in TNF-α concentration was reported in test samples diluted by 1/10, 1/50 and 1/100 ($p>0.05$). The absorbance of the sample diluted by 1/10 was reported as 1.63± 0.17, which exceeds the absorbance of the top standard at 500 pg/mL (0.89± 0.14) (Appendix C.1). Thus, this sample was too concentrated and a 1/10 dilution was not suitable for subsequent experiments. The absorbance of the test sample at 450 nm should lie within the

linear range of the graph, i.e. 0.0-0.5. Thus, a dilution in the range of 1/50 to 1/100 was deemed appropriate for further experiments.



(A) (B)

Figure 5.2 (A) Standard curve for TNF-α ELISA and (B) production of TNF-α (pg/mL) in diluted supernatants of THP-1 macrophages stimulated with lipopolysaccharide (LPS) for 2 h. **Notes:** Data represents mean of two replicates with error bars corresponding to standard deviation. Samples that do not share a letter are significantly different ($p<0.05$). Raw and statistical data is shown in the Appendix C.

5.4.2 Optimization of time course for LPS incubation

The enhanced expression of inflammatory cytokines (IL-6, IL-8, IL-10 and TNF-α) caused by treatment of THP-1 macrophages with LPS can be detected within 1 h to 6 h of incubation (Chanput et al., 2014). It is thus necessary to determine the optimum time period at which significant amounts of TNF-α are produced by macrophages upon stimulation with LPS.

Figure 5.3 shows the of levels TNF-α detected in the supernatant of differentiated THP-1 cells stimulated with LPS (1 µg/mL) for 0.5, 1, 2, 4 and 6 hours. The data revealed that treatment of THP-1 cells with LPS up-regulated the TNF-α cytokine levels in a time dependent manner, as reported in previous studies (Chanput et al., 2010; Chang et al., 2012; Shi et al., 2016; Ma et al., 2017). The levels of TNF-α detected in test samples stimulated with LPS for 1 h and 2 h were fairly low, and were statistically similar to levels of TNF-α detected in the negative control ($p>0.05$). Therefore, no stimulation was seen until 4 h of LPS incubation.

Concentration of TNF-α (pg/mL) was highest in test samples incubated with LPS for 6 hours as compared to other test samples and negative control ($p<0.05$). Similar results were reported by Chanput et al. (2010) and they further suggested THP-1 macrophages stimulated with LPS for 6 hours as an adequate model for studying the inflammatory activity of food compounds. However, in the current study, a significant up-regulatory response of LPS towards macrophages was achieved after 4 hours of stimulation, with TNF-α levels measured as 12.4± 4.00 ng/mL (Appendix C.2). Thus, stimulation of macrophages with LPS for 4 hours was considered an optimum model for further analysis in phase II.

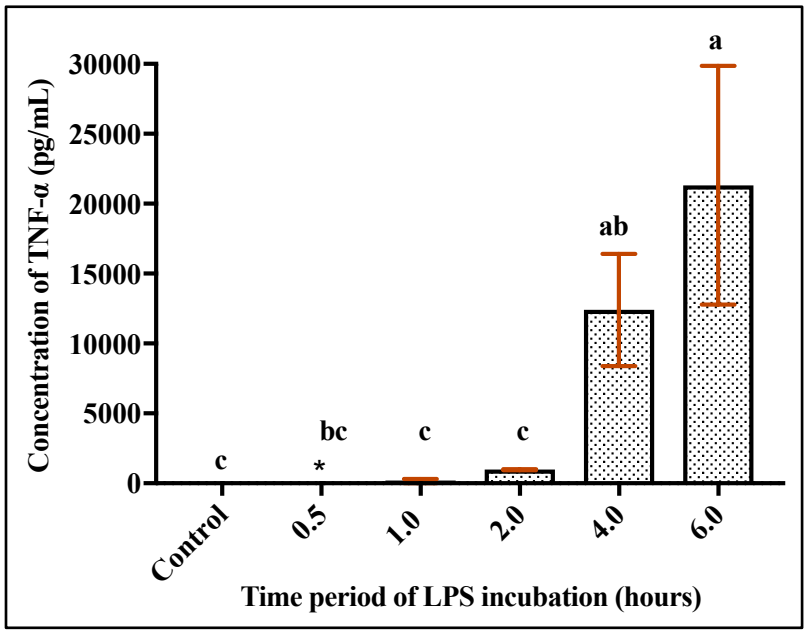


Figure 5.3 Production of TNF-α (pg/mL) in THP-1 macrophages stimulated with LPS for 30 minutes to 6 h. **Notes:** Data represents mean of three experiments with error bars corresponding to standard deviation. (*)- Data represents mean of two replicates. Samples that do not share a letter are significantly different ($p<0.05$). Raw and statistical data is shown in Appendix C.

5.4.3 Optimization of LPS dose-response for stimulation of THP-1 macrophages

Based on the literature, concentrations of LPS ranging from 50 ng/mL to 1000 ng/mL were tested to determine the optimum concentration of LPS for stimulation of THP-1 macrophages. The levels of TNF-α secreted into the supernatant of macrophages stimulated with LPS was tested by ELISA and compared to the negative control (cell supernatant without LPS).

Stimulation of differentiated THP-1 cells with LPS induced TNF-α gene expression even at doses as low as 50 ng/mL (Figure 5.4). The levels of TNF-α were highest in supernatants from the cells stimulated at 200 ng/mL LPS, and beyond this concentration, a decline in TNF-α levels was seen. No significance differences were found in the levels of TNF-α produced by cells stimulated with 50 ng/mL, 100 ng/mL or 500 ng/mL ($p>0.05$). However, the levels of TNF-α produced by cells stimulated with 1000 ng/mL were significantly lower ($p<0.05$) as compared to rest of the samples, with the average value calculated as 15.1± 3.54 ng/mL (Appendix C.3). Similar results were reported by Shi et al. (2016), where treatment of bovine mammary epithelial cells with LPS at final concentration of 1 μg/mL for 6 hours resulted in a decrease in cell viability by 22%.

Exposure of macrophages to LPS leads to enhanced production of proinflammatory cytokines and nitric oxide (NO) in dose-dependent manner (Kiemer, Müller & Vollmar, 2002). At high concentrations of LPS, the levels of NO increase significantly which results in cell toxicity leading to apoptosis (Zhuang & Wogan, 1997; Comalada et al., 2000). Further, Comalada et al. (2000) demonstrated that macrophage cell death occurs due to release of cytochrome C from the mitochondria, which activates apoptotic pathway. This pathway is activated by Bax, a Bcl-2-related pro-apoptotic gene whose transcription is regulated by NO activated p53 (Comalada et al., 2000). Therefore, it can be speculated that the decreased levels of TNF-α secreted by cells treated with LPS concentrations above 200 ng/mL may be due to the toxic and apoptotic action of nitric oxide on the macrophages. From this experiment it can be inferred that cells stimulated with 200 ng/mL LPS for 4 hours is an optimal model for inflammation related studies in present work.

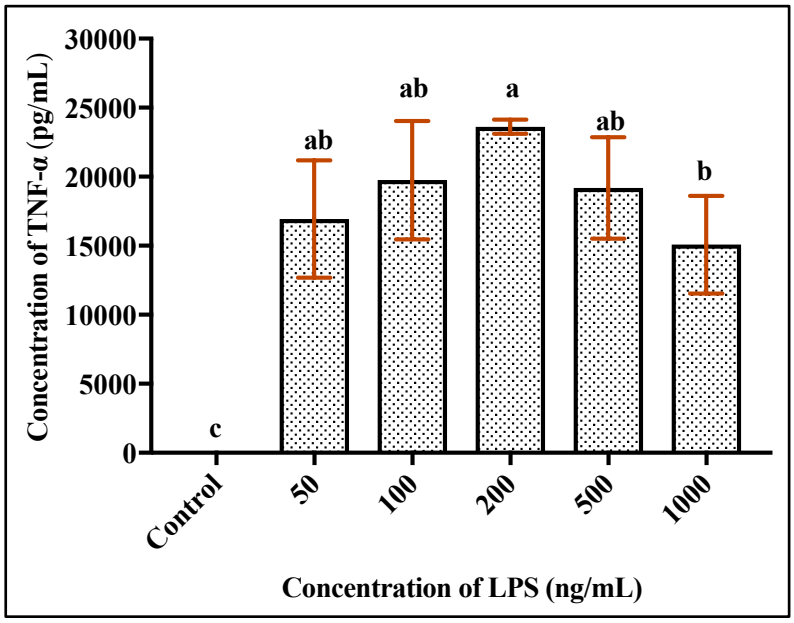


Figure 5.4 Production of TNF-α (pg/mL) by THP-1 macrophages stimulated with LPS (50 to 1000 ng/mL) for 4 h. **Notes:** Data represents mean of three experiments (n=3; three replicates in each assay) with error bars corresponding to standard deviation. Samples that do not share a letter are significantly different ($p<0.05$). Raw and statistical data is shown in Appendix C.

5.5 Treatment of LPS-stimulated THP-1 cells with curcumin/curcuminoids and DMSO

Experimental results obtained in Section 5.3 indicated that macrophages exposed to 200 ng/mL LPS for 4 h resulted in a significantly increase in secretion of TNF-α, as measured in the supernatant from these cells. This is an optimal model for examining the inflammation-immunomodulatory activity of curcumin/curcuminoids. Several *in vitro* studies have demonstrated the anti-inflammatory activity of curcumin/curcuminoids through their ability to suppress and inhibit the activation of inflammatory biomarkers in LPS stimulated cells, including transcription factors, growth factors, proinflammatory cytokines, enzymes, cell surface adhesion molecules and genes regulating cell proliferation and apoptosis (Section 2.6.2).

The current study was designed on the basis of pervious *in vitro* studies (Kumar, Dhawan, Hardegen & Aggarwal, 1998; Abe, Hashimoto & Horie, 1999; Hsu, Chu, Hua & Chao, 2008; Klawitter et al., 2012), where THP-1 cells were differentiated and stimulated with LPS to increase the levels of proinflammatory cytokines (TNF- α, IL-6, IL-8, IL-1) and transcription factor (NF-

kB) in the cells. To study the effect of food-grade curcuminoids (C7 and C9), the cells were pre-treated with these samples, as illustrated in Figure 3.2. The bioactivity of these food-grade samples was compared to cells treated with analytical grade curcumin (Pure C) and control vehicle DMSO. The results of this experiment are presented in Section 5.5.2.

Apart from the anti-inflammatory activity of curcumin, the cytotoxic effects of curcumin towards various cell lines have been reported (Section 2.5.1). Therefore, it is important to investigate whether the anti-inflammatory activity of curcumin is due to its apoptotic effects on the macrophages. To test the cytotoxicity of curcuminoids, the MTT assay was performed simultaneous to the TNF-α ELISA assay and the viability of cells treated with curcumin/curcuminoids was assessed. The results of the cell viability assay are presented in Section 5.4.1.

5.5.1 Cytotoxicity of curcumin/curcuminoids towards LPS-stimulated THP-1 macrophages

The effect of curcumin/curcuminoids on the viability of LPS stimulated macrophages exposed to curcumin/curcuminoids for 5 h was assessed by the MTT assay, as described in Section 3.3.2.5. The MTT colorimetric assay is one of the most common tests employed for the detection of cytotoxicity or cell viability following treatment with bioactive compounds (Fotakis & Timbrell, 2006). In the present work, the appropriate cell number and incubation time period for the MTT assay were determined prior to testing the cytotoxic effects of curcuminoids. The results of these experiments are discussed in the following sections.

5.5.1.1 Optimisation of cell density and incubation time for MTT assay

Differentiated THP-1 cells were seeded in 6-well plates at a density varying from 1.25x 10^5 cells/mL to 1x 10^6 cells/mL and incubated with the MTT reagent for three different incubation times (0.5- 3h), following the protocol described in Section 3.3.2.4.

The results obtained from the MTT assay performed on five different cell densities at three different incubation times are presented in Figure 5.5. Visual observation of the 96-well plates in Figure 5.5A, 5.5B and 5.5C showed a development of a purple color, which is due to reduction of yellow tetrazolium MTT dye into insoluble formazan crystals by the mitochondrial dehydrogenases of viable cells (Khattak et al., 2006; Werner et al., 2013). Further, the intensity of colour development increased in the time dependent manner, which was observed to be highest after 3 hours of incubation with MTT.

The results of visual observations were confirmed by measuring the absorbance of formazan product at 595 nm, as shown in Figure 5.5D. From the graph, a linear relationship of formazan absorbance with cell concentration and incubation time period can be observed. These results are in accordance with previous reports (Hussain, Nouri & Oliver, 1993; Khattak, Spatara, Roberts & Roberts, 2006; Gasque et al., 2014). Overall, the absorbance of formazan was highest in wells with a cell density of 1x 10^6 cells/mL at all three incubation times ($p<0.05$), with a value of 2.93± 0.08 at the end of a 3 h incubation with MTT in wells with cell density of 1x 10^6 cells/mL (Appendix B.4). This value lies outside the absorbance limit ($A_{595} \leq 1.5$) of this chemosensitive assay (Plumb, Milroy & Kaye, 1989; Fallon & Hellestad, 2008) and cell densities leading to absorbance values above 1.5 are not optimum for this assay (Moradi, Solgi, Najafi, Tanzadehpanah & Saidijam, 2018). Higher cell density interferes with the formation of formazan crystals, which results in incorrect absorbance values (Oostingh et al., 2011). In contrast, cells with lower density (up to 2.5x 10^5 cells/mL) are adversely affected by the high concentration of the test product, which also leads to false results (Gasque et al., 2014). Based on the absorbance data obtained in the current study (Appendix C.4), the macrophages seeded at a density of 5.0 x 10^5 cells/mL in a 6-well plate and incubated with MTT solution for 1.5 h was considered as the optimal conditions for assessing the cytotoxicity of curcumin samples.

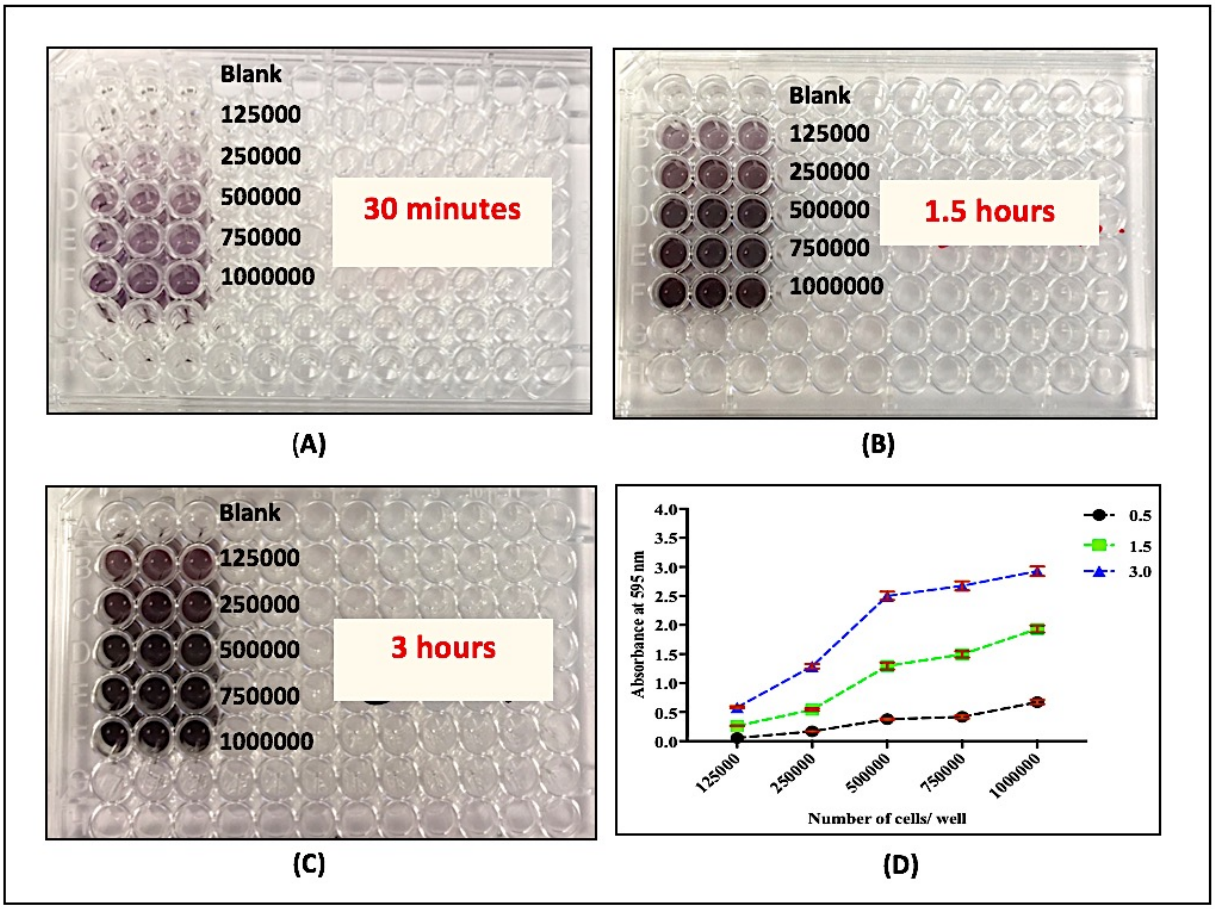


Figure 5.5 Formation of purple formazan by mitochondria of LPS stimulated macrophages seeded at a density of (1.25x 10^5/ ml) to (1x 10^6/ ml) and incubated with MTT solution for (A) 30 minutes, (B) 1.5 h and (C) 3 h. (D) Graphical representation of relation between number of cells seeded and the absorbance of formazan measured at 595 nm. **Notes:** Data represents mean of three replicates with error bars corresponding to standard deviation. Raw and statistical data is presented in Appendix C.

5.5.1.2 Effect on curcumin/curcuminoids on cell viability of LPS-stimulated macrophages

THP-1 macrophages were pre-treated with curcumin/curcuminoid-DMSO solution at final concentrations of 0.1µM to 50 µM for 1 h prior to LPS stimulation (Section 3.3.2.3) to determine the cytotoxicity of curcumin/curcuminoids and DMSO. Along with curcumin test samples, DMSO was used as a vehicle control and its cytotoxic effects on the LPS stimulated macrophages was tested at final concentrations of 0.001-0.5% using the MTT assay.

Cell viability was expressed relative to the negative control (causing no cell death) which was set at 100%. Stimulation of macrophages with curcumin samples for 5 h did not produce any toxic effects on the cells, with the exception of cells treated with 50 µM C7 and C9, which had decreased cell viability by approximately 50% (Figure 5.6A). A substance is classified as toxic if resultant cell number is reduced by half as compared to control (Meerloo, Kaspers & Cloos, 2011). Therefore, it can be interpreted that C7 and C9 are toxic to cells at concentration of 50 µM. The reason for the toxic effects of C7 and C9 towards THP-1 macrophages is unclear.

In this experiment, pure curcumin was found to be non-toxic up to a concentration of 50 µM upon 5 h of exposure to LPS stimulated cells (Figure 5.6A). This is in agreement with other findings which suggested that a 4-8 h of treatment with curcumin does not affect cell viability (Yue et al., 2012). In addition, it has been reported that treatment of cells with 50 µM curcumin for 48 h maintains the viability of THP-1 macrophages, without any significant toxic effects (Kong et al., 2016). In contrast, Yang et al. (2012) demonstrated that treatment of THP-1 macrophages with 50 µM curcumin for 24 h significantly induced apoptosis in these cells, resulting in cell death. Further, exposure of RAW 264.7 macrophages to curcumin concentrations above 20 µM for 24 to 48 h resulted in a significant decline in viable cell counts (Ma et al., 2017). Therefore, the effect of curcumin on cells appears to depend on the exposure time period, concentration of curcumin and type of cell line.

