The influence of cell-free *Lactobacillus rhamnosus* GG conditioned medium on macrophage functional activity

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Abstract

Macrophages as professional phagocytes have been considered as prominent participants in the response to acute infection and play a significant role in preventing infecting bacteria multiplying and damaging the host environment. Macrophages have complex mechanisms equipped with specialised receptors to recognise their targets. Upon internalisation of receptor-bacterial complex, macrophages initiate killing of the ingested microbes partly through the generation of free radicals species. However, the excessive production of reactive oxygen species (ROS) and nitric oxide (NO) during phagocytic activity may also contribute to the inflammatory process which although is beneficial in killing bacteria may result in deleterious consequences, especially following an interaction of NO with superoxide radicals to form reactive nitrogen species such as peroxynitrite.

Probiotics are beneficial bacteria which have been shown to activate host innate immune response by influencing phagocytic activity of macrophages. This immunomodulatory effect has also been shown due to release of a number of soluble proteins by probiotic bacteria in the culture medium. However, the mechanism of regulation is not clear. In this study, we have therefore evaluated the influence of cell free *Lactobacillus rhamnosus* GG conditioned medium (LGG-CM) on macrophage phagocytosis and also on ROS and NO production by the J774 macrophage cell line, and further evaluated the acute effect of LGG-CM on NO and ROS production during the process of macrophage phagocytosis of *E.coli* in real time.

Murine macrophage J774 cells were used in the study as these cells are readily activated by bacterial lipopolysaccharide (LPS) and produce NO. Gentamicin protection assay was employed in order to investigate the effect of LGG-CM on the ingestion and digestion phases of macrophages phagocytosis of *E. coli* HfrC. The cells were exposed to different dilutions of LGG-CM in the absence and presence of bacterial LPS, *E. coli* and the inhibitors or scavengers for ROS and NO. The J774 macrophages were loaded with free radicals sensitive fluorescent dyes, namely, H_2DCFDA for monitoring ROS or with DAFFM-DA for NO detection. Acute free radical production was measured using a fluorescence microplate reader and changes were analysed by cumulative sum (CuSum) calculations. The fluorescence measurements were taken every two minutes for the first 60 minutes to monitor free radicals production during the ingestion period and from 60 minutes to 280 minutes to monitor free radicals production during the bacterial digestion period. The fluorescence was measured at 485 nm excitation and 528 nm emissions.

The LGG-CM did not have any toxic effect on *E. coli* or macrophage viability, as determined by antibacterial studies and cell proliferation assays. The LGG-CM treated macrophages ingested significantly less bacteria both at 30 minutes and 60 minutes of incubation ($p < 0.05$). However, only higher concentrations of LGG-CM (75 % LGG-CM and 100 % LGG-CM) significantly increased the bacterial digestion rate of *E. coli* by the macrophages ($p < 0.01$). The LPS on its own did not have any effect on macrophage phagocytosis. The LGG-CM mediated enhanced bacterial digestion was inhibited by ROS and NO inhibitors and ROS scavengers. The LGG-CM at higher concentration induced increase expression of NADPH oxidase subunits (p47 phox and gp91 phox) at 6 hours ($p < 0.01$).

A low concentration of LGG-CM (10 % LGG-CM) or LPS did not cause any significant change in the basal levels of ROS or NO production. In contrast, a high concentration of

LGG-CM significantly enhanced ROS generation but also significantly reduced the NO level in both the ingestion and digestion phases of phagocytosis.

These findings are novel and suggest for the first time that probiotics may release factors in culture medium which enhances macrophage digestion capability. The LGG-CM also has the capability to increase ROS production and may additionally reduce deleterious effects associated with excessive nitrogen species by suppressing the production of NO. These events may account, in part, for the beneficial bactericidal and anti-inflammatory actions ascribed to probiotics and may be of clinical relevance especially in several pathological conditions associated with gut inflammation or bacterial infection and may prove useful in improving intestinal homeostasis.

Declaration

I confirm that this thesis is based on my own research effort. No part of the literature and data were submitted by me in the past for a degree or qualification to any other university or academic body. I certify that to the best of my knowledge, material from the other research is acknowledged in this thesis.

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Dedication

I dedicate this thesis to my husband and my dear son for their remarkable patience and unwavering love and support over the course of my research.

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Akt/P13K	Protein kinase B
Allo	Allopurinol
ANOVA	Analysis of variance
Apo	Apocynin
APS	Ammonium persulphate
BCA	Bicinchoninic acid assay
BH_4	Tetrahydrobiopeterin
BMM	Bone marrow derived macrophages
BSA	Bovine serum albumin
CFU	Colony forming units
CGD	Chronic granulomatous disease
CO_2	Carbon dioxide
COX	Cyclooxygenase
CpG	Cytosine plus Guanine
CuSum	Cumulative sum
DAFFM	Diamino fluorescein FM diacetate
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPI	Diphenylene iodonium chloride
eNOS	Endothelial nitric oxide synthase
ETC	Electron transport chain
FAD	Flavin adenine dinucleotide
FAO	Food and agriculture organisation
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
fMLF	N-formyl-methionyl-leucyl-phenylalanine
FMN	Flavin mononucleotide
GALT	Gut-associated lymphoid tissue
GAP	GTPase activating proteins
GAT	Gentamicin acetyl transferase
GDI	Guanine nucleotide dissociation inhibitors
GDP	Guanosine diphosphate

GEF	Guanine nucleotide exchange factors
GPA	Gentamicin protection assay
GTP	Guanosine triphosphate
H_2DCFDA-AM	2', 7'-Dichlorodihydrofluorescein diacetate diacetoxymethyl ester
HCl	Hydrochloric acid
HSP	Heat shock proteins
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cells
IgA	Immunoglobulin A
IKB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
IL1β	Interleukin 1 Beta
IL-8	Interleukin-8
iNOS	Inducible nitric oxide synthase
ITAM	Immunoreceptor tyrosine based activation motifs
JNK	Jun N-terminal kinase
LGG	*Lactobacillus rhamnosus* GG
LGG-CM	*Lactobacillus rhamnosus* GG conditioned medium
L-NMMA	NG-monomethyl-L –arginine
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
MAPK	Mitogen activated protein kinase
MDM	Monocyte derived macrophages
MOI	Multiplicity of infection
mRNA	Messenger RNA
MRS	de Man, Rogosa and Sharpe
MTT	(3-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NFkβ	Nuclear factor kappa B
NMDA	N-methyl D-aspartate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOD	Nucleotide binding oligomerisation domain receptor
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular patterns
PAO	Phenylarsideoxide

PBS	Phosphate buffered saline
PEG	Polyethylene Glycol
PEG-CAT	Polyethylene glycol conjugated catalase
PEG-SOD	Polyethyleneglycol conjugated Superoxide dismutase
PI3	Phosphatidylinositol
PIP3	Phosphatidylinositol (3, 4, 5)-triphosphate
PMN	Polymorphonuclear
PRR	Pathogen pattern recognising receptors
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCFA	Short chain fatty acids
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
TEER	Trans-epithelial electrical resistance
TEMED	Tetramethylethylenediamine
TLR	Toll like receptors
TNF-α	Tumor necrosis factor alpha
ULT	Ultra low temperature
UV	Ultraviolet
WHO	World health organisation

Contents

CHAPTER 1
INTRODUCTION

1.0. CHAPTER 1 Introduction

1.1. Microbes and the human body

The common assumption that- 'all microbes are harmful' is disproved by the presence of huge microbial population which exceed the number (10 to 1) of our own body cells (Zhu *et al*., 2011). Human body harbours nearly 10^{13}-10^{14} microorganisms which so far are classified in to 30 known genera and around 500 species (Wershil & Furata., 2008; Zhu *et al*., 2011; Purchiaroni *et al*., 2013).

The human intestinal microbiota is known to play a key role in metabolic, nutritional, physiological, psychological and immunological processes (Ottman *et al*., 2012). The human microbiota is established after birth and form a dynamic ecosystem dominated by *Lactobacillus* and *Bifidobacteria* that stabilizes during the first 2–3 years (Abt *et al*., 2012; Ottman *et al*, 2012). These microbes are also known as commensal bacteria. There is evidence that a stable, intimate, bidirectional interactions between commensal bacteria and the host are essential for the host's normal physiological process (Abt *et al*., 2012; Ganal *et al*., 2012; Rudin & Lundell, 2012). These bacteria have been shown to influence directly on the host health through modulation of gene expression or by preventing the colonisation of infectious bacteria and detoxification of toxic chemicals such as xenobiotics (Ley *et al*., 2006; Ottman *et al*., 2012; Pessione 2012; Honda and Littman, 2012).

A mutual beneficial relationship exists between the commensal bacteria and the host where the host provides food and a suitable environment for bacterial growth, and the microbes function in tandem with the host's defences and the immune system to protect against pathogen colonisation and invasion (Kraehenbuhl and Corbett, 2004; Winkler *et al*., 2007; Clavel & Haller, 2007; Manichanh *et al*., 2012). These microbes are also known to perform a number of essential metabolic functions of the hosts. The microbes actively metabolize non-digestible dietary carbohydrates, exfoliated epithelial cells, and mucus to provide essential nutrients such as short chain fatty acids (SCFA), amino acids and vitamins which profoundly influence host's intestinal epithelial functions and motility of the gut (Hooper and Gordon, 2001). They also help in the extraction of energy and maintain a host-microbial energy balance in the gut (Turnbaugh *et al*., 2006; Turnbaugh *et al*., 2009).

Alterations in the microbiota can result from exposure to various environmental factors, including diet, toxins, drugs, and pathogens. The importance of this indigenous microbiota

has been also demonstrated in germ free animals which were found to be more susceptible to diseases (Round *et al*. 2010). Altered intestinal microbial balance (dysbiosis) has been demonstrated in patients with several gastrointestinal disorders, including inflammatory bowel diseases (IBD), functional dyspepsia, non-alcoholic steatohepatitis and alcoholic liver disease. However it is still not clear whether the intestinal dysbiosis is a cause or a consequence of a given diseases (Sartor, 2008; Sartor, 2010).

Some of the commensal microbes are now commonly known as probiotic bacteria as they are readily available in the consumer market as food supplements.

1.2. Concept of probiotics

The concept of probiotics can be traced back to the work of Nobel Prize-winning Russian scientist Eli Metchnikoff. He first suggested in 1907 that consumption of fermented milk products increased the longevity of Bulgarian peasants (Fioramonti *et al.*, 2003). The word 'probiotics' is derived from the Greek words pro and biotos and translated as –'for life' (Hamilton *et al*., 2003).

A detailed history, basics and definitions of probiotics has been mentioned in detail by Azizpour *et al*. (2009) and are as follows: Vergin, (1954) had proposed that the imbalance in microflora in the body caused by antibiotics could have been restored by a probiotic rich diet. However, Lilly and Stillwell (1965) were first to define probiotics as "substances produced by one microorganism that promoted the growth of another microorganism".

Similar to this definition, Sperti (1971) and Fujii and Cook (1973) described probiotics as "compounds that either stimulated microbial growth or improved the immune response of the host without inhibiting the growth of the culture *in vitro*". Another definition offered by Parker (1974) resembles more recent descriptions of probiotics (Azizpour *et al*., 2009). Parker (1974) defined them as "organisms and substances, which contribute to intestinal microbial balance" (Azizpour *et al*., 2009).

The most cited definition in the 1990's is Fuller's (1992), who defined them as "live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance". This was not a clear statement as it was mostly applicable to animals.

Salminen *et al*. (1999) offered their view in incorporating non- viable bacteria in their definition. In 2002, Marteau *et al.* defined probiotics as 'microbial cell preparation or components of microbial cells that have a beneficial effect on health and well-being'. Gorbach, (2002) defined probiotics as 'living organisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition'.

Following the recommendations of a FAO/WHO, (2002) working group on the evaluation of probiotics in food, the suggested definition describes probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". Until today this is the most commonly used definition of probiotics in literature. Howarth in 2010 entitled the probiotics derived factors as –"probiotoceuticals". Although literature refers back to various terminologies and definitions- all together, in general, any live bacteria or probiotic derived factor are termed as probiotics.

1.3. Probiotics and their influence on the immune system.

Lactobacilli and *Bifidobacteria* are currently the most well-known probiotic species, due to their presence being essential for a healthy human microbiota (Arunachalam *et al*., 2000; Kullisaar *et al.,* 2002). These bacteria can survive in the intestine and promote recovery of normal gut microbiota. This, in addition to their long and safe history in the manufacture of dairy products (Fooks & Gibson, 2002), makes these species an ideal supplement to healthy or immune-compromised persons.

Several health benefits of probiotics, including the control of gastroenteritis and irritable bowel syndrome, have been previously proposed. Among these, much attention has been paid to their immune-regulatory activity (Shida & Nanno, 2008; O'Flaherty *et al*., 2010). A number of animal and human studies have shown the beneficial effects of probiotics on health through the maintenance of healthy gut microbiota [Azcarate –Peril *et al*., 2011; Akaza, 2012].

As early as 1907, probiotics were regarded as health promoting to human beings (Metchnikoff, 1907). The mechanisms by which these bacteria exert their effects are still largely unknown. The impact of probiotics on intestinal microflora was found to be limited in some studies as it only brought about a transient alteration in the composition of indigenous microflora (Venturi *et al*., 1999, Tannock *et al*., 2000). However, there is

mounting evidence that probiotics, by some means, directly influence the immune system (Cunningham–Rundles *et al*., 2000; Erickson and Hubbard, 2000).

When an individual gut flora composition fluctuates due to environmental changes in the gut, for example a change in the diet, acute diarrhoeal illness or antibiotic treatment (Antonopoulos *et al*., 2009; Sekirov *et al*., 2010; Willing *et al*., 2011), use of probiotics could shift the 'balance' of the bacterial communities back to the normal composition. *Lactobacilli* can out-compete pathogens for resources, or simply not allow pathogens or other bacteria to adhere to the epithelial cell wall (Collado *et al*., 2007).

Probiotics immunomodulatory effect can be derived by its metabolites, DNA and cell wall components interaction, with the host epithelial or immune cells (Oelschlaeger, 2010). Such recognition, either by cell adhesion or release of soluble factors, can trigger a signalling cascade (Oelschlaeger, 2010) which indeed regulates the innate and/or acquired immune system. Table 1.1 explains the effect of probiotics on immune functions in healthy adults and provides a general overview of the health benefits associated with probiotics.

For the pharmacobiotic approach the probiotics should be selected on the basis of the host responses depending upon the host condition. A recent study on human mucosal transcriptome responses to probiotics reveals how various cellular pathways are modulated by probiotics (van Baarlen *et al*., 2011). It also reveals a large person to person variation in response transcriptomes (set of all RNA molecules transcribed in cells) and large variations in genes encoding bioactive molecules.

Table 1.1: Effect of probiotics on immune function in healthy adults. [Fluorescein isothiocyanate (FITC), Multiplicity of infection (MOI), Polymorphonuclear cells (PMN)].

Probiotics used	Healthy adults (Age range)	Immune function effects	Reference
Lyophilized *Enterococcus faecium* M-74 (single capsule 5× 10^9 bacteria day^{-1}) for 6 weeks.	12 healthy adults (aged 44-66 years)	Decreased superoxide production by peripheral neutrophils incubated with zymosan or phorbol myristate acetate at the end of supplementation then increased 2 weeks after supplementation was stopped.	Mikes *et al*., (1995)
L. acidophilus La1 ($7x10^{10}$ CFU day^{-1}) or *B.bifidum* Bb12 ($1x10^{10}$ CFU day^{-1}) for 3 weeks.	28 healthy adults (aged 23-62 years)	Enhanced phagocytic activity of *Escherichia coli* by granulocytes and monocytes was observed.	Schiffrin *et al*., (1995), Schiffrin *et al*., (1997)
L.casei Shirota (10^9 CFU day^{-1}) for 4 weeks.	20 healthy adults (aged 40-65 years)	No effect on percentage of phagocytising neutrophils or percentage of neutrophils showing oxidative burst.	Spanhaak *et al*., (1998)
L. johnsonii La1 (10^9 CFU day^{-1}) or ($\approx 10^8$ CFU day^{-1}) for 3 weeks.	42 healthy adults (aged 21-57 years)	Increased leukocyte phagocytosis of FITC–labelled *E.coli* and increased respiratory burst from leukocytes treated with opsonised unlabelled *E.coli* with $\approx 10^9$ CFU day^{-1}. No significant effect of ($\approx 10^8$ CFU day^{-1}) on phagocytic activity and respiratory burst.	Donnet-Hughes *et al*., (1999)
Bifidobacterium lactis HN019 (1.5×10^{11} CFU day^{-1}) for 6 weeks.	25 healthy, elderly adults (aged 60-83 years)	Increased number of PMN cells showing phagocytic activity of *Staphylococcus aureus*.	Arunachalam *et al*., (2000)
Bifidobacterium lactis HN019 10^9 CFU *B. Lactis* twice daily.	50 healthy adults (aged 41-81 years)	Increase in percent PMN cell phagocytosis.	Chiang *et al*., (2000)
L. rhamnosus HN0001 DR20 ™ (5×10^{10} CFU day^{-1}) for 3 weeks.	13 healthy, elderly adults (aged 62- 77 years)	Increases phagocytosis of fluoresceinated *Escherichia coli* by PMN cells and monocytes.	Gill *et al*.,(2001)[a]
L. rhamnosus, *L. plantarum*, *L. Salivarius* and *B. bifidum* (2×10^9 CFU day^{-1}) for 8 weeks.	10 healthy adults (aged 24-54 years)	Increased percentage of phagocytic monocytes and neutrophils of fluorescent labelled *E.coli* K12.	Berman *et al*., (2006)

1.4. *Lactobacillus rhamnosus* GG (LGG)

Lactobacillus rhamnosus GG (LGG), ATCC 53103, a probiotic strain of human origin is by far the most thoroughly explored Gram-positive lactic-acid bacteria with widely evidenced health effects (Saxelin, 1997; Sheil, 2007). The LGG is known to influence immune response, both specifically by stimulating antibody production (Kaila *et al*., 1992) and non-specifically by impacting on phagocytic activity (Pelto *et al*., 1998). The LGG modulating various signalling pathways in macrophages and intestinal epithelial cells are shown in Table 1.2. The LGG was first isolated from a healthy person stool while screening bacterial strains that were stable to acid and bile (Silva *et al*., 1987; Lee and Salminen, 1995). Since its isolation, LGG has become one of the clinically best-documented probiotic strains (Doron *et al*., 2005). *In vivo* studies have shown the good persistence capacity of LGG in the human gastrointestinal tract (Alander *et al*., 1999) and are known to produce antimicrobial substance (Silva *et al*., 1987).

The LGG has shown to increase secretion of mucosal immunoglobulin A (IgA) levels in the gut (Malin *et al.*, 1996), improve intestinal permeability and reduce disease activity when administered to children with Crohn's disease (Gupta *et al.*, 2000). A double blind study performed on 105 infants aged 0 to 2 months when given enriched probiotic formula containing milk-based spray-dried powder and 10^7 CFU g^{-1} of LGG showed enhanced weight and size (Vendt *et al*., 2006). The LGG has previously shown to protect host tissues against the attachment, overgrowth and action of pathogenic microorganisms (Miettinen *et al*., 2000; Matsuguchi *et al*., 2003; Servin, 2004; Cotter *et al*., 2005; Deshpande *et al*., 2011; Yan and Polk, 2012).

Table 1.2: The LGG modulation of signalling pathways. [Protein kinase B (Akt / P13K), heat shock proteins (hsp), intestinal epithelial cells (IEC), Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IkBα), Jun N-terminal kinase (JNK), mitogen activated protein kinase (MAPK), nuclear factor kappa B (NFkβ)].

Signalling pathway	Cell Model	Effect of probiotic	Reference(s)
NFkβ	Macrophages	Induces NFkβ binding activity.	Miettinen *et al*., (2000)
MAPK	IEC	↑ Phosphorylation of p38 and JNK. ↑ of MAPK regulated genes.	Di Caro *et al.,* (2005) Tao *et al*., (2006) Lin *et al*., (2008)
NFkβ	IEC	Prevents IkBα ubiquitination and degradation.	Zhang *et al*., (2005) Kumar *et al*., (2007) Lin *et al*., (2009)
Apoptosis	IEC	Activates Akt / P13K.	Yan and Polk, (2002) Yan *et al*., (2007)

1.5. LGG derived factors

This section reviews the current knowledge on the effect of LGG components on the macrophages and the intestinal epithelial cells, which is a vital, interacting site for host-pathogen interactions.

1.5.1. Introduction to the role of LGG derived factors

A large number of experimental and clinical studies published in recent years have highlighted the beneficial effects of live probiotics (Malin *et al*., 1996; Sheil *et al*., 2007). The transition from the era of using live probiotics to the era of using probiotic components for the pharmacobiotic approach is still in the juvenile state. There are reports and concerns of bacteraemia and sepsis cases when live probiotics were used (De Groote *et al*., 2005; Kunz *et al*., 2004; Boyle *et al*., 2006). However, these reports were from immunocompromised patients or premature infants, for example from a child suffering from short gut syndrome ending up with bacteraemia after consuming LGG capsule (De Groote *et al*., 2005; Kunz *et al*., 2004; Besselink *et al*., 2008). These emerging concerns regarding the safety problems of using live probiotics have enhanced the opportunity to utilise the non- viable probiotic components as an alternative (Sanchez *et al*., 2010; Caselli

et al., 2011; Caselli *et al*., 2013). The use of probiotic components rather than whole live bacteria prevents the risk of bacterial translocation and infection (De Groote *et al*., 2005; Kunz *et al*., 2004; Boyle *et al*., 2006).

Literature review shows that not only the whole living bacteria, but also the probiotic components demonstrate a beneficial effect on host immune activity. These factors includes bacterial cell wall components, such as peptidoglycans and lipoteichoic acid (Matsuguchi *et al*., 2003; Pena and Versalovic, 2003), whole genomic DNA from lactic acid bacteria (Iliev *et al*., 2005; Lammers *et al*., 2003; Meier and Steuerwald., 2005), short DNA sequences containing unmethylated, phosphate-linked pairs of cytosine plus guanine, so-called CpG motifs (Bohle *et al*. 1999; Helwig *et al*. 2006; Hessel *et al*. 2005; Kline *et al*. 1998; Klinman *et al*. 1996), heat killed LGG (Li *et al*., 2009) and secreted proteins from probiotic supernatant and conditioned media (Hessel *et al*., 2005; Helwig *et al*., 2006; Vincenti, 2010; Hand *et al*., 2012; Rudin and Lundell, 2012;Yoda *et al*.,2012; Villena and Kitazawa, 2014).

The aim of this section is to gather together the current information and provide an overview of the scientific literature concerning studies which have employed both the structural and non-structural components (dead LGG, LGG components such as DNA, secreted proteins and cell wall) of *Lactobacillus rhamnosus* GG. Particular attention will be given to the downstream responses and the mechanisms by which the LGG components exert their effect. The role of various extracellular proteins secreted by LGG is reviewed and is listed in table 1.3.

Table 1.3: Role of various extracellular proteins secreted by LGG [Young adult mouse colonic epithelial cells (YAMC), heat shock proteins (HSP), Human colorectal adenocarcinoma cells (Caco-2)].

Active component secreted from LGG	**Role**	**Reference**
Unknown low molecular weight compound resistant to various proteases.	Active against broad range of Gram positive and Gram negative organisms including Lactic acid bacteria but non-inhibitory to *lactobacilli*.	Silva *et al*.,(1987)
Secreted proteins of approximate size 42 and 80 kDa	Prevents cytokine induced apoptosis in YAMC cells.	Yan &Polk, (2002)
Unidentified secreted proteins	Increased production of HSP25 and HSP 72 in YAMC cells.	Tao *et al*., (2006)
Unidentified proteins from probiotic supernatant	Increase of luminal chloride/ hydroxyl exchange activity in Caco-2 cells.	Borthakur *et al*., (2008)
p40 and p75 proteins	Intestinal epithelial cell growth promotion in both human and murine colon epithelial cells (HT-29 and YAMC cells). Inhibited epithelial cell apoptosis induced by cytokines.	Yan *et al*., (2007)
p40 and p75 proteins	Attenuation of transepithelial resistance decrease induced by hydrogen peroxide & reduction in TNF-α induced injuries.	Seth *et al*., (2008)
Peptide NPSRQERR and PDENK	Antimicrobial activity exerted against both Gram positive (*Staphylococcus aureus*) and Gram negative bacteria (*Escherichia coli* EAEC 042 and *Salmonella typhi*).	Lu *et al*., (2009)

1.5.2. LGG-conditioned medium and Cell free LGG supernatant

The components by which LGG exert their beneficial health effects are not clear. However, there is evidence that a number of low molecular weight proteins secreted by LGG might be responsible for these beneficial effects (Yan *et al*., 2007; Seth *et al*., 2008). The extracellular proteins secreted by these beneficial bacteria can be divided into two groups (Sanchez *et al*., 2010). These include proteins that contain a signal peptide, which is located in the N-terminal, part of the sequence to direct the protein to protein export machinery and the surface protein shredded off normally as a part of cell wall turn over (Sanchez *et al*., 2008).

Study by Pena and Versalovic. (2003) showed that LGG-CM could reduce the production of potent proinflammatory cytokine TNF-α in LPS activated RAW 264.7 murine macrophages. Their study identified that the putative immunomodulatory soluble factors in the conditioned medium might contain a peptide or protein component that inhibits TNF-α production from macrophages.

A study by Roselli *et al*. (2006) examined the effect of the LGG supernatant on *E. coli* induced inflammatory response in Caco-2 cells. Their study demonstrated that the LGG supernatant demonstrated the ability to reduce the inflammation associated with human intestinal Caco-2 cells as a response to enterotoxigenic *E. coli* K88. This was achieved by partly reducing pathogen adhesion, preventing increased expression of proinflammatory cytokines like IL1β, TNF-α and also by the inhibition of neutrophil migration.

Studies by Yan & Polk, (2002) demonstrated that products recovered from LGG-CM showed a concentration dependent activation of Akt (Protein Kinase B) - an apoptotic cellular process regulator and inhibitor of cytokine induced apoptosis. The LGG-CM activated antiapoptotic AKt/protein kinase B and inhibited p38 MAPK in cytokine (TNF, IL1α, IFNγ) stimulated human colonic epithelial carcinoma cell line (HT-29) and Young adult mouse colon cell line (YAMC). This process did not require bacterial- intestinal cell contact. The immunomodulin proteins identified from LGG-CM were of approximately 80 and 42 kDa based on SDS-PAGE. In the subsequent experiments the proteins were purified from LGG-CM and identified as p40 and p75 (40 and 75 kilodaltons respectively) (Yan *et*

al. 2007). These purified proteins from the LGG-CM reduced TNF-α, induced epithelial damage and activated Akt in a phosphatidyl inositol-3'-kinase (PI3K) dependent manner and prevented apoptosis which is consistent with their previous findings. Thus, proteins in LGG-CM regulate both anti and pro apoptotic signal transduction pathways and are beneficial for the gut epithelium.

In a similar study by Tao *et al*. (2006), LGG-CM protects murine intestinal epithelial cells (YAMC) against oxidative damage induced by NH_2Cl. As a stress response, LGG induced the production of heat shock proteins (hsp) by activating various signalling pathways. The hsp are induced during environmental stress conditions like heat, heavy metals and pathophysiological stress conditions like microbial infections. Inducible hsp like hsp25 and hsp 72 productions occurs as a response to LGG-cell free conditioned medium. Evidence indicates that even exposure of LGG-CM to intestinal epithelial cells for a few minutes triggered the production of heat shock proteins. The hsp-25 expression was seen after 18-20 hours of LGG-CM exposure while hsp 72 expressions began as early as 4-6 hours. The hsp72 is well known for stabilising and preventing denaturation of cellular proteins while hsp-25 acts as an actin stabilising agent by binding to actin filaments and fortifying filamentous actin (Tao *et al*., 2006).

1.5.3. *Lactobacillus rhamnosus* GG DNA

Iliev *et al*. (2005) found that the chromosomal DNA from LGG demonstrated beneficial properties (at a concentration of 50 μg ml^{-1}) by inducing a strong proliferation response in murine B-lymphocytes. Further they also demonstrated that both chromosomal DNA and derived TTTCGTTT oligodeoxynucleotide ID35 not only induced B- cell proliferation but also increased the expression of CD86 (proteins expressed on antigen presenting cells that provide costimulatory signals for cell-mediated immunity) in B-cells.

A study by Donato *et al*. (2010) carried out experiments utilising live LGG, heat killed LGG or LGG in presence of antibiotic chloramphenicol (20 μg ml^{-1}) and investigated their effect on proinflammatory cytokines induced epithelial barrier disruption on Caco-2bbe epithelial cell integrity. Their study showed that live LGG preserves epithelial barrier function against the effects of proinflammatory cytokines IFN-γ and TNF-α (which are known to disrupt epithelial cell integrity). However, heat killed LGG or antibiotic addition to culture medium abolished the protective response even when 10-fold more LGG was used. Thus, they concluded that heat killing might have denatured the LGG DNA, and

thereby the protective effect of LGG in preserving epithelial integrity is compromised. In a similar study by Yan & Polk, (2002), heat killed LGG did not demonstrate a role in maintaining epithelial cell integrity in both human colonic epithelial carcinoma cell line (HT-29) and Young adult mouse colon cell line (YAMC). This further fortifies that DNA denaturation attenuates the beneficial property of LGG.

Another study by Ghadimi *et al*., (2010) using HT-29 and T-84 cells (Human colonic adenocarcinoma cell-line) demonstrated that apically applied LGG DNA reverses the loss of epithelial integrity resulted by TNF-α treatment. As previously known, TNF-α applied at the apical surface leads in decreased trans-epithelial electrical resistance (TER), opening of the tight junction barrier, translocation of TNF-α from apical to basolateral surface and subsequent access to chemoreceptors. This access followed by recognition leads in to degradation of Ikβ and phosphorylation of p38. Once NFkβ is activated it translocates from cytoplasm to nucleus and transcribes various proinflammatory genes including proinflammatory cytokines. This epithelial damage is prevented by apically applying LGG DNA. The LGG DNA prevents TNF-α induced NFkβ activation by reducing Ikβ degradation and p38 MAP kinase phosphorylation.

1.5.4. Dead LGG

Most of the studies investigating the effect of dead LGG have either heat killed (Zhang *et al*., 2005) or UV-inactivated (Lopez *et al*., 2008) or glutaraldehyde fixed LGG (Miettinen *et al*., 1996). Miettinen *et al*., 1996 had demonstrated that glutaraldehyde fixed LGG were not as efficient as live bacteria in the production of TNF-α from human peripheral blood mononuclear leukocytes. They interpreted that glutaraldehye fixed LGG had undergone structural changes, as glutaraldehyde itself is a cross-linking agent. They concluded that this glutaraldehyde fixation process might have denatured the LGG structural proteins and therefore failed to produce any stimulatory effect.

Zhang *et al*. (2005) showed that heat-killed LGG decreases IL-8 (a proinflammatory cytokine) production from TNF-α induced Caco-2 intestinal cell-line. However, a high dose of live LGG actually increased the IL-8 production in their study. This effect of increase in IL-8 was much less pronounced with a high dose of heat-killed LGG indicating that the use of heat-killed LGG might be a safer option in certain situations. The mechanism of the decrease in IL-8 production was via the inhibition of translocation of NFkβ to the nucleus and the associated decrease in Ikβ. In a more recent study by Lopez *et*

al. (2008), uv-inactivated LGG was as effective as live LGG in down regulating IL-8 production from Caco-2 cell line stimulated with flagellin from *Pseudomonas aeruginosa.*

A similar conclusion was also seen from Li *et al*. (2009) where heat-killed LGG provided by the enteral route in infant rats decreased LPS-induced proinflammatory intermediaries and increased anti-inflammatory mediators (Li *et al*., 2009). Studies have also been carried out with human trophoblast cells where the heat killed LGG stimulates IL-4, IL-10 (anti-inflammatory cytokines) expression and reverses LPS induced TNF-α (a proinflammatory cytokine) release from human trophoblast cells without affecting basic trophoblast functions (Bloise *et al*., 2010).

1.5.5. Summary of LGG and its derived products

In this literature review, attention has been given to only LGG (our model probiotic bacteria); however in the literature, the same study might have employed several other probiotics. In this literature review, a range of effects for a single probiotic strain LGG has been observed. Several clinical studies have explained the multiple immunological actions of LGG in various medical conditions. For example, the LGG appeared to modulate the non-specific immune response differently in healthy and hypersensitive subjects. The LGG upregulated the phagocytic markers in healthy individuals but down-regulated the same markers in milk hypersensitive subjects (Pelto *et al*., 1998). These clinical studies using live LGG are significant but the answer for the varied responses remains unexplained.

Currently, the knowledge just supports that both live LGG and their structural or secreted components are beneficial (Sanchez *et al*., 2008; Kataria *et al*., 2009; Taverniti & Guglielmetti, 2011; Adams, 2010; Caselli *et al*., 2013). The utility and potential for LGG/LGG components is clear but in depth, further investigations are ultimately essential.

Several studies have described the soluble component secreted by the probiotics exhibiting the beneficial property is a protein (Yan & Polk, 2002; Pena & Versalovic, 2003; Yan *et al.,* 2007; Yan *et al*., 2011). Contradictorily, early studies by Silva *et al*. (1987), showed that the component exhibiting the antibacterial activity was of a low molecular weight (<1,000) compound and retained activity even after treatment with various proteases such as trypsin, proteinase K, α-chymotrypsin, bromelain and carboxypeptidase A. The LGG beneficial activity had a preference of acidic pH range suggesting that it might be a sort of short chain fatty acid (Silva *et al*., 1987).

1.6. Macrophages and their defence mechanism

The host response to microbial pathogens is conventionally divided into two distinct but complementary and interactive parts. It comprises the innate immunity which protects the hosts in an earlier stage of infection primarily through the activity of neutrophils and macrophages. These mechanisms, however, are not driven by specific antigen interaction but might significantly protect the host in the first few hours, at least in significantly preventing the infected bacteria to multiply within the host environment. This response feeds directly into the initiation of adaptive immune response which culminates in lasting specific immunity by also establishing immune memory (Karupiah *et al.*, 2000).

Macrophages are the phagocytic cells which are the key regulators of immunity. Phagocytosis is the process where the macrophages engulf the foreign particles and degrade them (Aderem and Underhill, 1999) (see Section 1.7). After bacterial recognition macrophages ingest bacteria. Ingested microbes are then killed within phagolysosome. The two main antimicrobial systems of these phagocytes involve:

1. NADPH Phagocyte oxidase [Responsible for the production of superoxide ($O_2^{\cdot-}$) and other oxygen derived intermediates termed Reactive oxygen species (ROS)]
2. Inducible nitric oxide synthase (iNOS) [Responsible for the generation of nitric oxide (NO) and its derivatives termed Reactive nitrogen species (RNS)].

1.6.1. Nicotinamide adenine dinucleotide phosphate-oxidase (NADPH-oxidase)

Macrophages constitute one of the most powerful means of host defence against invading pathogens (Segal, 2005; Nathan, 2006). During infection, phagocytes recognize and engulf the pathogen and activate the release of reactive oxygen species such as superoxide anion, hydrogen peroxide, hydroxyl radical and hypochlorous acid used for defensive purposes as microbicidal agents (Bylund *et al*., 2010). The ROS are produced by phagocytes in a powerful "oxidative burst", characterized by a rapid increase in oxygen uptake and high glucose utilization and rapid ROS release. The enzyme responsible for $O_2^{\bullet-}$ production is the multicomponent NADPH oxidase or respiratory burst oxidase and is located on plasma membrane and membrane of phagosomes (El-Benna *et al*., 2005; El-Benna *et al*., 2009; Bylund *et al*., 2010).

The phagocyte NADPH oxidase activation can be induced by a large number of soluble and particulate agents. Two of the subunits, p22phox (phox for phagocyte oxidase) and gp91phox (also known as NOX2), form a membrane bound, heterodimeric flavohemoprotein known as cytochrome b (cytochromeb558). The NADPH oxidase is dormant in the absence of cellular activation. Upon cellular activation, the cytosolic components, namely p40phox, p47phox, and p67phox translocate to the membrane and associate with cytochrome b to from a functional NADPH oxidase (Figure 1.1). The whole process is highly regulated involving one or more cofactors. In human phagocytes the predominant cofactor is Rac2 and in murine phagocytes it's Rac1. When activated, NADPH oxidase transfers electrons from intracellular NADPH to extracellular (or intraphagosomal) oxygen forming $O_2^{\cdot -}$ anion (Bylund *et al*., 2010).

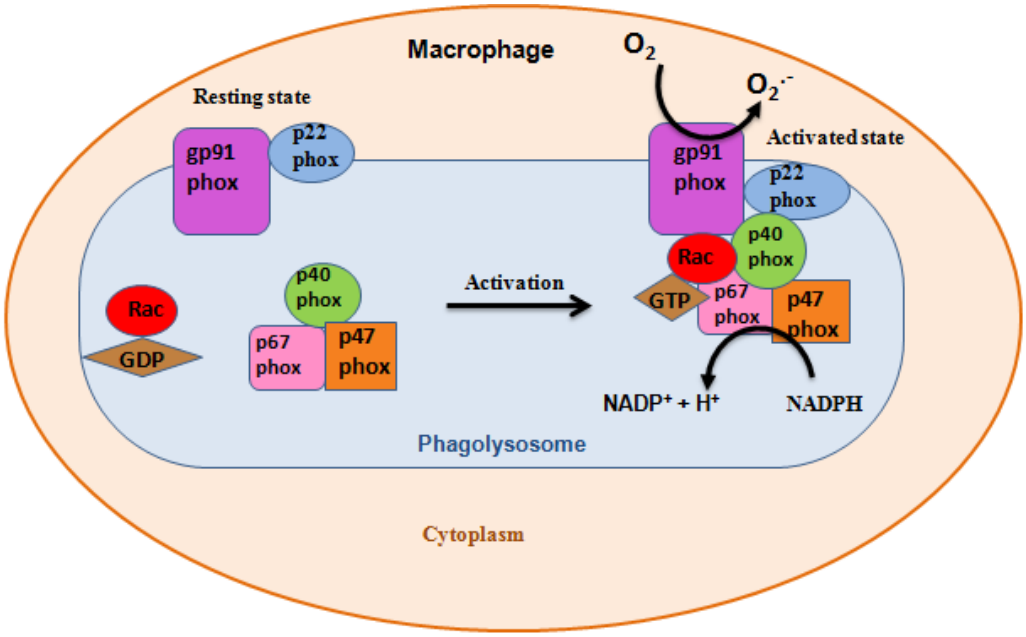

Figure 1.1: Phagocyte NADPH oxidase subunits in its active and resting state. Under normal conditions, gp91phox and p22phox are primarily found on the membrane of intracellular vesicles. Upon activation, there is an exchange of GDP to GTP on Rac cofactor leading to its activation. Phosphorylation of cytoplasmic p47phox results in the conformational changes allowing the interaction with p22phox. The p67phox and p40phox also moves to the membrane along with p47phox to form active enzyme complex. This active NADPH oxidase enzyme complex transfers electrons from cytoplasmic NADPH to extracellular or phagosomal oxygen to produce superoxide. [Guanosine diphosphate (GDP), Guanosine triphosphate (GTP), GTPase (Rac)]. Adapted from Bedard & Krause, 2007; Bylund *et al*., 2010).

The ROS are critical components of the antimicrobial repertoire of macrophages. The multi-subunit phagocyte oxidase (phox or NOX2) once assembled on the phagolysosome membrane, pumps electrons to reduce oxygen to superoxide anion (and derived species). Efficient production of ROS needs proper association of NADPH oxidase subunits.

Several studies suggest that phagocyte NADPH oxidase has a major contribution in the initiation and progression of many physiological and pathophysiological events, including thyroid hormone production in the thyroid gland, ischemia-reperfusion injury in multiple organs, septic shock, obesity, cancer, neuronal degeneration, and cardiovascular diseases (Caillou *et al*., 2001; Andreadou *et al*., 2009; Kleikers *et al*., 2012; Fu *et al*., 2014).

The important role of NADPH oxidase in host defence against invading microorganisms is demonstrated by an inherited human genetic disorder known as Chronic granulomatous disease (CGD), which is characterized by an absence of ROS production due to a deficiency in one of the components of the NADPH oxidase resulting in life-threatening microbial infections (Kannengiesser *et al*., 2008). Recurrent, often life-threatening, microbial infections usually start in the early stages of life. Different forms of CGD include gp91phox-deficient form (approximately 75%), p47*phox* deficient form (approximately 25%), p67phox deficient form (< 5%) and p22phox deficient form (< 5%) (Roos *et al*., 1996; Meischl & Ross, 1998; Kannengiesser *et al*., 2008; El –Benna *et al*., 2009). The importance of p47phox in host defence was also demonstrated in $p47^{phox}$ knockout mice. These mice developed lethal infections and granulomatous inflammation similar to those encountered in human CGD patients (Jackson *et al*., 1995).

1.6.2. Other sources of ROS in macrophages

Both generation and degradation of ROS is tightly regulated. In macrophages there are several vital sources of ROS, as well as multilevel antioxidant defences. Macrophage mechanisms of ROS generation and antioxidant defence are discussed below.

The physiological generation of ROS can occur as a by-product of several biological reactions. The ROS is mainly produced as a by-product within mitochondria or by the enzymes, namely, cyclooxygenase, lipooxygenase, xanthine oxidase, cytochrome p-450 and other cellular elements (Bedard & Krause 2007).

Mitochondria are thought to generate superoxide as a result of leakage from the electron transport chain (ETC). The ETC consists of a series of protein carriers found in the inner membrane of mitochondria. Electrons pass from a donor such as NADH and succinate along the electron transport chain to oxygen which is reduced to water. Most of the oxygen in respiring organisms is used this way, but a small percentage (1 to 2 %) is converted to

superoxide by one electron reduction. The dismutation of superoxide results in the formation of hydrogen peroxide. The formed hydrogen peroxide is broken down into water and oxygen by peroxidases. However, under normal conditions with functional mitochondrial antioxidant defence, ROS accumulating to have a physiological or pathophysiological effect is still unclear. The presence of a physiological inhibitor of the electron transport chain might explain this. Under *in vivo* conditions NO have been shown to inhibit complex IV of ETC and may modulate mitochondrial ROS production. Calcium uptake by mitochondria has been shown to increase mitochondrial metabolism and activate ETC and might thereby, increase stimulation of the superoxide molecule. (Poderoso *et al*., 1996; Moncada and Erusalimsky, 2002).

Cyclooxygenase (COX) and lipooxygenase are the enzymes involved in metabolism of unsaturated fatty acids such as arachidonic acid and are known to contribute to free radical production (Rice Evans & Bruckdorfer, 1992; Ricciotti & FitzGerald, 2011). The COX enzyme catalyzes the conversion of arachidonic acid to prostaglandins and thromboxane. Lipooxygenase catalyse the oxygenation of arachidonic acid to hydroxyeicosatetraenoic acid and hydroperoxyeicosatetraenoic acid. The ROS is produced as a by product during the fatty acid metabolism and also results from several studies suggest that COX metabolites are capable of stimulating NADPH oxidase and thereby ROS generation (Hong *et al*., 2008; Wang *et al*., 2010). These products are further known to be involved in the stimulation of NADPH oxidase (Hong *et al*., 2008) and thereby ROS production.

Xanthine oxidase is a form of xanthine oxidoreductase, which is known as a source of ROS in macrophages. The enzyme catalyses the oxidation of purines such as hypoxanthine and xanthine and can further catalyse the oxidation of xanthine to uric acid (Berry & Hare 2004; Nakai *et al*., 2006). During this process xanthine oxidase reduces oxygen to superoxide and thereby contributes to ROS production (Kelley *et al*., 2010).

Cytochrome p450 serves as a source of free radicals. The monooxygenase activity of this enzyme activates oxygen which is inserted into a substrate. A reactive intermediate might be formed during this process. However, this enzyme also exhibits an oxidase activity by molecular oxygen not being incorporated into the substrate but rather the oxygen may be released from oxy-complex of cytochrome p450 complex as superoxide anions (Zangar *et al*., 2004).

Most of the sources of ROS indicate the ROS production as by-product. The NADPH oxidase is an example of an enzyme system that generates ROS not as a by-product, but rather as a functional molecule of an enzyme system. The activated NADPH oxidase generates superoxide by reduction of molecular oxygen using NADPH as an electron donor (Babior *et al*., 2002). The non-phagocytic NADPH oxidase such as endothelial NAD(P)H oxidase also exists. But these unlike phagocyte oxidase are constitutively expressed and do not generate high level of superoxide. The endothelial NADPH oxidase however utilises both NADH and NADPH as a substrate unlike phagocyte oxidase which preferentially uses NADPH (Lasseguen and Clempus, 2003).

1.6.3. Inducible nitric oxide synthase

Nitric oxide is a small and short lived signalling molecule involved in various physiological and pathophysiological conditions in the human body (Korhonen *et al*., 2005). The NO is synthesized by a family of enzymes called the nitric oxide synthases (NOS), which convert the amino acid L-arginine into L-citrulline and NO (figure 1.2) (Korhonen, *et al*., 2005; He *et al*., 2006). The NOS belong to a family of three isoenzymes: neuronal [nNOS also known as NOS-1], inducible [iNOS also known as NOS-2] and endothelial eNOS [also known as NOS-3]. These isoforms differ in their tissue distribution, calcium sensitivity and upstream activators (see table 1.4).

The NO produced by the host phagocytic cells plays a major role in innate immunity, largely due to the ability of NO to inhibit or kill a broad range of microorganisms by its reactivity towards diverse cellular constituents such as [Fe–S] clusters and haem (Fang 2004). The half-life of NO in the biological system is very short, ranging from 1 to 30 seconds (Knowles and Moncada 1992; Feldman *et al*., 1993; Eich *et al*., 1996). It is rapidly oxidised to nitrite or nitrate (MacMicking *et al*., 1997). The NO, when combined with ROS can also form other radicals of even greater cytotoxicity such as peroxynitrite. Therefore, NO is regarded as one of the effective host immune responses against invading microorganisms (Fang 2004).

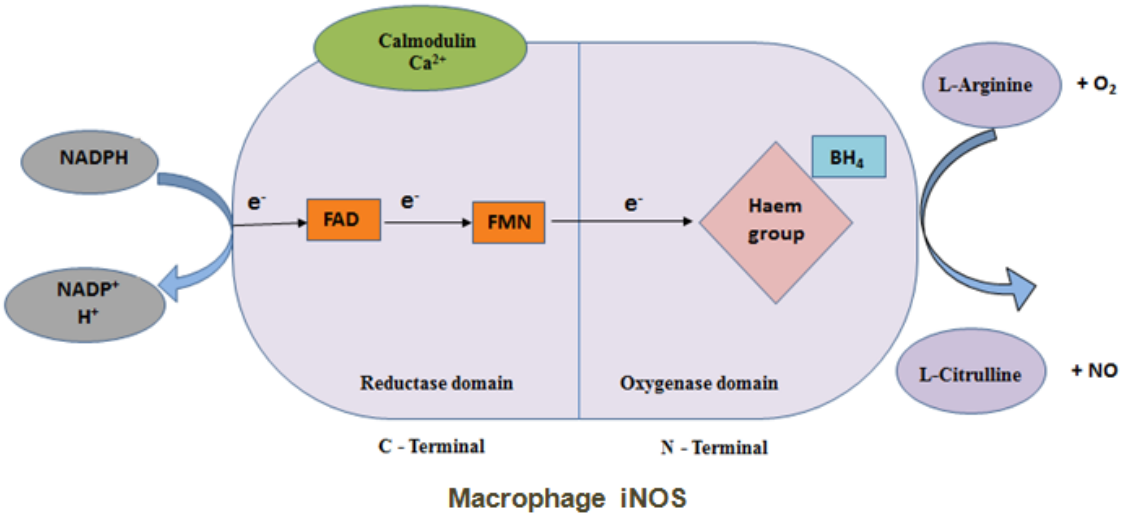

Figure 1.2 Synthesis of nitric oxide from L-arginine and oxygen by iNOS. The above figure shows the phases involved in the nitric oxide synthase reaction pathway. Electrons are donated from NADPH to the reductase domain and then progress through FAD and FMN to the oxygenase domain. Electron flow through the reductase domain requires the presence of bound Calmodulin. There the electrons interact with the haem and BH4 at the active site in order to catalyse the reaction of oxygen with L-arginine resulting in the formation of L-citrulline and NO. [Nicotinamide adenine dinucleotide phosphate (NADPH), Flavin adenine dinucleotide (FAD), Flavin mononucleotide (FMN), Tetrahydrobiopeterin (BH_4).

Table 1.4: Comparison of nitric oxide synthase isoforms. Adapted from Knowles and Moncada, 1994; Alderton *et al*., 2001 & Nahrevanian 2009 [N-methyl-D-aspartate (NMDA)]

NOS isoforms	Molecular weight	Tissue distribution	Activators	Expression & Calcium sensitivity
nNOS [NOS-1]	161kDa	Neurons	Steroid hormones, cytokines, NMDA	Constitutively expressed Calcium dependent
iNOS [NOS-2]	131KDa	Macrophages, endothelial cells, Smooth muscle cells	Cytokines Endotoxins	Inducible, Calcium independent
eNOS [NOS-3]	133kDa	Endothelial cells, cardiac myocytes, epithelial cells, reproductive organs	Shear stress, histamine, bradykinin, thrombin, estradiol	Constitutively expressed Calcium dependent

1.7. Macrophage phagocytosis

Phagocytosis is a primordial adaptation. In primitive organisms it is primarily used for the acquisition of nutrients and in higher organisms it is an important process which is a critical former line of defence against invading pathogens (Aderem and Underhill 1999). Phagocytosis of pathogens by macrophages initiates the innate immune response, which in turn orchestrates the adaptive response. Professional phagocytes have a vast and sophisticated arsenal of microbicidal features. Phagocytosis can be broken down into a series of defined steps (Flannagan *et al*. 2009; Rieger *et al*. 2010).

- Recognition of the particle
- Adherence and internalisation into the phagosome
- Phagosomal maturation and degradation of ingested particle.

1.7.1. Pathogen recognition

Macrophages are able to sense and migrate up the concentration gradients of chemoattractants in search of primary sites of infection and inflammation through a process known as chemotaxis (El-Benna *et al*, 2009). During inflammatory conditions, macrophage migrate towards the infection site attracted by chemoattractants such as the complement fraction C5a, the N-formyl-methionyl-leucyl-phenylalanine (fMLF) peptide, interleukin 8 (IL-8) and leukotriene B4 (LTB4) released by mitochondria or bacteria (El-Benna *et al*, 2009; Nathan and Cunningham -bussell 2013).

A primary challenge to the innate immune system is the discrimination of numerous potential pathogens from self by using a restricted number of germ-line encoded phagocytic receptors (Medzhitov & Janeway, 2002; Aderem 2003; Taylor *et al*., 2005). Macrophages can differentiate between the self and non-self-molecules by scanning the chemical structure of the microbes. Pathogens usually possess some common conserved moieties called pathogen associated molecular patterns (PAMP's) which are recognised by pathogen pattern recognising receptors (PRR) present over the macrophage surface.

On the basis of function, PRR are divided into signalling PRR and endocytic PRR. Signalling PRR include membrane bound Toll like receptors (TLR), cytoplasmic nucleotide binding oligomerisation domain receptors (NOD) and G-protein coupled

receptors known as formyl peptide receptors. Endocytic PRR recognise carbohydrate moieties and comprise mannose receptors, glucan receptors and scavenger receptors and are involved in microbial attachment, engulfment and destruction by macrophages (Taylor *et al*., 2005; Akira *et al*., 2006;Takeuchi & Akira, 2010).

Induction of phagocytosis requires receptor-mediated recognition of particles. Ten TLRs have been identified in humans and 12 in mice. Different TLRs recognize the different molecular patterns of microorganisms and self-components. For example, TLR-2 recognises peptidoglycan, TLR-3 recognises double stranded RNA, TLR-5 recognises flagellin and TLR-9 recognises unmethylated bacterial DNA. It is this receptor-ligand binding which orchestrate an immune response (Takeuchi and Akira, 2010). A diverse range of PRRs results in a rapid and efficient immune response to remove the microorganism from the host (Callol *et al*., 2013), however, species can evolve, changing targeted molecules on the cell surface to avoid detection and ingestion (Turvey and Broide, 2010; Cambier *et al*., 2014; Kugelberg, 2014).

Under normal conditions, the microbiota is the largest source of microbial stimulation in the gut. The recognition of microbial products may serve to prime the innate immune system, stimulation which is provided by the commensal bacteria (Clarke *et al*., 2010). Under these homeostatic conditions, there is a low expression of TLR2 and TLR4 and they are therefore unresponsive to TLR stimuli (Melmed *et al*., 2003; Abreu *et al*., 2001). However, under inflammatory conditions, TLR expression is increased, which contributes to both inflammation as well as immune tolerance (Abreu *et al*., 2002; Kivit *et al*., 2014). Ligation of suitable bacterial components with phagocyte receptors triggers the flushing of phagosome equipped with antimicrobial compounds.

1.7.2. Pathogen Ingestion

Once recognised by the macrophage's PRRs then particle bound ligand is ingested via the process of phagocytosis (Aderem and Underhill, 1999; Booth *et al*. 2001). The cytoplasmic domain of the receptors recruits Rho GTPases, although the exact enzyme is dependent on the type of receptor, for example; complement receptors recruit Rho whilst Fc receptors recruit Rac and CDC42 (Castellano *et al*., 2000; Heasman & Ridley, 2008).

Mammalian Rho GTPases comprise a family of 20 intracellular signalling molecules, well known for their important roles in regulating the actin cytoskeleton. Most Rho GTPases

switch between active GTP-bound and inactive GDP-bound conformations. The cycling of Rho GTPases between these two states is regulated by three sets of proteins; guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). The GDP bound form Rac1 and CDC42 are most likely to regulate actin polymerization, whereas Rac2 is activated in an F-actin depleted region at the base of the phagocytic cup (Castellano *et al*, 2000; Heasman & Ridley, 2008).

