



**Inhibition of Colon Cancer in Mice by
Microencapsulated Probiotic**

**Submitted in complete fulfillment for the Degree of Doctor of Philosophy in
Biotechnology in the Department of Biotechnology and Food Technology, Durban
University of Technology, Durban, South Africa**

Frederick Oluwasheyi Odun-Ayo

PROMOTER: Dr L. Reddy
CO-PROMOTER: Dr J. J. Mellem

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This study (thesis) presents original work by me. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Dr Lalini Reddy** and **Dr John Mellem**.

Frederick O. Odun-Ayo

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	i
LIST OF ABBREVIATIONS	iii
PUBLICATIONS	v
CONFERENCE PRESENTATIONS.....	vi
LIST OF FIGURES	vii
LIST OF TABLES	ix
ABSTRACT	x
CHAPTER 1 - INTRODUCTION AND AIM OF THE STUDY	1
1.1 Aim	2
1.2 Objectives	2
CHAPTER 2 - LITERATURE REVIEW.....	4
2.1 Colon cancer	4
2.1.1 Role of galectin-3 in colon carcinogenesis.....	4
2.1.2 Role of vascular endothelial growth factor in colon carcinogenesis.....	6
2.1.3 Azoxymethane as related to colon carcinogenesis	7
2.1.4 Colon cancer histopathology in a mouse model.....	9
2.1.5 Prevalence and risk factor of colon cancer.....	11
2.1.6 Treatment and prevention.....	12
2.2 Probiotics	14
2.2.1 Human colonic microflora.....	14
2.2.2 Definitions and concept of probiotics	16
2.2.2.1 Lactobacilli	17
2.2.2.2 Bifidobacteria	17
2.2.3 Potential role and health benefits of probiotic.....	18
2.2.3.1 Inflammation bowel diseases (IBD).....	18
2.2.3.2 Irritable bowel syndrome (IBS).....	19
2.2.3.3 Infectious diarrhoea	20
2.2.3.4 Obesity.....	20
2.2.4 Possible inhibition mechanism of colon cancer by probiotic bacteria	21
2.2.5 Probiotic encapsulation	23
2.2.5.1 Spray drying	25
2.2.5.2 Extrusion.....	25

2.2.5.3 Emulsification and polymerization.....	26
2.2.6 Probiotic applications in the food industry.....	26
2.2.6.1 Fermented food products.....	28
2.2.6.2 Non-dairy food products.....	30
2.3 Pectin	31
2.3.1 Historical discovery of Pectin	31
2.3.2 Structure of pectin	31
2.3.3 Properties of pectin.....	33
2.3.3.1 Pectin as a biopolymer agent and prebiotic.....	34
2.3.4 Modified Pectin (MP).....	35

CHAPTER 3 - VIABILITY OF A MICROENCAPSULATED PROBIOTIC IN

SIMULATED GASTRIC AND INTESTINAL JUICES.....37

3.1 Introduction.....	37
3.2 Materials and Methods	39
3.2.1 Plant materials	39
3.2.2 <i>Lactobacillus acidophilus</i> ATCC 4356 growth conditions and harvesting	39
3.2.3 Preparation of modified citrus pectin alginate <i>Lactobacillus acidophilus</i> ATCC 4356 microbeads.....	40
3.2.4 Enumeration of viable <i>Lactobacillus acidophilus</i> ATCC 4356 cells in microbeads .	41
3.2.5 Determination of survival of free and microencapsulated <i>Lactobacillus acidophilus</i> ATCC 4356 in simulated gastric and intestinal juice.....	41
3.3 Statistical analysis.....	42
3.4 Results.....	42
3.4.1 Microencapsulation yield and size of modified citrus pectin alginate and alginate calcium probiotic microbeads	42
3.4.2 Stability of free and microencapsulated <i>Lactobacillus acidophilus</i> ATCC 4356 in storage	43
3.4.3 Survival of free and microencapsulated <i>Lactobacillus acidophilus</i> ATCC 4356 in simulated gastric juice	45
3.5 Discussion.....	49

CHAPTER 4 - THE EFFECT OF A MICROENCAPSULATED PROBIOTIC ON

COLON LACTOBACILLIMICROFLORA IN A MOUSE MODEL.....53

4.1 Introduction.....	53
4.2 Materials and Methods	55
4.2.1 Animal model.....	55

4.2.2 Carcinogenic treatment of Balb/c mice with azoxymethane.....	55
4.2.3 Isolation and quantification of lactobacilli in faecal samples	56
4.2.4 DNA extraction of faecal bacteria.....	57
4.2.5 DNA amplification	57
4.2.6 Gene sequence analysis	58
4.2.7 Statistical analysis	58
4.3 Result	58
4.3.1 Morphological identification of colonies	58
4.3.2 Quantification of faecal lactobacilli analysis	61
4.3.3 Faecal bacteria revealed by 16S rRNA amplification BLAST search	63
4.4 Discussion.....	64
CHAPTER 5 - THE CHEMOPREVENTION OF AZOXYMETHANE-INDUCED COLON CARCINOGENESIS IN BALB/C MICE MODEL USING A MICROENCAPSULATED PROBIOTIC	69
5.1 Introduction.....	69
5.2 Materials and methods	71
5.2.1 Animal model	71
5.2.2 Carcinogenic treatment of Balb/c mice	71
5.2.2.1 Experiment I: Selection of optimum dose duration of azoxymethane	71
5.2.2.2 Experiment II: Investigation of chemopreventive efficacy of the modified citrus pectin alginate and alginate calcium microbeads treatment.....	71
5.2.3 Immunohistochemistry	73
5.2.3.1 Tissue preparation	73
5.2.3.2 Immunostaining	73
5.2.3.3 Semi quantitative image analysis	73
5.2.3.4 Data analysis	74
5.3 Results.....	74
5.3.1 Experiment I	74
5.3.1.1 Tumour type and incidence	74
5.3.1.2 Immunostaining	75
5.3.2 Experiment II.....	80
5.3.2.1 General observation.....	80
5.3.2.2 Tumour incidence	81
5.3.2.3 Immunostaining	81
5.4 Discussion.....	85
CHAPTER 6 - GENERAL DISCUSSION AND CONCLUSIONS	89

CHAPTER 7 - REFERENCES	94
APPENDICES	121
Appendix 1: Statistical analysis data for preliminary probiotic test.....	121
Appendix 2: Ethics application for animal study	122
Appendix 3: Statistical analysis of data for Balb/c mouse model	137
Appendix 4: Ethic application for extension of animal study	138
Appendix 5: Statistical analysis of immunoexpression data	144

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LIST OF ABBREVIATIONS

5-FU	-	fluorouracil
ACF	-	aberrant crypt foci
AP	-	alginate
Apc	-	adenomatous polyposis coli
AOM	-	azoxymethane
ATCC	-	American Type Culture Collection
ATP	-	adenosine triphosphate
BRU	-	Biomedical Research Unit
CP	-	citrus pectin
CRC	-	colorectal cancer
CRD	-	carbohydrate recognition domain
D-galA	-	1, 4-linked α -D-galacturonic acid
DE	-	degree of esterification
DMH	-	1, 2- dimethylhydrazine
DSS	-	dextran sodium sulphate
ECM	-	extracellular matrix
EPS	-	exopolysaccharides
FAP	-	familial adenomatous polyposis
FOBT	-	faecal occult blood test
FDA	-	Food and Drug Administration
Gal-3	-	galectin-3
GALT	-	gut associated lymphoid tissue
GRAS	-	generally regarded as safe
GIT	-	gastrointestinal tract
HG	-	homogalacturonan

HMP	-	high methoxyl pectin
IARC	-	International Agency for Research on Cancer
IBD	-	inflammatory bowel disease
IBS	-	irritable bowel syndrome
IDF	-	International Dairy Federation
IL-12	-	Interleukin 12
LAB	-	lactic acid bacteria
LMP	-	low methoxyl pectin
Mab	-	monoclonal antibody
MAM	-	methylazoxymethanol
MAP	-	modified apple pectin
MCP	-	modified citrus pectin
MCPA	-	modified citrus pectin alginate
MGMT	-	O6-methylguanine methyltransferase
MUC2	-	mucin
O4-MET	-	O4-methylthymine
O6-MEG	-	O6-methylguanine
RGI	-	rhamnogalacturonan 1
ROS	-	reactive oxygen species
SCFAs	-	short chain fatty acids
SGJ	-	simulated gastric juice
SIJ	-	simulated intestinal juice
VEGF	-	vascular endothelial growth factor
VEGFR1	-	vascular endothelial growth factor receptor 1
VEGFR2	-	vascular endothelial growth factor receptor 2

PUBLICATIONS

1. **Frederick Odun-Ayo**, John Mellem and Lalini Reddy. Improving the survival of probiotic in simulated conditions and tumour induced mice model using modified citrus pectin-alginate microencapsulation (2016). *African Journal of Traditional Complementary and Alternative Medicine* 13 (2): 101-109.
2. **Frederick Odun-Ayo**, John Mellem, Thajasvarie Naicker and Lalini Reddy (2015). Chemoprevention of azoxymethane-induced colonic carcinogenesis in Balb/c mice using a modified pectin alginate probiotic. *Anticancer Research* 35: 4765-4776.

CONFERENCE PRESENTATIONS

- Frederick Odun-Ayo, John Mellem and Lalini Reddy. Effect of a novel modified pectin and alginate encapsulation on *Lactobacillus acidophilus* ATCC 4356 in colon carcinogenic mice and simulated conditions. U6 International Conference “Research and innovation for sustainable development”, September 2014, Cape Town, South Africa.

LIST OF FIGURES

Fig 2.1	Signalling pathways associated with galectin-3 expression in the cell.	5
Fig 2.2	Azoxymethane model- colon carcinogenesis.	7
Fig 2.3	The morphology and genetic changes of aberrant crypt foci-adenoma-carcinoma sequence.	8
Fig 2.4	The histopathology of early colonic neoplasms developed in azoxymethane/dextran sodium sulphate treated mice.	10
Fig 2.5	The histopathology of late colonic neoplasms developed in azoxymethane/dextran sodium sulphate treated mice.	11
Fig 2.6	Colonic microbial showing differential distribution of bacteria.	15
Fig 2.7	Simplified schematic diagrams of the two proposed structures of pectin.	32
Fig 2.8	The molecular unit structure of pectin.	32
Fig 2.9	The schematic structure of modified pectin rhamnogalacturonan 1(RG1).	35
Fig 3.1	Schematic diagram of the emulsification polymerization procedure.	40
Fig 3.2	The morphology of modified citrus pectin alginate and alginate calcium probiotic microbead particles produced by emulsification.	43
Fig 3.3	The viability of <i>Lactobacillus acidophilus</i> ATCC 4356 as free cells and encapsulated cells during storage at 4°C.	44
Fig 3.4	The morphology of modified citrus pectin alginate microbead particles containing <i>Lactobacillus acidophilus</i> ATCC 4356 after exposure to simulated gastric juice at pH 2.	46
Fig 3.5	The viability of <i>Lactobacillus acidophilus</i> ATCC 4356 as free cells and encapsulated cells during exposure to simulated gastric juice at pH 2 and simulated intestinal juice at pH 8.	47
Fig 3.6	The morphology of alginate calcium microbead particles containing <i>Lactobacillus acidophilus</i> ATCC 4356 after exposure to simulated intestinal juice at pH 8.	48
Fig 4.1	A normal healthy male Balb/c mouse.	55
Fig 4.2	Gram reaction and cellular morphology of <i>Lactobacillus acidophilus</i> ATCC 4356 and isolated faecal bacteria.	60
Fig 4.3	Average counts of faecal lactobacilli (log ₁₀ cfu/g of faeces) in Balb/c mice model treated with modified citrus pectin alginate, alginate calcium probiotic microbeads and modified citrus pectin solution.	62
Fig 5.1	The administration of azoxymethane and colon tissue collection from the Balb/c mice model.	72

Fig 5.2	Colonic neoplasm from Balb/c mice treated with 15 mg/kg azoxymethane 4 weeks.	75
Fig 5.3	Immunostaining patterns of galectin-3 (gal-3) in azoxymethane treated Balb/c mouse model at 9, 12 and 16 weeks of colonic carcinogenesis.	77
Fig 5.4	Immunostaining patterns of vascular endothelial growth factor in azoxymethane treated Balb/c mouse model at 9, 12 and 16 weeks of colonic carcinogenesis.	77
Fig 5.5	Immunoexpressions for galectin-3(gal-3) in a Balb/c mouse model of colonic carcinogenesis treated with different azoxymethane (AOM) dose duration	79
Fig 5.6	Immunoexpression for vascular endothelial growth factor (VEGF) in a Balb/c mouse model at week 12 and week 16 of colonic carcinogenesis.	80
Fig 5.7	Azoxymethane treated Balb/c mice with eye inflammation and normal (untreated) Balb/c mouse.	81
Fig 5.8	Post-treatment immunoexpressions of galectin-3 and vascular endothelial growth factor markers in colon cancer induced Balb/c mice model.	82
Fig 5.9	The immunohistochemical features of galectin-3 expression in colon carcinogenesis Balb/c mice treated with probiotic microbeads and modified citrus pectin.	83
Fig 5.10	The immunohistochemical features of vascular endothelial growth factor expression in colon carcinogenesis Balb/c mice treated with probiotic microbeads and modified citrus pectin.	84

LIST OF TABLES

Table 2.1	General and histological features of aberrant crypt foci in rodents.	9
Table 2.2	Physical techniques of microencapsulation.	24
Table 2.3	Chemical techniques of microencapsulation.	24
Table 2.4	Applications of encapsulated probiotics in food products.	27
Table 2.5	Commercial probiotic products.	28
Table 3.1	Encapsulation yield of microencapsulated and free <i>Lactobacillus acidophilus</i> ATCC 4356 during storage at 4°C	44
Table 3.2	Encapsulation yield of microencapsulated and free <i>Lactobacillus acidophilus</i> ATCC 4356 exposed to simulated gastric and intestinal juice	49
Table 4.1	Category of treatments administered for the axozymethane-treated Balb/c mouse model.	56
Table 4.2	Category of treatments administered for the normal Balb/c mouse model.	56
Table 4.3	Morphological characterization of faecal bacteria isolated from Balb/c mice.	59
Table 4.4	Identification of faecal bacteria in the normal and axozymethane-treated Balb/c mouse model by genome sequence BLAST search.	64
Table 5.1	Category of treatments administered on the axozymethane-treated Balb/c mouse model.	72
Table 5.2	Semi quantitative analysis of galectin-3 in azoxymethane treated Balb/c mouse model.	76
Table 5.3	Semi quantitative analysis of vascular endothelial growth factor in azoxymethane treated Balb/c mouse model.	76

ABSTRACT

Colon cancer is the third most common cancer worldwide with a high morbidity and mortality rate. Therapies are less effective during metastasis, therefore prevention and earlier detection is key to reducing the risk of colon cancer. Increased dietary fibre and probiotic intake is known to lower the risk of colon cancer. Probiotics are defined as “live microorganisms which when administered orally in an adequate amount confer a health benefit on the host”. The International Dairy Federation recommends a viable minimum level of 6–7 log₁₀cfu/g in a probiotic product being consumed. Different biopolymer matrices have been used for encapsulation of probiotics; however, loss of viability is still a major challenge. Citrus pectin is a dietary fibre polysaccharide broken down into smaller fragments to form modified citrus pectin (MCP). The unique bioactivity of MCP against carcinogenesis is linked to its sugar β -galactose inhibiting the cell signalling protein marker, galectin-3 (gal-3), which is intimately involved in endothelial cell morphogenesis. The vascular endothelial growth factor (VEGF) signalling, which invariably drives angiogenesis can be activated when gal-3 binds to integrins. The bioactivity and uptake of MCP may be improved through a novel approach if conjoined with a supplement for example probiotic. Therefore, the synergistic inhibitory effect of modified citrus pectin alginate (MCPA) probiotic microbeads on gal-3 and VEGF in an azoxymethane (AOM) induced colon carcinogenesis Balb/c mouse model was investigated.

A microencapsulation process was used to produce a MCPA microbead containing probiotic, *Lactobacillus acidophilus* ATCC 4356. Efficiency of the microbead was evaluated *in vitro* (simulated conditions) and *in vivo* (Balb/c mouse model). Genomic identification of faecal lactobacilli samples from the treated mice was analyzed. Optimization of AOM dose-time with 10 and 15 mg/kg AOM intraperitoneal (*ip*) administered to Balb/c mice for 2 and 4 weeks were performed. The optimal AOM dose was initiated prior to intake of MCPA, AP (alginate calcium) probiotic microbeads and MCP in Balb/c mice for 16 weeks; samples were analyzed for colon histopathology and immunohistochemistry.

The MCPA probiotic microbeads significantly enhanced the viability of *L. acidophilus* ATCC 4356 compared to the AP microbeads *in vitro* ($p < 0.05$). Exposure of the MCPA probiotic microbeads to 3 h of simulated gastric juice (SGJ) resulted in 82.7% survival of *L. acidophilus* ATCC 4356. Also, the faecal lactobacilli count in the MCPA probiotic treated mice significantly increased after 28 days by 10.2% compared to the AP probiotic, MCP treated and control mice ($p < 0.0001$). A total of 4 DNA encoding 16S rRNA gene closest to the genera namely *Lactobacillus*, *Bacillus*, *Enterococcus* and *Bifidobacterium* were identified from faecal samples of the colon cancer-induced Balb/c mice. Azoxymethane at 15 mg/kg for 4 weeks induced optimal gal-3 and VEGF immunoexpression. Furthermore, MCPA probiotic treatment significantly reduced gal-3 immunoexpression in the colon of AOM induced cancer Balb/c mice compared to the control mice ($p < 0.0001$). The immunoexpression of VEGF in the MCPA and AP probiotic treated groups was weakly positive and significantly reduced when compared to the control group ($p < 0.05$), while the MCP treated group was barely positive ($p < 0.001$).

Modified citrus pectin alginate is a novel effective means of oral delivery of bacterial cells and bioactive compounds. It has a good biodegradability, inexpensive, non-toxic, proven efficiency, and stability at low temperatures warranting its use as a drug carrier by pharmaceuticals. Modified citrus pectin alginate probiotic microbeads increase bioactivity and chemoprevention against colon pre-cancerous lesions and adenocarcinoma through inhibition of gal-3 and VEGF in the mouse model. Modified citrus pectin alginate can be used in probiotic therapy, which may improve the prevention of colon cancer.

CHAPTER 1

INTRODUCTION AND AIM OF THE STUDY

There has been a marked increase in the overall incidence of colon cancer in African (for instance South Africa), Asian, North and South American, and Eastern European countries (Cronje *et al.*, 2009; American Cancer Society, 2011). Colon cancer has shifted from the 10th most common cancer diagnosed in males and females in 1989 in South Africa to the 5th most prevalent cancer in 1999 (Mqoqi *et al.*, 2004).

Modified citrus pectin (MCP) has emerged as one of the most promising naturally occurring anti-metastatic substance (Glinsky and Raz, 2009). A study reported relevant measurable clinical effects of the orally administered natural product MCP in pre-treated cancer patients and the role of dietary components in cancer progression and metastasis (Azémar *et al.*, 2007). There has been a series of experimental data which has indicated that galectin-3 (gal-3) is involved in carcinogenesis (Demetter *et al.*, 2008). In several tumour models, a correlation has been established between the level of degree of gal-3 and the stage of tumour progression (Liu, 2002; Liu and Rabinovich, 2005).

The use of dietary intervention has been indicated as a major strategy to lower and prevent the risk of colon cancer in the human population (American Cancer Society, 2013). A clinical trial indicated the anti-carcinogenic activity of *Lactobacillus acidophilus* in the colonic microflora of the human, while the viability of ingested probiotic bacteria in the gastrointestinal tract (GIT) is yet uncertain (Fooks *et al.*, 1999). The extensive application of coating alginate microcapsules with chitosan to control the release of an entrapped drug in the simulated intestinal condition has been envisaged in the pharmaceutical industry, but less with respect to probiotic encapsulation in the food industry counterpart. One of the purposes of this project was to improve the viability and controlled release of probiotic bacteria in the GIT using this novel ingredient MCP. In an attempt to advance the prevention of colon cancer via gal-3 and vascular endothelial growth factor (VEGF) inhibition, this research intends to improve the bioactivity, bioavailability and uptake of MCP through a novel approach if combined

with a probiotic. The improved MCP-based delivery system with bio therapeutic properties and modification of the probiotic functionality through synergistic effect was to contribute to reducing the risk of colon cancer in human. Hence, the consumption of functional foods or nutraceuticals containing MCP encapsulated probiotic may provide an immense benefit to human health.

1.1 Aim

The aim of this study was to investigate the inhibition of tumour growth in a colon cancer-induced Balb/c mouse model by a modified citrus pectin alginate (MCPA) microencapsulated probiotic.

1.2 Objectives

1. To produce a viable modified probiotic as follows:
 - 1.1. Culture *Lactobacillus acidophilus* ATCC 4356 and maintain growth using De Mann, Rogosa and Sharpe (MRS) agar and broth.
 - 1.2. Produce a microencapsulated probiotic with modified citrus pectin, alginate and *L. acidophilus* ATCC 4356 using emulsification polymerization
 - 1.3. Examine the viability of the microencapsulated probiotic in simulated conditions and storage using an automated colony Doc-It® imaging station.
2. To determine the optimum uptake and effect of the modified microencapsulated probiotic on the colon lactobacilli microflora in Balb/c mouse model by the examination of bacterial cocktail in faecal matter.
3. To optimize colon carcinogenesis in Balb/c mice by a dose and time response study with colon carcinogen, azoxymethane (AOM) via histopathological changes in the mice colon tissue and detecting the level of gal-3 and VEGF immunoexpressions with immunohistochemical staining.
4. To determine the effect of the modified microencapsulated probiotic on colon tumour proliferation, apoptosis, and angiogenesis in the Balb/c mouse model by

detecting the level of immunoexpressions of gal-3 and VEGF with histopathology and immunohistochemical (IHC) staining.

The limitations of this study are as follows:

1. Although the simulated intestinal and gastric juices used in this study may contain important similar gastric enzymes prepared at a minimal and optimal pH as represented in human GIT; differing pH effect at different regions of the human GIT may be considered.
2. This experimental study was conducted *in vitro* (simulated) and *in vivo* animal (Balb/c mice) therefore results have to be extrapolated to large animals and humans *in vivo* for clinical studies.
3. Some lactobacilli colonize limited areas in the mouse GIT and thus remain non-detectable.

CHAPTER 2

LITERATURE REVIEW

2.1 Colon cancer

The World Health Organization (WHO), through the cancer research agency, International Agency for Research on Cancer (IARC) reported 608,000 deaths were caused by colorectal cancer in 2008. Deaths attributed to cancer have been projected to continue rising worldwide with an estimated 13.1 million deaths in 2030 (International Agency for Research on Cancer, 2012). Colorectal cancer is recognized as the third most common cancer worldwide with high a morbidity and mortality rate (Hagggar and Boushey, 2009). The highest incidence rates were in North America, Australia, New Zealand, Eastern Europe, and Asia (Japan, Israel and Kuwait) including countries where risk was historically low. The variation in incidence and mortality rates by different geographical regions may be related to socioeconomic factors, legislative policies and easy access to medical services such as screening and testing (Siegel and Jemal, 2011).

2.1.1 Role of galectin-3 in colon carcinogenesis

Galectins are a family of carbohydrate binding proteins of which galectin-3 (gal-3) is a member of the family, a galactose-binding protein usually expressed in many human cells (epithelial and immune cells), a variety of human cancer cells from the head and neck, thyroid, stomach and brain (Bresalier *et al.*, 1997; Gillenwater *et al.*, 1996; Lotan *et al.*, 1994; Xu *et al.*, 1995; Yu, 2010). Gal-3 has a small molecular weight (30 kDa) and comprises of three main terminals namely; the -NH₂ and -COOH terminals and Asp-Trp-Gly-Arg (NWGR) anti-death motif (Maxwell *et al.*, 2012). The gal-3 carbohydrate recognition domain (CRD) has a special binding affinity to β -galactosides such as lactose and larger galacto-oligosaccharides. The endogenous galactoside-binding gal-3 is implicated in cell growth, proliferation, adhesion, differentiation, migration, angiogenesis, mRNA splicing promoter, malignant transformation and apoptosis (Yang *et al.*, 2008).

This multi-functional protein is synthesized in the cytoplasm as a cytosolic protein but can be expressed in the nucleus when transported to the multiple subcellular localization of the cell nucleus, or secreted into the extracellular matrix (ECM) (outside of the cell) (Hill *et al.*, 2010; Yu, 2010). Gal-3 regulates cell homeostasis both intracellular and extracellular (Demetter *et al.*, 2008). At the extracellular surface, gal-3 has the ability to bind glycoconjugates as an aggregate of cells coming together to form cell matrix interactions (Yang *et al.*, 1996) as shown in Figure 2.1.

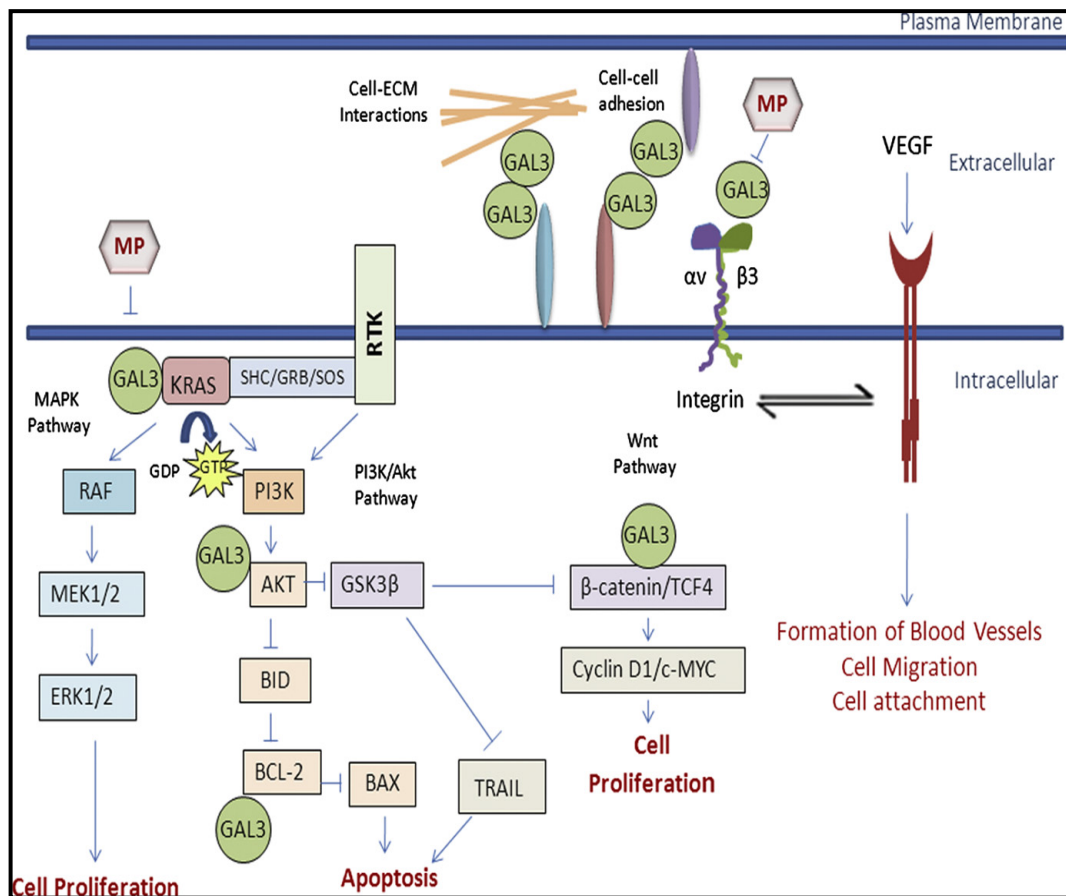


Fig 2.1 Signalling pathways associated with galectin-3 expression in the cell (Maxwell *et al.*, 2012).

The down regulation of cell surface gal-3 in later stages of colon and breast cancer enables them to interact with laminin which facilitates invasion and metastasis (Tsuboi *et al.*, 2007). Evidences has also shown that gal-3 is down regulated in prostate cancer but up regulated in cancer of the thyroid and colon cancer (Merseburger *et al.*, 2008; Htwe *et al.*, 2010; Povegliano *et al.*, 2011).

These suggest that the expression of gal-3 in carcinogenesis depends on the stage of the cancer and the tissue involved with gal-3 expressions and distribution altering the progression of cancer (Maxwell *et al.*, 2012). Gal-3 plays an important role in colon cancer metastasis and progression. Colon cancer cells with high levels of gal-3 also have high levels of mucin (MUC2), while those with low gal-3 levels have low MUC2. Extracellular gal-3's role involves angiogenesis, chemotaxis and cell aggregation (Markowska *et al.*, 2011; Nangia-Makker *et al.*, 2010).

2.1.2 Role of vascular endothelial growth factor in colon carcinogenesis

In the event of tumourigenesis, invaded cells utilize oxygen from the blood vessel for survival. The depletion of oxygen and nutrients in the blood vessel eventually demands for growth of new networks of blood vessels to the tumour sites. This process of regeneration is known as angiogenesis, which is one of the key events in colon carcinogenesis. The expression of vascular endothelial growth factor (VEGF) in colon tumour is usually on the increase as it fosters the growth of the tumour. VEGF receptors are prominently found on vascular endothelial cells which contribute to the survival and proliferation of the cells (Olsson *et al.*, 2006). Hypoxia and metabolic stress activates the response of hypoxia inducible transcription factor 1 α and 2 α to many cancers after VEGF expression (World Health Organisation, 2012). VEGF is a ligand for vascular endothelial growth factor receptor 1 (VEGFR1) and receptor 2 (VEGFR2) but the latter is expressed on elevated endothelial cells mainly mediating for VEGF signalling in angiogenesis (Shibuya and Claesson-Welsh, 2006).

One significant role of angiogenesis is an over expression of VEGF, signalling an increased release of leukocytes to the intestinal vascular endothelium thereby causing intestinal inflammation (Scaldaferri *et al.*, 2009). VEGF signalling which invariably drives angiogenesis can be activated when gal-3 binds to integrins causing cell clustering (Yang *et al.*, 1996). A poor prognosis in several cancers can be correlated to a high expression of intratumoural or circulating VEGF (Dvorak, 2002).

2.1.3 Azoxymethane as related to colon carcinogenesis

Azoxymethane (AOM) is a colonic pro-carcinogen that has been extensively used for the genetic study and pathogenesis of colon cancer. The underlying mechanism of AOM and the development of colon cancer in the mouse model are similar to the pattern of sporadic colon cancer envisaged in human. The development of cancer beginning from the polypoid growth (adenomas) is a histopathological feature which occurs similarly in the mouse model (Takahashi and Wakabayashi, 2004; Tanaka, 2009). Spontaneous colorectal cancer in humans is often predominantly found at the distal part of the colon, similar to tumours in AOM mouse models. Many studies have demonstrated the relevance of an AOM mouse model in the study of colon carcinogenesis, as an aberrant crypt foci (ACF) was identified at the precancerous stage (tumour growth) in AOM mouse models and in colorectal cancer (CRC) in humans (Chen and Huang, 2009).

The carcinogenesis of colon cancer is associated with genetic mutations as AOM metabolizes *in vivo* and then causes DNA mutation such as the TGF- β (transforming growth factor- β) receptor, K-ras, Apc (adenomatous polyposis coli), PIK3CA and P53 (Bachman *et al.*, 2004; Kinzler and Vogelstein, 1996). This activation process takes place in sequence of events beginning with an initiation step of hydroxylation of the methyl group of AOM into methylazoxymethanol (MAM) by CYP2E1 (Figure 2.2) (Sohn *et al.*, 2001).

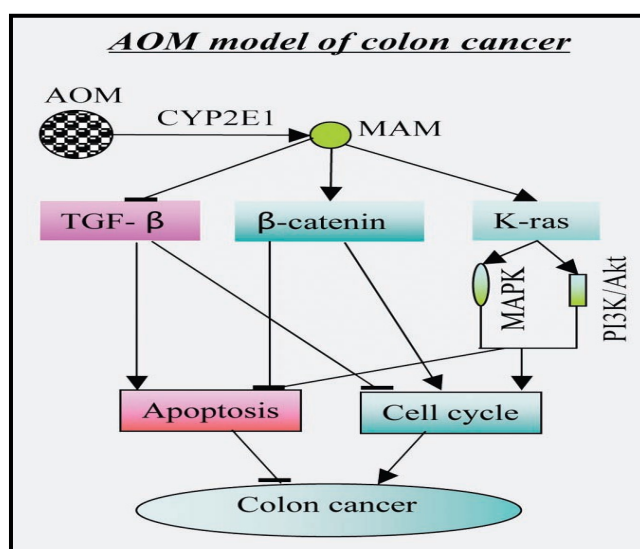


Fig 2.2 Azoxymethane model- colon carcinogenesis (Chen and Huang, 2009).

MAM disintegrates into methyldiaxonium, a highly reactive alkylation species and formaldehyde. Alkylation of the DNA guanine results in the formation of O⁶-methylguanine (O⁶-MEG) which can further wrongly pair with thymine to form O⁴-methylthymine (O⁴-MET) resulting in a change in the nucleotide from G:C to A:T (O'Toole *et al.*, 1993). However, the repair process of O⁶-MEG can be achieved by methylation of a protein O⁶-methylguanine DNA methyltransferase (MGMT) (Pegg and Byers, 1992). This initiation step of mutation begins tumourigenesis through some oncogenes in intracellular signal pathways. The development of colon cancer involves a multistep process that is characterized by 3 major phases namely the initiation, promotion and progression stage. Both morphological and genetic changes take place in the course ACF-adenoma-carcinoma sequence (Figure 2.3).

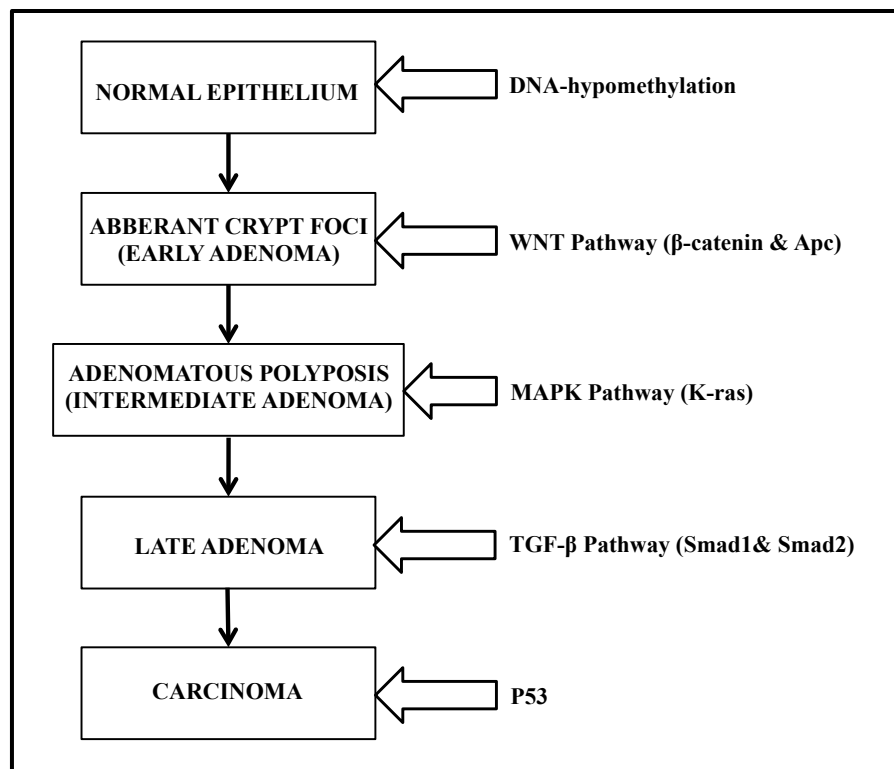


Fig 2.3 The morphological and genetic changes of ACF-adenoma-carcinoma sequence (Horkko, 2006).