Along with curcumin test samples, the effect of DMSO (0.001-0.5%) on cell viability was also examined. DMSO is a polar solvent with low toxicity and has been widely used for the dissolution of pharmacological compounds (Galvao et al., 2014; Costa et al., 2017). Results obtained from treatment of LPS stimulated macrophages with DMSO for 5 hours are presented in Figure 5.6B. From this graph, it can be seen that DMSO is not toxic to cells at a final concentration of 0.5% (v/v). These results are in accordance with previous studies (Yue et al., 2012; Galvao et al., 2014; Costa et al., 2017), which demonstrate that 0.5% (v/v) DMSO does not affect cell viability even after 24 h of incubation.

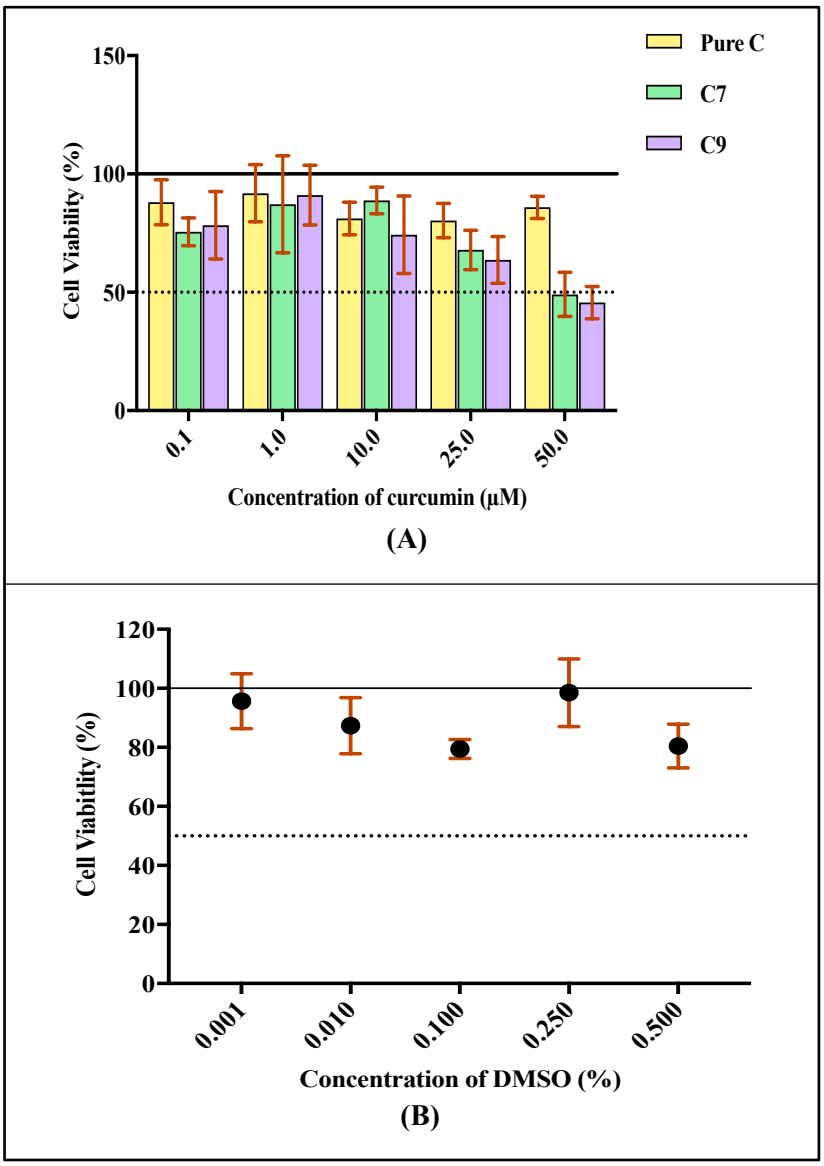


Figure 5.6 Cytotoxicity of (A) 0.5- 50 µM Pure C, C7 and C9 curcumin/curcuminoids and (B) 0.001- 0.5% (v/v) DMSO on exposure to differentiated macrophages for 1 h prior to stimulation of the cells with LPS for 4 h. Data was converted to % cell viability (Equation 2, Section 3.3.2.5) and expressed relative to the negative control (LPS stimulated cells without curcumin and DMSO) set at 100%. **Notes:** Data represents mean of three biological replicates (n=3; assayed in triplicate) with error bars corresponding to standard deviation. Raw and statistical data is shown in Appendix C.

5.5.2 Down-regulation of TNF-α expression by curcumin, C7 and C9

Investigation into the mechanisms by which curcuminoids cause anti-inflammatory effects revealed that curcuminoids may suppress inflammation by inhibiting the activation of transcription factors (NF-κB) and secretion of enzymes and proinflammatory cytokines (TNF-α, IL-6, IL-8 and

IL-1) (Section 2.6.). TNF-α is a 25 kDa transmembrane protein produced primarily by activated macrophages (Aggarwal, Gupta & Sung, 2013). The effect of curcumin/curcuminoids (Pure C, C7 and C9) on the TNF-α production in LPS stimulated macrophages was used as a measure to investigate the anti-inflammatory activity of these compounds. Simultaneously, the cells were treated with DMSO, at final concentrations of 0.001-0.5% (v/v) to investigate whether DMSO exhibits anti-inflammatory response in THP-1 macrophages (Figure 3.2). This concentration of DMSO used is equivalent to the concentration of DMSO in the curcumin working solution. The amounts of TNF-α accumulated in the supernatant of macrophages stimulated with LPS (with or without curcumin) was determined by ELISA.

The results showed that all tested curcumin/ curcuminoids down-regulated the secretion of TNF-α in a dose-dependent manner ($p<0.05$) (Figure 5.7). The levels of TNF-α produced by the cells stimulated with LPS were reported as 28.80± 5.43 ng/mL (Appendix C.7). However, pre-treatment of cells with 50 µM Pure C, C7 and C9 for 1 h prior to LPS incubation suppressed the secretion of TNF-α in activated macrophages, which a decrease in TNF-α levels by 21-fold, 7-fold and 10-fold respectively (Figure 5.7A). Further, a Tukey's pairwise comparison test (at 95% confidence level) indicated that all three types of curcumin samples presented a similar suppression effect on TNF-α production (Figure 5.7C). However, it must be noted that 50 µM C7 and C9 also resulted in a decrease in cell viability by approximately 50%, as previously discussed in Section 5.5.2. This decreased secretion of TNF-α may be partly due to the cytotoxic effect of these test samples on the cells. At lower concentration of 10 µM, C7 and C9 suppressed the TNF-α levels by approximately 2.5-fold without affecting the cell viability (Figures 5.6A & Figure 5.7A).

Several studies have demonstrated the ability of curcumin to suppress the expression of TNF-α secreted by activated macrophages (Abe et al., 1999; Giri, Selvaraj & Kalra, 2003; Yun, Jialal & Devaraj, 2011; Yang et al., 2012; Liu et al., 2014; Ma et al., 2017). In most of these studies, THP-1 cells have been treated with curcumin for 24 h or more to achieve an anti-inflammatory response. However, in the current study, treatment of LPS stimulated THP-1 macrophages with curcumin for only 5 h was shown to exhibit an anti-inflammatory response in these cells, by down-regulating the secretion of TNF-α.

Based on the TNF-α ELISA results, it can be speculated that food-grade curcuminoids C7 and C9 exhibited a down-regulatory effect on the expression of TNF-α in macrophages, and the anti-inflammatory activity of C7 was comparable to activity of C9, at all tested concentrations (Appendix C.7). C9 is a formulated curcumin complex containing 65% curcuminoids and 20% maltodextrin (Table 3.1). Previous studies have indicated that curcumin delivered in maltodextrin carrier improves the stability (Cano-Higuita, Malacrida & Telis, 2015; Wang, Ye, Wei & Zhao, 2017), solubility (Sousdaleff et al., 2013) and bioavailability (Chaurasia et al., 2015) of curcumin. Curcumin combined with phosphatidylcholine-maltodextrin increased the bioavailability of curcumin by 130-fold as compared to unformulated curcumin (Chaurasia et al., 2015). However, in this study t curcuminoids loaded in maltodextrin (C9) had similar bioactivity compared to unformulated curcumin, C7. Nevertheless, these findings require further investigation to examine the role of C7 and C9 in down-regulating other inflammatory biomarkers such as transcription factor (NF-kB), cytokines (IL-6, IL-8, IL-1) and NO production.

Treatment of LPS stimulated macrophages with DMSO (0.001 to 0.5%; v/v) did not affect the viability of macrophages (Section 5.4.1.2). From Figure 5.7B, it can be seen that DMSO did not have any effect on the TNF-α production at final concentration of up to 0.01%, with the levels of TNF-α produced being statistically similar ($p>0.05$) to TNF-α produced by control cells (macrophages stimulated with LPS). However, LPS stimulated THP-1 macrophages exposed to higher concentrations of DMSO (0.5%) for 5 h suppressed the secretion of TNF-α by almost two-fold (Figure 5.7B, as measured in the supernatant of these activated macrophages. In relation to this, some studies have demonstrated a possible anti-inflammatory activity of DMSO in various human cell lines. An *ex-vivo* study conducted by Elisia et al. (2016) demonstrated the ability of DMSO (0.5-2%, v/v) to suppress the production of IL-6, TNF-α and PGE_2, inactivation of ERK1/2, p38, JNK and Akt pathways in human blood cells infected with *Escherichia coli* and herpes simple virus-1 (HSV-1). In another study, it was reported that exposure to DMSO levels at 5% to 10% for 12 h significantly lowered the expression of TNF-α and IL-2 cytokine production in peripheral blood lymphocytes differentiated with PMA (Costa et al., 2017). However, the suppressing activity of DMSO is significantly lower as compared to that resulting from incubation with Pure C, C7 and C9 at a final concentration of 50 μM ($p<0.05$).

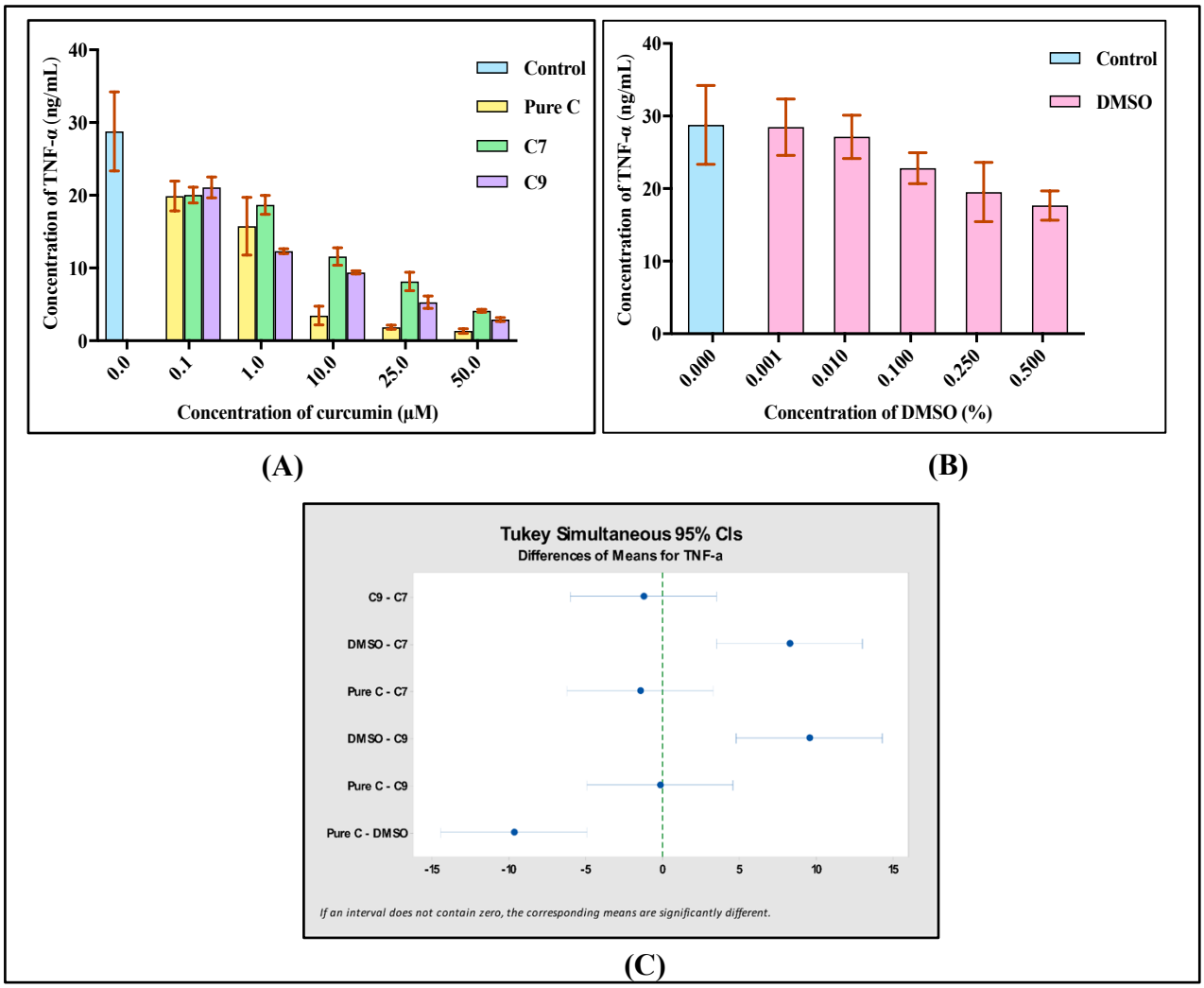


Figure 5.7 Amounts of TNF-α (ng/mL) measured in the supernatants of differentiated THP-1 macrophages exposed to (A) 0.1 to 50 µM Pure C, C7 and C9 curcumin/curcuminoids and (B) 0.001- 0.5% (v/v) DMSO for 1 h prior to stimulation of these cells with LPS for 4 h. The concentration of DMSO used are equivalent to concentration of DMSO in curcumin working solutions. Results showed the anti-inflammatory effects of DMSO is significantly lower as compared to curcumin/curcuminoids test samples, as calculated by (C) Tukey's multiple comparison test at 95% confidence level in Minitab 18. **Notes:** Data represents mean of three biological replicates (n=3; assayed in replicate) with error bars corresponding to standard deviation. Raw and statistical data is shown in Appendix C.

5.6 Summary of phase II

The main aim of phase II was to investigate the anti-inflammatory activity of food-grade curcuminoids by studying their ability to down-regulate TNF-α secreted by LPS stimulated macrophages *in vitro*. Results obtained from the experiments conducted in this phase demonstrated that treatment of THP-1 macrophages with food-grade curcuminoids (C7 and C9) for 1 h prior to exposure of these cells to LPS for 4 h, caused a dose-dependent suppression in TNF-α production, in the supernatant of these activated macrophages. Further, ELISA and MTT cell viability assay indicated that a final concentration 10 μM C7 and C9 suppressed the TNF-α levels by approximately 2.5-fold without any loss of the cell viability. Overall, these results indicate that the food-grade curcuminoids are bioactive, however, their anti-inflammatory activity is lower as compared to analytical grade curcumin but significantly higher as compared to the control vehicle DMSO. Thus, the down-regulation in TNF- α expression is solely due to the activity of curcuminoids. As C7 and C9 exhibited significantly similar bioactivity, both samples are suitable for addition to the yogurt prepared in phase III.

CHAPTER 6
DEVELOPMENT OF COCONUT YOGURT SUPPLEMENTED WITH FOOD-GRADE CURCUMINOIDS

6.1 Introduction

Yogurt can be produced from different type of milks including bovine, goat, soy and coconut milk, which contain different fermentable sugars. The fermented yogurt can be produced by fermenting one or more combinations of ingredients such as cream, milk, partially skimmed milk and skim milk with a characteristic bacterial culture containing lactic acid producing bacteria (*L. bulgaricus* and *S. thermophilus)* (Weerathilake, Rasika, Ruwanmali & Munasinghe, 2014). Coconut milk contains sugars (mainly sucrose), starch and the minerals (phosphorous, calcium and potassium) which makes it a suitable medium for growth of lactic acid bacteria (Yuliana, Rangga & Rakhmiati, 2010). Coconut milk is an opaque white protein-oil-water emulsion, extracted from the solid endosperm of the mature coconut (*Cocos nucifera* L.) fruit (Seow & Gwee, 1997). The term 'coconut milk' is often used interchangeably with 'coconut cream' (Seow & Gwee, 1997), however, these two terms vary due to differences in the fat content with 10% fat in former compared to 20% fat in latter (Codex Alimentarius Commission, 2003).

Yogurt is well-known for its high nutritional value and health promoting benefits (Zoidou et al., 2014). The health promoting properties of yogurt can be further enhanced by the addition of bioactives from green tea and coffee powder (Dönmez, Mogol & Gökmen, 2017), garlic powder (Cho, Kim & In, 2007), turmeric powder (Fu et al., 2016), olive fruit (Petrotos et al., 2012) and berries (Nile & Park, 2014). However, the addition of bioactives may adversely affect certain characteristics of the yogurt such as chemical and textural properties, microflora and sensory attributes (Petrotos et al., 2012). Therefore, one of the major objectives of phase III was to determine the effect of curcuminoids on the characteristics of yogurt during fermentation and storage at 4°C.

The factors which limit the usage of curcuminoids in food products have been covered in Chapter 2, which discussed the development of suitable food matrices for the delivery of bioactives. Curcuminoids are relatively stable in acidic media (Section 2.3.2) and lipid-based emulsions (Section 2.6.3). Therefore, the current study aims to investigate the application of fermented coconut milk as a potential delivery vehicle for curcuminoids. The detailed methodology of phase III is presented in Section 3.4, and the results are discussed in this chapter.

6.2 Fermentation of coconut milk and cream

In the preliminary experiment of phase III, various commercial coconut milks/ creams were fermented (Table 3.3) with YO MIX® 726 LYO 50 DCU starter culture containing *S. thermophilus, L. delbrueckii* spp. *bulgaricus, Biofidobacterium lactis* and *L. acidophilus.* Ultra-heat treated (UHT) or canned commercial coconut products were used in this study to ensure safe manufacture of yogurt, since fresh coconut milk is susceptible to contamination as it supports the growth of various spoilage microorganisms and coliforms (Seow & Gwee, 1997). The objective of this experiment was three-fold: to select a suitable coconut milk/cream as an ingredient for the production of yogurt; to determine the optimum fermentation time, and to characterise the yogurt gel.

Fermentation time is fundamental in yogurt manufacture (Soukoulis, Panagiotidis, Koureli & Tzia, 2007), as it provides information on the efficiency of the starter culture and rate of fermentation (Macedo, Soccol & Freitas, 1998). Fermentation of the different types of commercial coconut milk/creams reduced pH from 6.09 to 4.5 (Figure 6.1), which is considered as the end point of yogurt fermentation (Soukoulis et al., 2007). The decrease in pH indicated the successful fermentation of milk/cream to yogurt by the symbiotic activity of starter culture used (Jumah, Abu-Jdayil & Shaker, 2001). However, the fermentation of coconut yogurt varied among the commercial milks/creams (Figure 6.1). The shortest fermentation time (4 h) to the desired pH (4.58) was observed in sample R3 whereas sample R5 took 8 h. From an industrial point of view, shorter incubation times are preferred as this enables increased production output (Macedo et al., 1998).