In response to a chemotactic gradient, cells polarize and form a leading edge pointing toward the chemoattractant source and a posterior structure called the uropod. Actin polymerization within the leading edge drives up-gradient protrusion, whereas myosin activity in the uropod detaches at the rear of the cell. The proper coordination of these polarized events is essential for directional migration and depends on the asymmetrical distribution of specific molecular determinants. A hallmark of phagocyte polarization is the asymmetrical accumulation of the phospholipid phosphatidylinositol (3, 4, 5)-triphosphate [PIP3] in the leading edge. The formation of PIP3 is catalysed by Phosphatidylinositol kinases (PI3 kinase) (Chimini & Chavrier, 2000; Kuiper *et al*., 2011). The actin polymerisation underneath the receptor-ligand complex leads to the rearrangement of the cytoskeleton as two pseudopodia are driven around the target (Bokoch, 2005).

A phagocytic synapse (junction) like structure is formed by the lipid rafts produced by localisation of heat shock proteins (hsp70 and hsp 90), CD14 and TLR-4 (Triantafilou *et al*., 2002; Pfeiffer *et al*., 2001). Lipid rafts are the membrane surface domains consisting mainly of cholesterol and glycosphingolipids and are involved in cell signalling cascades, cell adhesion, migration and macrophage phagocytosis (Simons and Toomre, 2000; Gomez-Mouton *et al*., 2001; Nagao *et al*., 2011). These phagocytic synapses accumulate and cause the initial zippering of the membrane around the bacteria (May and Machesky, 2001; Silverstein, 1995).

The local reorganisation of F-actin that occurs at a phagosome is due, at least in part, to the recruitment and/or activation of actin-nucleating molecules like the seven subunit Arp2/3 complex (Greenberg, 1995; Aderem and Underhill, 1999; May and Machesky, 2001). It has been described that phosphatidylinositol (3, 4, 5) triphosphate (PIP3) along with Arp2/3 complex is involved in the scanning of surfaces for the particle to be taken up (Gerisch *et al*., 2009). Nucleation of the actin cytoskeleton, restructuring of filament

network and formation of the phagocytic cup occurs once Arp2/3 complex and integrin β2 are recruited under the lipid raft resulting in the formation of membrane phagosome. These activities would promote or stabilise the zipper-type interaction between the phagocytic receptors and the particle-bound ligands inducing a progressive engulfment of the particle and eventually the internalisation of the particle.

Once most of the target is surrounded by the phagocyte plasma membrane, PI3 kinase activity leads to the final engulfment which results in the internalisation of the pathogenic material in a membrane closed phagosome (Araki *et al*., 1996; Weber *et al*., 2006). Calcium is considered to be an important secondary messenger in the transduction of signals during bacterial ingestion. Cytosolic calcium levels have been found to increase as a result of the binding of an immune cell surface receptors which leads to the rapid activation of a number of molecules involved in the promotion of cellular functions, including, activation of a number of signalling pathways (Gronski *et al*., 2009).

Following the completion of the engulfing stage the actin is depolymerised and disassociates from the phagosome whilst the PRR, which is of no use to the macrophage internally, is recycled to the cell surface where it can once again bind its respective ligand. Such complex cytoskeletal remodelling during ingestion is accompanied by membrane remodelling locally, contributing to the pseudopod extension towards the cell and sealing into the phagosome (Greenberg & Grinstein, 2002).

1.7.3. Pathogen digestion

Pathogen digestion starts in mature phagosome. The phagosome maturation commences immediately after bacterial internalisation and sealing of the phagosome. Maturation of phagosomes occurs via a series of fusion events with organelles of the endocytic pathway (Niedergang & Chavrier, 2004; Stuart & Ezekowitz, 2005). Ultimately, this leads to the insertion of lysosomal membrane proteins (LIMPII, Lamps) in the phagosomal membrane and to the delivery of lysosomal lytic enzymes into the lumen of the phagosome (Henry *et al*., 2004). The fusion and fission events are regulated by Rab and SNARE proteins. The Rab proteins (Rab 5, 7, 11) are key constituents or regulators of phagosome fusion machineries and help in the recognition of membranes that should fuse specifically (Pei *et al*., 2012). The SNARE proteins act as downstream regulators and are involved in pulling the membranes together for fusion up to a few nanometres (Duclos *et al*., 2000; Duclos, 2003).

Accumulation of PI3P on the phagosomal membrane is vital and occurs soon after phagosome closure and is also essential for phagosome maturation (Desjardins *et al*., 1997; McBride *et al.,* 1999; Duclos *et al*., 2000; Vieira *et al*., 2002). At the core of these multi-layered changes, a degradative, highly acidic internal environment impeding microbial growth and metabolism is created (Flannagan *et al*., 2009). Several downstream regulators orchestrate phagosome maturation in a sequential manner. Syntaxin 7 and Syntaxin 13 (belonging to the SNARE proteins family) present on late and recycling endosomes are involved in phagosome maturation. The SNARE complex along with Rab5 facilitates the fusion of phagosome and lysosome resulting in the formation of phagolysosome (Duclos *et al*., 2000; Duclos, 2003). In addition, phagosome acidification and calcium fluxes modulates phagosomes maturation (Collins *et al.,* 2002; Harrison *et al*., 2003; Ungermann & Langosch, 2005; Yates *et al*., 2005; Verhaert *et al*.,2005; Haas, 2007).

Concomitantly with phagosomes maturation two important processes are brought into play. The first course of action is known as respiratory burst, comprising the rapid and striking activation of oxidative metabolism and the production of superoxide (Halliwell and Gutteridge, 2006; Niedergang and Chavrier, 2004) and concomitant toxic species. The second process, called degranulation, corresponds to the release of the contents of cytoplasmic granules into the endosomal vacuole.

The permutation of these processes results in the killing and digestion of the engulfed bacteria and the development of an inflammatory response. The secondary recruitment of circulating neutrophils and macrophages is triggered by a number of additional chemotactic factors released from the site of inflammation. It has been shown that unstimulated human phagocytes consume relatively little O_2 (less than 1 n mol O_2. min^{-1}. 10^7 cells at 37°C). Within a few seconds after contact with specific stimuli, the rate of O_2 consumption abruptly increases by a factor of 50 – 100 (Morel *et al*., 1991). The collective outcome of free radical production leads to impaired bacterial metabolism and irreparable DNA damage leading to bacterial killing (Flannagan *et al*. 2009; Dupre-Crochet *et al*., 2013).

Phagosomes are also equipped with a mixture of endopeptidases, exopeptidases and hydrolases that degrade various microbial components. Lactoferrin creates a bacteriostatic condition within phagosomes by creating a nutrient deprived environment. The low pH in the phagosomes results in bacterial degradation. Defensins and cathelicidins cause

membrane permeabilisation. Lysozyme causes hydrolysis of carbohydrates while phospholipase A2 causes hydrolysis of lipids. Several proteases including cathepsin D, cathepsin G and aminopeptidases are involved in bacterial protein hydrolysis (Underhill & Ozinsky, 2002; Flannagan *et al*., 2009).

1.8. Free radicals or Reactive species

A free radical can be defined as 'any species capable of independent existence (hence the term free) that contain one or more unpaired electrons (Halliwell and Gutteridge, 2006). Reactive oxygen species (ROS) is a collective term that may be either oxygen or non-radical oxygen derivatives. Hence, all oxygen radicals are ROS but not all ROS are oxygen radicals (Halliwell and Gutteridge 2006). Similarly, reactive nitrogen species are nitrogen derived radical or non-radical species. There is some degree of overlap or confusion in literature about the classification of free radicals. For example peroxynitrite ($ONOO^-$) and nitric oxide (NO or also referred as $NO^{\cdot}$) may be classified either way. A list of free radicals commonly mentioned in literature is listed in table 1.5. A role of free radicals on phagocytosis by macrophages is listed in table 1.6.

For several years ROS was considered as an inevitable but unwanted by-product of an aerobic existence. The ROS is now well recognised and are known to act as second messengers that regulate intracellular signal transduction pathways under a variety of physiological and pathophysiological conditions (Moraes *et al*., 2006; Dupre-Crochet *et al*., 2013). This occurs predominantly via oxidation of thiols (-SH) on protein cysteine residues, resulting in reversible protein posttranslational modifications such as glutathionylation, disulfide bond formation and sulfenic acid formation (Hattori *et al*., 2010).

The NADPH oxidase mediated ROS production also acts as a physiological regulator of pseudopod formation and was found highly enriched near the leading edge of migrating neutrophils (Hattori *et al*., 2010). In addition, the NADPH oxidase is also essential for chemoattractant-elicited depolarization of membrane potential and can regulate Ca^{2+}, K^+ homeostasis in neutrophils (Rada 2005; Hattori *et al*., 2010).

Table 1.5: Reactive oxygen and nitrogen species. Table 1.5 shows a list of reactive oxygen and nitrogen species produced in our body differentiated as radical and non-radical species.

Reactive oxygen and nitrogen species	
Radicals	**Non-radicals**
Superoxide [$O2^{-}$]	Hydrogen peroxide [H_2O_2]
Hydroxyl [OH^{-}]	Peroxynitrite [$ONOO^{-}$]
Nitric oxide [NO or $NO^{\cdot}$]	Organic peroxides[ROOH]
Alkoxyl [$RO^{\cdot}$]	Hypochlorous acid [HOCl]

Upon activation by various stimuli, the macrophage NADPH oxidase transfers electrons from the cytosolic NADPH to molecular oxygen releasing superoxide into the phagosomal lumen (see equation 1 below). Inside the phagosome, oxygen free radicals are then converted rapidly to hydrogen peroxide (H_2O_2). The H_2O_2 can also be converted by myeloperoxidase into hypochlorous acid and chloramines. These compounds appear in parallel in the phagosome at quite different concentrations (DeCoursey and Ligeti, 2005; Klebanoff, 2005). The ROS scavenging enzymes namely, superoxide dismutase (SOD) and catalase also plays a vital role in ROS production by macrophages. The SOD converts superoxide to hydrogen peroxide (see equation 2) while catalase catalyses the conversion of H_2O_2 to water and oxygen (see equation 3) (Slauch, 2011).

$$O_2 + e^- \xrightarrow{\text{NADPH Oxidase}} O_2 \quad \text{.....1}$$

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{\text{Superoxide dismutase}} H_2O_2 \quad \text{.....2}$$

$$2\,H_2O_2 \xrightarrow{\text{Catalase}} 2\,H_2O + O_2 \quad \text{.....3}$$

1.8.1. Role of ROS during bacterial ingestion

The ROS are identified as essential players for modulating chemotaxis and are known to regulate the process of chemotaxis by directly modulating actin glutathionylation and polymerization (Sakai *et al*., 2012). Studies by Sakai *et al*. (2012) found that the pharmacological inhibition of oxidative burst resulted in complete inhibition of actin glutathionylation, suggesting that NADPH-oxidase mediated ROS plays a key role in eliciting actin glutathionylation.

Evidence that ROS influence phagocytosis is provided by the observation that the amplification of ingestion during phagocytosis is deficient in neutrophils from patients with chronic granulomatous disease (Gresham *et al* 1988; Fialkow *et al*., 2007). The neutrophils with inhibited ROS production formed more frequent multiple pseudopodia and showed reduced chemotaxis efficiency as they migrated up a chemoattractant concentration gradient (Kuiper *et al*., 2011; Sakai *et al*., 2012).

Reactive oxygen intermediates such as H_2O_2 also enhance the Fcγ receptor (FcγR) signalling and amplify phagocytic capacity. The FcγR mediate the internalisation of opsonized particles by mononuclear phagocytes. Cross-linking of FcγR leads to the activation of protein tyrosine kinases and phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) within FcγR subunits, both obligatory early signals for phagocytosis (Pricop *et al*., 1999). In an inflammatory environment hydrogen peroxide is produced by activated macrophages at an estimated rate of 2–6 x 10^{-14} mol.hour^{-1} cell^{-1} and may reach a concentration of 10–100 μM in the vicinity of these cells (Nathan and Root 1977; Keisari *et al*., 1983; Droge 2002).

Studies by Kuiper *et al*. (2011) hypothesised that local ROS production in phagocytes such as neutrophils could regulate Phosphatidylinositol (3, 4, 5)-triphosphate (PIP3) levels through redox regulation of Phosphatase and tensin homolog-a phosphatase (PTEN). The PTEN negatively regulates PIP3 by dephosphorylating phosphoinositide substrates. They further identified PTEN to be a major target of chemoattractant induced ROS formation and demonstrate that ROS production down regulates PTEN activity (Shelton *et al*., 2005; Clavreul *et al* 2006; Hattori *et al* 2010). Decreased PTEN activity is found to increase PIP3, which could be reversed by ROS scavengers or genetic ablation of NADPH oxidase (Kuiper *et al*., 2011).

1.8.2. Role of ROS in bacterial digestion

The first role of ROS discovered in phagocytes was their microbicidal activity which can be attributed to a sequence of chemical reactions happening both inside and outside the phagosomes (Dupre-Crochet *et al*., 2013). Bacterial digestion or killing occurs due to production of ROS within the macrophage phagosome. The hydroxyl radical is perhaps the most reactive of ROS and as it rapidly combines with target molecule in its immediate vicinity, can initiate a free radical chain reaction leading to the peroxidation of lipids and proteins. Superoxide is less reactive than hydroxyl radicals and thus may diffuse some distance in the cell before it encounters an appropriate reaction partner. Hydrogen peroxide is even less reactive and therefore longer lived. Additionally, H_2O_2 is membrane permeable and thereby can easily reach the extracellular space from phagocytic cells and diffuse other cells and thus exhibits a paracrine role (Fialkow *et al*., 2007). The importance of ROS mediated bacterial killing by phagocytes is evidenced in CGD where the individual suffers from life threatening microbial infections (Meischl and Ross 1998; Kannengiesser *et al*., 2008, El-Benna *et al*., 2009; Dupre-Crochet *et al*., 2013).

Upon phagocytosis of bacteria, ROS is produced by NADPH oxidase in the small volume of phagosome. The ROS is not only produced on the phagosomal membrane, but is also observed on non-phagosomal intracellular organelles. Winterbourn *et al*., (2006) estimated the concentration of superoxide by modelling the steady state concentration of superoxide in phagosomes. The steady state concentration of superoxide and hydrogen peroxide were found to be 25 μM and 2 μM respectively (Winterbourn *et al*., 2006).

The fusion of azurophilic granules containing myeloperoxidase enzyme with the phagosomes results in the formation of HOCl from Cl and H_2O_2 (Klebanoff, 2005). Winterbourn *et al*. (2006) estimated that HOCl is produced at a rate of 134 mM min^{-1} and the concentration of phagosomal myeloperoxidase was ~1 mM. Nevertheless, the consequence of myeloperoxidase deficiency is not as drastic as expected as several major ROS producing myeloperoxidase independent killing mechanisms exist (Park *et al*., 2005; Dupre- Crochet *et al*., 2013).

The ROS not only kills the bacteria but also influences the overall environment of the phagocyte. Extracellular ROS production and the diffusion of ROS like H_2O_2 across the membrane enable ROS signalling in the extracellular space and in neighbouring cells. The ROS are often considered as a stimulator for inflammation and a mediator of inflammatory

tissue damage, however evidence for the anti-inflammatory role of ROS is also accumulating (Lambeth *et al*., 2008; Sareila *et al*., 2011).

1.8.3. Role of RNS in macrophage phagocytosis

The NO is a critical component of mammalian host defence that is produced in macrophages and other cells comprising the innate immune system. The discovery that, iNOS from activated macrophages produce copious amount of nitric oxide and their subsequent derivatives provided a new perspective of bacterial killing (Fang 2004).

The NO is toxic at high concentration, as it can inactivate critical enzymes essential for microbial physiological processes such as mitochondrial aconitase and NADH: ubiquinone oxidoreductase by interacting with iron sulphur centres to form iron sulphur nitrosyl derivatives, and inhibit both DNA and protein synthesis (Haddad *et al*., 1994; Stamler *et al*., 2001; Schapiro *et al*., 2003). In previous studies it is indicated that NO is an important, but possibly not essential, contributor in the control of the acute phase of parasitic infections (Qiu *et al*., 2009).

Macrophages have the opportunity to produce superoxide and NO in nearly equimolar amounts and thus can be proliferic generators of their joint and particularly destructive product peroxynitrite (Haddad *et al*., 1994; Fang, 1997). In the intraphagosomal environment, NO can combine with ROS and undergo either spontaneous or catalytic conversion to a range of RNS, including nitrogen dioxide, peroxynitrite, dinitrogen trioxide, dinitrosyl iron complexes, nitrosothiols and nitroxyl radicals (Dupre-crochet *et al* 2013).

Studies have shown that peroxynitrite was toxic to invading bacteria from the detection of 3-nitrotyrosine residues in the proteins of infected human cells or tissues (Beckman *et al*., 1992; Ischiropoulos *et al*., 1992; Evans *et al*., 1996). It is known that activated alveolar macrophages release both NO and superoxide which reacts at a near diffusion limited rate $6.7 \times 10^{9}\ M^{-1}s^{-1}$ to form peroxynitrite (Haddad *et al* 1994; Xia & Zweier, 1997; Nathan & Shiloh, 2000; Lundberg *et al*., 2004; Nioche *et al*., 2004).

Under mildly acidic conditions peroxynitrous acid (ONOOH) may form as the result of interaction between nitrite and hydrogen peroxide (H_2O_2) which can kill bacteria such as *E.*

coli (Kono *et al*., 1994). Another possible method of NO toxicity relies upon the formation of S-nitrosothiols (RSNO) (De groote *et al*., 1995; Vallance and Charles 1998). However, many RSNO have bacteriostatic effects on bacteria such as *E. coli* and *Salmonella* that are resistant to NO (Vallance and Charles 1998). Bacteria such as *Staphylococci*, which are deficient in low molecular weight thiols such as glutathione, are sensitive to S-nitrosothiols as they are unable to protect themselves. On the other hand bacteria such as *E. coli* with high thiol concentrations are normally resistant to NO itself but are sensitive to peroxynitrite and S-nitrosothiols (Vallance and Charles 1998).

The nitric oxide synthase activity of iNOS requires the enzyme to be in dimeric form. Work by Sun *et al*. (2010) found that the oxidative stress acts as a brake on the NO generation by conversion of the active iNOS dimer in to inactive monomer (NOS uncoupling). They also proposed that decrease in iNOS activity was also due to oxidation of tetrahydrobiopterin (BH_4) an important co-factor for NOS.

The L-arginine is the main substrate for NO production from iNOS. Study by Xia and Zweier, (1997) found that depleting the cytosolic L-arginine triggered production of both superoxide and NO generation from iNOS, which combined to form peroxynitrite. They observed that, this iNOS mediated increase in the peroxynitrite formation resulted in the increased antibacterial activity by RAW 264.7 macrophages. Thus iNOS mediated superoxide and peroxynitrite generation could be particularly important in the cytotoxic action of macrophages.

Studies by Murray and Nathan attempted to determine the *in vivo* macrophage microbicidal mechanism and tried to identify the relative contribution of ROS and RNS in macrophage mediated intracellular host defence. In early stage (2 weeks) of liver infection with *Leishmania donovani*, an intracellular parasite, they found that both $gp91^{phox-/-}$ and iNOS knockout mice displayed increased susceptibility to infection. They concluded that ROS and RNS probably act together during the first two weeks or the early stage of intracellular infection to regulate mononuclear inflammatory cells and the initial extent of microbial replication. However, they also found that RNS alone is sufficient for the eventual control of visceral infection (Murray & Nathan, 1999).

Table 1.6: Role of free radicals on phagocytosis by macrophages. [Bone-marrow derived macrophages (BMM); NG –monomethyl - L-Arginine (L-NMMA); Diphenylene iodonium chloride (DPI); Monocyte derived macrophages (MDM); Phenylarsideoxide (PAO)]

Study	Microbe/ Stimulus	Macrophage source	Treatment	Experiment condition	Effect on phagocytosis	Inference
Van der Goes *et al*., (1998)	Myelin	Rat peritoneal macrophages	100 mM Apocynin	*In vitro*	Decreased uptake of myelin	ROS regulates myelin phagocytosis
			100 µM DPI	*In vitro*	Decreased uptake of myelin	
			Allopurinol	*In vitro*	No effect on myelin uptake	
Gantt *et al*., (2001)	*Leishmania chagasi*	Human monocyte derived macrophages (MDM) and Murine bone marrow derived macrophages (BMM)	0.4 mM Tempol	*In vitro*	No effect on bacterial uptake. However, significantly increased level of infection in Human MDM. Murine BMM also had increased levels of infection but non-significant.	Both superoxide and NO contribute to intracellular killing of *L. chagasi* in human and murine macrophages.
			1 mM L-NMMA	*In vitro*	Significantly higher parasite burden compared with untreated control BMM and MDM	
Philippe *et al*., (2003)	*Aspergillus fumigatus* conidia	Murine macrophages	DPI PAO	*In vitro*	No effect on uptake. Significantly reduced *in vitro* killing	*A. fumigatus* killing is mediated by reactive oxygen intermediates.

Segal *et al*., (2003)	*Burkholderia Cepacia* *Chromobacterium violaceum*	Murine macrophages		*In vivo*	Despite the lower inoculum in p47 $^{phox-/-}$ mice, the bacterial burden was higher than those in iNOS$^{-/-}$ and wild-type mice.	Phagocyte NADPH oxidase but not iNOS is essential for early control of infection
Brennan *et al*., (2004)	*Coxiella burnetti*	Murine macrophages J774.16	Catalase L-NMMA	*In vitro*	J774.16 significantly inhibited bacterial replication. Catalase and L-NMMA reversed the effect	Both iNOS and NADPH oxidase contribute to the control of virulent phase-1 *C. burnetti* infection
		C57BL/6 C57BL/6 (p47 $^{phox-/-}$) C57BL/6 (iNOS-/-)		*In vivo*	iNOS$^{-/-}$ were less effective at controlling *C.burnetti* than wild type and that p47 $^{phox-/-}$ mice were even less effective than iNOS$^{-/-}$	
Qiu *et al*., (2009)	*Acinetobacter baumannii*	Murine macrophages C57BL/6 B6.129S-Cybb $^{tm1Din/J}$ gp91 $^{phox-/-}$ B6.129P2-NOS2$^{tm1\ Lau/J}$ iNOS$^{-/-}$		*In vivo*	gp91$^{phox-/-}$ but not NOS2$^{-/-}$ mice are highly susceptible to *A. Baumanni* infection.	NADPH phagocyte oxidase appears to have a crucial role in host defence against *A. Baumanni* infection.

1.9. Research gaps, novelty, aims and objectives

Though probiotics have been shown to have multiple effects on macrophages, the exact mechanism is still contested. One approach this study employed was to investigate the effect of probiotic conditioned media from a single probiotic strain *Lactobacillus rhamnosus* GG on macrophages and understand the bacterial-macrophage interactions.

Current literature on the role of probiotics into the phagocytic process by the phagocytic cells such as macrophages is mostly looked at the total number of phagosomes after a certain time period. Studies have described the effect of probiotics as either an increase or decrease in phagocytosis. None of the studies looked at the effect of cell free LGG-CM on phagocytosis as two phases of phagocytosis namely ingestion and digestion. The purpose of this study was to differentiate the effect of LGG-CM on bacterial ingestion and digestion as two separate entities.

There is also a gap in understanding of the role of LGG-CM mediated NO and ROS production and regulation by murine macrophages. Most of the studies investigating free radicals production from macrophages have investigated the ROS production as an end point study and have investigated ROS production at one or two time points. This study has identified this gap and investigated the effect of LGG-CM as a continuous study and has examined the pattern and kinetics of free radical production by macrophages.

So far, to the best of our knowledge, there are no studies that report or demonstrate whether probiotics exert their effect through the release of soluble factors with the inherent ability to induce ROS generation or indeed activate a signalling cascade that might regulate NO production in a parallel system. The hypothesis of this study was that the LGG-CM modulates macrophage phagocytic activity.

This research aims to:

- Investigate the effect of cell-free LGG-CM on bacterial macrophage interactions with respect to bacterial phagocytosis.
- Understand the physiological mechanisms of the observed functional attribute with respect to free radical production in presence of LGG-CM.

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To achieve the above aims, the following objectives are met by this research:

1. Understanding the role of probiotics as immunomodulatory agents from existing literature.
2. Preparation of an effective LGG-CM and determining the methods to investigate the influence of LGG-CM on murine macrophages using various microbiological and immunological techniques.
3. Identifying the effect of LGG-CM on macrophage and bacterial viability.
4. Understanding the effect of LGG-CM on NO production by macrophages.
5. Identifying the effect of LGG-CM on bacterial phagocytosis process by macrophages
6. Understanding the role of LGG-CM on signalling mechanisms modulation of observed functional attribute (phagocytosis).
7. Discussion and comparison of current findings with respect to existing literature and recommendations for future work.

Chapter 2

Materials and Methods

2.0. CHAPTER 2

A list of chemicals used in the experiments is listed in appendix A.

2.1. Culturing and sub-culturing of murine macrophage cell line J774

The murine macrophage cells J774 were gratefully received as a gift from Professor Anwar R Baydoun, University of Hertfordshire.

The J774 cells were cultured in T-75 tissue culture flasks which were labelled with cell type, date and passage number. The J774 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % Fetal Bovine Serum (FBS) and 1 % penicillin/streptomycin in a humidified tissue culture incubator at 37^0C and 5 % CO_2. The cells were sub-cultured every 2 to 3 days after examining the confluency under Olympus CKX41 inverted culture microscope at 400 x magnification.

2.1.1. Storage of macrophages

Sub-culturing of cells was performed to prevent the T-75 flask surface adhered macrophages to exceed the flask capacity. This process was performed depending upon cell growth, typically every 2 to 3 days.

The J774 cells were grown and made up a stock for future experiments and stored at -80^0C in liquid nitrogen. Approximately 5 x 10^6 cells ml^{-1} were stored in a cryovials containing 1 ml of freezing mixture (10 % Dimethyl sulfoxide and 90 % FBS). These vials were frozen at -80^0C in a Haier ULT freezer DW-86L490. After 24 hours the vials were transferred to storage in a liquid nitrogen container (CN509X7, Thermo Scientific Thermolyne) until required.

When needed, frozen cells were carefully removed from liquid nitrogen containers and rapidly defrosted in a 37^0C water bath taking care not to submerge the vial. The contents were then slowly pipetted out in to T-25 flasks containing 4 ml of prewarmed media (37^0C). The cells were then incubated at 37^0C in a humidified 5 % CO_2 atmosphere and allowed to attach. After a few hours, the flasks were removed from the incubator. The old

media containing any dead cells and traces of DMSO was removed and replenished with fresh macrophage culture media and incubated further to grow.

2.1.2. Enumeration of macrophages

Macrophages were counted by the trypan blue dye exclusion method. A confluent monolayer of J774 cells were scraped and then aspirated with a Pasteur pipette. Then, 100 µl of the sample was mixed with 100 µl of 0.4 % trypan blue and added to two chambers of the haemocytometer (Neubauer type) and covered by a cover slip. The cell suspension was allowed to settle for at least 15 seconds. The haemocytometer was placed onto a microscope stage and cells in the middle, large square of each chamber (with an area of 1 mm^2 and a depth of 0.1 mm) were counted. The averaged cell number was multiplied by 2 as it was diluted in trypan blue. The following equation was used to enumerate the total number of macrophages per ml.

≈ Number of cells per ml = [Mean cell count x Dilution Factor] x Conversion factor

$$= [\text{Mean cell count} \times 2] \times 10^4$$

2.2. Culturing and storage of *Lactobacillus rhamnosus* GG (LGG)

A pure culture of LGG was extracted from a Culturelle® probiotic tablet. The tablet was split open under sterile conditions and inoculated into 100 ml de Man, Rogosa and Sharpe (MRS) broth (52 g MRS L^{-1} distilled water) and incubated in an orbital incubator at 37^0C at 150 rpm. As it was not possible to start each time with a Culturelle® tablet, some cells were stored at -80^0C. To accomplish this, after confirmation of attainment of the exponential phase the culture was centrifuged for 10 minutes at 5000 rpm. The supernatant was discarded and bacterial cells were suspended in cryovials containing 15 % glycerol and 85 % MRS broth and stored at -20^0C.

2.2.1. Monitoring the bacterial growth and change in pH of LGG culture medium

The LGG were cultured in MRS broth at 37^0C according to ATCC guidelines. Frozen stocks (-20^0C) of LGG were inoculated in prewarmed MRS broth grown at 37^0C and 150 rpm for 16 hours. From this, a 1 ml sample of overnight LGG culture was transferred to a fresh and sterile MRS medium. An aliquot was collected every hour for optical density (600 nm) measurements and pH readings. The readings were taken every hour up to 12

hours. Due to laboratory time constraints the next readings were after 24 hours. The bacterial growth curve was constructed by plotting the absorbance against time.

2.2.2. Preparation of LGG conditioned cell culture medium (LGG-CM)

Cell-free LGG-CM was prepared using a slightly modified method from studies by Christensen *et al*. (2002) and Yan *et al*. (2007). The frozen stocks (-20^{0}C) of LGG were inoculated in MRS broth grown at 37^{0}C and oscillated at 150 rpm for 18 hours. From this culture, 1 ml of overnight LGG culture was transferred to a fresh and sterile MRS medium and incubated at 37^{0}C and 150 rpm for 18 hours.

The LGG culture in MRS broth was then centrifuged for 5 minutes at 10,000 rpm and the supernatant discarded. The bacterial pellet was washed twice with Phosphate buffered saline (PBS) and the pellet was suspended in 40 ml DMEM and incubated for a further 20 to 24 hours (late exponential or early stationary phase). The LGG culture in DMEM was then centrifuged for 5 minutes at 10,000 rpm. To obtain a cell-free conditioned medium; the supernatant extracted after centrifugation was filtered through 0.45 μm Minisart® syringe filters followed by 0.22 μm Millipore syringe filters. The supernatant was then aliquot into small Eppendorf tubes and stored at -20^{0}C for future use.

2.2.3. Enumeration of *Lactobacillus rhamnosus* GG

To enumerate the LGG count inoculated to prepare cell-free LGG-CM, a 100 μl aliquot of LGG culture was serially diluted and plated onto MRS agar plates and incubated at 37^{0}C for 24 hours. The bacterial colonies (white, convex, smooth, opaque and round colonies of LGG) were enumerated by colony counting technique.

2.3. Culturing, storage and enumeration of *E. coli* HfrC

To culture the *E.coli* cells nutrient broth was prepared by adding 13 g of nutrient broth in 1 litre of water. A 50 ml aliquot of media was added to each flask and autoclaved. Nutrient agar media was prepared by dissolving 28 g of nutrient agar in 1 litre of water and autoclaved. The agar was poured into sterile petridishes under sterile conditions and left to solidify overnight. The plates were then inverted to prevent any condensation and left to dry for at least 3 days prior to use. The *E. coli* frozen at -20^{0}C were thawed down and 1 ml of *E.coli* suspension from frozen stock was used to inoculate 50 ml of nutrient broth at

37^0C and 150 rpm. The media was incubated for 9 hours. Then 100 µl of fresh *E.coli* culture was inoculated into 50 ml nutrient broth and incubated overnight at 37^0C and 150 rpm. The bacterial cell number were enumerated by serial dilution and plating techniques. The optical density of bacterial suspension can be adjusted from growth curve and for *E.coli* OD_{600}=1, corresponds approximately to10^9 CFU ml^{-1}.

2.4. Experiments investigating antimicrobial properties of LGG-CM

To determine whether the LGG-CM demonstrated antibacterial properties within the specified time frame of incubation of *E. coli* HfrC, the *E. coli* was grown overnight in nutrient broth at 37^0C and 150 rpm. A 1 ml sample of bacterial culture was centrifuged and the pellet was washed with DMEM carefully and suspended in 1ml of DMEM/FBS devoid of penicillin/streptomycin. A 22 µl of this suspension was dispensed in 1 ml of varying concentrations of acid neutralised LGG-CM (5 %, 10 % and 75 % and 100 % LGG-CM, pH 7.4 ± 0.2) for 1 hour, 6 hours and 24 hours respectively. After the respective time periods, the cells were serially diluted and plated onto nutrient agar plates. The viable bacterial colonies were enumerated by colony counting technique after incubating the plates for 20 hours at 37^0C and were expressed as $\log_{10}$ CFU ml^{-1}.

2.5. Effect of LGG-CM on macrophage viability

2.5.1. Background to MTT cell proliferation assay

The MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole)] assay is a colorimetric assay, which is based on the cleavage of yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells (Mosmann, 1983; Vistica *et al*., 1991). This assay is mainly used to investigate the effect of any treatment on cell viability and proliferation.

The absorbance of the purple coloured formazan solution was measured at two different wavelengths 562 nm (test wavelength) and 620 nm (reference wavelength). Subtracting the readings of reference wavelength from test wavelength eliminates any background noise such as debris or scratches. The reduction of MTT takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable cells. By comparing the amount of purple formazan produced by cells in presence of treatments to the amount of formazan produced by untreated control cells, the effect of the treatments on cell viability and proliferation can be figured out. The MTT

solubilised in phosphate buffered saline (PBS) are yellowish in colour. Mitochondrial dehydrogenases of viable macrophages cleave the tetrazolium ring into purple MTT formazan crystals which are insoluble in aqueous solutions. The crystals can be dissolved in solvent like acidified isopropanol (originally described by Mosmann, 1983) or the method by Tada *et al*. (1986), where 10 % SDS in 0.01 M HCl was used as solvent. An increase in cell number results in an increase in the amount of MTT formazan formed and an increase in absorbance.

2.5.2. Macrophage number optimisation for MTT assay

Stock solutions of MTT were prepared by dissolving one vial of MTT (5 mg) in 1 ml sterile PBS. The solution was mixed and vortexed until completely dissolved. The MTT solutions were quickly aliquot and stored in dark at -20^0C.

Macrophages were scraped and cell number was adjusted to $5x10^5$ cells ml^{-1}. Cells were diluted and seeded at various cell densities from 1000 to 500000 cells ml^{-1} in a 96 well plate and were allowed to attach. After cell attachment, 10 µl of MTT (stock solution 5 mg ml^{-1} in PBS) was added to 100 µl of the medium in the 96 well plates. The microplate was incubated for 4 hours at 37^0C and 5 % CO_2 in a humidified incubator. The wells in the microplate were then viewed through an inverted microscope for the formation of intracellular purple punctate formazan precipitate. Then 100 µl of solubilisation solution (10 % SDS in 0.01 M HCl) was added making sure not to remove any crystals from the well. The plates were left in a humidified cell culture incubator overnight. The plates were visualised after confirming the complete solubilisation of formazan crystals. The optical density reading was read at 562 nm and 620 nm in a microwell plate reader (Asys Hitech expert plus, SLS). The reference wavelength values were subtracted from the respective test wavelength values. The percent viability was then calculated by using the formula:

$$\text{Percent cell viability} = [(\text{Sample OD}) / (\text{Control OD})] * 100]$$

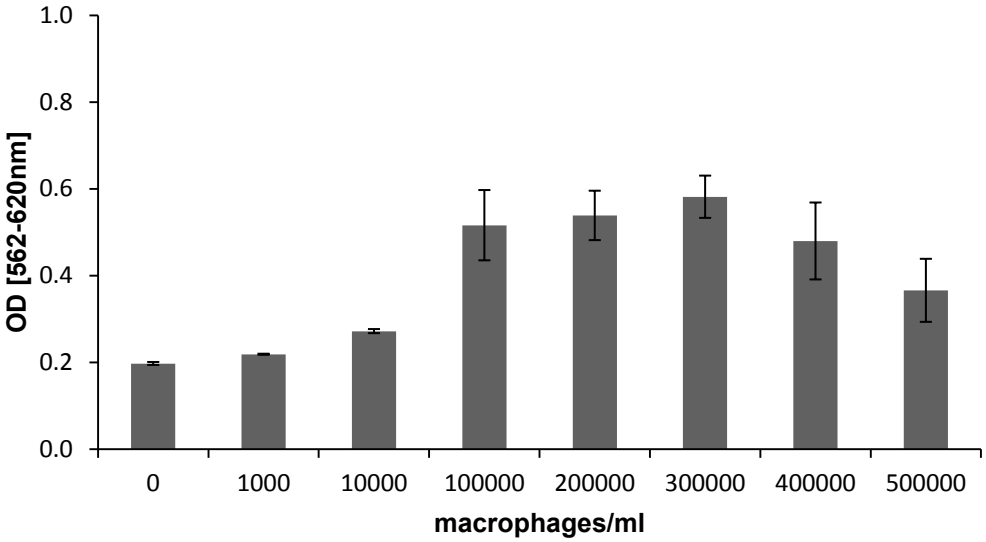


Figure 2.1: Cell optimisation for macrophage viability studies. The figure represents the cell number and respective optical density reading in the macrophage cell optimisation experiment by MTT assay. Results are expressed as the mean absorbance of 3 experiments performed in triplicate. Experiments were performed to determine optimal cell concentration for MTT assay. The optical density readings were taken at 562 nm and 620 nm respectively. Final absorbance was calculated by subtracting optical density readings from 562 nm with reference wavelength 620 nm.

2.5.3. Cell proliferation assay in presence of LGG-CM

Macrophages were cultured in T-75 flasks and sub-cultured when confluent. For MTT assay, the macrophages were scraped and enumerated by trypan blue assay as described in section 2.1.2. Macrophage optimisation for MTT assay was performed as described in section 2.5.2.

For investigating the effect of cell-free LGG-CM on macrophage viability and proliferation, macrophages were seeded at a cell density of $1x10^5$ cells ml^{-1} at least 4 hours before the addition of treatments. Cell free LGG-CM was prepared at the dilutions of 10 % LGG, 75 % LGG and 100 % LGG, both in the presence and absence of 20 μg ml^{-1} LPS. Macrophage viability was also assessed in the presence of *E. coli* in the above treatments. Macrophages were incubated with various treatments for various time periods. After the respective time periods MTT reagent was added and incubated for 4 hours. The formation of purple formazan crystals were observed under 400 x magnification in an inverted culture microscope. This was followed by the addition of 100 μl of solubilisation solution to dissolve the formazan crystals. The percent cell viability was calculated as in section 2.5.2 after measuring the absorbance at 562 nm and 620 nm in a microplate reader.

2.6. Measurement of total nitrite using Griess assay

The NO is synthesised in the biological system by the enzymes nitric oxide synthases. The NO is short lived and gets easily converted to nitrite, a stable end product by reacting with oxygen.

The total nitrite concentration in the cell culture medium is often used as an indication for total NO production (Green *et al*., 1982; Sun *et al*., 2003). Griess assay is therefore used to measure total nitrite in the cell culture medium. The principle is based on the diazotisation reaction where nitrite reacts with Griess reagents (1:1 ratio of 1 % sulfanilamide in 10 % orthophosphoric acid [reagent 1] and 0.2 % napthylethylenediamine [reagent 2] to form an azo dye (figure 2.2).

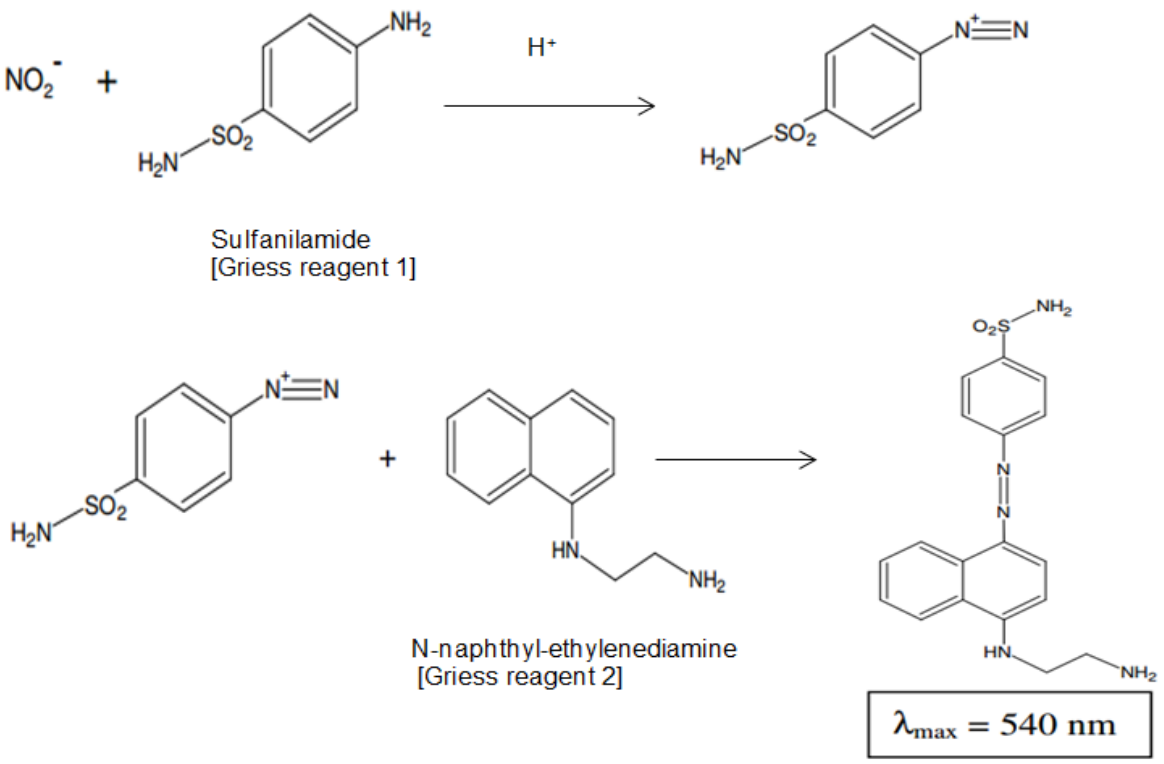


Figure 2.2: Chemistry of Griess reaction. Nitrite reacts with Griess reagents to form an azo product that absorbs strongly at 540 nm [Image adapted from Sun *et al*., 2003]

2.6.1. Preparation of nitrite standard curve

A stock solution of 0.1 M sodium nitrite ($NaNO_2$) was prepared by dissolving 34.5 mg of sodium nitrite in 5 ml of DMEM. A 1 mM working solution of sodium nitrite was then prepared by adding 40 µl of 1 M sodium nitrite to 3960 µl DMEM. Various dilutions of the working solutions were prepared as in table 2.1.

Table 2.1: Protocol for preparing a standard curve using sodium nitrite. Various concentrations of sodium nitrite solutions were then prepared by diluting working solutions of sodium nitrite as shown in table 2.1.

Volume of 1 mM $NaNO_2$ (µl)	Volume of DMEM (µl)	Final [$NaNO_2$] (nanomoles / 100 µl)
0	1000	0
10	990	1
20	980	2
30	970	3
40	960	4
50	950	5
100	900	10

A serial dilution of sodium nitrite solution (1, 2, 3, 4, 5 and 10 nmoles per 100 µl) in the cell culture medium was prepared to use as standards. Then, 100 µl of standards ranging from 0 to 10 nmoles per 100 µl were added to sterile 96 well plates. The Griess reagent (1:1 mixture of Reagent 1 and Reagent 2) was prepared and 100 µl added into each well. The plate was incubated for 15 minutes at room temperature and the absorbance was measured at 540 nm in a micro-plate reader (Asys Hitech microplate reader). The instrument was switched on 20 minutes before use and the calibrations were performed for the desired wavelength. Digiread software was used to control the instrument and collect the raw data and transfer to Microsoft excel. A standard curve of sodium nitrite was constructed by plotting the absorbance of serial dilutions of sodium nitrite against nitrite concentrations.

2.6.2. Calculations to measure nitrite produced from macrophages using a standard curve

A standard curve of sodium nitrite was constructed by plotting the concentration of sodium nitrite on the x-axis and related absorbance value on the y-axis. A linear trendline equation and R^2 value (co-efficient of determination) was then plotted on the graph.

A linear trendline equation $y = mx + c$ was used to calculate the unknown concentrations of nitrite produced from various treatments. The x-coordinate corresponds to the unknown concentration to be determined, m is the slope or the gradient of the trendline and C is the y-intercept. To determine the unknown concentration (x-value) from the optical density reading corresponding to the standard curve, the following steps were followed. (An example of slope and y-intercept values are taken from figure 2.3, where m=0.1123 and C=0.0331).

$$y = mx + c$$

$$x = (y - c)/m$$

Substituting the values from the standard curve,

$$Unknown\ [Nitrite]\ (x)\ =\ [(OD\ determined\ for\ the\ treatment) - 0.0331]\ /\ 0.1123$$

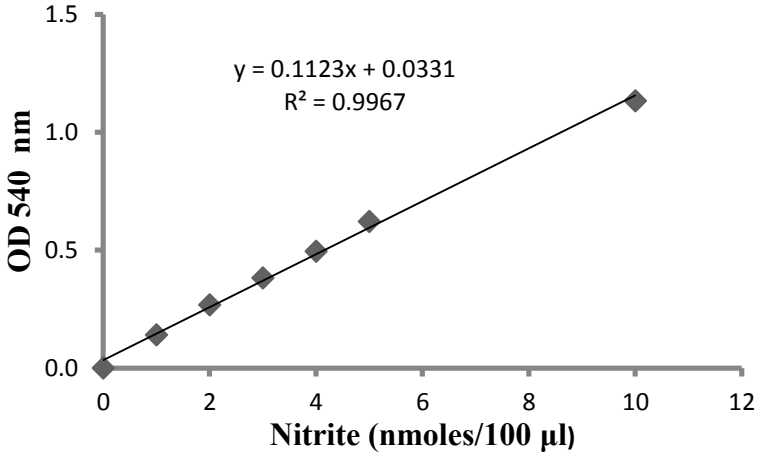


Figure 2.3: A standard curve of nitrite generated by Griess assay. The graph represents an example for a standard curve of nitrite. A linear trend line equation used to estimate the nitrite concentration of the unknown sample is depicted in the graph with its corresponding R^2 value.

2.6.3. Effect of LGG-CM on nitrite production from macrophages

To determine the effect of LGG-CM on nitrite production, macrophages were enumerated and plated in 24 well plates and incubated in a humidified cell culture incubator at 37^0C and 5 % CO_2. The macrophages were then treated with serial dilutions of LGG-CM both in the presence and absence of LPS for 24 hours. After the incubation period, the nitrite accumulated in the spent culture medium from each well was measured by Griess assay.

2.7. Experiments monitoring macrophage phagocytosis of bacteria by gentamicin protection assay.

The principle of gentamicin protection assay (GPA) is based on the inability of aminoglycoside antibiotic gentamicin to penetrate into eukaryotic cells. The GPA was used to study macrophage phagocytosis of *E. coli* in the presence of LGG-CM, LPS and LGG-CM+LPS combinations. Experiments were designed to investigate the effect of various concentrations of LGG-CM both in the presence and absence of LPS on bacterial ingestion and digestion.

2.7.1. Preparation of Bacterial culture

The *E.coli* HfrC cells were cultured in nutrient broth as described in section 2.3. On the day of the experiment, *E.coli* grown overnight from fresh bacterial inoculums was used in the experiment. A 22 µl ml^{-1} of 1:1 diluted (bacterial culture: sterile nutrient broth media resulting in approximately 2.5×10^7 CFU ml^{-1} *E.coli*) bacterial culture was centrifuged for 2 minutes at 10,000 rpm. The media was discarded and the pellet was washed twice with antibiotic free DMEM by alternate rounds of centrifugation at room temperature. The bacterial pellet was suspended in DMEM and was added to universals containing DMEM on its own (control), 20 µg ml^{-1} LPS; various dilutions of LGG-CM (10 %, 75 % and 100 % LGG-CM) and respective LGG-CM+LPS combination.

2.7.2. Preparation of macrophages

Macrophages were cultured in T-75 flasks as discussed in section 2.1. The cells were scraped and enumerated as in section 2.1.2. Following enumeration of macrophages from

the trypan blue dye exclusion technique, macrophages were plated in 24 well plates at a concentration of 5 x10^5 macrophages per well.

2.7.3. Experiments monitoring effect of LGG-CM on bacterial ingestion by gentamicin protection assay

On the day of the experiment, the media to be used for the experiment was left in the 37^0C water bath to warm up. The bacterial inoculums and macrophages were prepared as explained in section 2.7.1 and 2.7.2 respectively. The bacteria suspended in the control and treatments were then added to macrophages at a multiplicity of infection (MOI) of 50:1 in 24 well plate and the plates were gently shaken to initiate the bacterial contact with macrophages. A 100 µl of bacterial inoculums were serially diluted and plated to ensure the MOI reached approximately 50:1.

For monitoring bacterial ingestion, macrophages and bacteria were coincubated for a period of 30 minutes and 60 minutes in a humidified cell culture incubator at 37^0C and 5 % CO_2. After the respective incubation periods cells were washed with prewarmed PBS. Cells were then treated with 200 µg ml^{-1} gentamicin sulphate for 10 to 12 minutes to kill all extracellular bacteria. The cells were then lysed with 1x lysis buffer and immediately scraped using a cell scraper. The contents were quickly transferred to an Eppendorf tube. The bacteria were then enumerated by plating the lysed suspension onto the surface of agar plates by the spread plate method using plastic disposable spreaders. The plates were incubated at 37^0C for 18 to 22 hours and bacterial number was enumerated by colony counting assay. Figure 2.4 demonstrates the pictorial representation of steps involved in experiments monitoring bacterial ingestion by macrophages.

2.7.4. Experiments monitoring effect of LGG-CM on bacterial digestion by gentamicin protection assay

Macrophages were seeded in 24-well-plates (5 x 10^5 cells per well) and infected with *E. coli* at an MOI of 50:1 and incubated for 60 minutes at 37°C and 5 % CO_2.

After the bacterial ingestion time, medium was carefully removed, cells washed once with prewarmed PBS and medium containing 200 µg ml^{-1} gentamicin sulphate was added to kill all extracellular bacteria. Macrophages were then incubated in the respective treatments

containing 20 μg ml^{-1} gentamicin. The treatments included DMEM on its own (control), 20 μg ml^{-1} LPS; various dilutions of LGG-CM (10 %, 75 % and 100 % LGG) and respective LGG-CM+LPS combinations.

After specific time periods, cells were washed once with PBS and intracellular bacteria released by adding 1x lysis buffer. Undigested bacteria were subsequently recovered from lysed macrophages at an interval of 40 minutes for up to 280 minutes. Samples were diluted in saline and plated on nutrient agar plates for determination of the recovered colony forming units (CFU). Figure 2.5 demonstrates the pictorial representation of steps involved in experiments monitoring bacterial digestion by macrophages.

2.7.5. Analysing the bacterial digestion curve

In the studies monitoring bacterial digestion the first measurement of bacterial number recovered from macrophages was 15 minutes after gentamicin addition, after which the bacteria were recovered every 40 minutes up to 280 minutes. The recovered bacteria were expressed as CFU ml^{-1}. The bacterial killing curve from all the treatments appeared to follow the exponential pattern. The following steps were followed to calculate the slope from exponential equation: $a = a_0 e^{-kt}$.

1. The bacteria recovered at each time point (a) expressed as CFU ml^{-1} was divided by bacteria recovered at the first time point (a_0) for all the treatments.
2. Then, natural log of a/a_0 values were determined ($\ln a/a_0$).
3. The $\ln a/a_0$ values were plotted against time.
4. By converting an exponential graph into a linear one (y= mx + c), the slope or the rate of digestion could be directly calculated from the m value in the linear equation.
5. The slopes derived from each experiment were averaged and plotted against their respective treatment.

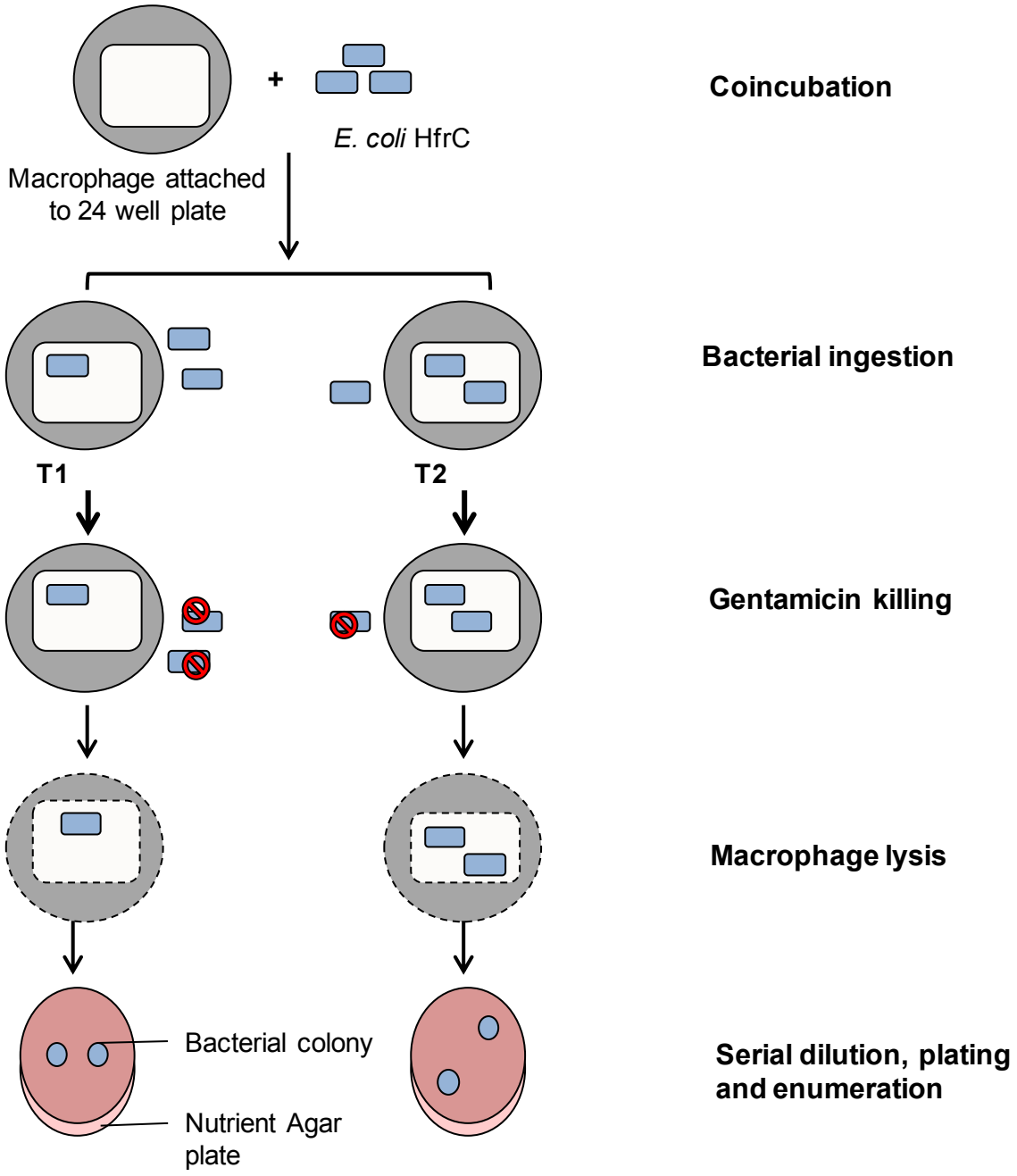


Figure 2.4: Schematic representation of experiments to monitor bacterial ingestion. Variable ingestion periods are represented as T1 and T2 in the figure. The red circle with a line through indicates bacterial killing by gentamicin.