The initiation begins with genetic alteration in the normal epithelium crypts, which causes crypts fission changes from aberrant single crypts to multiple aberrant crypts foci (ACF). This further proliferates to form microadenomas.

The latter enlarges and becomes macroscopically visible adenomatous polyps eventually progressing to adenocarcinomas (Robertis *et al.*, 2011). As there is yet to be an established explanation involved in the mechanism of AOM models, possible signalling pathways have been suggested and these involve β -catenin, K-ras and TGF- β (Chen and Huang, 2009; Robertis *et al.*, 2011).

2.1.4 Colon cancer histopathology in a mouse model

The colonic mucosae of a mouse model treated with an AOM dose (3-4 weeks) can be examined when the colon is fixed in buffered formalin and stained with methylene-blue (Orlando *et al.*, 2008). ACF is characteristically defined to be dysplastic, non-dysplastic or hyperplastic as shown in Table 2.1

Table 2.1: General and histological features of aberrant crypt foci (ACF) in rodents (Wargovich *et al.*, 2010)

Characteristics	Dysplastic ACF	Non-dysplastic ACF	Hyperplastic ACF
Darker staining	darkest	yes	yes
Size	increased	increased	increased
Topography	raised	raised	raised
Diameter	wide	widest	wide
Dilated lumen	thickened and closing	yes	mixed
Pericryptal area	non-serrated	serrated	mixed
Mucin status	depleted	present	mildly depleted
Polarity	lost	ordered	mixed
Nuclear morphometry	oval and stratified	round and non-stratified	mixed
Proliferation pattern	full progression throughout crypt	lower two-thirds of crypt	progression to upper crypt

Most studies have identified aberrant crypt in colonic mouse model displays as a high level of dysplasia (Raju, 2008; Roncucci *et al.*, 1991). This may be the effect of using chemical carcinogens (e.g. AOM) to induce the colon cancer in rodents (Wargovich *et al.*, 2010). Microscopically, the colonic tumour shows features ranging from intraepithelial neoplasm to ACF characterized with dark staining and increased crypt size, dilated lumen and aberrant crypt clustered together as foci (Gupta and Schoen, 2009) (Figure 2.4).

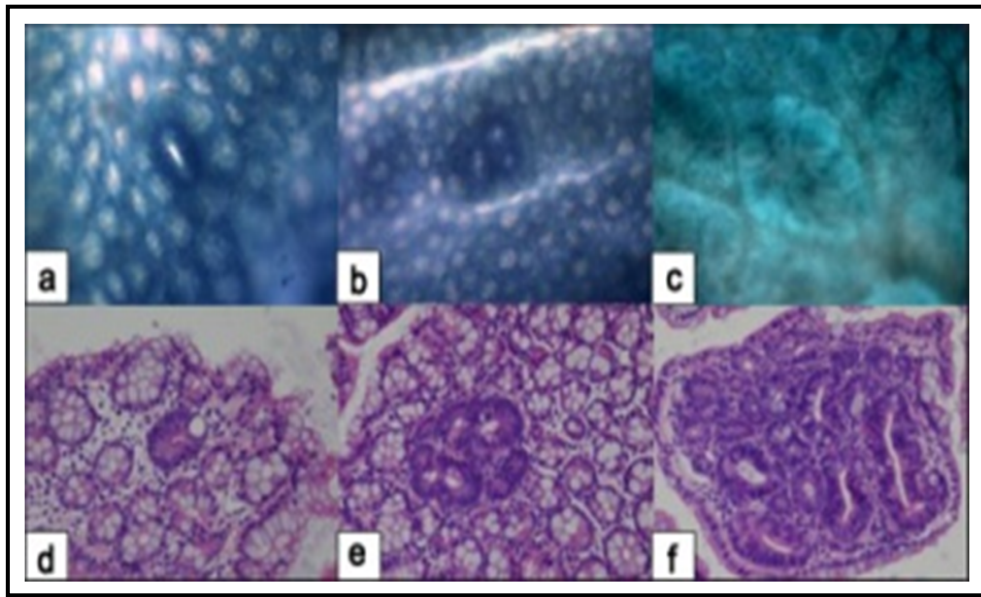


Fig 2.4 The histopathology of early colonic neoplasms developed in azoxymethane/dextran sodium sulphate (AOM/DSS) treated mice. (a, d) single aberrant crypt; (b, e) aberrant crypt focus - ACF; (c, f) microadenoma. (a, b, c) methylene blue and (d, e, f) hematoxylin-eosin stain. Original magnification (a, b, c) $\times 10$; (d, e, f) $\times 20$ (Robertis *et al.*, 2011).

Macroscopically, it is notable in AOM treated mice that the colonic tumours are observed in the middle and distal colon. Colonic tumours can be tubular adenomas or well/moderately differentiated tubular adenocarcinoma (Robertis *et al.*, 2011) (Figure 2.5). At week 20 after AOM treatment, the incidence of adenoma and adenocarcinoma is 100% and 38% with a multiplicity of 5.60 ± 2.42 and 0.20 ± 0.40 respectively (Tanaka, 2009). Similarly, at week 8 from AOM treatment the incidence of adenocarcinoma and tubular adenoma is 50% and 100% with a multiplicity of 4 ± 1.6 and 0.3 ± 0.7 respectively (De Robertis unpublished data). After week 21, only few adenocarcinomas invade the submucosa, muscularis propria, or serosa. The growth of adenomatous progression to malignancy after initiation of azoxymethane/dextran sodium sulphate (AOM/DSS) colon tumour in a mouse causes the regular invasion by immune cells and T lymphocytes on the bowel mucosal walls (Robertis *et al.*, 2011).

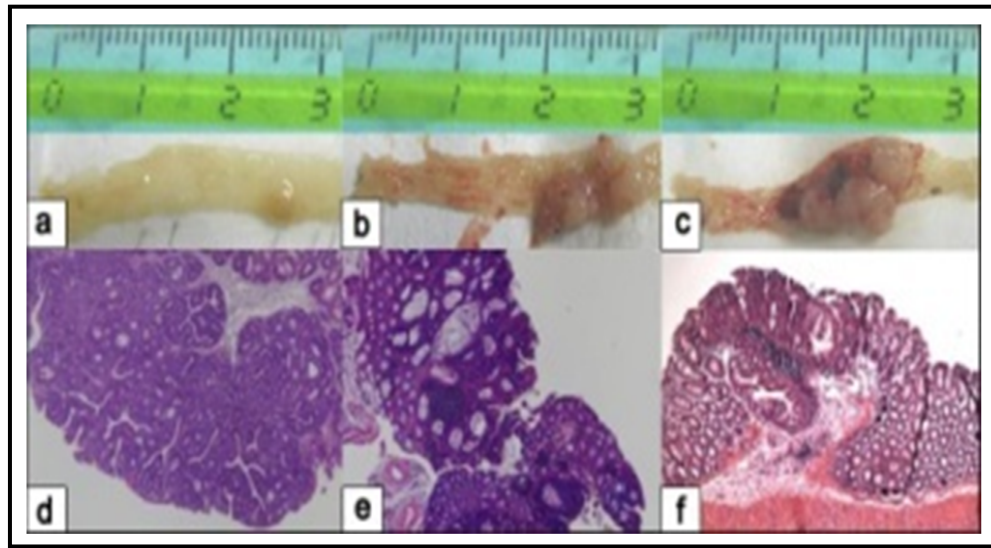


Fig 2.5 The histopathology of late colonic neoplasms developed in azoxymethane/dextran sodium sulphate (AOM/DSS) treated mice. (a, d) tubular adenoma; (b, e) moderately differentiated adenocarcinoma; and (c, f) moderately differentiated adenocarcinoma invading into the mucosa. (a, b, c) macroscopic analysis and (d, e, f) hematoxylin-eosin stain. Original magnification $\times 4$ (Robertis *et al.*, 2011).

2.1.5 Prevalence and risk factor of colon cancer

Colon cancer develops usually over a period of years, which may be estimated to be between 10 – 17 years (Jones *et al.*, 2008; Kelloff *et al.*, 2004). Most colon cancer develops from pre-existing adenomatous polyp (adenomas), which are common lesions that grow on the lining of the colon. About 50-75% of all people have the possibility of developing adenomas. (Bond, 2000; Schatzkin *et al.*, 1994). The prevalence of adenomas in Asia and Africa has been estimated between 10 - 15% and 35% in U.S. and Europe.

The incidence and mortality rates of colon cancer are 20% and 45% higher respectively in African American than in whites (American Cancer Society, 2011). The incidence rate of colon cancer in adults above 50 years is 15 times higher than those between 20 – 49 years. The risk increases with age and genetic mutation as the accumulating genetic events in ageing tissues explains the age-related exponential increase in colon cancer incidence (DePinho, 2000).

Inherited genetic mutations such as non-polyposis colon cancer, familial adenomatous polyposis (FAP), and ulcerative colitis are factors linked to family/hereditary history. About 20% of all colorectal cancer patients have a close relative diagnosed with the disease (Lynch and de la Chapelle, 2003).

Colorectal cases (1-2%) originate from patients with ulcerative colitis whose risk of developing malignancy is increased from a 2 to 8.2 fold. Environmental factors, such as living in an industrialized area physical inactivity, overweight, smoking, certain chemical exposure, consumption of alcohol and a low fiber diet are risk factors capable of developing colon cancer and increasing the rate of incidence. However, they are modifiable risk factors and therefore of greatest interest to decrease colon cancer risk. About 75% of colorectal cancer cases is associated with diet, which implies that diet contributes to the potential reduction of risk of the disease (Rafter, 2003). The great increase in incidence of colorectal cancer is associated with an increase in obesity rates as noted in most Northern European and Western countries (Larsson and Wolk, 2007).

2.1.6 Treatment and prevention

The early detection of precancerous lesions by regular screening and testing examination gives a higher chance of successful treatment of colon cancer. In the early stage of cancer detection, the removal of the tumour is done by surgical resection of the segment of the colon. An adjuvant radiation or chemotherapy is an added treatment especially when it is detected that the cancer has the chance of recurring in the patient. The use of chemotherapy (anticancer drugs) such as irinotecan and oxaliplatin has decreased the mortality rate associated with colon cancer. Fluorouracil (5-FU) principally reduces recurrence colorectal cancer, especially improving the survival of stage II or III patients (Sargent *et al.*, 2009). The spreading of cancer growth to adjacent tissues is reduced by the use of radiation therapy. This therapy may also be administered alone or combined with chemo and biologically targeted therapies but the survival rate from metastatic colon cancer for 5 years is less than 10% therefore, an effective therapy needs to be developed (Jemal *et al.*, 2006).

The treatment of metastatic colorectal cancer by the use of monoclonal antibody (Mab) targeted therapies [viz. Bevacizumab (avastin), cetuximab (erbitux) and panitumumab (vectibix)] was accepted by the US Food and Drug Administration (FDA). Bevacizumab obstructs the development of blood vessels to the tumour while cetuximab and panitumumab stop the outcome of cancer cell growth hormones. However, both cetuximab and panitumumab remain ineffective on certain tumours due to definite genetic alterations (Wang and Kelley, 2009). Bevacizumab anti-tumour activity is shown when combined with chemotherapy treatment for example intravenous 5-FU.

Despite promising results obtained from the use of bevacizumab in certain types of cancer that seems modest or irrelevant, toxicity effects profile were noted in clinical trials (Van Meter and Kim, 2010). This lead to the FDA's disapproval of bevacizumab as an indicative treatment for metastatic breast cancer. However, it has shown to have a beneficial effect on metastatic colorectal cancer but not on primary tumour stage 2 and 3, therefore suggesting that angiogenesis in these two phases differ in mechanisms requiring different means to target the mechanisms (Van Cutsem *et al.*, 2011).

Colon cancer is likely to be detected with tests such as flexible sigmoidoscopy, colonoscopy, barium enema with air contrast (double-contrast barium enema), stool DNA (sDNA) test and faecal occult blood test (FOBT). The long latency period of colon cancer is an opportunity to prevent the disease. Individuals who are at a high risk can begin screening and frequent testing at age 20 while those with an average risk should begin screening at age 50. However, it has been demonstrated that colon cancer is the only cancer that can be prevented by selecting an appropriate food and lifestyle (Chen and Huang, 2009). The choice of a physically active lifestyle, low consumption of alcohol, smoking cessation and high consumption of a healthy diet from plant sources specifically fruits and vegetables are recent recommendations to prevent the disease (International Agency for Research on Cancer, 2012). Therefore, high intake of dietary fibre (including prebiotics) and consumption of probiotics has been focused on to reduce the risk of colon cancer (Thantsha *et al.*, 2012).

2.2 Probiotics

2.2.1 Human colonic microflora

The human gut microflora consists of hundreds of types of microorganisms with an estimated value of over 10^{13} - 10^{14} bacteria playing an important role in maintaining the health of the body. Some of these bacteria grow and colonize the intestinal region of the host becoming the intestinal microflora, which stands as a line of defense against pathogenic organisms. These microorganisms are unevenly distributed at different sites of the digestive tract; stomach ($<10^3$), duodenum ($<10^3$), small intestine (10^2 - 10^3) and large intestine (10^{10} - 10^{12}) (Aureli *et al.*, 2011). A relatively small number of bacteria are enumerated in the stomach and small intestine compared to the large intestine owing to antimicrobial effect of the gastric acid and peristalsis motility in a healthy subject. The transition zone at the terminal of the ileum represented between the jejunum and the colon is predominated with aerobes and a dense population of Gram-negative anaerobes, which may count 10^9 cfu/ml. The human colon consists of a complex microbial composition mostly of bacteria which consist of more than 50 genera (Holdeman *et al.*, 1976). The bacterial concentration in the colon is estimated as high as 10^{12} cfu/ml. The colon comprises mostly of anaerobes such as *Bacteroides*, *Porphyromonas*, *Bifidobacterium*, *Lactobacillus* and *Clostridium* which outgrow the aerobes by a factor of 10^2 – 10^3 : 1 (Quigley, 2011). Some bacteria including *Bacteroides fragilis* and *Eubacterium rectale* inhabit discrete zones within the intestinal lumen of the human colon while some become adherent to the mucosal surface (Figure 2.6) (Swidsinski *et al.*, 2005).

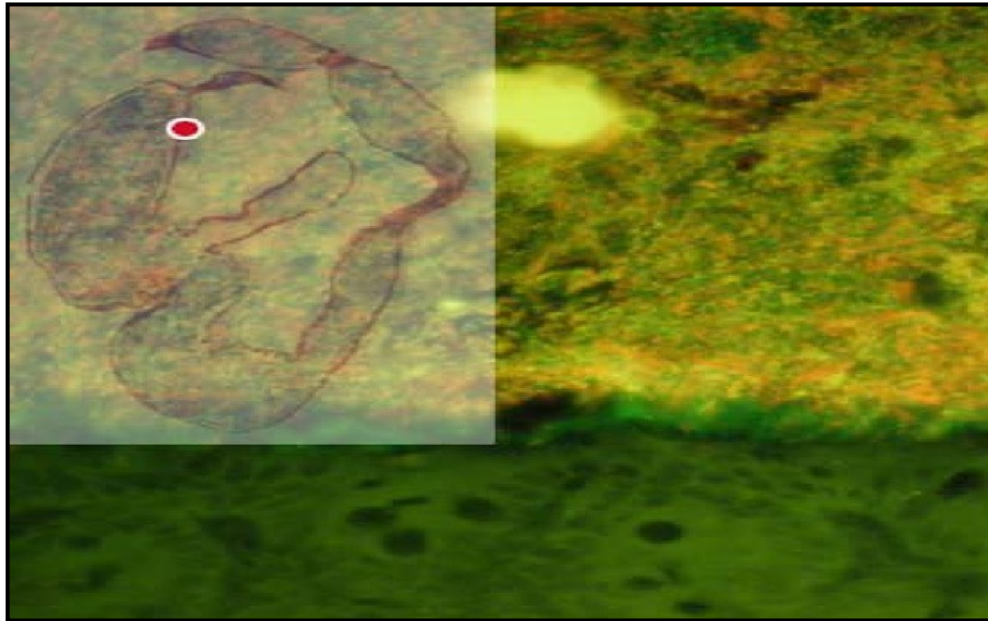


Fig 2.6 The differential distribution of bacteria in a colonic mucosae. FISH staining of the colonic wall microbiota reveals discrete zones for different bacterial species. Yellow staining designates the *B. fragilis*-specific Bfra probe conjugated to Cy3 dye; red staining designates the *E. rectale*-specific Erec probe conjugated to Cy5 dye, and green staining designates the universal bacterial Eub388probe conjugated to FITC dye (Swidsinski *et al.*, 2005).

The balance in interaction existing between the gut microbiota and the host is quite a fragile one demanding the balance be maintained. A slight disruption of the intestinal microflora or disturbance altering the interaction between the flora and the host can cause the host to be susceptible to infectious diseases (Sekirov *et al.*, 2010). A typical instance is related to the effect of broad-spectrum antibiotics, giving way to potential pathogenic organisms. At times microorganisms may find themselves in a favorable environment for their proliferation but different habitat from their normal flora causing overgrowth which eventually suppress the normal flora (Quigley and Abu-Shanab, 2010). It is therefore important that the integrity of the human gut microbiota and wellbeing is constantly protected. These organisms utilize the constituents of the food ingested for their metabolic activities obviously making the gut microbiota a target in the development and consumption of functional foods. Constant supply of functional food supplements consisting of probiotics and prebiotics has demonstrated a beneficial effect to the gut when sufficient amounts are consumed by the host (Roberfroid *et al.*, 2010).

2.2.2 Definitions and concept of probiotics

There has been a tremendous increase in knowledge about probiotics especially in the aspect of their function. Over the past few decades, probiotics have been defined based on different proposition, starting from being “a substance secreted by microorganism to stimulate the growth of another”, to “a substance that contribute to gut microbial balance” (Schrezenmeir and de Vrese, 2001). Probiotics have been defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Food and Agriculture Organisation, 2002). It was further described as a live microbial feed and food supplement that beneficially affect the host’s intestinal tract (Vasiljevic and Shah, 2008). The implantation or colonization of these viable microorganism(s) improves the microbial balance of the intestinal tract. One important point common to all the definitions is the ability of the probiotic to confer a beneficial effect on the health of the host. Probiotics are non-pathogenic microbes that exert a variety of beneficial effects such as antipathogenic effects, immunomodulatory factors, production of key nutrient and development of mucosal epithelium. Products derived from bacteria, or their end products cannot be considered as probiotic because they are not alive when administered or during consumption (Sanders *et al.*, 2007).

The term ‘probiotic’ includes a large range of microbial organisms, mainly bacteria such as lactic acid bacteria (LAB), non-lactic acid bacteria and also yeasts. The LAB includes the genera *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Enterococcus*, *Leuconostoc* and *Pediococcus* (Krasaekoopt *et al.*, 2003; Power *et al.*, 2008; Vandenplas *et al.*, 2007). Non lactic acid bacteria include *Escherichia coli nissle*, and also some yeasts (*Saccharomyces cerevisiae*, *Saccharomyces boulardii*). The most commonly used probiotic microorganisms associated with the human GIT are members of the genera *Lactobacillus* and *Bifidobacterium*. Species of the genus *Lactobacillus* include *L. acidophilus*, *L. casei*, *L. reuteri*, *L. rhamnosus*, *L. gasseri*, *L. brevis*, *L. amylovorous*, *L. crispatus*, *L. johnsonii*, *L. paracasei* and *L. plantarum* while the commonly used probiotics of the genus *Bifidobacterium* includes *B. longum*, *B. lactis*, *B. bifidum*, *B. infantis* and *B. breve* (Meurman and Stamatova, 2007).

2.2.2.1 Lactobacilli

Lactobacilli are Gram-positive, non-spore forming, rod shaped (sometimes coccobacilli), catalase negative, facultative anaerobe (sometimes aero-tolerant), fastidious, and acid-tolerant organism (Wood and Holzapfel, 1995). They have the ability to ferment glucose and produce solely lactic acid in the case of homolactic fermentation using the Embden-Meyerhof pathway metabolism. As heterolactic fermentation is concerned, carbon dioxide, ethanol, lactic acid and/or acetic acid are produced using the pentose phosphate pathway (Soccol *et al.*, 2010). Lactobacilli constitute the indigenous microflora widely distributed in the gastrointestinal and genital tract of humans and animals. They are inhabitants of carbohydrate rich-containing substrates especially in mucosal membranes. They can be considered as probiotic because they can stay alive in the GIT and give beneficial health effect to the host. The initial stage of colonization is the adhesion of the organisms to the intestinal epithelia wall, which provides the beneficial effects of the ingested probiotic bacteria. They are non-pathogenic and referred to as “generally regarded as safe” (GRAS) microorganisms (Salminen *et al.*, 1998).

2.2.2.2 Bifidobacteria

Bifidobacteria are Gram-positive, non-spore forming, non-motile, catalase negative and rod shaped organism with distinct club-shaped, bifurcated Y-shaped and curved rods morphologies. They are strictly anaerobes and grow at pH range of 4.5- 8.5.

Bifidobacteria are strictly carbohydrate fermentative producing acetic acid and lactic acid in a ratio of 3:2 (v/v) as the major end product but no carbon dioxide, butyric acid or propionic acid (Anal and Singh, 2007). They are distributed in various ecological niches in the human gastrointestinal and genitourinary tracts with exact ratio depending on the age and diet of the host. Their population in the gastrointestinal tract is about 25% of the colon microflora of an adult while they dominate the indigenous microflora in infants (Finegold *et al.*, 1983).

2.2.3 Potential role and health benefits of probiotic

The beneficial effects of probiotics are strain specific (Canani *et al.*, 2007). The health benefits vary for different strains of probiotic species. It is important to know that a considerable amount of viable probiotic should be consumed or administered by the host for effective functionality. The standard for any probiotic products must contain a minimum of 10^6 - 10^7 cfu/g products per day (Food and Agriculture Organisation, 2002; Krasaekoopt *et al.*, 2003). The ability of the probiotic bacteria to survive, multiply and become metabolically stable in the GIT strongly determines the benefits derived by the host. The benefits associated with probiotics can be of therapeutic (protective) or nutritional effect which depends on their mode of action (Prasad *et al.*, 1998; Sanders *et al.*, 2007).

Evidence from clinical data studies strongly substantiate the use of probiotics against inflammatory bowel disease (IBD), irritable bowel syndrome (IBS) and infectious diarrhea (Quigley, 2011; Saad *et al.*, 2013). Most diseases that are common to the human large bowel emanate from the distal colon, most notably colon cancer. The immune modulatory effect of probiotics on the intestinal gut is a bio therapeutic/preventive strategy against diabetes, obesity and colon cancer as they inhibit the colonization of the intestinal microbiota and translocation of pathogens (enterobacteria) to the liver (Arora *et al.*, 2013; Gregoret *et al.*, 2013; Yadav *et al.*, 2012).

2.2.3.1 Inflammation bowel diseases (IBD)

Inflammation bowel diseases such as Crohn's disease and ulcerative colitis are disorders characterized by chronic or recurrent inflammation of the mucosal lining marked by an auto immune response usually by the body's immune system. Probiotic administration regulates innate inflammatory responses in the mucosa by modulation of the gut microbiota composition through their effect on the epithelial and T cells on the surface of the lamina propria of the gut. The colon specific probiotic *L. rhamnosus* has been demonstrated to suppress inflammation and reduce apoptosis by activating the epithelial growth factor receptor (Yan *et al.*, 2011).

Secretory IgA antibody is one of the basic immunological responses induced by *Bifidobacterium* sp. binding to specific receptors on the intestinal epithelial surface and then released into the intestinal lumen (Aureli *et al.*, 2011). *Lactobacillus salivarius* Ls33 shows an anti-inflammatory effect in a colitis mouse model by the recognition of bacterial peptidoglycan and protein derived mucopeptides (Fernandez *et al.*, 2011). Probiotics such as non-pathogenic *Escherichia coli*, *Bifidobacterium* sp. and *Saccharomyces boulardii* has shown efficacy in reducing the post-operative recurrence and relapse in Crohn's disease. A meta-analysis evaluation was done to justify the efficacy of probiotics on Crohn's disease compared to a placebo for the prevention of post-operative recurrence, but the significant difference displayed by antibiotics reducing the clinical risk compared to the placebo as against probiotic requires more clinical trials to effectively define the preventative and therapeutic role of probiotic (Doherty *et al.*, 2010).

2.2.3.2 Irritable bowel syndrome (IBS)

Irritable bowel syndrome is a functional disorder in the colon that affects 8-22% of the population, especially women (Tuohy *et al.*, 2003). It is characterized by symptoms of abdominal discomfort usually related to disturbed defecation (motility of the colon), which causes a drastic reduction in beneficial gut bacteria. *L. rhamnosus* GG was reported to have little effect on IBS while *L. plantarum* 299V had a significant beneficial effect (Niedzielin *et al.*, 2001; O'Sullivan and O'Morain, 2000). A notable stability in the microbiota composition reduced abdominal pain and bowel distension which are basic symptoms of IBS when administered with a different species of probiotic supplements namely; *L. rhamnosus* Lc705, *L. rhamnosus* GG, *B. animalis* ssp. *lactis* Bb12 and *P. freudenreichii* ssp. *Shermanii* JS (Kajander *et al.*, 2008). A meta-analysis clinical trial was conducted on the effectiveness of probiotics on prevention of IBS and the probiotic therapy, in the clinical trial the probiotic sample (VSL#3) was shown to reduce the clinical symptoms and abdominal pain of IBS (McFarland and Dublin, 2008). The multispecies probiotic supplementation on IBS compared to single species stimulates the microbial composition in the colon thus affecting an improved bowel movement and replenishing the loss of beneficial gut bacteria.

2.2.3.3 Infectious diarrhoea

The efficacy of probiotics in several cases of infectious diarrhoea has been demonstrated in studies reducing the occurrence of diarrhoeal episodes, early symptoms and even rotavirus infection (Guandalini, 2006; Szajewska *et al.*, 2006). The administration of *L. rhamnosus* GG associated with oral rehydration therapy, once or twice daily, has effectively helped in the treatment of rotavirus associated diarrhoea, reducing the virus in stools thus offering an effective strategy to control the spread of nosocomial gastrointestinal infection (Hojsak *et al.*, 2010; Preidis *et al.*, 2011; Salazar-Lindo *et al.*, 2004; Szymański *et al.*, 2006). Sarker *et al.* (2005) reported that the treatment of gastroenteritis by probiotic *L. paracasei* ST1, though still controversial, improves non rotavirus diarrhoea condition in the children but had no effect on rotavirus gastroenteritis. Additional studies have also confirmed this through clinical evidences with the treatment of antibiotic associated diarrhoea with *L. rhamnosus* GG and *Saccharomyces boulardii*. It is however pertinent to mention that the strain specificity of probiotics is a subject of focus and relates to its efficacy in treatment of these infections (McFarland and Dublin, 2008). *Clostridium difficile* caused about 10-20% of antibiotic associated diarrhoea and a double-blind placebo controlled study was conducted as patients were administered with probiotic preparation of *L. bulgaricus*, *L. casei* and *S. thermophilus* which invariably reduced the incidence of *C. difficile* associated diarrhea (McFarland and Dublin, 2008).

2.2.3.4 Obesity

Obesity is closely related in terms of risk factor to colorectal cancer. It was suggested that infants presented with low level of *Bifidobacterium* sp. and higher number of *Staphylococcus aureus* in their stool are at a higher risk of obesity. A mouse model study demonstrated that the transplantation of microbial community within the gut could manipulate the propensity for deposition of fat. The administration of probiotics influences the intestinal microbiota inhibiting susceptibility to obesity. This depicts possible probiotic therapy which may prove to be a strategy for controlling childhood obesity (Gregoret *et al.*, 2013; Hsieh and Versalovic, 2008; Turnbaugh *et al.*, 2008).

In a randomized placebo controlled trial, fermented milk containing *L. gasseri* SBT2055 was administered to healthy and obese patients, with a decrease in fat, body weight and mass index was observed compared to the control group (Kadooka *et al.*, 2010). Probiotics interact with the endogenous bacteria in the gut by modification or regulation of fat metabolic pathways. Although the energy intake was less affected, the administration of *L. rhamnosus* PL60 in diet-induced obese mice lead to a significant loss in body weight by reducing the mass of white adipose tissue (Arora *et al.*, 2013).

2.2.4 Possible inhibition mechanism of colon cancer by probiotic bacteria

It is important to understand the mode/mechanisms by which probiotic bacteria perform their role to inhibit colon cancer. Zhu *et al.* (2011) surmises that possible mechanisms may involve cell cycle; apoptosis; reactive oxygen species (ROS); production of specific bacterial metabolic enzymes; and effects on host metabolism. However, some mechanisms were also suggested to be the possible ways by which this may occur which include alteration of quantitative and /or qualitative intestinal microflora involved in the production of carcinogen(s) and promoter(s); alteration in the physicochemical conditions in the colon; the production of anti-tumourigenic or anti-mutagenic compounds; enhancement of the host immune response system; effect on host physiology; and the binding and degradation of potential carcinogens (mutagenic compounds) (Rafter, 2003).

The G₁ phase is an important early phase of the cell cycle necessary for cell proliferation. *L. rhamnosus* GG induce an anti-proliferative effect in cancer cell lines by reducing the biosynthesis of polyamines. These may also be as a result of the lactobacilli ability to adhere to cells. Probiotic activity may bring about an induction of apoptosis possibly as an “oncologic surveillance” mechanism which eventually may prevent the proliferation of colon cancer, however more in-depth studies need to be carried out to confirm this hypothesis (Pagnini *et al.*, 2008). The presence of ROS is a prominent event in colon inflammation. Antioxidant properties of probiotic strains can inhibit/or reduce this effect and thereby increase probiotic gut colonization (Dolara *et al.*, 2005; Lin *et al.*, 2009; Park *et al.*, 2007). Interaction between the epithelial cells and microbiota gut is an active process that can be induced by the presence of probiotics in

production of pro-inflammatory cytokines (Paolillo *et al.*, 2009). Interleukin 12 (IL-12) can be induced after the activation of the innate immune system by some lactobacilli strains stimulating dendritic cells, native T cells in the lamina propria of the gut and macrophages (Shida *et al.*, 2006; Zhu *et al.*, 2011).

Probiotics ferment carbohydrates to synthesize short chain fatty acid (SCFA) products such as lactic, acetic and formic acids. The ability of this colonic microflora to produce SCFA during fermentation and low levels of some colonic enzymes, such as β -glucuronidase is suggested as the main process that prevents colorectal cancer (Fooks *et al.*, 1999; Holzapfel and Schillinger, 2002). The *Bifidobacterium* genome sequence has been shown to contain some metabolic enzymes which the bacteria produce to alter the nutrient composition of the environment which invariably adjusts their physiology, thereby adapting to new conditions (Schell *et al.*, 2002). *Bifidobacterium adolescentis* SPM0212 was reported to exhibit bacterial enzyme activities and some properties of anticancer which includes the inhibition of certain human cancer cell line proliferation, inhibition of harmful faecal enzymes such as β -glucuronidase, β -glucosidase, tryptophanase and urease, dose-dependently inhibits the production of TNF- α (Kim *et al.*, 2008).

Bacterial enzymes such as β -glucuronidase have the ability to hydrolyse many glucuronides thereby releasing carcinogens into the intestinal tract (Rowland *et al.*, 1998). Based on animal and human studies, the consumption of lactic acid bacterial cultures can reduce faecal enzyme levels, which may be involved in the formation of carcinogens. In a study carried out, the effect of *Lactobacillus acidophilus* strain NCFM and N-2 was evaluated on 21 healthy people for 10 days by checking the activity level of β -glucuronidase, nitroreductase and azoreductase, which resulted in the decrease of specific activities of these enzymes. The administration of *L. rhamnosus* LC705 and *Propionibacterium freudenreichii* ssp. *Shermanii* JS (PJS) to 38 healthy men for 4 weeks decreased the activity of β -glucosidase and increased the faecal count of lactobacilli and propionibacteria in the subjects (Hatakka *et al.*, 2008). Gastrointestinal tracts are dominated by the lactobacilli species, which contribute to the metabolic activities going on in this part of the host body.

A hypothesis with respect to colon carcinogenesis is that bile acids in the aqueous phase of the faeces exerts cytotoxic effects on colonic epithelium cells thereby causing an increase in cell proliferation in the intestine (Bruce, 1987). Some studies have shown significant decreases in bile acid with the intake of lactobacilli species. Mutagenic compounds in food diets bind to the cells of lactobacilli strains and the intestinal wall after exposure resulting in a reduction of the ratio of bound to free toxins within the intestine. The colonic mucosal has the ability to absorb mutagenic compounds and pass them into the bloodstream either as modified or unmodified metabolites from the intestinal lumen (Rafter, 2003).

2.2.5 Probiotic encapsulation

This is a relatively new and versatile technology that involves the entrapment of bioactive materials whose application ranges from cell therapy to drug delivery. In the past few decades, biotechnological immobilization applications involve the entrapment of bacteria in polymer matrices which serves as a physical barrier (Champagne *et al.*, 2008; Kailasapathy, 2002). This protects the probiotic cell against extrinsic conditions and separates the cell from their metabolites, but a small percentage of these cells may still be exposed at the surface. This technique was refined by forming a continuous coating around the inner matrix which contains the core encapsulated material (Kailasapathy, 2002). Microencapsulation segregates the core material from the environment until it is released, thereby stabilizing the cell and allowing small molecules like nutrients and metabolites to permeate through the semi permeable wall (membrane) contrary to immobilization. The capsule matrix maintains the core cells integrity during its passage through the GIT until the specified destination (colon) is reached where the probiotic bacteria is released following the breaking down of the microcapsule matrix/bead. Studies have shown that probiotics encapsulation has successfully protected cells against adverse environmental effects, improved their survival during storage and processing, releasing them in their viable and metabolic active states in the intestine under specified condition and extending their shelf life (Anal and Singh, 2007). Basically, there are two major methods used to produce microcapsules, namely, physical and chemical techniques (Table 2.2 and Table 2.3).

Table 2.2: Physical techniques of microencapsulation (Adapted from Anal and Singh, 2007)

Techniques	Feature	Advantages	Disadvantages
Spray-drying	Atomization of active material in polymer solution into a drying gas.	Rapid release of cell large amount of materials and economical.	High temperature
Spray freeze drying	Atomization of core material in frozen form and droplet dried in freeze dryer.	Heat sensitive bacteria, controlled size, large surface area capsule.	Expensive, long processing hours, high energy inputs
Coarcervation	Suspension of hydrophobic polymers in organic solvents, change in pH causes formation of shell and microcapsule.	Dispersed core materials in liquid are potentially coated.	Complex, difficult and agglomeration of capsules.
Extrusion	Centrifugal, nozzle vibration to disperse polymer solution.	Cheap, gentle on cell, simple flexibility, biocompatibility and uniformly shaped.	Difficult to scale up, slow production of beads, generally the diameter of bead formed is usually large (2-5mm).