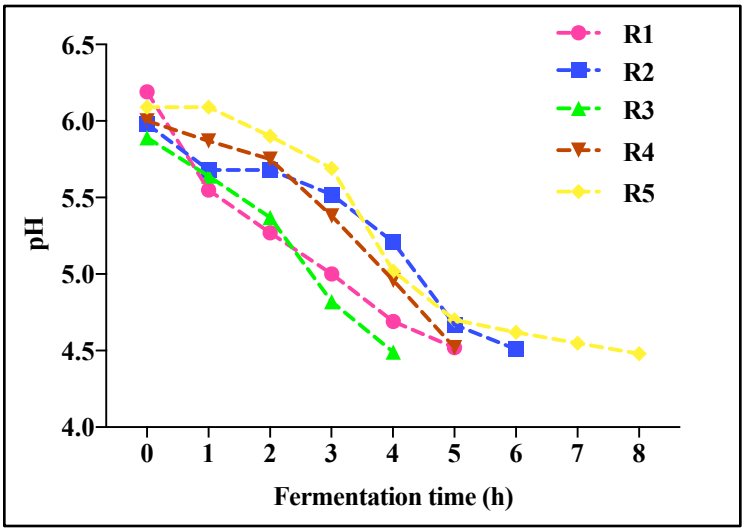


Figure 6.1 pH during fermentation of different commercial coconut milk/cream products at 42˚C. **Notes:** R1= So Good™ coconut milk, R2= Roar® coconut organic milk, R3= Pure Ceylon Kokonati™ organic coconut milk, R4= Spiral Foods™ organic coconut milk, R5= Kara™ coconut cream. Data represent mean of replicates (n=2).

Gel formation is the most important physical property of yogurt, and it is mainly governed by the protein content of the milk/cream (Bensmira, Nsabimana & Jiang, 2010). Nutritionally, coconut milk contains 2-4% protein (Table 4.1), of which >80% protein is characterised as albumins and globulins (Seow & Gwee, 1997). Only 30% of these albumins and globulins dissolve in the aqueous phase with the remaining protein acting as emulsifying agents (Seow & Gwee, 1997). During fermentation, the insoluble milk proteins bind to water and promote the coagulation of milk, resulting in the formation of a stable 3-D gel network at the isoelectric point (pH 4.5) with enhanced water absorption capacity, higher viscosity and a smooth consistency (Onsaard et al., 2006; Tamime & Robinson, 2007; Tangsuphoom & Coupland, 2008).

The composition of the milk/cream plays an important role in determining the structural properties of the final yogurt product (Walia, Mishra & Kumar, 2013). In this study, the final yogurt products prepared with coconut milk/ cream without additives (R2, R3 and R4) were unstable at pH 4.5 and formed a slightly turbid layer at the bottom with a lumpy middle layer and a hard opaque layer at the top (Figure 6.2A). Lumpiness is caused by protein aggregates with a size ranging from 1-5 mm (Isleten & Karagul-Yuceer, 2006). At pH 4.5, coconut proteins have low electric charge which influences the colloidal interactions between the proteins and leads to formation of aggregates (Onsaard et al., 2006). The lumps may also be formed due to oil droplet flocculation (Hagenmaier

et al.,1972), as the top layer of the fermented coconut milk/ cream stored at 4˚C was oily. According to Raghavendra & Raghavarao (2010), cooling of the coconut milk emulsion to 5˚C results in the separation of oil from the emulsion, thereby forming a solid layer of oil at the top of the fermented milk/cream.

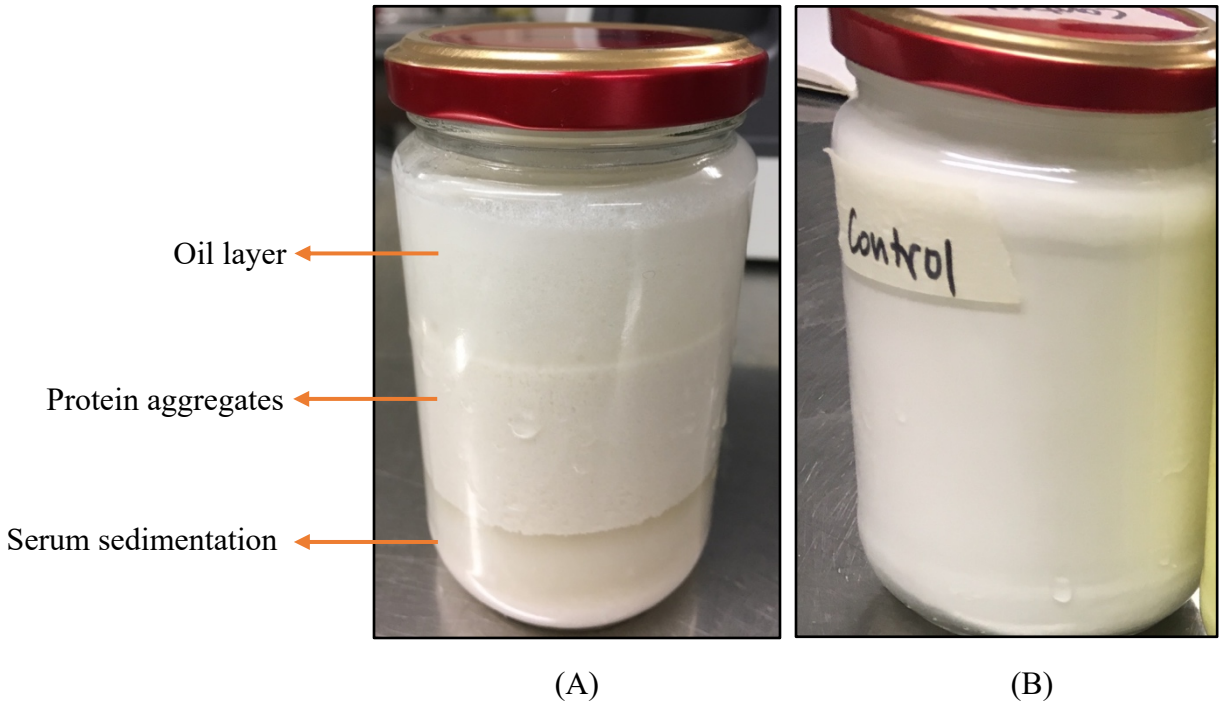


Figure 6.2 Coconut yogurt produced from R2: Roar® coconut organic milk (A) and R5: Kara™ coconut cream. Fermentation of R2 did not form yogurt gel and the milk constituents were separated into oil layer, protein layer and serum layer. However, fermentation of R5 formed a homogenized yogurt gel and no separation in oil-water emulsion or protein aggregation was observed. Image captured by Iphone 6S, Apple Inc., USA.

Two commercial products, R1 and R5 were stabilised with hydrocolloids (Table 3.3). R1 contained sunflower lecithin and gellan, while in R5, the coconut emulsion was stabilized with xanthan gum, guar gum and carrageenan (Table 3.3). Hydrocolloids promote gel stabilization by interacting with milk constituents (mainly proteins) and water, resulting in increased viscosity, which minimises syneresis and enhances texture and sensory properties (Soukoulis et al., 2007; Abdelmoneim, Sherif & Sameh, 2016). Apart from stabilizing the gel, gellan, carrageenan and xanthan gum act as gelling agents and form a three-dimensional gel network with the solvent entrapped in the interstices (Saha & Bhattacharya, 2010). After fermentation, R1 had a relatively weak and soft gel

while R5 was characterised by a semi-solid and consistent gel. Therefore, R5 has the potential to serve as a better ingredient for the production of yogurt for subsequent experiments.

6.3 Optimisation of curcuminoid concentration in coconut yogurt

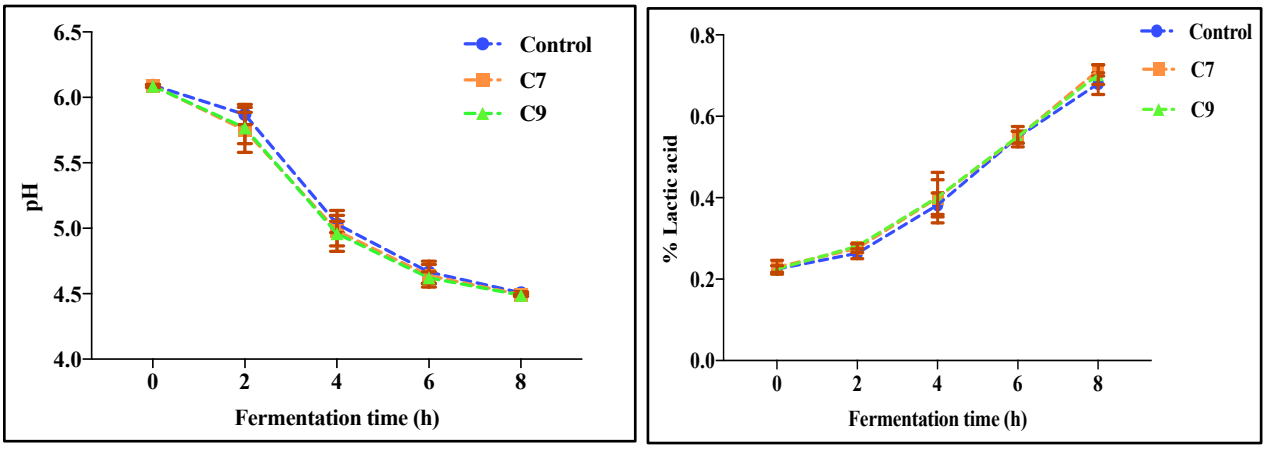
The results from phase II confirmed the potential anti-inflammatory activity of food-grade curcuminoids. Both C7 and C9 suppressed the levels of the inflammation biomarker, TNF-α, in the activated macrophages at 10 μM (Section 5.4.2). However, *in vitro* studies frequently display higher levels of bioactivity of the tested compounds that *in vivo* studies, therefore the results cannot be directly extrapolated to *in vivo* studies (Chanput, 2012). According to several reports, *in vivo* studies showed that only a minor percentage of curcuminoids are absorbed in the human body (Section 2.6.2). Therefore, the concentration of curcuminoids reported as effective in *in vitro* studies would not have a similar effect in the humans due to complexity of the body, which is due to numerous types of cells, tissues and organ systems (Chanput, 2012). These organ systems may respond differently to curcuminoids as compared to cells used in *in vitro* studies.

A dosage of curcuminoids as high as 8-12 g/day has been reported as safe for consumption with no adverse health effects, while however the recommended dosage of curcuminoids is 400-600 mg (three times/day) (Section 2.7.2). An average serving of yogurt is 150 g, and therefore it was decided that one serving of yogurt would contain 400 mg of curcuminoids. This yogurt can be consumed once to three times a day.

6.4 Effect of curcuminoids on fermentation of coconut cream

6.4.1 Acidity

During fermentation, the acidity of coconut yogurt (with and without curcuminoids) was measured by monitoring pH and titratable acidity. In general, the pH of coconut cream (with and without curcuminoids) inoculated with YO MIX® 726 LYO 50 DCU culture decreased ($p<0.05$) while the titratable acidity increased ($p>0.05$) during fermentation (Figure 5.3). These results are consistent with previous studies (Yaakob et al., 2012; Hamad, Ismail, El-Kadi & Zidan, 2016).



(A) (B)

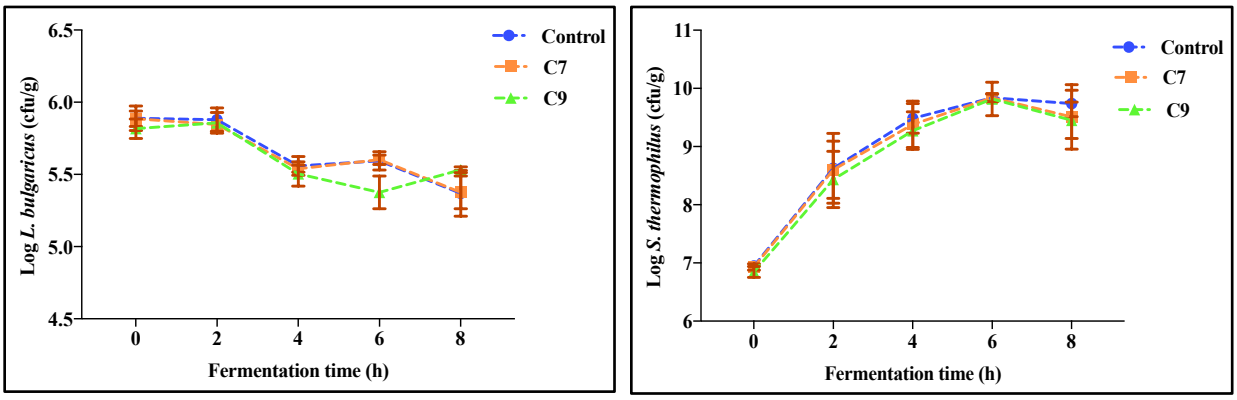
Figure 6.3 pH (A) and titratable acidity (B) of coconut cream (with or without curcuminoids) during fermentation at 42˚C. **Notes:** Sample C7= coconut yogurt containing 0.27% (w/w) curcuminoid; C9= coconut yogurt containing 0.27% (w/w) curcuminoid. Data represent mean of replicates (n=6) with error bars corresponding to standard deviation. Raw and statistical data are shown in Appendix D.

The initial pH of coconut cream was 6.09±0.01, while cream samples supplemented with 0.27% (w/w) C7 and C9 curcuminoids were 6.09±0.01 and 6.08±0.01, respectively (Appendix D.1). At the end of 8 h of fermentation, the pH of the yogurts decreased to 4.49-4.51 due to production of lactic acid and other organic acids (Kolapo & Olubamiwa, 2012). The trend of pH decrease in control yogurt was similar ($p>0.05$) to that of yogurts supplemented with curcuminoids. According to previous work of Foda et al. (2007) 1% (w/w) turmeric did not inhibit the proteinase activity of *L. bulgaricus* or *S. thermophilus*. Although in this study, proteinase activity was not measured, it appears that curcuminoids do not affect the fermentation of coconut cream yogurt.

The titratable acidity of yogurt is expressed as the amount of lactic acid and other organic acids produced by the starter culture microorganisms from metabolism of carbohydrates during fermentation (Tamime & Robinson, 2007). The titratable acidity of yogurt is inversely proportional to pH, and is expected to be >0.70%, as recommended by the International Dairy Federation (Yaakob et al., 2012). All three types of yogurts achieved acidity levels of more than 0.70% at the end of fermentation (Appendix D.1), and the acidity of yogurt increased with fermentation time, irrespective of the formulation (Figure 5.3B). There was also no difference ($p>0.05$) in the titratable acidity of coconut yogurt with and without curcuminoid enrichment (Appendix D.5).

6.4.2 Growth of *L. bulgaricus* and *S. thermophilus* during fermentation

The growth of *L. bulgaricus* and *S. thermophilus* was monitored at regular intervals during yogurt fermentation and the results are presented in Figure 5.4. The growth curves of *L. bulgaricus* (Figure 5.4A) and *S. thermophilus* (Figure 5.4B) were similar ($p>0.05$) in all three types of formulations, coconut yogurt and coconut yogurt enriched with C7 and C9. Thus, curcuminoids did affect the microbial activity of the starter culture.



(A) (B)

Figure 6.4 Data plotted as the logarithm of the microbial counts of (A) *L. bulgaricus* and (B) *S. thermophilus* in coconut cream (with or without curcuminoids) during fermentation at 42˚C. **Notes:** Sample C7= coconut yogurt containing 0.27% (w/w) curcuminoid, C9= coconut yogurt containing 0.27% (w/w) curcuminoid. Data represent mean of replicates (n=6) with error bars corresponding to standard deviation. Raw and statistical data are shown in Appendix D.

The growth pattern showed a steady increase in viable counts of *S. thermophilus* from an initial count of 6.83-6.94 log cfu/g to 9.83 log cfu/g till 6 h fermentation period, and remained constant ($p>0.05$) there after till the end of fermentation, in all three yogurt types (Appendix D.1). In contrast, the viable counts of *L. bulgaricus* were much lower compared to *S. thermophilus* during the fermentation period. However, this finding is consistent with previous studies (Ginovart et al., 2002; Walia et al., 2013). At the beginning of fermentation, the *L. bulgaricus* counts were 5.81-5.88 log cfu/g and reduced to 5.36-5.53 log cfu/g at the end of fermentation (Appendix D.1).

Traditionally, yogurt gels are formed by the fermentation of milk with starter culture consisting of *L. bulgaricus* and *S. thermophilus* in a 1:1 ratio (Lee & Lucey, 2004). As the fermentation commences, *S. thermophilus* grows more rapidly compared to *L. bulgaricus* as it utilises the amino acids produced by *L. bulgaricus* through fermentation of lactose into lactic acid and formic acid.

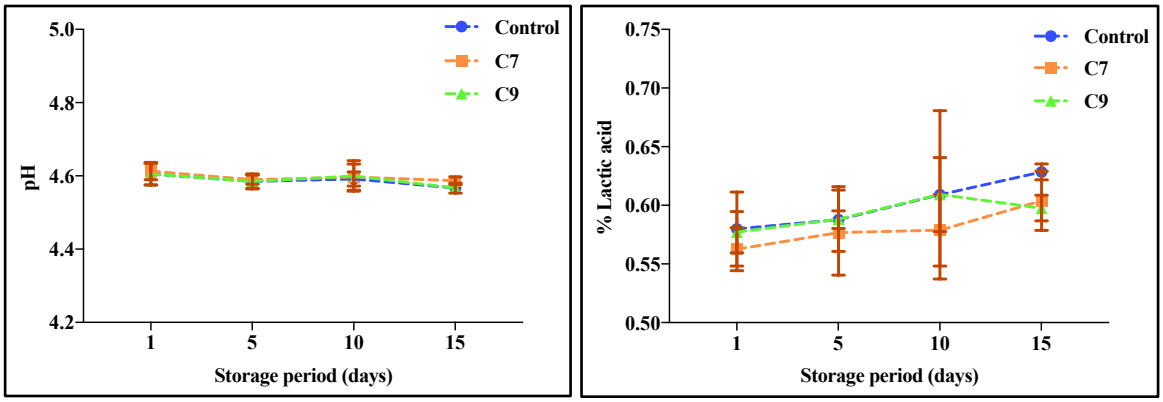
Normally the acidic environment favours the growth of *L. bulgaricus* while it limits the growth of *S. thermophilus* (Hamann & Marth, 1984). However, in the current study, no significant increase ($p>0.05$) in growth of *L. bulgaricus* was observed towards the end of fermentation. The contrasting results may be attributed to a difference in the carbohydrate substrate present in coconut milk. The main sugar in coconut milk is sucrose (Table 4.1) and the low ability of *L. bulgaricus* to metabolise sucrose has been reported (Yuliana et al., 2010). Meanwhile, *S. thermophilus* and *L. acidophilus* can metabolise sucrose into lactic acid via the Embden Meyerhof Parnas (EMP) pathway (Gomes & Malcata, 1999). Therefore, the fermentation of coconut cream yogurt may be mainly attributed to the activity of *S. thermophilus*.

6.5 Effect of curcuminoids on stability of coconut cream yogurt during storage (4°C)

6.5.1 Acidity

Changes in pH and titratable acidity (% lactic acid) of coconut cream yogurt (with or without curcuminoids) during storage at 4˚C for 15 days are shown in Figure 5.5. Overall, a decrease ($p<0.05$) in pH (Figure 5.5A) and an increase ($p<0.05$) in lactic acid (%) (Figure 5.5B) was observed during storage (4°C) for 15 days, irrespective of the formulation.

Furthermore, pH and titratable acidity decreased between day 1 and day 5 of storage, after which the two parameters remained stable ($p>0.05$). One of the possible reasons for the stability of the acidity of the yogurt may be the good buffering capacity of the minerals (mainly phosphates) present in the milk, post-fermentation (Lutchman et al., 2006). Another possible reason for stable acidity is the presence of anti-microbial compounds in the coconut cream, which restricts the rapid growth of starter culture in yogurt and prevents over-acidification during storage (Kolapo & Olubamiwa, 2012).