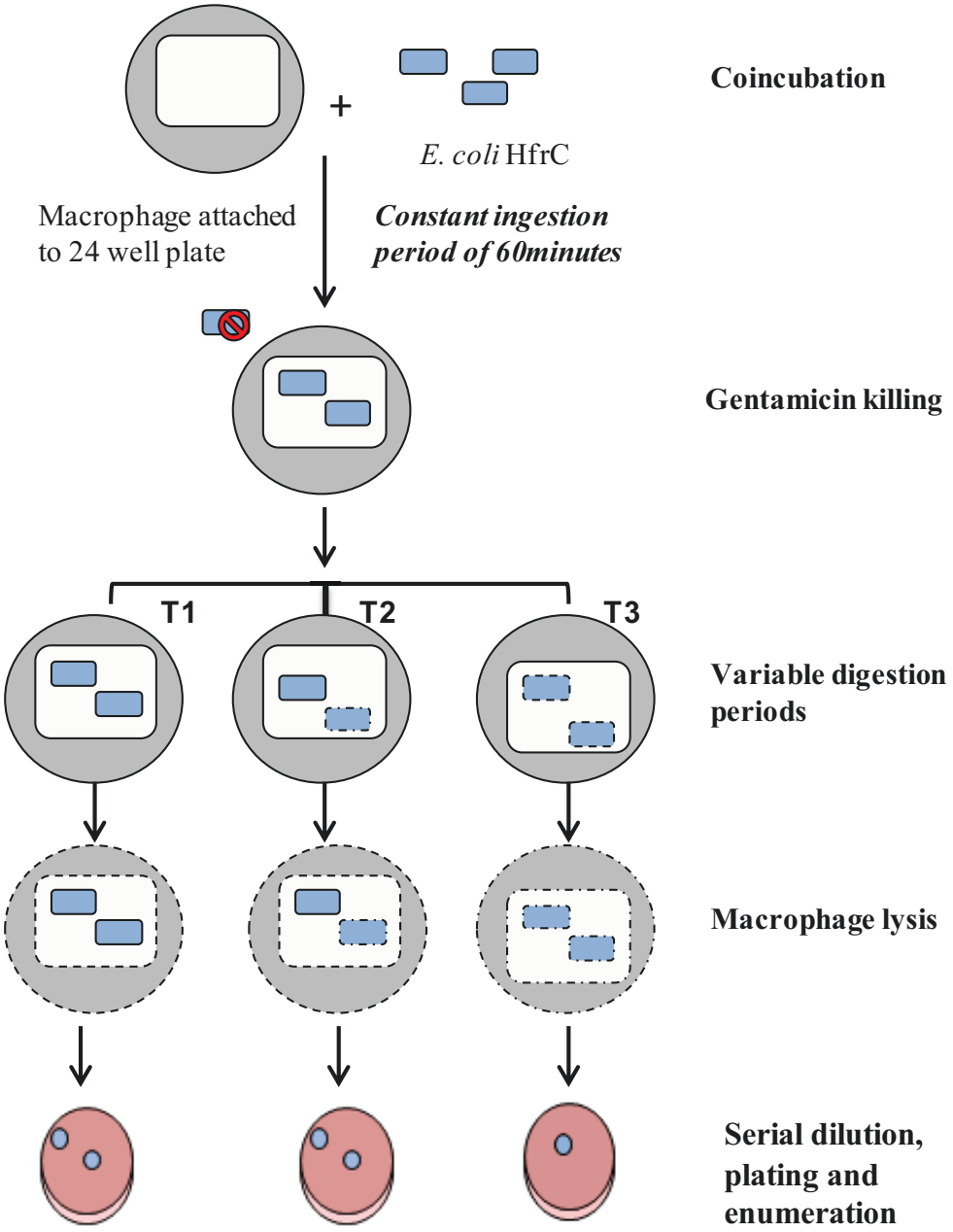


Figure 2.5: Schematic representation of experiments to monitor bacterial digestion. Variable digestion periods are shown as T1, T2 and T3 in the figure.

2.8. Acridine- orange crystal violet staining technique for monitoring macrophage phagocytosis of *E. coli*

The Acridine orange staining technique was used to image phagocytosis and digestion of *E. coli* coincubated with macrophages in the presence of LGG-CM. This technique has been widely used in previous studies investigating bacterial macrophage interactions (Zanetti *et al*., 1987; Pruzanski and Saito, 1988; Takao *et al*., 1996). The captured images were pseudo-coloured using the Image Hopper programme so that live bacteria appear green and dead bacteria appear bright red or yellow fluorescence. Images were captured at an excitation and emission wavelength of 485 nm and 528 nm respectively with a 60x objective. A bright field image was grabbed along with the fluorescence images.

2.8.1. Imaging of effect of LGG-CM on bacterial ingestion

Macrophages were grown in Mattek glass bottom culture dishes (P35G-0-14-C) for imaging phagocytosis. Briefly, 1ml of overnight grown *E. coli* culture was centrifuged for a minute at 10,000 rpm and the cells were washed with prewarmed PBS. Then the bacteria were labelled with 0.01 % acridine orange for 45 seconds. Excess dye was removed by washing the cells 3 times with PBS. The bacteria were observed under a microscope to confirm the dye uptake. For monitoring bacterial ingestion, *E.coli* was then added to the macrophages which were adhered to the bottom of Mattek dishes and incubated for 15, 30 and 60 minutes. After the respective time intervals, just before imaging, 0.05 % crystal violet dissolved in PBS was added to the petridishes to quench extracellular fluorescence. Crystal violet does not penetrate intact macrophage membrane and thereby don't quench fluorescence of intracellular bacteria (Takao *et al*., 1996).

2.8.2. Imaging of effect of LGG-CM on bacterial digestion

Macrophages and bacteria were prepared for imaging as described in 2.8.1. The acridine orange labelled *E.coli* was then added to the macrophages adhered to the Mattek dishes and incubated for 60 minutes. After coincubation period cells were washed once with prewarmed PBS and extracellular bacteria was killed by gentamicin treatment. For monitoring bacterial digestion, images were grabbed at 15, 60, 120, 180, 240 and 300 minutes after a coincubation period of 60 minutes. At respective time intervals, just before imaging, 0.05 % crystal violet dissolved in PBS was added to the petridishes to quench any extracellular fluorescence. Images were grabbed using Open lab software and analysed using Image Hopper 2 (Samsara research, UK).

2.9. Experiments investigating LGG-CM mediated free radicals production on macrophage phagocytosis using pharmacological inhibitors and scavengers

Free radicals inhibitors (ROS and NO inhibitors) and scavengers were used to investigate the role of LGG-CM induced changes in ROS and NO production on macrophage phagocytosis. Inhibitors used were L-NMMA (inhibitor of NO synthase), apocynin (NADPH oxidase inhibitor); allopurinol (xanthine oxidase inhibitors) and ROS scavengers namely, polyethylene glycol conjugated SOD and catalase as mentioned in Carter *et al*., (2004).

2.9.1. Experiments investigating LGG-CM mediated free radicals production on bacterial ingestion

Macrophages were enumerated and plated at $5x10^5$ cells ml^{-1}. Cells were preincubated for an hour with 100 μM apocynin and 100 μM allopurinol and ROS scavengers namely; polyethylene glycol conjugated 75 units ml^{-1} SOD and 100 units ml^{-1} catalase. After the preincubation period, bacteria in the respective treatments were added at 50:1 MOI and incubated for 60 minutes to allow macrophage ingestion of bacteria. The cells were washed with PBS after 60 minutes and gentamicin was added to kill the extracellular bacteria. The bacteria recovered from the various treatments were then enumerated by plating the liquid suspension by serially diluting and plating onto the surface of nutrient agar plates by the spread plate method using plastic disposable spreaders. The agar plates were incubated at 37^0C. The bacterial colonies were then enumerated by colony counting technique which provided a direct indication of the role and nature of free radicals involved in LGG-CM mediated bacterial ingestion. The experiment protocol for investigating LGG-CM mediated free radicals production on bacterial ingestion using specific inhibitors/scavengers is depicted in fig 2.6.

2.9.2. Experiments investigating LGG-CM mediated free radicals production on bacterial digestion

In order to investigate the potential role of free radicals involved in bacterial digestion in the presence of LGG-CM, macrophages were plated at a cell density of $5x10^5$ cells ml^{-1}. Cells were preincubated for an hour with 100 μM L-NMMA, 100 μM apocynin, 100 μM allopurinol and ROS scavengers namely; polyethylene glycol conjugated 75 units ml^{-1} SOD and 100 units ml^{-1} catalase. After the preincubation period, the macrophages and bacteria were coincubated in the respective treatment in the presence of scavengers or inhibitors for 60 minutes at 50:1 MOI to allow macrophage ingestion of bacteria. The cells were washed once with prewarmed PBS after 60 minutes of macrophage –bacterial coincubation and gentamicin was added to kill the extracellular bacteria. Various treatments were then added (various concentrations of LGG-CM, LPS, LGG-CM+LPS combination plus inhibitors/scavengers) and incubated for 280 minutes in the presence of 20 μg ml^{-1} gentamicin sulphate. The bacteria recovered from the various treatments at end of 280 minutes were then enumerated by plating the liquid suspension by serially diluting and plating onto the surface of nutrient agar plates by the spread plate method using plastic disposable spreaders. The plates were incubated at 37^0C. Enumeration of the recovered colonies by colony counting technique gave a direct indication of the role and nature of free radicals involved in LGG-CM mediated bacterial digestion. The experiment protocol for investigating LGG-CM mediated free radicals production on bacterial digestion using specific inhibitors or scavengers is depicted in figure 2.7.

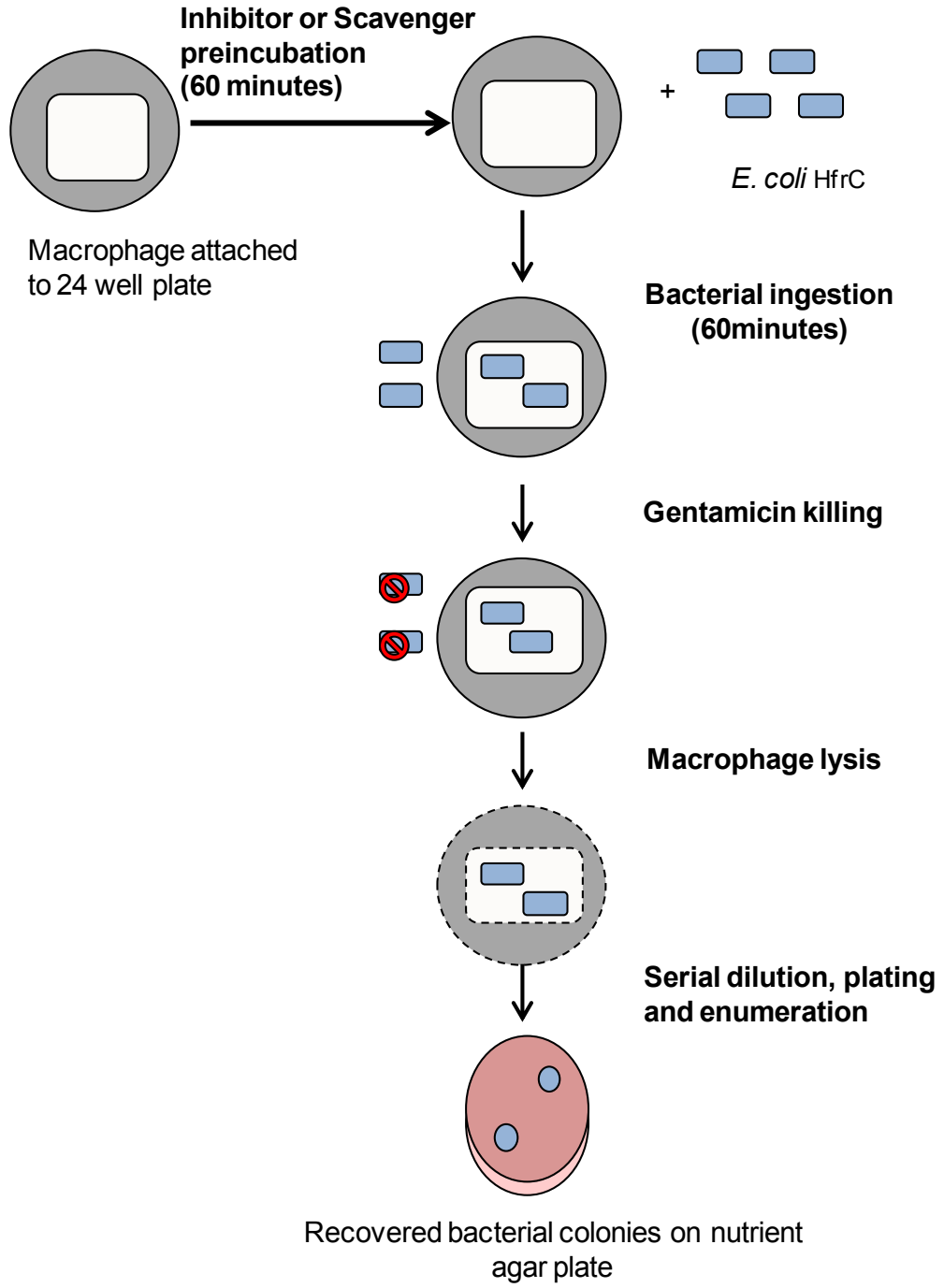


Figure 2.6: Diagrammatic representation of experiments monitoring bacterial ingestion in the presence of free radicals inhibitors and scavengers.

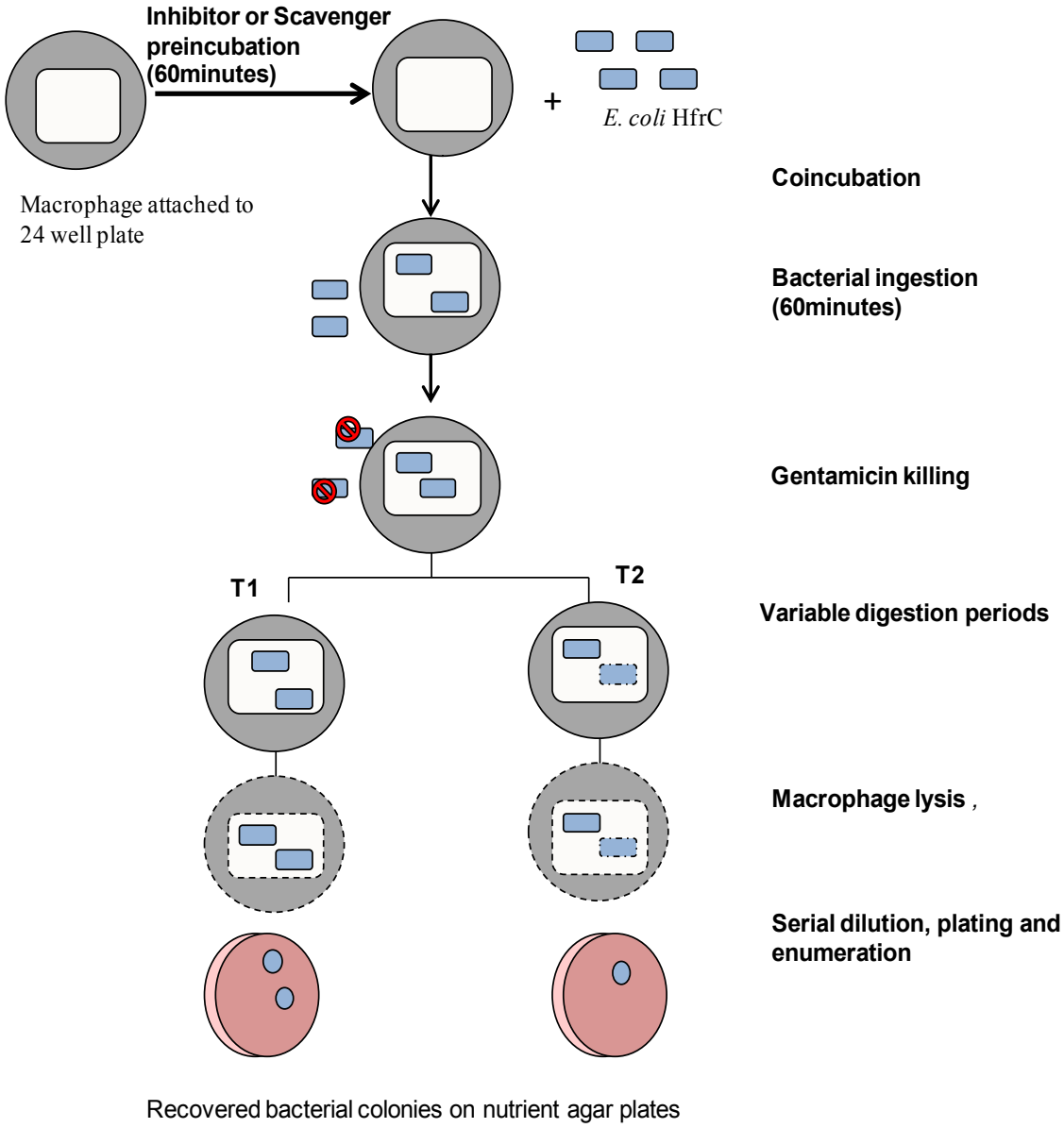


Figure 2.7: Diagrammatic representation of experiments monitoring bacterial digestion in the presence of free radicals inhibitors and scavengers. T1 and T2 represent 60 minutes and 280 minutes time points, at which the bacteria were recovered.

2.10. Fluorescence probes

2.10.1. ROS detection probe

The ROS production was monitored using a ROS specific dye, namely H_2DCFDA-AM (2', 7'-Dichlorodihydrofluorescein diacetate diacetoxymethyl ester). It is a membrane permeant molecule that passes through the cell membrane. After cell penetration, H_2DCFDA-AM is hydrolysed by cellular esterase to 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) that is non-fluorescent, but switched to fluorescent dichlorofluorescein (DCF) when oxidised by intracellular ROS (Rastogi *et al*., 2010).

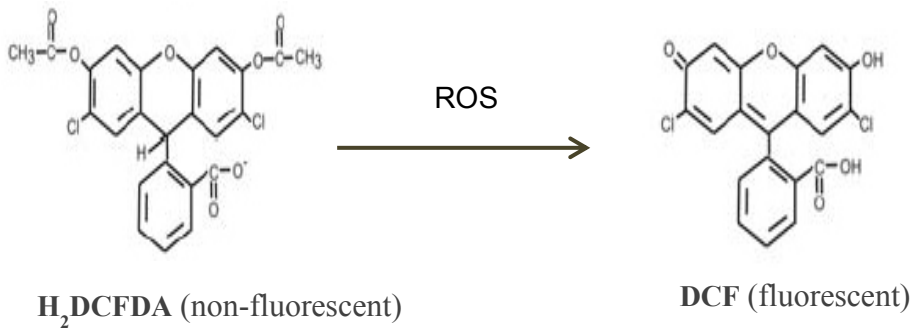


Figure 2.8: Formation of fluorescent compound DCF by Reactive oxygen species [Modified image from Held & Newick, 2008].

2.10.2. Nitric oxide detection probe

The NO production was monitored using cell permeable nitric oxide specific dye-Diaminofluorescein-FM diacetate (DAFFM-DA). It is an analog of diaminofluorescein and is more permeable and can be easily hydrolysed to DAF-FM via cellular esterases.

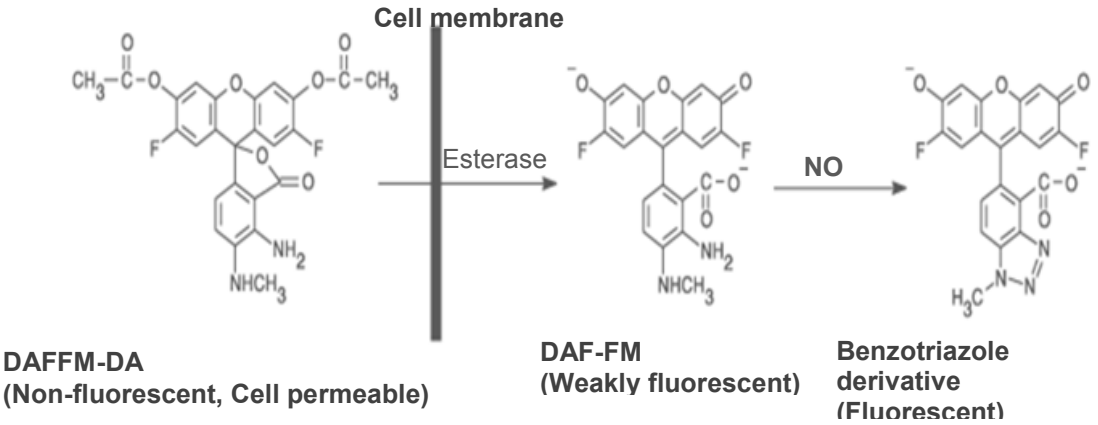


Figure 2.9: Formation of fluorescent derivative of DAF-FM from DAFFM-DA [Modified image from Molecular probes, 2001]

2.11. Imaging of free radical production using fluorescence microscope

Fluorescence imaging of free radicals production from macrophages treated with LGG-CM were performed using Nikon Eclipse TE-2000U fluorescence microscope. The microscope was switched on at least an hour before the start of experiment. Glass sterile cover slips (one per treatment) were placed in small Nunclon petri dishes.

Macrophages were scraped, enumerated and 1 ml of macrophage culture was added to small petridishes containing glass coverslips and incubated overnight in a humidified tissue culture incubator at 37^0C and 5 % carbon dioxide. On the day of the experiment, the media was replaced by 5 μM H_2DCFDA for ROS measurement or 5 μM DAFFM-DA for NO measurement for labelling the macrophages. The cells were then left in the cell culture incubator for 45 minutes. The medium was then removed; the cells washed three times with phosphate buffered saline and fresh prewarmed phenol red free DMEM was added.

The cover slip was then removed and attached to the bottom of the imaging chamber (macrophage affixed side facing up) before the medium was added onto the chamber. The chamber was then placed under an inverted microscope where a bright field filter was used to focus the cells prior to the fluorescence imaging. A single channel heater controller (model TC-324B) was used to control the temperature at 37^0C.

Both manual (Open lab) and time-lapse software was utilised for grabbing images. The fluorescence images of free radical production were grabbed from macrophages cultured on cover slips in the presence of untreated, 20 μg ml^{-1} LPS treated and 10 % LGG and 75 % LGG treated macrophages at various time periods. The time lapse measure plot 1 programme was used to measure ROS and NO using fluorescence filters which have an excitation and emission wavelength of 485 nm and 528 nm. The images were saved as tiff files and analysed using Image Hopper 2.

2.11.1. Experiment setup to measure the changes in free radical production using fluorescence microplate reader

For measuring the changes in free radical production using fluorescence microplate reader, macrophages were either labelled with 5 μM H_2DCFDA for ROS measurement or 5 μM DAFFM-DA for NO measurement.

The optimal cell loading concentration, time of incubation, temperature and dye retention time were determined by several pilot experiments. After macrophage attachment, the media was removed and cells were treated with respective fluorescent dyes for 45 minutes at 37^0C and 5 % CO_2. After 45 minutes, the loading media was removed and the cells were washed with PBS to remove any extracellular dye. The cells were returned with prewarmed phenol red free DMEM and incubated at 37^0C and 5 % CO_2 for 15 minutes more to allow a short recovery time for cellular esterases to hydrolyse the acetate groups and render the dye responsive to oxidation.

Various concentrations of LGG-CM were then added to wells (n=4). The baseline fluorescence intensity of the loaded samples was measured from untreated cells and the value was subtracted automatically from the programme from the sample fluorescent values. Protocol parameters were created and customised prior to the start of the experiment to avoid any possible error. Protocol parameters were stored in a protocol file with .PRT extension. The parameters and customisation process involved detection method as fluorescence, reading type as kinetic where readings were taken every 2 minutes, filter sets were defined at a wavelength selection of 485 nm excitation and 528 nm emission. The plate type was selected as a 96 well plate and the well details were defined. Well details mainly included labelling the wells as blank or as specific treatments. Wells were monitored throughout the experiment by the KC4 software programme, so that any fluctuations would be shown at the end. The plate layout for each experiment was designed so that it was possible to collect both the data obtained from treatments and treatment-blank. The audit trail logged in by KC4 software gave detailed information of events that happened at any time of the experiment. The programme was set to preheating mode so that optimal temperature of 37^0C was attained before the start of experiment. In the plate description parameter, details of experiment date and number of replicates was entered. A similar setup was used for measuring NO using DAFFM-DA.

2.11.2. Measurement of rate of ROS production by macrophages in the presence of LGG-CM during bacterial ingestion using fluorescence microplate reader

The macrophages were scraped from the flask and enumerated by trypan blue dye exclusion method as described in section 2.1.2. A 100 µl sample of cell suspension was then plated at a cell density of $5x10^5$ cells ml^{-1} in black clear bottom cell culture treated 96 well plates (n=4).

After cell attachment, cells were treated with 5 µM H_2DCFDA. The cells were incubated in dark at 37^0C for 45 minutes. After incubation period cells were washed with PBS to remove any excess dye. The cells were returned with prewarmed phenol red free DMEM and incubated at 37^0C and 5% CO_2 for 15 minutes more to allow a short recovery time for cellular esterase to hydrolyse the acetate groups and render the dye responsive to oxidation.

Then, 100 µl of treatments (various concentrations of LGG-CM, LPS, and LGG-CM + LPS combination both in the presence and absence of live *E. coli* HfrC) were added to the wells in quadruplicate and the fluorescence readings were started immediately in a fluorescence microplate reader with a pre-set temperature at 37^0C. The oxidation of the fluorescence dye by oxygen free radicals was detected by monitoring the increase in fluorescence by fluorescence microplate reader. The readings were taken every 2 minutes at a wavelength selection of 485 nm excitation and 528 nm emissions for 60 minutes. The experiment protocol for ROS measurement is depicted in Figure 2.10.

2.11.3. Measurement of rate of NO production by macrophages in presence of LGG-CM during bacterial ingestion.

For measuring NO production from macrophages treated with LGG-CM during ingestion period, experiments were performed in a similar manner to those described in section 2.11.2 for ROS measurement, the only difference being the macrophages were labelled with NO specific dye DAFFM-DA.

2.11.4. Measurement of rate of ROS production by macrophages in the presence of LGG-CM during bacterial digestion.

To further investigate the effects of LGG-CM on free radicals production by macrophages during bacterial digestion, a fluorescence microplate reader was used to monitor changes in free radicals production. Briefly, 100 µl of macrophage culture was plated at a density of $5x10^5$ cells ml^{-1} in black clear bottom cell culture treated 96 well plates.

After loading the macrophages with H_2DCFDA cells were washed with PBS. Then macrophages were coincubated with *E.coli* for 60 minutes. After 60 minutes, the macrophages were treated with 200 µg ml^{-1} gentamicin. Then, 100 µl of treatments (various concentrations of LGG-CM, LPS, LGG-CM +LPS combination both in presence and absence of live *E. Coli* HfrC) were added to the wells in quadruplicate and the fluorescence readings were started immediately in a fluorescence microplate reader preset to 37^0C and the fluorescence readings were taken for up to 280 minutes at 2 minute intervals.

The fluorescence data was plotted in an excel spread sheet. The rate of free radical production from the macrophages in the presence of various treatments was then calculated by Cumulative Sum (see section 2.11.6). The experiment protocol for free radical measurement during digestion period is depicted in figure 2.11.

2.11.5. Measurement of rate of NO production by macrophages in the presence of LGG-CM during bacterial digestion

For measuring NO production during digestion period, experiments were performed as described in section 2.11.4, the only difference being the macrophages were labelled with NO specific dye- DAFFM-DA.

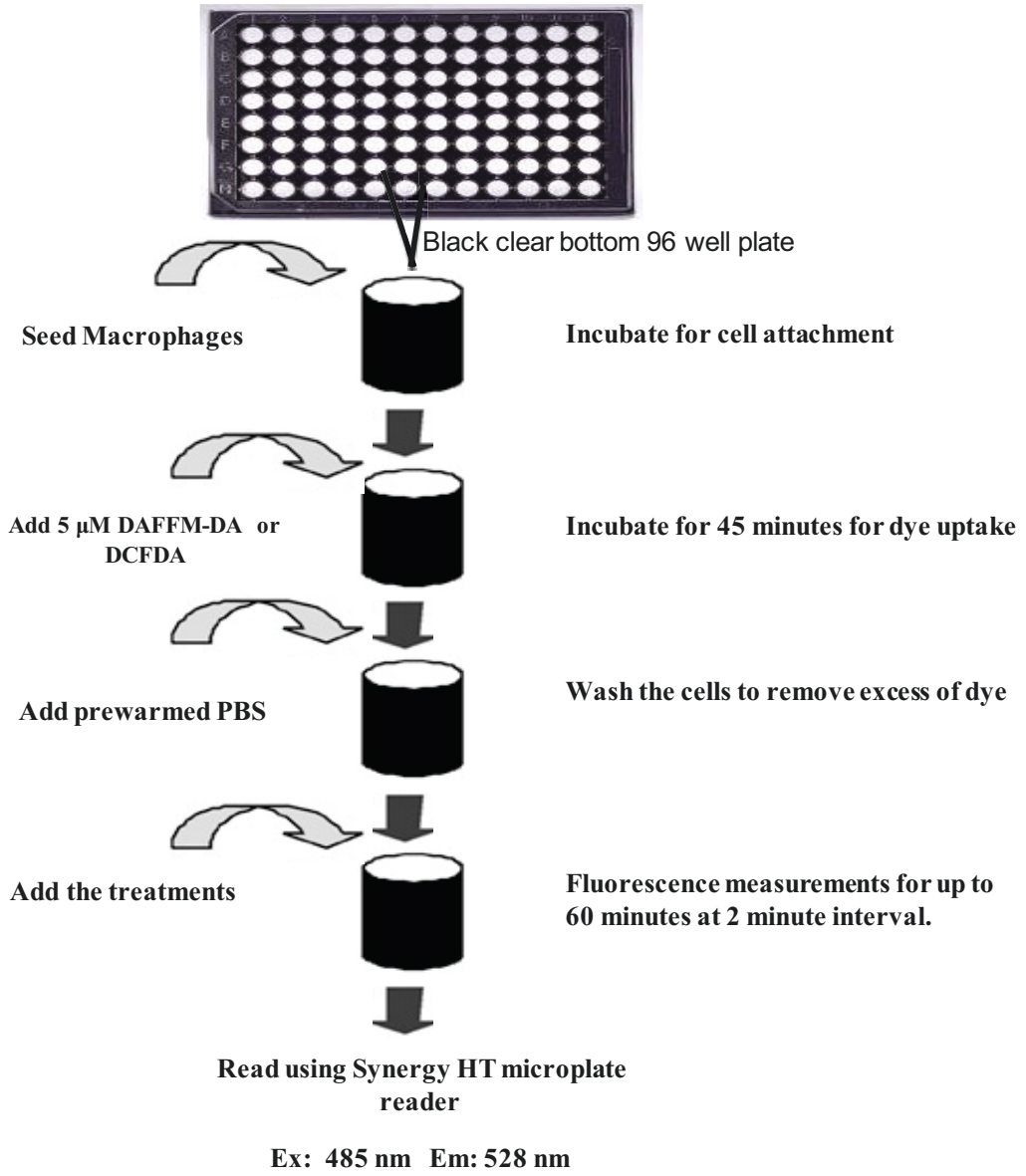


Figure 2.10: Monitoring free radicals production using a fluorescence microplate reader during bacterial ingestion period. The abbreviations used are MOI: multiplicity of infection, ex: excitation wavelength and em: emission wavelength

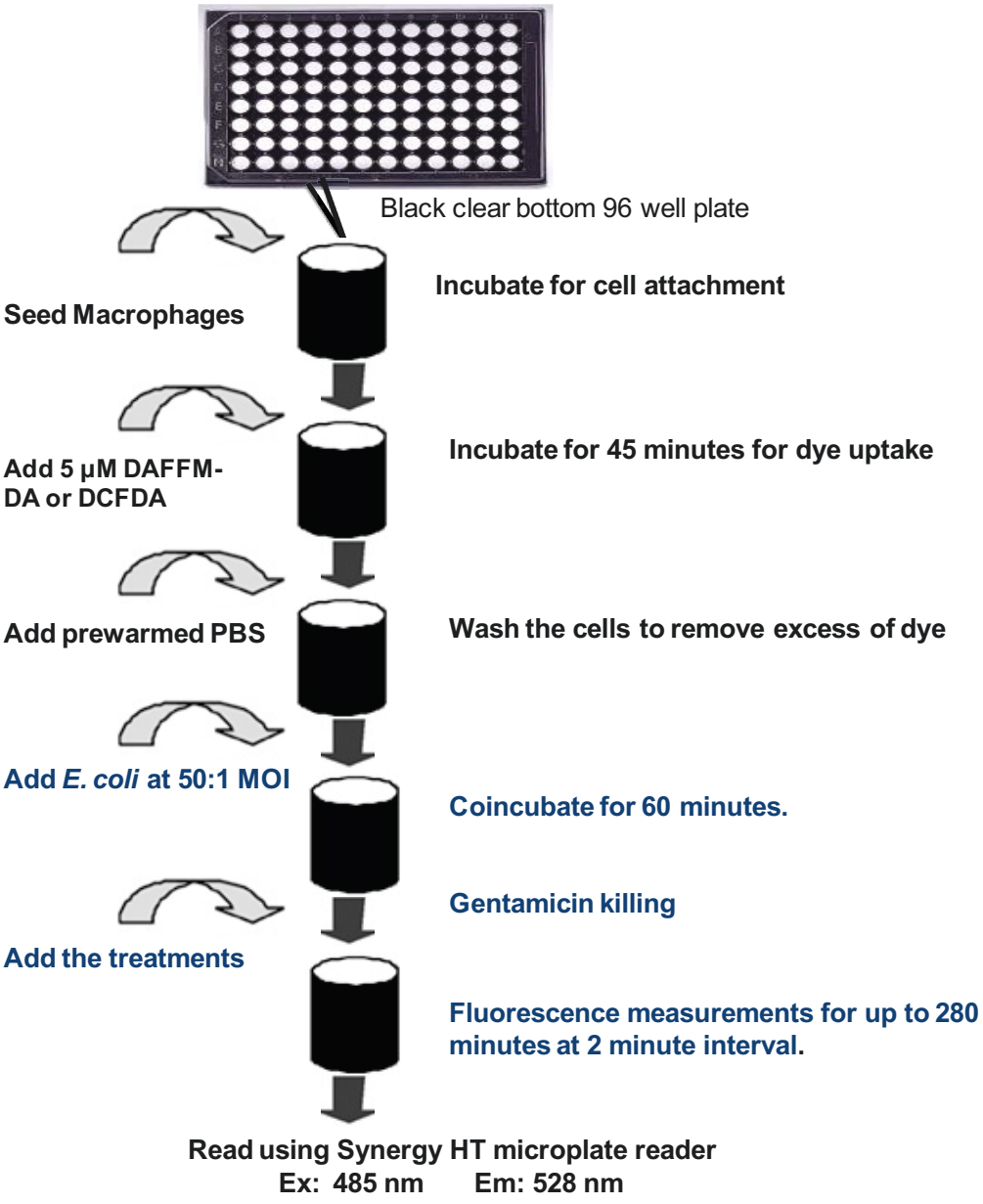


Figure 2.11: Monitoring free radicals production using a fluorescence microplate reader during bacterial digestion period. The abbreviations used are MOI: multiplicity of infection, ex: excitation wavelength and em: emission wavelength

2.11.6. CuSum analysis of free radical production by macrophages

For analysing the data sets from studies monitoring changes in free radical production using fluorescence microplate reader, raw experimental values for free radical production (test values) were plotted against time. The average of fluorescence measurements from control macrophages was used as the 'target value'. There was heterogeneity of pattern of free radical production in measured test (experimental) values between LGG-CM treated and untreated macrophages but the details were unclear.

CuSum was used in the analysis of fluorescence measurements to compare the rate of free radical production from macrophages from various treatments. The CuSum methodology is a simple technique that has frequently been applied to elucidate trends in time domain data (Stanton *et al*., 1992). It works by comparing each data point to a reference or target value (k) and then cumulatively summing the differences (Biau *et al*., 2007; Tam, 2009). If the underlying mean of the data points is constant the method produces a straight line whose slope is determined by the difference between the mean value and the chosen reference value. Small random variations are smoothed so the technique can reveal differences and changes in the mean value (Biau *et al*., 2007). When analysing the data sets, raw experimental values (test values) were plotted out against time. The average of the fluorescence measurements from the control macrophages was used as *k*, the 'target value'. This was then used to calculate the CuSum values.

The first CuSum C_o value is calculated using equation 1.

$$C_o = F_o - k \text{.....} 1$$

Where F_o is the first fluorescence reading in the time series and k is the 'target' or reference value. The next and subsequent CuSum values are calculated using equation 2 and 3

$$C_1 = C_o + (F_1 - k) \dots\dots 2$$

$$C_2 = C_1 + (F_2 - k) \dots\dots\dots 3$$

and so on, where C_n is the n^{th} CuSum value and F_n is the n^{th} fluorescence reading in the time series.

During an increase in free radical production from macrophages, the experimental values are mostly positive and so the CuSum values steadily increase and vice versa. As long as the free radical production is largely negative when compared to target value the CuSum value tends to remain close to zero.

2.12. Quantification of the expression of NADPH oxidase subunits by western blotting

The effect of LGG-CM on the expression of gp91 phox and p47 phox in J774 macrophages was investigated using SDS-PAGE and western blotting. This was performed to investigate the time dependent changes in NADPH oxidase activity. Macrophages were first treated with various dilutions of LGG-CM, 20 μg ml^{-1} LPS, LGG-CM+LPS combination for 2 hours, 6 hours and 24 hours. After the specific incubation time the treatments were removed, cells washed with ice cold PBS and lysed. The BCA (Bicinchoninic acid) assay was performed to identify the concentration of protein present in each lysed sample. The samples were stored in -20^{0}C until the proteins were separated by SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) (see detailed methodology for each step in section 2.13). The proteins were separated in a 0.75 mm discontinuous vertical slab minigel in an applied electric field. The separated proteins were blotted on to a PVDF membrane by a semidry transfer technique and the proteins of interest gp91 phox and p47 phox were identified using specific antibodies after blocking the membranes with 5 % non-fat dried milk (see detailed methodology for each step in section 2.14 and 2.15). Specific primary and secondary antibodies used to detect NADPH oxidase subunits gp91 phox and p47 phox are listed in table 2.4 (see details in section 2.16). The proteins are then detected by enhanced chemiluminiscence (see details in section 2.17).

2.13. Lysis of cells and extraction of samples for western blot

On the day of the experiment stock solution of lysis buffer (10 x) was diluted to 1 x lysis buffer and 5 μL of 200 mM sodium orthovanadate and 2 μl of protease cocktail inhibitor was added. The cells in the 24 well plate were then washed with ice cold PBS. Excess PBS was carefully aspirated without disturbing the cells. To extract the cell lysate, 100 μl of lysis buffer preheated at 95^{0}C was added to each well and the plate was left on the ice tray. The cells were scraped and transferred to individual pre-labelled eppendorf tubes placed in ice. The samples were then sonicated 5 times for 30 seconds, resting for 30 seconds between each run. The samples were then centrifuged at 10000 rpm for 3 minutes. The supernatant was carefully transferred to another set of pre-labelled eppendorf tubes and heated at 95^{0}C for 10 minutes. The BCA assay was performed to identify the concentration of protein present in each sample and the samples were stored at -20^{0}C.

2.13.1. Measurement of the total protein in cell lysate using BCA protein assay

The Bicinchoninic acid (BCA) protein assay is the colorimetric detection technique for quantitative estimation of the protein. The principle of the BCA assay is based on the reduction of cupric (Cu^{2+}) to cuprous (Cu^{1+}) ion by protein in an alkaline medium and formation of the purple coloured water soluble complex measurable by spectrophotometer. The BCA assay is a highly sensitive and selective assay for cuprous cation (Cu^{1+}) (Smith *et al*., 1985).

The BCA assay is 100 times more sensitive with respect to detection limit when compared to Biuret reagent (Smith *et al*., 1985; Wiechelman *et al*., 1988; Olsen and Markwell, 2007). In BCA assay BSA (Bovine Serum albumin) was used for preparing a protein standard curve.

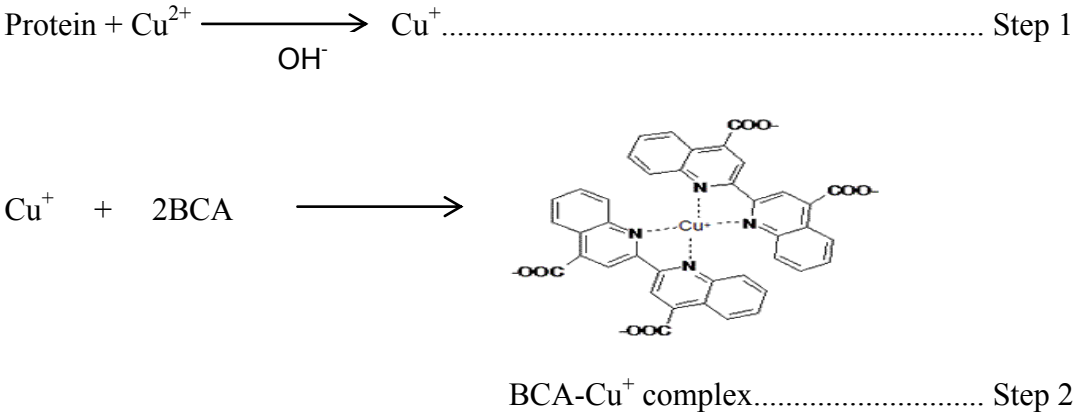


Figure 2.12: Reaction of BCA assay. This assay occurs in a two-step reaction. The first step is the biuret reaction, which involves the reduction of cupric ion to cuprous ion, in an alkaline environment, which results in a faint blue colour. In the second step of the colour development reaction, Bicinchoninic acid (BCA) reacts with the reduced cuprous cation that was formed in step one. The intense purple-coloured reaction product results from the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations.

Table 2.2: Protocol for preparing a standard curve using BSA. A stock solution (10 mg ml^{-1}) was prepared by dissolving 0.05 g of BSA in 5 ml of lysis buffer (1 x). A serial dilution of BSA solution was prepared using a 10 mg ml^{-1} BSA solution. In order to construct standard curve, absorbance was plotted as a function of total protein concentration. The protocol for preparing standard curve of BSA is shown in the table below.

Volume of 10 mg/ml BSA (µl)	Volume of 1X lysis buffer (µl)	Final [BSA] (µg/10µl)
0	100	0
10	990	1
20	980	2
30	970	3
50	950	5
100	900	10
150	850	15
200	800	20
250	750	25
300	700	30

2.13.2. Preparation of standard curve for protein.

A 10 µl of each concentration of BSA standard ranging from 0 to 30 µg per 10 µl was added in triplicate to the 96-well plate. An aliquot of 10 µl macrophage lysate from each treatment was also added in triplicate to the other empty wells in the same 96-well plate. Then BCA reagent was prepared by mixing BCA reagent A with BCA reagent B (50:1 ratio). After a homogenous colour formation of BCA reagent mixture, a 100 µl of BCA reagent was added into all the wells and incubated for 60 min at room temperature. The absorbance was measured at 562 nm using the Asys Hitech expert plus microplate reader. The absorbance of blank was subtracted from the BSA standard and the average absorbance of the standards was plotted against the respective protein concentration. The amount of total protein in the unknown macrophage lysate from untreated, LPS treated and LGG-CM ± LPS treated macrophages was then determined from the standard curve (Figure 2.13) using the values from the slope and y–intercept from the linear trendline equation $y = mx+c$. The unknown concentration of protein lysate from macrophages incubated with various treatments was calculated from the above linear trendline equation as described in section 2.6.2.

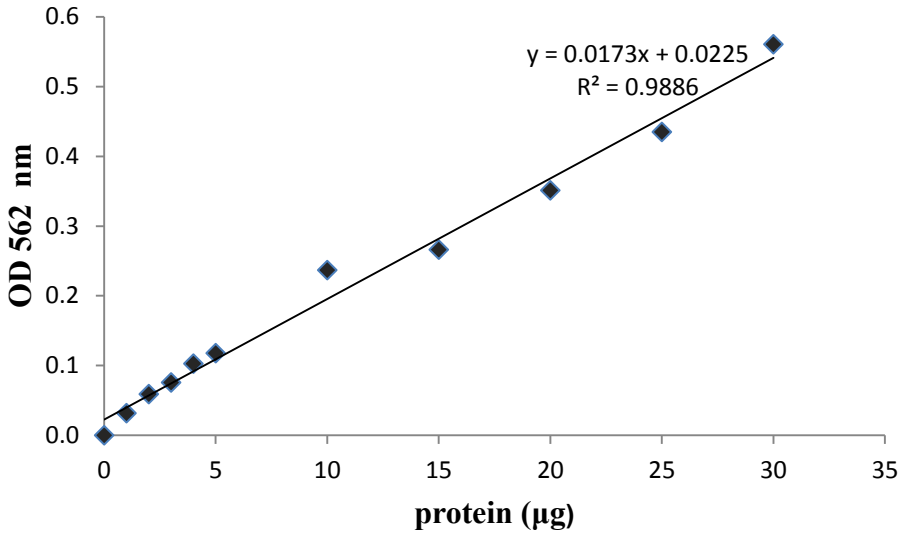


Figure 2.13: A representative standard curve of protein (BSA). The standard curve of protein can be used to convert optical densities of the unknown solutions to their respective protein concentration using a linear trendline equation.

2.14. Principle and background of SDS-PAGE and Western blotting

SDS-PAGE is based on the principle that a charged molecule will migrate in an electric field towards an electrode with an opposite sign. In SDS-PAGE proteins are separated based on their size or molecular weight, under the influence of an applied electric field. The SDS is a detergent used to remove the complex secondary, tertiary or quaternary protein structure. The linear polypeptide chains are then separated according to their molecular weight, which migrate in the gel. This migration rate again depends on the pore size of the gel and also the buffer system. The amount of SDS bound to the protein is proportional to the molecular weight of the protein and is independent of the protein sequence, so the proteins will migrate through the gel according to their size.

Western blot or the protein immunoblot is an analytical technique used to detect specific proteins from a cell lysate. The term blotting refers to the transfer of separated proteins from the gel to a second matrix which is generally PVDF (Polyvinylidine fluoride) or nitrocellulose membrane. Next the membrane is blocked to prevent any non-specific binding of antibodies to the membrane using fat free milk or BSA. The transferred protein is complexed with a primary antibody and suitable enzyme labelled (horseradish peroxidase or alkaline phosphatase) secondary antibody. An appropriate chemiluminiscent substrate is then added to the enzyme and together they produce light as a by-product. The

output will be proportional to the amount of secondary antibody bound to the protein of interest. When an x-ray film is exposed to the secondary antibody treated membrane, bands are formed on the x-ray film. Measuring the band size or intensity defines the concentration of the expressed protein of interest.

2.14.1. Preparation of gels

Discontinuous gels were constructed with two different acrylamide gels, one on top of other. Firstly, an 8 % resolving gel was prepared (see table 2.3). Gel at this concentration of acrylamide /polyacrylamide mixture was used for separating proteins generally in the range of molecular weight ranging from 40 to 200 kDa. For the gel to mould between the glass plates the glass plates were carefully cleaned, dried and the small glass plate was placed on the large plate with a spacer and inserted into the casting frame. The pressure clamps were locked and checked for any leakage before pouring the resolving mixture. Approximately 3.7 ml of resolving gel mixture was poured in between the plates and immediately covered with a layer of ultrapure water to prevent any bubbles or air traps. The gels were allowed to polymerise for 45 minutes.

Table 2.3: Composition of resolving and stacking gel

Ingredients	8 % Resolving gel	5 % Stacking gel
Gel volume	10 ml (2 gels)	5 ml (2 gels)
Ultra-pure water	4.68 ml	2.84 ml
30 % Acrylamide	2.67 ml	0.833 ml
1.5 M Tris pH8.8	2.50 ml	-
0.5 M Tris pH 6.8	-	1.25 ml
10 % SDS	0.1 ml	0.050 ml
10 % APS	0.075 ml	0.025 ml
TEMED	0.015 ml	0.005 ml

After solidification of the resolving gel, the stacking gel was prepared as shown in table 2.3. The water layer over the top of resolving gel was carefully removed and the stacking gel was carefully added on the top of the resolving gel. A 10 well comb was immediately

placed over the stacking gel making sure not to entrap any air bubbles. The stacking gel was then allowed to polymerise for 30 minutes before the samples were loaded.

After polymerization of the stacking gel, the plates were carefully removed and the wells washed with 1x tank Buffer (0.025 M Tris-HCl, 0.192 M Glycine, 0.1 % SDS). The whole gel cassette was placed in a mini-protean 3 electrophoresis tank and the tank buffer was poured into the tank. The gels were then left in the tank to equilibrate for 5 minutes.

2.14.2. Separation of proteins using SDS-PAGE

The protein samples were removed from the -20^{0}C freezer and mixed with 2 x loading buffer and heated for 5 minutes at 95^{0}C. The comb from the gel cassette was removed and the samples (15 µg) were loaded into the wells. A rainbow molecular weight marker (5 µl) was loaded in the first well in the first gel, and the 9th well in the second gel to mark the orientation and to identify the molecular weight of the proteins. The electrophoresis was run at 90 volts until the proteins moved out of the stacking gel and the voltage was increased to 200 volts for the rest of the time period. The electric supply was stopped when the tracker dye moved to the end of the gel.

2.15. Transfer of separated proteins from the gel to the PVDF membrane

The proteins from the gel were transferred to the PVDF membrane by the semi-dry transfer technique (V20-SDB Semi-Dry Western Blotting). The gels were removed from the glass plates in electrophoresis unit and the stacking gel was removed with a gel cutter. The gels were then rinsed in the transfer buffer. A PVDF (Polyvinyldifluoride) membrane was used for the protein transfer. The PVDF membranes were soaked in methanol for 20 seconds and placed in the transfer buffer for equilibration. The filter papers were cut according to the size of the gels and were also soaked in the transfer buffer. For transferring, 3 filter papers were placed on the blot unit, followed by the PVDF membrane and gel and the sandwich completed by placing 3 more pre-soaked buffer saturated filter papers on top. Any air bubbles were carefully removed using a glass pipette. The proteins were transferred for 2 hours at an applied current of 0.8 mA cm^{-2} and the voltage limited to less than 13 volts.

The PVDF membrane was blocked by placing the membrane in the blocking buffer (10 ml of 10 x washing buffer, 100 µl Tween-20, 5 % non-fat dried milk, 90 ml ultrapure water). The membrane was rocked on a shaker for 15 minutes at a moderate speed and placed

overnight at 4^0C to prevent the non-specific binding of antibodies. The membrane was again placed on a shaker for 15 minutes before being probed with a primary antibody.

2.16. Probing the membrane with antibodies

After blocking the PVDF membrane, the membrane was incubated with a primary antibody. The primary antibody is normally obtained by immunising an animal with the protein of interest, and collecting the antibodies the animal produces against that protein. The primary antibodies used for gp91phox , p47phox, β-actin are listed in table 2.4.

The PVDF membrane (after blocking) was sealed in a hybridisation bag filled with a primary antibody diluted in the blocking buffer. The membrane was then placed on a rotary shaker for 2 hours. After the incubation period, the membrane was washed three times with wash buffer (10 mM Tri-HCl pH 7.5, 100 mM NaCl, 0.1 % Tween-20) whilst shaking. The wash buffer was replaced, fresh, every 10 minutes and the membrane washed with gentle shaking. These washing steps removed any unbound, excessive primary antibody from the membrane. The membrane was again incubated with a secondary antibody and incubated for an hour (see table 2.4). This antibody binds to the primary antibody already bound to the protein of interest and is conjugated with horseradish peroxidase. After incubation with the secondary antibody, the membrane was again washed with wash buffer 3 times as described above.

Table 2.4: Antibodies used for identifying protein of interest. The table below lists the primary antibodies used for detecting various components of NADPH oxidase and a corresponding secondary antibody used for detection.