Table 2.3: Chemical techniques of microencapsulation (Adapted from Anal and Singh, 2007)

Techniques	Feature	Advantages	Disadvantages
Emulsification and interfacial polymerization	Dispersion of aqueous phase containing cells and polymer suspension (water soluble and insoluble monomer) in organic phase product.	Easy to scale up, high survival of cell, small diameter capsule (25m-2mm), produces smooth surface, final byproducts.	Large size range and shape of beads, residual oil or other adjuvant may remain in the polymer thus require exhaustive washing to remove, monomers, surfactants and other.
Dispersion polymerization	Monomers dissolved in dispersion medium to produce a spherical form of insoluble polymers by precipitation.	Can produce nanoparticles and microparticles sizes (<15µm).	Tendency of stabilizers covalently attached or adsorbed to the bead may render it undesirable.
Suspension polymerization	Monomer mixture polymerized in a liquid phase and dispersed by continuous mechanical agitation.	Final bead product is easy to recover and can be use directly.	Difficult to make small size due to coalesce of particle during polymerization.

2.2.5.1 Spray drying

Many of the techniques used for microencapsulation are based on spraying, as many bioactive material can be dispensed in liquid form. It involves the dispersion of the active material into a polymer solution which becomes entrapped in a dried particle by atomization of the mixture in a drying chamber (Jackson and Lee, 1991). This technique operates under high temperatures which may not be suitable for encapsulating probiotic bacteria other than the fact that the cell can be stress-adapted to the high processing temperature (Champagne and Fustier, 2007; Kailasapathy, 2002). The stability of the bacteria may be achieved by adding a thermoprotectant, e.g. trehalose, granular starch, and prebiotics/probiotics in combination or coated by an additional layer (Burgain *et al.*, 2011; Semyonov *et al.*, 2010). However, it has the advantages of rapid release of cells, production of large amounts of material and its economical (Anal and Singh, 2007; Champagne and Fustier, 2007). Spray-freeze-drying combines both spray drying and the freeze drying processes. The probiotic cell is atomized into a frozen droplets which is freeze dried (de Vos *et al.*, 2010; Della Porta *et al.*, 2012; Semyonov *et al.*, 2010). It has disadvantages similar to spray drying for example it is a more expensive process but provides controlled size and a larger surface area than spray drying. Spray coating involves the spraying of the liquid coating material over the core material which is allowed to solidify forming a layer at the surface.

2.2.5.2 Extrusion

Microencapsulation by extrusion is a physical method that involves the projection of the emulsion core containing the cell suspension through a nozzle at a high pressure. This is preferably done by the pulsation or the vibration of the jet nozzle. The technique is cheap, simple, gentle and causes no harm but rather gives viability to the probiotic bacterial cell (Krasaekoopt *et al.*, 2003). The main issue associated with this technique is during scaling up of production due to slow formation of microbeads (Burgain *et al.*, 2011).

2.2.5.3 Emulsification and polymerization

For encapsulation by emulsion, the capsules or beads are formed in a two-step procedure namely dispersion and hardening. Emulsification is a chemical technique that involves a discontinuous and continuous phase requiring an emulsifier, a surfactant, and a solidifying agent (calcium chloride) which is added to the emulsion (Chen and Chen, 2007; de Vos *et al.*, 2010; Kailasapathy, 2009). The aqueous phase containing the bacterial cells and the polymer suspension are dispersed into an organic phase e.g. oil, resulting in water in oil emulsion. The dispersed aqueous droplets are then hardened by cooling or by the addition of a gelling agent or cross linking agent. The beads may then be coated with a second polymer to create a coating layer that provides added protection to the cells. The microcapsule/bead size depends on the agitation speed and water/oil ratio (Kailasapathy, 2009). However, it results in smaller diameter beads and ease of scaling, but there are a large range of sizes and shapes possible with this technique. *In situ* polymerization, which is closely related to interfacial polymerization involves monomers that are oil soluble and water-soluble reacting to form a polymer at the interface. Direct polymerization occurs on the surface of the dispersed core particle to produce a microsphere/bead with a uniform coating and smooth surface.

2.2.6 Probiotic applications in the food industry

The use of probiotics is continuously expanding and attracting attention especially in the food sector. The consumption of food has gone beyond meeting only the nutritional needs of the consumer. Health, food safety and environmental consciousness are important issues gaining attention in order to meet with consumer's demands. This may be attributed to food containing probiotics, known as functional foods and tend to have psychological or physiological effects on the consumers (Anal and Singh, 2007). Encapsulation is capable of providing stability and improving the viability of the final products encouraging the possibility of incorporating encapsulated probiotics in a wide range of food products. There are many dairy food products on the market which make use of this technique, these include ice cream, milk powder, cheese, yoghurt, dairy dessert and non dairy foods namely cereals, beverages, juices, and chocolates (Table 2.4) (Anal and Singh, 2007). Table 2.5 shows some of the common commercial probiotic products available in the market.

Table 2.4: Applications of encapsulated probiotics in food products

Strains	Biopolymers	Products	Technology	References
<i>B. bifidum</i>	k-carrageenan	Cheese	Emulsification	(Dinakar and Mistry, 1994)
<i>L. acidophilus</i> <i>B. lactis</i>	Alginate/starch	Yoghurt	Emulsification	(Kailasapathy, 2006)
<i>L. acidophilus</i> <i>B. bifidum</i> <i>L. casei</i>	Alginate– chitosan	Yoghurt	Extrusion	(Krasaekoopt <i>et al.</i> , 2006)
<i>L. acidophilus</i> <i>B. longum</i>	Maltodextrin/ gum Arabic	Yoghurt	Spray-drying	(Ohkawara <i>et al.</i> , 2007)
<i>L. acidophilus</i>	Alginate- chitosan	Yoghurt	Extrusion	(Urbanska <i>et al.</i> , 2007)
<i>L. casei</i> <i>B. lactis</i>	Calcium Alginate/starch	Ice cream	Emulsification	(Homayouni <i>et al.</i> , 2008)
<i>L. casei</i>	Alginate-pectin	Yoghurt	Extrusion	(Sandoval-Castilla <i>et al.</i> , 2010)
<i>L. plantarum</i>	Alginate-pectin Chitosan	Yoghurt	Emulsification	(Brinques and Ayub, 2011)
<i>L. acidophilus</i>	Calcium alginate	Cheese	Extrusion	(Mirzaei <i>et al.</i> , 2012)

Probiotics such as those incorporated into foods are important functional foods as they represent about 65% of the world functional food market which invariably continues to expand the market for probiotic products (Jankovic *et al.*, 2010).

Table 2.5: Commercial probiotic products

Company	Product	Probiotic strain	Health benefits
Chr. Hansen, Denmark		<i>Bifidobacterium bifidum</i> Bb-11, <i>Bifidobacterium animalis</i> Bb-12, <i>Lactobacillus acidophilus</i> LA-1/LA-5	Improves gut health
Nebraska cultures	-	<i>Lactobacillus acidophilus</i> DDS 1	Anti-carcinogenesis, alleviates traveler's diarrhoea, inhibits stomach ulcer, reduces lactose intolerance
Yakult Honsha Co. Japan	Yakult	<i>Lactobacillus casei</i>	
Danone, South Africa	Activia (Actiregularis)	<i>Bifidobacterium essensis</i>	Improves digestion
Danone, France	Actimel Immunatis	<i>Lactobacillus casei</i> DN 014001, <i>Bifidobacterium infantis</i>	Immune booster
Microbial solution, South Africa	Biostart	-	Aquaculture
Nestle	LC1	<i>Lactobacillus johnsonii</i>	Gastrointestinal health
Tablets India (Pvt) Ltd, India	Bifilac	<i>Lactobacillus sporogenesis</i> , <i>Clostridium bytiricum</i> JPC, <i>Bacillus mesentricus</i>	Diarrhoea, IBS
Ranbaxy, India	Binifit	<i>Clostridium bytiricum</i> , <i>Streptococcus faecalis</i> , <i>Baccillus mesentricus</i> LAB	Antibiotic associated diarrhoea
Biocodex, USA	Florastor	<i>Saccharomyces boulardii</i>	Gastrointestinal health

2.2.6.1 Fermented food products

2.2.6.1.1 Yoghurt

Yoghurt products are one of the most common carriers of probiotics in the dairy food sector. Yoghurt is a coagulated milk product that is fermented by *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. This fermentation leads to the production of lactic acid, which lowers the pH causing major cell death of probiotic bacteria during storage and refrigeration. They are usually produced in a hydrated form and therefore possess a short shelf life even when refrigerated (Lourens-Hattingh and Viljoen, 2001).

2.2.6.1.2 Cheese

Cheese proffers an advantage of being a good carrier of probiotics over yoghurt products, due to its high fat content and the high pH buffering capacity which offers protection against the acidic environment of the GIT (Kasımoğlu *et al.*, 2004). The encapsulation of *B. bifidum* in the frozen and lyophilized form of a gel bead in cheese using emulsification retains the survival of the cell even after 24 weeks (Dinakar and Mistry, 1994). The same was reported for *L. acidophilus* La5 encapsulated in a calcium alginate gel and resistant starch using extrusion with a higher viable count of probiotic bacteria ($>10^7$ cfu/ ml) at the end of a six month period above that recommended by the International Dairy Federation (IDF) (Mirzaei *et al.*, 2012). Both probiotic strains *L. acidophilus* and *B. bifidum* were incorporated into kasar cheese and white-brined cheese using emulsification and extrusion technology which improves the bacterial count, proteolysis and organoleptic properties of the final product (Özer *et al.*, 2009; Özer *et al.*, 2008). The addition of immobilized cells though was not uniform and did not affect the survival of the *Bifidobacterium* in the cheese (Dinakar and Mistry, 1994).

2.2.6.1.3 Ice cream

The high total solid of cheese and ice cream may proffer protection to cells against harsh environment (Akin *et al.*, 2007; Kasımoğlu *et al.*, 2004). Encapsulation has improved the shelf life of probiotic bacteria in symbiotic ice cream containing resistant starch with *L. casei* and *B. lactis* encapsulated in calcium alginate beads using emulsification techniques, with results showing that the survival rate of the probiotic was enhanced over an extended shelf life (Homayouni *et al.*, 2008). The survival rate was raised about 30% when compared to free cells within the ice cream over the same duration of storage. On the contrary, it was reported that free cells survived better than encapsulated cells when studying the incorporation of probiotics (*L. acidophilus* and *B. infantis*) into ice cream in various forms while another study showed no significant difference (Godward and Kailasapathy, 2003).

2.2.6.2 Non-dairy food products

Non dairy foods are also good alternatives that are gaining attention and interest within the food industry although dairy foods are the most recognized carriers of probiotics. The large consumer market of fruit juice gives the option of exploring fruit juice as a medium of probiotic delivery. Fruit juices such as orange, peach, grapefruit, green apple, lemon and blackcurrant tolerate the survival of some free cells for about 5 – 6 weeks, but in pomegranates and cranberry juices, the probiotic cells died within 1 – 4 weeks (Rice *et al.*, 2005). The encapsulation of *L. acidophilus* and *L. casei* in calcium alginate with double coating sodium alginate had a higher viability when exposed to simulated gastric condition (Mokarram *et al.*, 2009). The double coating might have reduced the pore size of the matrix membrane, thereby inhibiting the distribution of gastric solutions from coming in contact with the probiotic cells within the microsphere (Brinques and Ayub, 2011). The potential of using tomato juice to convey encapsulated probiotics such as *L. acidophilus*, *L. casei*, *L. brevis* and *L. plantarum* to the consumers has been demonstrated with the quality of the product not being affected (Klewicka *et al.*, 2009).

The significance of dietary fibre functionality in relation to probiotics has been incorporated due to their prebiotic activity. Oat bran displayed a high potential to promote the viability of the probiotic *L. casei* during cold storage (Guergoletto *et al.*, 2010). Studies have shown that the entrapment of probiotic within chocolates is an effective means of protecting them against environmental stress during production and the harsh condition for optimal delivery. Chocolate is a protective carrier in the oral delivery of microencapsulated probiotic mixtures such as *L. helveticus* CNCM 1-1722 and *B. longum* CNCM 1-3470 (Possemiers *et al.*, 2010). Chocolate-coated breakfast cereals which are a combination of chocolate and dietary fibre, also offers potential protection to *L. rhamnosus* strains by enhancing its stability and viability (Saarela *et al.*, 2006). After a rheological study was conducted on a selection of gum mixture, edible gellan-xanthan gum was found suitable for encapsulation of *B. lactis* incorporated into soft foods and beverages thus recommending the mixture for microencapsulation at both small and large scale production (McMaster and Kokott, 2005).

2.3 Pectin

2.3.1 Historical discovery of Pectin

Pectin is a naturally occurring polysaccharide found in the cell wall of plants like fruits and vegetables. It is highly concentrated in the skin and core part of the fruit but mainly extracted from citrus peel and apple pomace. The biological function of pectin is ensuring adequate protection of the plant from mechanical injury, growth and development of the cells. Pectin can be found not only in the primary cell wall of plants, but also in the middle lamella binding cells together.

Fruits such as apple, citrus, grapes, blackberries and plums are high in pectin while apricots, blueberries, peaches, pears, raspberries and strawberries are low in pectin (Srivastava and Malviya, 2011). In 1790, Louis Nicolas Vauquelin discovered some fruit juices had the ability to gel. This property was later understood when Henri Braconnot in 1825, made new observations on pectic acid. The enzyme pectase in fruit causes coagulation to occur by converting pectin to pectic acid (from the Greek word “pexis”). Since this period, scientists have been working to unravel the potential functions and application of this polysaccharide “pectic substance” (Kondo *et al.*, 1994; Yapo, 2011).

2.3.2 Structure of pectin

Pectin is known to be a complex water soluble polysaccharide containing 1,4-linked α -D-galacturonic acid as the predominant residue with a part of methoxylated carboxyl group structure which makes it an acidic polysaccharide (Yapo, 2011). This is the ‘smooth region’ of the homogalacturonan (HG), which contains carboxyl groups, which are methyl-esterified. Also, another substantial component characterized with branch-points is the ‘hairy’ region of pectin (non-gelling area) composed of alternating α -(1-2)-L-rhamnose- α -(1-4)-D-galacturonic acid (1-20 residues). This region is composed mostly of neutral sugar side chains mainly L-arabinose, D-galactose (rhamnogalacturonan 1) (RG1), arabinans, galactans, arabinogalactans and other residues like D-xylose, D-glucuronic acid as shown in Figure 2.7 and Figure 2.8 (Vincken *et al.*, 2003; Yapo, 2009).

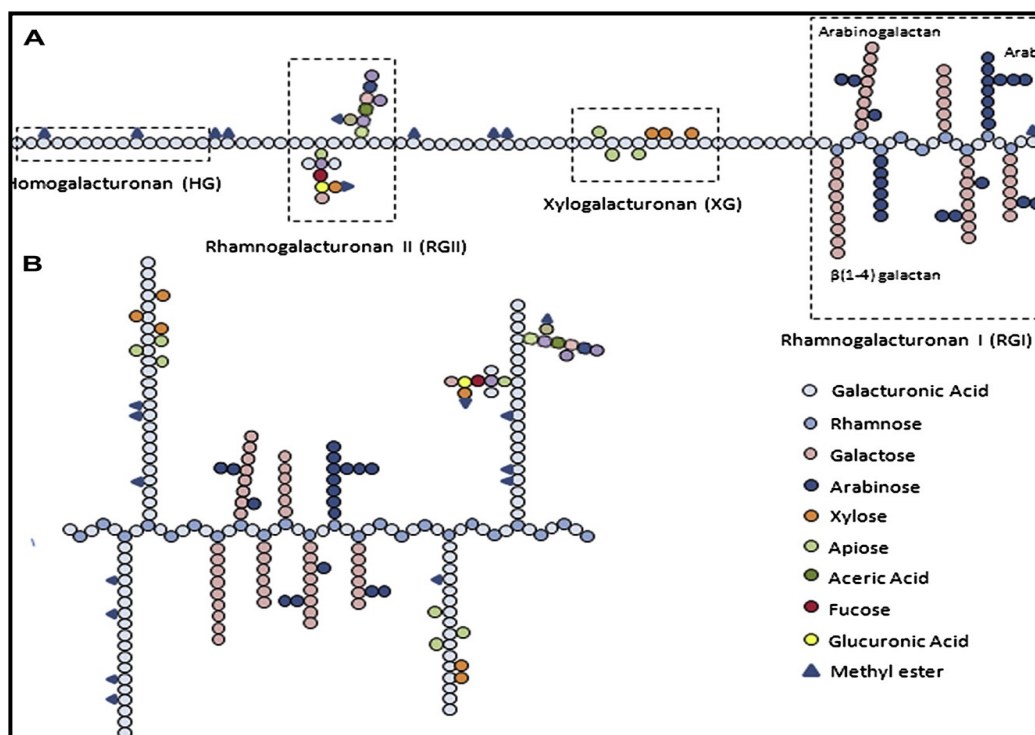


Fig 2.7 Simplified schematic diagrams of the two proposed structures of pectin. (A) The RG1 (hairy) region is considered attached to HG (smooth) regions. (B) An alternative structure whereby HG are side chains of RG1 (Maxwell *et al.*, 2012).

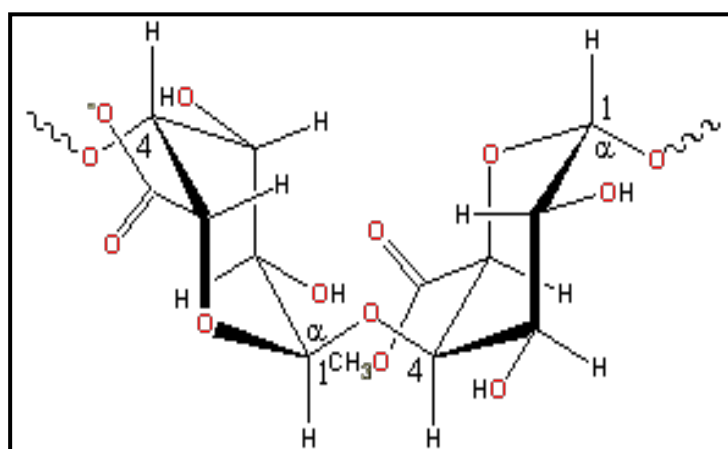


Fig 2.8 The molecular unit structure of pectin.

Rhamnogalacturonan 1 regions still require further characterization even though the composition varies in different species of plant and the isolation technique matters. The RG1 region (arabinans and galactans) adopt more flexibility which correlates more with the transition stages in the cell or/and tissue developments for instance, from cell division to cell elongation (Yapo, 2011).

This flexible hairy region is so important because galactan, arabinan and arabinogalactan side chains are located here and attached to the rhamnose residue. The relative flexibility and elasticity of the cell wall has been suggested to be likely due to the arabinans side chain attached to RG1(Moore *et al.*, 2008). There are two types of arabinogalactans attached to the rhamnose backbone residue: firstly, the linear β -(1-4)-D-galactan which is most likely a structural weapon with an affinity for binding with the carbohydrate recognition domain (CRD) gal-3 responsible for cancer proliferation and metastasis and secondly, the branched β -(1-3,6)-D-galactan (Maxwell *et al.*, 2012).

2.3.3 Properties of pectin

Pectin can be classified as a high methoxyl pectin (HMP) and low methoxyl pectin (LMP) depending on the degree of esterification, structure of pectin, molecular size and its gelling properties. The high methoxyl pectin has a degree of esterification (DE) above 50% while low methoxyl pectin is less than 50% DE (Rinaudo, 1996). Esterification is simply put as when a galacturonic acid group along the pectin chain has a large group of methyl attached to it while the DE is the ratio of galacturonic acid residues that has a methyl group attached to them to the ones that are free, in other words, the number of moles of methoxyl group in hundred moles of residue of galacturonic acid (Rinaudo, 1996). The acetyl- and methyl-esterification of HGs are mainly responsible for the gelling properties of pectin (Yapo and Koffi, 2006). Typically, the esterification of HMP gel at approximately 65% through hydrophobic interaction and hydrogen bonding requires an acidic medium (for electrostatic repulsion) and sugar (sucrose to reduce polymer water interaction). LMP gels through ionic interaction in the presence of polyvalent ions such as calcium. One advantage of LMP is that the strength of gelling increases as the DE value is reduced. This creates more chances for calcium to interact and bind with the carboxyl group, thus expanding the possibility of LMP to bind as the concentration of calcium ions increase. Low temperature (10°C) favours gel formation unlike high temperature and an increase in pH (less than 3) (Kohn, 1982; Srivastava and Malviya, 2011). The interaction of two fold helical chains forming an 'egg-box' structure as similarly envisaged in alginate and depicts the potential of pectin as an encapsulating agent (protecting core material from extrinsic factors).

Pectin in fruits and vegetables are consumed daily as dietary fibre, to prevent the occurrence of diseases such as diabetes and colorectal cancer (Yapo and Koffi, 2008). Pectin has a water retaining ability that makes it form a gel. This enables pectin to remove toxic waste from the colon and prevent constipation. When extracted, they are used as an oil-in-water emulsification in food formulations, water binder, stabilizer and thickening agent in the production of jams, jellies, fruit juices and milk drinks. Pectin is a high value functional food ingredient utilized in the nutraceutical industry (Srivastava and Malviya, 2011; Yapo *et al.*, 2007). It has emerged recently that pectin possesses the ability to inhibit tumourigenesis in cells with the discovery of a pro-metastatic protein gal-3 binding to galactan and has since become a major focus of research (Glinsky and Raz, 2009; Maxwell *et al.*, 2012). Most normal cells in the body have, on their surface few galectin molecules (particularly gal-3) that communicate messages to other cells and adhere to promote cell adhesions (Eliaz, 2001; Maxwell *et al.*, 2012; Morris, 2009).

2.3.3.1 Pectin as a biopolymer agent and prebiotic

Polysaccharides are biodegraded by the colonic microflora thus possessing the ability to control the release of drugs. They are used for target delivery means for drugs and bioactive living cells especially probiotics. Pectin is not degradable in the upper GIT and is a low cost polysaccharide and has therefore been utilized as a potential mechanism for the release of small drugs for colon specific delivery, but there have been limited trials on probiotic delivery (McConnell *et al.*, 2008). The water-soluble nature of pectin in an aqueous environment causes it to dissolve quickly thereby limiting its use. However, this major setback could be overcome by combining pectin with other material to form a composite (Lam *et al.*, 2008; Liu *et al.*, 2007). Prebiotics are nondigestible carbohydrates that, indirectly benefit the host, when consumed in substantial amounts by selectively influencing the growth and survival of the specific genera of beneficial bacteria in the colon (Ouweland *et al.*, 2007).

Prebiotic combination with probiotics is known as synbiotics. As the concept of synbiotics is new, it can be defined as 'a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the GIT, by selectively stimulating the growth and/or

activating the metabolism of one or a limited number of health promoting bacteria, and thus improving host welfare' (Burgain *et al.*, 2011; Roberfroid *et al.*, 2010). A food ingredient is classified as a prebiotic when it can resist acidity absorption of the GIT and hydrolysis of human enzymes, ability of the gut microbiome to ferment it, and selectively stimulate the growth and/or activity of the gut health promoting bacteria. The most common prebiotics used are inulin, fructo-oligosaccharides and more recently pectin, because of their ability to resist the effect of gastric acid and pancreatic enzymes (Gibson, 1998; Ramchandran and Shah, 2010).

2.3.4 Modified Pectin (MP)

The molecular weight of industrial pectin can be broken down into smaller fragments through the effect of pH modification by alkaline (sodium hydroxide) and/or with acid treatment or by enzymatic breakdown which gives uniform fragments of smaller size of about 10000-20000 daltons (Eliaz, 2001; Maxwell *et al.*, 2012). Ordinarily, the degree of esterification in pectin is as high as 70%, which can be determined by high performance liquid chromatography (HPLC) and gas chromatography (GC). This modification causes the β -elimination cleavage from the HG backbone thereby releasing oligomers of polygalacturonic. The rhamnogalacturonan 1 regions can be further split into galactans and arabinogalactan with less arabinose substitutes (Figure 2.9) as the acid treatment cleaves linkages between the neutral sugars and eventually modifies the RGI (Diaz *et al.*, 2007; Morris, 2009).

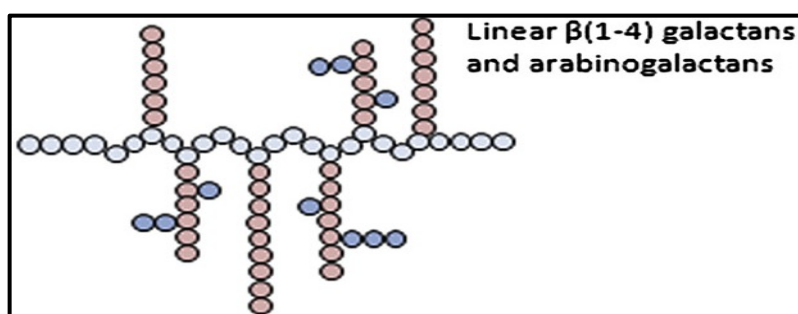


Fig 2.9 The schematic structure of modified pectin rhamnogalacturonan 1 (RG1). Alkali treatment breaks down the HG backbone and acid treatment cleaves the neutral sugars, preferentially leaving galactans and arabinogalactans (Maxwell *et al.*, 2012).

The DE in modified pectin has been defined to be as less than 10% which means that the removal of methoxyl group from HMP resulting in LMP creates more chance for the carboxyl group on the galactan CRD to interact more with calcium cations. DE is an important factor which determines the bioavailability of free galactans that binds with gal-3 (Morris, 2009). In a study carried out on pectin from different plant sources such as ginger, larch wood, citrus pectin (CP) and swallow root pectin polysaccharide, with CP discovered to have a higher gal-3 inhibiting effect when compared to others (Sathisha *et al.*, 2007).

The oral administration of MCP (dose dependent) has been shown to reduce liver metastasis in a mouse model of colon cancer (Liu *et al.*, 2008). The effect of modified apple pectin (MAP) on mouse model colitis-associated colon cancer induced apoptosis, reduced inflammation and prevented the formation of tumours. Surprisingly, MAP enhances the increase of gal-3 in the nucleus and cytoplasm of the cells (Li *et al.*, 2012). Invariably as a result of its competition with the cells for target gal-3, the cells continue to produce more gal-3 in order to replenish them for the bound ones (Li *et al.*, 2012).

CHAPTER 3

VIABILITY OF A MICROENCAPSULATED PROBIOTIC IN SIMULATED GASTRIC AND INTESTINAL JUICES

3.1 Introduction

The use of probiotic microorganisms in the food industry and complementary medicine has gained great interest (Gbassi *et al.*, 2011; Mitropoulou *et al.*, 2013). Probiotics are an important live supplement containing health beneficial microorganisms that the consumer derives from functional foods. These microorganisms when consumed pass through the gastrointestinal tract (GIT) to reach their target site during which survival can be compromised. Therefore, the viability of probiotic microorganisms during storage and transit in the GIT is of paramount importance (Anal and Singh, 2007; Gebara *et al.*, 2013).

Probiotic encapsulation has successfully protected cells against adverse environmental effects such as stress, oxygen and high temperature during processing storage (Anal and Singh, 2007; Rathore *et al.*, 2013) pH and intestinal enzymatic activity (Anal and Singh, 2007; Heidebach *et al.*, 2012). This improves the survival of the probiotic and remain metabolically in active states in the intestine under specified conditions. Various materials have been employed in probiotic encapsulation but fast and easy inflow of water and other liquids through some the matrices is a limitation (Anal and Singh, 2007). Different biopolymer matrix/systems have been used for encapsulation which includes k-carrageenan (Dinakar and Mistry, 1994), alginate and starch (Kailasapathy, 2006), arabic gum and maltodextrin, xanthan gum, chitosan (Nualkaekul *et al.*, 2012), whey protein (Gerez *et al.*, 2012; Souza *et al.*, 2012), cellulose acetate phthalate and pectin (Gebara *et al.*, 2013; Gerez *et al.*, 2012; Souza *et al.*, 2012). Porosity is however still an issue allowing fast and easy inflow of water and other liquids through the matrix (Anal and Singh, 2007). Alginate has been extensively studied and found suitable for probiotic encapsulation because of its non toxicity, its “generally recognized as safe” (GRAS) and it has the ability to entrap living microorganisms by forming mild matrices with calcium chloride (Dinakar and Mistry, 1994; Ding and Shah, 2007; Gombotz and Wee, 2012; Sheu and Marshall, 1993).

Calcium alginate has been shown to protect *Lactobacilli* spp. (Ortakci and Sert, 2012) in combination with resistant starch (Homayouni *et al.*, 2008; Mirzaei *et al.*, 2012) and even more effective when coated with chitosan (Chávarri *et al.*, 2010; Chen *et al.*, 2013; Cook *et al.*, 2011; Krasaekoopt *et al.*, 2006). Calcium alginate coated with chitosan is capable of controlling the alginate instability in high acidic condition ($\text{pH} < 2$) and in the presence of chelating agents (Gombotz and Wee, 2012; Sultana *et al.*, 2000). Studies have shown that unamidated or amidated pectin blend with alginate protects probiotic bacteria (Brinques and Ayub, 2011; Knaup *et al.*, 2008; Sandoval-Castilla *et al.*, 2010).

Modified pectin (MP) is a complex water soluble indigestible polysaccharide that has been treated by a low temperature, pH (7-8) and enzymatically to break down the pectin into smaller fragments. Pectin is derived from the pith of oranges, limes, lemons and grapefruit. Modified pectin, rich in β -galactose, is potentially safe, non toxic and possess a unique bioactivity for inhibiting carcinogenesis (Maxwell *et al.*, 2012; Morris, 2009). Modified citrus pectin (MCP) has been used as dietary supplement orally to promote cell growth. The microbial bacterial disintegration of MP at the colon compared to the ordinary pectin generates a distinctive effect accounting for a lower incidence of certain cancers upon oral consumption of MP (Azémar *et al.*, 2007; Li *et al.*, 2012; Liu *et al.*, 2008). Modified pectin could be more beneficial in the functional food industry, maybe as a polymer matrix but this remains uncertain. Amidated pectin was found to be stable in simulated gastric juice and human saliva but degraded by the metabolic activity of colon microflora (Knaup *et al.*, 2008). The sensitivity of free probiotic bacteria to extremely low pH in the stomach and bile salt reduces their survival considerably. The stomach gastric juice have a pH of 1.5–3.0 (Kos *et al.*, 2000). The loss of 6-8 \log_{10} cfu/g probiotic bacteria in simulated gastric digestion have been reported (Brinques and Ayub, 2011).

Therefore, providing effective protection to probiotic bacteria during gastric passage positively influences their health-promoting effects (Ortakci and Sert, 2012). Thus, the stability of the MP in simulated physiological (gastric and intestinal) conditions needs to be determined.

The aim of this study was to investigate the effect of modified citrus pectin alginate (MCPA) microencapsulation improving the survival of *Lactobacillus acidophilus* ATCC 4356 under *in vitro* simulated (acidic) gastric and intestinal (bile) conditions. In addition, the viability of microencapsulated cells under storage was also determined.

3.2 Materials and Methods

3.2.1 Plant materials

Modified citrus pectin is a natural supplement product obtained from ecoNugenics Inc. (CA, USA). It was extracted from the peel pith of orange fruit, *Citrus* sp. and processed in the laboratory. The pectin was enzymatically-modified into smaller fragments, which was finally obtained in the form of a fine powder that was stored at room temperature.

Sodium alginate is a natural polysaccharide product obtained from Sigma Aldrich (St. Louis, M.O. USA). It was extracted from the cell wall of brown seaweed mostly found in cold-water regions and finally obtained in powder form.

3.2.2 *Lactobacillus acidophilus* ATCC 4356 growth conditions and harvesting

Frozen stock culture of *L. acidophilus* ATCC 4356 (Microbiologics, St Cloud, MN) was rehydrated in De Mann, Rogosa and Sharpe (MRS) broth and incubated at 37°C for 48 h under aerobic conditions. The culture was transferred into fresh MRS broth and incubated under the same condition as before to obtain a cell count between 9– 10 log₁₀cfu/g. The cells were harvested by centrifugation at 3150 x g for 5 min at 4°C and washed twice in sterile saline (0.9% NaCl) solution. The cells were dispersed and serially diluted (10–10¹⁰) in sterile saline solution. Afterwards, 100 µl cell aliquots were plated onto MRS agar and incubated at 37°C for 48 h under anaerobic conditions using an Anaerocult® jar. Number of cells harvested were counted using the automated colony counter Doc-It® imaging station (UVP, C.A, USA) and recorded in log₁₀cfu/g. Fresh cell suspensions of *L. acidophilus* ATCC 4356 were prepared for each microencapsulation procedure and enumerated by pour plating in MRS agar. Plates were incubated under the same condition as before.

3.2.3 Preparation of modified citrus pectin alginate *Lactobacillus acidophilus* ATCC 4356 microbeads

Modified citrus pectin alginate (MCPA) and alginate calcium (AP) probiotic microbeads were produced aseptically at room temperature as illustrated in Figure 3.1 using emulsification polymerization method (Homayouni *et al.*, 2008). Modified citrus pectin (8.5%) and sodium alginate (2%) polymers, incorporated with hi-maize resistant starch (2%) (National Starch Food Innovation, Wadeville, Guateng) were agitated in distilled water for 10 min. Cell suspension (1 ml) of *L. acidophilus* ATCC 4356 containing $9.60 \log_{10}\text{cfu/ml}$ was added to the polymer matrix emulsion in canola oil. The mixture was emulsified by adding lecithin (0.1%) with a constant agitation at 1130 x g force for 40 min. Calcium chloride (0.1 M) solution was added to the polymer mixture to harden the beads and was agitated for 5 min. Thereafter, the MCPA or AP probiotic microbeads were collected by centrifugation at 3150 x g force for 5 min at 4°C and coated in chitosan (0.2 g) solution (Sigma Aldrich, St. Louis, M.O, USA). The microbeads were retrieved, washed and stored in sodium glycerol (0.9% NaCl, 5% glycerol) solution at 4°C.

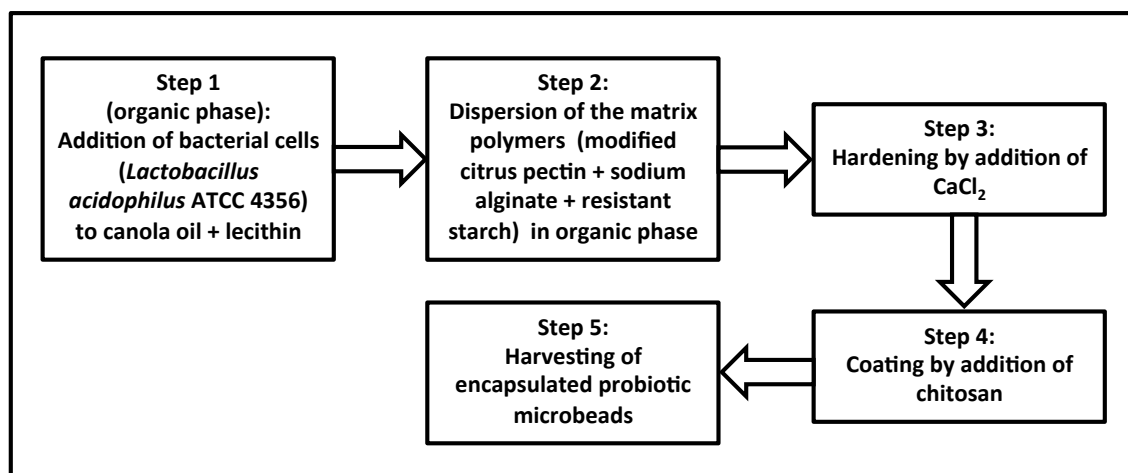


Fig 3.1 Schematic diagram of the emulsification polymerization procedure.