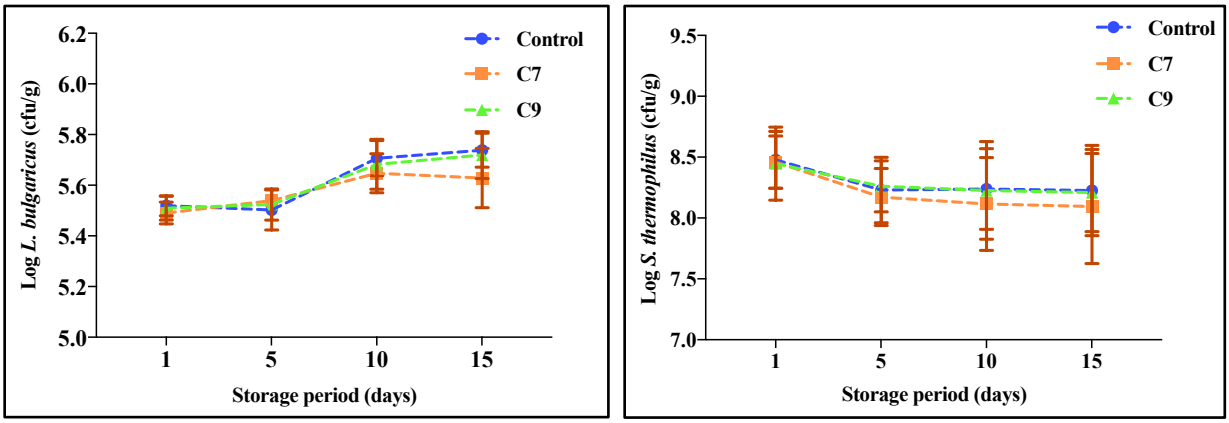
(A) (B)

Figure 6.5 pH (A) and titratable acidity (B) of coconut yogurt (with or without curcuminoids) during storage at 4˚C for 15 days. **Notes:** Sample C7= coconut yogurt containing 0.27% (w/w) curcuminoid, C9= coconut yogurt containing 0.27% (w/w) curcuminoid. Data represent mean of replicates (n=6) with error bars corresponding to standard deviation. Raw and statistical data are shown in Appendix D.

The pH of all yogurts reduced ($p<0.05$) from 4.61 at day 1 to 4.57 at the end storage, while lactic acid increased from 0.56% to 0.62% (Appendix D.2). The changes in pH and titratable acidity may be due to acid production during post-fermentation (Beal et al., 1999; Foda et al., 2007). However, changes in acidity of yogurts were not affected by the addition of curcuminoids as the acidity of coconut yogurt supplemented with 0.27% (w/w) curcuminoids was not different ($p>0.05$) from the control. The results are consistent with previous studies which reported that addition of bioactive spices to yogurt did not significantly affect the acidity of the final product during refrigerated storage (Foda et al., 2007; Behrad et al. 2009; Illupapalayam, Smith & Gamlath, 2014).

6.5.2 Survival of *L. bulgaricus* and *S. thermophilus* during storage

The survival of *L. bulgaricus* and *S. thermophilus* in coconut yogurt during storage is presented in Figure 5.6. A significant increase ($p<0.05$) in *L. bulgaricus* counts and decrease ($p<0.05$) in *S. thermophilus* counts were observed after 24 h of refrigerated storage in all yogurt types (Figure 5.6A& 5.6B). The decrease in *S. thermophilus* counts may be attributed to the increase in acidity (El-Kadi, Ismail, Hamad & Zidan, 2017) (Figure 5.5B). It is possible the increase in lactic acid, which occurred during storage may have established an environment which supported the growth of *L. bulgaricus* and suppressed the growth of *S. thermophilus* (Hamann & Marth, 1984).



(A) (B)

Figure 6.6 Data plotted as the logarithm of the microbial counts of (A) *L. bulgaricus* and (B) *S. thermophilus* in coconut cream (with or without curcuminoids) during storage at 4˚C for 15 days. **Notes:** Sample C7= coconut yogurt containing 0.27% (w/w) curcuminoid, C9= coconut yogurt containing 0.27% (w/w) curcuminoid. Data represent mean of replicates (n=6) with error bars corresponding to standard deviation. Raw and statistical data are shown in Appendix D.

In general, the microbial activity of starter culture bacteria decreases during refrigerated storage of food products (Yaakob et al., 2012). According to Food Standards Australia New Zealand (2015), yogurt must contain a viable microbial count of at least 10^6 cfu/g (or log 7 cfu/g) during its shelf-life period. In this study, *S. thermophilus* counts in the yogurt were > log 8 cfu/g during storage, while *L. bulgaricus* counts were 5.7 log cfu/g at the end of storage. Our results are in agreement with the findings of El-Kadi et al. (2017), who reported a higher survival rate of *S. thermophilus* in coconut yogurt, which may be attributed to lower acidity compared to bovine milk yogurt. The limited growth of *L. bulgaricus* in refrigerated yogurt may be attributed to the presence of non-fermented sucrose which can reduce the viability of lactic acid bacteria (Micanel, Haynes & Playne, 1997). Lastly, the low viable counts may be associated with the weakness of the strain or low proportion of *L. bulgaricus* present in the commercial starter culture, which allows production of yogurt with mild acidity and lower risk of post-acidification (Birollo, Reinheimer & Vinderola, 2000).

The growth patterns of the starter culture (Figure 5.6) showed that *L. bulgaricus* and *S. thermophilus* exhibited similar ($p>0.05$) metabolic activity in control yogurt and curcuminoid-supplemented yogurts. Therefore, it appears that the addition of curcuminoids (0.27%, w/w) did not influence the survival and acidification activity of the starter culture. These results are in

agreement with reports of a positive interaction of curcumin with lactic acid bacteria without any modification in its growth and acid gelation properties (Khanji et al., 2018). In contrast, Fu et al. (2016) reported one log reduction of the lactic acid bacteria counts in buttermilk yogurt containing 0.3% (w/w) curcuminoids. This difference may be due to use of different milks used for the yogurt as lactic acid bacteria have been reported to have higher survival rates in coconut milk yogurt compared to bovine milk yogurt (El-Kadi et al., 2017). Thus, components in coconut milk may be protective to the lactic acid bacteria against any anti-microbial actions of the curcuminoids.

6.5.3 Syneresis

Syneresis or serum separation from a gel matrix is a technological defect in set yogurt. It is therefore important to evaluate syneresis of set yogurt during storage (Dönmez, Mogol & Gökmen, 2017). Figure 6.7 shows data on syneresis of the fermented coconut yogurts produced in this study. Syneresis remained stable during storage ($p>0.05$), in all yogurt types. The addition of curcuminoids (0.27%, w/w) did not have a significant effect ($p>0.05$) on syneresis in the yogurts stored at 4˚C for up to 10 days (Figure 6.7). However, day 15 results showed an approximately two-fold increase in serum separation in the coconut yogurt supplemented with C9 curcuminoids compared to the control yogurt (Appendix D).

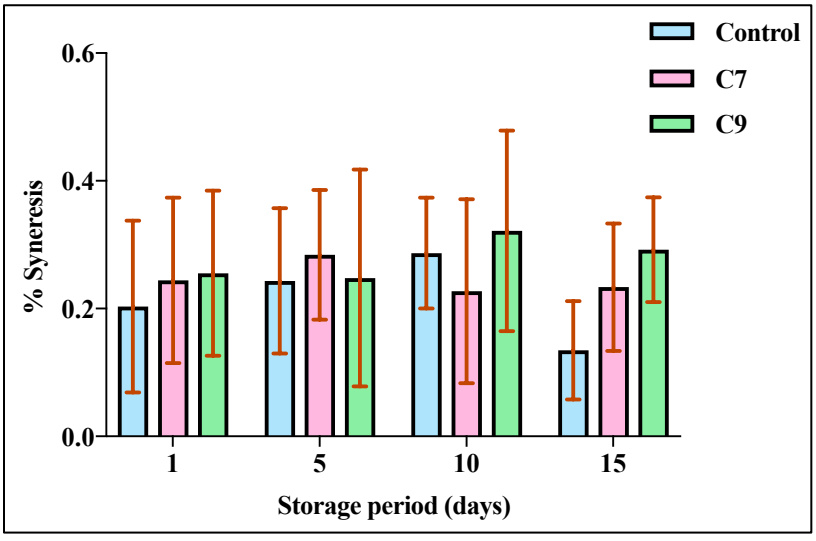


Figure 6.7 Syneresis (%) in coconut yogurt (with or without curcuminoids) during storage (4˚C) for 15 days. **Notes:** Sample C7= coconut yogurt containing 0.27% (w/w) curcuminoid, C9= coconut yogurt containing 0.27% (w/w) curcuminoid. Data represent mean of replicates (n=6) with error bars corresponding to standard deviation. Raw and statistical data are shown in Appendix D.

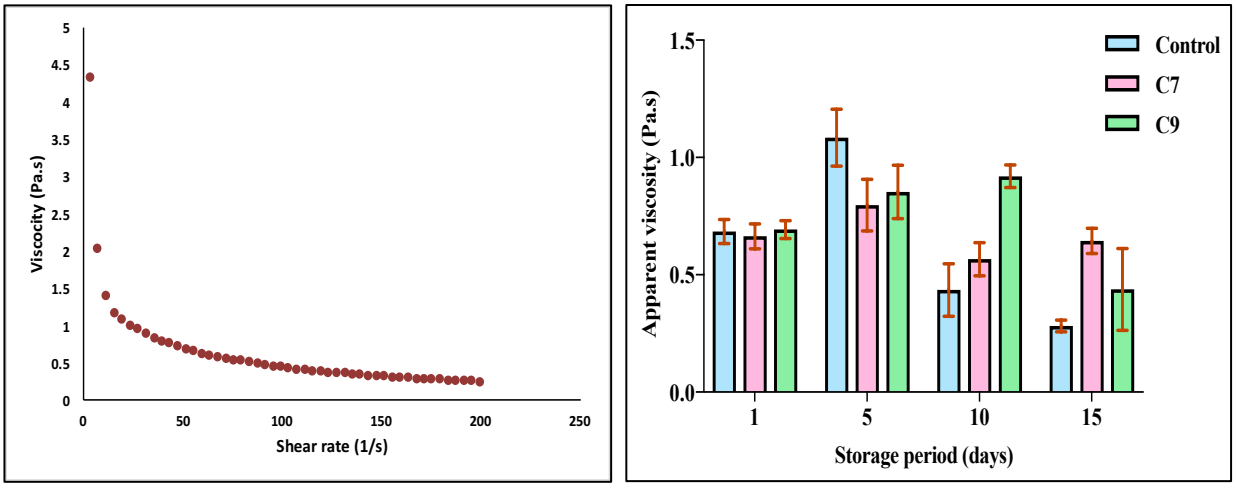
The level of syneresis in set yogurt acid gels depends upon several factors such as incubation temperature, excessive processing of the yogurt mix, total solids content (protein and/or fat), and acid production (pH>4.8) (Vareltzis et al., 2016). Previous studies have reported a significant co-relation between serum separation (syneresis) and post-acidification of yogurt gel (Foda et al., 2007; Dönmez et al., 2017). Small changes in pH could trigger instability of the 3-dimensional gel network, thereby promoting serum release from the gel (Dönmez et al., 2017). In the current study, a decrease ($p<0.05$) in pH from 4.61 after 24 hours of yogurt storage to 4.57 at the end of storage was observed in the coconut yogurt samples (section 6.6.1). However, this change in pH did not increase syneresis in the coconut yogurt. The low syneresis profile of coconut yogurt may be attributed to the high total solid content of coconut cream (26% (w/w) fat and 2% protein (w/w)), which reduces serum separation in yogurt during storage (Harwalkar & Kalab, 1983).

6.5.4 Apparent viscosity of broken yogurt gel

The flow behaviour of yogurt is generally characterized by its viscosity (Wu et al., 2009), which is the measure of internal resistance of yogurt to flow. The viscosity of yogurt gel is directly related to human perception of mouth feel or thickness of the product when the shear rate is 60 s^{-1} (Steffe, 1996; Henrysson, 2016). The apparent viscosity of yogurt samples was calculated at a shear rate of 60 s^{-1} and the results are presented in Figure 5.8. After 24 hours of storage, the average apparent viscosity of coconut yogurt samples was 0.67 Pa.s, (Appendix D). From day 1 to day 5 of storage, the viscosity of control yogurt increased markedly ($p<0.05$) and thereafter a steady decrease ($p<0.05$) was seen in the viscosity of control yogurt.

Viscosity analysis after 24 h of refrigerated storage showed that viscosity of control coconut yogurt was comparable ($p>0.05$) to viscosity of curcuminoid-coconut yogurts. However, day 5 analysis reported a higher ($p=0.042$) viscosity of control yogurt as compared to curcuminoid enriched yogurt. Furthermore, the viscosity of control yogurt decreased ($p<0.05$) by day 10 of storage while the viscosity of curcuminoid enriched yogurts remained stable. At the end of storage period, the viscosity of C9 enriched yogurt decreased ($p<0.05$) and was similar ($p>0.05$) to viscosity of control yogurt. In contrast, the viscosity of C7 enriched yogurt remained stable during the 15-day storage period (Figure 6.8). Therefore, in this study, the addition of curcuminoids to coconut yogurt increased or decreased the viscosity of yogurt in an irregular trend.

Yogurt is a non-Newtonian fluid system that shows a time-dependent 'shear thinning' behaviour (Ozer & Kirmaci, 2010; Joon et al., 2017), where a continuous and irreversible decrease in apparent viscosity occurs with increase in time and/or rate of shearing (Abu-Jdayil, Shaker & Jumah, 2000). The 'shear thinning' behaviour of coconut yogurt manufactured in this study is shown in Figure 5.8A. The apparent viscosity of yogurt was probably affected by factors such as the chemical composition of the milk, incubation and storage temperature, shear rate and/or time and presence of polyphenols (Wu et al., 2009; Dönmez et al., 2017). In this study, curcuminoids were seen to impact the viscosity of coconut yogurt during storage, following an irregular trend. Dönmez et al. (2017) reported similar results, where addition of polyphenols (green tea powder and coffee powder) increased or decreased the viscosity of set dairy yogurt during the storage period. In contrast, Fu et al. (2016) reported a continuous increase in viscosity of buttermilk yogurts supplemented with (0.3%, w/w) curcuminoids during storage. The reasons for the viscosity findings in the current study remain unclear.



(A) (B)

Figure 6.8 (A) Viscosity curve of C7 enriched coconut yogurt at day 5 of refrigerated storage. (B) apparent viscosity (at 60 s^{-1} shear rate) of coconut yogurt (with or without curcuminoids) during storage at 4˚C for 15 days. **Notes:** Sample C7= coconut yogurt containing 0.27% (w/w) curcuminoid; C9= coconut yogurt containing 0.27% (w/w) curcuminoid. Data represent mean of replicates (n=6) with error bars corresponding to standard deviation. Raw and statistical data are shown in Appendix D.

6.5.5 Firmness

The firmness of yogurt gel is one of the most important textural characteristics (Izadi, Nasirpour, Garoosi & Tamjidi, 2014), which impacts on appearance, mouth feel and overall acceptability of the yogurt (Ares, Paroli & Harte, 2006). The firmness of the control and curcuminoid enriched yogurts was determined from the highest peak of the graph on texture (Figure 6.9B) and the results are presented in Figure 6.9A. Overall, the firmness of control coconut yogurt was higher ($p<0.05$) as compared to curcuminoid enriched yogurts during the storage period (Figure 6.9A), which indicates a negative co-relation between coconut yogurt and curcuminoids. The lower firmness corresponds to a weaker gel network (Lee & Lucey, 2010), which may reflect the weak interaction of curcuminoids with coconut milk proteins (Sah, Vasiljevic, McKechnie & Donkor, 2016). However, no significant difference ($p<0.05$) was noted in the gel firmness of coconut yogurt supplemented with C7 and C9 curcuminoids during storage. During storage, the firmness of yogurt increased ($p<0.05$) until day 10, after which it remained stable, irrespective of the formulation (Appendix D). Similar results were reported in a vegetable oil-based yogurt, where the firmness of yogurts increased until 8 days of storage and remained constant till the end of 20-days storage period (Barrantes et al., 1996). The increase in gel firmness may be attributed to increased production of acidity in the yogurt gel (section 6.6.1), which causes the gel to shrink thereby increasing gel strength (Sah et al., 2016).

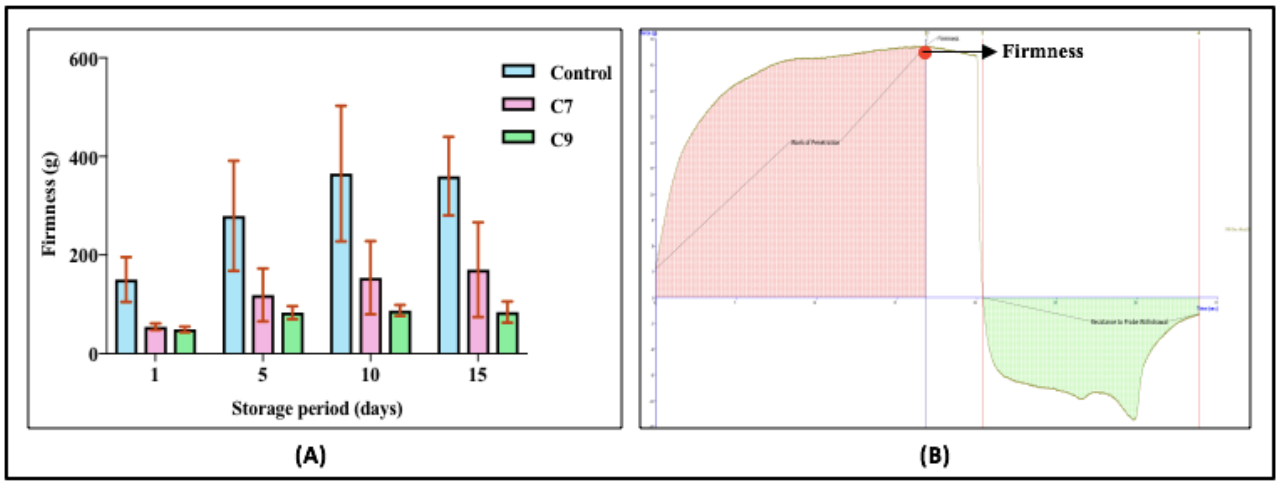


Figure 6.9 Firmness (g) (A) of coconut yogurt (with or without curcuminoids) during storage (4˚C) for 15 days. Figure (B) was obtained from penetration test of C7 sample at day 5. **Notes:** Sample C7= coconut yogurt containing 0.27% (w/w) curcuminoid, C9= coconut yogurt containing 0.27% (w/w) curcuminoid. Data represent mean of replicates (n=6) with error bars corresponding to standard deviation. Raw and statistical data are shown in Appendix D.

6.5.6 Sensory Evaluation

Sensory properties of foods are of utmost importance to the consumer as they contribute to the acceptance of the products (Day et al., 2009). In this study, the sensory characteristics of yogurt such as appearance, flavour, texture and colour, along with the overall acceptability were evaluated by consumer panellists (n=90). The sensory scores of control and curcuminoids enriched yogurts stored for 24 h and 15 days are presented in Figure 6.10. Overall, control yogurt (without curcuminoids) stored for 24 h and 15 days received the highest scores (7.07±0.22 and 7.01±0.22) for overall acceptability (Appendix D), on the 9-point hedonic scale. The average sensory scores for the control sample was >7 for the attributes comprising appearance, colour and texture. The average flavour scores for all three yogurt types were 6.93±0.57 and 6.87±0.27 for day 1 and day 15, respectively (Appendix D).

Overall, the sensory characteristics of all yogurts were stable ($p>0.05$) during storage (Appendix x). None of the panelists reported any 'turmeric like taste or flavour' in any of the fortified coconut yogurts. This may be attributed to the ability of coconut yogurt to mask the flavour of curcuminoids (Luckow, Moskowitz, Beckley, Hirsch & Genchi, 2005). However, it was noted that coconut yogurt supplemented with C7 was least preferred by the consumers and obtained lower scores ($p<0.05$) for appearance and colour compared to the control yogurt. The addition of C9 curcuminoids did not impact on the sensory attributes of the coconut yogurt ($p>0.05$). The lower scores of colour and appearance of C7 and C9 yogurt may be due to its yellow colour, induced by the bioactive curcuminoids.