Protein of interest	Molecular weight (KDa)	Antibody information (SantaCruz Biotechnology)	Dilution
$gp91^{phox}$	65	SC130543	1:500
$p47^{phox}$	47	SC17845	1:250
β-actin	43	SC47778	1:500
Goat-antimouse IgG-HRP	-	SC-2005	1:3000

2.17. Detection of protein by enhanced chemiluminescence (ECL) and quantification of western blots by scanning densitometry

The membrane was removed from the washing buffer and placed on clean cling film. Enhanced chemiluminescence detection solution was prepared (detection reagents 1 and 2 mixed at 1:1 ratio) as described by the manufacturer and applied to the membrane for 1 min at room temperature. Excess solution was drained and the PVDF membrane was carefully wrapped with cling film avoiding any trapped air bubbles. The membrane was then fixed to a cassette and was exposed to an autoradiography film for 1 to 3 minutes in the dark room. The film was immediately developed using Carestream® Kodak® autoradiography GBX developer and fixed with Carestream® Kodak® autoradiography GBX fixer.

The bands that developed on the autoradiography film were quantified using Image J, an image processing and analysing programme, where the film harbouring western blot bands were converted into values that are subsequently presented as graphs with accompanying statistics. Each band was scanned and its intensity noted by the Image J programme. The values obtained for the macrophages incubated with each treatment were compared to those of their respective controls.

2.18. Statistical analysis

Unless otherwise stated, the results are expressed as the mean ± S.E.M as indicated in individual experiments. Statistical differences between the means were determined by one-way analysis of variance (ANOVA) followed by Dunnett multiple comparison post-hoc tests. A p-value of less than 0.05 was considered to be statistically significant. The analyses were performed using prism version 3.00 for windows (Graph pad software, San Diego, USA).

CHAPTER 3

Effect of LGG-CM on cell-viability and nitric oxide production by macrophages

3.0. CHAPTER 3

3.1. Introduction

Activated macrophage produce NO and ROS (e.g. superoxide and hydrogen peroxide), which aid the destruction of ingested microbes. The previous work in the lab had demonstrated that J774 macrophages produce NO in the presence of VSL#3 (formulation containing 8 different probiotic strains) conditioned medium. This study also demonstrated that LGG-CM produced a low but slightly enhanced basal level of inducible nitric oxide synthase (iNOS) enzyme expression in J774 murine macrophages. However, when LGG-CM was applied in combination with LPS, iNOS expression from macrophages was apparently reduced (Sarker *et al*., 2011).

Our initial experiments were focussed on investigating the effect of LGG-CM on NO production by J774 murine macrophages. The mouse macrophage cell line J774 has been shown to respond to LPS by producing NO through the activation of iNOS gene (Hattori *et al*., 1996). The NO production is required to maintain normal physiology of the macrophages. However its production could be affected by the immunomodulatory substances that either increase or decrease the production of these molecules (Abbas *et al*., 2007). The NO can induce a cytoprotective or cytotoxic effect depending on its concentration level (Wiley, J.W. 2007). The LGG by itself or cell free conditioned medium has been shown to induce NO production via the iNOS pathway and the mechanism has shown to be similar to that of LPS mediated NO production by the macrophages (Korhonen *et al*., 2002).

The NO has a very short half-life and is normally oxidised to nitrite or nitrate end product. Studies have shown the expression of iNOS being induced in murine macrophages in the presence of 20 μg ml^{-1} LPS (Stuehr & Marletta, 1985), however, others suggested the additional requirement for IFN-γ (Lorsbach & Russell, 1992; Nathan, 1992; Lorsbach *et al*., 1993; Weisz *et al*., 1996; Kidd, 2003; Cunha *et al*., 1993; Fang, 1997) for successful nitrite detection from murine macrophages.

It has been generally accepted that the live probiotic bacteria are responsible for any health beneficial activities. However, currently the concept of probiotic derived factors induced health benefits is also extensively suggested (see section 1.5.2). It has been shown that the factors influencing the efficiency of the probiotic conditioned medium mainly depend on the growth conditions of probiotic bacteria; both physical and nutritional factors and

bacterial cell density for example. Variation in probiotics effects can also be attributed to the initial cell density used to prepare the probiotic conditioned medium. Most of the studies, including this study, have employed probiotic bacteria at a cell density of 10^9 CFU ml^{-1} (Donnet Hughes *et al*., 1999, Lee *et al*., 2012).

Several i*n vitro* evidences suggest that certain probiotic strains can inhibit the growth and adhesion of a range of enteropathogens (Bernet-Carnard *et al*., 1997; Coconnier *et al*., 1993; Coconnier *et al*., 1997; Hudault *et al*., 1997; Gopal *et al*., 2001; Demerdash and Mostafa, 2008; Ridwan *et al*., 2008; Lu *et al*., 2009). The effectiveness of probiotics to treat *Helicobacter pylori* gastroenteritis and urovaginal infections have also been demonstrated in well-designed *in vitro* and *in vivo* experimental studies (Reid *et al*., 2003; Servin, 2004). In relation to the infection in the intestinal tract, several studies have reported that probiotics are effective in decreasing the severity and duration of rotavirus infections in children as well as preventing acute and traveller's diarrhoea in adults (Ouwehand *et al*., 2002; Reid *et al*., 2003; Reid *et al*., 2011). For most of the competitive exclusion studies, and in conditions as mentioned above such as acute, rotavirus and traveller's diarrhoea, live probiotics are used as they compete for nutrition and space in the gut microenvironment.

Studies have demonstrated that the LGG produce antimicrobial substances with activity against potential pathogens (Silva *et al*, 1987; Ridwan *et al*., 2008; Lu *et al*., 2009). The mechanism of the antibacterial activity of the *Lactobacillus* strains appears to be multifactorial (Servin, 2004). Their antibacterial effectiveness depends on factors such as the secretion of antibacterial proteins and the secretion of metabolites like lactic acid and acetic acid (Richard *et al*., 2006). The *Lactobacillus* strains have also been shown to inhibit the growth of bacterial pathogens and sometimes even kill them (Vandenbergh 1993; Keersmaecker *et al*., 2006; Suskovic *et al*., 2010). However, health benefits that can be attributed to one probiotic strain cannot be extrapolated to other probiotic strains, or mixtures of strains as there is wide diversity in probiotic bacteria demonstrating antibacterial activity (Roselli *et al*., 2006; López *et al*. 2010; Snel *et al*. 2010; Van Hemert *et al*. 2010; Vissers *et al*., 2010, Vissers *et al*., 2011).

This study primarily aimed at the successful preparation of LGG-CM and confirming the presence of the secretion of active components in LGG-CM by detecting nitrite production from macrophages treated with LGG-CM. This study also aimed to test if cell free LGG-

CM demonstrate antibacterial activity on *E. coli* HfrC. At first, a growth curve of *Lactobacillus rhamnosus* GG was generated to identify the growth pattern of LGG over a period of time. This was followed by preparing the cell free LGG-CM and determining the effect of LGG-CM on bacterial viability. Next experiments were focussed on determining the effect of LGG-CM on macrophage viability and NO production by macrophages.

3.2. Results

3.2.1. LGG growth curve

Figure 3.1 represents the growth pattern of LGG. Optical density readings were taken every hour up to 12 hours and continued again at 24, 25, 26, 29 and 31 hours. The graph represents the sigmoidal pattern of LGG growth. When viable LGG was inoculated into the fresh MRS medium, it took some time to adjust to the new growth environment. This phase is termed as 'lag phase', in which cells increase in size, but the bacteria were not able to replicate and, therefore, a lag in increase in optical density reading was observed. For the first few hours, the bacterial growth was slow, as expected in the lag phase. The lag phase lasted for about 5 hours. Once the LGG entered the exponential phase (log phase) of growth, the metabolic activity of LGG increased and bacterial number started replicating and the nutrients in the growth medium were used at the maximal rate. The growth of LGG increased exponentially during this period. It was observed that at the 12^{th} hour bacteria were still in the log phase where bacterial numbers were increasing at an exponential rate. Experiments could not be continued from 12 hours to 24 hours due to laboratory time constraints. At 24 hours bacteria had already attained the stationary phase, which was indicated by no further increase in optical density reading (Figure 3.1). This was an equilibrium phase where the bacterial growth rate was almost stable as indicated with fairly constant optical density readings.

3.2.2. The pH Changes in the culture medium during LGG growth

The LGG grows in the liquid culture medium using available nutrients such as carbohydrates and aminoacids. The breakdown of these compounds to organic acids such as lactic acid results in a decrease in pH of the culture medium. As the bacterial number increases the available nutrients are used at a faster rate resulting in a reduction in pH. Figure 3.2 shows change in pH in the LGG culture medium during LGG growth. The pH readings were taken every hour up to 12 hours and then continued again at 24, 25, 26, 29 and 31 hours. In the control MRS medium, the pH had remained constant during all the measurements. In the LGG culture medium, the pH remained fairly constant (≈ pH 6.0) during lag phase. However, the pH in the LGG culture medium started to drop after 4 hours of incubation and continued to fall until the end of the log phase. The pH started to

reduce into acidic range during the log phase. At the stationary phase, the pH of the LGG culture medium was found to be static around 4. The drop in pH indicated the growth of inoculated LGG in the MRS culture medium. After 30 hours of incubation, the pH reading of the LGG culture medium was approximately 3.8.

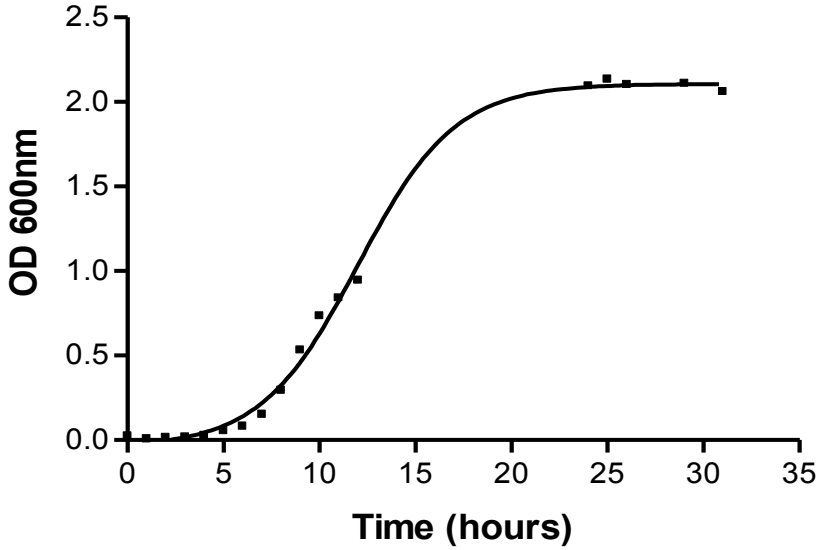


Figure 3.1: Growth phases of *Lactobacillus rhamnosus* GG. Experiments were performed in triplicate and repeated 3 times. Graph represents mean optical density readings of 3 separate experiments. Error bars indicate ± SE of the mean. Error bars where not seen are obscured by the data points. Sterile MRS medium was used as blank. Optical density readings were taken at an interval of 60 minutes for up to 12 hours at a wavelength of 600 nm using a spectrophotometer.

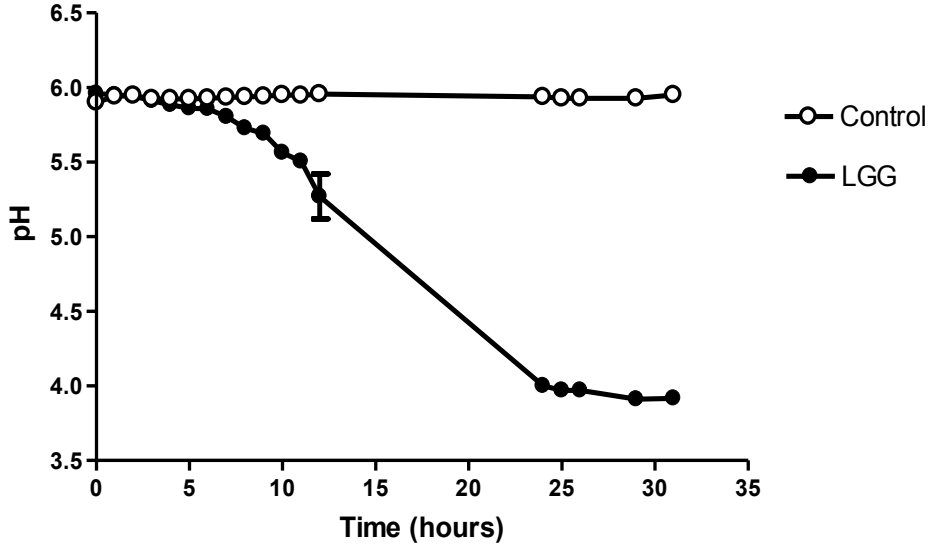


Figure 3.2: Change in pH against time of culture medium during LGG growth. Experiments were performed in triplicate and repeated 3 times. Graph represents the mean of 3 separate experiments. Error bars indicate ± SE of the mean. The pH readings were taken at an interval of 60 minutes for up to 12 hours and then again continued at 24, 25 , 26, 29 and 31 hours. Error bars where not seen are obscured by the data points.

3.2.3. Effect of cell-free LGG conditioned media on *E. coli* viability

The experiment to determine the antibacterial role of LGG-CM was performed as described in section 2.4 in chapter 2. The antibacterial activity of LGG was examined at 1 hour, 6 hours and 24 hours time points. Figure 3.3 shows the recovered bacterial colonies numbers from the control and the LGG-CM treatments. There was no significant difference in *E. coli* recovery between bacteria cultured in the control medium and in the various dilutions of the LGG-CM even after 24 hours of incubation time.

The bacterial recovery from the control medium at 1 hour incubation period was 7.51 ± 0.05 $\log_{10}$ CFU ml^{-1}. However, there was a small drop of bacterial recovery to 7.13 ± 0.12 $\log_{10}$ CFU ml^{-1} at 24 hours. There was no change in bacterial number from the highest concentration of LGG-CM tested (100 % LGG) ranging from 7.51 ± 0.06 $\log_{10}$ CFU ml^{-1} at 1 hour to 7.58 ± 0.09 $\log_{10}$ CFU ml^{-1} at 24 hours incubation. The LGG-CM (pH 7.4 ± 0.2) therefore tested for antibacterial activity against *E. coli* HfrC appeared to be non-toxic to bacteria.

There was no significant difference in bacterial number in control medium between 1 hour and 6 hours. However, the bacterial number was significantly lower from control medium at 24 hours when compared to the bacterial recovery at 1 hour and 6 hours ($p<0.05$). We also observed a significantly low bacterial number from 5 % LGG-CM between 6 hours and 24 hours incubation period ($p<0.01$).

There was no significant difference in bacterial number at 1 hour, 6 hours and 24 hours time point of incubation when bacteria were incubated in 10 % LGG-CM, 75 % LGG-CM and 100 % LGG-CM ($p>0.05$).

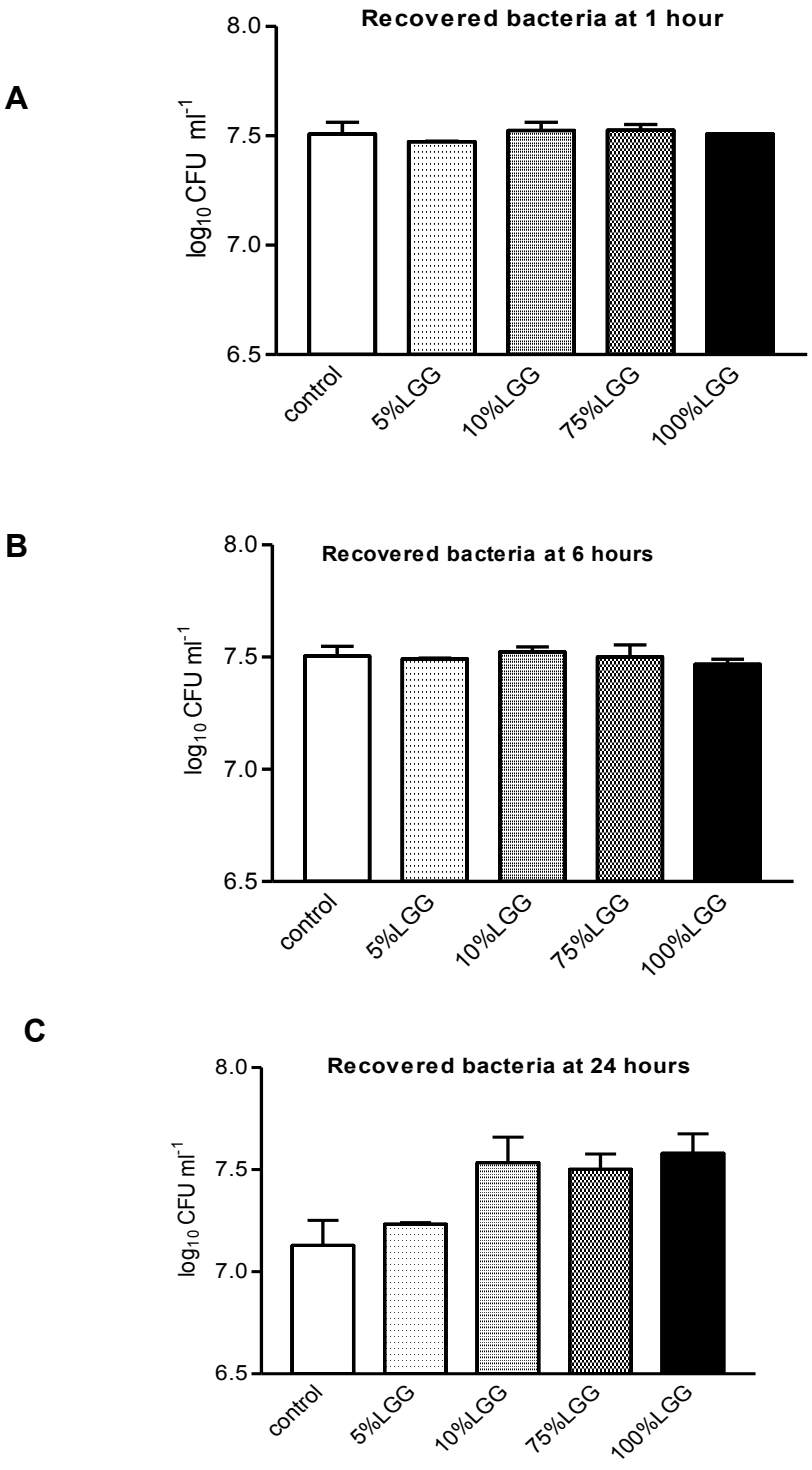


Figure 3.3: Bacterial recovery after incubation with LGG-CM. *E. coli* HfrC was incubated in various dilutions of LGG-CM for 1 hour (A), 6 hours (B) and 24 hours (C). Recovered bacteria were serially diluted, plated and enumerated by colony counting technique. Experiments were performed at least in duplicate and repeated at least 3 to 5 times. Statistical analysis was performed by One-way ANOVA and there was no significant difference between bacterial recoveries from control to various dilutions of LGG-CM at all the three time points ($P>0.05$).

3.2.4. Effect of LGG-CM on macrophage viability

To investigate the effect of LGG-CM on J774 macrophage viability, MTT experiments were performed as described in section 2.5.3. Experiments were performed to investigate the effect of LGG-CM (pH 7.4 ± 0.2) on macrophage viability at 1 hour, 6 hours and 24 hours to match other experimental protocols used in this study. The percent cell viability was calculated as described in section 2.5.2. The percent macrophage viability from LPS, 10 % LGG-CM, 75 % LGG-CM and 100 % LGG-CM and *E. coli* treated macrophages at 1 hour was found to be 126 % ± 5 %, 124 % ± 19 %, 105 % ± 30 %, 98 % ± 14 %, 105 % ± 2 % respectively (fig 3.4A). There was no significant difference in macrophage viability when macrophages were incubated with LGG-CM and LGG-CM ± LPS during the first 60 minutes of incubation (Fig 3.4B).

Experiments to investigate the effect of LGG-CM (pH 7.4 ± 0.2) on bacterial digestion were performed, approximately, for up to 6 hours. Thus, the effect of LGG-CM on macrophage viability was assessed after 6 hours of treatment incubation (Fig C & D). The percent macrophage viability from LPS, 10 % LGG-CM, 75 % LGG and 100 % LGG-CM and *E. coli* treated macrophages at 6 hours was 92 % ± 1 %, 108 % ± 5 %, 83 % ± 5 %, 99 % ± 15 %, 105 % ± 9 % respectively (Fig 3.4C). There was no significant difference in macrophage viability when macrophages were incubated with LGG-CM and LGG-CM ± LPS during the first 6 hours of incubation (Fig 3.4D).

Initial experiments of the effect of LGG-CM (pH 7.4 ± 0.2) and LPS on nitrite production by macrophages were measured by Griess assay at 24 hours incubation period. However, these experiments included treatments alone and were performed in the absence of live bacteria. To determine the effect of these treatments on macrophage viability, MTT assay was performed after incubating the macrophages with LGG-CM and LPS for 24 hours (Fig E & F). The percent macrophage viability from LPS, 10 % LGG-CM, 75 % LGG-CM and 100 % LGG-CM treated macrophages at 24 hours was 74 % ± 1 %, 102 % ± 10 %, 97 % ± 12 %, 94 % ± 7 % respectively (Fig 3.4E). The LPS in presence of LGG-CM did not have any effect on macrophage viability (Fig 3.4F).

There was significant difference in percent macrophage viability between macrophages treated with the LPS on its own at 6 hours and 24 hours when compared to macrophage viability at 1 hour incubation period ($p<0.01$). The 10 % LGG-CM, 75 % LGG-CM and 100 % LGG-CM treated macrophages had no effect on macrophage viability at 1 hour, 6 hours and 24 hours ($p>0.05$).

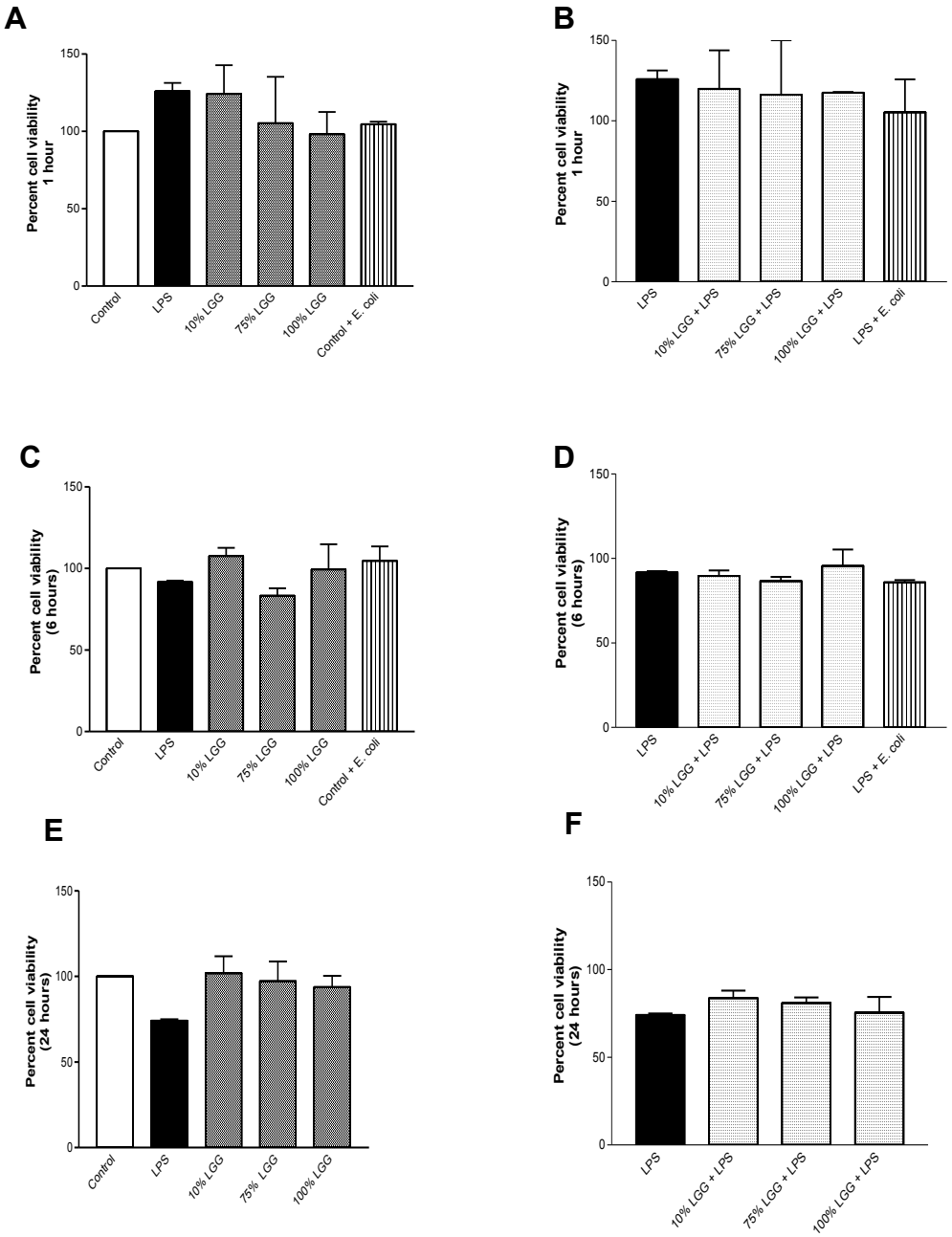


Figure 3.4: Effect of LGG-CM on macrophage viability. Macrophage viability was assessed in the presence of LGG-CM at 1 hour, 6 hours and 24 hours. The experiments were performed both in the presence and absence of LPS and *E. coli*. Experiments investigating macrophage viability at 1 hour were performed in triplicate and repeated 2 times (Figure A & B). Experiments investigating macrophage viability at 6 hours were performed in triplicate and repeated 3 times. (Figure C & D). Experiments investigating the macrophage viability at 24 hours were also performed in triplicate and repeated 3 times (Figure E & F). Error bars represents ± standard error of the mean. Statistical significance was determined by one-way ANOVA. There was no significant difference in macrophage viability between the treatments with respect to the control at any time point.

3.2.5. Effect of LPS on NO production in J774 macrophages

Confluent macrophages were treated for 24 hours with various dilutions of LPS (1 μg ml^{-1}, 10 μg ml^{-1}, 20 μg ml^{-1}, 50 μg ml^{-1} and 100 μg ml^{-1}). Macrophages produced variable amount of nitrite and there was no significant difference between nitrite produced from macrophages incubated with various concentrations of LPS.

The amount of nitrite produced from macrophages treated with 1 μg ml^{-1}, 10 μg ml^{-1}, 20 μg ml^{-1}, 50 μg ml^{-1} and 100 μg ml^{-1} for 24 hours was 25 ± 4 μM, 32 ± 4 μM, 31± 4 μM, 32 ± 2 μM, 28 ± 2 μM respectively.

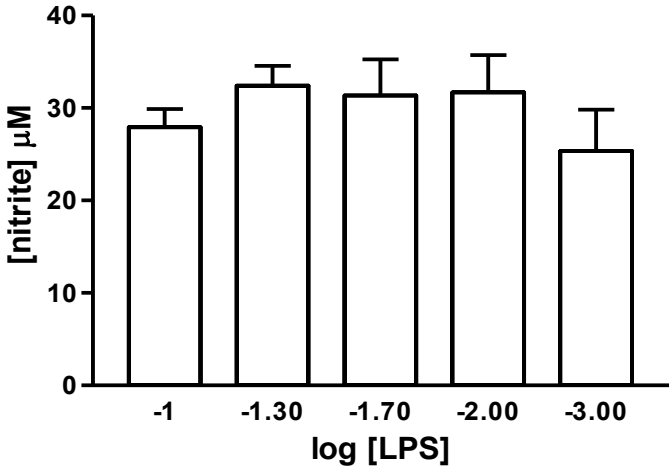


Figure 3.5: Nitric oxide production from macrophages treated with LPS. Various concentrations of LPS were used to treat confluent monolayers of J774 macrophage for 24 hours and NO production was measured by Griess assay. Data was from seven experiments (mean ± S.E.M) performed in triplicate. Statistical analysis was performed using One-way ANOVA. There was no significant difference in nitrite production between any of the LPS concentrations tested ($p < 0.05$).

3.2.6. Effect of LGG-CM on nitric oxide production in J774 macrophages

Confluent macrophages were treated for 24 hours with LGG-CM (pH 7.4 ± 0.2). Various dilutions of the LGG-CM (100 %, 75 %, 50 %, 25 % and 10 %) dose dependently increased a low level of NO production in J774 cells. The dose-response curve log mean effective dose (log EC_{50}) was -0.5417 ± 0.001. This lower EC_{50} value indicated the presence of active components even at the lower concentration of LGG-CM. The half maximal effective concentration (EC_{50}) for LGG-CM which induces a response halfway between the baseline and maximum NO production after 24 hours exposure period to macrophages is around 28.8 % LGG-CM. The maximal estimated nitrite production from undiluted LGG-CM at 24 hours was 2.5 ± 0.3 µM. Results indicate that 10 % LGG-CM treated macrophages significantly differed in nitrite production with respect to macrophages treated with a higher concentration of LGG-CM. There was no statistically significant difference in the nitrite production between macrophages treated with 25 % LGG-CM and higher concentrations of LGG-CM ($P > 0.05$). Figure 3.6 shows that the amount of nitrite produced from macrophages treated with 100 %, 75 %, 50 % and 25 % and 10 % LGG-CM at 24 hours was 2.5 ± 0.3 µM, 2.6 ± 0.5 µM, 2.5 ± 1.2 µM, 0.9 ± 0.3 µM, 0.2 ± 0.3 µM respectively. These results demonstrated the concentration dependent variation in the effect of LGG-CM on macrophage nitrite production.

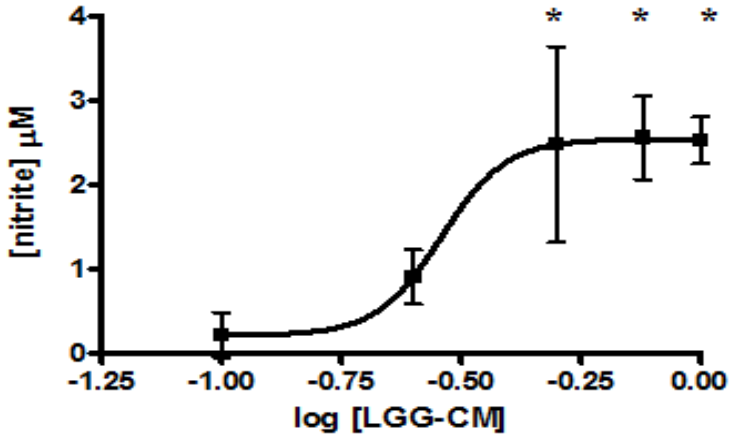


Figure 3.6: Basal NO production response to macrophages treated with LGG-CM. Various dilutions of LGG-CM were used to treat confluent monolayers of J774 macrophage for 24 hours and NO production was measured by Griess assay. Data was from seven experiments (mean ± SEM) performed in triplicate. The lower log EC_{50} value indicates the presence of strong active components in the LGG-CM which is effective even at a lower concentration. Statistical analysis was performed using one-way ANOVA followed by the Dunnett multiple comparison test. An asterisk indicates significant difference between nitrite production from macrophages treated with 10 % LGG-CM and macrophages treated with higher concentrations of LGG-CM (dilutions of 50 %, 75 % and 100 % LGG-CM).

3.2.7. Effect of LGG-CM on LPS induced NO production in J774 macrophages

The experiments were carried out to examine the effect of LGG-CM on LPS induced NO production in J774 macrophages. The macrophages were treated with a combination of various dilutions of LGG-CM (75 %, 50 %, 25 % and 10 %) and LPS (1 μg ml^{-1}). After a 24 hour incubation period, the nitrite accumulated in the spent culture medium from each well was measured by Griess assay as described in section 2.6.3. Different dilutions of LGG-CM inhibited the nitrite produced by LPS (1 μg ml^{-1}) in a concentration dependent manner.

Figure 3.7 shows that macrophages exposed to LGG-CM for 24 hours produced NO in a concentration dependent manner. The data from figure 3.5 and 3.6 is also plotted in figure 3.7 for comparison of NO production from LPS and LGG-CM. We observed that in the presence of the LGG-CM+LPS combination, the macrophages demonstrated a concentration dependent reduction of nitrite accumulation in the medium compared to LPS on its own. The concentration of LPS used for macrophage stimulation being constant, and increasing the concentration of LGG-CM in LGG-CM + LPS combination treatment, resulted in a decrease in the production of the nitrite end product (Fig. 3.7).

From the dose response curve, the amount of nitrite production from 20 μg ml^{-1} LPS was not significantly different from 1 μg ml^{-1} LPS. The amount of nitrite produced from 1 μg ml^{-1} LPS and 20 μg ml^{-1} LPS treated macrophages was 25 ± 4 μM and 31 ± 4 μM respectively (Figure 3.5). Therefore, in the LGG-CM+LPS combination treatment of macrophages 1 μg ml^{-1} LPS was used.

The amount of nitrite produced from the macrophages treated with 75 %, 50 %, 25 % and 10 % LGG-CM in the presence of LPS at 24 hours was 15 ± 3 μM, 17± 3 μM, 19 ± 3 μM, 23 ± 3 μM respectively. The LGG-CM at a higher concentration, therefore reduce the effect of LPS induced NO production from macrophages. This demonstrates the beneficial role of LGG-CM during inflammatory conditions as it prevents unnecessary host damage.

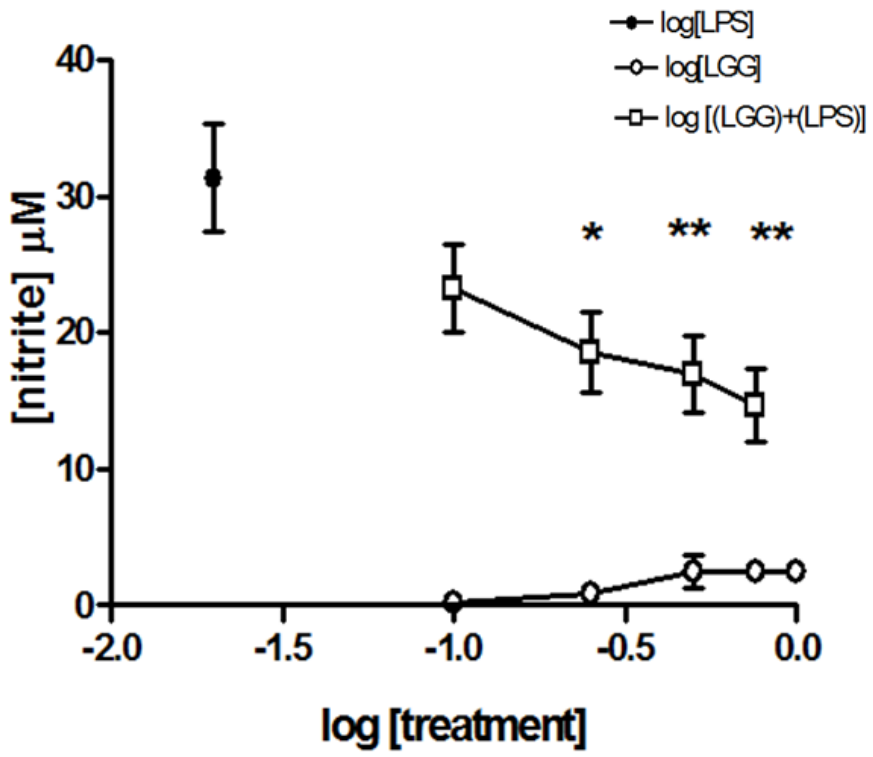


Figure 3.7: The effect of various concentrations of LGG-CM and LGG-CM + LPS combination on basal NO production from J774 macrophages. Macrophages were treated with different dilutions of LGG-CM both in the presence and absence of 1 µg ml^{-1} LPS. Figure 3.7 illustrates the nitrite production from macrophages stimulated with 20 µg ml^{-1} LPS on its own, various dilutions of LGG-CM on its own and in the presence of LPS (1 µg ml^{-1}). Accumulated nitrite was measured by Griess assay after 24 hours. Results are the mean of 7 separate experiments performed in triplicate. Error bars indicate ± standard error of the mean. Statistical analysis was performed using one-way ANOVA followed by Dunnett multiple comparison test. An asterisk indicates a significant difference between macrophages treated with various dilutions of LGG-CM + LPS combinations and LPS on its own (* $P<0.05$; ** $P<0.01$). A significant difference was observed in nitrite production between LPS and LGG-CM + LPS treated macrophages (dilutions of 75 %, 50 % and 25 % LGG-CM + LPS treatments).

3.3. Discussion:

3.3.1. Effect of LGG-CM on nitrite production by macrophages

Current knowledge on the inflammatory role of NO is poorly understood. In low or physiological concentration it may play a protective role by preventing host cellular damage by reducing inflammation (Alican & Kubes, 1996; Brown, 2003; Lundberg *et al*., 2004) increasing blood flow and also by stimulating mitochondrial biogenesis (Brown, 2003).

However, overproduction of NO during inflammation has been shown to cause cytotoxicity, especially if the inflammation becomes chronic (Brown, 2003). Increased iNOS expression and NO production are involved in inflammatory conditions such as IBD, asthma and rheumatoid arthritis (Alican & Kubes, 1996; ter Steege *et al*., 1997). Similar observations were also obtained from *in vitro* studies, where it was shown that up-regulation of NO biosynthesis is responsible for the increased epithelial cell lines permeability (Unno *et al*., 1995).

In this study, the production of NO by J774 macrophages following activation with LPS and LGG-CM was reflected by the accumulation of the nitrite end product in the culture medium. We found that the LGG-CM treated macrophages produced a low level and concentration dependant NO production at 24 hours. The LPS on its own treated macrophages produced 25 ± 4 μM NO. However, when LGG-CM was applied with LPS, the LGG-CM has dose dependently reduced the LPS induced NO production from J774 murine macrophages at 24 hours. These results were consistent from work by Lee *et al*. (2008), who demonstrated that supernatant prepared from several *Lactobacillus* and *Bifidobacterium* species demonstrated a decrease in LPS induced NO production in RAW 264.7 macrophages (Lee *et al*., 2008).

In our experiments monitoring nitrite production in the first 5 hours of macrophage incubation ($5x10^5$ cells ml^{-1}) with LGG-CM and LPS, it was not possible to detect nitrite accumulation. These results are consistent with previous studies by Weinberg *et al*., (1995) who demonstrated that the time course analysis of iNOS m-RNA expression showed a detectable expression of the iNOS protein only after 8 hours of stimulatory period in human blood monocytes and peritoneal macrophages (Weinberg *et al*., 1995).

3.3.2. LGG Growth curve and cell free-conditioned DMEM

The growth curves of LGG were established before the start of the experiment in order to correlate the optical density of the culture with the bacterial growth phase and pH. It was determined that the bacterial concentration of LGG, at the end of the log phase/ beginning of the stationary phase was approximately 2 x 10^9 CFU ml^{-1}. Studies by Pena and Versalovic have shown that the conditioned media prepared at these phases has most potent immunomodulatory activity (Pena and Versalovic, 2003).

Experiments were performed to investigate the effect of LGG-CM on the viability of *E. coli* HfrC (see section 3.2.3) and macrophages (see section 3.2.4). It was vital to confirm that LGG-CM is non-toxic to both the macrophage and the bacterial populations for the project. Otherwise, any reduction or increase in bacterial populations could imply that the bacteria being killed by LGG-CM or LGG-CM in itself, being toxic to macrophages. In the present study, antibacterial activity of LGG-CM was investigated by incubating the bacteria in various dilutions of LGG-CM and enumerating the bacterial number by serial dilution and plating. Macrophage viability in the presence of LGG-CM was assessed by MTT cell-proliferation assay. The LGG-CM was found to be non-toxic to both bacteria and J774 murine macrophages.

3.3.3. Effect of cell-free LGG-CM on bacterial viability

The *E. coli* viability assays in the presence of LGG-CM were performed with slight modifications as described previously (Coconnier *et al*., 1997; Keersmaecker *et al*., 2006). Briefly, colony count assays were performed by incubating bacteria with the different dilutions of cell free LGG conditioned DMEM (pH 7.4 ± 0.2) and the control media at 37^0C. At predetermined intervals, aliquots were removed, serially diluted and plated on nutrient agar plates to determine colony counts.

The antimicrobial activity of LGG-CM was examined at different time points (Figure 3.3). The *E. coli* viability remained unaffected at 1 hour and even after 6 hours of contact with LGG-CM (Fig 3.3A & 3.3B). The difference in results were statistically insignificant to demonstrate the antibacterial activity of LGG-CM, against bacterial strain *E. coli* HfrC ($p>0.05$) and no significant changes were observed after 24 hours treatments with the conditioned medium (Figure 3.3C).

Under *in vivo* conditions, several factors determine the efficiency of probiotics to demonstrate antibacterial activity including cell viability, cell concentration and the nature of the probiotic used (heat killed and live bacteria). In the *in vitro* experiments investigating the antibacterial activity of LGG-CM, factors such as the presence of organic acids and proteins demonstrating antibacterial activity determine the potency of cell-free LGG conditioned DMEM as an antibacterial agent (Servin, 2004).

Whole live LGG (Silva *et al*., 1987 & Ridwan *et al*., 2008) and the peptides secreted in the LGG culture media (Lu *et al*., 2009) have demonstrated antibacterial activity against a wide range of microbes. The effect of whole live LGG was suggested due to lactic acid produced by the bacteria. Lactic acid not only lowers the pH of the culture medium but also functions as a permeabilizer of the outer membrane of Gram negative bacteria (Servin 2004).

Bacteriocins are demonstrated as antibacterial and are secreted from a number of lactic acid bacteria (Messens *et al*., 2002; Neysens, *et al*., 2003, Servin 2004). The production of bacteriocins were growth associated, dependent on temperature, pH value, a complex nitrogen source and environmental conditions (Messens *et al*., 2002; Neysens, *et al*., 2003; Servin 2004). The maximum production of antimicrobial bacteriocins was found in the pH range of 4.0 and 6.0 (Messens *et al*., 2002).

Lu *et al*. (2009) demonstrated a dose-dependent antibacterial effect of LGG supernatant on *E. coli* growth. They identified novel proteins from probiotic supernatant namely, NPSRQERR and PDENK (molecular weight less than 1000 K Da) responsible for antibacterial activity. The LGG concentration used in their studies to prepare the LGG supernatant was in a range between 10^8 to 10^9 CFU ml^{-1}. They extracted and concentrated the active proteins in their experiments. Contrastingly, from literature, some studies have shown that, the active ingredient demonstrating antibacterial activity produced from LGG was a non-proteinaceous substance (Silva *et al*., 1987; Keersmaecker *et al*., 2006).

Studies by Sigrid *et al*., (2006) observed that LGG produces a pH dependent, heat-stable, non-proteinaceous, low molecular weight antimicrobial compound. They observed that when the probiotic supernatant was tested at a pH of 6.6, the antimicrobial activity was no longer present as it was reflected by the faster generation of *Salmonella* when compared to the *Salmonella* growth observed at pH 5. Our study supports the work of Keersmaecker *et*

al. (2006) where the antibacterial activity was not found from bacteria grown in LGG-CM at near neutral pH *(*7.4 ± 0.2).

To rule out the possibility that the antimicrobial activity of the LGG supernatant was only due to the low pH, they grew the bacteria in sterile MRS at pH 5. In this case, a shorter generation time (doubling time) and lag phase of *Salmonella* growth was observed as compared to LGG supernatant at pH 5.0. They attributed this property to the presence of undissociated organic acids in the LGG supernatant. The pH influences the ratio of dissociated to undissociated acid, as explained by the Henderson–Hasselbach equation. Although both forms can inhibit bacterial growth, the undissociated form of organic acids was reported to be further inhibitory per mole, than its corresponding dissociated form (Presser *et al*., 1997). This study found five compounds in the culture medium having antibacterial activity, which included an unidentified compound, acetic acid, pyroglutamic acid, formic acid and lactic acid. The first three compounds were identified by them even in sterile MRS medium. The formic acid concentration was not enough to bring antibacterial activity. They concluded that the observed antibacterial property was from lactic acid which is consistent with findings by Silva *et al*., 1987.

The role of pH in demonstrating the antibacterial activity of probiotics can also be explained by the findings of Ridwan *et al*. (2008) who demonstrated that the probiotic supernatant at pH 7 from multispecies probiotic ecologic 641 did not demonstrate any antibacterial activity. However, live bacteria demonstrated the bactericidal activity and therefore attributed the antibacterial property to organic acids produced.

However, not all the lactic acid producing probiotic bacteria demonstrate antibacterial activity (Asahara *et al*., 2001). Asahara *et al* found that *Lactobacillus* strains such as *L. fermentum* ATCC 14931^{T}, *L. jensenii* ATCC 25258^{T}, *L. plantarum* ATCC 14917^{T} and *L. reuteri* JCM 1112^{T} had no significant effect on antimicrobial activity against *Escherichia coli* in a murine urinary tract infection (UTI) model.

Another study by Roselli *et al*. (2006) found that *E. coli* K88 viability was unaffected by the presence of LGG or LGG supernatant, when 10^{8} CFU ml^{-1} *E. coli* were grown in DMEM with or without LGG (10^{9} and 10^{10} CFU ml^{-1}) or their equivalent spent culture supernatant fractions for 2 hours.

In our study investigating the effect of cell-free LGG-CM on macrophage bacterial interactions, it was important to investigate the antibacterial activity of LGG-CM at the same conditions as the gentamicin protection assay was performed. During gentamicin protection assay, the pH of cell free LGG-CM was adjusted to 7.4 ± 0.2 and the pH were adjusted in this range as this would be favourable for maintaining macrophage viability. However, most of the studies investigating for antibacterial activity have investigated at a far low pH.

We can therefore assume that, as the pH was not in the acidic range, the antibacterial activity of LGG-CM on *E. coli* was not observed. As determining the components or factors exhibiting antibacterial activity of cell-free LGG-CM was not the foremost interest or objective of this project, experiments were not oriented in a direction to change the pH of the conditioned medium or concentrate the LGG-CM and further investigate the effect of LGG-CM on bacterial viability.

3.3.4. Effect of LGG-CM on macrophage viability

From figure 3.4, LGG-CM did not demonstrate any effect on macrophage viability. The Gentamicin protection assay was performed for an approximate period of 6 hours. The LGG-CM and other treatments were non-toxic to macrophages during the first 6 hours of incubation. There was no evidence for any antiproliferative effects of LGG-CM on the macrophages within this time frame.

Experiments investigating for nitrite production by Griess assay were performed at a 24 hour incubation period in the presence of various dilutions of LGG-CM both in the presence and absence of 1 $\mu g\ ml^{-1}$ LPS. These experiments were performed in the absence of *E. coli*. The results from Figure 3.4 indicate that LGG-CM and other treatments do not demonstrate any toxic effects to macrophages even after 24 hour incubation period. There was no significant difference between the control and cells treated with LGG-CM and thus LGG-CM used at the concentration of 10 % LGG, 75 % LGG and 100 % LGG and their LPS combination are non-toxic and did not cause any significant cellular toxicity in this study.

3.4. Chapter Summary

The LGG-CM investigated at several dilutions (10 % LGG, 75 % LGG and 100 % LGG) were found to be non- toxic to both J774 macrophage cell line and *E. coli* HfrC. The LGG-CM produced a dual effect on NO production in J774 cells. The LGG-CM on its own produced a basal low level of NO production from the macrophages at 24 hours. The LPS by itself produced a high concentration of NO from the macrophages. However, when LGG-CM was co-applied with LPS, it produced a concentration dependent decrease in NO production from the macrophages when compared to LPS on its own treated macrophages. The basal low level of NO production to LGG-CM may contribute to regulate physiological processes in J774 macrophages to maintain a healthy environment. However, the property of LGG-CM to reduce the LPS induced excessive NO production is probably beneficial in inflammatory conditions, such as Ulcerative colitis and Crohn's disease, by cutting off excess NO production to the inflammatory mediator or bacterial infection and could reduce collateral tissue damage to the host.

CHAPTER 4

Effect of cell-free *Lactobacillus rhamnosus* GG conditioned medium on *E. coli* phagocytosis by macrophages

4.0. CHAPTER 4

4.1. Introduction

Macrophages function within the innate immune system by recognizing, internalizing, and destroying the invading microorganisms inside their phagosomes. Once formed, the intracellular phagosome undergoes maturation where a phagosome vacuole goes through a series of fission and fusion events, modifying both membrane and luminal contents (Huynh *et al*., 2007). The main changes include the modification of a phagosomes vacuole into an acidic environment and the production of hydrolytic enzymes and toxic free radical compounds (Fang, 2004; Cross and Segal, 2004; Yates *et al*., 2005). Additionally, phagosome maturation plays an important role in mounting a successful adaptive immune response by destroying microorganisms and presenting antigens to T-lymphocytes.

There is considerable interest in the role of probiotic bacteria in the modulation of immune functions (Round *et al*., 2010). Probiotics have long been known to contribute to the modulation of immune activity and play a vital role in maintaining overall intestinal health (Yan and Polk, 2012). Probiotic bacteria have also been suggested to activate the host innate immune function by influencing phagocytic activity of their immune cells including polymorphonuclear cells (Donnet-Hughes *et al*., 1999; Arunachalam *et al*., 2000; Sheih *et al*., 2001; Gill *et al*., 2001; Berman *et al*., 2006; Kotzamandis *et al*., 2010: Galdeano *et al*., 2011; Marranzino *et al*., 2012). Daily ingestion of probiotic supplements has been previously shown to enhance the innate immune function by significantly increasing the number of phagocytic monocytes and neutrophils and salivary immunoglobulin A levels in healthy non-elderly adults (Berman *et al*., 2006).

Current literature, on the role of probiotics into the phagocytic process by the phagocytic cells has mostly looked at the total number of phagosomes after a certain time period. Increased phagocytic ability might refer to either increased bacterial ingestion or increased bacterial killing. Very few works in the past have attempted to differentiate between ingestion and digestion phases of the phagocytic ability of macrophages, where, the phagocytosis assays focus on enumerating the recovery of ingested bacteria and killing assays determine the bacterial digestion by phagocytic cells (Hampton *et al*., 1994; Hampton and Winterbourn, 1999; Winterbourn, 2008).

The main aim of this chapter was to investigate the effect of LGG-CM on bacterial ingestion and digestion by macrophages. The cell free LGG-CM was used in this study

instead of whole live LGG. A challenge associated with stimulating macrophages with whole live LGG to measure phagocytosis is that the macrophages are equally as likely to ingest the live probiotic bacteria as they are the pathogen. As a result, the number of *E. coli* surviving inside the macrophages may well be lower, or the triggered immune response might be different, due to the co-ingestion of LGG. Thus, cell-free LGG-CM was employed as in previous studies from literature (Yan and Polk 2002, Vincenti, 2010).

4.2. Results

4.2.1. Effect of LGG-CM on *E. coli* ingestion by J774 macrophages

In these experiments macrophages and bacteria were co-incubated for 30 and 60 minutes at 37^0C in the presence of 20 μg ml^{-1} LPS, various dilutions of LGG-CM and also in combination of LGG-CM+LPS. There was no significant difference between the number of *E. coli* recovered after 30 minutes or at 60 minutes of ingestion period in control as well as from various treatments.

However, our results demonstrate that macrophage ingested significantly less bacteria in the presence of LGG-CM or LGG-CM+LPS combination treatments when compared to control at either 30 minutes or 60 minutes time points (Fig.4.1). The addition of LPS along with LGG-CM had no significant difference in bacterial ingestion compared to LGG-CM on its own.

Bacterial ingestion studies in presence of low and high LGG-CM were performed as 2 different sets of experiments (4.1A and 4.1 B as one set and 4.1C and 4.1D as another set). Bacterial recovery from low or high concentrations of LGG-CM treated macrophages were therefore compared to the bacterial recovery from the respective control macrophages.

From Figure 4.1A and 4.1B, the bacteria recovered (average $\log_{10}$ CFU ml^{-1}) from control macrophage, LPS, 10 % LGG and 10 % LGG-CM treated macrophages at 30 minutes (figure 4.1 A) and at 60 minutes (Fig. 4.1B) was 4.9±0.03, 4.81±0.03, 4.65±0.07, 4.69±0.04 and 4.92±0.04, 4.81±0.03, 4.66±0.07, 4.52±0.07 respectively.

From Figure 4.1C and 4.1D, the recovered bacteria (average $\log_{10}$ CFU ml^{-1}) from the untreated macrophages, LPS, 75 % LGG, 75 % LGG+LPS, 100 % LGG, 100% LGG-CM+LPS at 30 minutes and 60 minutes was 5.45±0.03, 5.23±0.02, 5.11±0.04, 5.09±0.02, 5.04±0.03, 4.87±0.12 and 5.49±0.04, 5.23±0.01, 5.1±0.03, 5.08±0.02, 5.05±0.02, 4.83±0.15 respectively.