The morphology and diameter size (μm) of the microbeads were measured and images captured using a MOTIC optical microscope (Motic images Plus 2.0 software, Moticam 2500 Hong Kong, Asia). The size of each microbead was presented as mean \pm standard deviation (SD).

3.2.4 Enumeration of viable *Lactobacillus acidophilus* ATCC 4356 cells in microbeads

After the probiotic microbeads were produced, the beads were broken to determine the count of viable entrapped *L. acidophilus* ATCC 4356 cells. The encapsulated bacteria in MCPA or AP microbeads were released by dissolving 1 g of the microbeads in 9 ml of sterile phosphate buffer solution (PBS) pH (7.4) in test tubes for 15 min at room temperature using a vortex for stirring (Annan *et al.*, 2008; Sheu and Marshall, 1993). Released *L. acidophilus* ATCC 4356 cells were retrieved by centrifugation at 3150 x g force for 5 min at 4°C. Cells were serially diluted in 0.9% saline solution as 100 µl aliquots of cells were plated on MRS agar and incubated at 37°C for 48 h under anaerobic condition. Colonies of *L. acidophilus* ATCC 4356 cells were enumerated using the automated colony counter Doc-It® imaging station and recorded in log₁₀cfu/g. Experiments were performed in triplicate. The microencapsulation yield (EY), which is a combined measurement of the efficacy of encapsulation and survival of viable cells during the microencapsulation procedure was calculated using the following formula:

$$EY = (N / N_0) \times 100$$

Where N is the number of viable encapsulated cells released from the microbeads (log₁₀ cfu/g) and N₀ is the number of free cells added to the biopolymer matrix emulsion.

3.2.5 Determination of survival of free and microencapsulated *Lactobacillus acidophilus* ATCC 4356 in simulated gastric and intestinal juice

Simulated gastric juice (SGJ) consisted of 9 g/l of NaCl and 3 g/l of pepsin (St. Louis, M.O. USA) with a final pH of 2.0 adjusted with 0.1 M hydrochloric acid (HCl) as described by Annan *et al.* (2008). Simulated intestinal juice (SIJ) was prepared by dissolving 3% (w/v) bile salt (Sigma Aldrich, St. Louis, M.O. USA), 6.5 g/l NaCl, 0.835 g/l KCl, 0.22 g/l CaCl₂ and 1.386 g/l NaHCO₃ with a final pH of 8.0 adjusted with 0.1 M NaOH. Freshly prepared probiotic microbeads (1 g of MCPA or AP) with entrapped 8.16 log₁₀cfu/g *L. acidophilus* ATCC 4356 or 1 ml (9.2 log₁₀cfu/ml) of free *L. acidophilus* ATCC 4356 cell mixed separately in test tubes containing 9 ml of SGJ or SIJ were incubated at 37°C for 30, 60, 120 and 180 min with a constant agitation.

At each specified time interval, free *L. acidophilus* ATCC 4356 cells were harvested by washing twice in 0.9% saline solution. The enumeration of free *L. acidophilus* ATCC 4356 cells was carried out by diluting serially (10^{-1} – 10^{-9}) in saline solution and plated out (100 μ l) aliquots onto MRS agar for incubation at 37°C for 48 h. Also, at each time intervals, MCPA or AP probiotic microbeads were harvested, washed and dissolved in 9 ml of PBS for 10 min on a shaker at room temperature. Released cells from microbeads were retrieved, serially diluted in saline solution and 100 μ l aliquots of cells were plated on MRS agar for incubation at 37°C for 48 h to determine the number of viable *L. acidophilus* ATCC 4356 cells in the microbeads. Experiments were performed in triplicate.

3.3 Statistical analysis

Data analysis were subjected to two way analysis variance and Tukey's test to determine the significant differences of the efficiency of the microbeads and survival of probiotic using Graphpad Prism software, SSPS version 17.0 for Windows (SSPS, Chicago, Illinois, USA). Results were presented as means \pm standard deviation (SD) and statistical significance was set as $p < 0.05$.

3.4 Results

3.4.1 Microencapsulation yield and size of modified citrus pectin alginate and alginate calcium probiotic microbeads

To determine the number of viable cells entrapped in the microbeads during microencapsulation, the average count of viable encapsulated *L. acidophilus* ATCC 4356 cells released from the MCPA microbeads was $8.43 \pm 0.08 \log_{10}\text{cfu/g}$ whilst AP microbeads contained $8.07 \pm 0.04 \log_{10}\text{cfu/g}$. The difference between the number of viable entrapped cells and released cells from the microbeads could indicate that not all cells were entrapped during microencapsulation procedure. However, both the MCPA and AP probiotic microbeads showed a high microencapsulation yield of $87.8 \pm 0.8\%$ and $84.0 \pm 0.4\%$ respectively. In this study, the average mean diameter of the MCPA microbeads ($220.89 \pm 2.6 \mu\text{m}$ to $685.19 \pm 0.7 \mu\text{m}$) was observed compared to the AP

microbeads ($147.61 \pm 2.6 \mu\text{m}$ to $358.09 \pm 2.6 \mu\text{m}$) with a significant difference ($p < 0.05$). The shape of microbeads produced was spherical and/or vermiform.

Although, MCPA microbead particles were vermiform shape, they tend to form a complete spherical shape after chitosan coating which results into having more of spherical shape than of a vermiform shape in the MCPA microbeads while AP microbead particles were more spherical (Figure 3.2).

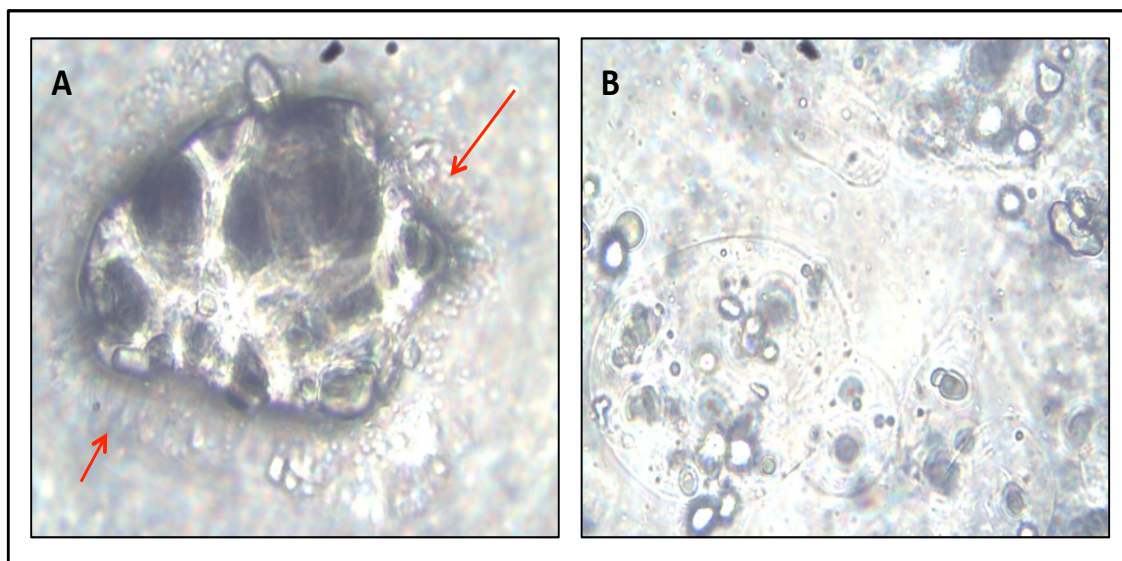


Fig 3.2 The morphology of (A) modified citrus pectin alginate (MCPA) probiotic microbead and (B) alginate calcium (AP) probiotic microbead particles produced by emulsification with arrows indicating the chitosan coating effect.

3.4.2 Stability of free and microencapsulated *Lactobacillus acidophilus*

ATCC 4356 in storage

The stability of the microencapsulated *L. acidophilus* ATCC 4356 at 4°C of storage was examined. A value $6.75 \pm 0.07 \log_{10}\text{cfu/g}$ (70.4%) of the free *L. acidophilus* ATCC 4356 was considerably lost after 28 days of storage ($p < 0.0001$). At day 28, the viability of entrapped *L. acidophilus* ATCC 4356 in the MCPA and AP microbeads was $7.30 \pm 0.12 \log_{10}\text{cfu/g}$ (86.6%) and $6.69 \pm 0.16 \log_{10}\text{cfu/g}$ (82.9%) respectively. The reduction in viable cells of *L. acidophilus* ATCC 4356 in MCPA or AP probiotic microbeads was $< 1.4 \log_{10}\text{cfu/g}$. However, microencapsulated *L. acidophilus* ATCC 4356 had a significantly higher survival compared to the free cells after 28 days of storage ($p < 0.0001$) (Figure 3.3). The difference in viability of *L. acidophilus* ATCC 4356 in MCPA

and AP microbeads from day 0 – 14 was insignificant till day 21 when the probiotic count in AP microbeads begins to reduce significantly ($p < 0.01$) (Appendix 1 - Table A1). The decrease in number of *L. acidophilus* ATCC 4356 in MCPA microbeads between day 7 and 21, however, remains insignificant ($p > 0.05$). The MCPA and AP microbeads showed a high microencapsulation yield during storage at 4°C as shown in Table 3.1.

Table 3.1 Encapsulation yield of microencapsulated and free *Lactobacillus acidophilus* ATCC 4356 during storage at 4°C

Time (days)	Encapsulation yield (%)		
	MCPA	AP	Free 4356
0	87.8 ± 0.08	84.0 ± 0.04	100 ± 0.06
7	83.3 ± 0.01	81.7 ± 0.02	82.8 ± 0.15
14	81.3 ± 0.2	80.4 ± 0.08	60.8 ± 0.32
21	80.4 ± 0.24	73.6 ± 0.15	36.1 ± 0.23
28	76.0 ± 0.12	69.6 ± 0.16	29.6 ± 0.13

MCPA- modified citrus pectin alginate, AP- alginate calcium, Free 4356- Free *Lactobacillus acidophilus* ATCC 4356. Values represent means ± S.D (n=3).

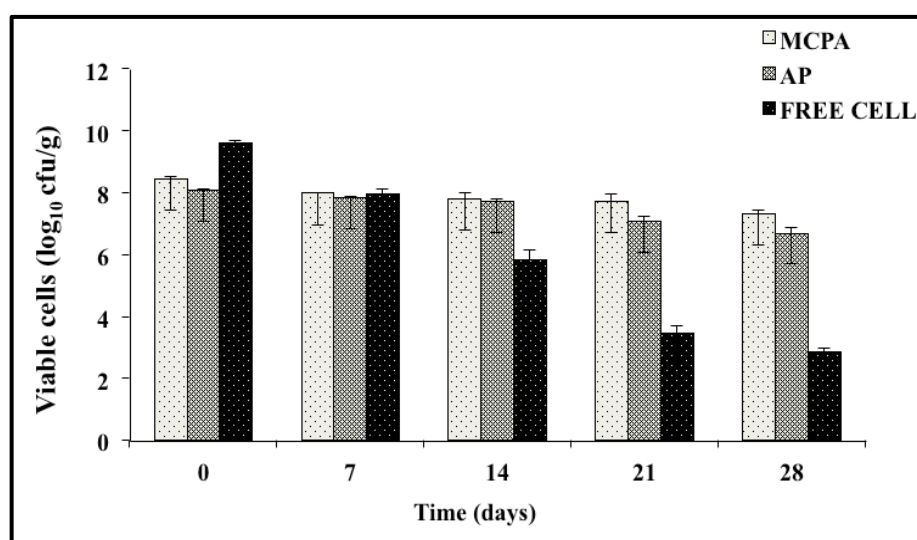


Fig 3.3 The viability of *Lactobacillus acidophilus* ATCC 4356 as free cells and encapsulated cells (modified citrus pectin alginate (MCPA) and alginate calcium (AP) probiotic microbeads) during storage at 4°C. Each bar denotes mean ± SD (n=3).

3.4.3 Survival of free and microencapsulated *Lactobacillus acidophilus*

ATCC 4356 in simulated gastric juice

To determine the likelihood of free and microencapsulated probiotic bacteria surviving passage through the stomach following oral administration, free and microencapsulated *L. acidophilus* ATCC 4356 were tested for stability *in vitro* simulated gastric juice (SGJ). At a pH 1.2 of SGJ, free *L. acidophilus* ATCC 4356 did not survive after 30 min. The viability of encapsulated *L. acidophilus* ATCC 4356 cells in both MCPA and AP was below the detection limit ($< 2 \log_{10}$ cfu/g) (result not shown). At pH 2.0 of SGJ, the number of free *L. acidophilus* ATCC 4356 reduced significantly by $4.01 \log_{10}$ cfu/g (43.6%) after 180 min ($p < 0.0001$). Also, $0.55 \log_{10}$ cfu/g (6.7%) and $0.91 \log_{10}$ cfu/g (11.2%) of *L. acidophilus* ATCC 4356 in the MCPA ($p < 0.05$) and AP microbeads ($p < 0.0001$) were lost respectively. The viability of encapsulated *L. acidophilus* ATCC 4356 in the MCPA and AP microbeads after 180 min in SGJ was $7.61 \pm 0.12 \log_{10}$ cfu/g (93.2%) and $7.19 \pm 0.16 \log_{10}$ cfu/g (88.7%) respectively. On the contrary to the MCPA, *L. acidophilus* ATCC 4356 in the AP microbeads was significantly reduced compared to the free *L. acidophilus* ATCC 4356 cells after 60 min of the probiotic exposure to SGJ ($p < 0.0001$). After 120 min, the difference in viable count of *L. acidophilus* ATCC 4356 in the MCPA compared to the AP microbeads becomes significant ($p < 0.05$). The morphology of MCPA microbead particle when exposed to SGJ over a period of 3 h is shown in Figure 3.4.

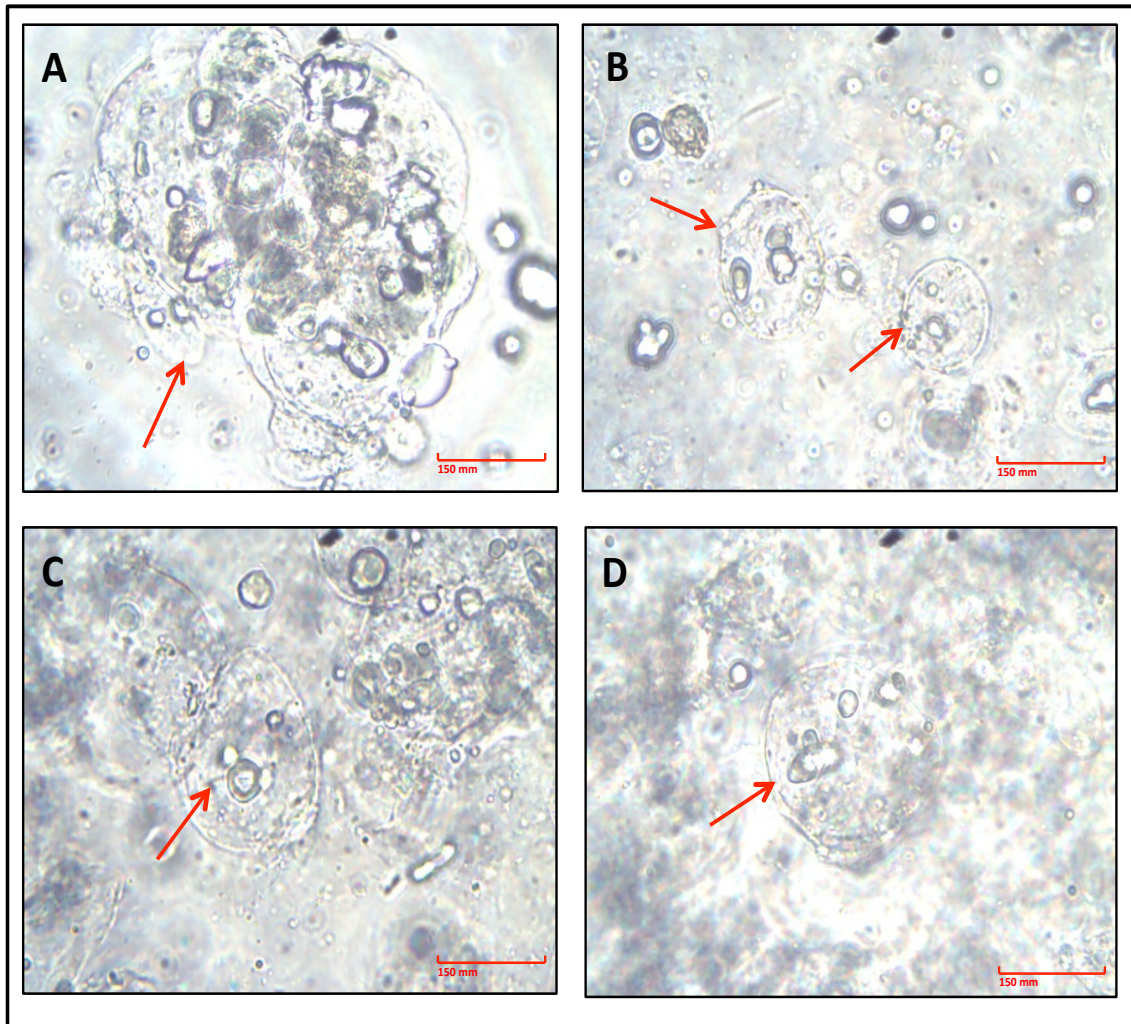


Fig 3.4 The morphology of modified citrus pectin alginate (MCPA) microbead particles containing *Lactobacillus acidophilus* ATCC 4356 after (A) 30 min (B) 60 min (C) 120 min and (D) 180 min exposure to simulated gastric juice (SGJ) at pH 2. Microbead particles encapsulating *Lactobacillus acidophilus* ATCC 4356 indicated by the arrows.

The reduction in viability of *L. acidophilus* ATCC 4356 in MCPA remains insignificant after 120 min of exposure in SGJ ($p > 0.05$) (Figure 3.5A) (Appendix 1 - Table A2).

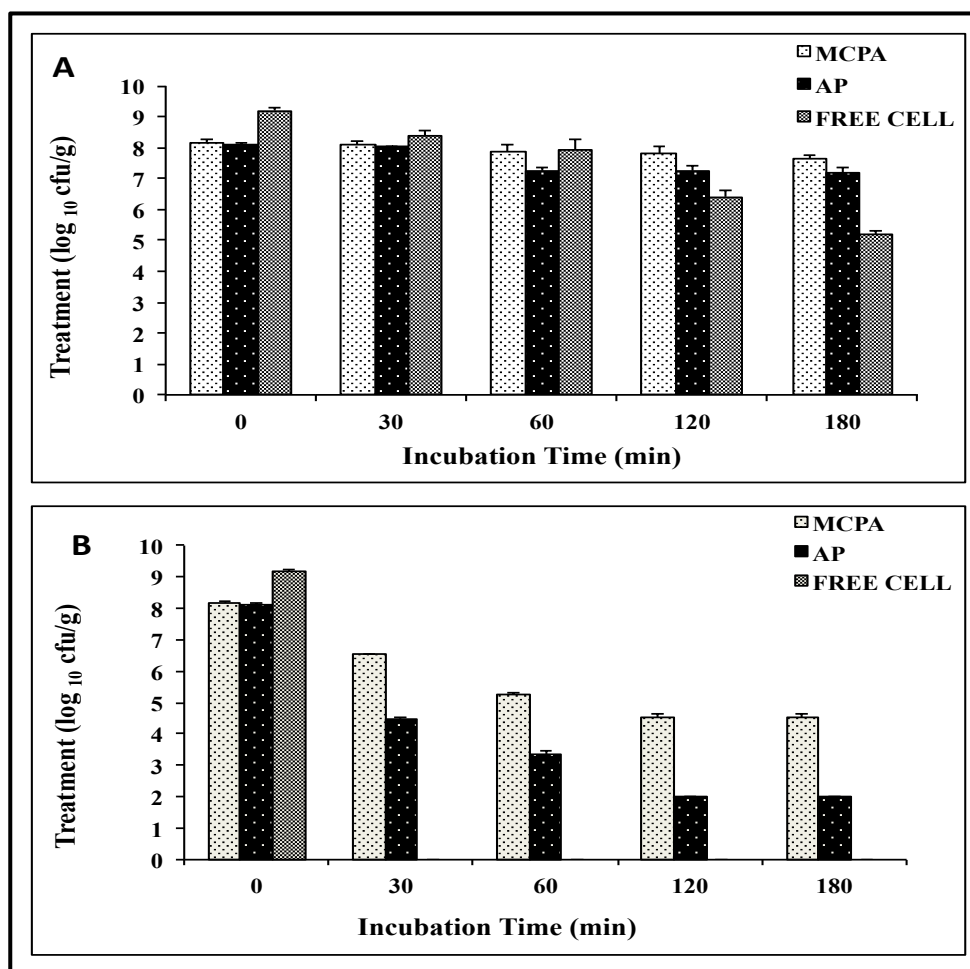


Fig 3.5 The viability of *Lactobacillus acidophilus* ATCC 4356 as free cells and encapsulated cells (modified citrus pectin alginate (MCPA) and alginate calcium (AP) probiotic microbeads) during exposure to (A) simulated gastric juice (SGJ) at pH 2 and (B) simulated intestinal juice (SIJ) at pH 8 for 30, 60, 120 and 180 min. Each bar denotes mean \pm SD (n=3).

3.4.4 Survival of free and microencapsulated *Lactobacillus acidophilus* ATCC 4356 in simulated intestinal juice

This is to determine the stability of both MCPA and AP probiotic microbeads in the presence of bile salt in SIJ (pH 8.0) (Figure 3.5B). The morphology of the AP microbead particles containing *L. acidophilus* ATCC 4356 in SIJ after sequential exposure for 3 h is shown in Figure 3.6. In this study, free *L. acidophilus* ATCC 4356 cells were found not to survive in SIJ after 30 min. The number of *L. acidophilus* ATCC 4356 cells in the AP microbeads decreased significantly from 8.10 ± 0.04 log₁₀ cfu/g to 3.35 log₁₀ cfu/g after 60 min and further reduced to 2 log₁₀ cfu/g at 180 min ($p < 0.0001$).

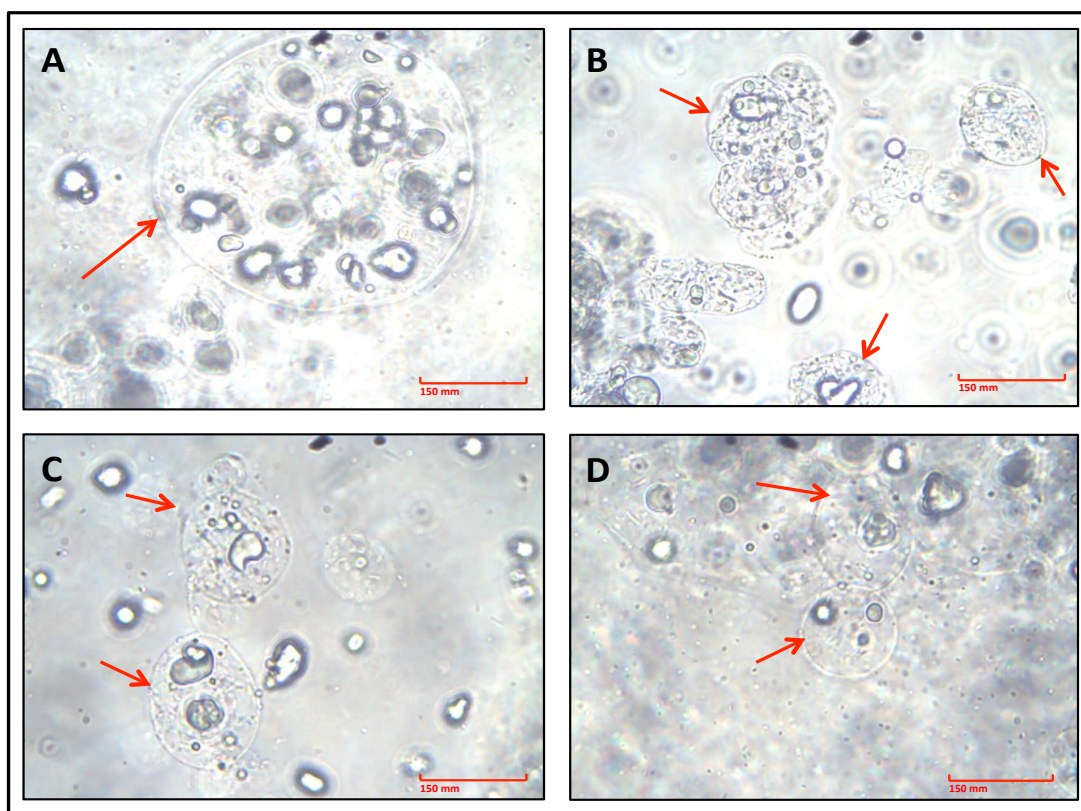


Fig 3.6 The morphology of alginate calcium (AP) microbead particles containing *Lactobacillus acidophilus* ATCC 4356 (as indicated by the arrows) after (A) 30 min (B) 60 min (C) 120 min and (D) 180 min exposure to simulated intestinal juice (SIJ) at pH 8.

In the MCPA microbeads, *L. acidophilus* ATCC 4356 reduced from $8.16 \pm 0.06 \log_{10}\text{cfu/g}$ to $4.53 \pm 0.10 \log_{10}\text{cfu/g}$ after 180 min in SIJ. The viability of *L. acidophilus* ATCC 4356 cells in the MCPA was significantly high compared to the AP microbeads after 30 min in SIJ ($p < 0.0001$). The difference in reduction of viable *L. acidophilus* ATCC 4356 in the MCPA and AP microbeads at 120 and 180 min was rather insignificant ($p > 0.05$) (Appendix 1 - Table A2). The MCPA microbeads showed a high microencapsulation yield during passage in SGJ and SIJ as shown in Table 3.2.

Table 3.2 Encapsulation yield of microencapsulated and free *Lactobacillus acidophilus* ATCC 4356 exposed to simulated gastric and intestinal juice

Incubation Time (min)	Encapsulation yield (%)					
	SGJ			SIJ		
	MCPA	AP	Free 4356	MCPA	AP	Free 4356
0	88.6 ± 0.08	88.0 ± 0.04	100 ± 0.06	88.6 ± 0.08	88.0 ± 0.04	100 ± 0.06
30	87.9 ± 0.1	87.0 ± 0.02	90.9 ± 0.15	70.8 ± 0.03	48.8 ± 0.03	0
60	85.6 ± 0.2	78.8 ± 0.08	86.1 ± 0.32	57.3 ± 0.02	21.7 ± 0.03	0
120	84.6 ± 0.24	78.8 ± 0.15	69.6 ± 0.23	49.3 ± 0.09	21.7 ± 0.0	0
180	82.7 ± 0.12	77.7 ± 0.16	56.4 ± 0.13	49.2 ± 0.1	0	0

MCPA - modified citrus pectin alginate, AP- alginate calcium, Free 4356- Free *Lactobacillus acidophilus* ATCC 4356, SGJ - simulated gastric juice and SIJ- simulated intestinal juice. Values represent means ± S.D (n=3).

3.5 Discussion

The use of the emulsification technique is relatively new in food encapsulation, easy to scale up for large scale production and with a 80–95% high encapsulation yield (Chen and Chen, 2007). However, encapsulation yield may be reduced due to the sensitivity of the probiotic strain to the prebiotic or biopolymer matrix (Chávarri *et al.*, 2010). Therefore, there is need to understand the interaction of MCPA with *L. acidophilus* ATCC 4356. This study utilizes MCP combined with alginate to entrapped *L. acidophilus* ATCC 4356 and determines the viability in gastric and intestinal conditions.

An important characteristic relevant to the stability and efficiency of microencapsulation is the mean particle size of the microbead (Shi *et al.*, 2013). Sandoval-Castilla *et al.* (2010) showed that alginate-pectin capsules formed a significantly higher entrapment efficiency compared to alginate capsule. Similarly, in our study, the encapsulation efficiency of MCPA microbeads was significantly higher in comparison to AP microbeads. Chávarri *et al.* (2010) and Shi *et al.* (2013) also found that polymer matrices with the highest concentration produce large microspheres.

Therefore, the high concentration of MCP could be responsible for the large diameter of the MCPA microbeads. However, the diameter size of the microbead may depend on the encapsulation technique. Emulsification mostly produces microbeads with small diameters ranging from 25 μm – 2 mm while extrusion forms a large diameter size (2 – 5mm). From previous studies, alginate calcium microbeads are more spherical in shape unlike the alginate/pectin calcium microbeads that are either vermiform shape or less spherical (Díaz-Rojas *et al.*, 2004; Pillay and Fassihi, 1999; Sandoval-Castilla *et al.*, 2010; Shi *et al.*, 2013). In this study, MCPA microbeads were mostly spherical which could be attributed to the coating effect of chitosan. The availability of carboxyl ions may increase the adsorption of chitosan to the MCPA-calcium particle surface thereby forming a spherical shape.

Brinques and Ayub (2011) found that under refrigerated storage (4°C) the loss of viability of entrapped *L. plantarum* was lowest in 4% (w/v) citric pectin, 3% (w/v) sodium alginate coated with chitosan or polymer mixture of 2% (w/v) sodium alginate and 2% (w/v) citric pectin respectively. On the contrary, from our study, the lowest loss of viability of encapsulated *L. acidophilus* ATCC 4356 was observed in MCPA microbeads compared to AP microbeads (both coated with chitosan). The efficiency of the MCPA microbeads ensuring the survival of *L. acidophilus* ATCC 4356 may be attributed to the large mean diameter of the particle size. Also, the abundance of carboxyl polyanions in the MCPA microbeads equivalently binding to calcium and chitosan polycations forming a strong polyelectrolyte complex improves the cross-linking cationic of polymers. In the AP microbeads, calcium and chitosan polycations compete to bind with the less carboxyl anions in alginate molecules. The formation of strong trapping matrix in the MCPA reduces the porosity of the microbead wall. This is an improved biopolymer mixture, which enhances the stability and survival of *L. acidophilus* ATCC 4356 under refrigeration condition. The cryogenic effect of the sodium glycerol during storage gives cryo-protective characteristics to the microencapsulated *L. acidophilus* ATCC 4356. The high viability (86.5%) of *L. acidophilus* ATCC 4356 in the MCPA microbeads corroborates with the findings by Sheu and Marshall (1993).

In our study, both the MCPA and AP microbeads significantly improved the survival of *L. acidophilus* ATCC 4356 in SGJ at pH 2 compared to the free cells ($p < 0.05$). The viable number of *L. acidophilus* ATCC 4356 in these polymer matrices was above $7 \log_{10}\text{cfu/g}$ which is the required minimum concentration of probiotic therapy able to confer beneficial health (Anal and Singh, 2007; Food and Agricultural Organization, 2002). The loss of viability of *L. acidophilus* ATCC 4356 in MCPA and AP microbeads was by $0.07 \log_{10}\text{cfu/g}$ and $0.09 \log_{10}\text{cfu/g}$ after 30 min in SGJ respectively. Ortakci and Sert (2012) found that *L. acidophilus* ATCC 4356 in alginate calcium microbeads were reduced by $0.25 \log_{10}\text{cfu/g}$ after 30 min in SGJ. A little more than $1 \log_{10}\text{cfu/g}$ of *L. gasseri* in alginate-chitosan capsules was lost after 120 min in artificial gastric juice (Chávarri *et al.*, 2010). The survival of *L. acidophilus* ATCC 4356 in the MCPA microbeads can be attributed to the resilient and cohesive cross-linking network between modified citrus pectin and alginate calcium polymeric nature.

Free *L. acidophilus* ATCC 4356 could not withstand the harsh conditions of bile salt at a high pH. Interestingly, the MCPA microbeads conferred a higher protection on *L. acidophilus* ATCC 4356 from the damaging action of intestinal juice containing bile salt compared to AP microbeads ($p < 0.05$). Shi *et al.* (2013) observed the total loss of free *L. bulgaricus* after 1 h exposure to a bile salt solution. Trindade and Grosso (2000) showed that alginate calcium beads did not protect *B. bifidum* and *L. acidophilus* from the 2 - 3% bile salt solutions. On the contrary, Ortakci and Sert (2012) found no reduction in the number of both free and encapsulated *L. acidophilus* ATCC 4356 in 1.2% bile juice. Studies demonstrated that some probiotic strains are tolerable to intestinal bile action (Ortakci *et al.*, 2012; Ortakci and Sert, 2012) while some are susceptible (Chávarri *et al.*, 2010; Clark and Martin, 1994; Hansen *et al.*, 2002; Shi *et al.*, 2013; Trabelsi *et al.*, 2013). Also, a high concentration of bile salt may disintegrate the bacterial cell wall integrity. In this regard, the varied outcome of studies as a result of different concentration of bile salt solutions (0.3 – 4%) and pH (6–8) makes the comparison of findings difficult. There are numerous publications on encapsulation of probiotics, which report little or no information on the nature of probiotic release in the GIT.

The evaluation and comparison of studies by the use of *in vitro* simplified models has provided inconsistent results due to different composition and pH of the simulated GI conditions (Cook *et al.*, 2012). Thus in this study, the ability to resist high bile concentrations is a good functional property of MCPA. The resilient and strong structural matrix formed by MCPA showed an effective biopolymer agent. Results thus far showed that the combination of MCP and alginate effectively protect and improve the survival of probiotic *L. acidophilus* ATCC 4356 in simulated gastrointestinal conditions.

CHAPTER 4

THE EFFECT OF A MICROENCAPSULATED PROBIOTIC ON COLON LACTOBACILLIMICROFLORA IN A MOUSE MODEL

4.1 Introduction

Behavioural and dietary risks (low intake of fruits and vegetable) are among factors that are responsible for about 30% of deaths caused by cancer (World Health Organisation, 2012). Epidemiological studies have shown that the origin of colon cancer may be associated with dietary intake and composition most especially high fat and low fibre diets (Ponz de Leon, 2002). These have a high influence on the metabolic activities and composition of the intestinal microflora since they are highly dependent on the substrates available to them. The end product of metabolism can be substances or chemicals that promote the activities of tumours in the colon (McGarr *et al.*, 2005). Therefore, dietary factors and emerging intestinal microflora seem to play a significant role necessary for the development of colon carcinogenesis in animal models which makes the protective role of probiotics of major interest (Kado *et al.*, 2001).

The alteration/manipulation of intestinal bacterial composition is associated with colon cancer by stimulating the immune system, regulating inflammations in the gut, decreasing incidence of infections and binding toxic substances (Capurso *et al.*, 2006). Studies have demonstrated that the inclusion of probiotics in a diet reduces the risk of cancer (Chen and Chen, 2007). Butyrate and other short chain fatty acid produced from colonic bacterial metabolism of undigested carbohydrate such as resistant starch (starch that isn't fully broken down and absorbed during digestion), lowers the risk of colon cancer (O'Keefe *et al.*, 2009; Zhu *et al.*, 2011). O'Keefe *et al.* (2009) reported that a low count of endogenous faecal lactobacilli in the colonic mucosal biopsies lead to a higher incidence of colon cancer in the African American population.