In addition to the quantitative sensory scores, some panellists provided written comments about the products. The most common comments given by sensory panellists were 'creamy and smooth texture' and 'mild sweet taste'. The high fat content of coconut cream contributes towards the creamy texture of the yogurt while the sweetness of coconut yogurt is mainly due to carbohydrates (monohydrates) present in the coconut milk (Sanful, 2009). The creamy texture of yogurt may be also attributed to the activity of *S. thermophilus* during fermentation, which produces diacetyl, a compound that gives yogurt its characteristic buttery flavour (Hamann & Marth, 1984). The panellists did not detect any undesirable flavour and texture of yogurt enriched with curcuminoids.

This result suggests that the fermentation produced a balanced curcuminoid-enriched yogurt. Storage (4°C) of yogurt for 15 days did not affect its sensory characteristics.

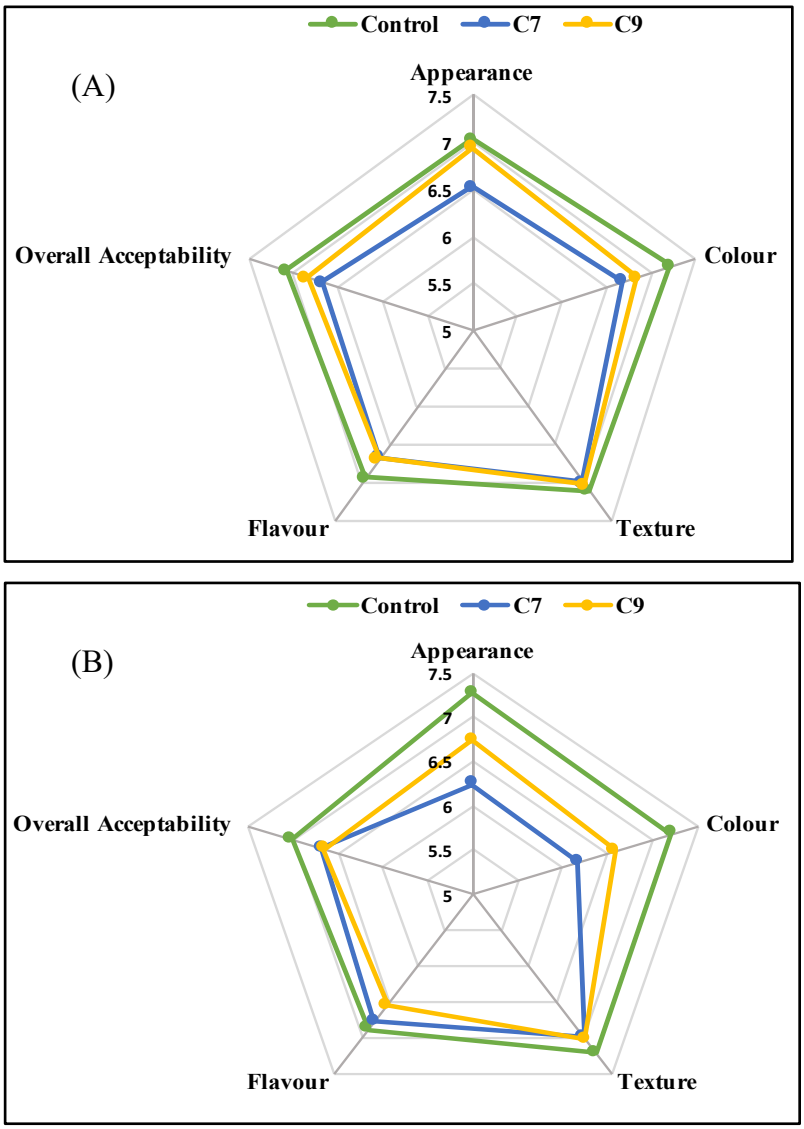
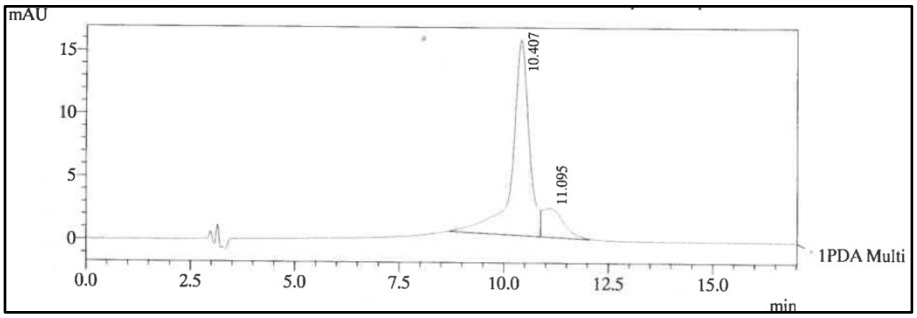


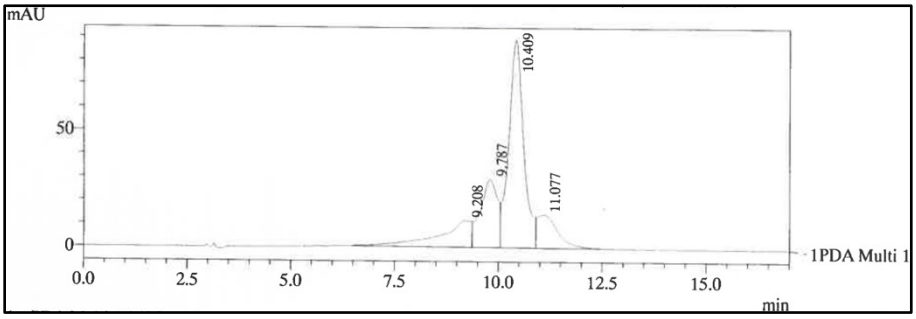
Figure 6.10 Sensory evaluation web of coconut yogurt (with or without curcuminoids) on 9-point hedonic scale after 24 h (A) and 15 days (B) storage. **Notes:** Sample C7= coconut yogurt containing 0.27% (w/w) curcuminoid; C9= coconut yogurt containing 0.27% (w/w) curcuminoid. Data represent mean of scores (n=90) with error bars corresponding to standard deviation. Raw and statistical data are shown in Appendix D.

6.5.7 Retention of curcuminoids in coconut yogurt during storage

The curcuminoids (C7 and C9) extracted from yogurt using acetone during storage (days 1, 5, 10 and 15) were analysed by HPLC (Section 3) . C7 and C9 curcuminoids were analysed separately and representative HPLC chromatograms are presented in Figure 5.11. For the C7 sample, two peaks were detected with a retention time of about 10 minutes, whereas in the C9 sample four peaks were detected around this time. The highest peak in both samples corresponds to the presence of curcumin, since curcuminoid is composed of 77% curcumin (Jayaprakasha, Rao & Sakariah, 2002). In the current study, these results were confirmed by testing the analytical grade curcumin (Table 3.2) sample.



(A)



(B)

Figure 6.11 HPLC chromatograms of standards of (A) C7 and (B) C9

Note: Injected concentration of standards = 0.08 µg/10 µL

The concentration of curcuminoids in yogurt stored for 24 h was calculated (Appendix D) from the standard curves of C7 and C9 curcuminoids respectively. The stability of curcuminoids in day 1 sample was assumed to be 100% and the HPLC results for yogurt stored for 5, 10 and 15 days

were expressed relative to day 1 results (100%), as shown in Figure 5.12. A decline in the stability of curcuminoids was seen (Figure 6.12) and at the end of storage, the amounts of curcuminoids present in the yogurts were 75.43±14.36 % for C7 coconut yogurt and 71.18±6.34 % for the C9 product. The low degradation of curcuminoids in yogurt was expected since previous studies have reported the stability of curcuminoids in acidic mediums (Wang et al., 1997; Rao & Rao, 2011; Akulov et al., 2014; Kharat, Du, Zhang & McClements, 2017). In addition, the high fat content in the coconut oil-in-water emulsion was expected to stabilise the curcuminoids in the yogurt. The oil droplets interact with curcuminoids and protect them against degradation (Vecchione et al., 2016; Kharat et al., 2017). This principle has been used to develop micro and nano emulsions to improve the stability, solubility and bioavailability of curcuminoids as discussed in Chapter 2. The reason for the decline in curcuminoids in the yogurt during storage warrants further investigations however, this was beyond the scope of this study. Based on the stability of curcuminoids in acidic and high fat medium, Kharat et al. (2017) reported that about 15% curcuminoids degraded in acidic oil-in-water emulsion stabilised with Tween-20 at the end of 31-day storage period. This present study did not add Tween-20 to the yogurt, which may have resulted in variation in findings.

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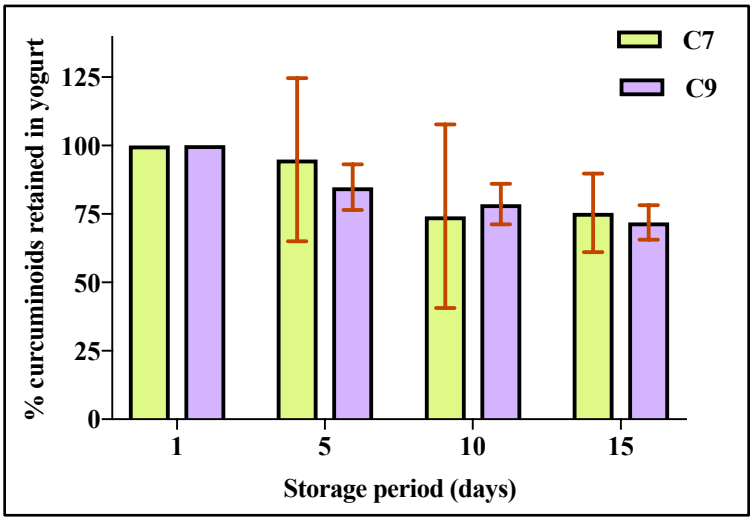

Figure 6.12 Retention of curcuminoids (C7 and C9) in coconut yogurts during storage (4˚C) for 15 days. **Notes:** Sample C7= coconut yogurt containing 0.27% (w/w) curcuminoid, C9= coconut yogurt containing 0.27% (w/w) curcuminoid. Data represent mean of replicates (n=6) with error bars corresponding to standard deviation. Raw data is shown in Appendix D.

6.6 Summary of phase III

The main aim of phase III was to develop coconut yogurt as a potential carrier of curcuminoids and to determine the stability of the curcuminoid-coconut yogurt during storage (4°C) for 15 days. Fermentation of coconut cream yogurt was not affected by the addition of curcuminoids (0.27%, w/w). Acidity, yogurt microflora and syneresis of the experimental yogurts were not significantly different from the control yogurts. However, addition of curcuminoids resulted in formation of a weaker gel compared to the control yogurt, and the viscosity of the gels varied during storage. At the end of storage, 70-75% of curcuminoids were retained in the yogurt, which indicates the stability of the bioactives in the fermented products. Overall, the presence of curcuminoids in the yogurts did not affect the sensory acceptability of the products. Therefore, coconut cream yogurt may serve as a potential food matrix for the delivery of curcuminoids.

CHAPTER 7
OVERALL CONCLUSION

Curcuminoids are the major bioactive of turmeric, a spice that has been used in Asian countries for cooking, cosmetic and medicinal purposes for centuries. Curcuminoids are renowned for their pharmaceutical properties, one of the most prominent being its ability to curtail chronic inflammation in body, thereby assisting in treatment of cardiovascular, neurodegenerative diseases and various types of cancers (as mentioned in Chapter 2). Various *in vitro* studies have validated the therapeutic potential of analytical grade (≥99% purity) curcuminoids in the treatment of chronic inflammation (as discussed in Chapter 2).

In this study, the anti-inflammatory potential of two food-grade curcuminoid powders (C7 and C9) containing variable amounts of curcumin/curcuminoids was investigated using inflammation biomarker, TNF-α, *in vitro* cell culture methods. Results showed a 2.5-fold decrease in TNF-α secretion in LPS-stimulated THP-1 macrophages on treatment with curcuminoids at a final concentration of 10 μM. Above a concentration of 10 μM, curcuminoids exhibited a toxic response in the cells, resulting in approximately 40% decrease in cell viability.

The major barriers for use of bioactive curcuminoids in the food processing industry are its poor water solubility and susceptibility to alkaline conditions, light, oxidation and heat, which also limits its clinical efficacy. Therefore, coconut cream yogurt was developed as a potential vehicle for delivery of curcuminoids. The presence of curcuminoids in yogurt samples did not affect the fermentation of the yogurts and the products were stable during storage for 15 days at 4°C. Results showed 70-75% retention of curcuminoids in yogurts at the end of storage. Overall, the physical and sensory characteristics of the yogurts containing curcuminoids were satisfactory.

CHAPTER 8
RECOMMENDATIONS

The following recommendations are suggested for future research:

1. In this study, curcuminoids were able to down-regulate the expression of TNF-α. It is however important to investigate the effect of the curcuminoids (C7 and C9) on other inflammatory biomarkers such as NF-kB, IL-6, IL-8, MAPK, JNK and lipid peroxides. Therefore, it is recommended to examine the effect of curcuminoids on these biomarkers in order to validate the anti-inflammatory activity of curcuminoids *in vitro* an *in vivo.*

2. A high retention of curcuminoids in yogurts was obtained during storage. However, the initial solubility of curcuminoids added in the coconut cream and the efficiency of acetone to extract curcuminoids from the yogurt mixture was not determined. Therefore, the concentration of curcuminoids in fresh and refrigerated yogurt could not be determined directly. Therefore, the results were expressed relative to day 1 sample. It is recommended to test the solubility and extraction efficiency of curcuminoids in future work. The stability of curcuminoids in coconut yogurt over prolonged storage period would be also useful.

3. The functional yogurt developed in the current study may be portrayed as health promoting product due to addition of curcuminoids. In addition, it is noteworthy to mention the occurrence of active compounds such as squalene, tocopherols and sterols in coconut milk, which are responsible for the anti-inflammatory activity of coconut milk (Zakaria et al., 2010). In the light of this, it is recommended to test the anti-inflammatory activity of the curcuminoid coconut yogurt to evaluate the synergistic effect of curcuminoids and coconut milk bioactives *via* addition *in vitro* studies as well as in clinical trials.

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APPENDICES

APPENDIX A

A.1 Sensory evaluation Information and consent form

INFORMATION SHEET

Project Title: Development of coconut yogurt supplemented with curcumin (turmeric)

Project Contacts:

Name	Contact Details
Tania Singh (Researcher)	taniasingh2610@icloud.com
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Dr. Martin Dickens (Co-supervisor)	M.Dickens@massey.ac.nz

You are invited to participate in the consumer sensory test to evaluate the coconut yogurt containing turmeric.

This work requires you to *taste* and *evaluate* the product.

Your participation in this task will take approximately *10 minutes*.

Ideal participants for this activity would be people consuming yogurt (any type) at least once a week.

The foods you will be tasting contains following components:
Coconut cream
Turmeric (spice)
You will be excluded from taking part in the test if you are allergic, or maybe adversely affected by the ingredients mentioned above.

The type of food you will be tasting is a *yogurt* containing following ingredients: coconut cream, turmeric powder and starter culture (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*).

The information collected in this study will be used to complete an assignment in partial fulfillment of the Masters in Food Technology. No data linked to an individual's identity will be collected.

You are under no obligation to accept this invitation. If you decide to participate, you have right to:

- Decline to answer any particular question
- Withdraw from study (specify timeframe)
- Ask any questions about the study at any time during the participation
- Provide information on the understanding that your name will not be used unless you give permission to the researcher.

- Be given access to summary of the project findings when it is concluded.

If you have any questions about the work, please contact one of the people indicated above.

- *This project has been reviewed and approved by the Massey University Human Ethics Committee (Application No.4000018843). If you have any concerns about the ethics of this research, please contact Dr. Brian Finch, Chairperson, Massey University Human Ethics at +6494140800 or email at humanethicsnorth@massey.ac.nz.*

CONSENT FORM

Development of coconut yogurt supplemented with curcumin (turmeric)

- I have read and understood the Information Sheet and have the details of the study explained to me. My questions have been answered to satisfaction, and I understand that I may ask further questions at any time.
- I agree to voluntarily participate in this study under the conditions set out in the Information Sheet.
- I understand that I have the right to withdraw from the study at any time and to decline to answer any particular questions.
- I have discussed and advised the Researcher of any potentially relevant cultural, religious or ethical beliefs that may prevent me from consuming the Foods under consideration.

Participants Signature: _____
Full Name: _____
Date: _____

A.2 Sensory evaluation questionnaire

Name: Sample No. 489

Please evaluate the product given to you and select by ticking (✔) the attribute that best reflects your feelings about the respective property of the product. You're welcome to provide additional comments regarding the sample.

1. How would you rate the **OVERALL APPEARANCE** of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
{ }	{ }	{ }	{ }	{ }	{ }	{ }	{ }	{ }

Comments: .....

2. How would you rate the **COLOR** of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
{ }	{ }	{ }	{ }	{ }	{ }	{ }	{ }	{ }

Comments: .....

3. How would you rate the **TEXTURE** of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
{ }	{ }	{ }	{ }	{ }	{ }	{ }	{ }	{ }

Comments: .....

4. How would you rate the **FLAVOUR** of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
{ }	{ }	{ }	{ }	{ }	{ }	{ }	{ }	{ }

Comments: .....

5. How would you rate the **OVERALL ACCEPTABILITY** of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
{ }	{ }	{ }	{ }	{ }	{ }	{ }	{ }	{ }

Comments: .....

APPENDIX B
DATA ANALYSIS OF PHASE I

Table B.1 pH of yogurts containing commercial curcuminoid samples (0.4% w/w) at 0 h, 2 h, 6 h and 24 h.