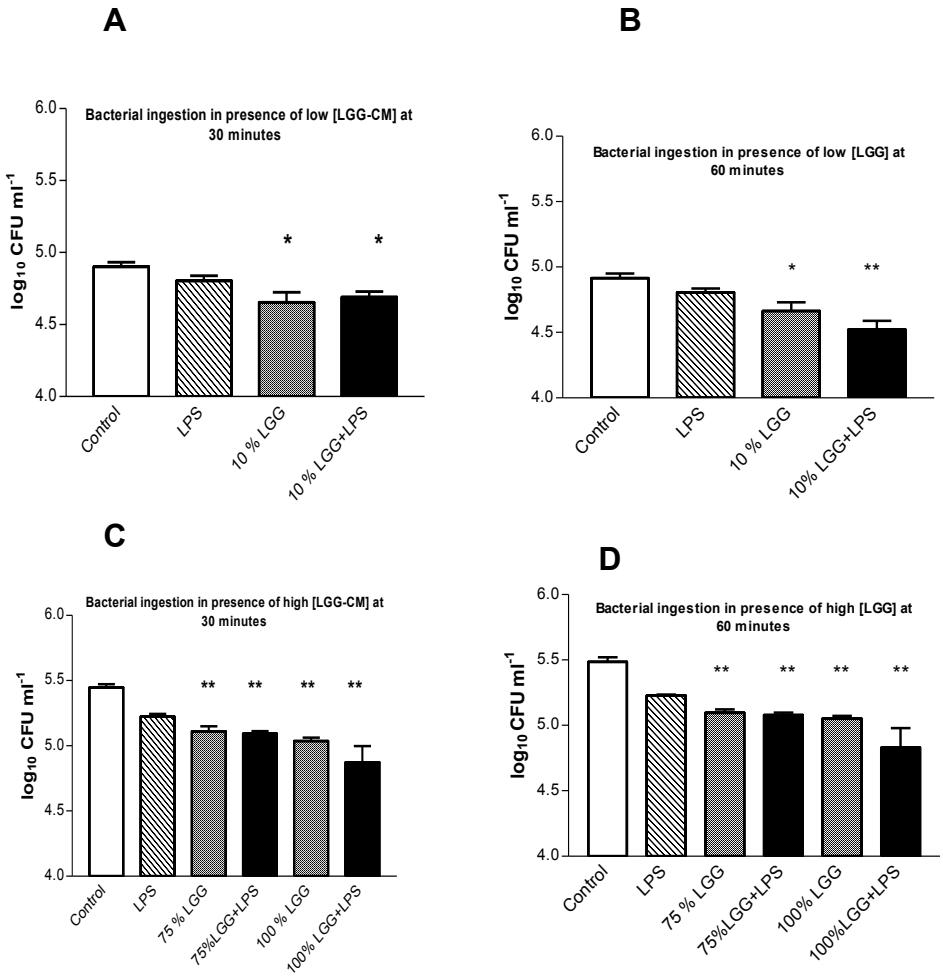


Figure 4.1: Bacterial ingestion in presence of LGG-CM and LPS. Results demonstrate *E. coli* recovered from macrophages expressed as $\log_{10}$ CFU ml^{-1} from various treatments. Figure A and B represents the bacterial recovery from control, LPS and 10 % LGG ± LPS at 30 and 60 minutes respectively. Figure C and D represents the bacterial recovery from control, LPS, 75 % LGG ± LPS and 100% LGG± LPS at 30 and 60 minutes respectively. Experiments were performed in duplicate and repeated 3 times. Statistical analysis was carried out using One way ANOVA followed by Dunnett's multiple comparison tests using Graphpad prism 3. Error bars represents ± standard error of the mean. Asterisks indicate significant difference to control (* $p< 0.05$; **$p<0.01$).

4.2.2. Monitoring the effect of cell free LGG-CM on bacterial ingestion by macrophages using acridine orange crystal violet staining.

To further investigate the above findings (Figure 4.1) produced by gentamicin protection assay that macrophages ingest less bacteria in the presence of LGG-CM, fluorescently labelled *E.coli* ingestion was monitored by using a fluorescence microscope (figure 4.2).

The fluorescently labelled *E.coli* was added to the confluent macrophages and incubated for 15, 30 and 60 minutes. After the respective time intervals, just before imaging 0.05 % crystal violet dissolved in PBS was added to the petridishes to quench extracellular fluorescence (see Chapter 2, Section 2.8.1).

In the pseudocoloured images viable ingested bacteria appeared green and any bacteria killed appeared red. It was possible to view that macrophages treated with LGG-CM had lower fluorescence intensity indicating the lower number of bacteria residing in the macrophages. We detected a negligible number of dead bacteria during this bacterial ingestion period (Figure 4.2).

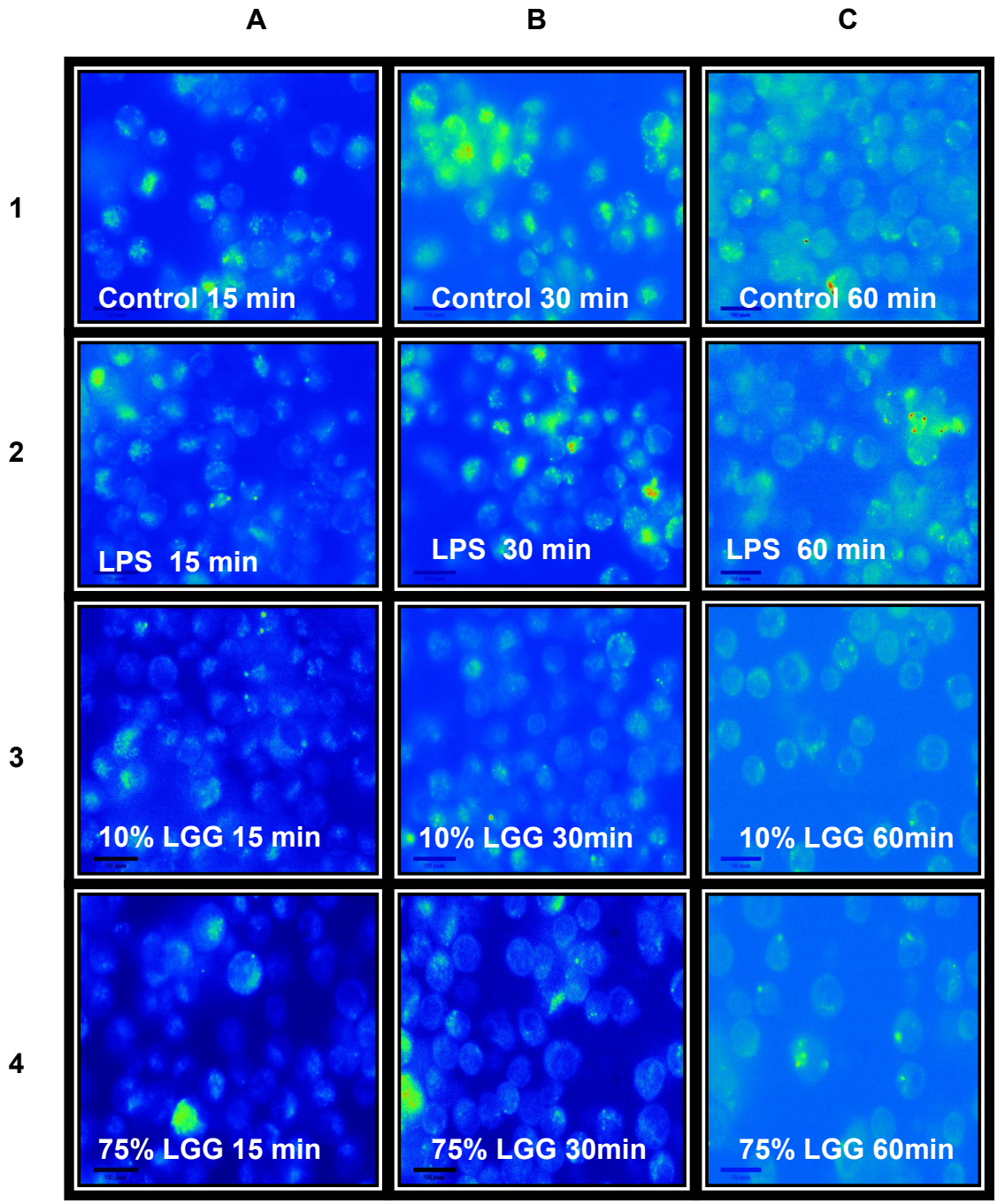


Figure 4.2: Monitoring bacterial ingestion in the presence of LGG-CM and LPS by acridine orange and crystal violet staining using a fluorescence microscope. Macrophages were coincubated with acridine orange labelled *E. coli* in control (row 1), 20 µg ml^{-1} LPS (row 2), 10 % LGG (row 3) and 75 % LGG (row 4). Images are representative of one of the three experiments performed. Vertical panels illustrate pseudo coloured fluorescence images grabbed at variable ingestion periods of 15, 30 and 60 minutes (columns A, B & C).

4.2.3. Bacterial digestion curves

To investigate the effect of LGG-CM on *E.coli* digestion by macrophages, bacterial recovery was monitored for set periods of time after a constant ingestion period of 60 minutes. At 60 minutes, gentamicin (200 µg ml^{-1}) was added to kill the remaining extracellular *E. coli* and intracellular *E. coli* was recovered within 15 minutes. The bacteria were recovered every 40 minutes up to 280 minutes.

The bacteria recovered at each time point (a) expressed as CFU ml^{-1} was divided by the bacteria recovered at the first time point (a_0) for all the treatments (see Figure 4.3). The results showed that in the control and as well as in all the treatments, there was a time dependant decline in the number of viable *E. coli* recovered from the macrophages over the period of 280 minutes.

In the presence of higher concentration of LGG-CM, there were reduced bacterial recovery compared to the control or 10 % LGG-CM treated macrophages. This reduction in bacterial number was pronounced during the first 100 minutes of the bacterial digestion period (see Figure 4.3). It is evident in Figure 4.3 that the bacterial killing by the macrophages was slower in 10 % LGG-CM treated macrophages. At the end of the digestion period (280 minutes), macrophages treated with LPS, 10 % LGG, 10 % LGG±LPS and also untreated macrophages had a greater number of undigested bacteria compared to macrophages treated with a high concentration of LGG-CM.

The time dependent bacterial recovery from control, LPS, 10 % LGG-CM, 10 % LGG-CM+LPS treated macrophages appears to be similar (see Figure 4.3 A, B, C & D). However, the bacterial recovery from the higher concentration of LGG-CM shows a sharp decline of bacterial number within the first 100 minutes of digestion period both in the presence and absence of LPS (see Figure 4.3 E, F, G & H).

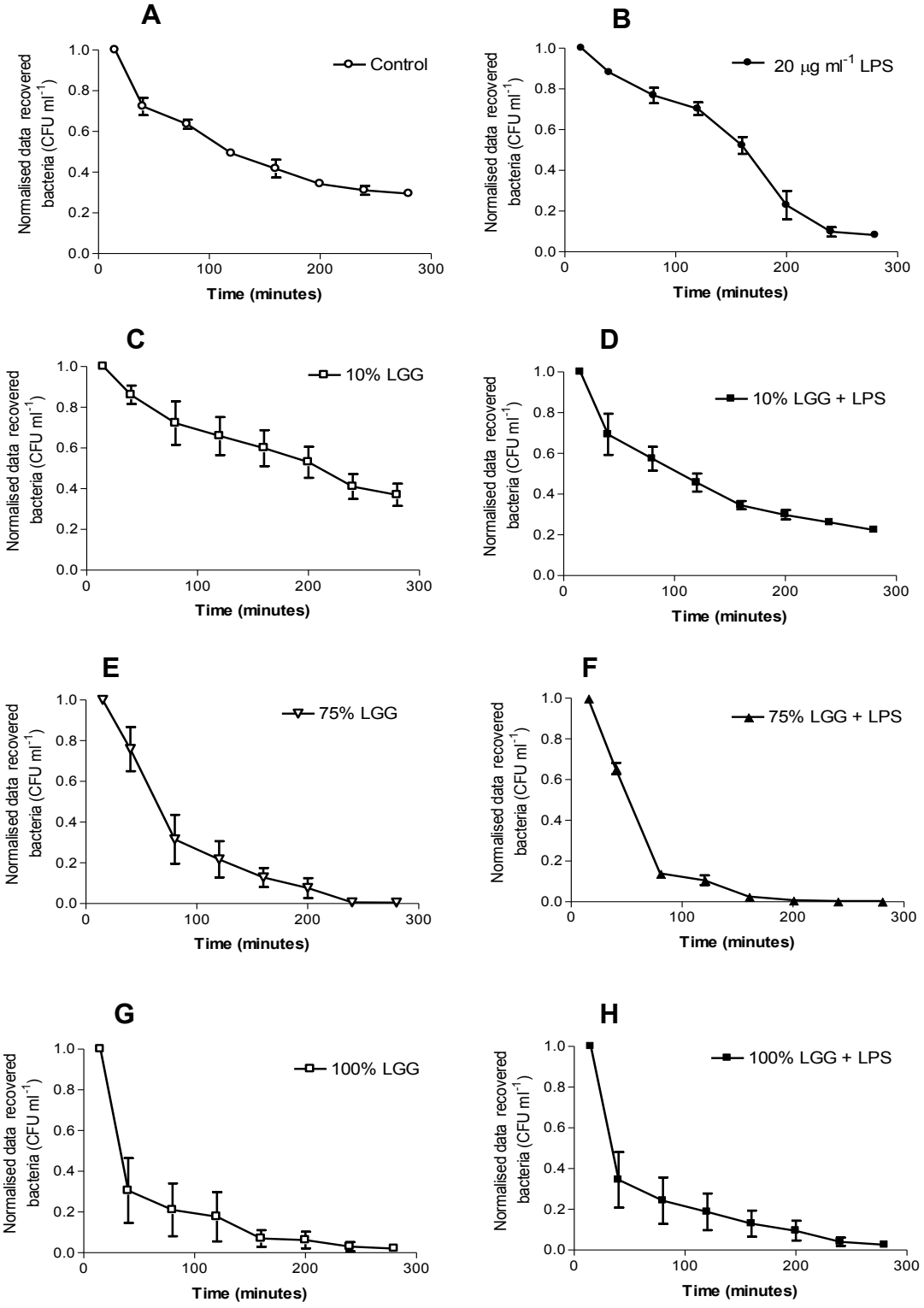


Figure 4.3.Recovered bacteria from macrophages during bacterial digestion period. The graphs represent the normalised data expressed as CFU ml^{-1}. Experiments were performed in duplicate and repeated at least three times. Error bars indicate ± standard error of the mean. Where the error bars are not seen they are obscured by the data.

4.2.4. Bacterial digestion rate by macrophages to LGG-CM treatment

The methodology for calculating the digestion rate from the recovered bacterial colonies is discussed in Chapter 2 Section 2.7.5. It was found that, the macrophages treated with high concentration of LGG-CM demonstrated a significantly increased rate of bacterial digestion compared to the control. However, a lower concentration of LGG-CM (10 % LGG-CM) did not show a significant difference in bacterial digestion rate when compared to the control.

The digestion rate (min^{-1}) of control, 20 μg ml^{-1} LPS, 10 % LGG-CM and 10 % LGG-CM+LPS treated macrophages was 0.004 ± 0.0004, 0.007 ± 0.001, 0.003±0.0005 and 0.005 ±0.0005.

The digestion rate of macrophages from 75 % LGG-CM, 75 % LGG-CM+LPS, and 100 % LGG-CM, 100 % LGG-CM + LPS treated macrophages was 0.022 ±0.0028, 0.025 ± 0.0029, 0.017 ±0.0047, and 0.018 ±0.0070 min^{-1}.

In figure 4.4, the digestion rate was non-significant between LPS and 10 % LGG ± LPS treated macrophages in comparison to the control macrophages. However, the higher concentration of LGG-CM (75 % LGG ± LPS and 100% LGG ± LPS) treated macrophages demonstrated a significantly enhanced bacterial digestion rate when compared to the macrophages treated with LPS. Similarly, the digestion rate of macrophages from 10 % LGG ± LPS treated macrophages were significantly different from the higher concentration of LGG-CM treated macrophages.

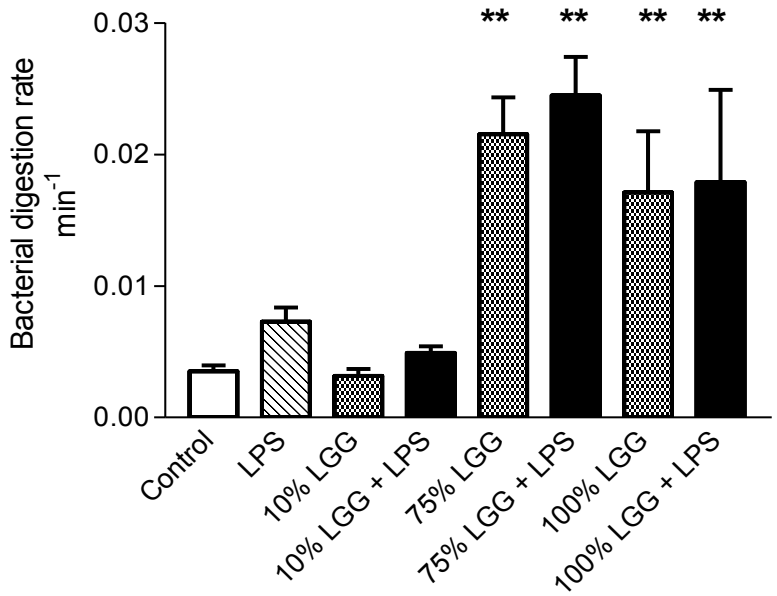


Figure 4.4: Macrophage digestion rate. The graph represents the bacterial digestion rate expressed as min^{-1}. Experiments were performed at least in duplicate and repeated at least three times. Error bars indicate ± standard error of the mean. Statistical analysis was carried out using Graph pad Prism 3. The Dunnett multiple comparison post-hoc test was performed after observing a significant difference from One way ANOVA. A ** ($p < 0.01$) indicates a significant difference in the bacterial digestion rate when compared to the control.

4.2.5. Monitoring the effect of cell free LGG-CM on bacterial digestion by macrophages by acridine orange staining.

It was found from experiments monitoring bacterial ingestion by acridine orange staining that LGG-CM treated macrophages ingested less bacteria (figure 4.2). To further monitor and image the fate of ingested bacteria in the presence of LGG-CM, bacterial cells were fluorescently labelled with acridine orange and bacterial digestion was monitored using a fluorescence microscope. Experiments were performed as described in Section 2.8.2 in Chapter 2.

In the pseudocoloured images viable ingested bacteria appeared green and any bacteria killed appeared red. In Figure 4.5, it was possible to view that, the macrophages treated with 75 % LGG-CM demonstrated a rapid bacterial killing which was evident by increased number of dead cells (appearing red) within the macrophages (see column 'C' in Fig. 4.5).

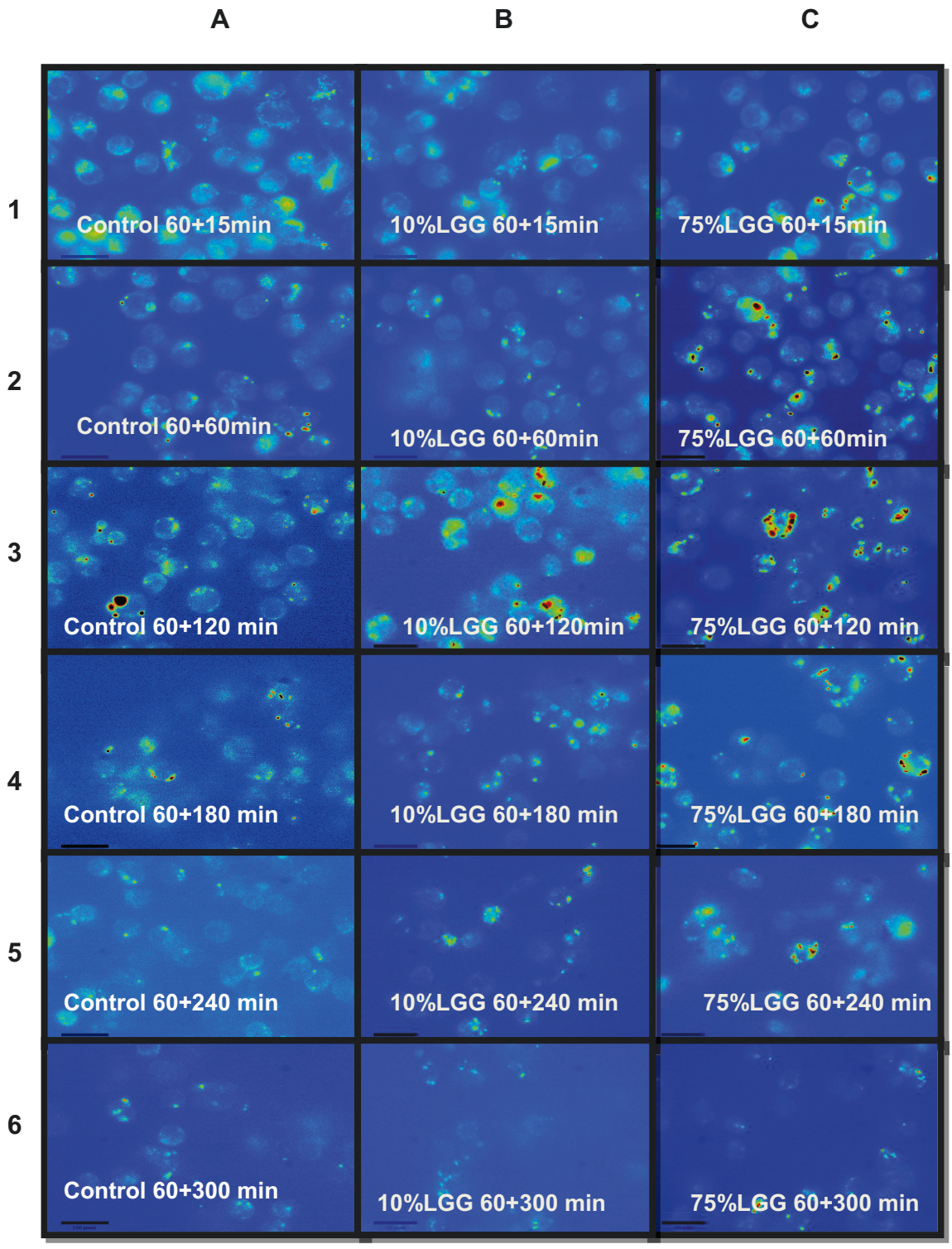


Figure 4.5: Monitoring bacterial digestion in the presence of LGG-CM by acridine orange and crystal violet staining. Images were grabbed during various time periods during bacterial digestion from the control, 10 % LGG & 75% LGG treated macrophages. Vertical panels illustrate pseudo coloured fluorescence images placed adjacently (column A, B & C). Images are representative of one of the three experiments performed. Images were grabbed at various time points during digestion period (row 1 to 6). Viable bacteria are shown in green and dead bacteria are shown in red.

4.3. Discussion

4.3.1. Reduction in bacterial ingestion by macrophages treated with LGG-CM

In this study, a cell-free extract of LGG-CM was used to ensure that only the effect belonging to products which are constitutively released from the LGG were investigated. Since macrophages were exposed to a model bacterium (*E. coli*), the use of LGG cells, rather than the cell free extract, would have complicated the interpretation of the number of bacteria ingested by the macrophages.

Microbes possess certain patterns on their cell membrane known as microbe associated molecular patterns (MAMP) which are recognised by certain pattern recognising receptors (PRR) present on immune cells such as macrophages. Toll like receptors (TLR) are a type of pattern recognition receptor that are broadly shared by microbes. Gram negative bacteria are generally recognised by TLR-4 and Gram positive bacteria are sensed by TLR-2 either as a dimer with TLR-1 or TLR-6 (Takeuchi and Akira, 2010).

Macrophages differentially regulate the phagocytosis of Gram positive and Gram negative bacteria. A study by Paul-Clark *et al*., (2006) investigated the phagolysosome activity of murine macrophages as a response to *Staphylococcus aureus* (Gram positive bacteria) and *E. coli* (Gram negative bacteria). They observed that Gram negative bacteria significantly increased the NO production mediated by TLR-4 at 24 hours. However, *S. aureus* produced a delayed (48-72 hours) NO production mediated by collaborative interactions between TLR-2, TLR-1 and TLR-6. The live LGG has previously been shown to enhance TLR-2 expression in human macrophages. This interaction is known to be essential for NFkβ activation (Miettinen *et al*., 2008), which regulates the induction of the iNOS gene (Aktan, 2004).

This study has analysed the interactions of murine macrophage J774 with *E. coli* HfrC in the presence of various concentrations of cell free LGG-CM. Gentamicin protection assay was used as a quantitative method to enumerate the viable bacterial recovery after the bacterial ingestion period and also during different digestion periods by the macrophages. The internalised bacteria were released by lysing the macrophages and enumerated them after serial dilution and plating. The viable bacteria recovered after 15 minutes of ingestion was very low for the controls as well as for the treatments. However, after a 30 minute

ingestion period, the recovered intracellular bacterial number was found to be a premium level and there was no further ingestion of *E. coli* at 60 minutes of incubation (Figure 4.1). The results above were further supported by imaging the bacterial ingestion using the Acridine-orange crystal violet staining technique. In the images, it could be observed that nearly all intracellular bacteria appeared viable at 30 minutes and 60 minutes and the *E. coli* were unevenly distributed in the macrophages (Figure 4.2). Clumped bacteria in the phagosomes or bacterial spots in the cytosol was found to be difficult to enumerate since the images were captured at 600X magnification and this resolution was found to be insufficient for manual quantification. Thus acridine orange and crystal violet staining was used only as a semi-quantitative approach.

At both 30 minutes and 60 minutes, the macrophages treated with LGG-CM ingested significantly less bacteria. A similar effect was also observed by Vincenti (2010), who investigated phagocytosis of *E. coli* HfrC by a J774 murine macrophage cell line in the presence of cell-free LGG-CM. His study found that LGG-CM treated macrophages recovered significantly less bacteria compared to untreated cells at both 30 and 60 minutes. He suggested that this reduced recovery of bacteria from macrophages as a response to LGG-CM was due to the enhanced bacterial digestion within 60 minutes of incubation.

Our findings indicated that macrophages as a response to LGG-CM ingested significantly less bacteria. Given, that the significant killing in this period (within 60 minutes) is not apparent, as shown by fluorescence images by acridine orange-crystal violet staining in Figure 4.2, it could be interpreted that probiotics interfere or suppress the ingestion of *E. coli* by macrophages.

Campbell *et al*. (2001) had described that 30 minutes is the minimum time required before any significant killing of the bacteria occur. Bacterial killing usually occur in the later stages of phagosomes maturation, when the phagosomes reach the final maturation stage to become phagolysosome. At this stage phagolysosome gain a range of weapons for bacterial destruction such as antimicrobial peptides and free radicals (Flannagan *et al*., 2009). The process of bacterial killing in the phagolysosome requires from several minutes to few hours depending upon the bacterial strain (Nusse, 2011).

It is important to consider the effect a bacterial population, or the phagocyte concentration, can have on bacterial recovery results. The bacteria's ability to survive in a macrophage

intracellular environment also determines the final recovery and multiplicity of infection, as some pathogenic bacteria might induce macrophage cell death (Hamrick *et al*., 2000). Without the ability of *E. coli* HfrC to survive within a macrophage intracellular environment, and extracellular bacteria being killed by gentamicin, there is a negligible chance of *E. coli* being multiplied or increased during a short interval period of bacterial ingestion in the control group. The first assumption of LGG-CM being an antibacterial agent was negated by a non-significant difference in the bacterial recovery between bacteria grown in the presence and absence of LGG-CM (Figure 3.3).

The killing of bacteria in steadily maturing phagosomes could have an impact on viable cell recovery. Thus, in our experiments, while investigating bacterial ingestion for an extra time point at 60 minutes was also investigated. The *E. coli* recovered from the control and LGG-CM treated macrophages at 60 minutes was nearly identical to the number of *E. coli* recovered at 30 min suggesting no bacterial killing occurs within this time frame (Figure 4.1).

It is well recognised that LPS stimulates the macrophages to induce an iNOS expression through the NFκβ pathway which is further responsible for NO production (Raetz *et al*., 1991; Alderton *et al*., 2005; Reddy & Reddanna, 2009). Thus, 20 μg ml^{-1} LPS were used as a positive control and from previous studies and at the concentration utilised LPS was supposed to illicit an immediate effect (Vincenti., 2010; Hatcher and Lambrecht, 1993). In our studies macrophage treatment with 20 μg ml^{-1} LPS resulted in the reduced ingestion of bacteria but the reduction was not significantly different compared to the control macrophages.

There are studies which have shown that LPS is ingested by phagocytes as a phagocytic target and that they induce the signal mediated by the TLR-4 receptor. When bacteria containing a LPS moiety in their outer cell wall are incubated along with LPS on its own with the macrophages, LPS might potentially act as an alternative target for the macrophages as they share the common recognising receptor TLR-4 (Hornef *et al*., 2003; Dunzendorfer *et al*., 2004).

Studies by Sivagnanam *et al*. (2010) used a quantitative real time PCR to investigate the regulation of 13 mRNA sequences involved in various inflammatory responses in rat microglial cells as a response to *E. coli* and LPS treatments. Their results showed that *E.*

coli and LPS stimulated different cytokines production separately. However, when *E. coli* and LPS was applied in combination, 12 of the13 genes monitored were expressed at the same level as compared to *E. coli* alone as a sole immunomostimulator. As LPS alone did not demonstrate the immunomodulatory effect when in LPS and *E. coli* combination, Sivagnanam *et al*. (2010) concluded that the ingestion of *E. coli* dominates over LPS, during macrophage co-stimulation. It is, therefore, possible that in our studies, the lower recovery from LPS treated macrophages might be due to shared TLR-4 receptor occupancy by both LPS and live *E. coli*.

In our study we observed a significant reduction in bacterial ingestion in the presence of LGG-CM irrespective of the concentration tested. However, studies from Kim *et al*., (2006a), demonstrated significantly enhanced phagocytosis of fluorescent microparticles by peritoneal macrophages from mice orally administered with heat killed whole probiotic *Lactococcus lactis* ssp *lactis* when compared to groups administered with the PBS, cell wall or cytoplasmic fraction. The microparticles incorporated into the cells were measured by flow cytometry. However it has to be noted that, in our phagocytosis studies macrophages were challenged with live *E. coli* cells in the presence of cell free LGG-CM which might have a different effect on the macrophage ingestion process.

Earlier researches have not focussed much on the effect of LGG-CM on bacterial ingestion and digestion as two separate physical entities. The term phagocytosis assay is slightly misapprehended as this might be either the combined uptake and killing assays or only one of them. This study has, therefore, investigated the effect of LGG-CM on bacterial ingestion and digestion as two separate experimental set ups using murine macrophage J774 and a model bacterium *E. coli*. In our study we have explored phagocytosis further as ingestion and digestion phases. Our study protocol supports the work by Zhou and Kobzik. (2007) who had investigated the effect of concentrated ambient particles on uptake and digestion of *Streptococcus pneumonia* by J774 macrophage as two separate studies. The comparison of literature reporting various parameters of gentamicin protection assay has been shown in Table 4.1.

Princivalli *et al*. (2009) tested live LGG and LGG supernatant for their ability to prevent group A streptococci (GAS) infections. They found that both live LGG and LGG supernatant significantly prevented GAS infecting the human respiratory cell line both in competition and displacement assays. The mechanism described for the reduced bacteria in

respiratory cells was mediated through the cell surface proteins expressed on live LGG, and also present in LGG supernatant which competes for the same Fn-receptor necessary for GAS binding in respiratory cells.

It is unlikely that organic acids released by LGG (Keersmaecker *et al*., 2006) might play a role in the modulation of bacterial ingestion by macrophage as the pH of the LGG-CM used in the study was set to neutral (pH 7). The LGG is known to express cell surface proteins which are present both on the live bacterial surface and also secreted actively in the supernatant (Princivalli *et al*., 2009). The first possible mechanism of observed reduction in bacterial ingestion in presence of LGG-CM might be due to these secreted bacterial proteins being present in the cell free conditioned medium which might be actively blocking the receptors either through stearic hindrance or through specific receptor–target interactions (Bernet Camard *et al*., 1997; Coconnier *et al*., 1997; Princivalli *et al*., 2009).

The second possible mechanism might be due to the regulation of immune responses and downstream signalling pathways, such as free radicals production, which are known to regulate the process of chemotaxis by directly modulating actin glutathionylation and polymerization (Sakai *et al*., 2012).

4.3.2. LGG-CM increases digestion rate of macrophages

Experiments investigating the bacterial digestion by gentamicin protection assay were performed for up to 280 minutes post ingestion (see Figure 4.3). The duration of monitoring bacterial digestion is in agreement with previous literature (Levitz *et al*., 1986; Michaliszyn *et al*., 1995; Marr *et al*., 2001; Philippe *et al*., 2003). It is well documented that macrophages undergo apoptosis approximately 8 to 16 hours after bacterial ingestion. This is clearly after the intracellular destruction of most of the ingested bacteria approximately 4 hours post ingestion period (Baran *et al*., 1996; Hamrick *et al*., 2000; Hacker *et al*., 2002; Kirschnek *et al*., 2005). So, in our studies approximately 4 hours time period of monitoring bacterial digestion was sufficient to determine the rate of bacterial digestion.

Bacterial digestion in macrophages, as a response to various treatments and in control cells, was also investigated by acridine orange crystal violet staining technique (see section

2.8.2 in Chapter 2). Acridine orange staining has previously been used in study by Takao *et al*., (1996) to monitor bacterial phagocytosis. The *E.coli* was labelled with acridine orange and fluorescence measurements were made every hour for up to 300 minutes.

The J774 macrophages were quite efficient at taking up the bacteria that were visible as fluorescent spots inside the cells. On pseudo-colouring the grey scale images, viable bacteria appeared green and any bacteria killed appeared red. After approximately one hour of bacterial ingestion, the bacteria were largely localised in cellular compartments around the nucleus. It was possible to see that the macrophages treated with 75 % LGG-CM demonstrated a rapid rate of bacterial killing which was evident by the increased number of dead cells (appearing red) from the macrophages treated with a higher concentration of LGG-CM (see Figure 4.5).

Experiments investigating the effect of LGG-CM on bacterial digestion were performed at a multiplicity of infection of 50:1. To avoid under evaluation of the killing rate as determined independently from the rate of phagocytosis, the number of extracellular bacteria present during the killing assay must be sufficiently low; otherwise the macrophages will continue to phagocytise bacteria during the assay. At these bacteria to macrophage ratio, the plating of extracellular media did not reveal a presence of extracellular bacteria. From these findings it may be concluded that, the results of the bacterial digestion studies which measure the decrease in the number of viable bacteria during the incubation of the macrophages containing ingested bacteria, represent intracellular bacterial killing.

The rate of decrease in the total number of bacteria within the macrophages is a measure of the rate of intracellular killing. In figure 4.4 the bacterial recovery data during different digestion period were normalised and digestion rate was calculated (see chapter 2, Section 2.7.5). As the comparison for bacterial digestion rate between treatments was performed by calculating the rate by dividing recovered bacterial number at various time points by the initial recovered bacterial number (a/a_0), the increased bacterial digestion rate observed from the LGG-CM treated macrophages was due to rapid bacterial killing.

The LPS and 10% LGG-CM treated macrophages ingested less bacteria compared to control, however they did not have any effect on the bacterial digestion rate by the macrophages. The rate of bacterial killing in the untreated cells were significantly low ($p<0.01$) compared to macrophages treated with 75% or 100% LGG-CM. The bacterial

digestion rate from 10 % LGG-CM was significantly low when compared to 75 % LGG-CM or 100 % LGG-CM treated macrophages. The rate of bacterial digestion from the macrophages treated with 75 % LGG-CM and 100 % LGG-CM was 0.022 ± 0.003 and 0.017 ± 0.005 respectively. This observed rate was significantly different from the rate of bacterial digestion from control macrophages, which was 0.004 ± 0.0004. The rate of bacterial digestion from both LPS (0.007 ± 0.001) and 10 % LGG-CM (0.003 ± 0.001) treated macrophages were non-significant from the control (Figure 4.4). The possible mechanisms that a higher concentration of LGG-CM induced an increased bacterial digestion rate are investigated in Chapter 5.

In this study, the rate of intracellular killing is calculated by fitting an exponential curve to the bacterial digestion data (see Section 2.7.5). The trendline plotted from the recovered bacteria versus time showed an exponential trend in bacterial killing. Similar exponential decrease in bacterial number was also found by Van Dissel *et al*., (1985). They found that opsonised *Salmonella* enteric serovar *Typhimurium* underwent an exponential decrease after being taken up by murine macrophages over a 90 minute period of their assay. In contrast Hamrick *et al*., (2000) showed that the digestion of *E. coli* by murine macrophage follows a parabolic shape, started with an initial exponential pattern in bacterial killing followed by a slight upward slope, as time progressed indicating the bacterial number increasing slightly. They concluded that the parabolic curve might be due to inefficiency in extracellular bacterial killing by low concentration of gentamicin (5 $\mu g\ ml^{-1}$).

It's quite irrational to compare the rates from previous studies as the variation is expected due to the difference in assay conditions and the use of different macrophages and bacterial strains. The comparison of the bacterial digestion rate from previous studies has been made irrespective of the variability in experimental conditions and is listed in Table 4.2. In summary, and to the best of our knowledge, this is the first study investigating the effect of LGG-CM on bacterial digestion rate and these findings can be used as a comparison on the effect of various probiotics on bacterial digestion rate in future.

4.4. Chapter Summary

Macrophages treated with LGG-CM ingest significantly less bacteria during both 30 minutes and 60 minutes of macrophage-bacterial coincubation irrespective of the concentration tested. However, an increased bacterial digestion rate was observed only from macrophages treated with a higher concentration of LGG-CM.

Table 4.1: Comparison of literature reporting various parameters of GPA. [*: Strains which are pathogenic and exhibit characters like invasiveness and are capable of surviving in macrophages.] Colony forming units (CFU); Multiplicity of infection (MOI); Not defined (ND)

Study reference	Macrophage origin	Bacterial strain	Initial inoculums CFU ml^{-1}	MOI	Incubation period [minutes]	Recovery CFU ml^{-1}	Bacteria Per macrophage
This study	Murine	*E. coli* HfrC	$2.5x10^7$	50:1	30	$2.8x10^5$	0.56
This study	Murine	*E. coli* HfrC	$2.5x10^7$	50:1	60	$3.0x10^5$	0.62
Buchmeier and Heffron, (1989)	Murine	*Salmonella typhimurium* *	Undefined	10-20:1	0,60,240, 1440	Undefined	Undefined
Boyce and Adler (2000)	Murine	*Pasteurella multocida* M1404	$1.0x10^7$	Undefined	30	$1.8x10^3$	Undefined
		Pasteurella multocida PBA875	$1.0x10^7$	Undefined	30	$7.1x10^3$	Undefined
		Pasteurella multocida PBA1514	$1.0x10^7$	Undefined	30	$1.2x10^3$	Undefined
Hatcher and Lambrecht, (1993)	Murine	*Salmonella typhimurium* *	$1x10^6$	20:1	120	Undefined	Undefined
Wells *et al.* (1990)	Murine	*Proteus mirabilis* M13	$8 X 10^7$	100:1	3 *(in vivo)*	Undefined	0.03
		Proteus mirabilis H1	$8 X 10^7$	100:1	3 *(in vivo)*	Undefined	0.02
		Escherichia coli M14	$8 X 10^7$	100:1	3 *(in vivo)*	Undefined	0.48
		Escherichia coli A-D M5	$8 X 10^7$	100:1	3 *(in vivo)*	Undefined	0.45
		Escherichia coli H40	$8 X 10^7$	100:1	3 *(in vivo)*	Undefined	0.28
Nagl *et al*. (2002)	Human	*Staphylococcus aureus* ATCC25923	$\approx 6 X 10^5$	10:1	30	Undefined	21.90
		Escherichia coli ATCC 11129	$\approx 2X10^5$	10:1	30	Undefined	19.00

Poermadjaja and Frost, (2000)	Poultry	*Pasteurella multocida* VP161	10^7	100:1	30	5 X 10^5	1.00
		Pasteurella multocida VP138	10^7	100:1	30	6 X 10^5	1.2
		Pasteurella multocida VP17 *	10^7	100:1	30	9 X 10^5	1.8
		Pasteurella multocida VP21 *	10^7	100:1	30	8 X 10^5	1.6
		Pasteurella multocida VP161	10^7	100:1	60	6 X 10^5	1.2
		Pasteurella multocida VP138	10^7	100:1	60	Undefined	Undefined
		Pasteurella multocida VP17	10^7	100:1	60	Undefined	Undefined
		Pasteurella multocida VP21	10^7	100:1	60	Undefined	Undefined
De Koning - Ward *et al*. (1998)	Murine	*Yersinia enterocolitica* JP273v	2 X 10^7	10:1	30	6 X 10^3	0.003
		Yersinia enterocolitica 8081c *	2 X 10^7	10:1	30	2.6 X 10^5	0.13
		Escherichia coli HB101	2 X 10^7	10:1	30	2.0 X 10^4	0.010
		Escherichia coli HB101(pVM101)*	2 X 10^7	10:1	30	3.3 X 10^5	0.17
Fernandez Prada, (1997)	Murine	*Shigella flexneri* M90T-W, M90T-A3, SC403, 2457-T, M4243A, M90T-55, BS103	ND	3-30:1	30	Undefined	Undefined
Tabrizi and Robins –Browne, (1993)	Murine	*Escherichia coli* HB101	$5x10^6$	5:1	30	Undefined	Undefined
		Yersinia enterocolitica 8081c *	ND	5:1	30	Undefined	Undefined
Laroux *et al*. (2005)	Murine	*Escherichia coli* (F18)	$1x10^7$	10:1	120	≈$4.5x10^4$	Undefined

Larsen & Boesen, (2001)	Fish	*Vibrio anguillarum*	$1.75x10^{9}$	50:1	180	$1.3x10^{3}$	Undefined
		Vibrio anguillarum	$1.75x10^{8}$	5:1	180	$0.026x10^{3}$	Undefined
Vincenti, (2010)	Murine	*E. coli HfrC*	$5x10^{7}$	100:1	15	$3.0x10^{3}$	0.005
		E. coli HfrC	$5x10^{7}$	100:1	30	$3.3x10^{6}$	6.40
		E. coli HfrC	$5x10^{7}$	100:1	60	$3.3x10^{6}$	6.40
Sjölinder *et al*. (2008)	Murine	*Neisseria meningitidis* FAM20 nhhA	$5x10^{8}$	100:1	180	Undefined	Undefined
		Neisseria meningitidis FAM20	$5x10^{8}$	100:1	180	Undefined	Undefined
Gille *et al*. (2009)	Human	*E.coli* DH5α*	$5x10^{7}$	50:1	180	Undefined	0.4
		Group B *Streptococci* BSU98*	$5x10^{7}$	50:1	180	Undefined	0.31

Table 4.2: Rate of intracellular killing of bacteria by phagocytes. All values are means of rates of the bacterial killing with their standard deviation. A * indicates rate ± SEM, otherwise it is standard deviation. All experiments are performed under *in vitro* conditions.

Bacterial strain	**Mouse strain/ macrophage cell line**	**Rate constant of bacterial killing K_k (min-1)**	**Reference**
E. coli HfrC	J774	0.004 ± 0.0004 *	This study
E.coli M14	Swiss Webster mice	0.017 ± 0.006 *	Wells *et al*., (1990)
Proteus mirabilis M13		0.020 ± 0.003 *	
E. coli A-D M5		0.016 ± 0.003 *	
E. coli H40		0.026 ± 0.009 *	
Proteus mirabilis H1		0.029 ± 0.003 *	
Salmonella typhimurium	Swiss	0.022 ± 0.005	van Dissel *et al*., (1985)
	CBA	0.020 ± 0.007	
	C57BL/10	0.012 ± 0.003	
E. coli O54	CBA	0.039	van Dissel *et al*., (1987a)
E. coli O54	C57BL/10	0.036	
E. coli O2K1	CBA	0.063	
E. coli O2K1	C57BL/10	0.060	
Salmonella typhimurium	Peritoneal macrophages	0.035 ± 0.015	Langermans *et al*., (1990)
Listeria monocytogenes		0.026 ± 0.009	
Listeria monocytogenes	CBA	0.021 ± 0.004	van Dissel *et al*., (1987b)
	C57BL/10	0.022 ± 0.005	
Salmonella typhimurium	CBA	0.036 ± 0.008	
	C57BL/10	0.019 ± 0.009	
Staphylococcus aureus	CBA	0.025 ± 0.006	
	C57BL/10	0.023 ± 0.004	
E. coli 054	CBA	0.031 ± 0.007	
	C57BL/10	0.033 ± 0.008	
Salmonella typhimurium	Granulocytes of CBA mice	0.017 ± 0.006	van Dissel *et al*., (1986)
	Granulocytes of C57BL/10 mice	0.007 ± 0.004	
Listeria monocytogenes	Granulocytes of CBA mice	0.012 ± 0.003	
	Granulocytes of C57BL/10 mice	0.013 ± 0.002	
Staphylococcus aureus	Human granulocytes	0.026	Leigh *et al*., (1980)
E. coli (O54)		0.036	
Staphylococcus aureus	Human neutrophils	0.11 ± 0.02	Hampton *et al*., (1994)
E. coli ATCC 25922		0.36 ± 0.06	

Chapter 5

Mechanisms for *Lactobacillus rhamnosus GG* conditioned medium induced enhanced macrophage phagocytosis

5.0 CHAPTER 5

5.1. Introduction

In chapter 4 it has been evident from both the GPA and fluorescent data (acridine orange – crystal violet staining) that LGG-CM plays a role in the activation of macrophage enhancing *E.coli* digestion. In the current chapter we will investigate the possible physiological mechanisms which are activated in macrophages to LGG-CM treatment to enhance the bacterial digestion process.

Studies have demonstrated that both ROS and RNS play an important role in digesting the trapped bacteria in the macrophage phagosomes. This digestion process involves oxidizing and damaging bacterial cell wall proteins and DNA, resulting in bacterial killing. Both NO and ROS combine to form a potent oxidant peroxynitrite (ONOO-) which has been demonstrated to contribute into the bacterial killing process by macrophages (Beckman *et al*., 1992; Ischiropoulos *et al*., 1992; Evans *et al*., 1996).

There is evidence that NO and ROS can also work independently in bacterial killing process (Nathan and Shiloh, 2000; Fang 2004; Flannagan *et al*., 2009; Qiu *et al*., 2009). Other free radicals independent mechanisms such as defensins, lysozymes, antimicrobial peptides including lactoferrin and condition like reduced pH (approximately 5.5 or lower) in the phagosome are also suggested to contribute in bacterial killing process by macrophages (Flannagan *et al*., 2009; Slauch, 2011).

The LGG has shown that it can induce rapid ROS generation in murine intestinal epithelial cells (Lin *et al*., 2009), human (Caco-2, T-84 and HeLa) and rat (IEC-6) intestinal epithelial cell lines (Kumar *et al*., 2007). Farnell *et al*. (2006) demonstrated an increased ROS production from chicken heterophils when they were treated with heat killed *Bacillus subtilis*, *Lactococcus Lactis* and *Lactobacillus acidophilus* bacteria. Studies by Ichikawa *et al*. (2012) have shown an increased production of reactive oxygen metabolites from murine peritoneal macrophages fed with heat killed LGG. The induction of ROS from macrophages have been suggested as beneficial actions of probiotics as ROS is involved in the killing of invading microorganisms (Farnell *et al*., 2006; Flannagan *et al*., 2009).

Probiotic mediated NO production has also been shown in murine macrophage cell line J774 and in human epithelial cell line –T84 as a response to live LGG (Korhonen *et al*.,

2001) and in intestinal pig epithelial cell line (IPEC-J2) by several lactobacillus strains (Pipenbaher *et al*., 2009). However, there are studies which have demonstrated that probiotic bacteria such as VSL-3 demonstrate the anti-inflammatory response by reducing the expression of the iNOS enzyme from *in vivo* studies (Esposito *et al*., 2009). Studies have been shown to reduce nitrite production (a stable end product of NO) from mouse fed with *Lactobacillus acidophilus* cell-free supernatant (Rishi *et al*., 2009). Probiotics are previously also known to attenuate proinflammatory TNF-α which has known to have a profound effect on NO production in intestinal epithelial cells (Madsen *et al*., 2001; Gopal *et al*., 2001).

Current literature are in agreement with the hypothesis that probiotic bacteria enhances the phagocytic activity of macrophage by activating oxidative burst resulting in the increased killing of pathogens (Schiffrin *et al*.,1995, Donnet –Hughes *et al*., 1999, Farnell *et al*., 2006; Ichikawa *et al*., 2012; Marranzino *et al*., 2012). However, the mechanisms accounting for the observed beneficial effects remain to be fully elucidated.

The traditional pathways of free radical production are mainly observed through the NADPH oxidase (first few hours of infection) and the iNOS (later stages of infection). Xanthine oxidase is another source of ROS production (Crane, 2013). However, there are controversial reports where one suggests that peritoneal macrophages possess scant amount of xanthine oxidase (Jarasch *et al*., 1986) while the other demonstrates a substantial amount of xanthine oxidase in murine peritoneal macrophages (Takao *et al*., 1996). From the literature review, the precise source and mechanism of ROS production for LGG-CM mediated bacterial killing is undefined.

The exact mechanism via which the LGG-CM cause their actions is currently not known, nor is it clear whether they could regulate ROS generation and mediate bacterial killing as seen in the chapter 4. In this study, we have therefore evaluated the influence of cell free LGG-CM on ROS and NO production by the J774 macrophage cell line, and further evaluated the acute effect of LGG-CM on NO and ROS production during the process of macrophage phagocytosis of *E.coli* in real time. Fluorescence analysis was used to monitor ROS or NO generation on a real time basis. Data was analysed using CuSum analysis which offers a simple and rapid method for identifying sustained changes in real time under experimental situations.

The Cumulative sum (CuSum) technique was first proposed by Page, in 1954 (Tam, 2009; Hu *et al*., 2012) but now widely used in several biological fields in investigating medical outcomes (Kabir *et al*., 2013; Cho *et al*., 2014), quantitative analysis of circadian blood pressure profiles (Stanton *et al*., 1992), monitoring epidemics (Menotti *et al*., 2009; Hu *et al*., 2012) and in several clinical studies (Knight *et al*., 2001; Kverka *et al*., 2007). This is however the first study to investigate a real time changes in ROS and NO productions using CuSum in *in vitro* experiments.

In this chapter, the role of both NO and ROS in LGG-CM mediated macrophage digestion were also investigated using selective pharmacological inhibitors for NO and ROS. This was followed by attempts to delineate precisely which toxic oxidants are primarily responsible for the observed LGG-CM mediated phagocytic modulation by using cell permeable free radical scavengers such as PEG conjugated SOD and catalase.

5.2. Results: Ingestion Phase

In the ingestion phase, the involvement of both NO and free radicals during bacterial ingestion was tested by using pharmacological inhibitors and scavengers. The rate of ROS and NO production during the ingestion period was also measured using a fluorescence microplate reader.

5.2.1. Effect of ROS inhibitors on bacterial ingestion

Macrophages pretreated with 100 μM apocynin (apo), 100 μM allopurinol (allo) and a combination of apocynin and allopurinol (apo+allo) were coincubated with *E. coli* for 60 minutes. The recovered bacteria were expressed as $\log_{10}$ CFU ml^{-1}. Recovered bacteria at 60 minutes from untreated macrophages (control) were 5.61 ± 0.1. Macrophages in the presence of 75 % LGG-CM ingested significantly less bacteria at 60 minutes co-incubation period. Bacteria recovered from the macrophages treated with apocynin, allopurinol, apocynin + allopurinol was 5.77 ± 0.09, 5.72 ±0.15, and 5.64 ± 0.17 $\log_{10}$ CFU ml^{-1}.

We did not observe any significant difference in the bacterial recovery from macrophages treated with 75 % LGG-CM when compared to the bacterial recovery from 75 % LGG-CM and inhibitor treatments. However, we observed a significant difference in the bacterial recovery between control macrophages and macrophages treated with some of the inhibitor treatments in the presence of 75 % LGG-CM as shown in Figure 5.1.

Bacteria recovered from macrophages treated with 75 % LGG, 75 % LGG + apo, 75 % LGG+ allo, 75 % LGG (apo+allo) at 60 minutes were 5.19 ± 0.07, 5.22 ± 0.06, 5.14 ± 0.03, 5.09 ± 0.03 $\log_{10}$ CFU ml^{-1} respectively.

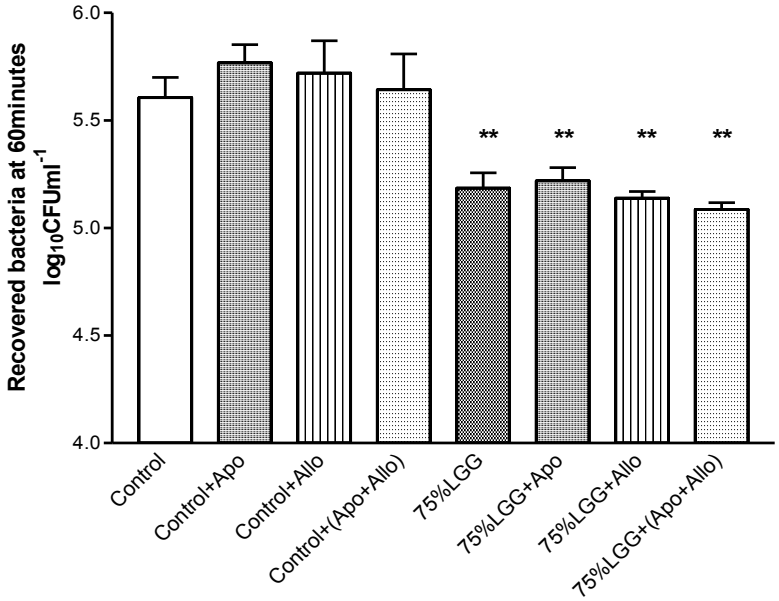


Figure 5.1: Effect of ROS inhibitors on bacterial ingestion. The effect of NADPH oxidase and xanthine oxidase inhibitors on bacterial ingestion were investigated in the presence of LGG-CM. Cells were pretreated for an hour with 100 μM apocynin (apo) a NADPH oxidase inhibitor; 100 μM allopurinol (allo)- a specific xanthine oxidase inhibitor and 100 μM apocynin-allopurinol combination (apo+allo). Recovered bacteria at 60 minutes were expressed as $\log_{10}$ CFU ml^{-1}. Experiments were performed at least in duplicate and repeated at least four times. Error bars indicate ± standard error of the mean. Statistical analysis was carried out using one way ANOVA followed by Dunnett multiple comparison post-hoc tests. Treatments which significantly differed with the control are shown with an asterisk [(*$p<0.05$); (**$p<0.01$). However, there was no difference in the bacterial recovery between treatments and their respective inhibitors during the first 60 minutes of incubation with LGG-CM.