Probiotics are defined as “live microorganisms which when administered orally in adequate amount confer a health benefit on the host” (Food and Agriculture Organization, 2002). Modified citrus pectin (MCP) is a polysaccharide dietary fibre

broken down into smaller fragments. The potential role of MCP and probiotics in the prevention of carcinogenesis has prompted the need to understand their synergistic influence on colon microflora. Amongst various polysaccharides, however, pectin has been shown to be a good biopolymer matrix (Wong *et al.*, 2011) due to its resistance against intestinal enzymes such as protease and amylase which are active in the upper gastrointestinal tract (GIT) and its suitability for colon specific delivery with controlled release properties (McConnell *et al.*, 2008).

Azoxymethane (AOM), a metabolite of 1,2- dimethylhydrazine (DMH), is a potent specific carcinogen used to induce colon cancer in mice and rats (Bissahoyo *et al.*, 2005; Tanaka, 2009). The AOM mouse model has been extensively used in a study of underlying mechanism of sporadic colon cancer in humans. The response of AOM induced colorectal cancer (CRC) in a mouse model mimics the occurrence of non-familial colon tumours particularly sporadic colon cancer in humans (Chen and Huang, 2009).

Although, few animal studies have shown that probiotic alone or symbiotically (probiotic and prebiotic) can reduce the incidence of preneoplastic or neoplastic. The mechanism by which this health benefits comes into play remains unclear and may however be dynamic in nature (Capurso *et al.*, 2006). The potential role of modified pectin (MP) and probiotics in the prevention of carcinogenesis has prompted the need to understand their synergistic influence on colon microflora. Azoxymethane (15 mg/kg body weight) dose administered intraperitoneally has been shown to induce early aberrant crypt foci in Balb/c mouse model (Bissahoyo *et al.*, 2005; Chen and Huang, 2009).

Therefore, the aim of this study is to investigate the synergistic influence of *Lactobacillus acidophilus* ATCC 4356 and resistant starch encapsulated with alginate with or without MCP (PectaSol-C) (ecoNugenics Inc., Santa Rosa, California, USA) to form biopolymer probiotic microbeads, on the colonic milieu lactobacilli microflora from the faecal matter of Balb/c mouse model pre-treated with or without AOM.

4.2 Materials and Methods

4.2.1 Animal model

Post institutional ethics approval (063/13/Animal) (Appendix 2) was obtained prior to commencement of the animal trial. The experiment was carried out at the Biomedical Research Unit (BRU), University of KwaZulu-Natal (UKZN). The BRU approved standard protocols for animal treatment were followed. Seven-week-old male Balb/c mice weighing 20 – 25 g (Figure 4.1) were bred in-house under a controlled condition of humidity ($50 \pm 10\%$) and temperature ($23 \pm 2^{\circ}\text{C}$) on a 12 h light/dark cycle. The mice were allowed to acclimatize to a new room for a week after weaning prior to the experiment with free access to water and food. During the experiment mice were carefully observed for any toxic effects, abnormal behavior and rectal bleeding. Additionally, their body weight was monitored and recorded in the humane endpoint form (Appendix 2 - Table A3).

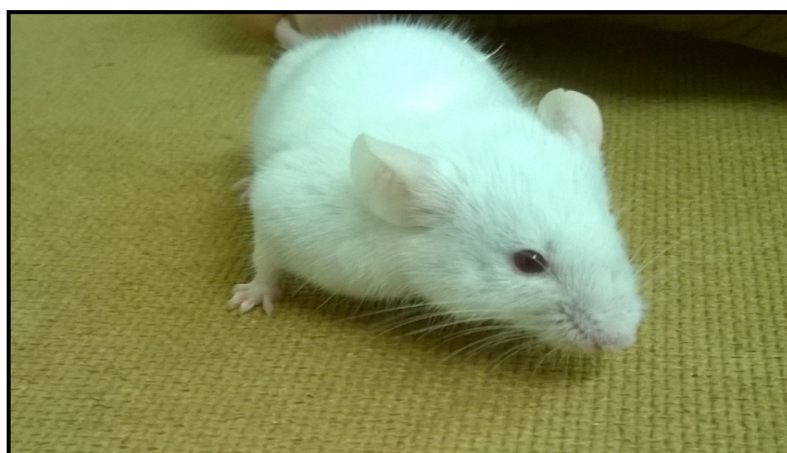


Fig 4.1 A normal healthy male Balb/c mouse.

4.2.2 Carcinogenic treatment of Balb/c mice with azoxymethane

The total study population (n=80) consisted of eight groups of Balb/c mice (n=10 each). Groups 1, 2, 3 and 4 were pre-treated with 15mg/kg AOM (13.4Molarity, $\geq 98\%$, Sigma-Aldrich Co., St. Louis, USA) intraperitoneally (*i.p.*), once a week for 4 consecutive weeks (Table 4.1). Groups 5, 6, 7 and 8 were not treated with AOM (Table 4.2).

Table 4.1: Category of treatments administered for the AOM-treated Balb/c mouse model

Groups	Treatments
1	Modified citrus pectin alginate (MCPA) probiotic microbeads
2	Alginate calcium (AP) probiotic microbeads
3	Modified citrus pectin (MCP)
4	Water (Control)

Table 4.2: Category of treatments administered for the normal Balb/c mouse model

Groups	Treatments
5	Modified citrus pectin alginate (MCPA) probiotic microbeads
6	Alginate calcium (AP) probiotic microbeads
7	Modified citrus pectin (MCP)
8	Water (Control)

4.2.3 Isolation and quantification of lactobacilli in faecal samples

Each group of mice was orally administered either 0.2 ml MCPA- or AP probiotic microbeads, MCP or water, once daily for 28 days as shown in Table 4.1 and 4.2. Water and food were available to the animals *ad libitum* throughout the experiment. Faecal samples were obtained for each group of mice at the initial day (day 0) prior the probiotic microbeads treatments and on days 7, 14 and 28. Faecal samples, collected in sterile disposables tubes containing sterile saline solution kept in ice packs, were transported to the laboratory and processed within 2 to 12 h of collection. Samples were serially diluted in saline (0.9% NaCl) from 10^1 up to 10^{10} and inoculated onto MRS agar (selective medium to isolate lactobacilli) incubated anaerobically for 24 to 48 h at 37°C. Colonies obtained between 30 – 300 cfu/ml were counted from the faecal sample plates using the automated colony counter Doc-It® imaging station and recorded in \log_{10} cfu/g. Total number of viable lactobacilli were enumerated in triplicate. Plates were subcultured on MRS agar to obtain pure colonies. Shiny, entire, smooth/rough, circular and opaque colonies in anaerobic condition were noted.

Cell morphology and Gram stain reactions were also determined. However, all isolates that were catalase negative (using 5% hydrogen peroxide solution) rod were also selected as lactobacilli.

4.2.4 DNA extraction of faecal bacteria

Bacterial samples were added directly to Zymo Research (ZR) bashing bead lysis tubes and lysed by bead beating. The filtered lysate was bonded, washed, isolated and purified and finally the DNA eluted with Zymo-spinTM IIC for PCR analysis. The gDNA was extracted using ZR fungal/bacterial DNA MiniPrepTM (Zymo Research Corporation). Lysis solutions (750 µl) were added to the bacterial isolates and suspended in 200 µl of PBS in a ZR bashing bead lysis tube. Secured in a Disruptor GenieTM, samples were processed at maximum speed for 5 min then centrifuged in a microcentrifuge at 11 500 x g force for 1 min. The supernatant (400 µl) was transferred to a Zymo-spinTM IV spin filter in a collection tube and centrifuged at 8600 x g force for 1 min. Bacterial DNA (1200 µl) binding buffer was added to the filtrate in the collection tube and 800 µl of this mixture transferred to a Zymo-spin IIC column and centrifuge at 11 500 x g force for 1 min. The latter stage was repeated after the removal of the flow through the collection tube. The mixture was then washed with DNA pre wash buffer, centrifuged followed by an additional wash using a bacterial DNA wash buffer and centrifuged at 11 500 x g force for 1 min. Finally, 100 µl of DNA elution buffer was added directly to the column matrix to elute and obtain ultra pure DNA.

4.2.5 DNA amplification

PCR amplification from the DNA sample fragments of the 16S region was obtained using DreamTaq (Thermoscientific Fermentas) and primers; 27F: 5'GAGTTTGATCCTGGCTCAG and 1492R: 5'GGTTACCTTGTTACGACT 3'. A total volume of 50 µl containing 25 µl of DreamTaq Green PCR master mix (DreamTaq DNA polymerase, optimized DreamTaq green buffer, MgCl₂ and dNTPs), 1.0 µM of each primers, 1 µg of template DNA and nuclease-free water. Samples were gently vortexed and spun. PCR was started by initial denaturation of template DNA at 95°C for 3 min at 1 cycle then 25 cycles of denaturation at 95°C for 30 s followed by primer annealing at 72°C for 30 s, extension at 72°C for 15 min and final extension for 15 min

at 72°C. The resulting amplicons were separated on a 1% agarose gel followed by staining with GRGreen.

4.2.6 Gene sequence analysis

Amplicon (USB, Staufen, Germany) purification was used directly on the PCR products after the completion of the PCR reaction. Ten microlitres aliquots of PCR mixtures, 0.5 µl of exonuclease I and 2 µl of thermosensitive alkaline phosphatase were mixed thoroughly and incubated at 37°C for 15 min. The reaction was stopped by heating the mixture at 85°C for 15 min (Werle *et al.*, 1994). A sequencing chromatogram of DNA was generated by first labeling the DNA with ABI Big Dye V3.1 terminators and cleaned with a ZR DNA sequencing clean-up kitTM (Zymo Research Corporation, USA) before the ultra pure DNA fragment was loaded into the ABI 3500 XL sequence analyzer. Consensus sequence and Basic Local Alignment Search Tools (BLAST) algorithm were performed in CLC Bio (Inqaba biotech, SA) as the sequences obtained were compared with the known ones available in the Genbank database.

4.2.7 Statistical analysis

Data was subjected to Tukey's test and ANOVA, to determine significant differences and comparison among the group treatments. Graphpad Prism software and SPSS version 17.0 for Windows (SSPS, Chicago, Illinois, USA) was used. Results were presented as means ± standard deviation (SD), $p < 0.05$ was considered significant.

4.3 Result

4.3.1 Morphological identification of colonies

Apart from the colonial morphology similar to *Lactobacillus* sp. described earlier, catalase negative cocci, smooth, irregular, flat colonies were observed. The colonies were characterized based on the cellular, colonial morphology from Gram reaction and catalase test of the isolated faecal bacteria. Similar colonial and cellular morphology were observed thus reducing the total number of isolated colonies to five as shown in (Figure 4.2 and Table 4.3).

Table 4.3: Morphological characterization of faecal bacteria isolated from Balb/c mice

	Gram stain	Cell shape	Colony Shape	Elevation	Margin	Texture	Colour	Opaque/ Transparent	Catalase
A.	positive	rod	circular	raised	entire	smooth/ glossy	milky	opaque	negative
B.	positive	rod	circular	flat	entire	rough	milky	opaque	negative
C.	positive	rod	circular	flat	entire	smooth/ glossy	milky	transparent	negative
D.	positive	rod	circular	umbonate	entire	smooth/ glossy	milky	opaque	negative
E.	positive	cocci	irregular	flat	eroded	smooth	milky	transparent	negative

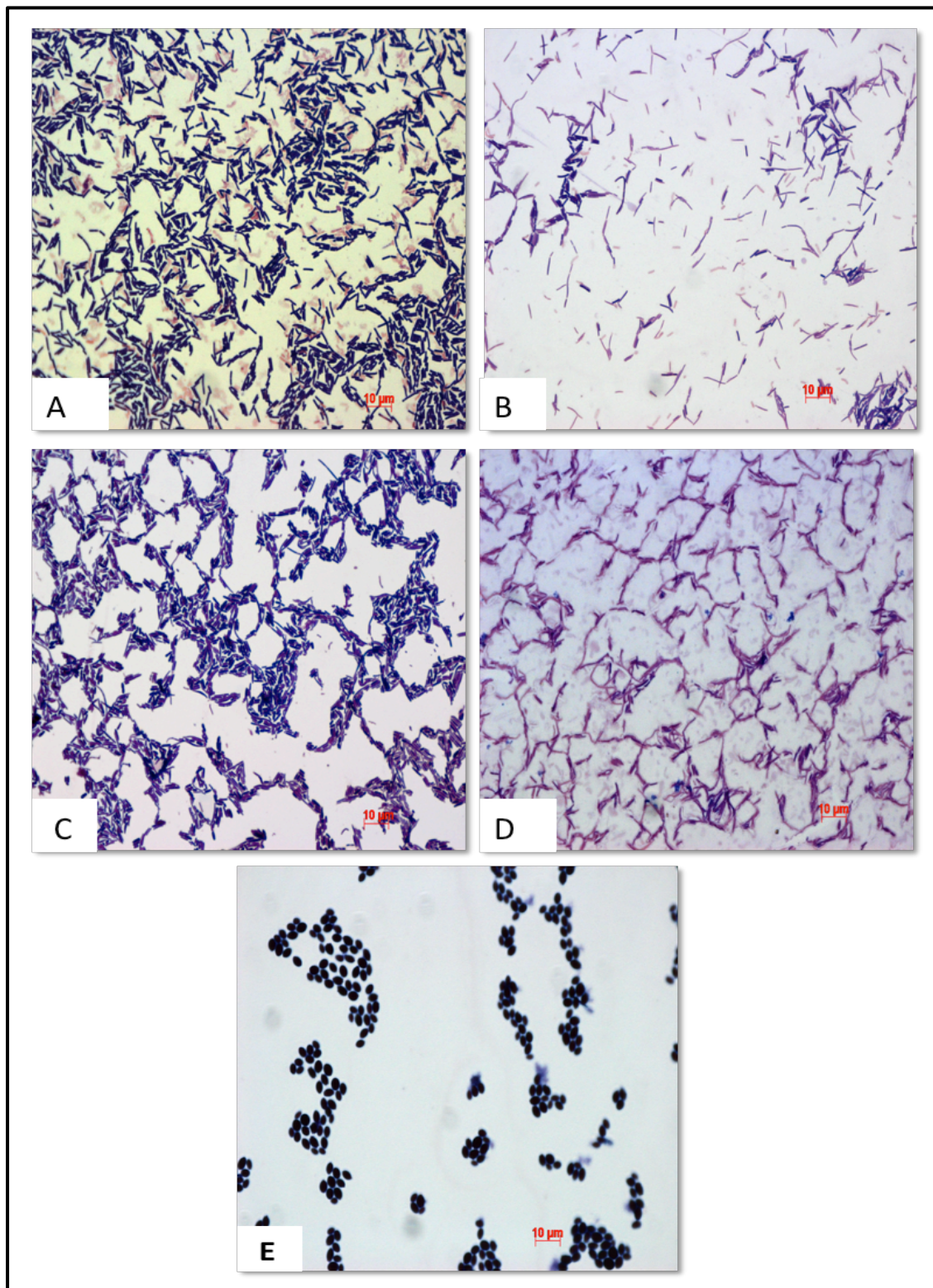


Fig 4.2 Gram reaction and cellular morphology of (A) the microencapsulated probiotic bacteria, *Lactobacillus acidophilus* (B, C) *Lactobacillus* (D) *Bacillus* (E) *Enterococcus*

4.3.2 Quantification of faecal lactobacilli analysis

The faecal lactobacilli count from the microencapsulated probiotic-and MCP-treated mice injected with or without AOM are shown in Figure 4.3A, 4.3B and 4.3C. At day 7 of probiotic microbeads treatment, the increase in percentage of faecal lactobacilli count in the AOM+MCPA (4.9%) and MCPA (normal) (4.4%) probiotic-treated groups of mice was high compared to 0.2% in both the AOM+AP probiotic and AOM+MCP treated mice. The highest number of faecal lactobacilli count ($8.13 \pm 0.22 \log_{10}\text{cfu/g}$) was observed in the AOM+MCPA probiotic-treated mice. However, there was reduction in the number of faecal lactobacilli in normal mice administered with AP probiotic or MCP and in the control groups (AOM and normal) over the 7 days of treatment ($p>0.05$).

At day 14 of probiotic microbeads treatment, the number of faecal lactobacilli in the AOM+MCPA and MCPA (normal) probiotic-treated mice increased by $0.75 \pm 0.4 \log_{10}\text{cfu/g}$ (9.7%) and $0.68 \pm 0.15 \log_{10}\text{cfu/g}$ (9%) respectively with no significant difference. At this period, there was 1.4% and 1.2% increase in the number of faecal lactobacilli in the normal (AP probiotic-treated and control) groups of mice respectively. Although an increase was also observed in the faecal lactobacilli count in the AOM+MCP treated mice $0.02 \log_{10}\text{cfu/g}$ (0.2%), it was still low compared to the rise in AOM+AP probiotic-treated mice by $0.32 \pm 0.04 \log_{10}\text{cfu/g}$ (4%) ($p>0.05$). Conversely, the normal mice treated with MCP and the AOM-treated groups of mice were further reduced in faecal lactobacilli count ($p>0.05$).

At day 28 of probiotic microbeads treatment, the increase of faecal lactobacilli count was more obvious in the AOM+MCPA and MCPA (normal) probiotic-treated groups ($p< 0.0001$). The count in AOM+MCP treated mice was reduced insignificantly by 0.6% below the initial baseline level (before probiotic treatment) ($p>0.05$). In the AOM+AP probiotic-treated mice, the faecal lactobacilli count was also reduced. The highest number of faecal lactobacilli was observed in the MCPA probiotic-treated mice in comparison to the AP probiotic- and MCP-treated groups of mice. While the number of faecal lactobacilli in the AOM-treated (control) group of mice was reducing, a slight increase was observed in the normal (control) mice, however, the changes were not

statistically significant ($p>0.05$) (Appendix 3 - Table A4). There was no animal death during the experiment.

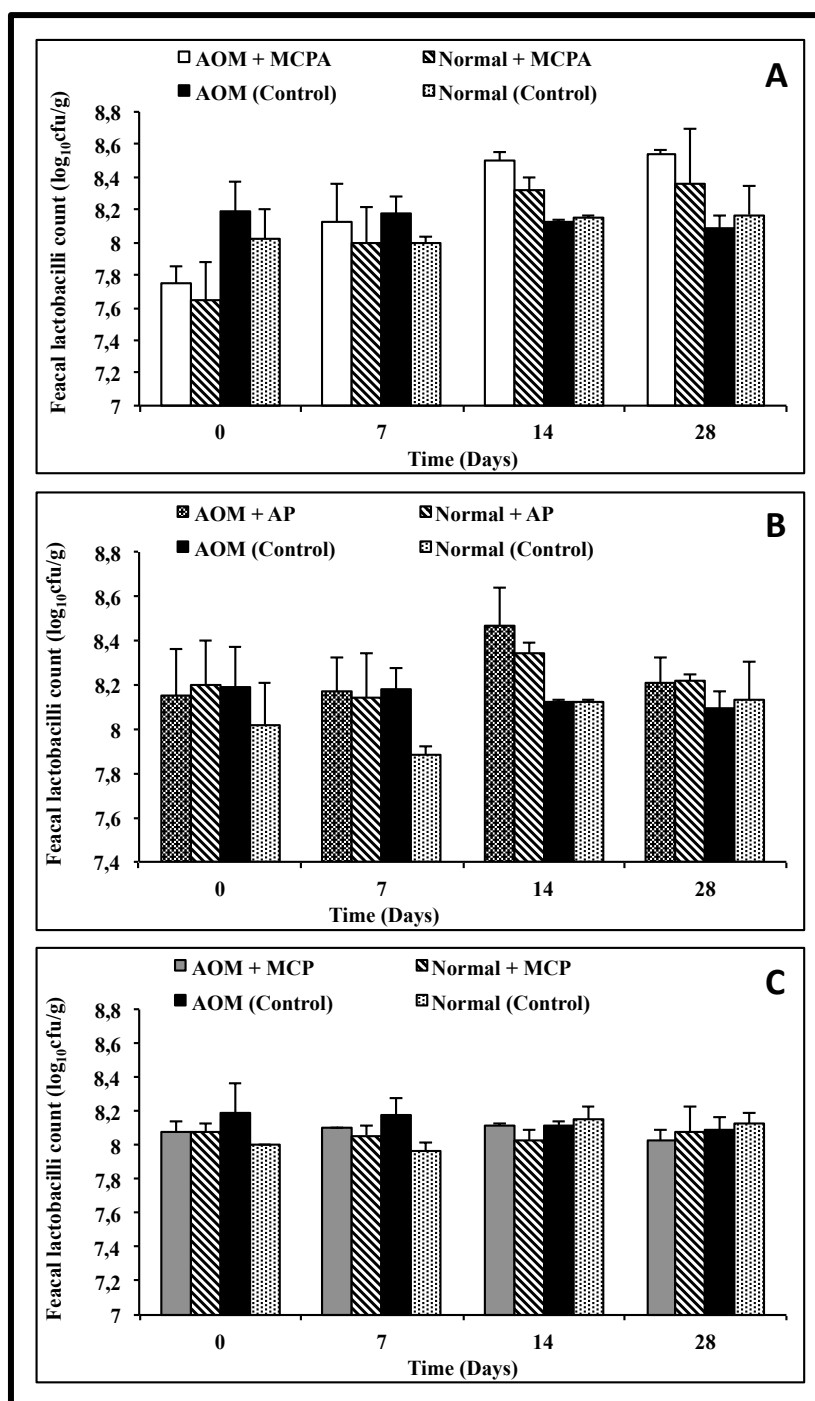


Fig 4.3 Average counts of faecal lactobacilli ($\log_{10}\text{cfu/g}$ of faeces) in both normal and azoxymethane (AOM) treated-Balb/c mice model treated with; A. modified citrus pectin alginate (MCPA) probiotic microbeads, B. alginate calcium (AP) probiotic microbeads and C. modified citrus pectin (MCP) solution. Each column represents mean \pm SD ($n = 10$).

4.3.3 Faecal bacteria revealed by 16S rRNA amplification BLAST search

The DNA sample fragments encoding 16S region were processed from the MCPA-, AP probiotic-, MCP-treated mouse model with or without AOM and control (AOM and normal) after 28 days of treatment respectively (Table 4.4). The blast hits showed high significant alignments (with Expected values 0.00) and regions of 16S rRNA gene homology across the query (unknown faecal bacteria) sequence despite mismatched genes and gaps in some cases. In the MCPA probiotic-treated mice, four of the DNA samples encoding 16S rRNA gene were closest to the genus *Lactobacillus*. In the AP probiotic- and MCP treated groups; two and one *Lactobacillus* was found respectively. The rest belonged to other genera including one *Bacillus* and one *Enterococcus faecium* (both in MCPA, AP and MCP groups of mice treated with and without AOM) and one *Bifidobacterium* (AOM+MCP only). The final was an uncultured bacteria found in the AOM-treated group (control). Generally, similar bacteria encoding 16S rRNA gene was found in both the AOM-treated and healthy mice irrespective of the treatment with exception to the genus *Bifidobacterium*. A large number of bacteria were revealed by blast search gene sequence with the possibility of identifying the faecal isolates. However, Table 4.4 shows the faecal bacteria whose gene alignments are significantly high (100%) homology closest to the queried isolates and expected value of 0.0 with no or one gap in the gene sequence analysis.

Table 4.4: Identification of faecal bacteria in the normal and azoxymethane (AOM)-treated Balb/c mouse model by gene sequence BLAST search

Treatment groups	Closest relatives with significant alignment identified by BLAST	Maximum score (bits)	Expect value	Sequence identity (%)	No of gaps	GenBank accession number
MCPA	<i>Lactobacillus acidophilus</i> LM3 16S rRNA gene	2793.6	0.0	1412/1413 (100)	0/1413	HG518158.1
	<i>Lactobacillus acidophilus</i> ATCC 4356 gene for 16S rRNA*	2783.7	0.0	1411/1412 (100)	1/1412	AB008203.1
	<i>Lactobacillus johnsonii</i> 16S ribosomal RNA gene	2694.5	0.0	1359/1359 (100)	0/1359	KC158587.1
	<i>Bacillus</i> sp. 16S ribosomal RNA gene	1703.3	0.0	859/859 (100)	0/859	KJ849835.1
	<i>Enterococcus faecium</i> 16S rRNA gene	2773.8	0.0	1399/1399 (100)	0/1399	KJ702551.1
	<i>Lactobacillus reuteri</i> 16S ribosomal RNA gene	2765.9	0.0	1409/1416 (100)	0/1416	KC700337.1
AP	<i>Bacillus</i> sp. 16S ribosomal RNA gene	1703.3	0.0	859/859 (100)	0/859	KJ849835.1
	<i>Lactobacillus</i> sp. 16S ribosomal RNA gene	2803.5	0.0	1414/1414 (100)	0/1414	EU600905.1
	<i>Enterococcus faecium</i> 16S rRNA gene	2773.8	0.0	1399/1399 (100)	0/1399	KJ702551.1
	<i>Lactobacillus casei</i> 16S ribosomal RNA gene	2750.0	0.0	1392/1394 (100)	0/1394	JN560892.1
MCP	<i>Enterococcus faecium</i> 16S ribosomal RNA gene	2773.8	0.0	1399/1399 (100)	0/1399	KJ702545.1
	<i>Lactobacillus reuteri</i> 16S ribosomal RNA gene	2765.9	0.0	1409/1416 (100)	0/1416	KC700337.1
	<i>Bifidobacterium</i> species 16S ribosomal RNA gene*	2758.0	0.0	1405/1412 (100)	0/1412	JFS19689.1
CONTROL	<i>Enterococcus faecium</i> 16S ribosomal RNA gene	2773.8	0.0	1399/1399 (100)	0/1399	KJ702545.1
	<i>Bacillus</i> sp. 16S ribosomal RNA gene	1703.3	0.0	859/859 (100)	0/859	KJ849835.1
	Uncultured organism clone small subunit 16S rRNA gene, partial sequence*	2759.9	0.0	1399/1400 (100)	1/1400	HQ749588.1
	<i>Lactobacillus reuteri</i> 16S ribosomal RNA gene	2765.9	0.0	1409/1416 (100)	0/1416	KC700337.1

*- not found in normal group of mice, MCPA - modified citrus pectin alginate probiotic microbeads, AP - alginate calcium probiotic microbeads and MCP – modified citrus pectin solution.

4.4 Discussion

One of the key roles of the intestinal bacterial flora is to influence and maintain the integrity of the epithelia mucosa (Serban, 2014). An important factor in the risk of colon cancer is the composition of the intestinal bacterial community. However, the manipulation of the gastrointestinal tract (GIT) microbiota may give a preventive therapeutic measure against the risk of colon cancer (Orlando and Russo, 2013). Previous studies have reported differences between the microflora of colorectal cancer (CRC) and healthy patients (Wang *et al.*, 2012).

Also studies have indicated the effect of oral consumption of probiotic products on the faecal microbiota in human adults (Wang *et al.*, 2014) and rats (Mountzouris *et al.*, 2009). For instance, the administration of *Lactobacillus rhamnosus* GG with inulin to treat polypectomy showed an increased lactobacilli count in the faecal samples (Rafter *et al.*, 2007). Therefore, this study utilised microencapsulated *L. acidophilus* ATCC 4356 probiotic (in MCPA or AP) to determine the effect on the faecal lactobacilli microflora in colonic cancer induced mouse model.

In this study, highest number of lactobacilli in the faeces of mice treated with/without AOM mice model was observed after 28 days of probiotic microbeads treatment. A dosage of 15 mg/kg AOM administered *i.p* once in a week for 4 weeks to initiate colonic carcinogenesis was tolerable and non-toxic as indicated by the survival of all the animals. Previous studies aimed at evaluating the amount of probiotic bacteria in faeces of rat model treated with carcinogen reported significantly higher amount of viable bacteria compared to untreated animals (not given probiotic) (Capurso *et al.*, 2006). In this present study, the most significant increase relative to the initial count of faecal lactobacilli was observed in the AOM-treated and normal mice administered with MCPA probiotic microbeads compared to all other groups ($p < 0.0001$). Similarly, the viable faecal lactobacilli count in the AOM-treated mice administered with AP probiotic microbeads was significantly higher at day 14 of probiotic treatment. This may probably be due to the stimulating effect of the two prebiotics, MCP and the resistant starch incorporated into the microbeads which corroborates with Yen *et al.* (2011). The absence of MCP in the AP probiotic microbeads administered to both the normal and AOM-treated mice may cause the insignificant increase in the faecal lactobacilli which later decline after 14 days.

The actual differences in the increase of faecal lactobacilli after 28 days ($0.72 \log_{10}\text{cfu/g}$ and $0.8 \log_{10}\text{cfu/g}$ for the normal MCPA probiotic- and AOM+MCPA probiotic-treated mice respectively) is fairly small, however, statistically significant in comparison with other groups. The probiotic, *L. acidophilus* ATCC 4356 was expected to become competitive for colonization with the indigenous bacteria population that utilizes the carbohydrates (prebiotics) as substrates.

One important function of gut microflora is the ability to catabolize complex polysaccharides into SCFAs (butyrate, lactic acids, acetic acid and propionate) which are major energy source to the epithelium cells of the colon (Macfarlane and Macfarlane, 2012). It is reasonable to presume that the fermentation of the MCP and resistant starch by *L. acidophilus* ATCC 4356 and intestinal microflora may increase the production of SFCAs, gases and butyrate. Faecal lactobacilli utilize butyrate as the preferred energy source for their growth and proliferation of the colonic epithelial cells. These SFCAs reduce the effect of bile in the intestine by initiating bile salt hydrolase which deconjugates bile acid (Ooi and Liong, 2010).

The influence on the faecal lactobacilli count may be associated with probiotic intake. It has been generally recommended that probiotic concentration of $10^6 - 10^9$ cfu/ml or $10^8 - 10^{10}$ cfu/day is considerably sufficient to exert the beneficial effect (Shah, 2001). The changes in faecal lactobacilli count observed between the normal (control) mice and MCPA- or AP probiotic-treated mice were significant ($p < 0.05$) (Appendix 3 - Table A4). The limitation of this study, however, is the need for *in vivo* sampling of the colon tissue as some lactobacilli may colonized discrete areas in the mouse GIT or remain adherent to the mucosal surfaces and thus remain non-detectable from the faeces, in order to confirm colonic lactobacilli concentration.

Studies have reported that lactobacilli in the gut lower the risk of colon cancer by displacing other bacteria such as *Bacteriodes* and *Clostridium* genera that may produce faecal enzymes or toxins (carcinogen) during metabolism (De Preter *et al.*, 2007; Nakamura *et al.*, 2002). Lactobacilli species were reported to have a protective effect on colon cancer induced DNA damage in the colon cells of rats (Pool-Zobel *et al.*, 1996). The metabolic activity of lactobacilli lowers the level of colonic/faecal enzymes such as β -glucosidase and β -glucuronidase which hydrolyse many glucuronides releasing carcinogens into the intestinal tract (Kim *et al.*, 2008; Rowland *et al.*, 1998).

In the present study, the daily intake of MCPA probiotic *L. acidophilus* ATCC 4356 may have increased the faecal lactobacilli in the colon cancer induced mice after 28 days of probiotic consumption.

The effect of the different treatments (MCPA- and AP- probiotic or MCP) on the faecal bacteria count of the AOM-treated and normal groups of mice indicated that the faecal lactobacilli was probably influenced by the interaction of colon microbiota with the probiotic bacteria, *L. acidophilus* ATCC 4356 supplemented with resistance starch and MCP. Although, there was no significant difference in the number of faecal lactobacilli between MCP-treated mice and the control ($p > 0.05$), the reduced faecal lactobacilli presented by MCP-treated mice corroborates with Doyle (2007) and Zhong *et al.* (2014) which suggested that pectin stimulates bacteria other than lactic acid bacteria. This may also suggest why the 16S rRNA gene sequence alignment relative to the genus *Bifidobacterium* may possibly be present in the MCP-treated colon cancer-induced mouse model compared to other treatment groups.

The colon is the primary site of microbial colonization in humans and animals, harboring a tremendous number and species of bacteria (Serban, 2014). The mucosal surface of a colon adenoma harbours an increased number of bacteria compared to non-adenoma (Shen *et al.*, 2010). In this study, there was no significant change in the lactobacilli count between the AOM-treated and normal mice ($p > 0.05$). However, the influence of the MCPA probiotic on the faecal bacteria count in the AOM-treated mouse model compared to the normal group of mice may corroborate with data obtained by Mountzouris *et al.* (2009); Wang *et al.* (2012) and Wang *et al.* (2014). It is important to reiterate that the objective of this study was to identify the *Lactobacillus acidophilus* ATCC 4356. According to the 16S rRNA sequencing BLAST result, *Lactobacillus acidophilus* may be present in the AOM-treated mouse model compared to the normal group of mice (Table 4.4). Furthermore, bacteria other than lactobacilli present in the GIT were reported from the BLAST outcome, which may require further tests. Therefore, it cannot be conclusively stated that the number and species of bacteria identified were the total bacteria present in the faecal sample of the mouse model.

However, it is plausible to assume that the MCPA probiotic may have influenced the stimulation of colonic microflora in the colon cancer-induced mice compared to the AP probiotic and MCP (Appendix 3 - Table A4).

This may also be due to indirect effects of other bacteria concentrations, level of short-chain fatty acid, colonic enzyme levels and reduction of DNA damage or DNA adducts formation (De Preter *et al.*, 2007; Pool-Zobel *et al.*, 1996). As the application of the *in vitro* findings into the *in vivo* systems would provide a better idea on the efficacy of this formulation (MCPA), it is also paramount that this new biopolymer matrix must be further investigated and developed to advance probiotic microencapsulation. Thus, the functionality and physiological properties of probiotic and the colonic microflora make it a promising target for the development of a colon cancer therapeutic. Also, the breakdown of the encapsulation matrix by the colon microflora rather than the effect of pH in the GIT may however be better targeted as an approach to release probiotics in the colon.

CHAPTER 5

THE CHEMOPREVENTION OF AZOXYMETHANE-INDUCED COLON CARCINOGENESIS IN BALB/C MICE MODEL USING A MICROENCAPSULATED PROBIOTIC

5.1 Introduction

Colonic cancer is the third most common cancer worldwide, with high morbidity and mortality (Hagggar and Boushey, 2009). About 1.4 million new cases of the disease were reported in 2012 alone. Worldwide deaths from colonic cancer have increased from 608,000 in 2008 to 694,000 in 2012 (World Health Organisation, 2014). This trend is projected to rise, with an estimated 13.1 million deaths in 2030 (World Health Organisation, 2012). Treatments include either single or combination therapies such as chemotherapy, radiation, surgery and biologically targeted management (Jemal *et al.*, 2006). However, these therapies are less effective during metastasis, therefore prevention and earlier detection are key to reducing the risk of colonic cancer (World Health Organisation, 2014). Notably an improved dietary fibre uptake through fruit and vegetable is capable of reducing colon cancer (Doyle, 2007). Recent attention has been focused on increasing dietary intake of probiotic fibre in order to reduce colonic cancer risk (Thantsha *et al.*, 2012). *In vitro* trials have suggested that lactobacilli may reduce the progression of colonic cancer through increased apoptosis, immunomodulation, antioxidant activity and antiproliferative effect (Zhong *et al.*, 2014). Probiotics are defined as “live microorganisms which when administered orally in adequate amount confer a health benefit on the host” (Food and Agriculture Organisation, 2002). They are non-pathogenic microbes that exert beneficial effects via their immunomodulatory role and influence on the mucosae (Sanders *et al.*, 2007). Probiotics can be encapsulated with a coating matrix to protect the cell from adverse environmental effects such as stress, oxygen tension (Anal and Singh, 2007) high temperature during processing and storage (Anal and Singh, 2007; Rathore *et al.*, 2013), as well as pH and intestinal enzymatic activity (Heidebach *et al.*, 2012). Modified citrus pectin (MCP) is a polysaccharide dietary fibre broken down into smaller fragments. In humans, the unique bioactivity of MCP against carcinogenesis is linked to sugar β -galactose inhibiting the cell signalling protein marker, galectin-3 (gal-3) (Maxwell *et al.*, 2012; Morris, 2009).