Yogurt type	Curcuminoids	pH			
		t=0 h	t= 2h	t= 6h	t= 24 h
Coconut cream yogurt	Control	4.25± 0.01	4.18± 0.01	4.16± 0.00	4.15± 0.01
	C1	4.22± 0.01	4.18± 0.01	4.16± 0.00	4.13± 0.01
	C2	4.18± 0.00	4.15± 0.00	4.15± 0.01	4.12± 0.00
	C3	4.18± 0.01	4.17± 0.01	4.16± 0.00	4.12± 0.01
	C4	4.19± 0.01	4.16± 0.00	4.15± 0.01	4.11± 0.00
	C5	4.17± 0.01	4.15± 0.01	4.14± 0.01	4.11± 0.01
	C6	4.16± 0.01	4.17± 0.01	4.16± 0.01	4.1± 0.00
	C7	4.18± 0.01	4.18± 0.00	4.17± 0.01	4.12± 0.00
	C8	4.23± 0.01	4.2± 0.01	4.18± 0.01	4.24± 0.01
	C9	4.26± 0.01	4.22± 0.01	4.2± 0.01	4.14± 0.01
	C10	4.51± 0.01	4.41± 0.01	4.38± 0.01	4.13± 0.00
Goat's milk yogurt	Control	3.96± 0.01	3.95± 0.01	3.96± 0.00	4.08± 0.00
	C1	3.99± 0.01	3.99± 0.01	3.92± 0.01	4.02± 0.00
	C2	3.97± 0.00	3.98± 0.03	3.98± 0.00	4.00± 0.01
	C3	3.94± 0.01	3.97± 0.00	3.99± 0.01	3.98± 0.03
	C4	3.97± 0.01	3.96± 0.03	3.99± 0.01	3.94± 0.00
	C5	3.9± 0.01	3.96± 0.00	3.97± 0.00	3.96± 0.01
	C6	3.9± 0.01	3.9 ± 0.01	3.9± 0.01	3.86± 0.01
	C7	3.93± 0.00	3.9± 0.00	3.91± 0.01	3.87± 0.00
	C8	3.96± 0.05	4.01± 0.01	3.98± 0.01	3.94± 0.01
	C9	3.94± 0.01	3.92± 0.01	3.93± 0.01	3.89± 0.01
	C10	3.94± 0.01	3.96± 0.00	3.96± 0.01	3.92± 0.00
Cow's milk yogurt	Control	4.07± 0.01	4.05± 0.00	4.05± 0.01	4.03± 0.01
	C1	4.02± 0.00	4.02± 0.01	4.02± 0.00	3.99± 0.01
	C2	4.01± 0.01	4.00± 0.01	3.99± 0.01	3.98± 0.00
	C3	4.01± 0.01	4.00± 0.01	4.00± 0.00	3.98± 0.01
	C4	4.01± 0.01	4.00± 0.01	3.99± 0.01	3.98± 0.01
	C5	4.03± 0.01	4.02± 0.01	4.01± 0.01	4.00± 0.01
	C6	4.01± 0.00	4.01± 0.01	4.00± 0.01	3.99± 0.01
	C7	4.02± 0.01	4.00± 0.01	3.99± 0.01	3.99± 0.01
	C8	4.06± 0.01	4.04± 0.01	4.04± 0.01	4.04± 0.01
	C9	4.03± 0.01	4.01± 0.01	4.01± 0.00	4.02± 0.01
	C10	4.00± 0.01	4.00± 0.00	3.99± 0.01	3.98± 0.01

Table B.2 Pugh decision-making matrix for screening commercial curcuminoids in Phase I

Curcuminoids	Coconut cream yogurt				Goat's milk yogurt				Cow's milk yogurt				Overall score
	Appearance	Colour	Flavour	Overall accetability	Appearance	Colour	Flavour	Overall accetability	Appearance	Colour	Flavour	Overall accetability	
C1	-1	1	1	0	-1	1	-1	-1	-1	0	1	0	-1
C2	-1	0	-1	-1	-1	0	-1	-1	-1	1	0	0	-6
C3	-1	-1	-1	-1	-1	1	-1	-1	0	0	-1	-1	-8
C4	-1	1	-1	-1	-1	1	-1	-1	-1	-1	-1	-1	-8
C5	0	0	-1	-1	-1	1	-1	-1	0	0	-1	0	-5
C6	0	1	0	1	-1	1	-1	-1	0	1	-1	-1	-1
C7	1	1	1	1	1	1	-1	0	1	1	1	1	9
C8	0	-1	-1	-1	1	-1	-1	0	1	-1	-1	-1	-6
C9	1	1	1	1	1	1	-1	0	1	1	0	1	8
C10	1	-1	1	1	1	0	-1	-1	1	-1	1	0	2

B.3 Statistical analysis of phase I

General Linear Model: pH versus Time (h), Type of curcuminoids

Factor Information

Factor	Levels	Values
Type of yogurt	3	Coconut cream, Cow milk, Goat milk
Curcuminoids	11	C1, C10, C2, C3, C4, C5, C6, C7, C8, C9, Control

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	33	3.07877	0.09330	82.69	0.000
Covariates	1	0.05068	0.05068	44.92	0.000
Time (h)	1	0.05068	0.05068	44.92	0.000
Linear	12	2.75686	0.22974	203.63	0.000
Type of yogurt	2	2.60273	1.30137	1153.49	0.000
Curcuminoids	10	0.15413	0.01541	13.66	0.000
2-Way Interactions	20	0.27124	0.01356	12.02	0.000
Type of yogurt*Curcuminoids	20	0.27124	0.01356	12.02	0.000
Error	230	0.25949	0.00113		
Lack-of-Fit	98	0.24564	0.00251	23.89	0.000
Pure Error	132	0.01385	0.00010		
Total	263	3.33825			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0335886	92.23%	91.11%	89.69%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	4.05877	0.00270	1500.94	0.000	
Time (h)	-0.001460	0.000218	-6.70	0.000	1.00
Type of yogurt					
Coconut cream	0.13621	0.00292	46.59	0.000	1.33
Cow milk	-0.03856	0.00292	-13.19	0.000	1.33
Curcuminoids					
C1	0.00500	0.00654	0.76	0.445	1.82
C10	0.05000	0.00654	7.65	0.000	1.82
C2	-0.00542	0.00654	-0.83	0.408	1.82
C3	-0.00792	0.00654	-1.21	0.227	1.82
C4	-0.01208	0.00654	-1.85	0.066	1.82
C5	-0.01458	0.00654	-2.23	0.027	1.82
C6	-0.03667	0.00654	-5.61	0.000	1.82
C7	-0.02708	0.00654	-4.14	0.000	1.82
C8	0.02667	0.00654	4.08	0.000	1.82
C9	-0.00333	0.00654	-0.51	0.611	1.82

APPENDIX C
DATA ANALYSIS OF PHASE II

Table C.1 TNF-α (pg/mL) produced by differentiated THP-1 macrophages stimulated with LPS for 2 h, as measured in cell supernatant diluted by a factor of 10, 50, 100 and 200.

Dilution factor	Replicate	A-450 nm	Conc. of TNF-α (pg/mL)	Mean TNF-α (pg/mL)	SD
10	1	1.509	1510.381	1787.869	392.428
	2	1.752	2065.357		
50	1	0.733	321.488	357.413	50.805
	2	0.804	393.337		
100	1	0.53	157.096	152.868	5.979
	2	0.517	148.641		
200	1	0.259	32.406	34.02	2.283
	2	0.271	35.634		

Table C.2 TNF-α (pg/mL) produced by differentiated THP-1 macrophages stimulated with LPS (1000 ng/mL) for 0 to 6 h.

Incubation time (h)	Replicate	A-450 nm	Conc. of TNF-α (pg/mL)	Mean TNF-α (pg/mL)	SD
0	1	0.003	0.233	0.364	0.164
	2	0.004	0.311		
	3	0.007	0.549		
0.5	1	0.002	0.155	0.194	0.055
	2	0.003	0.233		
	3	-	-		
1	1	0.041	348.057	88.31	226.472
	2	-0.002	-15.333		
	3	-0.009	-67.795		
2	1	0.105	1019.912	977.32	37.332
	2	0.1	961.78		
	3	0.099	950.269		
4	1	0.598	11448.138	124000.687	4003.415
	2	0.757	16794.464		
	3	0.512	8959.461		
6	1	0.599	11478.739	21320.866	8538.192
	2	0.976	25741.809		
	3	0.998	26742.051		

Table C.3 TNF-α (pg/mL) produced by differentiated THP-1 macrophages stimulated with LPS (50 to 1000 ng/mL)

Conc. of LPS (ng/mL)	Replicate	A-450 nm	Conc. of TNF-α (pg/mL)	Mean TNF-α (pg/mL)	SD
0	1	-0.015	11.586	13.813	0.968
		0.018	15.724		
		-0.011	12.076		
	2	0.008	14.446		
		0.035	17.945		
		-0.019	11.1		
	3	-	-		
		-	-		
		-	-		
50	1	0.915	16167.105	16936.683	4252.76
		0.812	13803.785		
		0.859	14861.521		
	2	0.797	13473.514		
		0.879	15322.146		
		0.791	13342.395		
	3	1.402	29597.941		
		0.981	17769.069		
		0.994	18092.673		
100	1	1.087	20485.115	19753.493	4292.321
		1.038	19207.648		
		1.175	22874.029		
	2	1.277	25795.208		
		0.566	8833.647		
		0.65	10423.89		
	3	1.373	28693.841		
		1.132	21691.521		
		1.06	19776.539		
200	1	1.454	31252.166	23622.826	513.148
		1.144	22018.602		
		0.967	17423.541		
	2	1.386	29097.494		
		1.233	24515.048		
		1.025	18875.059		
	3	1.506	32948.866		
		1.192	23349.543		
		0.781	13125.12		
500	1	0.833	14272.102	19182.112	3676.289
		0.898	15765.56		
		0.871	15137.142		
	2	1.251	25035.074		
		0.837	14362.091		
		1.133	21718.691		
	3	1.278	25824.656		
		1.067	19959.145		
		1.09	20564.552		
1000	1	1.118	21312.786	15075.271	3548.319
		0.642	10267.663		
		1.222	24199.76		
	2	0.928	16477.232		
		0.845	14542.822		
		0.838	14384.627		
	3	0.76	12673.955		
		0.665	10719.525		
		0.684	11099.07		

Table C.4 Absorbance of formazan obtained at 595 nm, the purple dye produced by LPS stimulated macrophages seeded at a density of (1.25×10^5 cells/mL) to (1×10^6 cells/mL) on exposure to MTT reagent for 0.5 h, 1.5 h and 3 h.

Incubation time (h)	No. of cells	Replicate	A-595 nm	Mean A-595 nm	SD
0.5	125000	1	0.55	0.056	0.001
		2	0.057		
		3	0.056		
	250000	1	0.163	0.167	0.003
		2	0.169		
		3	0.169		
	500000	1	0.364	0.379	0.013
		2	0.387		
		3	0.385		
	750000	1	0.389	0.418	0.031
		2	0.416		
		3	0.45		
	1000000	1	0.637	0.673	0.037
		2	0.671		
		3	0.711		
1.5	125000	1	0.257	0.263	0.005
		2	0.267		
		3	0.264		
	250000	1	0.517	0.543	0.022
		2	0.558		
		3	0.553		
	500000	1	1.227	1.294	0.06
		2	0.316		
		3	1.34		
	750000	1	1.432	1.494	0.054
		2	1.526		
		3	1.525		
	1000000	1	1.862	1.931	0.061
		2	1.958		
		3	1.974		
3	125000	1	0.568	0.583	0.018
		2	0.577		
		3	0.603		
	250000	1	1.243	1.288	0.039
		2	1.315		
		3	1.305		
	500000	1	2.421	2.503	0.071
		2	2.547		
		3	2.542		
	750000	1	2.597	2.674	0.077
		2	2.674		
		3	2.75		
	1000000	1	2.833	2.928	0.082
		2	2.975		
		3	2.975		

Table C.5 Cytotoxicity of (0.1 to 50 µM) Pure C, C7 and C9 towards differentiated macrophages pre-treated with curcumin for 1 h prior to stimulation with LPS for 4 h.

Test sample	Conc. of curcumin (µM)	Replicate	A-595 nm	Cell viability %	Mean cell viability %	SD	Test sample	Conc. of curcumin (µM)	Replicate	A-595 nm	Cell viability %	Mean cell viability %	SD
			1.54							0.447	79.642		
		1	1.516						1	0.486	83.769		
			1.133							0.495	83.546		
			1.509							0.448	89.546		
Control	0	2	1.574	100	100	0		10	2	0.473	94.773	88.812	5.642
			1.362							0.481	94.635		
			1.399							0.645	87.758		
		3	1.178						3	0.699	91.884		
			1.067							0.69	93.398		
			1.258	93.881						0.418	59.422		
		1	1.351	100.821					1	0.443	63.824		
			1.356	101.194						0.422	64.237		
			1.044	77.91						0.407	61.214		
	0.1	2	1.094	81.642	88.035	8.605	C7	25	2	0.428	64.787	67.905	8.288
			1.09	81.343						0.412	65.337		
			1.109	82.761						0.52	74.966		
		3	1.16	86.567					3	0.565	78.955		
			1.155	86.194						0.553	78.404		
			1.322	98.657						0.275	33.425		
		1	1.403	104.702					1	0.307	46.492		
			1.443	107.687						0.313	47.318		
			1.189	88.721						0.288	46.217		
	1	2	1.264	94.328	91.847	10.903		50	2	0.315	37.689	49.106	9.285
			1.259	93.955						0.325	51.719		
			1.028	76.71642						0.353	63.549		
		3	1.093	81.562					3	0.414	54.058		
			1.076	80.299						0.4	61.231		
			1.152	85.97						0.496	69.464		
		1	1.201	89.627					1	0.522	72.765		
			1.221	91.119						0.526	72.765		
			1.023	76.343						0.574	65.612		
Pure C	10	2	1.077	80.373	81.202	6.856		0.1	2	0.605	68.638	78.313	14.217
			1.073	80.075						0.612	71.664		
			1	74.627						0.514	92.022		
		3	1.02	76.119					3	0.55	96.699		
			1.026	76.567						0.544	95.048		
			1.131	84.403						0.48	83.319		
		1	1.205	89.925					1	0.508	89.271		
			1.208	90.149						0.518	88.582		
			1.012	75.522						0.576	78.129		
	25	2	1.067	79.627	80.299	7.291		1	2	0.612	82.118	91.056	12.591
			1.096	81.791						0.623	81.843		
			0.944	70.448						0.767	100.275		
		3	1.008	75.224					3	0.81	107.703		
			1.013	75.597						0.81	107.153		
			1.041	77.687						0.579	61.486		
		1	1.133	84.552					1	0.609	66.85		
			1.12	83.582						0.608	68.088		
			1.097	81.866						0.651	61.623		
	50	2	1.156	86.269	85.912	4.679	C9	10	2	0.689	65.062	74.324	16.411
			1.153	86.045						0.688	66.162		
			1.171	87.388						0.638	88.721		
		3	1.243	92.761					3	0.668	96.149		
			1.247	93.06						0.679	94.911		
			0.505	68.223						0.432	57.497		
		1	0.529	71.802					1	0.464	60.935		
			0.529	72.352						0.467	58.047		
			0.477	78.955						0.445	5.983		
	0.1	2	0.499	83.219	75.562	5.859		25	2	0.471	58.872	63.732	9.882
			0.521	84.182						0.475	56.671		
			0.669	70.702						0.545	71.529		
		3	0.703	75.653					3	0.574	77.166		
C7			0.691	74.828						0.57	76.066		
			0.613	66.025						0.243	37.827		
		1	0.649	69.876					1	0.338	42.228		
			0.644	71.252						0.344	43.054		
			0.568	79.23						0.336	39.615		
	1	2	0.597	84.182	87.208	20.533		50	2	0.274	43.329	45.667	6.832
			0.595	85.695						0.376	44.704		
			0.729	105.502						0.462	48.556		
		3	0.783	111.417					3	0.393	56.946		
			0.779	111.417						0.448	55.021		

Table C.6 Cytotoxicity of (0.001 to 0.5%, v/v) DMSO towards differentiated macrophages pre-treated with DMSO for 1 h prior to stimulation with LPS for 4 h.

Test sample	Conc. of DMSO (%)	Replicate	A-595 nm	Cell viability %	Mean cell viability %	SD
Control	0	1	1.54	100	100	0
			1.516			
			1.133			
		2	1.509			
			1.574			
			1.362			
		3	1.399			
			1.178			
			1.067			
DMSO	0.001	1	1.407	105	95.638	9.306
			1.462	109.105		
			1.407	105		
		2	1.163	84.791		
			1.228	91.642		
			1.218	90.896		
		3	1.167	87.09		
			1.249	93.209		
			1.233	92.015		
	0.01	1	1.253	93.507	87.338	9.486
			1.33	99.254		
			1.343	100.224		
		2	1.015	75.746		
			1.077	80.373		
			1.084	80.896		
		3	1.097	81.866		
			1.188	88.657		
			1.146	85.522		
	0.1	1	1.068	79.701	79.46103	3.197
			1.116	83.284		
			1.156	86.269		
		2	0.996	74.328		
			1.051	78.433		
			1.05	78.358		
		3	1.015	75.746		
			1.071	9.925		
			1.06	79.104		
	0.25	1	1.282	95.672	98.537	11.444
			1.342	100.149		
			1.34	100		
		2	1.42	106.343		
			1.497	111.716		
			1.496	111.641		
		3	1.123	83.806		
			1.201	89.629		
			1.174	87.612		
	0.5	1	0.92	68.657	80.464	7.394
			0.974	72.687		
			0.998	74.478		
		2	1.077	80.373		
			1.156	86.369		
			1.19	88.806		
		3	1.198	89.403		
			1.129	84.352		
			1.062	79.254		

Table C.7 TNF-α produced in differentiated macrophages pre-treated with curcumin (Pure C, C7 and C9) for 1 h at final concentration of 0.1-50 μM prior to stimulation with LPS for 4 h.

Test sample	Conc. of curcumin (μM)	Replicate	Conc. of TNF-α (ng/mL)	Mean of TNF-α (ng/mL)	SD	Test sample	Conc. of curcumin (μM)	Replicate	Conc. of TNF-α (ng/mL)	Mean of TNF-α (ng/mL)	SD
Control	0	1	25.126	28.797	5.431	C7	10	1	9.806	11.573	1.195
			26.428						2.648		
			25.999						12.36		
		2	36.507					2	12.753		
			23.94						2.859		
			27.821						12.648		
		3	36.507					3	10.039		
			26.094						10.681		
			30.414						10.37		
Pure C	0.1	1	21.052	19.885	2.05		25	1	7.935	8.152	1.27
			21.116						8.616		
			24.482						8.879		
		2	23.768					2	8.638		
			17.161						9.922		
			16.285						9.123		
		3	18.831					3	7.281		
			19.192						6.77		
			17.075						6.203		
	1	1	11.195	15.751	3.951		50	1	4.117	4.111	0.202
			13.875						4.163		
			9.727						3.776		
		2	29.812					2	4.378		
			16.424						4.473		
			12.16						4.178		
		3	27.045					3	5.643		
			6.517						3.123		
			15.002						3.15		
	10	1	2.768	3.461	1.292		0.1	1	21.295	21.081	1.424
			3.393						19.304		
			2.668						19.99		
		2	5.608					2	24.065		
			4.55						23.386		
			4.637						20.721		
		3	2.046					3	19.596		
			3.368						20.755		
			2.113						20.62		
	25	1	1.972	1.866	0.27		1	1	12	12.315	0.339
			1.654						12.257		
			1.285						12.543		
		2	3.089					2	12		
			1.344						12.912		
			2.055						11.096		
		3	1.696					3	12.283		
			1.935						12.965		
			1.765						12.779		
	50	1	0.91	1.344	0.314		10	1	9.146	9.392	0.224
			1.935						9.101		
			1.221						9.553		
		2	1.014					2	9.326		
			0.971						9.484		
			1.091						8.968		
		3	1.235					3	10.322		
			1.571						8.638		
			2.152						9.992		
C7	0.1	1	21.159	20.035	1.08		25	1	4.81	5.296	0.84
			20.62						5.66		
			20.889						5.227		
		2	18.598					2	4.378		
			19.045						4.488		
			18.821						4.6		
		3	21.125					3	4.826		
			19.271						7.707		
			20.788						5.965		
	1	1	19.142	18.699	1.299		50	1	2.901	2.922	0.258
			21.842						3.109		
			19.204						3.189		
		2	17.999					2	2.991		
			18.502						2.529		
			18.917						2.352		
		3	18.949					3	3.126		
			1.558						3.189		
			17.072						2.901		

Table C.8 TNF-α produced in differentiated macrophages pre-treated with control vehicle DMSO for 1 h at final concentration of 0.001 to 0.5% (v/v) prior to stimulation with LPS for 4 h.

Test sample	Conc. of DMSO (%)	Replicate	Conc. of TNF-α (ng/mL)	Mean of TNF-α (ng/mL)	SD
Control	0	1	25.126	28.797	5.431
			26.428		
			25.999		
		2	36.507		
			23.94		
			27.821		
		3	36.507		
			26.094		
			30.414		
DMSO	0.001	1	30.458	28.47	3.879
			30.803		
			30.496		
		2	31.188		
			31.149		
			30.153		
		3	24.447		
			30.458		
			17.075		
	0.01	1	34.318	27.146	2.981
			24.721		
			32.556		
		2	24.482		
			24.825		
			25.449		
		3	24.929		
			23.971		
			29.061		
	0.1	1	29.313	22.813	2.13
			18.891		
			15.012		
		2	25.206		
			24.869		
			27.275		
		3	13.016		
			19.991		
			28.741		
	0.25	1	19.192	19.531	4.075
			16.036		
			15.678		
		2	24.111		
			20.36		
			22.402		
		3	14.631		
			14.921		
			25.45		
	0.5	1	10.537	17.675	2.018
			12.908		
			19.99		
		2	17.103		
			24.036		
			11.873		
		3	11.897		
			19.961		
			29.172		

C.9 Statistical analysis of phase II

General Linear Model: TNF- ∝ concentration versus Dilution

Factor Information

Factor	Type	Levels	Values
Dilution	Fixed	4	0.005, 0.010, 0.050, 0.100

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Dilution	3	3972423	1324141	33.82	0.003
Error	4	156590	39147		
Total	7	4129013			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
197.857	96.21%	93.36%	84.83%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	1788	140	12.78	0.000	
Dilution					
0.010	-1430	198	-7.23	0.002	1.50
0.050	-1632	198	-8.25	0.001	1.50
0.100	-1754	198	-8.86	0.001	1.50

One-way ANOVA: TNF- ∝ concentration versus Dilution

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
197.857	96.21%	93.36%	84.83%

Means

Dilution	N	Mean	StDev	95% CI
0.005	2	1788	392	(1399, 2176)
0.010	2	357.4	50.8	(-31.0, 745.9)
0.050	2	156.110	1.400	(-232.332, 544.552)
0.100	2	34.02	2.28	(-354.42, 422.46)

Pooled StDev = 197.857

Grouping Information Using the Tukey Method and 95% Confidence

Dilution	N	Mean	Grouping
0.005	2	1788	A
0.010	2	357.4	B
0.050	2	156.110	B
0.100	2	34.02	B

Means that do not share a letter are significantly different.