5.2.2. Effect of free radical scavengers on bacterial ingestion

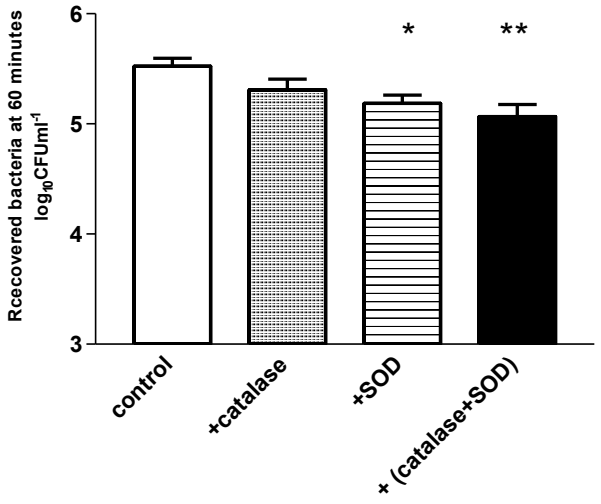
Macrophages were pre-treated with PEG-conjugated SOD and catalase for 60 minutes. The bacterial ingestion was then monitored from control cells ± scavengers and 75 % LGG-CM ± scavengers. As observed previously, LGG-CM treated macrophages ingested significantly less bacteria when compared to the control.

We also observed significant differences between the bacterial recoveries from control macrophages to macrophages treated with SOD on its own and SOD + catalase combination (figure 5.2 A). The recovered bacteria from control macrophages were 5.52 ± 0.07 $\log_{10}$ CFU ml^{-1}. The recovered bacteria from the catalase, SOD and catalase + SOD treated macrophages was 5.31 ± 0.1 $\log_{10}$ CFU ml^{-1} , 5.19 ± 0.07 $\log_{10}$ CFU ml^{-1}, 5.07 ± 0.11 $\log_{10}$ CFU ml^{-1} respectively.

In the presence of LGG-CM, macrophages treated with PEG-SOD and PEG-catalase significantly reduced bacterial ingestion compared to macrophages treated with LGG-CM on its own. Both the PEG-SOD and PEG-catalase treated macrophages yielded similar bacterial recovery. However, in combination, they did not have an additive effect and the bacterial recovery was non-significant between the scavengers themselves.

The recovered bacteria from the LGG-CM treated macrophages were 4.88 ± 0.09 $\log_{10}$ CFU ml^{-1}. The recovered bacteria from LGG-CM + catalase, LGG-CM + SOD and LGG-CM + (catalase + SOD) treated macrophages were 4.56 ± 0.08 $\log_{10}$ CFU ml^{-1} , 4.56 ± 0.04 $\log_{10}$ CFU ml^{-1} and 4.52 ± 0.02 $\log_{10}$ CFU ml^{-1} respectively.

A



B

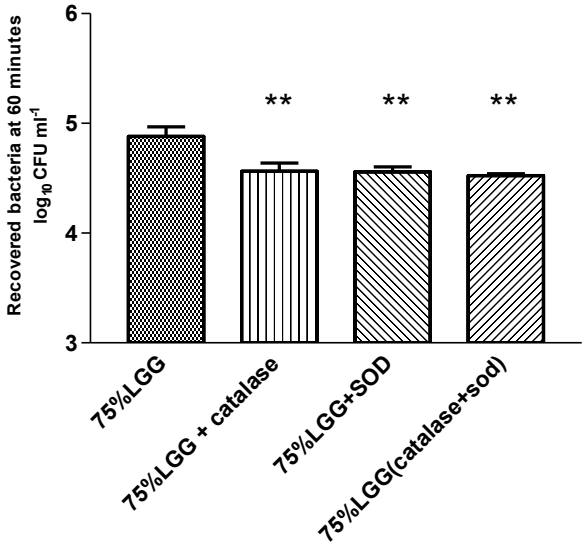


Figure 5.2: Monitoring the effect of free radical scavengers on bacterial ingestion. The effect of catalase, superoxide dismutase and catalase + superoxide dismutase combination on bacterial ingestion were investigated in the presence of untreated (figure A) and LGG-CM (figure B) treated macrophages. Experiments were performed at least in duplicate and repeated at least four times. Error bars indicate ± standard error of the mean. Statistical analysis was carried out using Graph Pad Prism 3. The Dunnett's multiple comparison post-hoc tests was performed after observing a significant difference from One way ANOVA. Error bars represent ± standard error of the mean. An asterisk indicates a significant difference to the control (**$p<0.01$).

5.2.3. Generation of free radicals as a response to menadione and arginine

Macrophages were exposed to 100 μM arginine and 0.5 mM menadione and continuous measurements (every 2 minutes) of NO and ROS were made for 60 minutes (ingestion period). Menadione and Arginine were used as a positive control for ROS and NO production respectively.

Figure 5.3A and 5.3C show the characteristics of ROS generation from untreated macrophages (control) and 0.5 mM menadione treated macrophages (raw data and CuSum plot). The Figure 5.3B and 5.3D represent the characteristics of NO production by J774 cells over a 60 minute period from untreated and 100 μM arginine treated macrophages (raw data and CuSum plot).

There were small differences in the extent of the change of fluorescence readings between the control and experimental plates from the raw data. However, the random variation in the measurements made these differences difficult to follow when the raw data of fluorescence measurements were plotted against time.

To address this, CuSum statistics was used to understand the pattern of real time ROS production data. The smoothing of the data in this way elucidated the difference between the control and experimental results.

In the Figure 5.3C, the rate or gradient of ROS production from the untreated macrophages was zero. However, the gradient of 0.5 mM menadione treated macrophages was 0.3 fluorescence arbitrary units min^{-1}. The gradient of NO production (figure 5.3D) from the untreated cells was 0.05 fluorescence arbitrary units while from 100 μM arginine this was found to be 0.29 fluorescence arbitrary units. The gradients are the m values from the linear trendline equation y= mx+c derived from CuSum values (see Section 2.11.6 in Chapter 2).

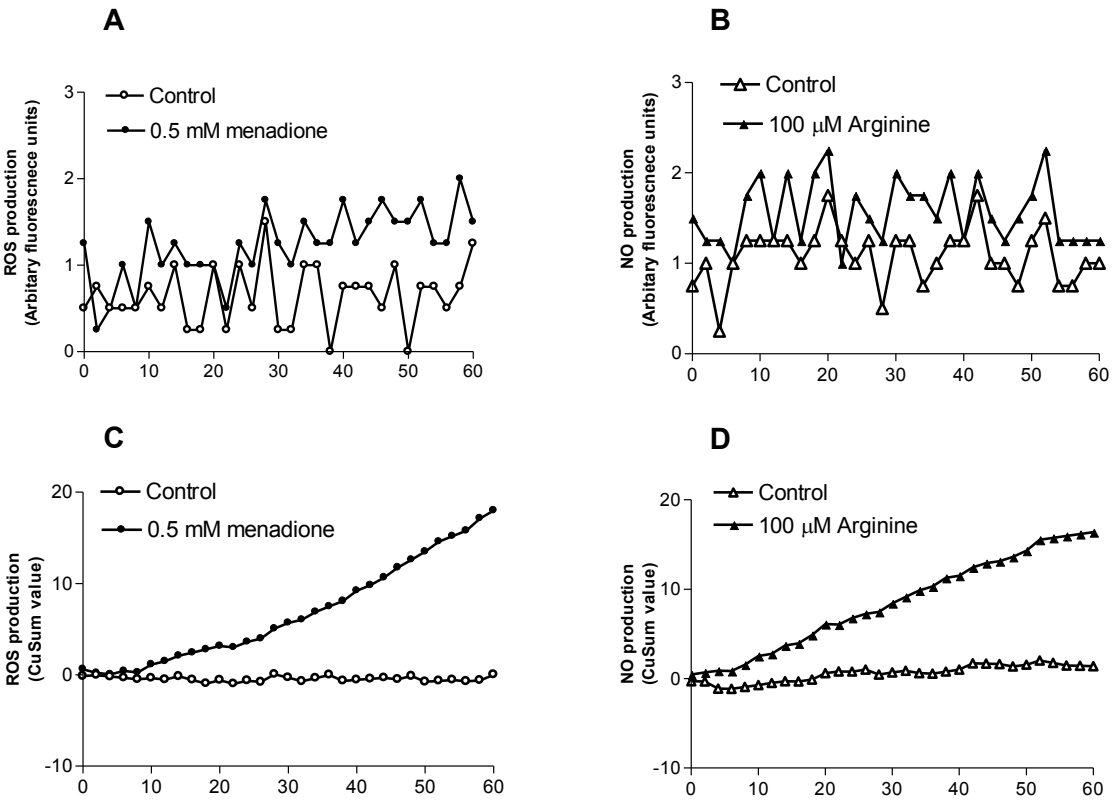


Figure 5.3: Raw data and CuSum plot for J774 macrophages incubated with menadione and arginine. Figure A illustrates the profile of ROS production from control and menadione treated macrophages and Fig C represents the corresponding CuSum plots. Figure B illustrates the profile of NO production from arginine treated macrophages and Figure D represents the corresponding CuSum plots. In figure C and D, menadione and arginine treated macrophages demonstrate an increase in free radical production and thereby increased fluorescence values. The CuSum plot shows a sharply rising trend with these positive treatments. The measurements were made over 60 minutes at two minute intervals. The above graphs are representative of at least 3 independent experiments (n=4).

5.2.4. CuSum gradient of ROS production from macrophages during incubation of treatments for 60 minutes

In the next series of experiments, the effect of various dilutions of LGG-CM, LPS, and LGG-CM + LPS combination on ROS production was investigated during first 60 minutes in J774 murine macrophage cell line by labelling the macrophages with H_2DCFDA as described in Figure 2.10.

As shown in Figure 5.4, there was a significant difference in the rate of ROS production from macrophages treated with 75 % LGG-CM ± LPS and 100 % LGG-CM ± LPS treatments when compared to the control. The rate of ROS production from macrophages treated with 75 % LGG-CM, 75% LGG-CM +LPS, and 100 % LGG-CM, 100 % LGG-CM + LPS were 3.27 ± 1.03, 4.00 ± 1.12, 5.2 ±1.06, and 5.13 ± 1.08 fluorescence arbitrary units per minute. However, LPS and 10 % LGG-CM ± LPS treatment of the macrophages did not differ significantly when compared to untreated macrophages.

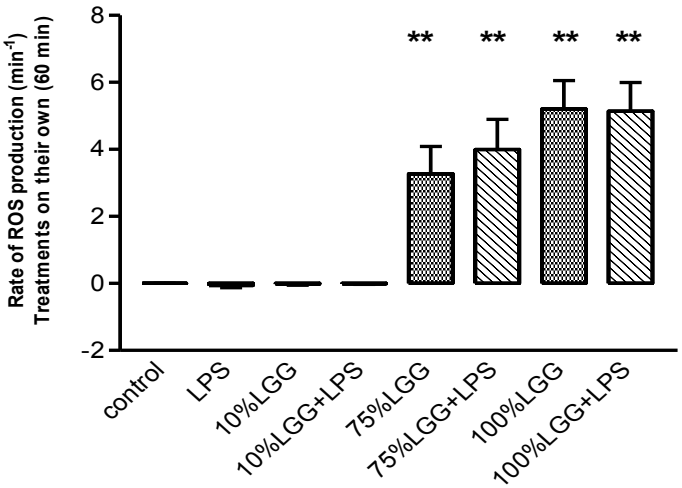


Figure 5.4: Fluorescence measurement of ROS production during the ingestion period. The macrophages were labelled with H_2DCFDA and fluorescence measurements were taken every 2 minutes for up to 60 minutes. Experiments were repeated at least 7 times (n=4) and the rate of ROS production was calculated by CuSum analysis. Error bars indicate ± standard error of the mean. Dunnett's multiple comparison post-hoc tests was used to identify significantly differing groups after observing a significant difference from One-way ANOVA. Error bars represent ± standard error of the mean. An asterisk indicates a significant difference to control (**$p<0.01$).

5.2.5. CuSum gradient of ROS production from macrophages in the presence of bacteria during the ingestion period

The effect of LPS, various dilutions of LGG-CM and LGG-CM + LPS combination on ROS production was investigated by co-incubating J774 murine macrophage cell line with viable *E. coli* HfrC during first 60 minutes (ingestion phase).

Increased levels of intracellular ROS production was observed from macrophages co-incubated with bacteria in the presence of 75 % LGG-CM ± LPS and 100 % LGG-CM ± LPS. The LPS and 10 % LGG-CM ± LPS treatment did not significantly influence ROS production from the macrophages when compared to the control (Figure 5.5).

Similar results were observed from the macrophages in the presence of 10 % LGG-CM in the absence of bacteria (Figure 5.4). The differential level of ROS production from a lower concentration of LGG-CM when compared to higher [LGG] is suggestive of a potential anti-inflammatory role of LGG-CM at low concentrations. The slope or rate of ROS production from macrophages treated with 75 % LGG-CM, 75% LGG-CM +LPS, 100 % LGG-CM and 100 % LGG-CM +LPS in the presence of bacteria were 3.4 ± 0.78, 3.7 ± 0.92, 4.7 ±0.92 and 5.8 ± 1.4 fluorescence arbitrary units per minute.

The rate of ROS production from control macrophages incubated with *E. coli* and macrophages incubated with LPS and *E. coli* were zero. The slope or rate of ROS production from 10 % LGG-CM and 10% LGG-CM +LPS was 0.7 ± 0.29, 1.0 ± 0.56 fluorescence arbitrary units per minute.

There was no significant difference ($p>0.05$) in ROS production at 60 minutes from any of the treatments on its own (Figure 5.4) and in presence of bacteria (Figure 5.5).

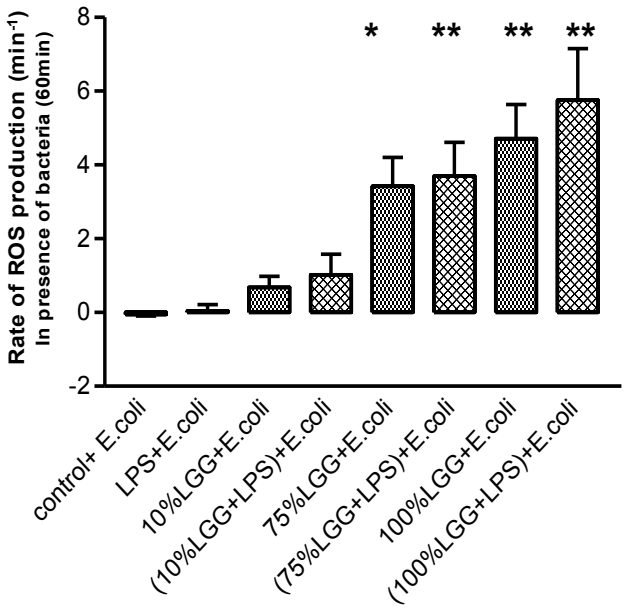


Figure 5.5: Fluorescence measurement of ROS production during the ingestion period. Macrophages were labelled with H_2DCFDA and fluorescence measurements were taken every 2 minutes for up to 60 minutes. Experiments were performed in the presence of bacteria. Experiments were repeated at least 7 times (n=4) and the slope of ROS production was calculated by CUSUM analysis. Error bars indicate ± standard error of the mean. Dunnett's multiple comparison tests was used to identify significantly differing groups after observing a significant difference from One-way ANOVA. Error bars represent ± standard error of the mean. An asterisk indicates a significant difference to the control [(*$p<0.05$); (**$p<0.01$).

5.2.6. Visualization of ROS production using a fluorescence microscope

To visualise the ROS production from macrophages treated with LGG-CM, the macrophages were first loaded with fluorescent dye H_2DCFDA. Various dilutions of LGG-CM (10 % LGG and 75 % LGG) and 20 μg ml^{-1} LPS were applied and the images were captured using a fluorescence microscope as described in section 2.11. The images were then pseudocoloured with Image Hopper 2 software.

In the pseudocoloured images blue represented the coolest fluorescent spot, followed by shades of green, yellow, orange and red for brighter intensity. Panel A shows the bright field images while panel B and panel C shows the fluorescence images at 30 minutes and 60 minutes respectively.

It was evident from the images that macrophages treated with 75 % LGG-CM demonstrated higher ROS production both at 30 minutes and 60 minutes (panel B4 and C4 in figure 5.6) compared to control (panel B1 and C1), LPS treated (panel B2 and C2) or 10 % LGG-CM treated macrophages (panel B3 and C3). The bright brick red/orange fluorescence from 75 % LGG treated macrophages demonstrate increased ROS production when compared to the control (blue/green fluorescence).

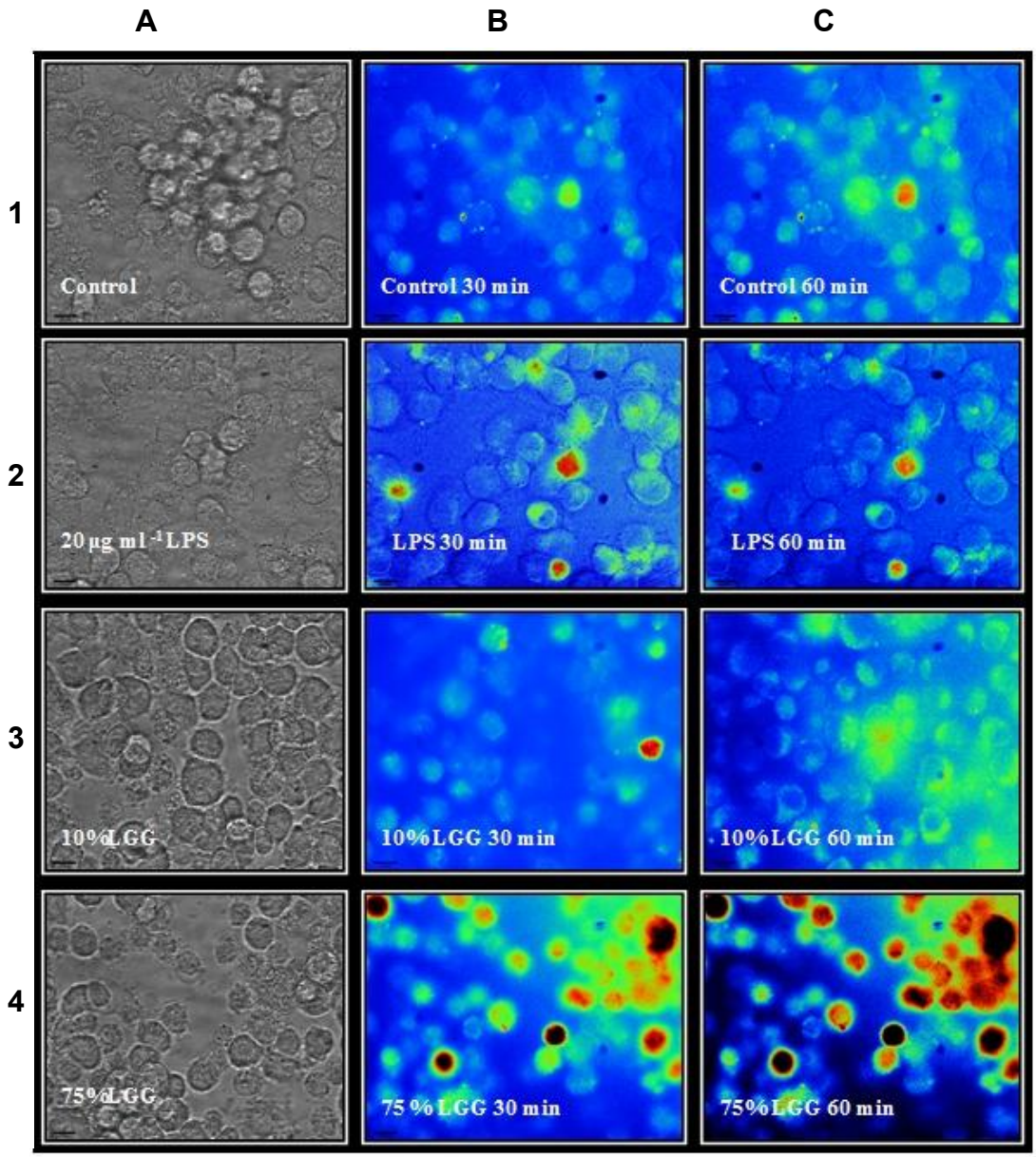


Figure 5.6: Fluorescence imaging of ROS production from macrophages. The macrophages were labelled with H_2DCFDA and treated with 20 µg ml^{-1} LPS, 10 % LGG-CM and 75 % LGG-CM for 30 and 60 minutes. The images are representative of one of the three experiments performed. The vertical panels illustrate adjacently placed brightfield (A) and pseudo coloured fluorescence images (B and C).

5.2.7. CuSum gradient of NO production from macrophages during first 60 minutes

After observing an increase in ROS production from LGG-CM treated macrophages, we next studied the effect of LGG-CM on the extent of NO production. Macrophages were labelled with DAFFM-DA. Upon covalent modification by NO, DAFFM forms a fluorescent triazolo-fluorescein analog which provides an integrated measure of intracellular NO concentration.

In order to study the effect of LGG-CM on NO production by murine macrophages, the macrophages were incubated with LPS and various dilutions of LGG-CM ± LPS combinations. The gradient of NO production from untreated macrophages, LPS treated macrophages and 10 % LGG±LPS treated macrophages were zero in the first 60 minutes.

However, we observed a negative slope in NO production indicating a reduction in NO when compared to the control cells. The rate of NO production from the macrophages treated with 75 % LGG-CM, 75 % LGG-CM +LPS, 100 % LGG-CM and 100 % LGG-CM +LPS were 0.26 ± 0.07, -0.27 ± 0.07, -0.28 ± 0.08 and -0.34 ± 0.08 respectively. The LPS did not potentiate the NO production from LGG-CM treated macrophages acutely.

We had previously observed a positive trend in NO production from cells treated with 100 μM arginine (figure 5.3D). However, in this study, where monitoring NO production from macrophages in the presence of LPS and LGG-CM ± LPS, both of the treatments did not potentiate NO production. The results indicate a significant reduction in slope (rate) of NO production from the higher concentration of LGG-CM treated macrophages (Figure 5.7).

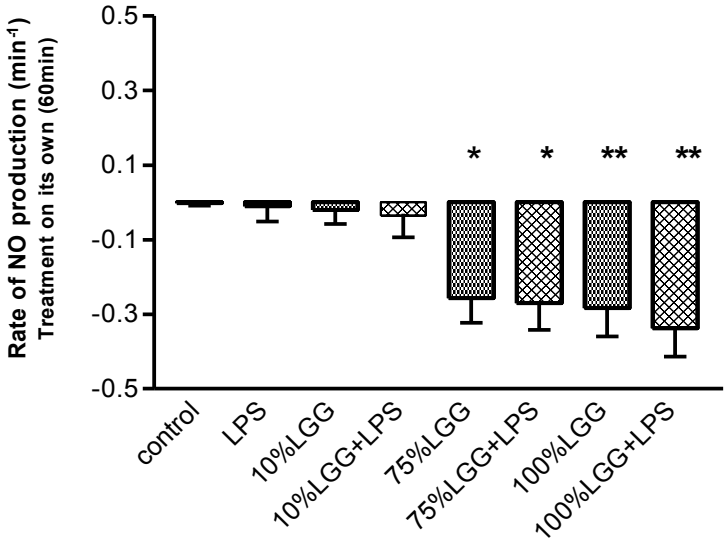


Figure 5.7: Fluorescence measurements of NO production during the ingestion period. The macrophages were labelled with DAFFM-DA and fluorescence measurements were taken every 2 minutes for up to 60 minutes. Experiments were repeated at least 7 times (n=4) and the rate of NO production was calculated by CuSum analysis. Error bars indicate ± standard error of the mean. Dunnett's multiple comparison tests was used to identify significantly differing groups after observing a significant difference from One-way ANOVA. Error bars represent ± standard error of the mean. An asterisk indicates a significant difference to the control [(*$p<0.05$); (**$p<0.01$).

5.2.8. CuSum gradient of NO production from macrophages in the presence of bacteria during ingestion period

In order to study the effect of LGG-CM on NO production by murine macrophages in the presence of viable *E. coli* HfrC, macrophages were coincubated with *E. coli* in the presence of LPS and various dilutions of LGG-CM ± LPS combinations. The NO production was then investigated by measuring the fluorescence intensity of DAFFM-DA every 2 minutes as described in Figure 2.10.

Results indicate a significant reduction in slope (rate) of NO production from 100 % LGG-CM ± LPS treated macrophages compared to the control. The 75 % LGG-CM ± LPS reduced the NO production from the macrophages; however, it was not statistically significant (Figure 5.8).

The rate of NO production from macrophages treated with, 75 % LGG-CM, 75 % LGG-CM + LPS, 100 % LGG-CM, 100 % LGG-CM + LPS in the presence of *E. coli* were -0.26 ± 0.07, -0.24 ± 0.07, -0.27 ± 0.07, -0.31 ± 0.09 respectively. The LPS did not potentiate the NO production in LGG-CM treated macrophages.

Macrophages coincubated with *E. coli* in the presence of LPS and 10 % LGG-CM ± LPS treatments did not significantly differ in NO production compared to the control and the gradient was approximately zero.

There was no statistically significant difference in NO production at 60 minutes from any of the treatments on its own (Figure 5.7) and in presence of bacteria (Figure 5.8) except umtreated macrophages ($p<0.05$).

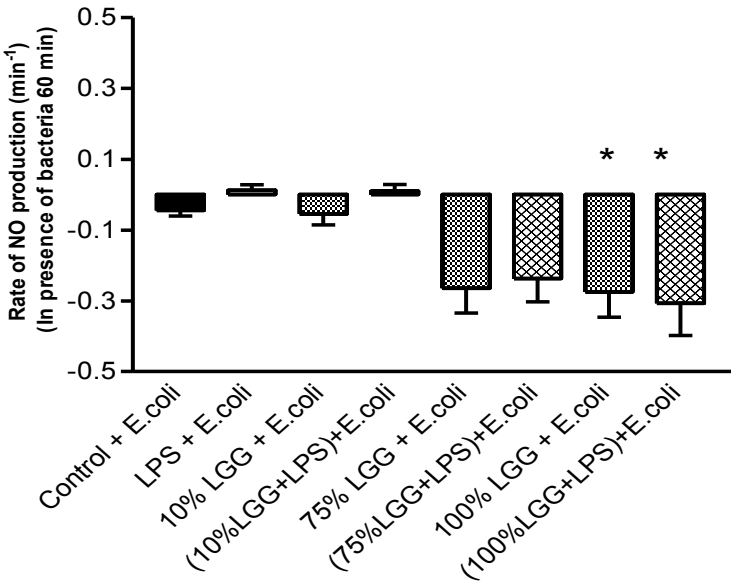


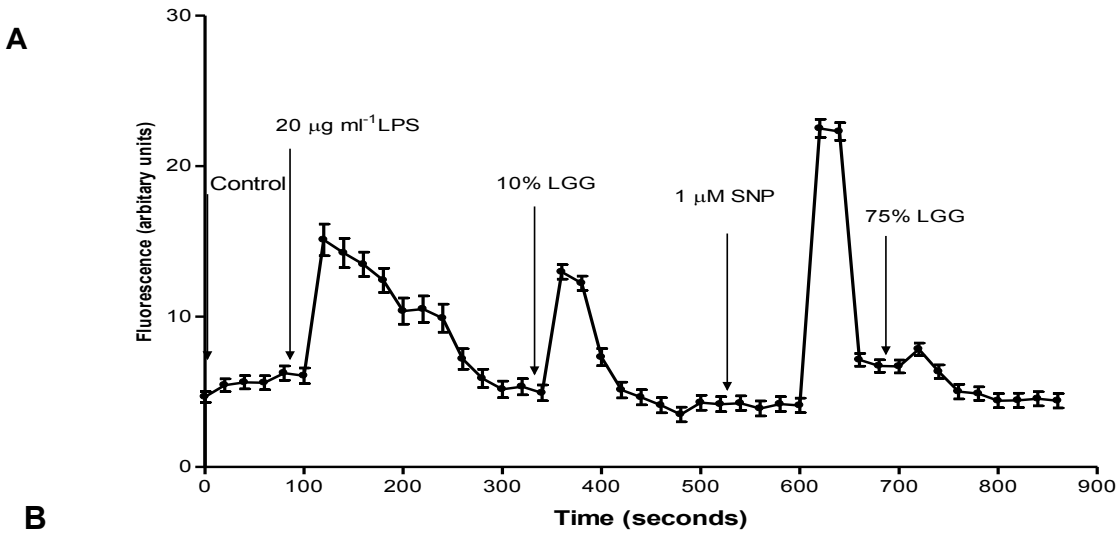
Figure 5.8: Fluorescence measurements of NO production during the ingestion period. The macrophages were labelled with DAFFM-DA and fluorescence measurements were taken every 2 minutes for up to 60 minutes in presence of bacteria. Experiments were repeated at least 7 times (n=4) and the rate of NO was calculated by CuSum analysis. Error bars indicate ± standard error of the mean. Dunnett's multiple comparison tests was used to identify significantly differing groups after observing a significant difference from One-way ANOVA. Error bars represent ± standard error of the mean. An asterisk indicate a significant difference to the control [(*$p<0.05$); (**$p<0.01$)].

5.2.9. Nitric oxide production following acute exposure of LGG and LPS to macrophages

To visualise the changes in NO production, unstimulated murine macrophages labelled with DAFFM-DA were exposed to 20 μg ml^{-1} LPS and the NO production was observed in a Nikon Eclipse TE2000-U fluorescent microscope equipped with fluorescence filters (ex: 485nm; em 515nm) using time lapse imaging.

Figure 5.9 shows the time course investigations of effect of LGG-CM and LPS on NO production by the macrophages. Images were grabbed every 20 seconds. The images (zero through 100 seconds) are in the presence of control media only, whilst images 100 -340 seconds was in the presence of 20 μg ml^{-1} LPS, 350-520 seconds in presence of 10 % LGG, 520 -680 sec in the presence of sodium nitroprusside (SNP) and 680-860 sec in the presence of 75 % LGG. These image sets show that LPS only slightly increase the NO production when compared to SNP. The NO production was transient and necessitated the investigation to be performed a few seconds after the addition of treatments.

In Figure 5.9, the NO production from control cells ranged from 4.9 ± 0.52 to 15.1 ± 1.05 fluorescence arbitrary units. The onset of the increase in NO levels peaked just 20 seconds after the addition of LPS. However, this was a spike rather than a continuous increase of fluorescence production. A similar observation was made with response to the addition of 10 % LGG from 350 sec -520 sec. Treatment with LGG did not have a lag phase and a spike of DAF-FM oxidation was evident in the images taken at 360 seconds. The fluorescence intensity ranged from 3.49 ± 0.4 to 12.96 ± 0.48. The NO production from macrophages treated with 1 μM SNP ranged from 3.89 ± 0.49 to 22.51 ± 0.60 fluorescence arbitrary units. The fluorescence intensity after addition of SNP peaked within a minute to the highest value of 22.51 arbitrary units and this peak was sustained for 20 seconds and then dropped back to a nominal value of 6.7 fluorescence units at 680 seconds. The 75 % LGG treatment was added at around 690 seconds. The magnitude of fluorescence remained fairly constant ranging from 4.4 ± 0.47 to 7.82 ± 0.41 between 690 to 860 seconds. The changes in magnitude and the onset of fluorescence intensity varied from cell to cell.



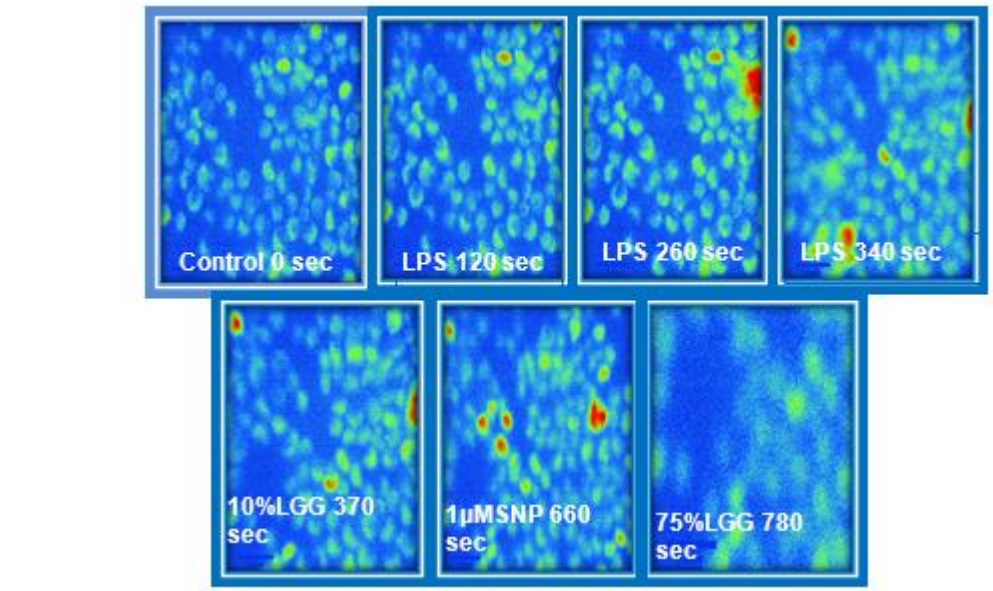


Figure 5.9: Fluorescence measurements of NO production from macrophages subjected to acute exposure of LGG-CM, LPS and SNP. Figure A is a semi-quantitative representation of the images shown in figure B. Experiments was performed under complete darkness to avoid any background noise. In figure A, 47 cells were selected and their fluorescence output was measured by grabbing images every 20 seconds. Error bars represents the fluorescence output from 47 selected cells. The NO formation was visualised by labelling the macrophages with DAFFM-DA. Images are representative of one of 3 experiments. Basal level or nominal fluorescence was observed initially in the presence of the control media. The fluorescence intensity was high when macrophages were treated with 1 μM SNP.

5.3. Results: Digestion phase

In the digestion phase, the involvement of both NO and ROS during bacterial digestion was tested by using pharmacological inhibitors and scavengers. The rate of ROS and NO production during the digestion period was measured using fluorescence microplate reader.

5.3.1. Effect of ROS inhibitors on bacterial digestion

To establish the potential role of free radicals in the LGG-CM mediated increased bacterial digestion rate, the macrophages and bacteria were coincubated for a period of 60 minutes in the presence of various pharmacological inhibitors and scavengers. Gentamicin (200 μg ml^{-1}) was used to kill the extracellular bacteria (see Section 2.7.4.). The recovered bacteria from macrophage lysate at 280 minutes represented any remaining undigested bacteria from the macrophages.

Figure 5.10 shows that NADPH oxidase inhibitors apocynin (100 μM) inhibited bacterial digestion activity both in control and also in 75% LGG-CM treated macrophages. The recovered bacteria from control macrophages at 280 minutes were 5.12 ± 0.04 log_{10} CFU ml^{-1}. However the bacterial recovery from apocynin treated macrophages at 280 minutes was 5.57 ± 0.12 log_{10} CFU ml^{-1}. Similarly in the presence of 75 % LGG-CM treated macrophages, the macrophages treated with apocynin and apocynin + allopurinol combination yielded significantly higher bacterial recovery than LGG-CM on its own. The bacterial recovery from LGG-CM + apocynin and LGG-CM + (apocynin+allopurinol) combination treated macrophages were 5.17 ± 0.01 log_{10} CFU ml^{-1} and 5.19 ± 0.02 log_{10} CFU ml^{-1} at 280 minutes.

In the control and LGG-CM treated macrophages, allopurinol (xanthine oxidase inhibitor) had no effect on the bacterial recovery at 280 minutes. We also did not observe any summative effect on the bacterial recovery from apocynin and allopurinol combination treatments in the presence of LGG-CM when compared to apocynin on its own.

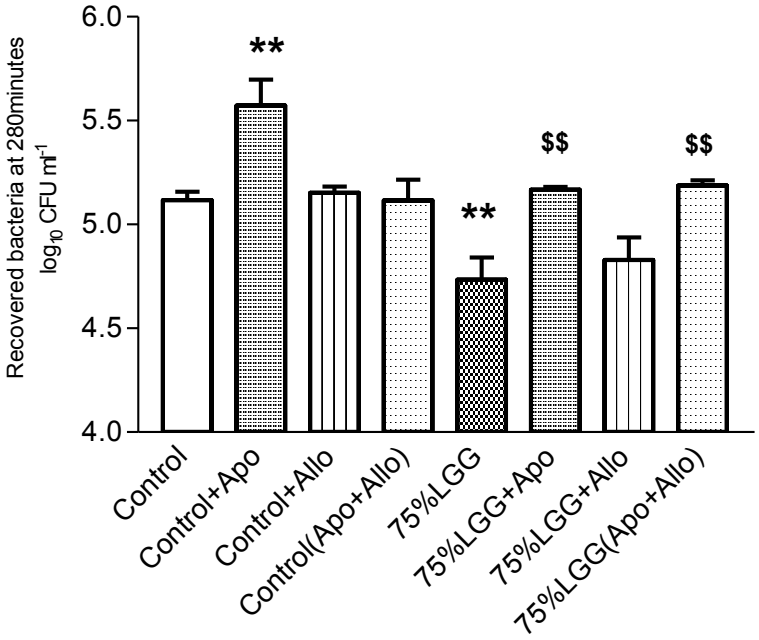


Figure 5.10: Role of ROS in bacterial digestion in the presence of LGG-CM. The effect of NADPH oxidase inhibitors on bacterial digestion was investigated in the presence of LGG-CM. Cells were pretreated for an hour with 100 μM apocynin (apo); 100 μM allopurinol (allo); 100 μM apocynin and allopurinol combination (apo + allo). Recovered bacteria at 280 minutes were expressed as $\log_{10}$ CFU ml^{-1}. Experiments were performed at least in duplicate and repeated at least three times. Error bars indicate ± standard error of the mean. Statistical analysis was carried out using Graph pad Prism 3. Statistical analysis was performed using One-way ANOVA followed by Dunnett multiple comparison post-hoc test. An asterisk (*) indicates a significant difference between the treatments and the control. A ($) indicates a significant difference between inhibitors and 75 % LGG-CM treated macrophages.

5.3.2. Effect of free radical scavengers catalase and SOD on bacterial digestion

The effect of the free radical scavengers PEG-SOD and PEG-CAT were investigated on bacterial digestion by macrophages from both control and LGG-CM treated macrophages at 280 minutes.

The bacterial recovery from the control macrophages was 5.19 ± 0.02 $\log_{10}$ CFU ml^{-1}. The bacterial recovery from catalase, SOD and catalase+SOD treated macrophages similar to control were 5.19 ± 0.02 $\log_{10}$ CFU ml^{-1}, 5.13 ± 0.02 $\log_{10}$ CFU ml^{-1} and 5.13 ± 0.08 $\log_{10}$ CFU ml^{-1}. Although these values are fairly similar to control, however as shown in figure 5.2, that macrophages ingest less bacteria in the presence of ROS scavengers. Hence any increase of *E. coli* recovery from ROS scavengers treated macrophages at 280 minutes, could be masked due the fact of initial lower uptake of bacteria to ROS scavengers.

The LGG-CM + catalase and LGG-CM + (Catalase + SOD) combination treatment of macrophages demonstrated a significant increase in bacterial number (shown as a dollar sign in Figure 5.11) compared to LGG-CM on its own. There was no additive effect on the bacterial recovery when catalase and SOD were applied in combination. The SOD appears to have small influence on inhibiting digestion in LGG-CM treated macrophages. This could be due to the nature of the data as the error bar seems to have masked the effect of SOD. The bacterial recovery from 75 % LGG-CM treated macrophages at 280 minutes was significantly less (4.35 ± 0.09 $\log_{10}$ CFU ml^{-1}). The bacterial recovery from LGG-CM + catalase, LGG-CM+SOD and LGG-CM + (catalase +SOD) combination treated macrophages were 4.78 ± 0.03 $\log_{10}$ CFU ml^{-1}, 4.66 ± 0.14 and 4.79 ± 0.04 $\log_{10}$ CFU ml^{-1}.

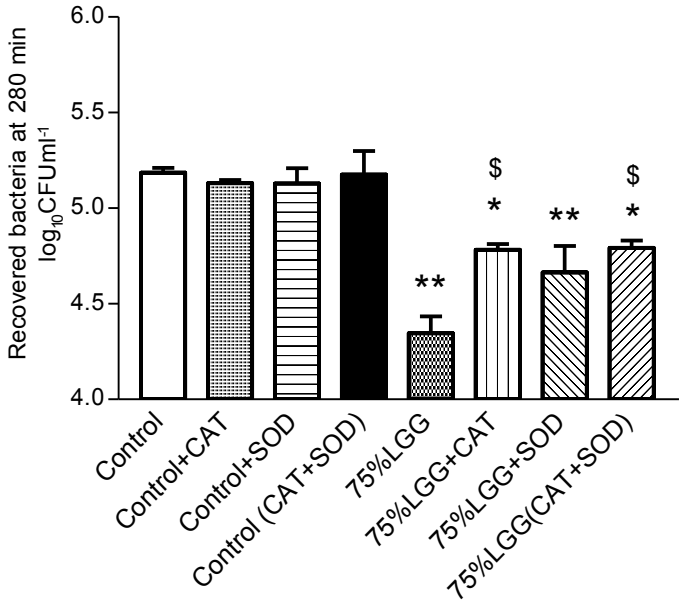


Figure 5.11: Effect of free radical scavengers on bacterial digestion. The effect of superoxide dismutase (SOD) and catalase (CAT) on bacterial digestion was investigated in the presence of LGG-CM. After bacterial ingestion, the macrophages were allowed to digest bacteria for 280 minutes in the presence of PEG conjugated free radical scavengers. Recovered bacteria were expressed as $\log_{10}$ CFU ml^{-1}. Experiments were performed at least in duplicate and repeated at least three times. Error bars indicate ± standard error of the mean. Statistical analysis was carried out using Graph Pad Prism 3. The Dunnett's multiple comparison post-hoc tests was performed after observing a significant difference from One way ANOVA. An asterisk (*) indicates significant difference between the treatments and the control. A significant difference in the bacterial recovery was also observed between LGG-CM treated macrophages and LGG-CM treated either with catalase or with SOD + catalase groups ($P<0.05$) shown as $.

5.3.3. Effect of nitric oxide inhibitor on bacterial digestion

To investigate if NO has any effect on LGG-CM mediated bacterial killing, the macrophages and bacteria were coincubated for a period of 60 minutes in the presence of L-NMMA, after which gentamicin was used to kill the extracellular bacteria. The recovered bacteria at 280 minutes represented any remaining undigested bacteria from the macrophages. Figure 5.12 shows that blocking the iNOS activity of the macrophages using 100 μM L-NMMA had significant effect on the bacterial digestion by macrophages.

The bacterial recovery from the control and LGG-CM treated macrophages at 280 minutes were 5.04 ± 0.09 and 4.44 ± 0.16 $\log_{10}$ CFU ml^{-1}. The bacterial recovery from L-NMMA treated macrophages was 5.5 ± 0.06 $\log_{10}$ CFU ml^{-1}. The bacterial recovery from LGG-CM treated macrophages in presence of L-NMMA was 5.21± 0.02 $\log_{10}$ CFU ml^{-1}.

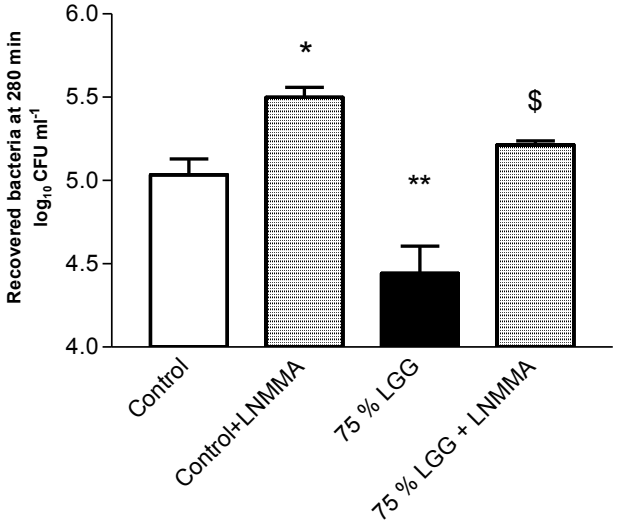


Figure 5.12: Role of NO in bacterial digestion in the presence of LGG-CM. The effect of iNOS inhibitors on bacterial digestion were investigated in the presence of LGG-CM. Macrophages was pretreated for an hour with 100 μM L-NMMA. Recovered bacteria at 280 minutes were expressed as $\log_{10}$ CFU ml^{-1}. Experiments were performed in duplicate and repeated atleast three times. Error bars indicate ± standard error of the mean. Statistical analysis was carried out using Graph Pad Prism 3. The Dunnett's multiple comparison post-hoc tests was performed after observing a significant difference from one-way ANOVA. An asterisk (*) indicates significant difference between the treatments and control. Significant difference in the bacterial recovery was also observed between LGG-CM treated cells and LGG-CM + L-NMMA is shown as $ as indicated by two-tailed unpaired t-test.

5.3.4. Effect of nitric oxide inhibitor and NADPH oxidase inhibitor combination on bacterial digestion

To investigate the effect of both apocynin and L-NMMA combination on bacterial digestion, the macrophages and bacteria were coincubated for a period of 60 minutes in presence of the control ± inhibitors and 75 % LGG-CM ± inhibitors. After killing the extracellular bacteria by gentamicin, the macrophages were further incubated for 280 minutes. The recovered bacteria at 280 minutes represented any remaining undigested bacteria from the macrophages. Figure 5.13 shows that blocking the iNOS and NADPH oxidase activity of the macrophages using 100 μM L-NMMA and 100 μM apocynin combinations had a significant effect on bacterial killing from 75 % LGG-CM treated macrophages. From figure 5.13, the bacterial recovery from the control and LGG-CM treated macrophages at 280 minutes was 5.04 ± 0.09 and 4.44 ± 0.16 $\log_{10}$ CFU ml^{-1}. The LGG-CM treated macrophages at 280 minutes demonstrated significantly less bacteria at 280 minutes from unpaired t-test. The bacterial recovery from L-NMMA + Apo treated macrophages was 5.63 ± 0.15. The bacterial recovery from LGG-CM treated macrophages in the presence of L-NMMA +Apo inhibitors was 5.19 ± 0.06 $\log_{10}$ CFU ml^{-1}.

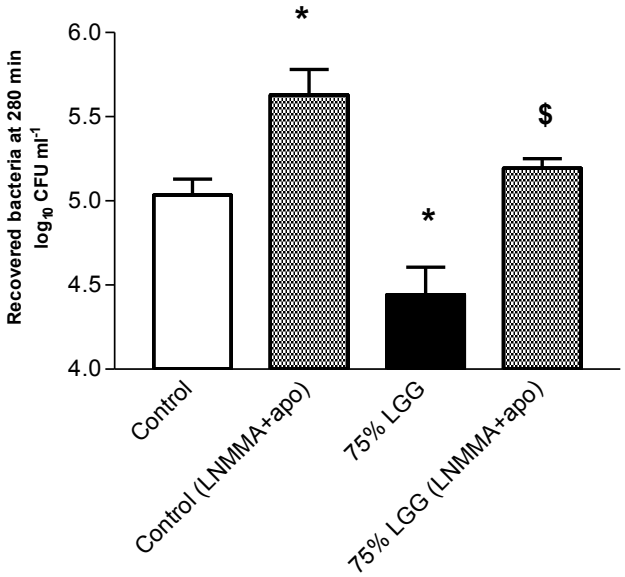


Figure 5.13: Role of NO+ROS inhibitor combination in bacterial digestion in the presence of LGG-CM. The effect of iNOS + NADPH oxidase inhibitors on bacterial digestion were investigated in the presence of LGG-CM. Experiments were performed in duplicate and repeated three times. Error bars indicate ± standard error of the mean. Statistical analysis was carried out using Graph Pad Prism 3. The Dunnett's multiple comparison post-hoc tests was performed after observing a significant difference from One way ANOVA. An asterisk (*) indicates significant difference between the treatments and control. Significant difference in the bacterial recovery was also observed between LGG-CM treated cells and LGG-CM + (L-NMMA + apo) ($P<0.05$) shown as $ from a two-tailed unpaired t-test.

5.3.5. Effect of menadione on ROS production from macrophages

Menadione was used as a positive control for monitoring ROS production by macrophages. The macrophages were either incubated for 60 minutes or 280 minutes with 0.5 mM menadione. Fluorescence readings were taken every 2 minutes and the rate of ROS generation from menadione was calculated as described in section 2.11.6. The rate of ROS production from menadione treated macrophages was 0.24 ± 0.05 min^{-1} (n=3 experiments) during the first 60 minutes (figure 5.14A). However, the rate of ROS production from menadione treated macrophages (3 experiments) was 0.5 ± 0.12 min^{-1} at 280 minutes incubation period (figure 5.14B). The rate of ROS production during 280 minute incubation almost doubled when compared to the rates during the first 60 minutes of incubation. However, it was not statistically different (p=0.1)

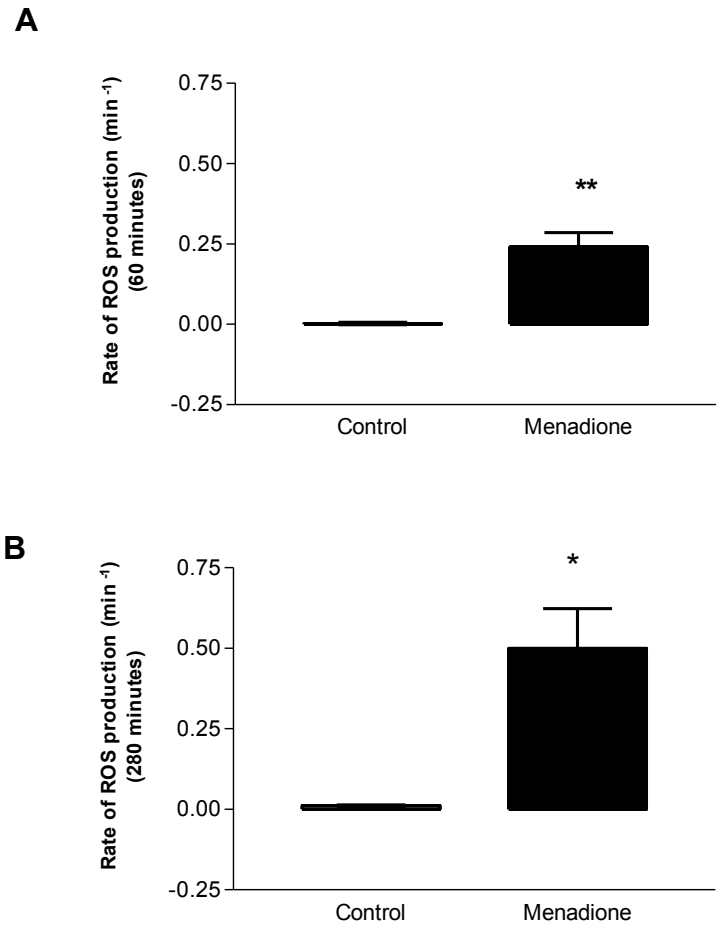


Figure 5.14: Effect of menadione on ROS production by macrophages. The effect of menadione on ROS production by macrophages was investigated by incubating the macrophages for 60 minutes and 280 minutes. Experiments were repeated at least three times (n=4). Error bars indicate ± standard error of the mean. Statistical analysis was carried out by two-tailed unpaired t-test using Graph pad Prism 3. An asterisk indicates a significant difference to the control [(*$p<0.05$); (**$p<0.01$).

5.3.6. Effect of arginine on NO production from macrophages

Arginine was used as a positive control for monitoring NO production by macrophages. Arginine is an essential substrate for iNOS enzyme. Macrophages were either incubated for 60 minutes or 280 minutes with 100 μM arginine and the fluorescence readings were taken every 2 minutes. The rate of NO generation from arginine was calculated as described in section 2.11.6. The average rate of NO production from macrophages treated with 100 μM arginine during the first 60 minutes of incubation was 0.23 ± 0.03 min^{-1} (figure 5.15A). The average rate of NO production from macrophages incubated with arginine for 280 minutes was 0.17 ± 0.02 min^{-1} (Figure 5.15 B). The rate of NO production decreased slightly when compared to the ingestion period. However this decrease in NO production during the 280 minutes incubation period was statistically non-significant (P=0.06). This decrease in NO production might possibly be due to the reduced arginine substrate availability for iNOS over a period of time, as arginine is consumed for NO generation.