The degree of gal-3 expression has been related with the stage of colon cancer development in several animal models (Liu, 2002; Liu and Rabinovich, 2005).

Increased expression of gal-3 is associated with metastatic colon cancer cells and poor patient prognosis (Endo *et al.*, 2005; Hill *et al.*, 2010). Gal-3 is intimately involved in endothelial cell morphogenesis and angiogenesis (Nangia-Makker *et al.*, 2000). Vascular endothelial growth factor (VEGF) receptors contribute to the survival and proliferation of endothelial cells (Possemiers *et al.*, 2010). The up-regulation of VEGF signalling can lead to intestinal inflammation and the release of leukocytes in the vascular endothelium thus upsetting the cytokine milieu in favour of cancer growth (Possemiers *et al.*, 2010).

Azoxymethane (AOM) is a potent specific carcinogen used to induce colonic cancer in mice and rats (Chen and Huang, 2009; Escribano *et al.*, 2004; Marotta *et al.*, 2003; Orii *et al.*, 2003). Diverse dosage regimens of AOM administered through several routes have revealed aberrant crypt foci (ACF) in mice several weeks (12-36 weeks) after AOM initiation (Bissahoyo *et al.*, 2005; Chen and Huang, 2009) with post-exposure subcutaneous doses of 15 mg/kg and intraperitoneal (*i.p.*) dose of 20 mg/kg being toxic (Alizadeh *et al.*, 2012; Bissahoyo *et al.*, 2005).

The bioactivity, bioavailability and uptake of MCP may be improved through a novel approach if conjoined with a supplement such as a probiotic. In an attempt to improve colon cancer outcome via gal-3 and VEGF inhibition, the aim of this study was to investigate the synergistic inhibitory effect of *L. acidophilus* ATCC 4356 combined with alginate with/without MCP using a commercial representative preparation, PectaSol-C (ecoNugenics Inc. CA, USA) to form biopolymer microbeads in a Balb/c mouse model of AOM-induced colonic carcinogenesis. Preceding this investigation the optimal AOM dosage and duration of treatment that elevated gal-3 and VEGF immunoreactivity within the colon of the Balb/c mouse model was determined.

5.2 Materials and methods

5.2.1 Animal model

After institutional ethics approval (063/13/Animal and 046/14/Animal), this experiment was carried out at the Biomedical Research Unit (BRU), University of KwaZulu-Natal (Appendix 4). The BRU approved standard protocols for animal treatment were followed. Male Balb/c mice (aged 7 weeks) weighing 20-25 g were bred in-house under a controlled condition of humidity ($50 \pm 10\%$) and temperature ($23 \pm 2^\circ\text{C}$) on a 12 h light/12 h dark cycle with free access to water and food. During the experiment, mice were carefully observed for any toxic effect and abnormal behaviour. Additionally, their body weight was recorded.

5.2.2 Carcinogenic treatment of Balb/c mice

5.2.2.1 Experiment I: Selection of optimum dose duration of azoxymethane

The study population (n=36) consisted of six groups of Balb/c mice (n=6 each). Groups 1 and 2 received 10 and 15mg/kg AOM *i.p.*, respectively, once a week for 2 consecutive weeks. Groups 4 and 5 received 10 and 15mg/kg AOM *i.p.*, respectively, once a week for 4 weeks. Group 3 and 6 (control mice) were injected with a 0.9% saline solution for 2 and 4 weeks, respectively. All groups were observed for 9, 12 and 16 weeks, at the end of which colonic samples were obtained.

5.2.2.2 Experiment II: Investigation of chemopreventive efficacy of the modified citrus pectin alginate and alginate calcium microbeads treatment

The optimum dose and time response selected from Experiment I was adopted in Experiment II. The total study population (n=40) consisted of four groups (n=10 each). Each group were administered either 0.2 ml MCPA, AP probiotic microbeads, MCP or water once daily for 16 weeks (Table 5.1). Water and food were available to the animals *ad libitum* throughout the experiment. Following euthanasia, macroscopic tumour detection and histopathological analysis of the entire gastrointestinal tract was performed (Figure 5.1). Immunohistochemistry for gal-3 and VEGF were also performed.

Table 5.1: Category of treatments administered on the AOM-treated Balb/c mouse model

Groups	Treatments
1	Modified pectin alginate (MCPA) probiotic microbeads
2	Alginate calcium (AP) probiotic microbeads
3	Modified citrus pectin (MCP)
4	Water (Control)

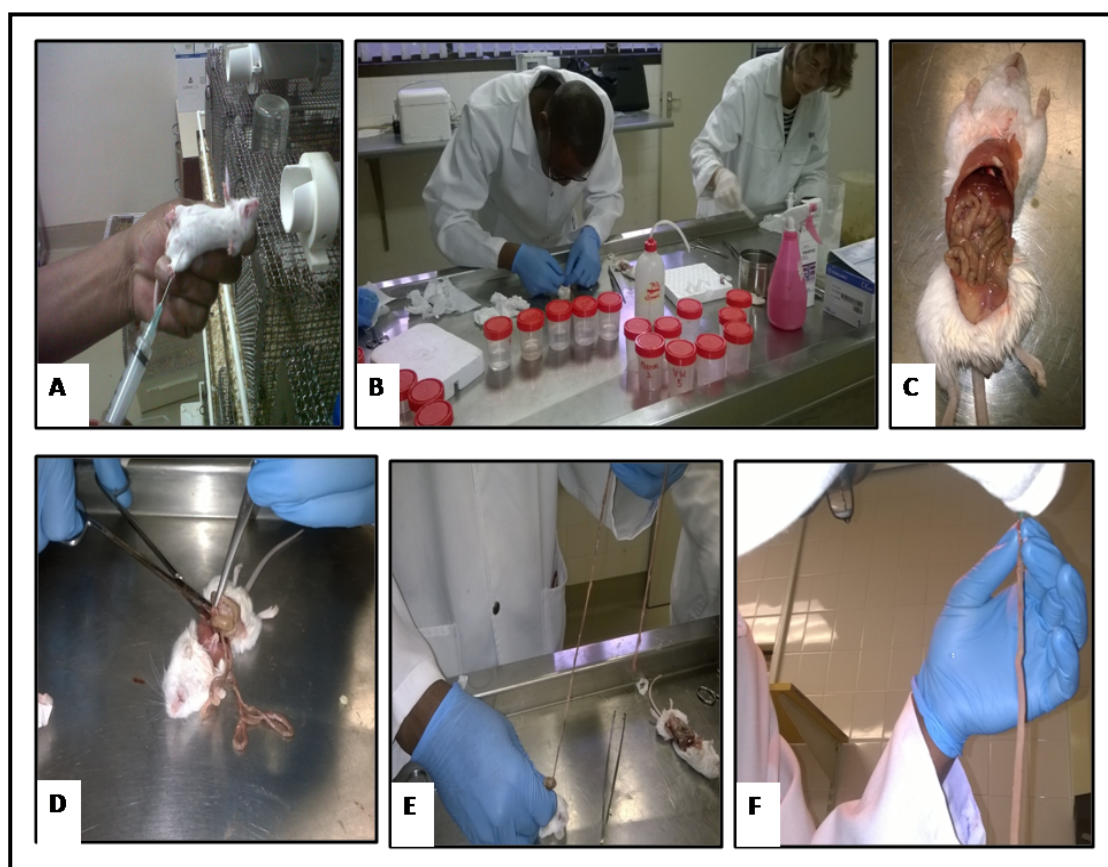


Fig 5.1 The administration of azoxymethane (AOM) and colon tissue collection from the Balb/c mice: (A) AOM *ip* treatment (B) euthanasia of animal (C-F) macroscopic examination of colon tumour.

5.2.3 Immunohistochemistry

5.2.3.1 Tissue preparation

The distal colonic tissues were fixed in 10% buffered formalin. Tissue processing was performed on a LEICA ASP 200S (SMM Instruments (Pty) Ltd., Midrand, South Africa) and wax embedding was carried out on a LEICA EG 1150H embedding station. Sections (3-5 µm) were cut on a rotary microtome (LEICA RM 2135) and collected onto poly-L-lysine-coated slides. This was followed by deparaffinisation and rehydration in a descending series of ethanol containing aqueous solution.

5.2.3.2 Immunostaining

Immunostaining was performed using the EnVision™ FLEX reagents (Dako, Glostrup, Denmark) and EnVision™ FLEX staining technique. Sections were incubated in a preheat EnVision™ FLEX target antigen retrieval solution for 20 min at 95-99°C. Endogenous peroxidase was blocked using 3% hydrogen peroxide blocking for 5 min. Post washing, sections were incubated at 4°C in primary monoclonal antibodies (Mab) against gal-3 (1:50 dilution, clone A3A12; Thermoscientific, Waltham, MA, USA) and VEGF (1:100 dilution, clone VG1; DAKO) for 24 h and 30 min respectively. A pre-formed avidin biotinylated horseradish peroxidase macromolecular complex was then linked with a secondary antibody for 15 min at room temperature. Detection of reaction was carried out with diaminobenzidine as the chromogen. Slides were viewed with an Axioscope A1 photomicroscope (Zeiss, Gillitts, Durban, South Africa).

5.2.3.3 Semi quantitative image analysis

Expression levels of gal-3 Mab A3A12 and VEGF markers were based on the proportion of immunopositive cells in a field and the average staining intensity of immunopositive cells. The semiquantitative score adopted for the percentage of immunopositive cells was: 0: 0-10%, 1: 10-39%, 2: 40-69% and 3: >70%; B, and for staining intensity was: 0: no staining, 1: weak staining, 2: moderate staining and 3: strong staining. The sum of A + B will give; 0-2 = negative, 3-4 = weakly positive and 5-6 = strongly positive.

5.2.3.4 Data analysis

The statistical package Graphpad Prism software SSPS version 17.0 for Windows (SSPS, Chicago, Illinois, USA) was used for all statistical analyses. Both Turkey's test and analyses of variance (ANOVA) were used to compare expression of antibodies and probiotic treatments amongst groups. A value of $p < 0.05$ indicated statistical significance.

5.3 Results

5.3.1 Experiment I

5.3.1.1 Tumour type and incidence

A high percentage of pre-cancerous lesions in the AOM-treated mice were recorded in the distal compared to the proximal region of the colon. Normal colon and ACF to microadenoma were noted (Figure 5.2). Crypts were found to be abnormally large with prominent nuclei. Moderately to severely dysplastic microadenoma was observed in which goblet cells were markedly reduced or absent. Colonic mucosal ulceration with focal dysplasia was marked with deep erosion inflammation mostly of crypt abscess formation. ACF developed in 4/12 (10 mg/kg) and 3/12 (15 mg/kg) AOM-treated mice, while microadenomas were observed in 5/12 mice treated with 15 mg/kg AOM; no microadenomas developed in mice treated with 10 mg/kg AOM. Almost 67% of mice in the 4 weeks AOM treated group administered with 15 mg/kg AOM developed pre-cancerous tumours ($p < 0.05$). The control group had morphologically normal colon with no signs of pathology.

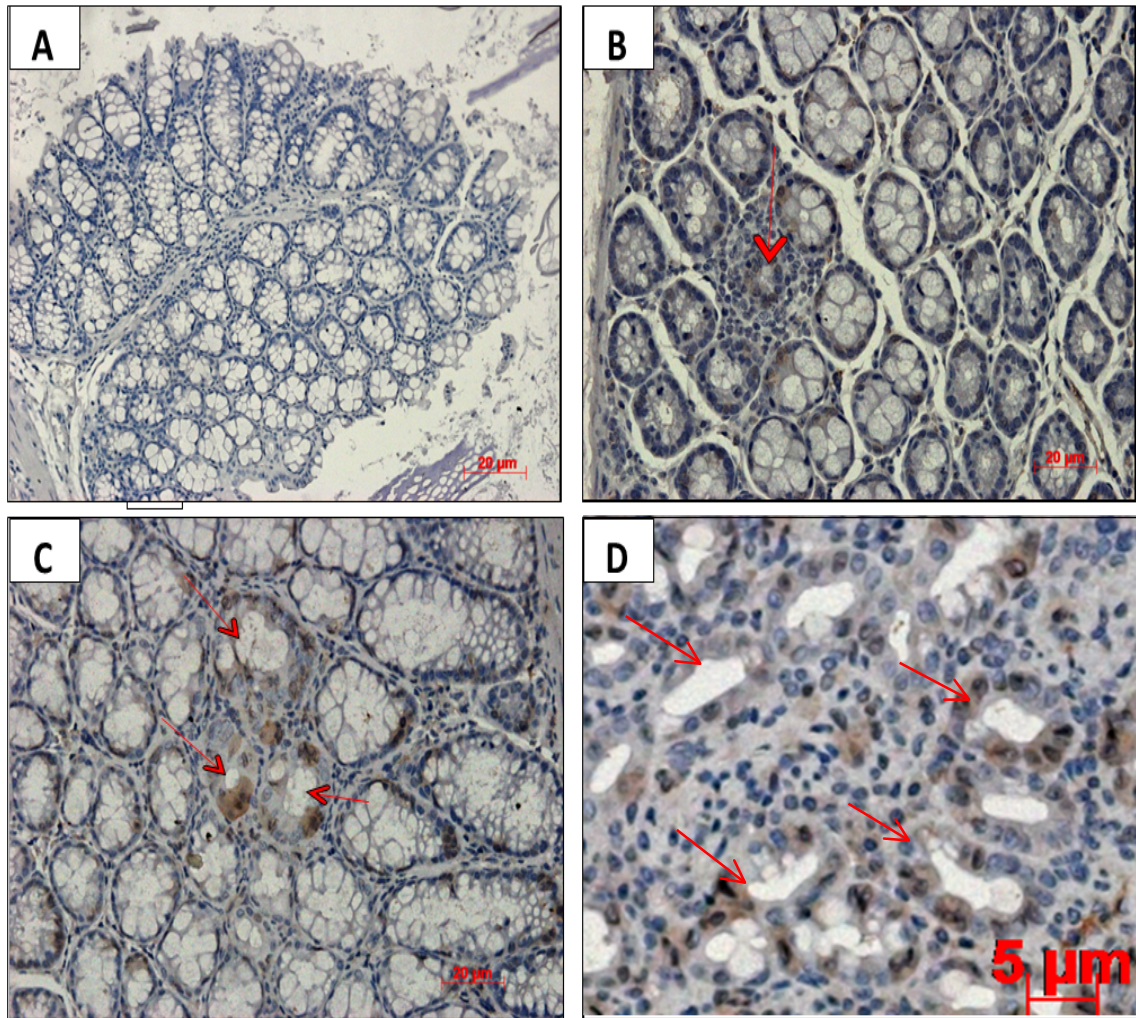


Fig 5.2 Colonic neoplasm from Balb/c mice treated with 15 mg/kg azoxymethane (AOM) 4 weeks. (A) A normal colonic mucosa showing negative expression of galectin-3. (B) A single aberrant crypt at week 9 showing weak expression of galectin-3. (C) Aberrant crypt foci (indicated by arrow) at week 12 showing moderate expression of galectin-3. (D) Microadenomas (indicated by arrow) invading the submucosa showing moderate expression of galectin-3 at week 16.

5.3.1.2 Immunostaining

Gal-3 and VEGF immunostaining was observed in the colonic mucosae, crypts and endothelial cells of vessels within the *lamina propria*. The subsequent semiquantitative evaluation of immunoexpression of gal-3 and VEGF in the colon of AOM-treated Balb/c mice is shown in Table 5.2 and 5.3, Figures 5.3 and 5.4.

Table 5.2: Semi quantitative analysis of galectin-3 (gal-3) in azoxymethane (AOM) treated Balb/c mouse model

Dose (mg/kg)/Treatment	9 weeks				12 weeks				16 weeks			
	C		E		C		E		C		E	
	%	SI	%	SI	%	SI	%	SI	%	SI	%	SI
10 mg/kg/ 2 weeks	1.5	2	1.5	2	1	1	2	2	2	2.5	1.5	2
10 mg/kg/ 4 weeks	3	3	1	1	0.5	0.5	0.5	0.5	3	2	2	1
15 mg/kg/ 2 weeks	1.5	2	2	2	3	2.5	2	1	2.5	2.5	2	2
15 mg/kg/ 4 weeks	2	2	2.5	1.5	1.5	1.5	2	1.5	3	3	3	1
Control/ 2 weeks	0	1	0	0	0	0	0	1	0	1	0	0
Control/ 4 weeks	1	1	0	0	0	0	1	2	1	1.5	0	1

(A) % - percentage of positive cells (0-10%: 0, 10-39%: 1, 40-69%: 2 and > 70%: 3), (B) SI- Staining intensity (No staining: 0, weak staining: 1, moderate staining: 2, strong staining: 3. Number of mice in each groups (n=6) and values represent means.

Table 5.3: Semi quantitative analysis of vascular endothelial growth factor (VEGF) in azoxymethane (AOM) treated Balb/c mouse model

Dose (mg/kg)/ Treatment	9 weeks		12 weeks		16 weeks	
	%	SI	%	SI	%	SI
10 mg/kg/ 2 weeks	1	2	2.5	3	2.5	3
10 mg/kg/ 4 weeks	1	1.5	2	2	2.5	3
15 mg/kg/ 2 weeks	1	2	2	2	2	2
15 mg/kg/ 4 weeks	2.5	3	3	3	3	3
Control/ 2 weeks	1	1	1	1	1	1
Control/ 4 weeks	1	2	1	1	1	2

(A) % - percentage of positive cells (0-10%: 0, 10-39%: 1, 40-69%: 2 and > 70%: 3), (B) SI- Staining intensity (No staining: 0, weak staining: 1, moderate staining: 2, strong staining: 3. Number of mice in each groups (n=6) and values represent means.

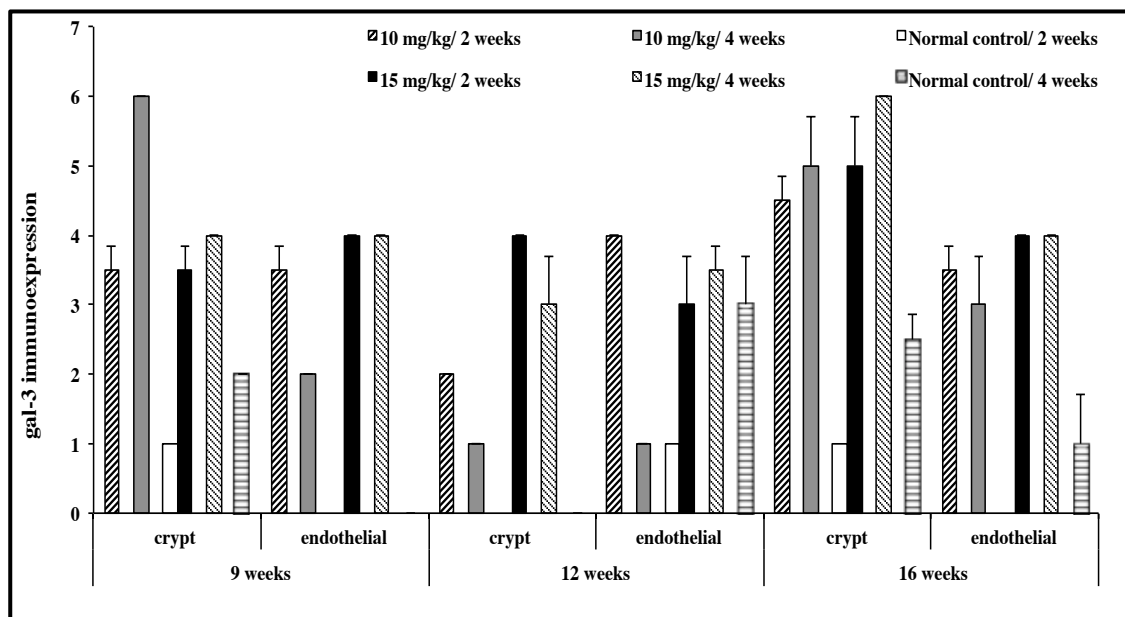


Fig 5.3 Immunostaining patterns for galectin-3 (gal-3) in azoxymethane (AOM) treated Balb/c mouse model at 9, 12 and 16 weeks of colonic carcinogenesis. [(A) % - percentage of positive cells, (B) SI- Staining intensity: A+B- 0-2 = negative, 3-4 = weakly positive and 5-6 = strongly positive. Dose (mg/kg)/duration (weeks) treatment - 10 mg/kg/ 2 weeks; 10 mg/kg/ 4 weeks; 15 mg/kg/ 2 weeks; 15 mg/kg/ 4 weeks]

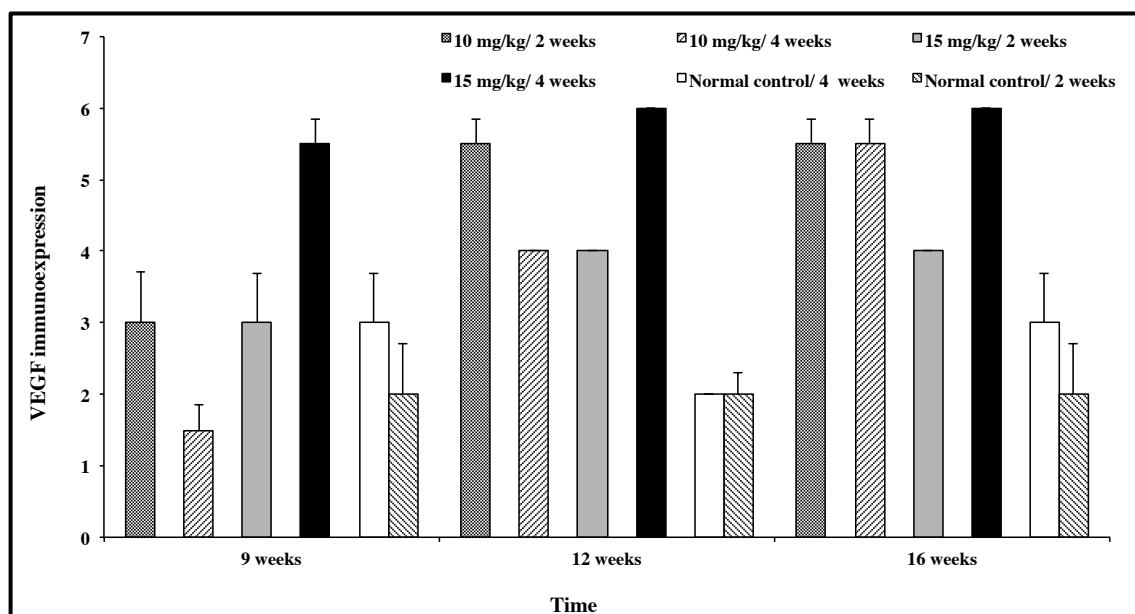


Fig 5.4 Immunostaining patterns for vascular endothelial growth factor (VEGF) in azoxymethane (AOM) treated Balb/c mouse model at 9, 12 and 16 weeks of colonic carcinogenesis. [(A) % - percentage of positive cells, (B) SI- Staining intensity: A+B- 0-2 = negative, 3-4 = weakly positive and 5-6 = strongly positive. Dose (mg/kg)/duration (weeks) treatment - 10 mg/kg/ 2 weeks; 10 mg/kg/ 4 weeks; 15 mg/kg/ 2 weeks; 15 mg/kg/ 4 weeks]

At week 9 (treatment + observation) of carcinogenesis, the expression of gal-3 in the groups treated with AOM for 2 weeks was weakly positive. Immunoexpression, however, varied from moderate to strongly immunopositive in the groups treated for 4 weeks. At week 12 (treatment + observation), although gal-3 immunopositivity was observed in groups treated with AOM at both 10 and 15mg/kg (2 and 4 weeks), it was not intense (Appendix 5 - Table A5). At week 16 (treatment + observation), gal-3 was more obvious in the superficial crypt of the colonic mucosa (Figure 5.5; I-L). Immunostaining of gal-3 was intense in 25% of the mice, which were dosed with 15 mg/kg AOM-, and 17% of the 10 mg/kg AOM (4 weeks)-treated groups ($p<0.05$). The expression of gal-3 in the control group was weakly positive in 33% and negative in 67% of the mice. The goblet cells in the colonic mucosae were not stained in AOM-treated or control groups.

The immunostaining of VEGF in both AOM-treated groups increased progressively between weeks 9-16, with the results ranging from weak to strongly positive (Figure 5.6). VEGF expression in both AOM treated groups was intensely positive in 50% of the colon tissue. A weakly immunopositive reaction was observed in 50% of mice, which were dosed with 15 mg/kg AOM-, and 33% of 10 mg/kg AOM-treated groups ($p<0.05$). None of the mice within the control group showed an intense immunopositivity for VEGF. A significant intense immunopositive VEGF in the 15 mg/kg AOM treated groups (weeks 4) was observed (Appendix 5 - Table A6). There was no animal death during the experiment.

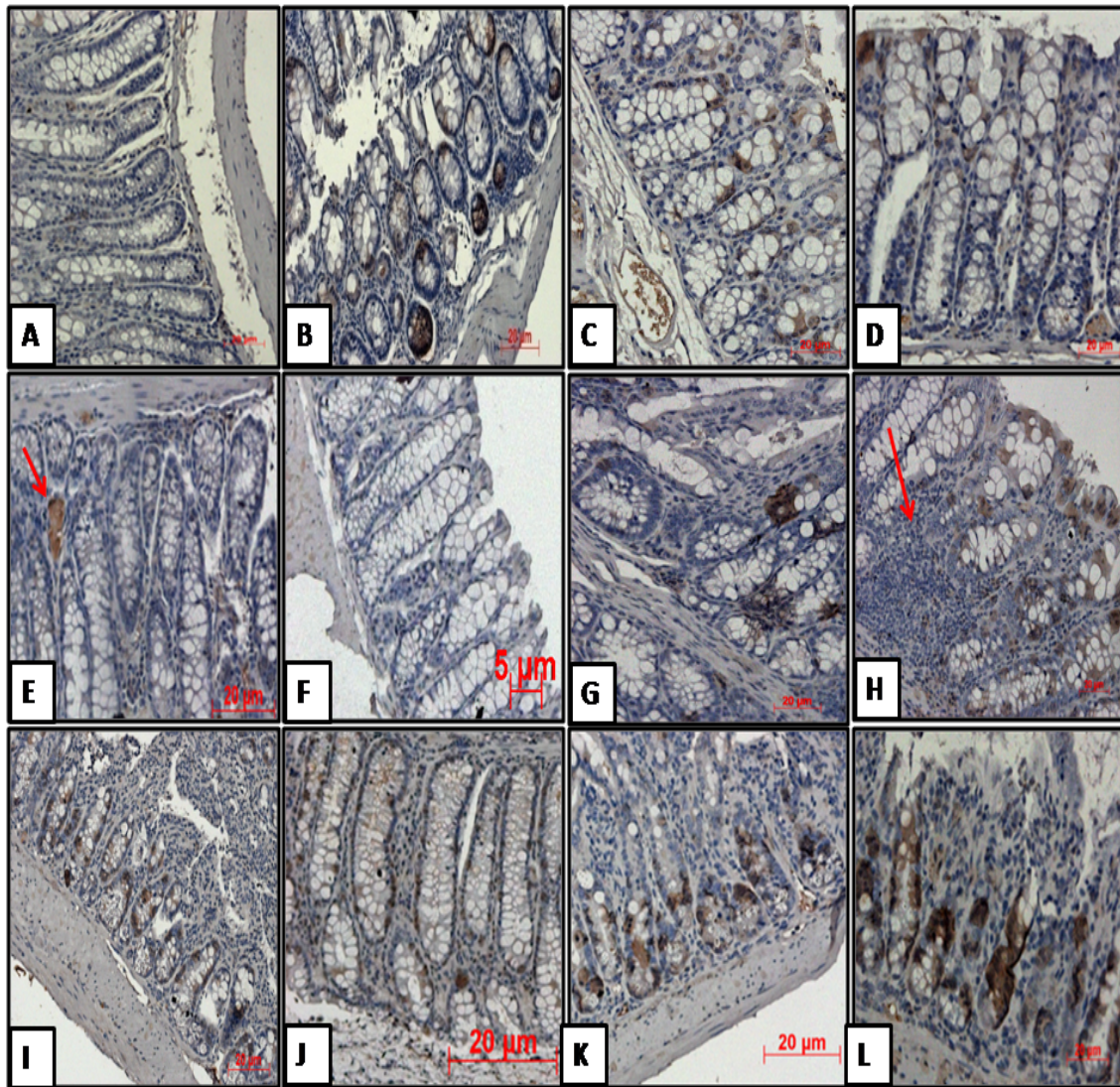


Fig 5.5 Immunoexpressions for galectin-3 (gal-3) in a Balb/c mouse model of colonic carcinogenesis treated with different azoxymethane (AOM) dose duration: (A,E,I) 10 mg/kg AOM; 2 weeks treatment (B,F,J) 10 mg/kg AOM; 4 weeks treatment (C,G,K) 15 mg/kg AOM; 2 weeks treatment and (D,H,L) 15 mg/kg AOM; 4 weeks treatment; at (A-D) 9 weeks (E-H) 12 weeks and (I-L) 16 weeks. The arrow (E) indicates galectin-3 expression in the endothelial cells within the lamina propria close to the mucosa muscularis. Arrow indicates aberrant crypt foci (ACF) (at H) and galectin-3stains (at E).

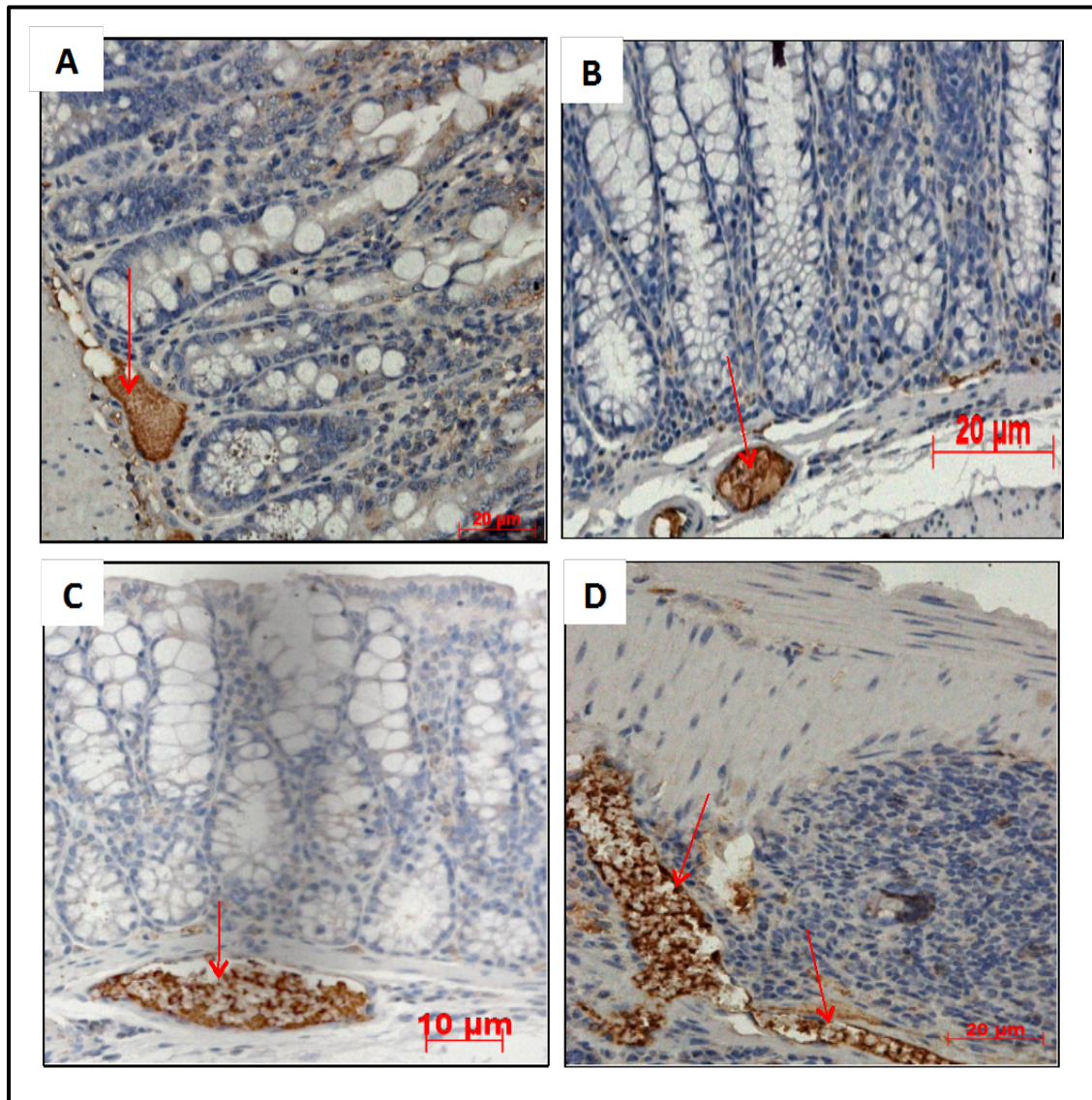


Fig 5.6 Immunoexpression for vascular endothelial growth factor (VEGF) in a Balb/c mouse model at: (A, B) week 12 and (C, D) week 16 of colonic carcinogenesis. The arrow indicates stained endothelial cells located at the submucosa muscularis.

5.3.2 Experiment II

5.3.2.1 General observation

The body weight of mice in the MCPA/AP probiotic, MCP-treated and control groups increased with no significant difference ($p > 0.05$). Some mice from the treatment and control groups developed dim vision and eye inflammation. Vision was improved and inflammation reduced in the probiotic and MCP-treated groups, while the control group showed no sign of improvement (Figure 5.7). No rectal bleeding was observed and animal behaviour did not change in the course of probiotic treatment.