General Linear Model: TNF- α concentration versus LPS incubation time

Factor Information

Factor	Type	Levels	Values
Time	Fixed	6	0.0, 0.5, 1.0, 2.0, 4.0, 6.0

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time	5	1187273187	237454637	14.68	0.000
Error	11	177961477	16178316		
Total	16	1365234663			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
4022.23	86.96%	81.04%	70.67%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	5798	987	5.88	0.000	
Time					
0.0	-5798	2137	-2.71	0.020	1.69
0.5	-5798	2523	-2.30	0.042	1.94
1.0	-5710	2137	-2.67	0.022	1.69
2.0	-4821	2137	-2.26	0.045	1.69
4.0	6603	2137	3.09	0.010	1.69

One-way ANOVA: TNF-a conc. versus Time
Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
4022.23	86.96%	81.04%	70.67%

Means

Time	N	Mean	StDev	95% CI
0.0	3	0.3643	0.1646	(-5110.8388, 5111.5675)
0.5	2	0.1940	0.0552	(-6259.7259, 6260.1139)
1.0	3	88	226	(-5023, 5200)
2.0	3	977.3	37.3	(-4133.9, 6088.5)
4.0	3	12401	4003	(7289, 17512)
6.0	3	21321	8538	(16210, 26432)

Pooled StDev = 4022.23

Grouping Information Using the Tukey Method and 95% Confidence

Time	N	Mean	Grouping		
6.0	3	21321	A		
4.0	3	12401	A	B	
2.0	3	977.3			C
1.0	3	88			C
0.0	3	0.3643			C
0.5	2	0.1940		B	C

Means that do not share a letter are significantly different.

General Linear Model: TNF- α concentration versus LPS dose Method

Factor Information

Factor	Type	Levels	Values
LPS	Fixed	6	0, 50, 100, 200, 500, 1000

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
LPS	5	2265522407	453104481	16.41	0.000
Error	45	1242642045	27614268		
Total	50	3508164452			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
5254.93	64.58%	60.64%	55.17%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	15764	744	21.18	0.000	
LPS					
0	-15750	1903	-8.28	0.000	1.94
50	1173	1612	0.73	0.471	1.69
100	3989	1612	2.47	0.017	1.69
200	7859	1612	4.87	0.000	1.69
500	3418	1612	2.12	0.040	1.69

One-way ANOVA: TNF- α concentration versus LPS dose Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal

Significance level α = 0.05

Equal variances were assumed for the analysis.

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
5254.93	64.58%	60.64%	55.17%

Means

LPS	N	Mean	StDev	95% CI
0	6	13.81	2.70	(-4307.07, 4334.70)
50	9	16937	5057	(13409, 20465)
100	9	19753	6491	(16226, 23281)
200	9	23623	6612	(20095, 27151)
500	9	19182	4510	(15654, 22710)
1000	9	15075	4855	(11547, 18603)

Pooled StDev = 5254.93

Grouping Information Using the Tukey Method and 95% Confidence

LPS	N	Mean	Grouping		
200	9	23623	A		
100	9	19753	A	B	
500	9	19182	A	B	
50	9	16937	A	B	
1000	9	15075		B	
0	6	13.81			C

Means that do not share a letter are significantly different.

General Linear Model: A-595 nm versus Time and No. of cells

Factor Information

Factor	Type	Levels	Values
Time	Fixed	3	0.5, 1.5, 3.0
No. of cells	Fixed	5	125000, 250000, 500000, 750000, 1000000

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time	2	19.9757	9.98783	67.91	0.000
No. of cells	4	13.5624	3.39060	23.05	0.000
Error	38	5.5892	0.14708		
Lack-of-Fit	8	4.7382	0.59227	20.88	0.000
Pure Error	30	0.8510	0.02837		
Total	44	39.1272			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.383514	85.72%	83.46%	79.97%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	1.1350	0.0572	19.85	0.000	
Time					
0.5	-0.7634	0.0809	-9.44	0.000	1.33
1.5	-0.0966	0.0809	-1.19	0.240	1.33
No. of cells					
125000	-0.780	0.114	-6.82	0.000	1.60
250000	-0.469	0.114	-4.10	0.000	1.60
500000	0.146	0.114	1.28	0.209	1.60
750000	0.394	0.114	3.44	0.001	1.60

One-way ANOVA: A-595 nm versus Time

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Means

Time	N	Mean	StDev	95% CI
0.5	15	0.3716	0.2138	(0.0197, 0.7235)
1.5	15	1.038	0.667	(0.687, 1.390)
3.0	15	1.995	0.937	(1.643, 2.347)

Pooled StDev = 0.675270

Grouping Information Using the Tukey Method and 95% Confidence

Time	N	Mean	Grouping
3.0	15	1.995	A
1.5	15	1.038	B
0.5	15	0.3716	C

Means that do not share a letter are significantly different.

One-way ANOVA: A-595 nm versus No. of cells
Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Means

No. of cells	N	Mean	StDev	95% CI
125000	9	0.3554	0.2231	(-0.1831, 0.8940)
250000	9	0.666	0.494	(0.127, 1.204)
500000	9	1.281	0.992	(0.742, 1.820)
750000	9	1.529	0.978	(0.990, 2.067)
1000000	9	1.844	0.980	(1.305, 2.383)

Pooled StDev = 0.799450

Grouping Information Using the Tukey Method and 95% Confidence

No. of cells	N	Mean	Grouping		
1000000	9	1.844	A		
750000	9	1.529	A	B	
500000	9	1.281	A	B	C
250000	9	0.666		B	C
125000	9	0.3554			C

Means that do not share a letter are significantly different.

General Linear Model: Cell viability % versus Pure C, C7, C9 and DMSO & their concentrations

Factor Information

Factor	Type	Levels	Values
Test Sample	Fixed	4	C7, C9, DMSO, Pure C
Conc.	Fixed	6	0.0, 0.1, 1.0, 10.0, 25.0, 50.0

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Test Sample	3	7531	2510.2	12.33	0.000
Conc.	5	26321	5264.2	25.85	0.000
Error	207	42154	203.6		
Lack-of-Fit	15	16222	1081.5	8.01	0.000
Pure Error	192	25932	135.1		
Total	215	76005			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
14.2703	44.54%	42.39%	39.61%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	104.55	2.91	35.89	0.000	
Test Sample					
C7	-8.51	2.75	-3.10	0.002	1.50
C9	-12.03	2.75	-4.38	0.000	1.50
DMSO	2.32	2.75	0.84	0.399	1.50

Conc.					
0.1	-15.68	3.36	-4.66	0.000	1.67
1.0	-10.68	3.36	-3.17	0.002	1.67
10.0	-15.68	3.36	-4.66	0.000	1.67
25.0	-25.75	3.36	-6.24	0.000	1.67
50.0	-34.71	3.36	-10.32	0.000	1.67

One-way ANOVA: TNF- α versus Conc.

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis

Means

Test Sample	N	Mean	StDev	95% CI
C7	54	78.08	18.91	(73.26, 82.90)
C9	54	12.66	22.23	(69.74, 79.38)
DMSO	54	88.91	8.93	(84.08, 93.73)
Pure C	54	86.59	14.28	(81.77, 91.41)

Pooled StDev = 17.9720

Grouping Information Using the Tukey Method and 95% Confidence

Test Sample	N	Mean	Grouping
DMSO	54	22.21	A
C7	54	13.94	A B
C9	54	12.66	B C
Pure C	54	12.49	C

Means that do not share a letter are significantly different.

General Linear Model: TNF- α versus Test Sample and Conc.

Factor Information

Factor	Type	Levels	Values
Test Sample	Fixed	4	C7, C9, DMSO, Pure C
Conc.	Fixed	6	0.0, 0.1, 1.0, 10.0, 25.0, 50.0

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Test Sample	3	3482	1160.76	61.59	0.000
Conc.	5	15437	3087.42	163.81	0.000
Error	207	3901	18.85		
Lack-of-Fit	15	1014	67.61	4.50	0.000
Pure Error	192	2887	15.04		
Total	215	22821			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
4.34135	82.90%	82.24%	81.39%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	15.325	0.295	51.88	0.000	
Test Sample					
C7	-1.387	0.512	-2.71	0.007	1.50
C9	-2.665	0.512	-5.21	0.000	1.50

DMSO	6.887	0.512	13.46	0.000	1.50
Conc.					
0.0	13.472	0.661	20.40	0.000	1.67
0.1	7.042	0.661	10.66	0.000	1.67
1.0	2.733	0.661	4.14	0.000	1.67
10.0	-5.571	0.661	-8.43	0.000	1.67
25.0	-7.614	0.661	-11.53	0.000	1.67

One-way ANOVA: TNF- α versus Conc.
Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis

Means

Test Sample	N	Mean	StDev	95% CI
C7	54	13.94	7.94	(11.38, 16.50)
C9	54	12.66	8.14	(10.10, 15.22)
DMSO	54	22.21	8.93	(19.65, 24.77)
Pure C	54	12.49	12.48	(9.93, 15.05)

Pooled StDev = 9.55089

Grouping Information Using the Tukey Method and 95% Confidence

Test Sample	N	Mean	Grouping
DMSO	54	22.21	A
C7	54	13.94	B
C9	54	12.66	B
Pure C	54	12.49	B

Means that do not share a letter are significantly different.

One-way ANOVA: TNF- α versus Conc.
Means

Conc.	N	Mean	StDev	95% CI
0.0	36	28.80	7.11	(26.85, 30.75)
0.1	36	22.368	4.582	(20.419, 24.316)
1.0	36	18.06	7.54	(16.11, 20.01)
10.0	36	9.754	5.594	(7.806, 11.703)
25.0	36	7.711	5.486	(5.763, 9.659)
50.0	36	5.263	4.605	(3.315, 7.211)

Pooled StDev = 5.92962

Grouping Information Using the Tukey Method and 95% Confidence

Conc.	N	Mean	Grouping			
0.0	36	28.80	A			
0.1	36	22.368		B		
1.0	36	18.06			C	
10.0	36	9.754				D
25.0	36	7.711				D	E
50.0	36	5.263					E

Means that do not share a letter are significantly different.

APPENDIX D
DATA ANALYSIS OF PHASE III

Table D.1 Acidity and microbiological analysis of coconut yogurt (with or without curcuminoids) during fermentation at 42˚C.

Test sample	Time (h)	Replication	pH	TA	LB counts (cfu/g)	ST counts (cfu/g)
Control	0	1	6.09±0.00	0.23±0.01	5.91±0.02	6.93±0.04
		2	6.1±0.00	0.22±0.00	5.79±0.05	7.03±0.02
		3	6.09±0.01	0.22±0.01	5.96±0.01	6.88±0.02
	2	1	5.85±0.01	0.27±0.02	5.92±0.07	8.19±0.18
		2	5.8±0.01	0.27±0.01	5.79±0.03	9.39±0.02
		3	5.97±0.01	0.26±0.01	5.94±0.11	8.30±0.03
	4	1	5.1±0.04	0.37±0.02	5.59±0.02	9.81±0.07
		2	4.95±0.01	0.41±0.01	5.60±0.14	9.29±0.04
		3	5.05±0.01	0.36±0.03	5.48±0.01	9.36±0.01
	6	1	4.77±0.01	0.55±0.01	5.57±0.11	9.89±0.06
		2	4.58±0.00	0.56±0.01	5.67±0.11	9.77±0.05
		3	4.65±0.00	0.53±0.01	5.54±0.00	9.86±0.01
	8	1	4.51±0.01	0.67±0.01	5.50±0.07	9.95±0.04
		2	4.51±0.01	0.7±0.01	5.19±0.21	9.81±0.05
		3	4.51±0.01	0.71±0.03	5.42±0.06	9.46±0.04
C7	0	1	6.08±0.00	0.22±0.01	5.83±0.01	6.87±0.01
		2	6.1±0.01	0.25±0.01	5.94±0.02	6.97±0.03
		3	6.1±0.01	0.22±0.01	5.89±0.02	6.96±0.02
	2	1	5.68±0.01	0.27±0.01	5.82±0.05	8.42±0.04
		2	5.61±0.00	0.29±0.01	5.85±0.10	9.21±0.02
		3	5.97±0.00	0.27±0.00	5.86±0.07	8.18±0.04
	4	1	4.94±0.01	0.41±0.01	5.52±0.04	9.89±0.02
		2	4.83±0.00	0.46±0.02	5.57±0.05	9.08±0.00
		3	5.17±0.03	0.33±0.02	5.54±0.12	9.18±0.03
	6	1	4.68±0.01	0.54±0.02	5.61±0.10	9.87±0.05
		2	4.53±0.01	0.56±0.01	5.63±0.05	9.82±0.08
		3	4.71±0.02	0.54±0.01	5.75±0.04	9.84±0.12
	8	1	4.5±0.02	0.72±0.02	5.47±0.04	9.90±0.04
		2	4.49±0.01	0.69±0.02	5.25±0.36	9.83±0.08
		3	4.5±0.00	0.71±0.01	5.40±0.11	8.80±0.14
C9	0	1	6.08±0.01	0.23±0.01	5.78±0.07	6.83±0.05
		2	6.09±0.01	0.29±0.01	5.78±0.05	6.93±0.05
		3	6.1±0.01	0.22±0.00	5.90±0.00	6.79±0.14
	2	1	5.77±0.04	0.28±0.01	5.79±0.04	8.20±0.03
		2	5.64±0.01	0.28±0.01	5.86 ±0.03	9.05±0.01
		3	5.9±0.01	0.28±0.00	5.93±0.03	8.06±0.11
	4	1	4.93±0.04	0.4±0.01	5.55±0.03	9.68±0.03
		2	4.88±0.01	0.45±0.01	5.55±0.05	8.98±0.05
		3	5.08±0.01	0.36±0.01	5.41±0.04	9.16±0.03
	6	1	4.65±0.01	0.55±0.01	5.54±0.06	9.73±0.06
		2	4.57±0.01	0.58±0.01	5.56±0.08	9.55±0.03
		3	4.65±0.03	0.52±0.00	5.55±0.17	10.17±0.02
	8	1	4.49±0.01	0.72±0.01	5.52±0.01	9.79±0.03
		2	4.5±0.01	0.68±0.01	5.52±0.02	9.33±0.35
		3	4.49±0.01	0.71±0.02	5.56±0.03	9.23±0.04

Table D.2 Chemical, rheological and microbiological characteristics of coconut yogurt (with or without curcuminoids) during 15-day storage period at 4˚C.

Test sample	Day	Replication	pH	TA (%)	LB counts (cfu/g)	ST counts (cfu/g)	Syneresis (%)	Apparent viscosity	Firmness (g)
Control	1	1	4.64±0.01	0.58±0.01	5..55±0.03	8.59±0.01	0.34±0.08	0.72±0.02	146.31±24.56
		2	4.61±0.01	0.61±0.04	5.52±0.03	8.18±0.01	0.14±0.03	0.71±0.01	198.2±33.69
		3	4.57±0.00	0.55±0.01	5.48±0.02	8.66±0.01	0.13±0.16	0.62±0.1	105.9±1.66
	5	1	4.61±0.01	0.59±0.00	5.53±0.08	8.22±0.00	0.32±0.11	1.18±0.18	322.05±28.28
		2	4.58±0.01	0.59±0.00	5.41±0.07	7.99±0.05	0.12±0.06	1.13±0.32	364.94±156.84
		3	4.57±0.01	0.59±0.01	5.56±0.04	8.23±0.03	0.29±0.05	0.95±0.14	169±5.41
	10	1	4.61±0.01	0.68±0.10	5.67±0.03	8.10±0.04	0.32±0.08	0.51±0.03	490.545±38.15
		2	4.59±0.02	0.58±0.03	5.67±0.07	8.06±0.03	0.30±0.05	0.49±0.16	360.37±176.49
		3	4.58±0.01	0.58±0.02	5.78±0.04	8.93±0.04	0.24±0.15	0.31±0.03	244.81±37.02
	15	1	4.58±0.01	0.63±0.00	5.66±0.01	8.05±0.04	0.11±0.03	0.27±0.00	308.92±26.9
		2	4.57±0.01	0.63±0.01	5.74±0.02	8.02±0.01	0.22±0.02	0.27±0.00	441.98±61.55
		3	4.56±0.01	0.63±0.01	5.81±0.02	8.28±0.03	0.07±0.06	0.31±0.02	328.93±82.63
C7	1	1	4.64±0.00	0.55±0.00	5.54±0.06	8.70±0.01	0.35±0.2	0.72±0.08	53.75±4.55
		2	4.59±0.00	0.56±0.03	5.49±0.18	8.26±0.01	0.22±0.02	0.64±0.10	50.29±0.14
		3	4.61±0.01	0.57±0.02	5.45±0.15	8.56±0.02	0.16±0.06	0.62±0.10	58.93±11.26
	5	1	4.59±0.00	0.59±0.00	5.52±0.04	8.21±0.01	0.30±0.10	0.88±0.03	82.85±0.24
		2	4.6±0.01	0.61±0.01	5.54±0.02	7.94±0.07	0.36±0.03	0.83±0.18	166.54±83.69
		3	4.58±0.01	0.53±0.02	5.56±0.03	8.18±0.01	0.18±0.08	0.67±0.10	107.34±4.15
	10	1	4.64±0.01	0.56±0.05	5.72±0.04	7.91±0.03	0.07±0.02	0.49±0.19	131.35±10.89
		2	4.59±0.01	0.59±0.00	5.56±0.02	7.92±0.06	0.18±0.012	0.61±0.01	198.9±112.07
		3	4.57±0.01	0.58±0.03	5.67±0.01	8.92±0.09	0.31±0.18	0.60±0.00	131.42±93.85
	15	1	4.6±0.01	0.61±0.01	5.51±0.05	7.80±0.08	0.27±0.13	0.58±0.01	160.63±19.96
		2	4.58±0.01	0.62±0.04	5.68±0.13	7.87±0.02	0.29±0.06	0.66±0.02	281.26±17.93
		3	4.59±0.01	0.59±0.02	5.69±0.08	8.27±0.05	0.15±0.08	0.69±0.01	68.88±8.57
C9	1	1	4.63±0.00	0.57±0.00	5.56±0.02	8.68±0.06	0.15±0.04	0.72±0.11	52.65±7.25
		2	4.61±0.03	0.57±0.01	5.51±0.05	8.12±0.01	0.36±0.18	0.71±0.05	50.58±2.47
		3	4.58±0.02	0.59±0.03	5.46±0.02	8.71±0.10	0.25±0.08	0.65±0.11	43.83±5.35
	5	1	4.61±0.01	0.59±0.03	5.59±0.12	8.27±0.03	0.43±0.33	0.96±0.01	67.09±2.17
		2	4.59±0.02	0.61±0.01	5.48±0.04	8.06±0.02	0.43±0.08	0.86±0.04	92.41±4.91
		3	4.57±0.01	0.56±0.03	5.50±0.12	8.27±0.01	0.19±0.18	0.74±0.02	89.76±9.29
	10	1	4.65±0.01	0.58±0.00	5.58±0.10	8.12±0.05	0.34±0.34	0.95±0.05	80.59±2.4
		2	4.59±0.00	0.61±0.01	5.73±0.06	7.93±0.03	0.34±0.05	0.94±0.02	88.44±1.18
		3	4.56±0.01	0.63±0.05	5.74±0.03	8.91±0.13	0.23±0.09	0.86±0.06	93.24±21.47
	15	1	4.58±0.01	0.60±0.01	5.60±0.02	8.07±0.01	0.30±0.02	0.62±0.34	86.03±15.59
		2	4.58±0.01	0.59±0.00	5.79±0.02	7.99±0.04	0.21±0.04	0.43±0.02	71.82±0.01
		3	4.55±0.00	0.60±0.02	5.77±0.01	8.27±0.07	0.37±0.09	0.27±0.01	94.55±39.13

Table D.4 Average scores of sensory evaluation of coconut yogurts conducted after 24 h and 15 days of storage at 4˚C.