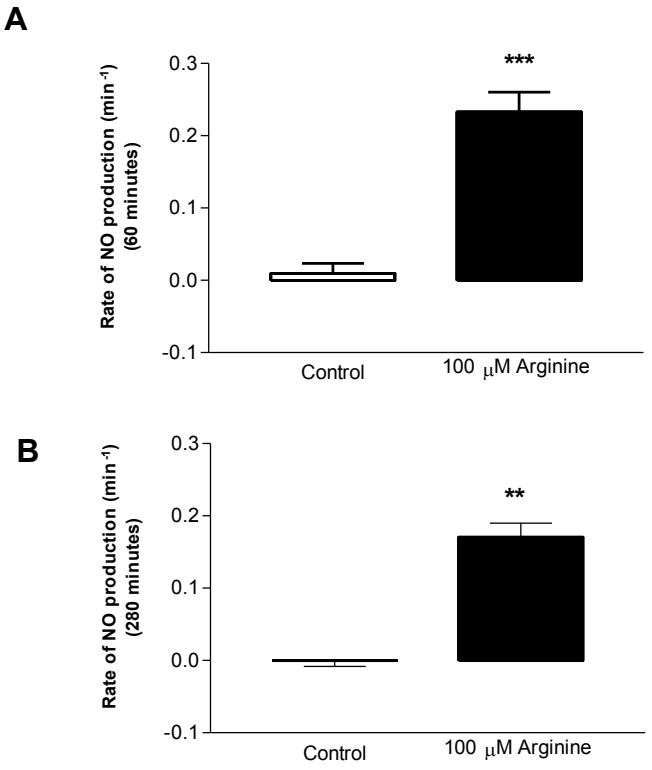


Figure 5.15: Effect of arginine on NO production by macrophages. The effect of arginine on NO production by macrophages was investigated by incubating the macrophages for 60 minutes and 280 minutes. Experiments were repeated at least 3 times. Error bars indicate ± standard error of the mean. Statistical analysis was carried out by two-tailed unpaired t-test using Graph pad Prism 3. An asterisk indicate a significant difference to the control [(***p<0.001); (**p<0.01)

5.3.7. Analysing the effect of LGG-CM mediated ROS production by macrophages during the digestion period

Macrophages exhibited an increased digestion rate in the presence of a high concentration of LGG-CM (see figure 4.4). Monitoring the bacterial recovery using ROS inhibitors and scavengers also demonstrated the importance of LGG-CM mediated ROS production in bacterial killing, as the inhibition of ROS production resulted in an increased number of bacterial recovery from the macrophages (see figure 5.10 and 5.11). To further validate the above findings, ROS production was monitored acutely and continuously in real time for a period of 280 minutes. Figure 5.16A in the panel represents an example of raw data of ROS production and figure B represents its corresponding CuSum lines.

The variation of ROS generation in the raw data makes it very difficult to detect differences between the patterns of ROS production from the various treatments. Figure B shows the CuSum analysis of the same data from Figure A plotted as CuSum, using the overall average for the ROS production in the control wells as the target. These experiments were performed in quadruplicate and repeated at least 3 times. The CuSum values in figure B are based on 2 minute intervals, but only 10 minute points are plotted for clarity. The graph demonstrates the gradient, or slope, from 75 % or 100 % LGG-CM treated macrophages were higher when compared to the control or cells treated with only 10 % LGG-CM.

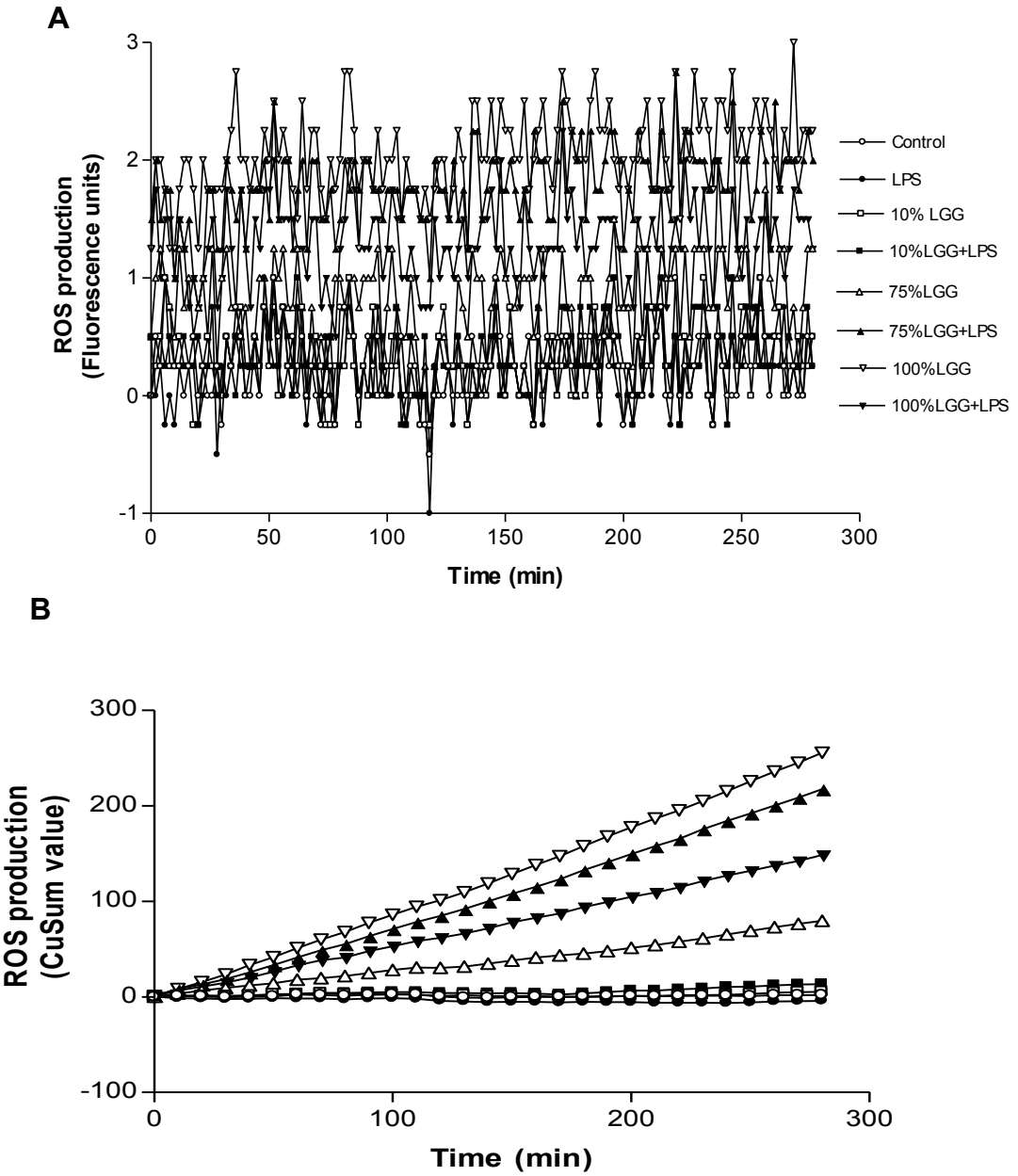


Figure 5.16: Raw data and CuSum plots of ROS production from murine macrophages in the presence of LGG-CM. Figure A shows a representative example of raw data for ROS production recorded over 280 minutes for control cells and cells exposed to various concentrations of LGG-CM, 20 μg ml^{-1} LPS and LGG-CM + LPS combinations. Figure B represents the same data expressed as CuSum values.

5.3.8. CuSum gradient of ROS production from macrophages during the incubation of treatments for 280 minutes

The ROS production from LGG-CM treated macrophages were measured every 2 minutes for up to 280 minutes using a fluorescence microplate reader. From Figure 5.17, increased levels of intracellular ROS production were observed from macrophages coincubated with bacteria in the presence of 75 % LGG-CM ± LPS and 100 % LGG-CM ± LPS. However, the rate of ROS production decreased at least by 2 times compared to the rate of ROS production during the first 60 minutes (Fig 5.4).

The LPS on its own and 10 % LGG-CM ± LPS treatment for 280 minutes did not affect ROS production from the macrophages (Figure 5.17). The differential level of ROS production from a lower concentration of LGG-CM is suggestive of a potential anti-inflammatory role of LGG-CM at low concentrations. The rate of ROS production from the control macrophages, LPS, 10 % LGG-CM and 10 % LGG-CM + LPS treated macrophages were almost zero. The slope or rate of ROS production from macrophages treated with, 75 % LGG-CM, 75 % LGG-CM + LPS, 100 % LGG-CM and 100 % LGG-CM + LPS were 1.3 ± 0.3, 1.4 ± 0.4, 2.1 ±0.5, and 1.8 ± 0.5 fluorescence arbitrary units min^{-1}.

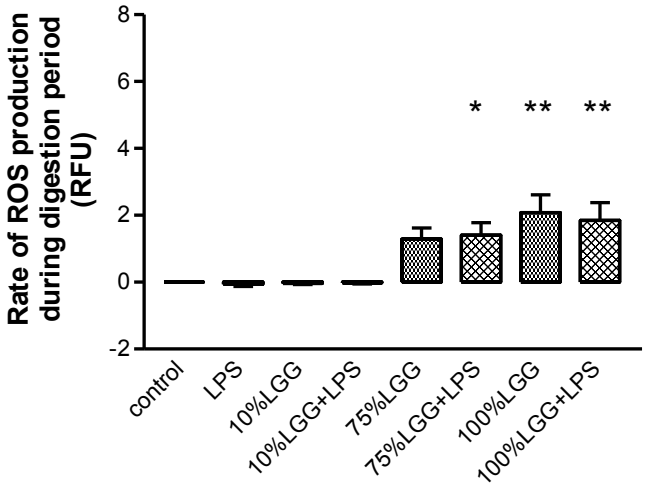


Figure 5.17: Fluorescence measurements of rate of ROS production during digestion period. Macrophages were labelled with H_2DCFDA and fluorescence measurements were taken every 2 minutes for up to 280 minutes. Experiments were performed in the absence of bacteria. Experiments were repeated at least 5 times (n=4) and the rate of ROS production was calculated by the fluorescence values from 60 minutes to 280 minutes by CuSum analysis. Error bars indicate ± standard error of the mean. Statistical analysis was carried out by using One-way ANOVA followed by Dunnett's multiple comparison tests. An asterisk (*) indicates a significant difference between the treatments and the control [(*) ($P<0.05$); (**) ($P<0.01$)].

5.3.9. CuSum gradient of ROS production from macrophages in the presence of bacteria during the digestion period

The ROS production from LGG-CM treated macrophages coincubated with viable *E. coli* HfrC were measured every 2 minutes for up to 280 minutes using a fluorescence microplate reader as described in Section 2.11.4. Figure 5.18 demonstrates the effect of LGG-CM on the extent of ROS production by macrophages during the bacterial digestion period. The rate of ROS production from the control macrophages incubated with *E. coli* and macrophages incubated with LPS and *E. coli* were 0.06 ± 0.09 and 0.19 ± 0.25 respectively. The slope or rate of ROS production from 10 % LGG-CM and 10 % LGG-CM + LPS were 0.34 ± 0.16 and 0.99 ± 0.47 fluorescence arbitrary units min^{-1}. The slope or rate of ROS production from macrophages treated with the 75 % LGG-CM, 75 % LGG-CM +LPS, 100 % LGG-CM and 100 % LGG-CM +LPS in presence of bacteria were 1.53 ± 0.34, 1.40 ± 0.35, 1.55 ± 0.49 and 1.73 ± 0.58 fluorescence arbitrary units min^{-1}. However, the rate of ROS production decreased at least by 2 times compared to the rate of ROS production during the ingestion period (Fig 5.5). There was no statistically significant difference in ROS production from any of the treatments on its own (Figure 5.17) and in presence of bacteria (Figure 5.18).

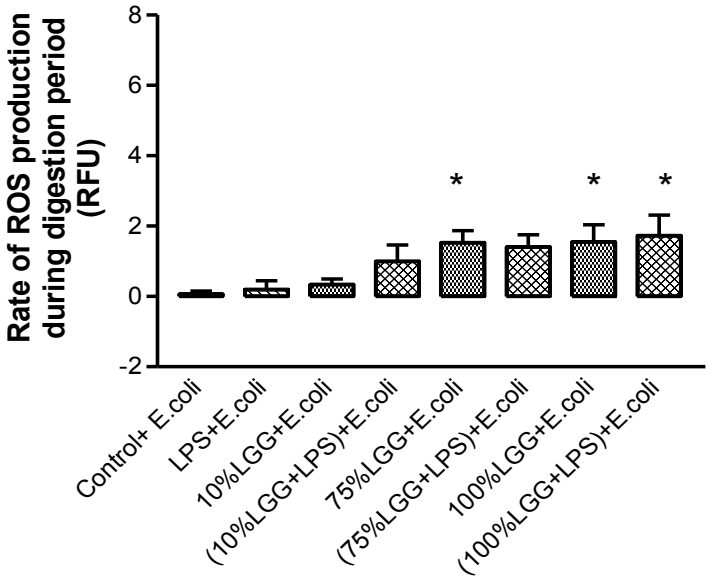


Figure 5.18: Fluorescence measurements of the rate of ROS production during the digestion period in presence of bacteria. Macrophages were labelled with H_2DCFDA and fluorescence measurements were taken every 2 minutes for up to 280 minutes. Experiments were performed in the presence of bacteria. Experiments were repeated at least 5 times (n=4) and the rate of ROS production was calculated by the fluorescence values from 60 minutes to 280 minutes by CuSum analysis. Error bars indicate ± standard error of the mean. Statistical analysis was carried out by using ANOVA followed by Dunnett's multiple comparison tests. An asterisk (*) indicates a significant difference between the treatments and the control [* ($P<0.05$)].

5.3.10. CuSum gradient of NO production from macrophages during the incubation of treatments for 280 minutes

The LGG-CM treated macrophages demonstrated only a basal effect on NO production at 24 hours from Griess assay (Figure 3.7). To further investigate whether LGG-CM had any effect on NO production during 280 minutes of treatment incubation period, the macrophages were labelled with NO specific dye DAFFM-DA and the fluorescence measurements were performed using a fluorescence microplate reader. Figure 5.19 demonstrates the effect of LGG-CM on the rate of NO production by macrophages during 280 minutes of treatment incubation period. The slope of the control and 10 % LGG + LPS was zero. The rate of NO production from LPS and 10 % LGG were 0.02 ±0.05 and -0.01 ±0.03 respectively. The rate of NO reduction from 75 % LGG, 75 % LGG+LPS, 100 % LGG and 100 % LGG-CM + LPS were -0.25 ± 0.08, -0.25 ± 0.07, -0.29 ± 0.07 and -0.33 ± 0.08 fluorescence arbitrary units min^{-1}. The rate of NO production from 100 μM arginine was 0.14±0.02 fluorescence arbitrary unit min^{-1}.

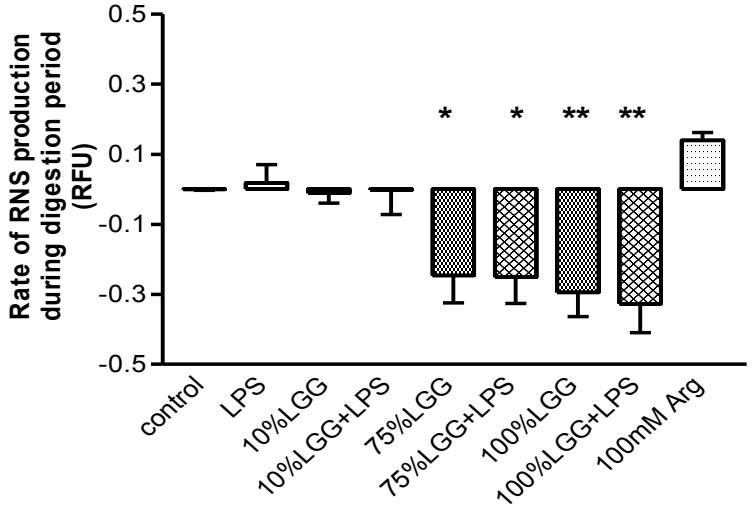


Figure 5.19: Fluorescence measurements of rate of NO production during the digestion period. Macrophages were labelled with DAFFM-DA and fluorescence measurements were taken every 2 minutes for up to 280 minutes. Experiments were repeated at least 3 times (n=4) and the rate of NO production was calculated by the fluorescence values from 60 minutes to 280 minutes by CuSum analysis. Error bars indicate ± standard error of the mean. Statistical analysis was carried out by using One way ANOVA followed by Dunnett's multiple comparison tests.

5.3.11. CuSum gradient of NO production from macrophages in presence of bacteria during the digestion period

The NO production from LGG-CM treated macrophages coincubated with viable *E. coli* was measured every 2 minutes for up to 280 minutes. From Figure 5.20, there was no difference in NO production from macrophages coincubated with *E. coli* in the presence of LPS or 10 % LGG-CM. Both LPS and LGG-CM ± LPS produced NO to a similar extent as those from untreated macrophages. The gradient of NO production from untreated macrophages, LPS treated macrophages, 10 % LGG and 10 % LGG-CM +LPS treated macrophages in the presence of *E. coli* were 0.06 ±0.08, 0.11 ± 0.02, 0.09 ± 0.04 and 0.11± 0.04 fluorescence arbitrary units min^{-1}. However, we observed a negative slope in NO production from a higher concentration of LGG-CM treated macrophages in the presence of bacteria indicating a reduction in NO when compared to the control cells. The rate of reduction of NO production from macrophages treated with 75 % LGG-CM, 75 % LGG-CM +LPS, 100 % LGG-CM and 100 % LGG-CM +LPS in presence of bacteria were -0.24 ± 0.04, -0.20 ± 0.07, -0.29 ± 0.05 and -0.35 ± 0.09 fluorescence arbitrary units min^{-1} respectively. The LPS did not potentiate the NO production in LGG-CM treated macrophages. There was no statistically significant difference in NO production from any of the treatments on its own (Figure 5.19) and in presence of bacteria (Figure 5.20).

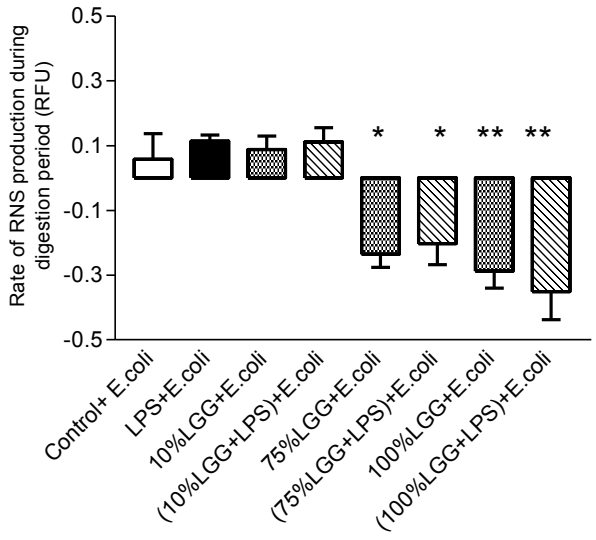


Figure 5.20: Fluorescence measurements of the rate of NO production during the bacterial digestion period. Macrophages were labelled with DAFFM-DA and fluorescence measurements were taken every 2 minutes for up to 280 minutes. Experiments were performed in presence of bacteria. Experiments were repeated at least 3 times (n=4) and the extent of NO production was calculated by the fluorescence values from 60 minutes to 280 minutes by CuSum analysis. Error bars indicate ± standard error of the mean. Statistical analysis was carried out by using One-way ANOVA followed by Dunnett's multiple comparison tests.

5.4. NADPH Oxidase expression to LGG-CM

Experiments were performed to investigate the effect of LGG-CM on NADPH oxidase expression. Two subunits of NADPH oxidase p47 phox and gp91 phox were investigated in the presence of LPS and various dilutions of LGG-CM at 2 hours, 6 hours and 24 hours.

5.4.1. Effect of LGG-CM on the expression of p47 phox at 2 hours.

Figure 5.21A shows the percent change in expression of p47 phox from untreated macrophages, LPS and various dilutions of LGG-CM ± LPS with respect to the control at 2 hours incubation period. The figure 5.21B shows the p47 phox protein bands from various treatments. The sequence in the bands (left to right) is the same as in figure 5.21A. The protein β-actin was used as a loading control to ensure that treatments were loaded equally across the wells and were used as a guide for effective protein transfer (Fig 5.21C).

The expression of p47 phox in J774 macrophages was very low at 2 hours. The figure 5.21 shown is from one successive experiment. We also could not detect the expression of p47 phox at 24 hours from macrophages. However, at 6 hours of incubation period we were successful in detecting the expression of p47phox as the protein expression was high compared to the 2 hours of incubation period (Fig 5.22).

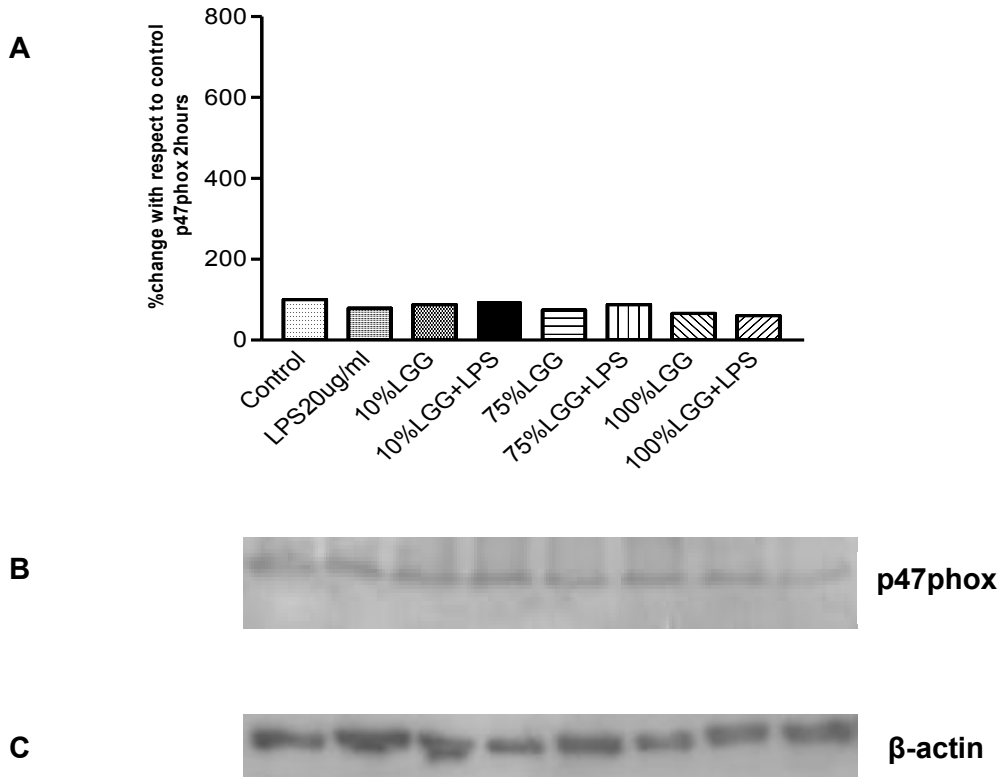


Figure 5.21: Effect of LGG-CM on the expression of p47 phox at 2 hours incubation. A confluent monolayer of macrophages was treated with various concentrations of LGG-CM and LGG-CM + 20 μg ml^{-1} LPS combination. The result represents one experiment's data demonstrated as the percent change in percent area with respect to the control (A). Image J was used to analyse the bands of p47 protein expression (B). The β-actin expression was used as a control to indicate the sample loading efficiency(C). The western blot bands shown in figure B for p47phox represents the protein expression as a response to various treatments which are in the same series as in the graph A.

5.4.2. Effect of LGG-CM on the expression of p47phox at 6 hours

It was challenging to detect the p47 expression at the initial 2 hours of incubation with LGG-CM (see figure 5.21). The next attempt was to investigate the effect of LGG-CM on the p47phox expression (cytosolic subunit of NADPH oxidase) at 6 hours of incubation.

A confluent monolayer of macrophages was treated with various concentrations of LGG-CM, LPS (20 μg ml^{-1}) and LGG-CM+LPS combination for 6 hours. Figure 5.22A shows the percent change in the expression of p47 phox from untreated macrophages, LPS and various dilutions of LGG-CM ± LPS with respect to the control at 6 hours. The figure 5.22B shows the p47 phox protein bands from various treatments. The sequence in the bands (left to right) is the same as in figure 5.22A. The figure 5.22C represents the expression of β-actin protein from the macrophages incubated with the respective treatments and was used to indicate protein loading efficiency.

We observed a significant increase in the expression of p47 phox from macrophages treated with 75 % LGG-CM ± LPS when compared to untreated macrophages. A low concentration of LGG-CM ± LPS and LPS expressed p47phox to same extent as untreated macrophages. 100 % LGG-CM ± LPS treated macrophages demonstrated an enhanced expression of p47 phox but was not statistically different from the control at 6 hours.

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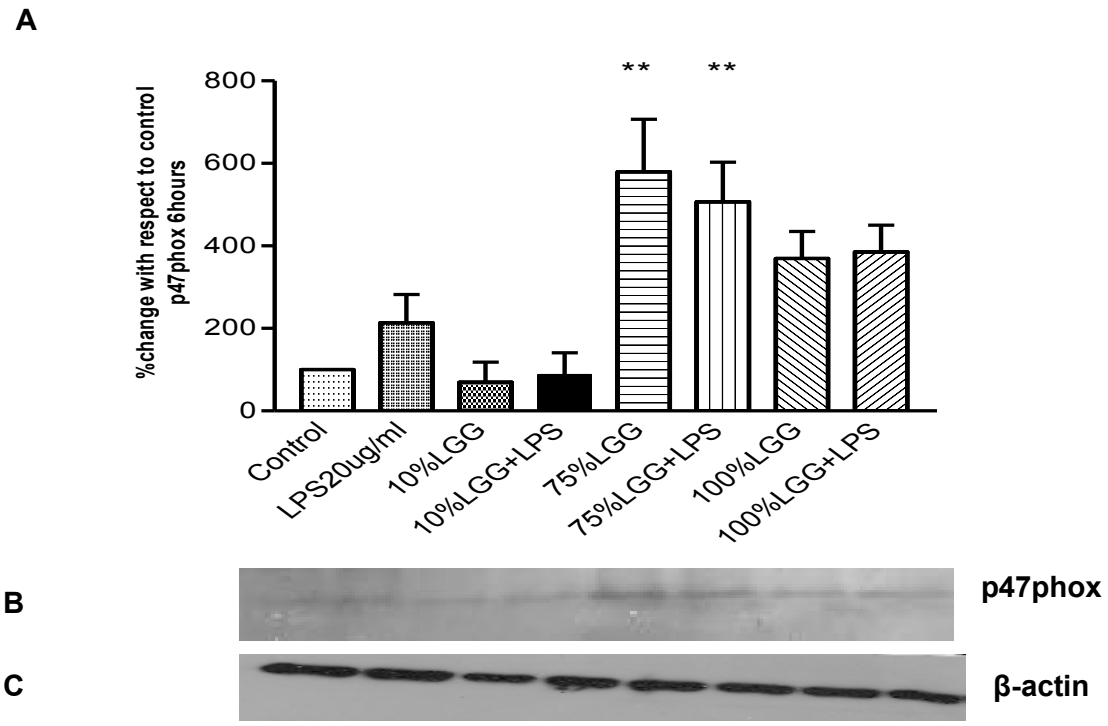

Figure 5.22: Effect of LGG-CM on the expression of p47 phox at 6 hours incubation in macrophages. The result represents the percent change in percent area with respect to the control (A). Image J was used to analyse the bands of p47 protein expression (B). The β-actin expression was used as a control to indicate the sample loading efficiency (C). The graph represents the mean of 3 independent experiments. Error bars indicate ± standard error of the mean. Statistical analysis was performed using One-way ANOVA followed by Dunnett's multiple comparison post-hoc tests. An asterisk (*) indicates a significant difference to control (**$p<0.01$).The western blot bands shown in figure B for p47phox represents the protein expression as a response to various treatments which are in the same series as in the graph A.

5.4.3. Effect of LGG-CM on the expression of gp91phox at 2 hours

These experiments were performed to investigate time dependent changes in the NADPH oxidase subunits expression in the presence of LGG-CM. We observed the expression of protein subunits gp91 phox (Figure 5.23) after a 2 hour incubation period of the macrophages with LPS and various dilutions of LGG-CM ± LPS.

Figure 5.23A shows the percent change in the expression of gp91 phox from untreated macrophages, LPS and various dilutions of LGG-CM ± LPS with respect to the control at 2 hours. The figure 5.23B shows the gp91 phox protein bands from various treatments. The sequence in the bands (left to right) is the same as in figure 5.23A. The figure 5.23C represents the expression of β-actin protein from macrophages incubated with the respective treatments.

The LPS and various dilutions of LGG ± LPS treated macrophages expressed gp91 phox protein to a similar extent. There was no significant change in expression of gp91 phox from macrophages treated with any of the treatments with the control at 2 hours of incubation.

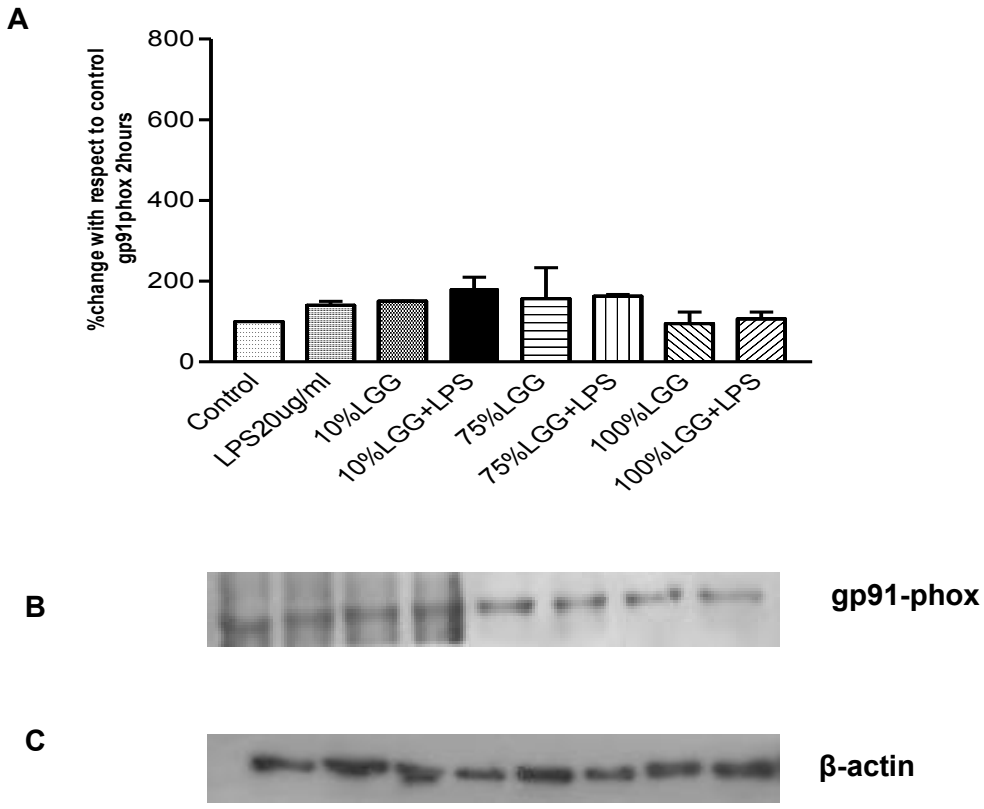

Figure 5.23: Effect of LGG-CM on the expression of gp91 phox at 2 hours incubation. The macrophages were treated with various concentrations of LGG-CM and LGG-CM + LPS (20 μg ml^{-1}) combination for 2 hours. The result represents the percent change in percent area with respect to control (A). Image J was used to analyse the bands of protein expression (B). The β-actin expression was used as a control to indicate the sample loading uniformity. The graph represents the mean of 3 independent experiments. The western blot bands shown in figure B for gp91phox represents the protein expression as a response to various treatments which are in the same series as in the graph A. Error bars indicate ± standard error of the mean. Statistical analysis was performed using one -way ANOVA. There was no significant difference between the treatments.

5.4.4. Effect of LGG-CM on the expression of gp91 phox at 6 hours

We had previously observed a non-significant difference in gp91phox NADPH oxidase subunit expression at 2 hours incubation in the presence of various dilutions of LGG-CM (Figure 5.23). A confluent monolayer of macrophages was treated with various concentrations of LGG-CM, LPS (20 μg ml^{-1}) and LGG-CM+LPS combination for 6 hours. Following 6 hours incubation, cells were lysed and the protein concentration of cell lysate was determined by BCA assay. After separating the protein on 8 % gel, the proteins were transferred to a PVDF membrane and incubated with a gp91 specific primary antibody followed by incubation with a goat-antimouse secondary antibody.

Figure 5.24A shows the percent change in the expression of gp91 phox from untreated macrophages, LPS and various dilutions of LGG-CM ± LPS with respect to the control at 6 hours. The figure 5.24 B shows the gp91 phox protein bands from various treatments. The sequence in the bands (left to right) is the same as in figure 5.24A. The figure 5.24C represents the expression of β-actin protein from the macrophages incubated with respective treatments and was used to indicate protein loading efficiency.

From figure 5.24, there was a significant increase in the expression of gp91 phox from the macrophages treated with 75 % LGG-CM ± LPS and 100 % LGG-CM ± LPS when compared to control macrophages. A low concentration of LGG-CM ± LPS and LPS expressed gp91phox to the same extent as control macrophages.

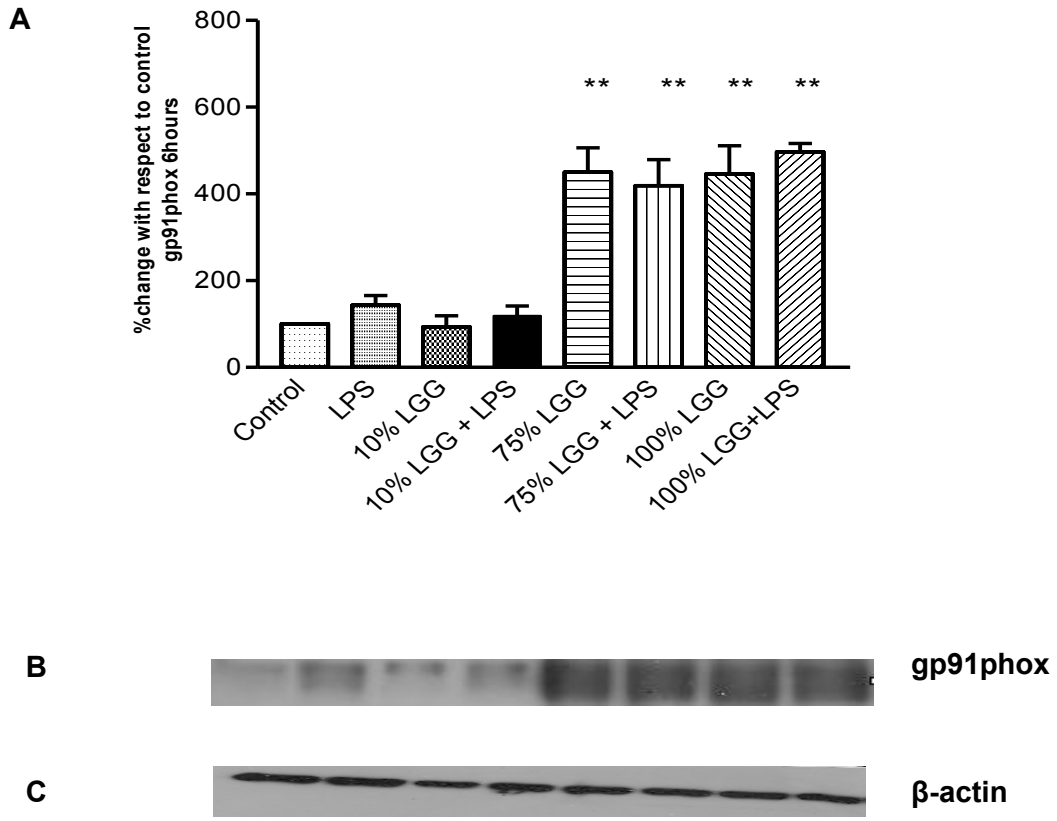

Figure 5.24: Effect of LGG-CM on the expression of gp91 phox at 6 hours incubation in macrophages. The result represents the percent change in percent area with respect to the control (A). Image J was used to analyse the bands of gp91phox protein expression (B). The β-actin expression was used as a control to indicate the sample loading consistency (C). The graph A represents the mean of 3 independent experiments. The western blot bands shown in figure B for gp91phox represents the protein expression as a response to various treatments which are in the same series as in the graph A. Error bars indicate ± standard error of the mean. Statistical analysis was performed using One-way ANOVA followed by Dunnett's multiple comparison post-hoc tests. An asterisk (**) indicates a significant difference to the control ($p<0.01$).

5.4.5. Effect of LGG-CM on the expression of gp91 phox at 24 hours

A significantly increased expression of gp91 phox and p47 phox subunits of NADPH oxidase was observed when the macrophages were incubated with LGG-CM for 6 hours (see Figure 5.22 and 5.24). These results are supportive with the observations made with respect to an increased bacterial digestion rate from macrophages (Figure 4.4) and increased rate of ROS production in the presence of a high concentration of LGG-CM from macrophages (Figure 5.18).

To further monitor the changes in the NADPH oxidase activity at 24 hours, a confluent monolayer of macrophages were treated with various concentrations of LGG-CM, LPS (20 $\mu g\ ml^{-1}$) and LGG-CM+LPS combination. Following 24 hours incubation, cells were lysed and the protein concentration of cell lysate was determined by BCA assay. After separating the protein on 8 % gel, the proteins were transferred to a PVDF membrane and incubated with a gp91phox specific primary antibody followed by incubation with a goat-antimouse secondary antibody (see Section 2.12 for methods).

From Figure 5.25, we found that at 24 hours there was no difference in the gp91phox expression between control, and LPS and LGG-CM ± LPS, treated macrophages.

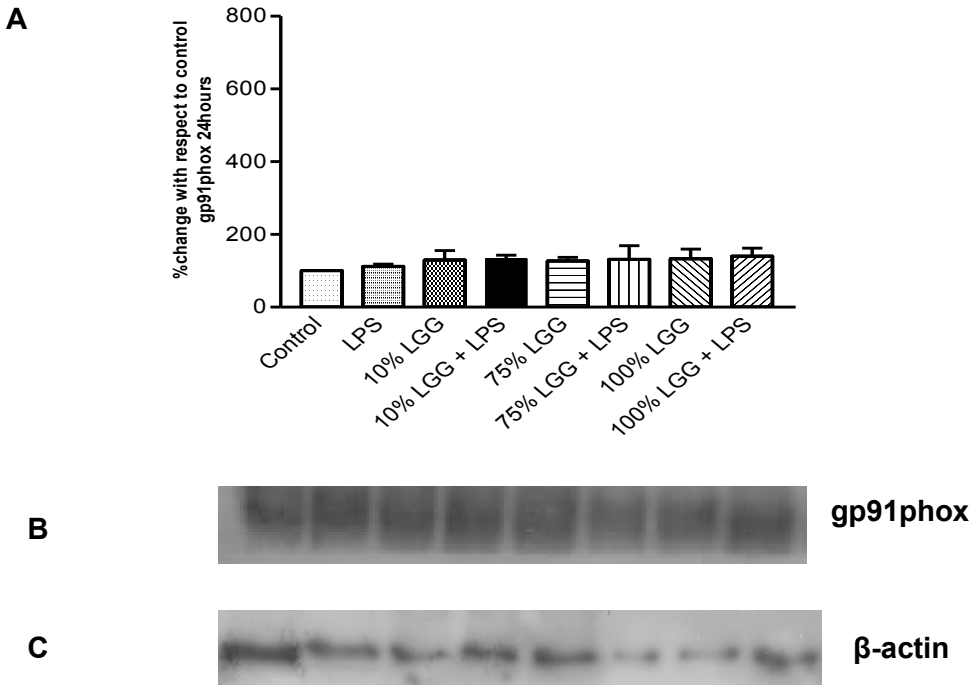

Figure 5.25: Effect of LGG-CM on the expression of gp91phox at 24 hours incubation in macrophages. The result represents the percent change in percent area with respect to the control (A). Image J was used to analyse the bands of gp91phox protein expression (B). The β-actin expression was used as a control to indicate the sample loading efficiency (C). The graph represents the mean of 3 independent experiments. The western blot bands shown in figure B for gp91phox represents the protein expression as a response to various treatments which are in the same series as in the graph A. Error bars indicate ± standard error of the mean. Statistical analysis was performed using One-way ANOVA and there was no significant difference between the treatments.

5.3. Discussion

In chapter 4, the LGG-CM has been shown to have increased the rate of *E.coli* digestion. Both NO and ROS are known to be involved in macrophage digestion. In this chapter role of NO and ROS to LGG-CM induced increased digestion by macrophages was further investigated by using various ROS scavengers and inhibitors for ROS and NO production. The ROS and NO productions from macrophages were also monitored using fluorescence microplate reader. The results indicated that ROS is involved in the process of increased bacterial digestion rate and is produced by NADPH oxidase. To further support these findings western blotting was performed to investigate the expression of NADPH oxidase subunits.

We observed that the treatment of macrophages with higher concentrations of LGG-CM significantly increased the rate of ROS production both in the presence and absence of *E. coli*. Higher concentrations of LGG-CM significantly reduced the rate of NO production both in the presence and absence of *E. coli*. Expressions of NADPH oxidase protein subunits gp91 and p47 phox were found to be enhanced significantly to higher concentration of LGG-CM treatments at 6 hours.

Macrophages phagocytosis has been shown to be associated with the activation of phagocyte NADPH oxidase and iNOS. The NADPH oxidase is a membrane associated enzyme that catalyses the production of superoxide ($O_2^{\cdot -}$) which can be metabolised into a variety of other ROS. The steady state concentration of $O_2^{\cdot -}$ and H_2O_2 in phagosome was found to be 25 μM and 2 μM respectively (Winterbourn *et al*., 2006). Studies have shown that phagocytes produce approximately around 2 μM $O_2^{\cdot -}$ even at pH 4.5. The *E.coli* membrane damage is normally observed at approximately 0.5 μM $O_2^{\cdot -}$ concentration (Imlay., 2009). The *E. coli* DNA and other intracellular protein damage were observed at approximately 0.5 μM H_2O_2 (Slauch, 2011). Thus, ROS produced from activated macrophages acts as a potential antibacterial agent.

The iNOS activates the production of NO from arginine. Unlike superoxide, NO is synthesized on the cytoplasmic side of phagosomes, but it has the ability to diffuse across membranes to reach intraphagosomal targets (Denicola *et al*., 1996) where it encounters ROS. In the intraphagosomal environment, ROS and NO can undergo either spontaneous or catalytic conversion to a range of intermediate compounds such as nitrite (NO_2^-), nitrate (NO_3^-), dinitrogentrioxide (N_2O_3) and dinitrogen tetroxide (N_2O_4) (Darrah *et al*., 2000;

Aktan 2004). The combination of NO and $O_2^{\cdot -}$ to form peroxynitrite ($ONOO^-$) from murine macrophages has previously been shown as potently bactericidal to *Rhodococcus equi* (Darrah *et al*., 2000).

In this study, the NADPH oxidase inhibitor apocynin (apo) and xanthine oxidase inhibitor allopurinol (allo) were used to investigate the role of free radicals on *E.coli* ingestion by macrophages to LGG-CM treatment. There was no significant difference in bacterial recovery at 60 minutes between LGG-CM treated macrophages and macrophages treated with LGG-CM in the presence of apocynin, allopurinol and apo+allo combination (Figure 5.1). These results support the work by Takao *et al*. (1996) where murine peritoneal macrophages had no effect on *Candida parapsilosis* ingestion when treated with allopurinol (xanthine oxidase inhibitor) and Diphenyleneiodonium (DPI) (NADPH oxidase inhibitor).

In our study cell permeable free radical scavengers such as polyethylene glycol (PEG) conjugated SOD, PEG-conjugated catalase and PEG-conjugated SOD + catalase combination were used to investigate the effect of free radicals produced by LGG-CM on bacterial ingestion by macrophages. These experiments demonstrated a significant reduction in bacterial ingestion between 75 % LGG-CM and 75 % LGG-CM and scavenger combinations, indicating the role of free radicals in bacterial ingestion (Figure 5.2). However, Takao *et al*., (1996) found that phagocytosis of *Candida parapsilosis* by murine peritoneal macrophages was unaffected by the antioxidant activity of SOD and/or catalase. The difference in the macrophage response observed in our study compared to Takao *et al*., may be mainly due to the use of PEG conjugated SOD and catalase. The PEG-Catalase and PEG-SOD binds to the cell membrane and rapidly penetrates into the cell, whereas, unconjugated SOD and catalase has limited cellular penetration (Beckman *et al*., 1998). Thus, the PEG conjugated SOD and catalase scavengers can even neutralise the intracellular ROS as they can easily penetrate the macrophage. In our study, macrophages in the presence of scavengers demonstrated a significant reduction in the bacterial ingestion compared to either the control or the LGG-CM treated cells.

The further reduction in the bacterial recovery in the presence of ROS scavengers from control macrophages indicates its involvement in the normal phagocytic process. The ROS are known to play a key role in phagocyte chemotaxis. During chemotaxis, actin polymerisation occurs within the leading edge of the cell membrane driving up-gradient

protrusion, whereas myosin activity in the uropod detaches the rear of the cell. These include a localised polymerisation of F-actin at the site of the cell cortex closest to the chemoattractant source, a morphological change characterised by cell elongation, and the formation of pseudopodia. The ROS has been shown to be involved in the coordination of these polarised events for the directional migration of macrophages and neutrophils (Gresham *et al*., 1988; Fialkow *et al*., 2003, Kuiper *et al*., 2011; Sakai *et al*., 2012).

In this study, bacterial digestion was found to be unaffected by SOD only treatments both in the control or the LGG-CM treated macrophages at 280 minutes. The catalase on its own and in combination with SOD significantly inhibited LGG-CM induced bacterial digestion. However, we did not observe an additional effect on bacterial digestion from LGG-CM treated macrophages coincubated with the catalase and SOD combination (Figure 5.11). Brummer & Stevens, (1989) found that SOD inhibited the killing of *C. parapsilosis* but the catalase did not. They found that *C. parapsilosis* was resistant to killing at physiological concentrations of H_2O_2 and the killing mechanism did not appear to involve hydroxyl radicals. However, the main drawback of their study was that they did not study the bacterial killing in the presence of SOD + catalase combination. Studies by Takao *et al*. (1996) found that the killing of *C. parapsilosis* by murine peritoneal macrophages IC-21 was inhibited by pretreatment with SOD. Catalase did not affect the bacterial killing in their studies. However, they found that the SOD+catalase combination potentiated the inhibitory effect of SOD.

We have found that the higher concentration of LGG-CM significantly increased the rate of ROS production in the macrophages during both the bacterial ingestion and digestion period. This finding is similar to a number of previous studies which have also shown an increased ROS production in macrophages and also in epithelial cells to live probiotic treatments (Farnell *et al*., 2006; Kumar *et al*., 2007). Farnell *et al*. (2006) demonstrated a significant increase in the oxidative burst from chicken heterophils treated with *Bacillus subtilis*, *Lactococcus lactis* and *Lactobacillus acidophilus* bacteria. Donnet –Hughes *et al*. (1999) also observed an enhanced respiratory burst and phagocytic activity from peripheral blood leukocytes in the presence of 10^7 CFU ml^{-1} live *L. johnsonii*.

During the first 60 minutes of incubation, both 75 % LGG-CM and 100 % LGG-CM treated macrophages (both in the presence and absence of *E.coli* and/or LPS) produced a massive amount of ROS. However, macrophages treated with LPS, live *E. coli* and a low

concentration of LGG (10 % LGG) had no effect on ROS production from the macrophages (see Figure 5.4 and 5.5). The ROS measurements from the macrophages during the digestion period from the same treatments showed a diminished rate, but still significantly higher than the rate of ROS production from the control macrophages. The rate of ROS produced from the macrophages were approximately 2 times less during the digestion period when compared to ROS produced during the bacterial ingestion period (see Figure 5.17 and 5.18). This increase in ROS production from macrophages was found to be due to enhanced expression of NADPH oxidase subunits as a response to higher concentration of LGG-CM treatment.

We observed that the macrophages treated with L-NMMA demonstrated a significant reduction in their ability to kill bacteria (Figure 5.12). This result indicates that even the basal level of NO plays a vital role in bacterial killing by both LGG-CM treated and the control macrophages. This effect might be partly due to the synergistic effect of ROS and NO due to formation of their combination product, namely, peroxynitrite. Complete inhibition of NO production from L-NMMA might block the production of peroxynitrite and thereby partly, weaken the ability of macrophages to kill bacteria. There is evidence that both reactive oxygen and nitrogen intermediates are involved in bacterial killing, which is mediated by peroxynitrite (Darrah *et al*., 2000; Brennan *et al*., 2004).

We were not successful in measuring any nitrite by Griess assay within 4 hours of treatments due to it's sensitivity limit in the culture media is in micromolar range (Sun *et al*., 2003). This issue was resolved by using a fluorophore DAFFM-DA to measure NO. It was found that the limit of detection of NO by DAF-FM DA *in vitro* was approximately 3 nM (Kojima *et al*., 1998). In order to study the effect of LGG-CM on NO production, the macrophages were incubated with LPS, various dilutions of LGG-CM and combination of LGG-CM and LPS, both in the presence and absence of *E. coli* for 60 minutes. A significant reduction in the rate of NO level was observed from the macrophages treated with a higher concentration of LGG-CM relative to the control macrophages. The LPS and 10 % LGG-CM alone or in combination, did not have any effect on the NO production by macrophages. This finding remained unchanged by the addition of *E. coli* in the treatments. However, we observed a significant increase in NO production from macrophages treated with 100 μM arginine compared to the control macrophages (see Figure 5.15).

The rate of NO production was further investigated until 280 minutes in the presence of above treatments. It was also observed that the higher concentration of LGG-CM brought

about a significant reduction in the rate of NO production (see Figure 5.19 and 5.20) from the macrophages during the further 280 minutes of incubation time.

There is evidence that NO possibly is not an essential contributor in the control of the acute phase of *Acinetobacter Baumannii* infections in mice (Qiu *et al*., 2009). Weinberg *et al*. (1995) demonstrated negligible expression of iNOS mRNA in the first few hours from human blood monocytes and peritoneal macrophages to LPS treatments. Study by Hattori *et al*. (1996), observed that iNOS mRNA, was barely detectable in unstimulated J774 macrophages. However, it became detectable after 4 hours, when stimulated with 1 μg ml^{-1} LPS. Similar conclusion was also drawn by Stuehr & Marletta (1985) who showed that maximum NO production was achieved in murine macrophages approximately after 10 hours of incubation with the LPS. They observed that within 10 to 12 hours of the macrophage incubation with LPS, the nitrite production increased by 5 to 6 times compared to the control macrophages.

The kinetics of ROS production in our study is quite similar to the findings of Pearson *et al*. (1982). They have shown that an increased respiratory burst occurs within 1 hour of initial phagocytosis of serum-opsonized *Leishmania donovani* promastigotes by the human monocyte derived macrophages. Vanderven *et al*. (2009) also demonstrated a similar kinetics of ROS production in macrophages. In their study ROS production rate was peaked between 20 to 30 minutes after ingestion of oxidation sensitive fluorochrome coated microbeads by macrophages.

Thus, from our findings and existing literature, we can conclude that the J774 macrophages treated with a higher concentration of LGG-CM modulate bacterial phagocytosis by modulating free radicals production. We observed an enhanced NADPH oxidase activity at 6 hours (which is essential for bacterial killing in the acute phase), which is consistent with increased rate of ROS production from higher concentration of LGG-CM (75 % and 100 % LGG-CM). In this study, we did not observe a significant difference in NADPH oxidase expression at 2 hours to LGG-CM treatments (see Figure 5.23). Although we observed an enhanced rate of acute ROS production from the macrophages in response to higher concentration of LGG-CM treatments, this could be due to phosphorylation of the enzyme subunits (Li & Shah., 2004; Ray & Shah., 2005). Thus we have not have seen any increased expression of gp91phox within 2 hours. The antibody used in our study to detect gp91phox was not able to differentiate the phosphorylated and non-phosphorylated state of

enzyme subunits and could be therefore used only for overall relative quantitative expression of these proteins.

Due to the nature of experimental protocols, monitoring ROS production by macrophages using a microplate reader could not be performed for up to 24 hours. In our study 24 hours treatment of macrophage by LGG-CM have failed to produce any increase in expression of NADPH enzyme. This could be due to activation of a protective antioxidant mechanism activated by LGG-CM to reduce the host tissue damage. The excessive ROS production acutely from macrophages might be beneficial for bacterial killing by macrophages but can potentially damage the host if it continues for longer duration.

5.4 Chapter Summary

Free radical production during phagocytosis has significant implications for immune functions. In light of the established role for ROS as a physiological signalling molecule and the emerging role of LGG-CM induced ROS production in macrophage phagocytic activity, this study has investigated the potential regulatory role of LGG-CM in physiological interactions between the host macrophage and the bacteria. Higher concentration of LGG-CM treated macrophages demonstrated a decreased rate of NO production during the bacterial ingestion and digestion period of macrophage phagocytosis. However, macrophages treated with a high concentration of LGG-CM demonstrated a significantly increased rate of ROS production both during the bacterial ingestion and digestion periods. The rate at which ROS was produced due to LGG-CM treatment during the initial 60 minutes treatment is significantly higher than the rate of ROS production after 60 minutes to 280 minutes of incubation. The increased ROS production was found to be due to increased expression of NADPH oxidase components to LGG-CM treatments. Thus, we conclude that the effect of LGG-CM observed is more acute and is elegantly balanced, during the first few hours of bacterial contact with the macrophages. This physiological modulation of free radicals production and enhanced bacterial digestion by LGG-CM by modulating the NADPH oxidase activity is an added advantage to the host during innate immune responses.

Chapter 6

General discussion, Conclusion and Future work

6.0. CHAPTER 6 General Discussion

There is an increasing interest in the field of probiotics to understand the mechanism of probiotic action. This section will address the analysis of the experimental techniques employed in this research and explore the scope, novelty and limitations of the research findings and further understand the mechanism of LGG mediated immunomodulation.
The J774 murine macrophage cell line is an immortalised cell line established from female BALB/c mice. The cell-line has been widely used in studies of macrophage bacterial interactions for a variety of microorganisms including *Brucella abortus* (Ko and Splitter, 2003), *Mycobacterium tuberculosis* (Zhang *et al*., 2005), *Cryptococcus neoformans* (Mukherjee *et al*., 1995) and *E. coli* (Vincenti, 2010) and also responds to probiotic bacteria from human origin (Morita *et al*., 2002). This cell line is also known to respond to LPS treatment and produce NO (Hattori *et al*., 1996; Korhonen *et al*., 2002).