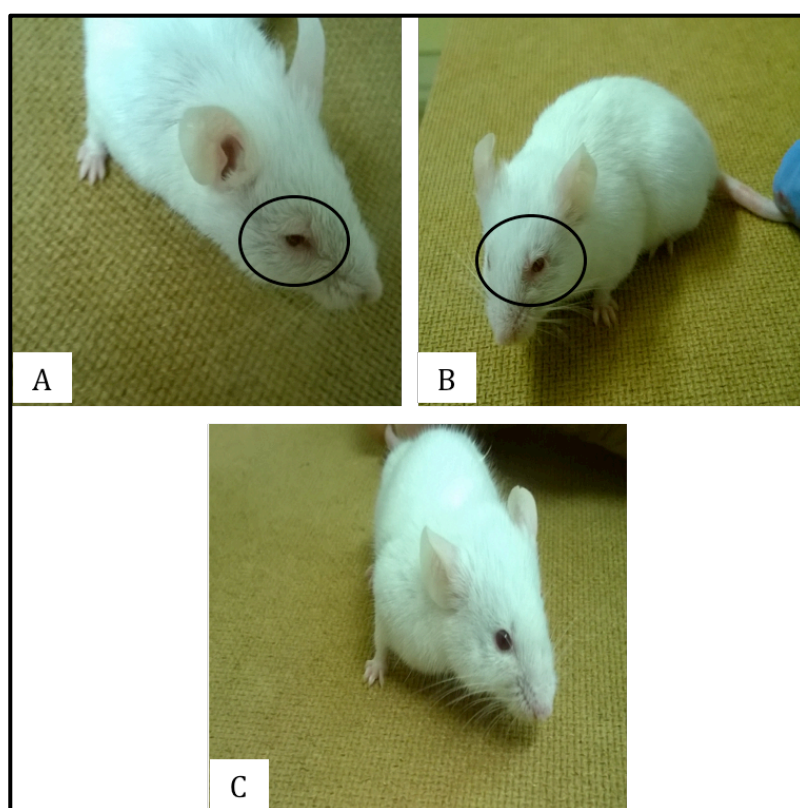


Fig 5.7 (A-B) Azoxymethane (AOM) treated Balb/c mice model with eye inflammation (C) Normal (untreated) Balb/c mouse

5.3.2.2 Tumour incidence

The percentage of colon pre-cancerous lesions in the control (untreated) and MCP-treated mice groups was high compared to the MCPA and AP probiotic-treated groups. The lowest percentage of lesions was observed in the MCPA-treated group (20%) compared to AP- and MPC-treated groups (40-50%). However, there was significant reduction of tumour in the treatment groups compared to the control group ($p < 0.05$).

5.3.2.3 Immunostaining

The immunoexpression of gal-3 and VEGF after MCPA, AP probiotic and MCP treatments is shown in Figures 5.8, 5.9 and 5.10. In the MCPA probiotic-treated group, the immunoexpression of gal-3 in the crypt and endothelium was almost negative, with 15-20% of the crypt cells barely stained.

The epithelium of the villi was not stained, while that close to the submucosa was faintly positive. In the case of AP probiotic- and MCP-treated groups, about 30% of the crypts and the epithelial luminal surface were weakly immunopositive. The intensity of gal-3 was less expressed at the crypt of the MCP treated group. Conversely, almost the entire crypt and endothelium were strongly immunopositive in the control group. The immunoexpression of gal-3 at the endothelial cells in the MCPA probiotic treated group was negative while the AP probiotic and MCP treated mice showed weakly positive compared to the strong positive in the control group (Appendix 5 - Table A7). Treatment with MCPA, AP probiotic, and MCP, reduced the expression of gal-3 in AOM-treated mice in comparison to the control group (Appendix 5 - Table A8).

The immunoexpression of VEGF in the MCPA- and AP probiotic-treated groups was weakly positive. Although the staining intensity of the endothelial cells in the MCP-treated group was barely positive ($p < 0.001$), the percentage of positive cells was less than 10% in both AP probiotic- and MCP-treated groups. VEGF expression in the control group was intense (Figure 5.8).

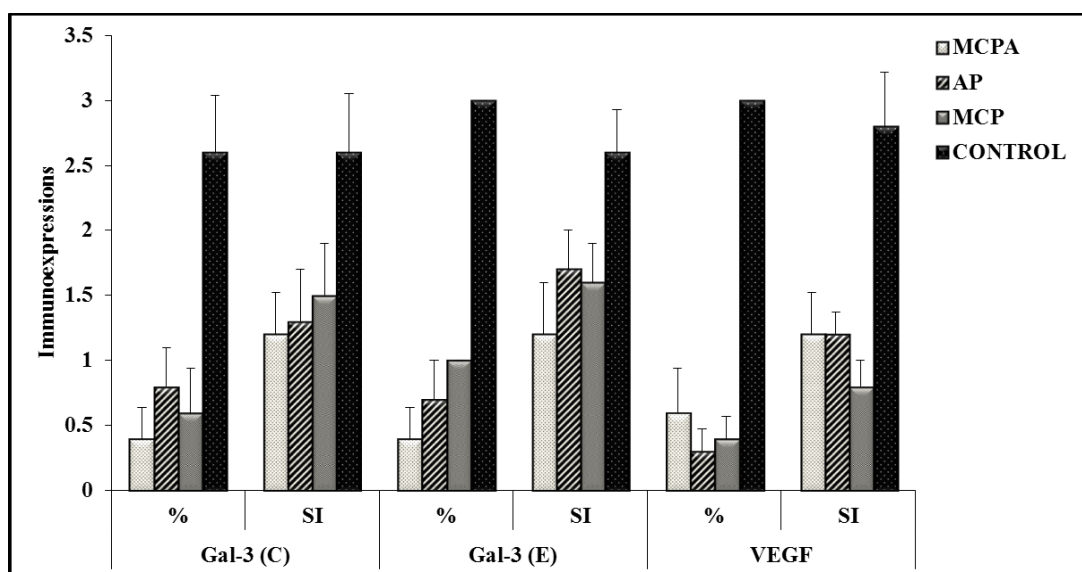


Fig 5.8 Post-treatment immunoexpressions of galectin-3 (gal-3) and vascular endothelial growth factor (VEGF) markers in a colon cancer induced Balb/c mouse model. Columns denote mean \pm SD (n=10). [C- crypt; E- endothelial]

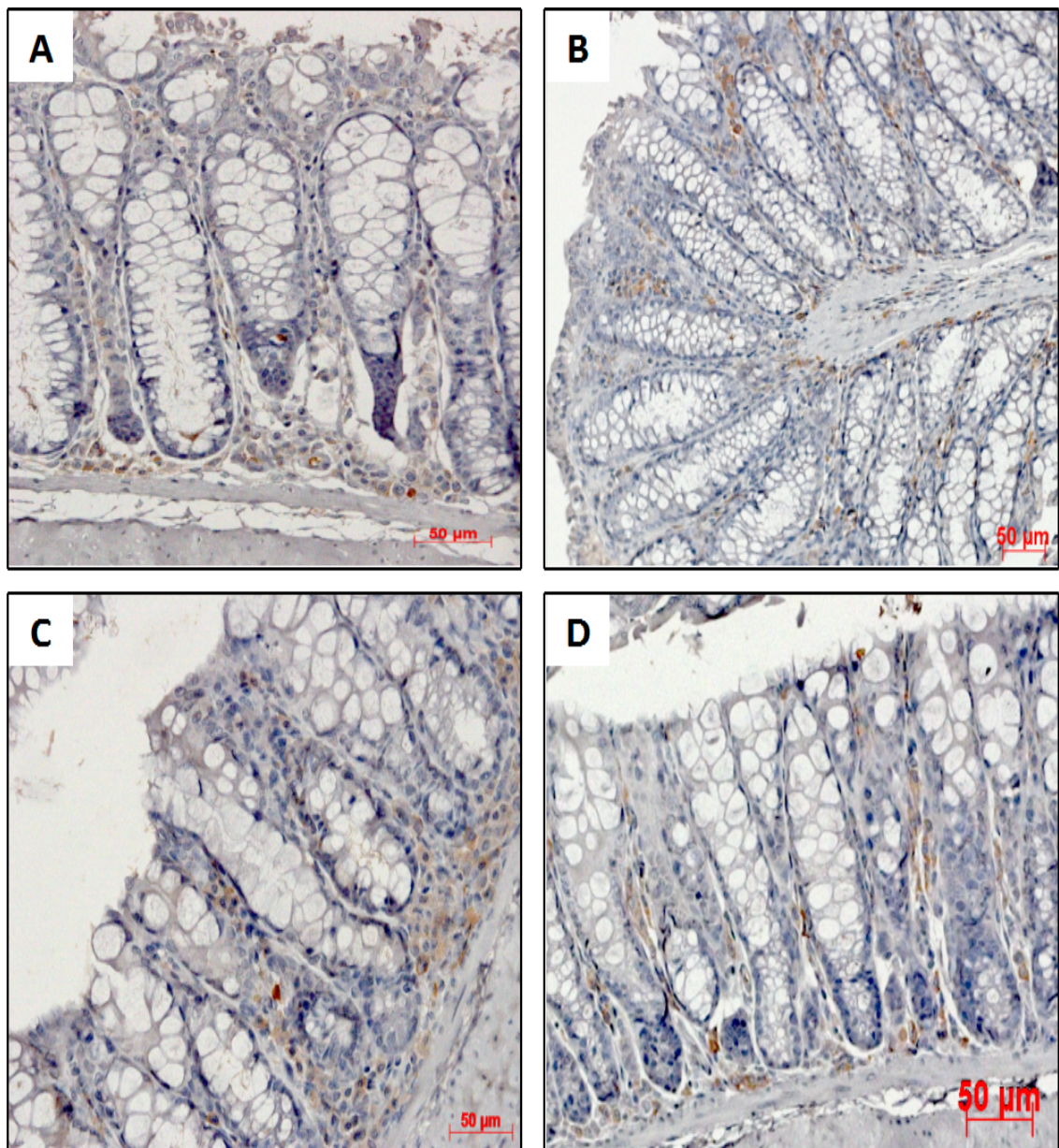


Fig 5.9 The immunohistochemical features of galectin-3 (gal-3) expression in a colonic carcinogenesis Balb/c mice treated with probiotic microbeads and modified citrus pectin (MCP): (A) modified citrus pectin alginate (MCPA) treated colon (B) normal (untreated) colon (C) alginate calcium (AP) treated colon (D) modified citrus pectin (MCP) treated colon.

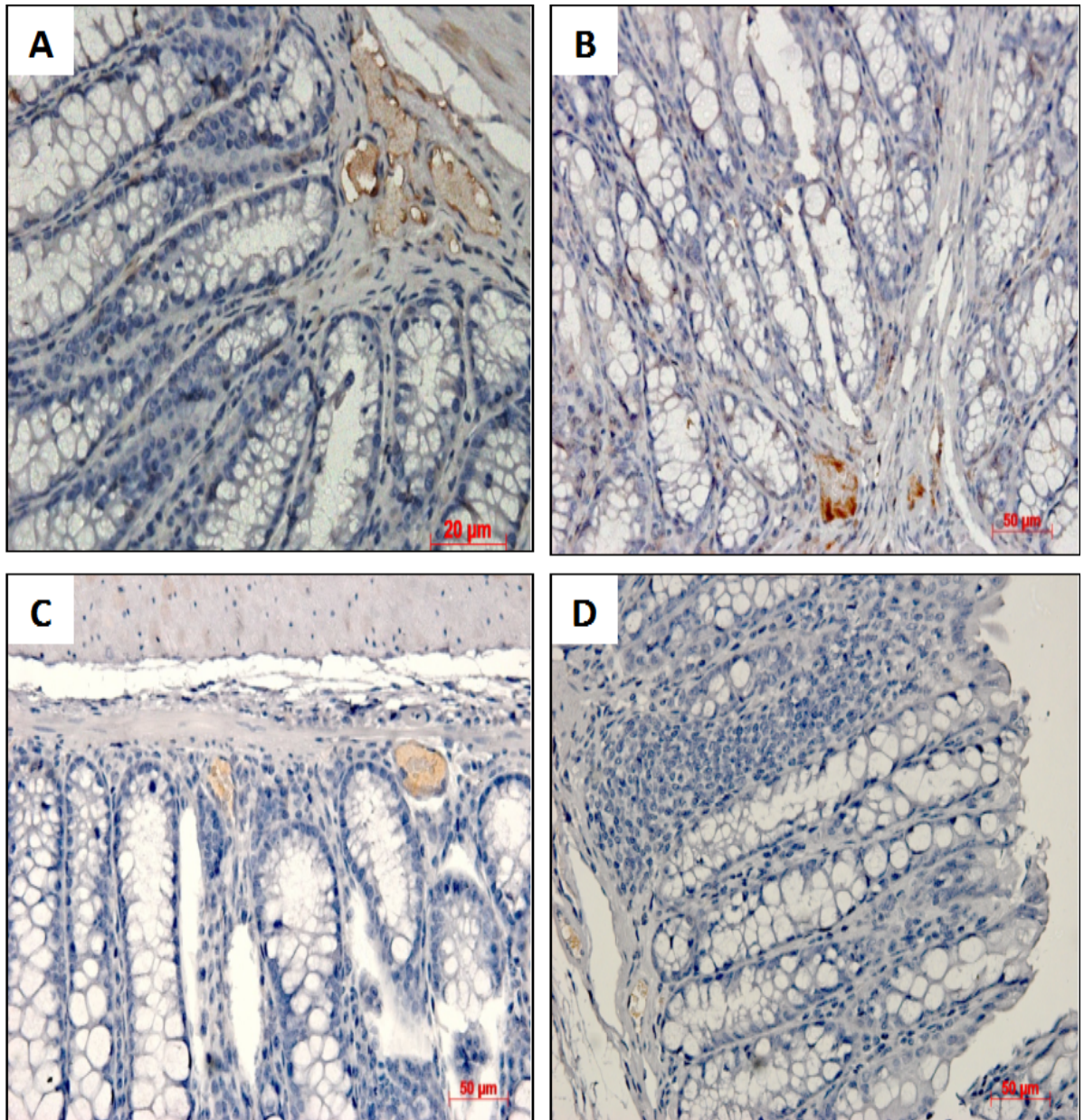


Fig 5.10 The immunohistochemical features of vascular endothelial growth factor (VEGF) expression in a colonic carcinogenesis Balb/c mouse treated with probiotic microbeads and modified citrus pectin (MCP): (A) Normal (untreated) colon (B) modified citrus pectin alginate (MCPA) treated colon (C) alginate calcium (AP) treated colon (D) modified citrus pectin (MCP) treated colon.

5.4 Discussion

The optimal dose of AOM required to induce colon carcinogenesis in mice is dependent on the genetic constitution of the rodent strain as well as environmental factors (Alizadeh *et al.*, 2012; Bissahoyo *et al.*, 2005; Escribano *et al.*, 2004; Tanaka *et al.*, 2003). Previous studies have reported the histological progression of colonic cancer in AOM-treated rodents (Hill *et al.*, 2010). There is however, an urgent need to standardize the dosage of AOM used to induce colonic cancer. This novel study utilised gal-3 and VEGF markers to determine the optimal AOM dose required to initiate colon carcinogenesis in a mouse model.

In experiment 1 of the study, intense immunoexpression of gal-3 and VEGF in the colon of Balb/c mouse model was observed after 16 weeks of AOM treatment. This optimal and tolerable dosage was equivalent to 15 mg/kg AOM administered *ip.* once every 4 weeks. However, in Wistar rats a similar dose of 1,2- dimethylhydrazine administered for 8 weeks led to strong immunoexpression of gal-3 and developed dysplastic ACF at week 32 of colon carcinogenesis (Hill *et al.*, 2010). The survival of all animals in the model indicates that both dose regimens (10 mg/kg and 15 mg/kg) were non-toxic to the Balb/c mice. In this study, the strong expression of gal-3 in both the mucosal crypts and blood vessels within the lamina propria of AOM treated mice implies the absorption and elevated free circulation of the protein marker associated with disease progression. Evidently, the distribution and immunoexpression of gal-3 in colonic carcinogenesis is dependent on the stage of cancer and metastasis. Thus these results corroborate the association of immunoexpression of gal-3 with the severity of the colon lesion.

Whilst some studies report a decline in gal-3 expression during the development of colonic cancer, others show an increase in gal-3 expression (Povegliano *et al.*, 2011; Tsuboi *et al.*, 2007). This study found that there was a significant increase of gal-3 in the AOM-treated groups at weeks 9 and 16 ($p < 0.05$). However, there was no significant difference between weeks 9 and 16 (Appendix 5 - Table A5 and A6). This advocates a prognostic significance of gal-3 as a reliable biomarker for colorectal cancer detection.

A limitation to this, however, is the need for *in vivo* sampling at different time intervals in order to confirm disease prognosis. Hence, the prognostic value of gal-3 as an indicator of colorectal cancer remains uncertain.

Similarly, in the present study, VEGF immunoexpression was up-regulated with tumour progression. The incidence of tumour formation has been associated with elevated expression of gal-3 and VEGF; consequently they are both good indicators of cancer cell proliferation and angiogenesis (Hill *et al.*, 2010; Kondo *et al.*, 1994). VEGF expression varies in that some studies have shown the presence of VEGF after tumour development whilst others show VEGF before detection of ACF (Escribano *et al.*, 2004). This study demonstrates an association between gal-3 and VEGF expression at the early stage of colonic carcinogenesis.

An increased concentration of free gal-3 has been seen in the circulating blood of patients with colonic cancer has been reported, thereby contributing to the rapid metastatic spread of cancerous cells (Yu, 2010). Circulating gal-3 has the ability to metastatic cell proliferation (Yu, 2010). Cell surface gal-3 can interact with laminin and promote tumour cell release from the primary site (Yang *et al.*, 1996). The over expression of cell surface associated extracellular gal-3 in epithelial cells can trigger cancer cells interaction by binding glycoconjugates to integrins, thereby activating intracellular VEGF signalling (Yang *et al.*, 1996). In the present study, 12 weeks after the initiation of AOM in the mice, there was an elevated expression of gal-3 together with an up-regulation of VEGF in the colonic tissue compared to the control group.

With regards to experiment II, the administration of the MCPA probiotic microbeads to the mouse model demonstrated a reduction in gal-3 and VEGF immunoexpression with lower tumour incidence. Likewise, the AP probiotic microbeads also inhibited gal-3 expression during carcinogenesis together with a low incidence of precancerous lesions. The combination of the probiotic and MCP was more effective in reducing gal-3 immunoreactivity in colonic carcinogenesis than both the single therapy of MCP and AP probiotic alone. Furthermore, in all three treatment regimens, the immunoexpression of gal-3 was lower in the mucosal cells of the crypts compared to the endothelial cells.

The immunoexpression of VEGF ranged from low in the colon of mice receiving the MCPA treatment to low/absent in the AP- and the MCP-treated groups (Appendix 5 - Table A7). This is indicative of MCP bioactivity regulating cell proliferation and angiogenesis in cancer progression. Azémar *et al.* (2007) demonstrated the inhibition of tumour associated angiogenesis by MCP in animal model.

In this present study, the staining pattern of gal-3 was cytoplasmic. Gal-3 is synthesized as a cytosolic protein that is translocated to the mitochondria and acts as an inhibitor of apoptosis (programmed cell death) (Lemasters, 2005; Yen and Klionsky, 2008).

Both the reactive oxygen species and antioxidant properties of probiotic play a key role in the prevention of colonic cancer (Lin *et al.*, 2009). The reduction of gal-3 expression in the MCPA and AP treated groups may be a function of their apoptotic activity. The availability of orally ingested MCP and resistant starch in the MCPA may influence the glycolytic and metabolic activities of the *L. acidophilus* ATCC 4356 in the gastrointestinal tract. This leads to increased production of ATP, which stabilizes the mitochondria by reducing oxidative damage and the release of apoptotic molecules thereby maintaining colonic mucosal integrity.

Gal-3 inhibits the inflammatory response of the intestinal system via the gut associated lymphoid tissue (GALT). GALT modulates macrophage signalling recruitment (Rice *et al.*, 2005). Additionally, gal-3 can bind to cell surface receptors creating a clustering effect (Yang *et al.*, 1996). Consequentially, a higher concentration of gal-3 at this binding site activates T cells with possible evasion of the immune surveillance system. Gal-3 has three domains namely the -NH₂ and -COOH terminals and Asp-Trp-Gly-Arg (NWGR) anti-death motif. The β -1,4-galactan of the neutral sugar chain in MCP binds specifically to the COOH terminal carbohydrate-recognition domain (CRD) of cytoplasmic gal-3 (Maxwell *et al.*, 2012). However, the S-glycoprotein layer of *L. acidophilus* ATCC 4356 also comprises of both the amino and carboxyl terminal domains (Smit *et al.*, 2001) which would compete to bind to the gal-3 COOH terminal. The initiation of colonization/adhesion of the probiotic bacteria to specific receptors on the epithelial cell surface of the colon may competitively inhibit gal-3 extracellular matrix interactions in addition to the MCP-gal-3 binding.

Extracellular macromolecules or exopolysaccharides synthesized by the bacteria may also contribute in modulating the immune response (Liu *et al.*, 2011; Ruas-Madiedo *et al.*, 2010). Biopolymers and prebiotic agents' such as the modified pectin, alginate and resistance starch may also modulate intestinal homeostasis (Sánchez *et al.*, 2012).

Tumours produce proteins or hormones that circulate and spread to other tissues or organs away from the initial site thus initiating a paraneoplastic syndrome. The blurred vision or the loss of vision acuity as was observed in the Balb/c mouse model has also been noted in other human studies and is indicative of an early sign in cancer development (Asteriou *et al.*, 2010). In this study, the uptake and bioavailability of MCPA reduced the blurred vision, probably via an autoimmune response. However, the bioavailability of MCPA was higher than MCP. The uptake of β -glycan by the macrophage has been suggested as the proposed mechanism of MCP absorption (Maxwell *et al.*, 2012; Rice *et al.*, 2005). Orally ingested MCPA modifies the functional properties of *L. acidophilus* ATCC 4356 cell envelope. The *L. acidophilus* ATCC 4356 surface associated proteins interacting with MCPA may easily be internalized by the intestinal epithelial cell or GALT (macrophage), thus, improving the bioavailability and the anti-cancer effect of MCP.

In conclusion, this novel study utilizes the combination of a probiotic *L. acidophilus* ATCC 4356 and MCP with alginate as a chemopreventative cancer therapy. The chemopreventative ability of MCPA is significantly demonstrated in this study via the inhibition of gal-3 and VEGF immunoexpression in the Balb/c mice colon model of AOM-Induced colonic cancer. MCPA probiotic dramatically inhibits the precancerous lesions. This inhibition is associated with reduced cell proliferation cell apoptosis, and angiogenesis. Further studies will clarify the mode of action of MCPA probiotic in *in-vivo* cancer and preclinical models.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

Previous studies have demonstrated the role of probiotics in the prevention of colon cancer using animal models. The inhibition of colon cancer by probiotics may involve the presence of reactive oxygen species, apoptosis, production of specific bacterial enzymes and effect on host metabolism (Zhu *et al.*, 2011). The influence of probiotic on the epithelial cells and native T cells in the lamina propria of colon mucosa stimulates the adaptive immune system in 1,2- dimethylhydrazine (DMH) treated Balb/c mice (Perdigón *et al.*, 1998). Although probiotic such as lactic acid bacteria present in many foods (for example yogurt) are often consumed, their viability in the host gut to improve biological functions of the host remains uncertain (Zhu *et al.*, 2011). Prebiotics have proven to improve the viability of probiotics and contributed to the colon cancer prevention trials (Roberfroid *et al.*, 2010). Also, the administration of microencapsulated *Lactobacillus acidophilus* to MIN mice resulted in a lower incidence of intra epithelial neoplasms with dysplasia tumour (Urbanska *et al.*, 2009). Thus a similar approach i.e. to microencapsulate the probiotic, *Lactobacillus acidophilus* ATCC 4356 with biopolymer and prebiotic ingredients (alginate, modified citrus pectin and resistant starch) was used in this study.

MCP is an oral dietary supplement consumed primarily to promote cell growth (Azémar *et al.*, 2007). In this study, the administration of MCP as biopolymer and prebiotic is not only to ensure the viability of *L. acidophilus* ATCC 4356 but also to investigate its synergistic effect in an attempt to improve colon cancer prevention via galectin-3 (gal-3) and vascular endothelial growth factor (VEGF) inhibition. Though there are few published studies on MCP alone, currently there is none on the effect of MCP probiotic microencapsulation. *L. acidophilus* ATCC 4356 was chosen owing to the probiotic properties such as inflammatory response and reducing oxidative stress (Chen *et al.*, 2013), anti-proliferative, apoptosis and antioxidant activities (Choi *et al.*, 2006) and recent research significance (Ortakci *et al.*, 2012; Ortakci and Sert, 2012).

In this study, the efficiency of the modified citrus pectin alginate (MCPA) microbeads was attributed to the large mean diameter of the particle size and enhanced prebiotic functionality of resistance starch. The abundance of carboxyl polyanions in the MCPA microbeads binds equivalently to calcium and chitosan polycations thus forming a strong polyelectrolyte complex. In the alginate calcium (AP) microbeads, calcium and chitosan polycations compete to bind with carboxyl anions in alginate molecules. The formation of strong trapping matrix in the MCPA reduces the porosity of the microbead wall. Chitosan-alginate microencapsulation is known to have potential for colon targeting (Hill *et al.*, 2010). Site-specific delivery for a specific target site is necessary using the specific property of that target site (Huang and Liu, 2008). Degradation of the chitosan and alginate gel through sequestering of the calcium ions occur by the action of microflora in the colon (Hejazi and Amiji, 2003; Simonoska *et al.*, 2008). Therefore, the coating of MCPA microbeads by chitosan is to specifically target the colon in order to ensure the release of metabolically active *L. acidophilus* ATCC 4356 and adsorption of MCP in the colon. Maxwell *et al.* (2012) suggested that orally ingested MCP is absorbed into the blood stream in the small intestine.

The manipulation of the gastrointestinal tract (GIT) microbiota may be beneficial as a preventive therapeutics measures from colon cancer (Orlando and Russo, 2013). In this study, the count of faecal lactobacilli microflora in the AOM-treated Balb/c mouse model increased significantly by 10% ($0.8 \pm 0.08 \log_{10}\text{cfu/g}$) ($p < 0.05$) after 4 weeks of daily oral administration of the MCPA *L. acidophilus* ATCC 4356 microbeads. The difference in number of faecal lactobacilli after probiotic consumption was highly significant in the MCPA probiotic treated group compared to the AP probiotic and control groups ($p < 0.01$). The changes in the faecal count of the treated mice may support the observation that colon microflora responds dynamically to change in dietary intake (Mountzouris *et al.*, 2009). In this present study, the administration of MCP alone to the colon cancer-induced mice may not have significantly influence the faecal lactobacilli count. This result corroborates with Doyle (2007). The 16S rRNA gene of the isolated faecal bacteria was amplified, sequenced and identified for *L. acidophilus* which may possibly be present in the faeces of the colon cancer-induced Balb/c mouse model treated with MCPA and AP probiotic.

The MCPA and AP microbeads are to ensure the viability of *L. acidophilus* ATCC 4356 to the point of release in the colon. The fermentation of the MCP and resistant starch by *L. acidophilus* ATCC 4356 and intestinal microflora may improve the production of SFCAs, gases and butyrate. These SFCAs reduce the effect of bile in the intestine by initiating bile salt hydrolase which deconjugates bile acid. Also, butyrate is one of the preferred energy source for the growth of faecal lactobacilli and proliferation of the colonic epithelial cells (Ooi and Liong, 2010).

MCPA ensures the viability of the probiotic bacteria to the point of target delivery (colon), thus its efficiency is significant. It is plausible to assume that in addition to the protective role of MCPA as a biopolymer, it also modifies the functionality and physiological properties of the *L. acidophilus* ATCC 4356 during gastric transit or adhesion/colonization. It is very important that indigenous microflora should displace exogenous pathogens from colonizing the GIT (Brassart and Schiffrin, 1997). Therefore, probiotic *L. acidophilus* ATCC 4356 would compete with the indigenous flora for survival in the colon cancer-induced Balb/c mice model. Lactobacilli compete with many pathogenic bacteria for adhesion in the gut by producing antagonistic substances such as lactic acid, reuterin and bacteriocin (Hao and Lee, 2003; Socol *et al.*, 2010). Lactobacilli possess surface adhesins like most bacterial pathogen in the intestine thereby compete for the specific receptors on the mucosal surface. For instance, *L. johnsonii* shares the carbohydrates binding specificities with many enteropathogens (Neesser *et al.*, 2000) while *L. reuteri* strains compete for glycolipids specificity with *Helicobacter pylori* (Mukai *et al.*, 2002). Although the composition of bacteria in the human GIT may be different from the mouse, similar principle may be involved in the bacterial competition and interactions (Hao and Lee, 2003). All these factors may explain the colonization and adhesion of the *L. acidophilus* ATCC 4356 in the faecal microflora of the colon cancer induced mice model.

However, in the process of metabolism, these indigenous bacteria can convert dietary substances into pre-carcinogens or carcinogens (Gorbach and Goldin, 1990). Studies have demonstrated some specific bacterial strains such as *Lactobacillus* sp., *Bacillus* sp. and *Bifidobacterium* could inhibit toxic faecal enzymes (β -glucuronidase, β -

glucosidase, nitroreductase and urease) capable of causing colon cancer (De Preter *et al.*, 2007; Piscaglia *et al.*, 2010; Zhu *et al.*, 2011). The interaction of lactobacilli with intestinal epithelia reduces the absorption of mutagenic compounds in the lumen thereby inhibits tumour cells growth (Challa *et al.*, 1997). Both MCP and alginates belong to a class of polysaccharides called polyuronides (Eliaz, 2001). The combination of these two polyuronides forms a very strong matrix chain (in MCPA), which effectively attracts heavy metal cations. This synergistic effect prevents the re-adsorption of heavy toxic metals (mutagenic compounds) in the colon. Thus, MCPA probiotic microbead is a more effective anti-mutagenic agent via reduced tumour development in the Balb/c mouse model.

This novel study utilises gal-3 and VEGF markers to determine the reduction of colon carcinogenesis in a mouse model. The administration of MCPA probiotic microbeads to our Balb/c mice model demonstrated a reduced gal-3 and VEGF immunoexpression with lower tumour incidence. The degree of gal-3 immunoexpression is associated with the incidence of tumour development in the Balb/c mice model which corroborates with Liu and Rabinovich (2005) and Demetter *et al.* (2008). Gal-3 plays role of tumour promoter in most cancers and suppressor in other cancers (Song *et al.*, 2014). Gal-3 regulates tumour cell adhesion by direct binding to glycolipids (Krześlak and Lipińska, 2004) or glycoproteins such as laminin (Warfield *et al.*, 1996). The carbohydrate β -galactoside in MCP blocks this interaction by specifically binding to the gal-3 CRD (Maxwell *et al.*, 2012; Morris, 2009).

VEGF is up-regulated in many tumours (Detmar, 2000). MCPA blocks the VEGF and gal-3 markers in endothelial cells of the colon mucosa. The administration of MCP and MCPA probiotic microbeads to the Balb/c mouse model demonstrated a low-absent immunoexpression of VEGF but incidence of ACF/ tumour was lower in MCPA treated mice. The reduced immunoexpression of VEGF demonstrated the inhibition of tumour associated angiogenesis by MCPA in our animal model. This shows the improved bioactivity of MCPA as a regulator of pathological and physiological angiogenesis.

According to this study, MCPA probiotic microbeads presents to be a novel and effective oral delivery of bacterial cells, bioactive compounds and also an adjunct (combined with health beneficial bacteria) to supplementary dietary. MCPA has a good biodegradable ability, is inexpensive, non-toxic, proven efficiency, easy to use and stability at low temperatures warranting its use as a drug carrier by pharmaceuticals. MP combined with alginate could be used as a probiotic carrier to improve the viability and survival of the probiotic microorganisms in functional foods and other gastrointestinal beneficial bacteria for the prevention of intestinal diseases such as colon cancer. The combination of MCP and probiotic improved anti-adhesive, apoptosis-promoting and anti-tumour properties. This increases the efficiency of MCP as a conventional chemotherapy in colon cancer. As the potential and necessity of developing of MCP-based drugs and nutraceuticals is becoming more and more commonly known (Jackson *et al.*, 2007; Nangia-Makker *et al.*, 2007), the addition of MCPA and probiotic to anticancer products or dairy foods holds the promise of improving the treatment of colon cancer in human.

Future recommendations

This study focused on the inhibition of tumour growth in a colon cancer mouse model exposed to a chemical carcinogen, AOM through post-treatment by the novel product MCPA probiotic microbeads. Further research would be required to determine the therapeutic aspect of this product by first developing the colon cancer to an advanced phase in the mouse and subsequently treating the mouse with the MCPA probiotic microbeads. Although, the efficiency of the microbead to maintain the stability and viability of the probiotic bacteria was subjected to both *in vitro* and *in vivo* GIT effect, other molecular techniques should be employed to further investigate its efficacy most especially in food or drug products. Also, gal-3 and VEGF protein biomarkers were recognized to be involved in colon carcinogenesis, other biomarkers and the possible mechanisms of action of MCPA probiotic should be investigated.

CHAPTER 7

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APPENDICES

Appendix 1: Statistical analysis data for preliminary probiotic test

Table A1: Statistical analysis of the viability of free and microencapsulated probiotic in storage

Tukey's multiple comparison test					
	Storage (4°C)				
	MCPA	AP		FREE CELL	
0 day vs 7 days	ns	ns		****	
0 day vs 14 days	ns	ns		****	
0 day vs 21 days	*	****		****	
0 day vs 28 days	**	****		****	
7 day vs 14 days	ns	ns		****	
7 day vs 21 days	ns	***		****	
7 day vs 28 days	**	****		****	
14 day vs 21 days	ns	**		****	
14 day vs 28 days	*	****		****	
21 day vs 28 days	ns	ns		ns	
	0 day	7 days	14 days	21 days	28 days
MCPA vs AP	ns	ns	ns	**	**
MCPA vs FREE CELL	****	ns	****	****	****
AP vs FREE CELL	****	ns	****	****	****

Table A2: Statistical analysis of the viability of free and microencapsulated probiotic during exposure to simulated gastric and intestinal juices

Tukey's multiple comparison test						
	SGJ			SIJ		
	MCPA	AP	FREE CELL	MCPA	AP	FREE CELL
0 min vs 30 min	ns	ns	****	****	****	****
0 min vs 60 min	ns	***	****	****	****	****
0 min vs 120 min	ns	****	****	****	****	****
0 min vs 180 min	*	****	****	****	****	****
30 min vs 60 min	ns	**	ns	****	****	ns
30 min vs 120 min	ns	***	****	****	****	ns
30 min vs 180 min	ns	****	****	****	****	ns
60 min vs 120 min	ns	ns	****	****	****	ns
60 min vs 180 min	ns	ns	****	****	****	ns
120 min vs 180 min	ns	ns	****	ns	ns	ns
SGJ	0 min	30 min	60 min	120 min	180 min	
MCPA vs AP	ns	ns	ns	*	*	
MCPA vs FREE CELL	****	ns	ns	****	****	
AP vs FREE CELL	****	ns	*	****	****	
SIJ	0 min	30 min	60 min	120 min	180 min	
MCPA vs AP	ns	****	****	****	****	
MCPA vs FREE CELL	****	****	****	****	****	
AP vs FREE CELL	****	****	****	****	****	

ns = not significant ($p > 0.05$), * = $p < 0.05$ (significant), ** = $p < 0.01$ (very significant), *** = $p < 0.001$ (extremely significant), **** = $p < 0.0001$ (extremely significant).

Appendix 2: Ethics application for animal study

SURNAME AND INITIALS OF APPLICANT:

Animal Ethics Research (AER) 2013 p1

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UNIVERSITY OF KWAZULU-NATAL ETHICS COMMITTEE ANIMAL ETHICS SUB-COMMITTEE

APPLICATION FOR APPROVAL OF RESEARCH PROTOCOLS USING ANIMALS

Please note that approval must be obtained for ALL work involving animals
irrespective of the source of funding.

This form is to be completed in typescript and one signed, hard copy submitted to Animal Ethics Administration, School of Life Sciences, Rm 105, John Bews Building, Pietermaritzburg Campus AND an electronic copy submitted to animalethics@ukzn.ac.za. Please enter your surname between the marks at the top of each page.