Day	Formulation	Appearance	Color	Texture	Flavour	Overall Acceptability
1	Control	7.03±1.16	7.00±1.34	6.77±1.85	7.00±1.76	7.03±1.30
		7.30±1.29	7.33±1.21	7.9±0.99	7.47±1.52	7.30±1.39
		6.77±1.70	7.33±1.54	6.67±2.19	6.63±2.33	6.87±1.72
	C7	5.83±2.00	6.4±2.04	6.37±1.75	6.37±1.81	6.47±1.89
		6.73±1.31	6.6±1.67	7.33±0.86	7.07±1.51	6.97±1.33
		7.00±1.43	7.07±1.68	6.87±1.75	6.57±2.13	6.57±1.90
	C9	5.83±2.00	6.4±2.04	6.37±1.81	6.37±1.75	6.47±1.89
		6.73±1.31	6.6±1.67	7.33±0.86	7.07±1.1.51	6.97 ±1.33
		7±1.43	7.07±1.68	6.87±1.76	6.57±2.13	6.57±1.87
15	Control	7.27±1.41	7.23±1.66	7.07±1.70	6.61±0.98	6.80±1.68
		7.43±0.95	7.3±1.03	7.37±1.37	7.13±1.56	7.23±1.29
		7.20±1.31	7.07±1.10	7.20±1.39	6.90±1.89	7.00±1.63
	C7	5.93±2.20	6.03±2.08	6.9±1.45	6.37±1.69	6.30±1.68
		6.43±2.05	6.17±2.34	7.33±1.54	7.23±1.30	7.17±1.44
		6.37±1.93	6.30±1.99	6.73±1.41	6.73±1.72	6.6±1.50
	C9	6.33±2.07	6.37±2.04	6.5±1.97	6.2±1.99	6.23±1.79
		6.87±2.04	6.77±2.09	7.30±1.76	6.80±1.88	6.90±1.86
		7.00±1.05	6.60±1.59	7.27±1.57	6.63±1.71	6.83±1.46

Table D.4 HPLC analysis of % of curcuminoids retained in yogurt during storage at 4˚C for 15 days

Product	Day	Replication	Peak Area	µg injected (10µl)	AV	% remaining C	Average %	SD
C7	1	1	989139	0.133386194	0.145190899	100	100	0
			1164217	0.156995604				
		2	1137051	0.153332247	0.166137141	100		
			1326963	0.178942034				
		3	948751	0.12793984	0.126935336	100		
			933853	0.125930833				
	5	1	1252717	0.168929901	0.173457449	118.7350767	94.86115007	29.80927387
			1319866	0.177984998				
		2	1120759	0.151135258	0.152512557	104.3978807		
			1141186	0.153889855				
		3	302744	0.040825273	0.089771667	61.45049286		
			1028678	0.138718061				
	10	1	1214843	0.163822561	0.158326312	108.377512	74.16015927	33.55152227
			1133327	0.152830063				
		2	573230	0.077300529	0.060358615	41.31667356		
			321961	0.043416701				
		3	814562	0.109844345	0.106331886	72.78629221		
			762468	0.102819427				
	15	1	334781	0.045145489	0.087112342	59.6301317	75.42657974	14.36750883
			957200	0.129079194				
		2	851494	0.114824655	0.115312882	78.93396305		
			858735	0.11580111				
		3	891762	0.120254827	0.128141846	87.71564446		
			1008736	0.136028866				
C9	1	1	1098408	0.024800831	0.02401329	100	100	0
			1028649	0.02322575				
		2	1125977	0.025423308	0.026340462	100		
			1207217	0.027257617				
		3	1226137	0.027684809	0.02805438	100		
			1258873	0.028423952				
	5	1	1104109	0.024929553	0.024596627	94.13031208	84.65861526	8.345039911
			1074619	0.024263701				
		2	873214	0.019716201	0.020483149	78.38819504		
			941149	0.021250097				
		3	920103	0.020774902	0.021285128	81.45733865		
			965298	0.021795355				
	10	1	1051890	0.023750506	0.02242508	85.81988793	78.51214804	7.398772009
			934486	0.021099654				
		2	808563	0.018256453	0.01855927	71.02558576		
			835386	0.018862086				
		3	818074	0.018471201	0.020562265	78.69097044		
			1003297	0.02265333				
	15	1	796536	0.017984897	0.017729586	67.85041892	71.17901846	6.339683826
			773921	0.017474275				
		2	746786	0.016861597	0.017558822	67.19691141		
			808545	0.018256046				
		3	859373	0.019403686	0.020509679	78.48972503		
			957340	0.021615672				

D.5 Statistical analysis of phase III

(A) During Fermentation of coconut cream

General Linear Model: pH versus Test Sample, Time

Factor Information

Factor	Type	Levels	Values
Test Sample	Fixed	3	C7, C9, Control
Time	Fixed	5	0, 2, 4, 6, 8

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Test Sample	2	0.0429	0.02143	3.22	0.045
Time	4	35.9682	8.99205	1350.54	0.000
Error	83	0.5526	0.00666		
Lack-of-Fit	8	0.0311	0.00389	0.56	0.807
Pure Error	75	0.5215	0.00695		
Total	89	36.5637			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0815973	98.49%	98.38%	98.22%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	6.1207	0.0228	268.96	0.000	
Test Sample					
C7	-0.0430	0.0211	-2.04	0.044	1.33
C9	-0.0490	0.0211	-2.33	0.022	1.33
Time					
2	-0.293	0.0272	-10.81	0.000	1.60
4	-1.098	0.0272	-40.40	0.000	1.60
6	-1.448	0.0272	-53.25	0.000	1.60
8	-1.593	0.0272	-58.60	0.000	1.60

General Linear Model: Titratable acidity versus Test Sample, Time

Factor Information

Factor	Type	Levels	Values
Test Sample	Fixed	3	C7, C9, Control
Time	Fixed	5	0, 2, 4, 6, 8

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Test Sample	2	0.00323	0.001617	2.61	0.080
Time	4	2.76336	0.690841	1113.27	0.000
Error	83	0.05151	0.000621		
Lack-of-Fit	8	0.00252	0.000315	0.48	0.865
Pure Error	75	0.04898	0.000653		
Total	89	2.81810			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0249109	98.17%	98.04%	97.85%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	0.21808	0.00695	31.39	0.000	
Test Sample					
C7	0.01339	0.00643	2.08	0.040	1.33
C9	0.01192	0.00643	1.85	0.067	1.33
Time					
2	0.04708	0.00830	5.67	0.000	1.60
4	0.16832	0.00830	20.27	0.000	1.60
6	0.32286	0.00830	38.88	0.000	1.60
8	0.47214	0.00830	56.86	0.000	1.60

General Linear Model: *L. bulgaricus* counts (log cfu/g) versus Test Sample, Time
Factor Information

Factor	Type	Levels	Values
Test Sample	Fixed	3	C7, C9, Control
Time	Fixed	5	0, 2, 4, 6, 8

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Test Sample	2	0.0217	0.01084	0.84	0.434
Time	4	12.6715	3.16787	246.23	0.000
Error	83	1.0678	0.01287		
Lack-of-Fit	8	0.0120	0.00150	0.11	0.999
Pure Error	75	1.0558	0.01408		
Total	89	13.7610			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.113425	92.24%	91.68%	90.88%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	5.8795	0.0316	185.87	0.000	
Test Sample					
C7	-0.0102	0.0293	-0.35	0.728	1.33
C9	-0.0368	0.0293	-1.26	0.212	1.33
Time					
2	0.1041	0.0378	2.75	0.007	1.60
4	0.6702	0.0378	17.73	0.000	1.60
6	0.8805	0.0378	23.29	0.000	1.60
8	0.8514	0.0378	22.52	0.000	1.60

General Linear Model: *S. thermophilus* counts (log cfu/g) versus Test Sample, Time
Factor Information

Factor	Type	Levels	Values
Test Sample	Fixed	3	C7, C9, Control

Time	Fixed	5	0, 2, 4, 6, 8

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Test Sample	2	0.399	0.1996	1.87	0.160
Time	4	101.133	25.2832	237.23	0.000
Error	83	8.846	0.1066		
Lack-of-Fit	8	0.186	0.0233	0.20	0.990
Pure Error	75	8.659	0.1155		
Total	89	110.378			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.326461	91.99%	91.41%	90.58%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	6.9876	0.0910	76.75	0.000	
Test Sample					
C7	-0.0743	0.0843	-0.88	0.381	1.33
C9	-0.1629	0.0843	-1.93	0.057	1.33
Time					
2	1.646	0.109	15.13	0.000	1.60
4	2.472	0.109	22.72	0.000	1.60
6	2.924	0.109	26.87	0.000	1.60
8	2.659	0.109	24.43	0.000	1.60

(B) During storage at 4˚C for 15 days

General Linear Model: pH versus Test Sample, Day
Factor Information

Factor	Type	Levels	Values
Test Sample	Fixed	3	C7, C9, Control
Day	Fixed	4	1, 5, 10, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Test Sample	2	0.001219	0.000610	1.10	0.340
Day	3	0.011493	0.003831	6.89	0.000
Error	66	0.036719	0.000556		
Lack-of-Fit	6	0.000969	0.000162	0.27	0.948
Pure Error	60	0.035750	0.000596		
Total	71	0.049432			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0235872	25.72%	20.09%	11.60%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	4.59097	0.00278	1651.56	0.000	
Test Sample					
C7	0.00569	0.00393	1.45	0.152	1.33
C9	-0.00181	0.00393	-0.46	0.648	1.33
Day					
1	0.01681	0.00481	3.49	0.001	1.50
5	-0.00431	0.00481	-0.89	0.374	1.50
10	0.00514	0.00481	1.07	0.290	1.50

General Linear Model: Titratable acidity versus Test Sample, Day
Factor Information

Factor	Type	Levels	Values
Test Sample	Fixed	3	C7, C9, Control
Day	Fixed	4	1, 5, 10, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Test Sample	2	0.005242	0.002621	2.82	0.067
Day	3	0.014110	0.004703	5.06	0.003
Error	66	0.061361	0.000930		
Lack-of-Fit	6	0.003144	0.000524	0.54	0.776
Pure Error	60	0.058217	0.000970		
Total	71	0.080713			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0304912	23.98%	18.22%	9.52%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	0.59164	0.00359	164.64	0.000	
Test Sample					
C7	-0.01110	0.00508	-2.18	0.033	1.33
C9	0.00144	0.00508	0.28	0.777	1.33
Day					

5	-0.00731	0.00622	-1.17	0.245	1.50
10	0.00736	0.00622	1.18	0.241	1.50

General Linear Model: *L. bulgaricus* counts versus Test Sample, Day

Factor Information

Factor	Type	Levels	Values
Test Sample	Fixed	3	C7, C9, Control
Day	Fixed	4	1, 5, 10, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Test Sample	2	0.05014	0.025069	3.30	0.043
Day	3	0.25156	0.083852	11.04	0.000
Error	66	0.50131	0.007596		
Lack-of-Fit	6	0.04650	0.007750	1.02	0.420
Pure Error	60	0.45481	0.007580		
Total	71	0.80300			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0871525	37.57%	32.84%	25.70%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	5.7387	0.0103	558.73	0.000	
Test Sample					
C7	-0.0275	0.0145	-1.89	0.063	1.33
C9	-0.0081	0.0145	-0.56	0.577	1.33
Day					
1	0.0918	0.0178	5.16	0.000	1.50
5	0.0110	0.0178	0.62	0.537	1.50
10	-0.0596	0.0178	-3.35	0.001	1.50

General Linear Model: *S. thermophilus* counts versus Test Sample, Day

Factor Information

Factor	Type	Levels	Values
Test Sample	Fixed	3	C7, C9, Control
Day	Fixed	4	1, 5, 10, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Test Sample	2	0.07617	0.03809	0.49	0.613
Day	3	1.90739	0.63580	8.22	0.000
Error	66	5.10277	0.07731		
Lack-of-Fit	6	0.06157	0.01026	0.12	0.993
Pure Error	60	5.04120	0.08402		
Total	71	7.08633			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.278055	27.99%	22.54%	14.30%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	8.2562	0.0328	251.95	0.000	
Test Sample					
C7	-0.0458	0.0463	-0.99	0.326	1.33
C9	0.0263	0.0463	0.57	0.572	1.33
Day					
1	0.2385	0.0568	4.20	0.000	1.50
5	-0.1047	0.0568	-1.84	0.070	1.50
10	0.0539	0.0568	0.95	0.346	1.50

General Linear Model: Syneresis versus Test Sample, Day
Factor Information

Factor	Type	Levels	Values
Test Sample	Fixed	3	C7, C9, Control
Day	Fixed	4	1, 5, 10, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Test Sample	2	0.09002	0.04501	2.78	0.069
Day	3	0.05348	0.01783	1.10	0.355
Error	66	1.06791	0.01618		
Lack-of-Fit	6	0.07677	0.01280	0.77	0.593
Pure Error	60	0.99114	0.01652		
Total	71	1.21141			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.127203	11.85%	5.17%	0.00%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	0.2515	0.0150	16.78	0.000	
Test Sample					
C7	-0.0142	0.0212	-0.67	0.505	1.33
C9	0.0486	0.0212	2.29	0.025	1.33
Day					
1	-0.0173	0.0260	-0.67	0.508	1.50
5	0.0403	0.0260	1.55	0.125	1.50
10	0.0082	0.0260	0.32	0.752	1.50

General Linear Model: Viscosity versus Test Sample, Day
Factor Information

Factor	Type	Levels	Values
Test Sample	Fixed	3	C7, C9, Control
Day	Fixed	4	1, 5, 10, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Test Sample	2	0.1327	0.06635	2.03	0.140
Day	3	1.8822	0.62742	19.18	0.000

Error	66	2.1585	0.03271		
Lack-of-Fit	6	1.3106	0.21843	15.46	0.000
Pure Error	60	0.8479	0.01413		
Total	71	4.1735			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.180846	48.28%	44.36%	38.45%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	0.6729	0.0213	31.57	0.000	
Test Sample					
C7	-0.0033	0.0301	-0.11	0.912	1.33
C9	0.0542	0.0301	1.80	0.077	1.33
Day					
1	0.0082	0.0369	0.22	0.825	1.50
5	0.2388	0.0369	6.47	0.000	1.50
10	-0.0312	0.0369	-0.85	0.400	1.50

General Linear Model: Firmness versus Test Sample, Day

Factor Information

Factor	Type	Levels	Values
Test Sample	Fixed	3	C7, C9, Control
Day	Fixed	4	1, 5, 10, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Test Sample	2	32953351	16476675	1.32	0.273
Day	3	37277969	12425990	1.00	0.399
Error	66	820975763	12439027		
Lack-of-Fit	6	74177249	12362875	0.99	0.438
Pure Error	60	746798514	12446642		
Total	71	891207083			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
3526.90	7.88%	0.90%	0.00%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	578	416	1.39	0.169	
Test Sample					
C7	-454	588	-0.77	0.443	1.33
C9	-502	588	-0.85	0.396	1.33
Day					
1	-494	720	-0.69	0.495	1.50
5	1243	720	1.73	0.089	1.50
10	-376	720	-0.52	0.603	1.50

(C)Sensory evaluation of coconut yogurt

General Linear Model: Appearance versus Test Sample, Day
Factor Information

Factor	Type	Levels	Values
Test Sample	Fixed	3	C7, C9, Control
Day	Fixed	2	1, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Test Sample	2	1.78508	0.89254	6.96	0.008
Day	1	0.03268	0.03268	0.25	0.622
Error	14	1.79645	0.12832		
Lack-of-Fit	2	0.23162	0.11581	0.89	0.437
Pure Error	12	1.56482	0.13040		
Total	17	3.61421			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.358215	50.29%	39.64%	17.83%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	6.7907	0.0844	80.43	0.000	
Test Sample					
C7	-0.408	0.119	-3.41	0.004	1.33
C9	0.048	0.119	0.40	0.692	1.33
Day					
1	0.0426	0.0844	0.50	0.622	1.00

General Linear Model: Colour versus Test Sample, Day
Factor Information

Factor	Type	Levels	Values
Test Sample	Fixed	3	C7, C9, Control
Day	Fixed	2	1, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Test Sample	2	1.8868	0.94339	15.90	0.000
Day	1	0.3289	0.32886	5.54	0.034
Error	14	0.8306	0.05933		
Lack-of-Fit	2	0.1878	0.09389	1.75	0.215
Pure Error	12	0.6428	0.05357		
Total	17	3.0462			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.243571	72.73%	66.89%	54.93%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	6.7834	0.0574	118.16	0.000	
Test Sample					
C7	-0.3556	0.0812	-4.38	0.001	1.33
C9	-0.0721	0.0812	-0.89	0.390	1.33
Day					
1	0.1352	0.0574	2.35	0.034	1.00

General Linear Model: Texture versus Test Sample, Day

Factor Information

Factor	Type	Levels	Values
Test Sample	Fixed	3	C7, C9, Control
Day	Fixed	2	1, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Test Sample	2	0.09820	0.049100	0.25	0.785
Day	1	0.00393	0.003931	0.02	0.890
Error	14	2.79799	0.199857		
Lack-of-Fit	2	0.01125	0.005625	0.02	0.976
Pure Error	12	2.78674	0.232228		
Total	17	2.90012			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.447053	3.52%	0.00%	0.00%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	7.059	0.105	66.99	0.000	
Test Sample					
C7	-0.071	0.149	-0.47	0.643	1.33
C9	-0.032	0.149	-0.21	0.836	1.33
Day					
1	-0.015	0.105	-0.14	0.890	1.00

General Linear Model: Flavour versus Test Sample, Day

Factor Information

Factor	Type	Levels	Values
Test Sample	Fixed	3	C7, C9, Control
Day	Fixed	2	1, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Test Sample	2	0.25628	0.128140	1.06	0.372
Day	1	0.00402	0.004020	0.03	0.858
Error	14	1.68672	0.120480		
Lack-of-Fit	2	0.05039	0.025196	0.18	0.834
Pure Error	12	1.63633	0.136361		
Total	17	1.94702			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.347102	13.37%	0.00%	0.00%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	6.7482	0.0818	82.48	0.000	
Test Sample					
C7	-0.026	0.116	-0.22	0.827	1.33
C9	-0.131	0.116	-1.14	0.275	1.33
Day					
1	0.0149	0.0818	0.18	0.858	1.00

General Linear Model: Overall Acceptability versus Test Sample, Day

Factor Information

Factor	Type	Levels	Values
Test Sample	Fixed	3	C7, C9, Control
Day	Fixed	2	1, 15

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Test Sample	2	0.44740	0.22370	2.82	0.094
Day	1	0.02730	0.02730	0.34	0.567
Error	14	1.11157	0.07940		
Lack-of-Fit	2	0.03111	0.01556	0.17	0.843
Pure Error	12	1.08046	0.09004		
Total	17	1.58627			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.281776	29.93%	14.91%	0.00%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	6.8204	0.0664	102.69	0.000	
Test Sample					
C7	-0.1479	0.0939	-1.57	0.138	1.33
C9	-0.0706	0.0939	-0.75	0.465	1.33
Day					
1	0.0389	0.0664	0.59	0.567	1.00