6.1. Experimental methodology: advantages and limitations

6.1.1. Gentamicin protection assay

Gentamicin protection assay, or gentamicin survival assay, is usually used for the measurement of bacterial ingestion by eukaryotic cells (Srivastava & Isberg, 2002; Edwards & Massey, 2011). The method is generally used to study bacterial macrophage interactions because of its simplicity and sensitivity relative to techniques such as Giemsa staining or direct microscopic observation. The principle of the assay is based on the concept that gentamicin cannot penetrate eukaryotic cells. Once the bacteria have been ingested by the macrophage; the intracellular phagosomal bacteria are protected from the antibacterial effect of extracellular gentamicin. Non-internalised but adhered or extracellular bacteria will be exposed to gentamicin and killed. After removal of gentamicin, the eukaryotic cells are lysed to release intracellular bacteria which are then enumerated by colony counting technique (Elsinghorst, 1994; Mandell, 1973; Srivastava & Isberg, 2002; Edwards & Massey, 2011).

Based on the current understanding of the bacterial macrophage interactions, in this thesis it is considered appropriate to use the term ‘ingestion’ to specify bacterial entry into the macrophages. Several studies have used the term ‘invasion’ to indicate bacterial uptake. However, it seems inappropriate in this study as the bacterial target *E.coli* HfrC is a non pathogenic laboratory strain. In the present study gentamicin protection assay was adopted

with some modifications. In the experimental setup the macrophage phagocytosis of *E. coli* was divided into two different stages-

1. Ingestion of bacteria and
2. Digestion of bacteria

Gentamicin protection assay is highly sensitive and a small fluctuation of experimental conditions can interrupt with bacterial phagocytosis. Various factors need to be monitored and maintained in optimum conditions during the gentamicin protection assay. These factors include pH, temperature, CO_2 level, macrophage-bacterial co-incubation time, multiplicity of infection (MOI) and antibiotic concentration.

In our experiments the ingestion studies were performed at two time points. The bacterial ingestion was studied at 30 minutes and 60 minutes. Similar to Vincenti's report (2010) we also observed that macrophages needed at least 30 minutes co-incubation with *E. coli* to recover any countable number of ingested bacteria. Studies by De Koning-Ward *et al.* (1998) have found that 30 to 60 minutes was sufficient for bacteria to be ingested by macrophages without a significant number being digested. Our results are in agreement with this finding that there is no significant difference of *E. coli* recovery between 30 minutes and 60 minutes of incubation. The bacterial killing by macrophages is a culmination of the bacterial ability to bind to the surface of macrophages and the macrophage ability to ingest and finally kill the bacteria within the phagosome. These ingestion and digestion steps can occur as independent steps in sequence and therefore can be easily distinguished by careful experimental procedures.

The J774 macrophages were maintained at a temperature of approximately 37^0C. It was really important to maintain the media in the 24 well plates at approximately 37^0C as changes in temperature would significantly affect the process of phagocytosis (Li *et al*., 2002). A study by Peterson *et al*., (1976) demonstrated a significant reduction of *Staphylococcus aureus* and *S. epidermidis* ingestion by human leukocytes when the incubation temperature was raised from 37^0C to 41^0C. Similarly, Li *et al*., (2002) showed that phagocytosis of *M. tuberculosis* by human macrophages was significantly decreased when the incubation temperature was reduced to 23^0C and 30^0C.

Generally, gentamicin protection assay uses a MOI of about 10 bacteria per eukaryotic cell (see Table 4.1). In the present study the MOI was optimised by using a number of MOI to

suit the J774 cell line (Elsinghorst, 1994). Several pilot experiments were performed at a higher MOI (100:1) which yielded inconsistent bacterial recovery in our study. This was assumed to be due to the increased susceptibility of macrophages to bacteria at higher bacterial inoculums which in turn lead macrophages rendering to detach easily during the washing steps. At this MOI, we also observed an increase in the background levels of bacteria. This was possibly due to release of *E. coli* from the dead macrophages.

Therefore, modification to the gentamicin protection assay was essential in order to retrieve the actual bacterial count. Following this, experiments were performed at a lower MOI of 10:1. However, at this MOI, the macrophages treated with LGG-CM yielded a very low bacterial count. The next attempt was to use a MOI of 50:1 as mentioned in Gille *et al*. (2009). This MOI was found to be a balanced standard bacterium to macrophage ratio without affecting macrophage viability at least during the time course of the experiment. The background bacterial recovery observed from plating the extracellular medium was negligible. This could be due to the effect of lower concentration of gentamicin (20 $\mu g\ ml^{-1}$) in the media which had killed any accidental release of bacteria from any morbid cells. Lower concentration of gentamicin would also ensure no further bacterial replication in the media.

The gentamicin is a broad-spectrum antibiotic that exerts its bactericidal activity by selectively binding to the bacterial ribosome and interfering with protein synthesis (Magnet and Blanchard, 2005). The minimum inhibitor concentration (MIC) for *E. coli* ATCC 25922 and *E. coli* LU2 from literature was found to be 0.5 mg L^{-1} (Isaksson *et al*., 1988).

The gentamicin concentration used in this study was found to be an important factor for the reproducibility of the data. A higher concentration (200 $\mu g\ ml^{-1}$) of gentamicin was initially used for approximately 10 minutes to kill most of the extracellular bacteria as in Gille *et al*. 2009. Following this initial killing, 20 $\mu g\ ml^{-1}$ gentamicin was constantly maintained in the medium until the macrophages were lysed. This lower concentration of gentamicin was found to be essential to maintain macrophage health and its attachment capability during the time-course of the experiment. This was also found to be necessary to recover live *E. coli* reproducibly from the lysate. The killing of extracellular bacteria in our experiments was confirmed by direct microscopic observation and also by the inoculation of an extracellular medium on to nutrient agar plates.

Observations of the inability of the gentamicin and other antibiotics to kill the intracellular bacteria were used in early studies investigating the invasion of host cells by bacteria (Vaudaux & Waldvogel, 1979; De Melo & Pechere, 1988; Hardt *et al*., 1998). However, there are reports suggesting that gentamicin added to the extracellular media can actually cross the cell membrane and kill the internal bacteria (Ohya *et al*., 1998; Eze *et al*., 2000; Tsang *et al*., 2000). Gentamicin is thought to enter eukaryotic cells through pinocytosis. It is shown to accumulate within the pinocytotic endosomes, reaching the phagosomes by fusion of the endosomes with the pathogen-containing phagosomes (Drevets *et al*., 1994). However, studies by Menashe *et al*. (2008) demonstrated that gentamicin at a concentration of between 15 and 150 μg ml^{-1} did not kill intracellular *Salmonella*. After infecting the macrophages with *Salmonella*, the cells were treated with gentamicin for one hour. When 50 μg ml^{-1} or a lower concentration of gentamicin was used, the intracellular viable bacterial counts did not change significantly. Higher antibiotic concentrations (up to 150 μg ml^{-1}) resulted in a log reduction in the viable counts ($P < 0.01$) at 60 minutes. Their study did not increase the antibiotic concentration beyond 150 μg ml^{-1}, as gentamicin at higher concentrations and a longer incubation period changed the morphology of the host cells (Menashe *et al*., 2008).

Studies have demonstrated that the antimicrobial activity of aminoglycosides is pH dependent and is strongly inhibited at a low pH (Menashe *et al*, 2008). Low pH changes the electrostatic potential across the bacterial cytoplasmic membrane, and this electrostatic potential is crucial for the uptake of aminoglycosides into the bacterial cell (Eisenberg *et al*, 1984; Xiong *et al*., 1996; Taber *et al*, 1987). It has been observed that the bacteria-containing phagosomes acidify within less than 60 minutes after their formation (Rathman *et al*., 1996). Thus, the low pH in phagosomes could affect both the uptake of aminoglycosides into the bacterial cell and their antibacterial activity. Indeed, a previous observation indicated that a direct increase in the pH of the phagosomes was sufficient to significantly increase the intracellular killing effect of *Staphylococcus aureus* by the aminoglycoside amikacin (Maurin and Raoult, 1994).

Studies by Kim *et al*., (2006b) estimated the number of internalised *Salmonella enterica serovar typhimurium* by employing bacteriophage SP6, a *Salmonella* specific lytic bacteriophage, by immunostaining and fluorescence microscopy. They demonstrated that gentamicin at a concentration of more than 20 μg ml^{-1} can accumulate inside the macrophage influencing the bacterial number. However, they concluded that gentamicin might still be useful at concentrations upto 20 μg ml^{-1} but not above (Kim *et al*., 2006b).

Thus, we can summarise that gentamicin can still be used at a high concentration but not for an extended period of time. Using a lower concentration of gentamicin after 60 minutes also ensures the minimal or negligible role of gentamicin in intracellular bacterial killing.

Several other approaches are used in different studies to remove the extracellular bacteria. This includes the use of complement and bacteriophages. The use of complement is least satisfactory because it facilitates phagocytosis. On the other hand bacteriophages lyse the cell wall of susceptible bacteria by their lytic enzymes (Shaw *et al*., 1983; Kim *et al*, 2006b). However, this needs the bacteriophage recognising specific receptors and attaching to the surface of bacteria. This poses a problem when the bacteriophage fails to eradicate all the extracellular bacteria as any remaining bacteria can easily multiply in the nutritive macrophage culture medium. The rapid development of phage resistant bacteria was shown to be difficult to eradicate with this method. Studies by Kim *et al*., (2006b) used the bacteriophages to eradicate *Salmonella* by replacing the media containing SP6 bacteriophage every 2 hours to remove any phage resistant *Salmonella*. They concluded by saying that this problem can be overcome by employing a cocktail of different types of lytic phages. Another method to overcome these technical difficulties is by utilising the bacteria that are unable to replicate in the media used to maintain the macrophages (Straley and Harmon, 1984). However, challenges may still exist as the above system might just provide bacteriostatic conditions as one won't be efficiently able to remove bacteria adhered to macrophages.

A study by Tabrizi and Robins-Browne, (1993) quantified the bactericidal capacity of phagocytes by removing the extracellular bacteria from the reaction mixture before intracellular bacteria were released by macrophage cell lysis. Gentamicin was used to kill the extracellular bacteria. However they enzymatically inactivated the antibiotic using the enzyme gentamicin acetyl transferase (GAT). This reduced the number of washing steps and therefore reduced the probability of the inadvertent removal of macrophages from the reaction mixture.

To summarise, these assays are variable on a day to day basis. This variability is attributed to an overall physiological state of both prokaryotic and eukaryotic cells (Elsinghorst, 1994). The uptake of gentamicin by bacteria is influenced by the bacterial membrane potential, cell membrane integrity and it also requires active protein synthesis. Several environmental conditions such as low pH, low oxygen and the accumulation of metabolic products in the cultured media may render an antibiotic less effective (Davis *et al*., 1987).

This is evident from the variation in the bacterial recovery observed in previous studies as shown in Table 4.1).

6.1.2. Use of Pharmacological inhibitors and scavengers

In this study a number of pharmacological inhibitors and scavengers were used in the gentamicin protection assay to identify the effect of LGG-CM on ROS and NO production during bacterial macrophage interactions. The ROS inhibitors used were apocynin (an NADPH oxidase inhibitor) and allopurinol (a xanthine oxidase inhibitor) and L-NMMA was used to inhibit NO production. Cell permeable SOD and catalase (PEG conjugated) were used to scavenge both extra and intracellular free radicals from the macrophages.

Apocynin is a reversible inhibitor of NADPH oxidase. Preincubation of human primary macrophages with apocynin has been shown to prevent both membrane translocation of p47phox and superoxide production by human primary macrophages (Ye *et al*., 2011). It has been reported that apocynin does not act directly as an inhibitor of NADPH oxidase; it must be pre-activated by hydrogen peroxide or peroxidase for its effect (Simons *et al*., 1990). Phagocytic cells, such as macrophages, have a low level of hydrogen peroxide as well as myeloperoxidase (Kumar *et al*., 2004; Tseng & Kung, 2012) and hence apocynin does not require to be activated in these cells.

Apocynin has been shown to inhibit ROS production in both phagocytic and non-phagocytic cells (Stefanska & Pawliczak, 2008). However, a study by Vejrazka *et al*. (2005) using apocynin to inhibit NADPH oxidase activity, found that treatment with apocynin, surprisingly bought about a significant increase in ROS production from vascular fibroblasts. Their study concluded that apocynin inhibits ROS production in phagocytes such as macrophages, but stimulates ROS production in non-phagocytic cells since they do not have any constitutive peroxidase activity.

On the other hand, studies have also proved the inhibitory effects of apocynin on ROS formation in non-phagocytes as well (Holland *et al*., 2001; Beswick *et al*., 2001; Thabut *et al*., 2002). The conditions, under which these experiments were performed, however, were different. Cells or tissues were treated with apocynin several hours, or even several days before ROS measurement began. It can be assumed that the slower production of H_2O_2 by non-phagocytes was sufficient in these cases, and that the peroxidase activity was probably

replaced by the pseudo-peroxidase effect of iron and other transition metals present as ubiquitous contaminants (Vejrazka *et al*., 2005).

In summary, the activation of apocynin depends on its oxidation by H_2O_2 in the presence of a peroxidase activity, either externally added or produced by macrophages. It is known that symmetrical 5, 5′ dimer (that can be formed in such a reaction) is a potent inhibitor of NADPH oxidase assembly (Johnson *et al*., 2002). Thus, the effect of apocynin varies amongst various experimental conditions and should be carefully monitored and tailored if it is used as a ROS inhibitor. In our study, macrophages were pre-treated with apocynin for 60 minutes prior to the addition of bacteria while investigating the role of NADPH oxidase mediated ROS production on bacterial phagocytosis.

The L-NMMA competitively inhibits the generation of NO from arginine and is a useful tool for the inhibition of NO mediated effects. It is cell-permeable and is an L-arginine analog that acts as a competitive inhibitor of all three isoforms of NOS and is readily available. It is probably still the most widely used, investigative tool in elucidating the potential role of iNOS. Studies by McCall *et al*. (1991) have found that L-NMMA (100 μM) needs a preincubation period of 50 minutes to achieve its full inhibitory activity in both J774 murine macrophages and rat peritoneal neutrophils. In this study, the macrophages were preincubated with 100 μM L-NMMA for 60 minutes prior to the addition of bacteria while investigating the role of NO on bacterial killing by gentamicin protection assay. The fact that L-NMMA undergoes slow processing at the active site of NOS being metabolised several times to inactivate the enzyme, probably contributes to the necessity of the preincubation period (Olken and Marletta, 1993).

In macrophages, iNOS is thought to account for most of the observed NO dependent microbial killing (Stuehr and Nathan 1989; Green *et al*., 1990; Schapiro *et al*., 2003). Study by Lorsbach *et al*. (1993) showed that iNOS m- RNA was not detected until 4 hours after induction with LPS and 2 hours after induction with LPS and interferon-γ in murine macrophages. Given the time course of our study it is unlikely that iNOS could account for the observed macrophage mediated bacterial ingestion and digestion in our model. However, it is difficult to rule out that the basal level of NOS potentiating the bactericidal activity, perhaps via the formation of peroxynitrite (Zhu *et al*., 1992; Evans *et al*., 1996; Darrah *et al*., 2000; Brennan *et al*., 2004).

Allopurinol is a selective inhibitor of xanthine oxidase (Das *et al*., 1987; Moorhouse *et al*., 1987; Takao *et al*., 1996). Allopurinol was used in this study to investigate the role of xanthine oxidase in bacterial killing by J774 macrophages. Xanthine oxidase is one of the sources of ROS in the biological system, however a previous study shows that peritoneal macrophages contain a scant amount of xanthine oxidase (Jarasch *et al*., 1986) and might not exist at the biologically important concentrations.

At a low concentration, allopurinol is a substrate and a competitive inhibitor of enzyme xanthine oxidase and at higher concentration; it is a non-competitive inhibitor (Pacher *et al*., 2006). *In vitro* studies by Joh *et al*. (1994) have shown that allopurinol (100 μM) completely inhibits the activity of xanthine oxidase (Joh *et al*., 1994). Consistent with the studies by Joh *et al*. (1994), experiments to investigate bacterial ingestion and digestion in the presence of allopurinol were performed after preincubating the inhibitor (allopurinol) with macrophages for 60 minutes.

In this study we have used PEG conjugated SOD and catalase to scavenge ROS. The enzyme SOD catalyses the production of H_2O_2 from superoxide which is then split into water and oxygen by the enzyme catalase. The PEG conjugation of SOD and catalase enhances the cell association of these enzymes in a manner which increases cellular enzyme activities and provides prolonged protection from intracellular proteases (Beckman *et al*., 1988). The PEG conjugation of these enzymes brings about an effective scavenging of free radical activity both *in vivo* and *in vitro* (Carter *et al*., 2004; Franchini *et al*., 2013).

6.1.3. Use of fluorescent dye to measure ROS and NO

Macrophages were grown on an optically accessible surface, such as glass bottomed cell culture wells or 96 well plates. The black 96 well plates were used, as the background fluorescence levels because of auto-fluorescence is low in black 96 well plates. The use of black 96 well plates also resolves the cross-talk in fluorescence measurements in adjacent wells and results in better sensitivity.

For ROS measurements, the macrophages were labelled with H_2DCFDA, a dye which has been widely used to monitor intracellular ROS production in general from intact cells (Kalyanaraman *et al*., 2012; Winterbourn, 2014). A loading concentration of 5 μM was

used as it gave consistent results and is similar to that which has previously been used in human intestinal epithelial cells (Lin *et al*., 2009). At this concentration the fluorescent dye was non-toxic and suitable for detecting the signals with negligible background noise. Experiments were performed using a medium without phenol red as it reduces the interference of fluorescent dyes.

The H_2DCFDA is cell permeable and is hydrolysed intracellularly to the DCFH anion which after two electron oxidation results in the formation of fluorescent dichlorofluorescein (DCF). The fluorescent intensity of DCF is directly proportional to intracellular ROS generation (Klien *et al*., 2012). Although the fluorescence dye H_2DCFDA has been widely used to measure oxidative bursts in phagocytes such as macrophages and neutrophils (Zielinska *et al*., 2001; Caldefie-Chézet *et al*., 2002), there are reports that peroxynitrite can also interfere with the formation of DCF in cellular systems (Possel *et al*., 1997; Crow,1997; Wang and Joseph 1999; Kalyanaraman *et al*., 2012) and therefore the probe H_2DCFDA is mainly used as a marker of general ROS production rather than as a specific marker of any one of the ROS products (Gomes *et al*., 2005). The advantages, pitfalls and limitations of these fluorescent dyes have previously been well discussed in a number of reports, but this dye is still considered as a convenient fluorescent probe for measuring the general aspects of intracellular ROS production (Halliwell & Whiteman, 2004; Dikalov *et al*., 2007; Kalyanaraman *et al*., 2012; Dikalov & Harrison 2014; Winterbourn 2014).

Reliable measurements of NO and its derivatives in biological systems presents significant challenges due to its short half life. The majority of studies investigating the role of RNS during bacterial macrophage interactions mainly concentrate on the stable end products of NO such as nitrite and nitrate (Sun *et al*., 2003). The NO does not fluorescence itself and it needs to be labelled with NO specific fluorophores such as DAFFM-DA. In our study, the requisite incubation time for the DAF probes with macrophages was 45 minutes at 5 μM concentration. Earlier studies have also demonstrated that the requisite incubation time for DAF probes was less than 2 hours (Nakatsubo *et al*., 1998). The vast majority of studies incorporating the use of DAF's have only reported relative changes in the fluorescent signal (Nagy *et al*., 2004; Planchet *et al*., 2006; Nyberg *et al*., 2010). The diacetate derivative of DAFFM has some advantages over another commonly used NO sensing fluorescence probe DAF-2. The main advantage is that the spectra of NO adduct of DAFFM-DA are independent of pH above 5.5 and it is more photostable than DAF-2.

These fluorescent probes may be used to measure nanomolar levels of NO both *in vitro* and *in vivo* (Namin *et al*., 2013). The detection limits of DAFFM-DA is ~3nM compared to DAF-2 which is ~5 nM.

6.1.4. Use of Griess assay to measure nitric oxide.

This is a widely used technique used as an index of integrated NO production estimated from determining the concentrations of nitrite and nitrate end products. In this study we could not detect nitrite accumulation within the first 5 to 6 hours of macrophage incubation with various treatments. However, nitrite production from macrophages as a response to various treatments was easily detectable at 24 hours. This could also be attributed to the sensitivity of Griess assay. The two main limitations of Griess assay is the sensitivity limit and experiment duration. Detecting nitrite production from macrophages requires an incubation time of approximately 9 to 40 hours (Nakatsubo *et al*., 1998). There is a variation in the reported sensitivity limit for Griess assay in the literature. In biological fluids the sensitivity limit for Griess assay is about 0.4 to 300 μM (Green *et al*., 1982; Pratt *et al*., 1995; Sun *et al*., 2003).

6.1.5. Strength of Cusum analysis

In this study, CuSum analysis has provided useful evidence regarding the acute change of the rate of free radicals generation from pre-existing levels. The interpretation is most robust when detecting a change, or irregular fluctuations, or shifts in free radical production (Stanton *et al*., 1992). The CuSum method is quite simple as it perceives the variation without assuming any functional form of the data. If the measured fluorescence value of free radical production from the experiment is equal to the target value (average fluorescence from untreated cells) the slope would be zero. But if the measured fluorescence value of free radical production from the experiment is greater than the target value, the slope will be a positive and vice versa. The greater the difference between the measured fluorescence value and the target value, the greater the slope will be. The slope of the plot over any given time period (the change in the CuSum over that period divided by the period duration) indicates the average deviation of free radicals generation from the mean control ROS generation during that period. The plot facilitates the calculation of the relative rate of ROS production and the derivation between the treatments.

6.2. General discussion on experimental findings

The results in this thesis have demonstrated the role of LGG-CM on the modulation of macrophage phagocytic activity. The MTT assay demonstrates that LGG-CM is non-toxic to the J774 macrophage cell line. It also has no acute antibacterial activity on *E. coli*. There are reports suggesting that, the antibacterial activity of LGG-CM was mainly because of low pH. However, in this study LGG-CM was neutralised to pH 7.4 ± 0.2 and no antibacterial activity was observed at this pH. In this study macrophage phagocytosis was investigated separately as ingestion (uptake) and a digestion (killing) phase. The LGG-CM treated J774 macrophages demonstrated a significant reduction of *E.coli* ingestion. Reduced bacterial uptake was also observed when the macrophages were incubated with LPS. However, the bacterial digestion rate by macrophages was found to be enhanced when the macrophages were incubated with a higher concentration of LGG-CM. The LPS had no effect on the bacterial digestion rate by the macrophages. The present study also explored the role of ROS and NO generation in both the ingestion and digestion phases of phagocytosis modulated by LGG-CM. This was investigated by using a number of pharmacological inhibitors of ROS such as apocynin, allopurinol; NO inhibitor L-NMMA and also cell permeable free radicals scavengers PEG-SOD and PEG-catalase.

The results from our experiments have demonstrated that LGG-CM increased the *E.coli* digestion rate by enhancing ROS production. Enhanced ROS production to LGG-CM was found to be due to an increase in the expression of NADPH oxidase subunits in J774 macrophages.

6.2.1. Variation of Bacterial ingestion by macrophages

Studies monitoring the bacterial ingestion by macrophages at 30 and 60 minutes in the presence of LPS demonstrated a lower bacterial ingestion. The LPS mediated reduction in bacterial ingestion by macrophages has been shown previously (Eder *et al*., 2009; Sivagnanam *et al*., 2010). The Gram negative bacteria possess LPS in its outer cell membrane component and are recognised by TLR-4 on the macrophages. Thus, when the macrophages are exposed to LPS and live bacteria, there is the possibility of competition for TLR-4 receptors on the macrophages (Hornef *et al*., 2003; Dunzendorfer *et al*., 2004).

A possible similar mechanism could explain the lower bacterial ingestion in the presence of LGG-CM treated macrophages. A growing amount of evidence indicates that LGG-CM contains a number of components, or factors, which are released either from the cell membrane or secreted constitutively from the bacteria (Oelschlaeger, 2010). However, it is not known if any of the secreted components of LGG could compete for TLR-4 receptors and hence the reduced uptake of *E. coli* in the presence of LGG-CM. Generally, recognition of Gram positive bacterial cell wall components by innate immune cells occurs through TLR-2 (Yoshimura *et al*., 1999). Thus one possible mechanism of reduction in bacterial ingestion in the presence of LGG-CM in the present study may be due to the non-specific inhibition of certain receptors by components of LGG-CM instead of receptor competition. However, there are studies where probiotic bacterial components have been shown to block bacterial binding sites in mice (Aiba *et al*., 1998) and humans (Rolfe 2000; Hamilton –Miller 2003; Wang *et al*., 2004). But the mechanism is yet to be established.

The lower recovery of bacteria in the presence of the LGG-CM might also be due to the down-regulation of macrophage signalling mechanisms involved in phagocytosis rendering the macrophages to a less active state. It is quite challenging to argue that reduced bacterial ingestion could be due to the down-regulation of the activation state of macrophages as we have seen a significant difference in the acute ROS production to LGG-CM treated macrophages. Therefore, in this study, reduced bacterial ingestion in the presence of LGG-CM might be attributed to the activities of LGG components at receptor level (Roselli *et al*., 2006; Wong *et al*., 2013).

Pharmacologic inhibitors such as apocynin and allopurinol had no effect on bacterial ingestion. Apocynin and allopurinol block the activities of the enzymes namely, NADPH oxidase and xanthine oxidase and, thereby, prevents ROS production. However, these inhibitors would not be able to stop ROS production from macrophages completely since the arachidonic acid pathway also contributes to ROS production in macrophages (Griendling *et al*., 2000; Sakuma *et al*., 2012) and could maintain a basal level of ROS production to regulate its physiological functions such as calcium regulation (Klebanoff 2005). Interestingly, in this study, macrophages in the presence of PEG-SOD and PEG-catalase treatments demonstrated a significant further reduction in bacterial ingestion. This can be attributed to the effectiveness of cell permeable PEG-SOD and PEG-catalase to scavenge the free radicals from any source and thereby resulting in reduced bacterial recovery.

6.2.2. Role of LGG-CM induced ROS in bacterial digestion

In the present study an increased rate of *E.coli* digestion was observed when macrophages were treated with LGG-CM. This increased rate of digestion is found to be due to enhanced ROS production in the LGG-CM response.

In this study, the macrophages increased ROS production in response to LGG-CM is primarily due to the activation of NADPH oxidase. Phosphorylation of p47 phox is shown to be essential for NADPH oxidase activation in phagocytes such as neutrophils (Li & Shah, 2004). Ray and Shah, (2005) suggested that unphosphorylated p47 phox would have inhibitory effects on NADPH oxidase activity. Phosphorylation of p47 phox relieved these inhibitory effects and at the same time increased NADPH oxidase activity.

In the present study NADPH oxidase subunits gp91 phox and p47 phox were measured at different time points. We did not observe any significant change in the gp91 phox and p47 phox expression in the first 2 hours of incubation with LGG-CM treatments; wherein at 6 hours of incubation time, LGG-CM treated macrophages demonstrated a significant increase in the expression of gp91 phox and p47 phox compared to untreated macrophages. This is in line with the findings from previous studies that NADPH oxidase activation occurs over the short term through phosphorylation of the enzyme subunits and over the long term by increased subunit expression (Li and Shah., 2004; Ray and Shah., 2005). We also observed no significant difference of gp91 phox and p47 phox expression between the control and the LGG-CM treated cells at 24 hours.

CuSum data analysis also demonstrated that there is a variable rate of ROS production from macrophages during the first hour (ingestion period) and also approximately 280 minutes of LGG-CM treatment (digestion period). Despite the sustained elevation of ROS production observed in the presence of LGG-CM compared to the control cells throughout the experiments, there is a differential rate of ROS production between these two phases. When the macrophages were treated with a higher concentration of LGG-CM, the ROS production rate during the first hour of treatment was approximately 2 times higher than the ROS production rate during the next 280 minutes. Similarly, from the macrophages treated with the positive control menadione (0.5 mM), the rate of ROS production from the

macrophages almost doubled during same period of incubation when compared to the rate of ROS production during first 60 minutes.

In this study the LGG-CM mediated ROS production in the macrophages appears to happen in a highly controlled manner where the macrophages probably regulate ROS production by oscillating the ROS production. This sort of ROS regulation has been previously demonstrated in plant root hairs and guard cells and also during pollen stigma interactions (Monshausen *et al*., 2007; Takeda *et al*., 2008; McInnis *et al*., 2006; Jammes *et al*., 2009). Detection of the oscillating pattern of ROS signals in plants clearly supports our current findings where our raw data for free radical production demonstrates an oscillatory pattern. It has been postulated that maintaining a nontoxic steady-state level of ROS, while allowing for the transient accumulation of ROS in particular subcellular locations, could have a physiological significance and could act as signals (Mittler *et al*., 2004).

Excessive ROS generation causes oxidative stress which has been implicated in many disease processes (Veal *et al*., 2007). Disproportionate, or prolonged, ROS generation may result in the non-specific oxidation of biomolecules and contribute to tissue injury. High ROS production has also been implicated as a cause of the deleterious suppression of NFκβ mediated cell survival factors (Storz & Toker 2003). In gut epithelial cells, LGG has been shown to induce an increased ROS production through NADPH activation. However, this increase in ROS has been suggested to play a role as an anti-inflammatory by inhibiting NFκβ through oxidative inactivation of the key regulatory enzyme Ubc12 (Kumar *et al*., 2007; Lin *et al*., 2009). A similar suppression of NFκβ activation was also demonstrated in liver cells to probiotic treatment (Li *et al*., 2003). This suppression was due to the reduced activity of Jun N-terminal kinase (JNK); a regulatory enzyme found to be modulated by ROS (Zhang & Chen, 2004). It seems that ROS mediated suppression of NFκβ may account to regulate physiological inflammatory susceptibility of the cells to probiotic bacteria.

6.2.3. Modulation of NO by LGG

In this study, we investigated the role of LGG-CM on NO production by J774 macrophages acutely and also after 24 hours of treatments. Macrophages treated with

LGG-CM for 24 hours produced a basal low level of NO. This is consistent with a study by Pipenbaher *et al*. (2009) where several live *Lactobacilli* demonstrated a low basal level of NO production from pig alveolar macrophages.

In our study, the macrophages produced a massive amount of NO to LPS treatment at 24 hours. However, when LGG-CM was added to LPS, there was dose dependent reduction of NO production by macrophages at 24 hours. A low level of NO plays an important role by protecting the intestinal mucosa and resolving gut inflammation (Brown, 2003; Pipenbaher *et al*., 2009). Under pathogenic stress, or as a response to inflammatory stimuli, macrophages induce increased NO production from iNOS (Korhonen *et al*., 2001). Over expression of NO has been found to be involved in condition such as ulcerative colitis (Alican and Kubes 1996). Numerous studies have shown that the chronic inflammation associated with IBD is due to the prolonged activation of iNOS and thereby NO production. The NO produced is further known to contribute to tissue injury (Rachmilewitz *et al*., 1995). Studies by Toumi *et al*. (2014) have shown that probiotic supplementation reduced NO production and iNOS expression in colitic mice.

In the present study a reduced rate of NO production was observed acutely from macrophages when treated with the higher concentration of LGG-CM. Previous studies have reported differential kinetics of ROS and NO production by macrophages in response to parasite infections. A study by Gantt *et al*. (2001) detected the ROS production (within 40 minutes) occurring early after *Leishmania chagasi* phagocytosis, whereas the effect of NO occurred later (48–72 hours after infection) from murine and human macrophages.

These observations are consistent with our studies where we also observed a rapid ROS production from macrophages, but failed to detect any enhanced NO production. The reduction in the rate of NO production from the higher concentration of LGG-CM treated macrophages in our study is probably due to the inactivation of NO by ROS. Interestingly, there is evidence in the central nervous system that SOD could increase the toxic effects of NO by inhibiting the superoxide mediated inactivation of NO (Oury *et al*., 1992). The NO can react rapidly with superoxide to generate the stable peroxynitrite anions. This pattern of elevated levels of ROS leading to a decrease in NO bioavailability has been described in endothelial cells (Hsieh *et al*., 2014). The scavenging of superoxide by SOD has also been shown to increase the half-life of the endothelium-derived relaxing factor (NO) and to preserve the biological activity of NO measured by platelet aggregation (Gryglewski *et al*.,

1986; Rubanyi *et al*., 1986). Thus, it is likely that the acute reduced rate of NO production to LGG-CM, in our study, is probably due to the enhanced ROS generation in the macrophages.

It has been shown that peroxynitrite might further decrease iNOS expression by nitration reactions (Connelly *et al*., 2001; Aktan 2004). Peroxynitrite has been shown to oxidise the iNOS cofactor BH_4 into inactive dihydro-L-biopterin and, thereby, results in NOS reactions being uncoupled (Milstien & Katusic, 1999; Kopincova *et al*., 2011). Uncoupling of the iNOS further enhance ROS production as NADPH consumption increases independently of L-arginine oxidation reactions. Thus, it seems NO and ROS together determines the fate and reactions of their own production or inhibition. Thus, the production of low level NO, after 24 hours of treatment, could be a balancing act of LGG-CM between NO and ROS production to regulate the homeostatic mechanism of macrophage phagocytosis and the prevention of collateral tissue injury. A summary figure of modulation of macrophage phagocytosis by LGG-CM has been shown in figure 6.1.

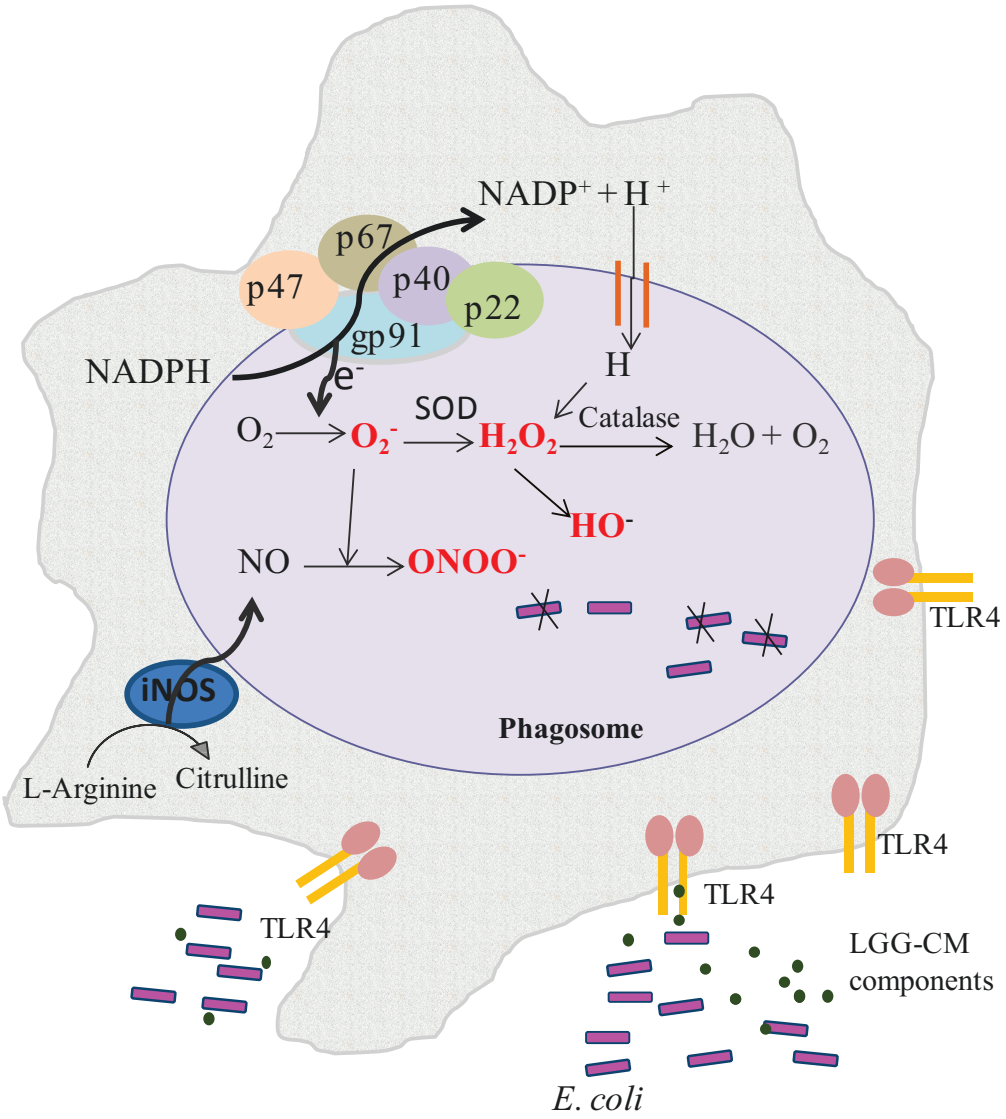

Figure 6.1: Modulation of macrophage phagocytosis by LGG-CM. In the presence of LGG-CM macrophages ingest significantly less bacteria. This might be due the components secreted by the LGG in the culture medium which might block the bacterial ingestion by non specific receptor interactions. The LGG-CM stimulated macrophages produced elevated level of ROS. This ROS then combines with NO to form peroxynitrite. Elevated peroxynitrite further reduces the NO bioavailability by inhibiting NO production by iNOS by decoupling reactions. The production of free radicals highlighted in red are probably accelerated in presence of LGG-CM which results in increased bacterial killing. [Hydrogen peroxide (H_2O_2), Hydroxyl (OH), Nitric oxide (NO), Peroxynitrite ($ONOO^-$), Reactive oxygen species (ROS), Toll-like receptor 4 (TLR-4).

6.3. Recap and Conclusions

This section provides an overview of the research by restating the objectives along with a description on how they have been met. The first chapter was concerned with introducing the background knowledge of the research question and sets the background for the research through establishing the aims and objectives. The subsequent chapters were designed to achieve these objectives and thereby meet the aims of our project. This study was set out to explore the effect of cell free LGG-CM on bacterial macrophage interactions. This study has also sought to understand the mechanisms involved in LGG-CM mediated bacterial-macrophage interactions, particularly with respect to LGG-CM mediated ROS production from macrophages. Major findings in the course of achieving these objectives have been listed below. The final section in this chapter states how this research generates a platform for future work for the advancement of knowledge.

From the literature review in chapter 1 we found that probiotic bacteria have been shown to demonstrate immunomodulatory activity, however the functional properties attributing these effects were unclear. The research, therefore, aimed to investigate the effect of probiotic conditioned media from a single probiotic strain *Lactobacillus rhamnosus* GG on immune cells like macrophages and understand the bacterial-macrophage interactions.

The research aimed at investigating and understanding the following:

1. The effect of cell-free LGG-CM on bacterial phagocytosis by macrophages
2. The physiological mechanisms attributed by LGG-CM in modulating macrophage phagocytosis with respect to free radical production.

Understanding the LGG-CM mediated immunomodulatory activity enables the host to achieve the probiotic health benefits in a more informed manner. The step by step conclusion approach reiterates the objectives set out in Chapter 1 and describes vital findings during the process of accomplishing these objectives towards meeting the purpose or aim of the research.

6.3.1. Understanding the role of probiotics as immunomodulatory agents

The Objective 1 is achieved through the literature review conducted in Chapter 1. The literature review on the effect of probiotics and their influence on the immune system

mainly concentrated on the effect of LGG-CM on immune cells such as macrophages at the component level. Information was also gathered on macrophages and their defence mechanisms such as iNOS and NADPH oxidase. The literature review was then oriented towards the role of free radicals in macrophage phagocytosis. Attempts have been made to summarise the literature in the form of table to provide more collective information. Each section in the literature review chapter (to achieve Objective 1) has a vast diversity in the terms of details included. Careful decisions were made to include or exclude the topics in the introductory chapter as this form the basis for the future five chapter objectives. The major findings from the literature review was that although an enormous amount of data is available to demonstrate probiotics modulates phagocytosis, there is no specific mounting evidence on the physiological modulation of the whole process. This study fills the gap in the existing literature by explaining the functional mechanisms behind the observed probiotic mediated phagocytic modulation by using LGG- CM.

6.3.2. Designing various approaches to investigate the bacterial macrophage interactions.

The achieve Objective 2, various experimental techniques were established which were paramount to achieving the rest of the objectives. This chapter described the methodological framework for preparing cell free LGG-CM, experiments to perform macrophage and bacterial cell-viability studies and methods to determine the effect of LGG-CM on NO production by J774 murine macrophages. The background knowledge such as the principle of an experimental technique and steps involved in general, are described for a number of microbiological and immunological methods used to monitor bacterial ingestion and digestion, along with the experimental techniques to modulate free radical production. The methods adapted in this thesis provide an efficient way to monitor changes in the bacterial number and free radical production from macrophages. These also helped to determine the effect of LGG-CM induced free radical production on bacterial number recovered from macrophages both during ingestion and digestion periods. The developed fluorescence microplate reader technique for the continuous monitoring of free radical production (real time kinetic approach) along with the CuSum technique, adds a significant knowledge and tool for measuring free radicals. Specifically, data reliability was significantly improved as the overall assay protocol employed multiple techniques to support any findings. This multiparameter accessing protocols increases the accuracy of

the findings rather than simple measurements taken from the single type of experimental measurements.

6.3.3. Determining the effect of LGG-CM on cell viability and nitric oxide production.

The preliminary, but most important step, of the present study performed to achieve Objective 3 and 4 was preparing an effective cell free LGG-CM. The main findings from this section is that LGG-CM do not demonstrate antibacterial activity, at least on our model bacterium *E.coli* HfrC. Findings from macrophage viability assays also demonstrated that LGG-CM at a concentration and duration utilised in the experiment do not demonstrate toxic effects. A key advance in knowledge in this study with respect to NO production from macrophages at 24 hours is the observed dual effect of LGG-CM on NO production.

6.3.4 Determining the effect of LGG-CM on bacterial ingestion and digestion

This work was performed in order to achieve Objective 5. The methods developed by modifying existing protocol in this thesis helped in understanding the effect of LGG-CM on the bacterial uptake by macrophages. Bacterial recovery by gentamicin protection assay was monitored both during bacterial ingestion and digestion. Gentamicin protection assay performed in the presence of LGG-CM demonstrated that LGG-CM treated macrophages ingest significantly less bacteria. A higher concentration of LGG-CM demonstrated enhanced bacterial digestion rate.

6.3.5 Determining the effect of LGG-CM on physiological mechanisms

To achieve Objective 6, experiments were performed in the presence and absence of pharmacological inhibitors and free radical production was monitored on a real-time approach by fluorescently labelling the macrophages with ROS or NO specific fluorophores. Studies monitoring free radical production have demonstrated increased ROS production and decreased NO production from macrophages treated with LGG-CM during bacterial phagocytosis.

Western blotting results during the first 6 hours of LGG-CM treated macrophages demonstrated an enhanced expression of NADPH oxidase subunits. These results supported the increased rate of ROS production observed in presence of high concentration of LGG-CM. Bacterial killing monitored in the presence of ROS and NO inhibitors, also supports the role of ROS and basal NO mediated bacterial killing in the presence of LGG-CM. Taken together; LGG-CM modulates *E. coli* phagocytosis by modulating free radical production in J774 murine macrophages.

6.3.6. Summary

In conclusion, we have observed that the LGG-CM treated macrophages modulate macrophage phagocytosis by enhancing bacterial digestion. We have investigated the role of LGG-CM in the regulation of ROS and NO production in macrophages during phagocytosis. It has been clear that LGG-CM may have some soluble components which activate macrophage to significantly increase the rate of ROS production both in presence and absence of *E. coli*. In contrast, macrophages treated with high concentration of LGG-CM demonstrated a significant decrease in the rate of NO production. This skewing modulation of ROS and NO production is probably beneficial to the host as excessive production of NO might not only be useful to accelerate bacterial killing mechanism but could also potentially damage the host tissue. Here we have also shown for the first time that by using CuSum it has become possible to detect subtle changes in the trend in free radical production from macrophages treated with various treatments and thus can be used as a successful tool in analysing noisy *in vitro* data. The ability of probiotic to differentially regulate NO and ROS generation may have clinical implications in several pathological conditions associated with bacterial infection and may prove useful in improving intestinal homeostasis.

6.4. Future work

1. This study demonstrated that the LGG-CM modulates the macrophage phagocytosis and thereby could be used as a potential immunomodulating agent. However, in this study, work was not carried out on isolation, identification and quantification of the active components in LGG-CM. In future work, considerable

attention has to be given to identifying and quantifying the active components from the LGG-CM.

2. It is not known if the LGG-CM can also activate bacterial digestion in the macrophages from human origin. Therefore the effects of LGG-CM should be investigated from both *in vitro* using human primary cell line and also *in vivo* in animal models.

3. Future work should also consider the use of pathogenic bacteria instead of a non-pathogenic *E. coli* HfrC to see if the LGG-CM can potentiate pathogenic bacterial digestion as well.

4. Our study demonstrated the potential role of LGG-CM mediated ROS and NO production in macrophage phagocytosis. An important aspect that should be considered in the future is to detect and analyse the potential role of peroxynitrite (combined product of ROS and NO) during bacterial macrophage interactions. Peroxynitrite is known to have potent bactericidal effect. It is not known if concentration of peroxynitrite plays a role into this effect. It would be interesting to see if low peroxynitrite level has any protective or anti-inflammatory effect. Future work therefore needs to be directed towards identifying the role of peroxynitrite mediated phagocytosis and physiological regulatory mechanisms in the presence of the LGG-CM.

5. Increase in ROS has also been suggested to play a role as an anti-inflammatory agent by inhibiting NFκβ through oxidative inactivation of the key regulatory enzyme Ubc12. We have found that LGG increased ROS production through NADPH activation. It would be interesting to see if this increase in ROS to LGG-CM treatment has a role as anti-inflammatory along with its bacterial killing effect.

6. In this study, the raw data for free radical production demonstrated an oscillatory pattern. More work is needed to investigate whether this pattern is also observed in other non-immune cells such as epithelial cells, as these are also known to produce free radicals to LGG treatments.

7. Pattern of NADPH oxidase expression to LGG-CM treatments need to be investigated. It is not known if NADPH oxidase expression can be regulated on a switch on and off basis for skewing of ROS production.

8. The LGG-CM antibacterial role needs to be further investigated at acidic pH range since in this study we have observed no antibacterial activity of LGG-CM to *E.coli* at neutral pH.

9. Finally, it is important to highlight the extended necessary amount of preparation time and the demanding number of agar plates and other equipments necessary for gentamicin protection assay. In future, this work can be improved by employing alternative techniques such as flow cytometer and also by improving the acridine orange crystal-violet staining technique. A higher magnification lens would possibly enable one to enumerate the fluorescently labelled bacteria with in the macrophages. This would also simplify the steps involved in the tedious and time consuming gentamicin protection assay.

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Appendix A

List of chemicals

Acridine orange hydrochloride (Sigma 318337)

Acrylamide/bis solution (Biorad 161-0156)

Allopurinol (Sigma A8003)

Ammoniun persulphate (Sigma A3678)

Apocynin Santa Cruz Biotechnology (CAS 498-02-2)

Autoradiography film (Ultracruz SC-201696)

BCA (Thermoscientific-Pierce BCA protein assay kit-23225)

Blocking buffer (10 ml of 10 x wash buffer, 100 µl Tween-20, 5 % non-fat dried milk, 90 ml ultrapure water).

Carestream® Kodak® autoradiography GBX developer

Carestream® Kodak® autoradiography GBX fixer.

DAFFM-DA (Santa Cruz biotechnology SC-205940).

de Man, Rogosa and Sharpe (MRS) broth (OXOID CM0359)

Dimethyl sulfoxide (Sigma D8418 >= 99.9 % purity)

DMEM (1000 mg L^{-1} glucose, 4 mM L-glutamine and 110 mg L^{-1} sodium pyruvate Gibco 31885)

ECL lumiglo reserve^{TM} 547101 KPL

FBS (Sigma F6178)

Gentamicin sulphate (Sigma G1264)

Glycerol (Fischer Scientific G/0650/17)

Glycine (Sigma G8898)

gp91phox antibody (Santa Cruz biotechnology SC130543)

H_2DCFDA-AM (Molecular probes, D399).

L-Arginine hydrochloride (Sigma A92600)

L-NMMA (Sigma M7033)

Lysis buffer (10 x) [(100 mM tris –HCl pH 7.4, 10 % SDS, 1.5 M NaCl)]

Menadione (Santa Cruz biotechnology CAS 58-27-5)

MTT (Cell proliferation Kit, Roche, 1465007)

N-Napthylethylene-diamine (S222488)

Nutrient agar media (Oxoid, CM0003)

Nutrient broth (Oxoid, CM0001)

p47phox antibody (Santa Cruz biotechnology SC17845)

PEG conjugated Catalase (Sigma C4963)

PEG conjugated SOD (Sigma S9549)

Penicillin-streptomycin (Gibco 10378-016)

Phosphate buffered saline (Sigma P4417)

Protease cocktail inhibitor (Sigma P8340)

Sodium dodecyl sulphate (Sigma L3771)

Sodium chloride (Sigma S3014)

Sodium nitrite (Sigma 237213)

Sodium nitroprusside dehydrate (SNP) Sigma 31444

Sodium orthovanadate (Sigma S6508)

Sulfanilamide (Sigma S9251)

Tank Buffer (1x) (0.025 M Tris-HCl, 0.192 M Glycine, 0.1 % SDS)

TEMED (SigmaGE17-1312-01)

Tris-HCL (Sigma RES3098T-B7)

Trypan blue (Sigma T6146)

Tween 20 (Sigma P1379)

Wash buffer (10 mM Tri-HCl pH 7.5, 100 mM NaCl, 0.1 % Tween-20)

β-actin antibody (Santa Cruz biotechnology SC47778)

Appendix B

Conference Presentations:

Poster presentations

2010 Y. Seenappanahalli Nanjundaiah, Z. Ali, D. A. Wright & M. Sarker The influence of a cell -free probiotic supernatant on bacterial macrophage interactions. 5 Febraury 2010
North East Postgraduate research Conference, King's road centre, Newcastle university,

2011 Y.S. Nanjundaiah, Z.Ali, D.A.Wright, M.Sarker. The influence of a cell-free probiotic supernatant on bacterial macrophage interactions. 17 May 2011
TFI research Day, Teesside University, Middlesbrough

2012 Yashaswini Seenappanahalli Nanjundaiah, Ali Z., Wright D.A., Sarker M.H. Influence of cell-free probiotic supernatant on bacterial macrophage interactions 8 May 2012
Vitae yorkshire and North East hub Public engagement conference and competition at the Royal York hotel, York.

2012 Y. Seenappanahalli Nanjundaiah, Z. Ali, D. A. Wright & M. Sarker. The influence of cell free probiotic supernatant on bacterial macrophage interactions. 5-8 Sep2012
European congress of Immunology, 5-8 September 2012, Glasgow, Scotland
Immunology Volume 137, Issue Supplement s1, Article first published online: 12 SEP 2012

2012 Y.S. Nanjundaiah, Z.Ali, D.A.Wright, M.Sarker. Influence of probiotic supernatant on macrophage phagocytic activity. 26 October 2012
North East Postgraduate research Conference.

2013 Y.S. Nanjundaiah, Z.Ali, D.A.Wright, M.Sarker. Differential Immunomodulatory properties of cell free probiotic supernatant: relevance of phagocytosis and free radical production by macrophages. 28 October 2013
North East Postgraduate research Conference, Hancock museum, Newcastle upontyne.

2014 Mosharraf H. Sarker, Yashaswini S. Nanjundaiah, David A wright, Nasima S. Chowdhury and Anwar R Baydoun. Modulation of *E. coli* phagocytosis by LGG conditioned medium in macrophage J774 cells. International scientific conference on Probiotics and Prebiotics, Budapest, Hungary.24th-26th June 2014

2015 Y.S. Nanjundaiah, D.Wright, L.O, Hare, A.Baydoun, Z.Khaled & M. Sarker. Probiotic *Lactobacillus rhamnosus* GG conditioned media enhances acute ROS production, but reduces nitric oxide in J774 macrophages.Physiology 2015, 6th-8th July. Motorpoint Arena, Cardiff

Oral presentations

2012 Y.S. Nanjundaiah, Z.Ali, D.A.Wright, M.Sarker. The influence of cell free probiotic supernatant on bacterial macrophage interactions. 15 May 2012
research Day, Teesside University, Middlesbrough

2013 Yashaswini Seenappanahalli Nanjundaiah, Ali Z., Wright D.A., Sarker M.H. Immunomodulatory effects of cell free probiotic supernatant on murine macrophages. 21 May 2013. Annual research day and postgraduate symposium best oral communication award
TFI research Day, Teesside University, Middlesbrough

2014 Yashaswini Seenappanahalli Nanjundaiah, Ali Z., Wright D.A., Sarker M.H. Modulation of *E. coli* phagocytosis by murine macrophages in the presence of probiotic conditioned medium is mediated by NADPH-oxidase activity. 31 October 2014

2014 Yashaswini S.Nanjundaiah, David A. Wright, Nasima S. Chowdhury, Anwar R. Baydoun & Mosharraf H.Sarker. Modulation of macrophage phagocytosis by probiotic bacteria *in vitro*. Invited Lecture, 4th Biennial conference of South Asian Association of physiologists and 3rd National convention of Bangladesh society of Physiologists, 5-7 December 2014, Dhaka, Bangladesh

List of Papers

1. Yashaswini Seenappanahalli Nanjundaiah, Anwar R Baydoun, Zahangir Khaled, David A Wright, Liam O'Hare, Mosharraf Sarker. *Lactobacillus rhamnosus* GG conditioned media enhances acute free radical production in J774 murine macrophages. (Submitted to Free radical Biology and Medicine)

2. Yashaswini Seenappanahalli Nanjundaiah, David A. Wright, Zahangir Khaled, Mosharraf Sarker. *Lactobacillus rhamnosus* GG conditioned medium enhances bacterial killing by macrophage *in vitro*. (Under revision)

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