1. TITLE OF PROJECT

Max. 50 characters including spaces

Inhibition of colon cancer in mice by probiotics

2. DETAILS OF APPLICANT

2.1 Title (e.g. Dr): Mr

2.2 Surname: Odun-Ayo

2.3 Full name: Frederick Oluwasheyi

2.4 Qualifications: B. Sc, M.Sc.

2.5 Position: D. Tech Student

2.6 School: Durban University of Technology

2.7 Campus: Steve Biko campus

2.8 Internal mailing address: Dr L Reddy, Dept. of Biotechnology and Food
Technology, Durban University of Technology, P O Box 1334, Durban, 4000.

2.9 Tel ext.: 0787849231, 0833829455

2.10 Fax: 0866870186

2.11 Email: (1) fasytctlogsp@yahoo.com; (2) redDYla@dui.ac.za

3. STAFF, RESEARCH ASSOCIATES, STUDENTS AND TECHNICIANS AUTHORISED TO CARRY OUT THE PROPOSED HANDS-ON ANIMAL STUDIES.

Name (initials and surname)	Academic qualification	Animal training
Dr S.D. Singh	B.V.Sc & AH (Mumbai); MS Animal Sc (Illinois, USA)	Yes
Ms Linda Bester	M.Vet Tech	Yes

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Dr L. Reddy	D. Tech (Biotechnology)	No
Mr J Mellem	M. Tech (Biotechnology)	Yes

4. EXPERIENCE IN WORKING WITH ANIMALS RELEVANT TO THE APPLICATION

I have no prior laboratory work experience in the use of laboratory animals for research purposes. I am fully aware of the ethical and legal considerations relevant to the use of laboratory animals in research and teaching. However, I will be trained and assisted by the senior staff of BRU (especially Dr. S Singh and Ms L Bester) with dissection procedures. The BRU staff will also provide assistance in anaesthesia and euthanasia of the experimental animals.

5. ANIMAL HOUSING FACILITIES WHERE WORK WILL BE CARRIED OUTANIMAL SPECIES

Balb/C mice

HOUSING FACILITY**5.1. University of KwaZulu-Natal Centres**

Biomedical Resource Unit (W)

☒

School of Life Sciences (SLS) (P) Animal House

☐

Ukulinga Research Farm (P)

☐

Other (specify)

☐**5.2 Non-University of KwaZulu-Natal Centres***

PLEASE SPECIFY _____

☐

*N.B. If ALL your work involving animals is performed at a Non-University of KwaZulu-Natal Centre, you need not complete the rest of this form, but you HAVE TO attach a letter of ethical approval obtained from the relevant authority at the Non-University of KwaZulu-Natal Centre.

6. BACKGROUND, OBJECTIVES AND POTENTIAL BENEFITS OF THE PROJECT

Please give a clear and succinct statement of the background, objectives and potential benefits of the project under these three separate headings.

BACKGROUND

World Health Organization, through the cancer research agency, International Agency for Research on Cancer (IARC), reported 608,000 deaths were caused by colorectal cancer in 2008. Cancer is recognized as one of the leading cause of death worldwide (WHO, 2012). Cancer death has been projected to continue rising worldwide, with an estimated 13.1 million deaths in 2030 (WHO, 2012). However, there has been a marked increase in the overall incidence of colon cancer in South Africa, over the past decades (Cronje *et al.*, 2009). Colon cancer has shifted from being the 10th most common cancer diagnosed in males and females in 1989 in South Africa to the 5th most prevalent cancer in 1999, based on the 2004 NCR report which was the last reported by CANSA as at 2012.

The behavioural and dietary risks (low intake of fruits and vegetable) are responsible for about 30% of deaths caused by cancer (WHO, 2012). Attention has been focused on decreasing the risk of cancer, particularly through the consumption of probiotics and increase of dietary fibre intake (Brady *et al.*, 2000). Studies show that the inclusion of probiotics in a diet prevents and reduces the risk of cancer (Sanders, 1999; Sanders *et al.*, 2007; Thantsha

Ref	Date
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et. al., 2012). Chen and Yao (2002) showed the activity of probiotic against certain tumours. A clinical trial was conducted where colonic microflora, *Lactobacillus acidophilus* showed anti-carcinogenic activity in humans (Fooks *et. al.*, 1999). The ability of this colonic microflora to produce short fatty acid chains during fermentation and low levels of some colonic enzymes, such as β -glucuronidase is suggested as the main process that prevents colorectal cancer (Adams and Moss, 2000; Chen and Yao, 2002; Fooks *et. al.*, 1999; Holzapfel and Schillinger, 2002). Prebiotics are non-digestible food ingredients known for their ability to selectively stimulate the growth and/or activity of one or a limited number of microbes in the colon thus promoting colonic health, when sufficient quantity is consumed (Ouweland *et. al.*, 2007).

Research has envisaged the potential role of modified pectin in the reduction and prevention of carcinogenesis. Modified citrus pectin (MCP) is an altered form of pectin (pH and temperature modified) which is a polysaccharide extracted from citrus peel broken down into smaller groups of simple sugar galactose. This effect exhibited by MCP is as a result of the binding ability of a structural element (a neutral sugar chain) in the MCP to the galectin-3 (GAL3), a pro metastatic protein whose expression is up-regulated in many cancers (Maxwell *et. al.*, 2012). There is usually an increase in concentration of free GAL3 circulating in the blood of patients with many types of cancer including colon cancer (Yu, 2010). In several tumour models, a correlation has been established between the level of degree of galectin-3 and the stage of progression of tumour (Liu, 2002; Liu and Rabinovich, 2005). Glinesky and Raz (2009) reported that MCP has emerged as one of the most promising naturally occurring anti-metastatic substance.

The first Good Clinical Practices (GCP) study carried out showed relevant measurable clinical effects of the orally administered natural product MCP in a cancer population of heavily pre-treated patients and the role of dietary components in cancer progression and metastasis as an emerging field of clinical importance (Azemar *et. al.*, 2007; Nangia-Makker *et. al.*, 2002). However, Yan and Katz (2010) reported that Pectasol-C MCP can inhibit cell proliferation and apoptosis in prostate cancer cell line. Furthermore, a study was carried out on a mouse model of Colitis-associated colon cancer and the effect of modified apple pectin (MAP) enhanced apoptosis, decreased inflammation and prevented tumour formation (Li *et. al.*, 2011). Even though, MCP has to be small enough to be absorbed into the body but at the same time, the fragments must not be disrupted for it to exhibit its effect (Maxwell *et. al.*, 2012). More so, Maxwell *et. al.* (2012) states that its optimal form to be consumed needs to be understood for it to generate the required oligosaccharides and ensure that the intended target is reached and it is important to know also if a particular food supplements are needed for MCP to achieve its bioactivity and optimal form as an anti-cancer agent.

Studies have demonstrated the presence aberrant crypt foci several weeks (12-36 weeks) after first exposure of mice to azoxymethane (AOM) to induce colon tumour (Chen and Huang, 2009; Escibano *et. al.*, 2004; Marotta *et. al.*, 2003; Orii *et. al.*, 2003). In a study to establish an optimal dosing regime for detecting experiment-dependent differences in tumourigenesis, A/J, AKR/J and SWR/J strains of mice were used. 5, 10, and 20 mg AOM per kg body weight was given once a week for 2, 4, and 8 weeks both subcutaneously and intraperitoneally. Although subcutaneous and intraperitoneal administration resulted in similar levels of tumour induction, significant dose- and strain-dependent effects of AOM were observed (Bissahoyo *et. al.*, 2005). However, the study showed that four weekly intraperitoneal administrations with 10mg/kg body weight of AOM were optimal to maximize inter-strain differences for multiplicity and penetrance of colon tumours. The study also identified 10 mg/kg AOM as the maximal tolerated dose for tumour induction and A/J strain mice to be highly susceptible to AOM-induced colon tumourigenesis in comparison to AKR/J and SWR/J strains. Bissahoyo *et. al.* (2005) reported that all mice treated with 20 mg/kg of AOM died shortly after the first injection suggesting acute toxicity of AOM at this dose; though no premature losses of mice was recorded for the other dose regimen.

The percentage of tumour-bearing mice (penetrance), tumour multiplicity and diameter were 10-fold higher in the 10 mg/kg treated group than the 5 mg/kg AOM group. Also, three to five months old mice were used as it was reported that mice dosed at younger than three month

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Animal Ethics Research (AER) 2013 p4

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of age displayed a high sporadic incidence of acute toxicity (Bissahoyo *et. al.*, 2005). Suzuki *et. al.* (2006) reported Balb/c mice to be the most sensitive to intraperitoneal administration of AOM (10 mg/kg body weight) induced colon tumourigenesis among the 5 week old mice of four inbred strains (Balb/c, C3H/HeN, C57BL/6N and DBA/2N) with no record of death. However, in a study to determine the preventive effect of polymeric nanocarrier-curcumin (PNCC), Alizadeh *et. al.*, (2012) reported that 15 mg/kg, subcutaneous administration of AOM- induced colon cancer was injected in 40 male Wistar rats for two consecutive weeks and four died as a result of AOM's toxicity two to three weeks after AOM's injection.

Site-specific delivery for a specific target site is necessary using the specific property of that target site (Vaidya, *et. al.*, 2012). Picot and Lacroix (2004) as cited by Burgain *et. al.* (2011) shows that encapsulation is to ensure that the viable cells (encapsulated materials) remain metabolically active while they are released in the intestine (the target site) and also to protect them against adverse conditions. However, this can be enhanced by the prebiotic functionality of resistance starch (Anal and Singh, 2007; Crittenden *et. al.*, 2001; Mortazavian *et. al.*, 2008). Chitosan-alginate microencapsulation is known to have potential for colon targeting (Chavarri *et. al.*, 2010). Degradation of the chitosan and solubilising alginate gel by sequestering of the calcium ions happens by the action of microflora in the colon (Hejazi and Amiji, 2003; Simonoska *et. al.*, 2008).

Therefore, the aim of this study is to produce a modified microencapsulated probiotic to inhibit the growth of tumour in the colon of mice.

OBJECTIVES

To investigate the effect of microencapsulated probiotic on tumour growth in Balb/C mice and to monitor morphological changes using immunohistochemistry and histopathology with light and electron microscopy. Statistical analysis will be carried out using student's t- test and ANOVA.

POTENTIAL BENEFITS

Experimental findings obtained in this study give a better understanding of the role of probiotics and modified citrus pectin in the prevention of carcinogenesis. It is hoped that the specially prepared probiotic will produce good uptake and medicinal value in the animal model. This could then be suggested as a human probiotic with potential anticancer activity.

Ref	Date
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7. DESCRIPTION OF YOUR PLAN OF WORK

An animal model is proposed to test the therapeutic effect of the probiotic in the treatment and prevention of colon cancer. The objective of this study is to determine the optimization of colon carcinogen, azoxymethane (AOM) by conducting a preliminary dose and time response study in the animal model, to investigate the uptake and transport mechanism of the modified microencapsulated probiotic following oral consumption, and its effect to inhibit cancer (angiogenesis and tumour growth) in the colon.

Animal model

In this study, 6 week-old male Balb/C mice will be used for these experiments. They will be humanely euthanized with overdose of halothane by qualified BRU staff, after which the following will be removed/ isolated;

1. The small and large intestine.
2. The abdominal tumour mass.
3. Liver nodules

However, the following will be noted;

1. Evidences of ACF and blood vessel stain (angiogenesis)
2. Characterization of the colon tumours i.e. the tumour number, size and location
3. Presence of liver nodules
4. Histological evaluation

Animal procedures

1. Optimization of colon carcinogen dose-time response in mice

Carcinogen treatment:

Each 100 mg vial of azoxymethane (AOM) will be resuspended in 2 ml phosphate-buffered saline (PBS) and individual 250 µl aliquots will be stored at -80°C until use. A working stock of 1.25 mg/ml AOM will be prepared by diluting individual 250 µl aliquots of AOM into 10 ml of saline (0.9% NaCl). There will be six groups of mice, (n=6 for each group). The mice in each group 1, 2, and 3 will be treated by injecting them intraperitoneally once a week for two (2) consecutive weeks with 10 mg/kg, 15 mg/kg body weight of AOM and saline injection as control respectively, while group 4, 5, and 6 will be injected intraperitoneally, once a week for four (4) consecutive weeks with 10 mg/kg, 15 mg/kg body weight of AOM and saline injection respectively. These groups of mice will be fed normally with diet and water, check their weight and carefully observed for any toxic effects or abnormality throughout the period. One mouse from each group will be euthanased at week 9, week 12, and week 16 consecutively, after the first AOM dose, dissected and isolate the small and large intestine to observe for the presence of aberrant crypt foci (ACF) and primary tumour in the colon. See Figure 1.

2. Optimization of the probiotic uptake by mice

The mice will be divided into three (3) experimental groups (n=3 for each group). The mice will be fed orally, once in a day. The groups 1, 2, and 3, will be fed with 0.2ml of the modified encapsulated probiotic (MEP), encapsulated probiotic (EP) and citrus pectin solution (P) as control, for two weeks. However, faecal samples of the mice from each group will be collected before probiotic ingestion (control samples) and after two week's ingestion of the probiotic (test sample). The faecal samples will be collected in a sterile disposable container and between 2-6h after collection, it will be sent to the laboratory where microbial analysis of

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the faecal bacterial will be done by culturing to determine whether the probiotic strain and the strain(s) isolated from the faeces will be the same or different. See Figure 2.

3. Effect of the encapsulated probiotic on the growth of tumour

This experiment will involve eight (8) groups of mice. The mice in each group (n=5) will be treated with intraperitoneal injection of azoxymethane (AOM) once a week. However, the applicable dose (mg/kg body weight) of AOM, the specific number of period for which the AOM will be injected and the optimum duration of feeding with the probiotic will be determined based on the optimum dose result from the preliminary dose-time response study described above. After the last injection of AOM, the mice in group 1 will be given, once in a day oral administration of 0.2 ml of the modified encapsulated probiotic (MEP), group 2 will be given 0.2 ml of encapsulated probiotic (EP) once daily, group 3 will be given 2% (w/v) of citrus pectin (P) solution, once daily and group 4, the control will take 0.2 ml of distilled water. Also, the same volume of MEP, EP, P and distilled water will be orally given once daily to mice in group 5, 6, 7, and 8 respectively. The mice will be fed with their normal standard chow (diet) and housed separately. The body weight variation of the control and treated mice will be checked on daily basis. At the optimum week for angiogenesis (primary tumour), group 1 – 4 will be euthanased while at the optimum week for tumour growth, group 5 – 8 will be euthanased and isolate the small and large intestine. Aberrant crypt foci will be checked out for and the primary tumour will be removed, fixed with 10% formalin in PBS and processed for immunohistochemical (IHC) staining of blood vessel. However, the abdominal tumour mass, and liver will be removed, while the number of mice with tumour and macroscopic liver nodules will be determined and compared with that of control animals placed on MCP and water. Tumour 1 mm or larger will be counted and measured and their location along the ceecal to rectal axis will be noted. See Figure 3.

4. Immunohistochemical (IHC) studies

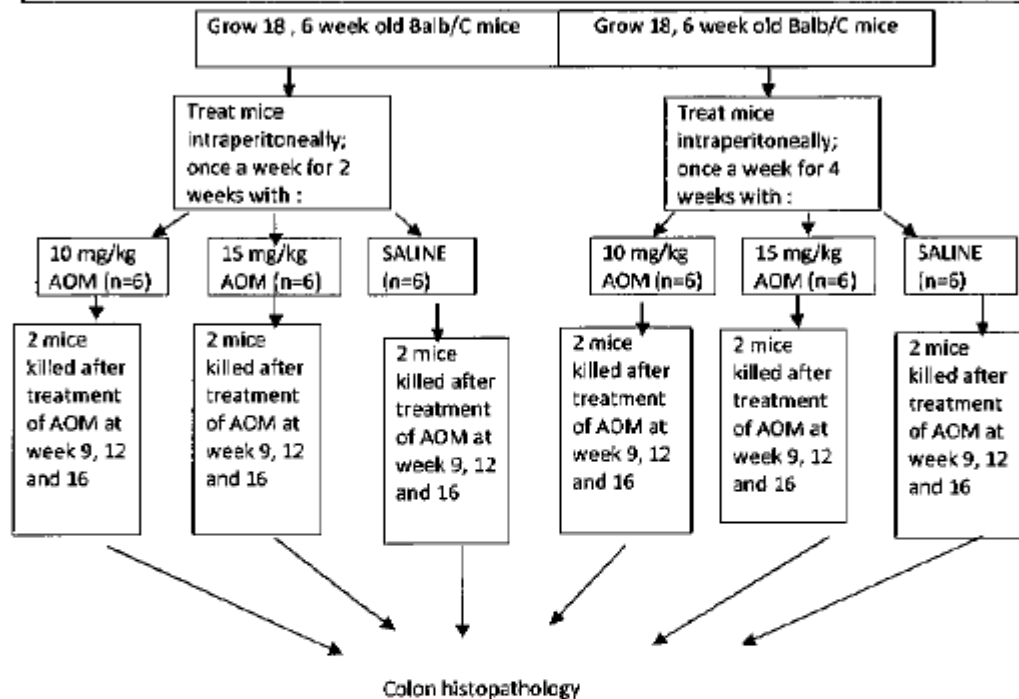
The tumours will be fixed, 10% formalin in PBS, after removal. The alpha smooth muscle actin stains the smooth muscle cells of the vessels. A modification of the avidin-biotin-peroxidase complex technique will be used to perform the immunohistochemistry as described by Nangia-Makker *et al.*, (2002). A thin section of the tissue, 4 µm will be deparaffinised and rehydrated. Thereafter, it will be treated with 3% hydrogen peroxide solution. 0.1% of trypsin and calcium chloride will be used to treat the tissue section for 30 min at 37°C to facilitate the exposure of the antigenic sites, then masked with 3% normal goat serum for 1hr and will be incubated with monoclonal mouse anti-human alpha smooth muscle actin at 1:100 dilution, overnight at 4°C. After 24 h of incubation, biotinylated secondary antibody will be applied on the tissue section for 30 min at room temperature. For another 30 min, avidin-biotinylated horseradish peroxidase (HRP) complex will be added, followed with diaminobenzidine for 1 min and finally, the section will be counter-stained with haematoxylin and a cover slip will be placed on it.

5. Tumour histopathological (HP) study

The distal colonic tissue removed from the caecum to the rectum will be gently flushed with PBS and sprayed open along the longitudinal axis on Whatman 3 MM filter paper. Tumour 1 mm or larger will be counted and measured and their location along the ceecal to rectal axis will be noted. Each colon loosely rolled will be held by spearing using a 30-gauge needle before fixing overnight in 10% formalin, then dehydrated in ethanol and placed in paraffin. 4 µ thick cross-sections will be cut and stained with hematoxylin and eosin to evaluate the tumour histopathology. The grade of histological abnormality will be semi-quantitatively scored using the following five parameters a) nuclear/cytoplasmic ratio (<25%: 0, 25–50%: 1, >50%: 2); (b) epithelial stratification (none: 0; mild: 1, severe: 2); (c) nuclear dipolarity (none: 0, mild: 1, severe: 2); (d) goblet depletion (null to mild: 0, moderate: 1, severe: 2); (e) structural abnormality (none: 0, mild: 1, severe: 2).

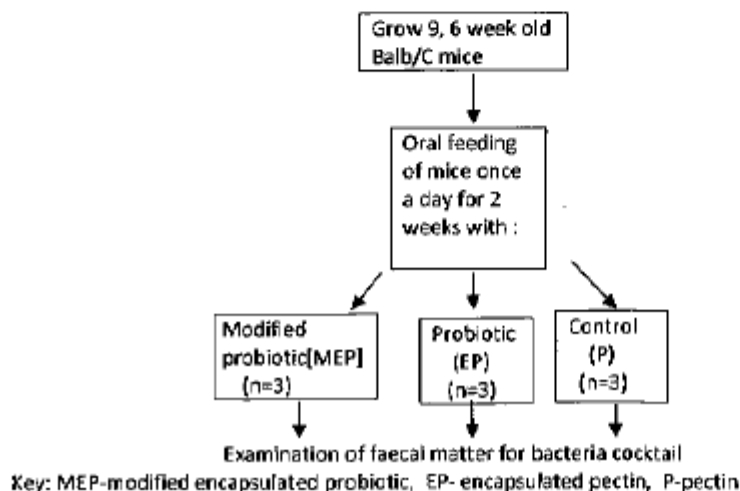
6. Statistical analysis

The result of the angiogenesis and tumour growth will be recorded. Optimization experiments carried out in duplicate and final experiment carried out in triplicate. The student's t test (paired or unpaired) and ANOVA will be used, as appropriate, for the comparison between group treatments and to analyze the statistical significance of the results. A $p < 0.05$ will be considered significant.



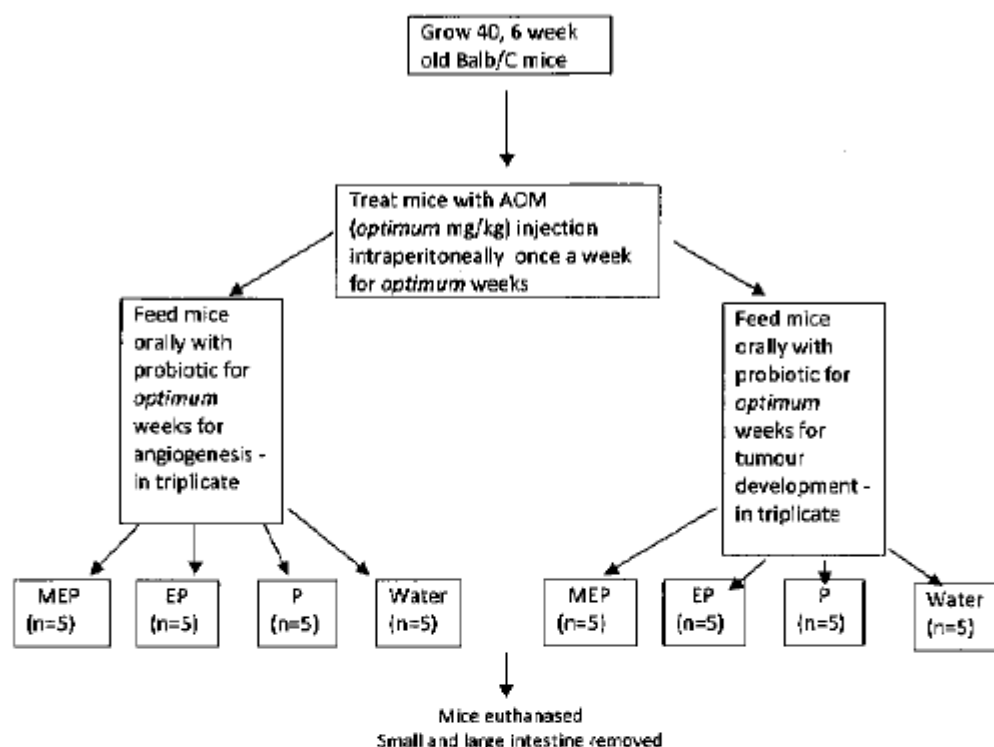
Key: AOM-azoxymethane

Fig 1: Optimization study of colon carcinogenesis in mice using dose and time response



Key: MEP-modified encapsulated probiotic, EP-encapsulated pectin, P-pectin

Fig 2: Optimization study of probiotic uptake by mice



Key: MEP-modified encapsulated probiotic, EP- encapsulated pectin, P-pectin, AOM-azoxymethane

Fig 3. Study design of effect of encapsulated probiotic on colon cancer induced mice

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Ref

Date

8. INDEX OF PROCEDURES

Consult the **Approved Standard Procedures Booklet** (obtainable from your School's representative on the Animal Ethics Sub-committee)

- 8.1 Experimental procedures (other than antibody production-see Table 8.2) included in the Approved Standard Procedures Booklet: Using the *Approved Standard Procedures Booklet*, note by title and code the protocols to be used for each of the experimental procedures other than antibody production in your proposed studies.

Species ¹	Balb/C mice	Balb/C mice	Balb/C mice		
Strain	Balb/C mice	Balb/C mice	Balb/C mice		
Age/weight	6 weeks	6 weeks	6 weeks		
Sex	male	male	male		
Number of animals	n=36	n=9	n=40		
Handling (code)	MH	MH	MH		
Code(s) for procedure(s)	MIIP	MIIG	MIIP&MIIG		
Pain level	moderate	Moderate	moderate		
Euthanasia (code)	MEOD	MEOD	MEOD		
Name of anaesthetic/analgesic	halothane	halothane	halothane		

¹ In the case of amphibians and reptiles, indicate genus and fish genus or other convenient grouping.

- 8.2 Antibody production: Antibody production follows the general format of animal handling, immunisation, bleeding and eventually euthanasia, with each researcher using a number of unique schedules. To expedite review, use the *Approved Standard Procedures Booklet* code numbers and simply indicate the species, route of injection, total number of injections, type of adjuvant, method of bleeding, including volume and frequency, and method of euthanasia.

Species ¹					
Strain					
Age/weight					
Sex					
Number of animals/immunogen					
Handling (code)					
Injection (code)					
Total number of immunisations					
Adjuvant(s)					
Bleeding route (code)					
Blood volume					
Bleeding frequency					
Euthanasia (code)					

<Odun-Ayo F. O.>

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8.2 Other Experimental procedures included in the Approved Standard Procedures Booklet: Using the *Approved Standard Procedures Booklet*, note by title and code the protocols to be used for each of the experimental procedures other than antibody production in your proposed studies.

Species*	Balb/C Mice	Balb/C Mice	Balb/C Mice	Balb/C Mice	
Age/weight	6 weeks old	6 weeks old	6 weeks old	6 weeks old	
Sex	Male	Male	Male	Male	
Number of animals	N=18	N=6	N=24	N=24	
Code	MISQ	MIG	MIG	MISQ	
Procedure	Subcutaneous injection	Intragastric (oral)	Intragastric (oral)	Subcutaneous injection	
Pain level	Moderate	moderate	moderate	moderate	
Euthanasia (code)	MEOD	MEOD	MEOD	MEOD	
In the case of amphibians and reptiles indicate genus and fish genus or other convenient grouping					

8.3 Experimental procedures **NOT** included in the *Approved Standard Procedures Booklet*: Please give details of all procedures using the same format as that in the *Booklet*. Please use additional sheets if necessary. - N/A

9. What is your assessment of the overall pain severity of this project?

Please enter a cross in one box only to indicate your assessment.

- ☐ Substantial
- ☒ Moderate
- ☐ Mild
- ☐ Unclassified

10. DECLARATION BY THE APPLICANT AND HEAD OF SCHOOL

I have considered the feasibility of achieving the purpose of the project by means other than those using animals and, in my opinion, no such alternatives would achieve the objectives of this project. I agree to follow the Approved Standard Protocols and any delineated modifications as approved by the Animal Ethics Sub-committee. I will also supervise and assure compliance and training by my co-workers and students as listed above.

[Signature]
SIGNATURE APPLICANT

12-11-2012
DATE

[Signature]
SIGNATURE SUPERVISOR*

12-11-2012
DATE

[Signature]
INITIALS AND SURNAME OF SUPERVISOR*

12-11-2012
DATE

[Signature]
SIGNATURE HEAD OF SCHOOL

30/11/12
DATE

[Signature]
INITIALS AND SURNAME OF HEAD OF SCHOOL

*Signature of supervisor required if application submitted by research student



UNIVERSITY OF
KWAZULU-NATAL

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YAKWAZULU-NATALI

**Research Office
Animal Ethics Research Committee**

Govan Mbeki Centre, Westville Campus,
University Road, Chiltern Hills, Westville, 3629, South Africa
Telephone 27 (031) 260-2273/35 Fax (031) 260-2384
Email: animalethics@ukzn.ac.za

23 January 2013

Reference: 063/13/Animal

Mr FS Odun-Ayo
Dept of Biotechnology and Food
Technology
Durban University of Technology
P O Box 1334
Durban
4000

Dear Mr Odun-Ayo

Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Ethics Sub-committee of the University Ethics Committee has granted ethical approval for 2013 on the following project:

"Inhibition of colon cancer in mice by probiotics."

Yours sincerely



**Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee**

Cc Registrar – Prof. J Meyerowitz
Research Office – Dr N Singh
Supervisor – Dr L Reddy
BRU – Dr S Singh



Founding Campuses:

- Edgewood
- Howard College
- Medical School
- Pietermaritzburg
- Westville

Table A3: Score sheet and humane endpoint form for mouse model animal study
SCORE SHEET AND HUMANE ENDPOINT FORM FOR AOM STUDY

DATE OF STUDY					ANIMAL NO:		
WEIGHT (g)							
DATE							
DAY							
TIME							
UNDISTURBED OBSERVATION							
Inactive							
Mobility							
Hunched posture							
Grooming							
Alertness							
Presence of a mass							
Ruffled coat							
ON HANDLING							
Not inquisitive & alert							
Not eating							
Food intake (g/day)							
Not drinking							
Body temperature (°C)							
Vocalization on gentle palpation							
Body weight (g)							
Dehydration							
Type of breathing *							
Condition scoring 4 to 1 **							
SPECIFIC CLINICAL SIGNS							
Size of tumour							
Necrosis of tumour							
Bleeding of tumour							
Ulceration							
Bloody stooling							
Nothing Abnormal Detected (NAD)							
OTHER							
SIGNATURE							

Special Husbandry Requirements:

After the tumour becomes visible, the frequency of observation and sizing of the tumour should be increased. They should be maintained under controlled condition of humidity ($50 \pm 10\%$), 12-h light / 12-h dark cycle and constant temperature ($23 \pm 2^\circ\text{C}$). Particular attention should be paid to the growth mice of the tumour.

Scoring Details

* Breathing: R = rapid; S = Shallow; L = laboured; N = normal.

**Condition: 4 = Normal, 1 = emaciated.

Humane Endpoints and Actions:

1. Any animal weighing less than the starting weight after 7 days will be euthanized, or losing more than 20% than start weight at any time will be euthanized.
2. Tumour size of 40cm^3
3. Poor condition.
4. Necrosis and bleeding of the tumour few days after full dose of tumour induction.
5. Inform veterinarian and principal investigator if more than one clinical sign occurs.
6. Any animal showing tiptoe or slow pondering gait should be reported immediately to the veterinarian and the principal investigator.

Scientific Measures:

Tissues to be ISOLATED— the small and large intestine will be fixed with 10% formalin in PBS and processed for immunohistochemical (IHC) and histopathology (HP).

Appendix 3: Statistical analysis of data for Balb/c mouse model

Table A4: Statistical analysis of faecal lactobacilli count in Balb/c mouse model

Tukey's multiple comparison test				
	0 days	7 days	14 days	28 days
AOM + MCPA vs AOM + AP	*	ns	ns	ns
AOM + MCPA vs AOM +MCP	*	ns	*	**
AOM + MCPA vs AOM (control)	**	ns	*	**
AOM + AP vs AOM + MCP	ns	ns	ns	ns
AOM + AP vs AOM (control)	ns	ns	ns	ns
AOM + MCP vs AOM (control)	ns	ns	ns	ns
Normal + MCPA vs Normal + AP	**	ns	ns	ns
Normal + MCPA vs Normal +MCP	**	ns	ns	*
Normal + MCPA vs Normal (control)	*	ns	ns	ns
Normal + AP vs Normal + MCP	ns	ns	*	ns
Normal + AP vs Normal (control)	ns	ns	ns	ns
Normal + MCP vs Normal (control)	ns	ns	ns	ns
AOM + MCPA vs Normal + MCPA	ns	ns	ns	ns
AOM + MCPA vs Normal + AP	*	ns	ns	*
AOM + MCPA vs Normal + MCP	*	ns	**	**
AOM + MCPA vs Normal (control)	ns	ns	ns	*
AOM + AP vs Normal + MCPA	*	ns	ns	ns
AOM + AP vs Normal + AP	ns	ns	ns	ns
AOM + AP vs Normal + MCP	ns	ns	*	ns
AOM + AP vs Normal (control)	ns	ns	ns	ns
AOM + MCP vs Normal + MCPA	*	ns	*	*
AOM + MCP vs Normal + AP	ns	ns	ns	ns
AOM + MCP vs Normal + MCP	ns	ns	ns	ns
AOM + MCP vs Normal (control)	ns	ns	ns	ns
AOM (control) vs Normal (control)	ns	ns	ns	ns

	AOM + MCPA	AOM + AP	AOM + MCP	Normal + MCPA	Normal + AP	Normal + MCP	AOM (control)	Normal (control)
0 day vs 7 days	*	ns	ns	*	ns	ns	ns	ns
0 day vs 14 days	***	*	ns	***	ns	ns	ns	ns
0 day vs 28 days	****	ns	ns	****	ns	ns	ns	ns
7 day vs 14 days	*	*	ns	*	ns	ns	ns	ns
7 day vs 28 days	*	ns	ns	*	ns	ns	ns	ns
14 day vs 28 days	ns	ns	ns	ns	ns	ns	ns	ns

ns = not significant ($p > 0.05$), * = $p < 0.05$ (significant), ** = $p < 0.01$ (very significant), *** = $p < 0.001$ (extremely significant), **** = $p < 0.0001$ (extremely significant).

Appendix 4: Ethic application for extension of animal study

SURNAME AND INITIALS OF APPLICANT:

Animal Ethics Research Renewal (AERR) 2013 p1

<< Odun-Ayo F. O.

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Date

UNIVERSITY OF KWAZULU-NATAL RESEARCH COMMITTEE

ANIMAL ETHICS SUB-COMMITTEE

RENEWAL

OF APPLICATION FOR APPROVAL OF RESEARCH PROTOCOLS USING ANIMALS

Please note that approval must be obtained for ALL work involving animals irrespective of the source of funding.

This form is to be completed in typescript and one signed, hard copy submitted to Animal Ethics Administration, School of Life Sciences, Rm 105, John Bews Building, Pietermaritzburg Campus AND an electronic copy submitted to animalethics@ukzn.ac.za. Please enter your surname between the marks at the top of each page.

1. TITLE(S) OF PROJECT(S)

Inhibition of colon cancer in mice by probiotics

Reference number on last approval letter: 063/13/Animal

2. DETAILS OF APPLICANT

2.1 Title (e.g. Dr): Mr

2.2 Surname: Odun-Ayo

2.3 Full name: Frederick Oluwasheyi

2.4 Qualifications: B. Sc., M. Sc.

2.5 Position: D. Tech

2.6 School: Durban University of Technology

2.7 Campus: Steve Biko campus

2.8 Internal mailing address: Dr John Mellem, Dept. of Biotechnology and Food Technology, Durban University of Technology, P O Box 1334, Durban, 4000

2.9 Tel. Ext.: 031-373 5592 Cell: 0787849231, 0782256215 2.10 Fax: 0866740589

2.11 Email: (1) fasytotogsp@yahoo.com (2) johnm@dut.ac.za.

3. STAFF, RESEARCH ASSOCIATES, STUDENTS AND TECHNICIANS AUTHORISED TO CARRY OUT THE PROPOSED HANDS-ON ANIMAL STUDIES.

Name (initials and surname)	Academic qualification	Animal training
Dr S.D. Singh	B.V.Sc & AH (Mumbai); MS Animal Sc (Illinois, USA)	Yes
Dr Linda Bester	M.Vet Tech	Yes
Dr L. Reddy	D. Tech (Biotechnology)	No

SURNAME AND INITIALS OF APPLICANT:

Animal Ethics Research Renewal (AERR) 2013 p2

<< Odun-Ayo F. O.

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Dr J. Mellem

D. Tech (Biotechnology)

Yes

4. ANIMAL HOUSING FACILITIES WHERE WORK WILL BE CARRIED OUT

HOUSING FACILITY

ANIMAL SPECIES (Balb/C mice)

4.1. University of KwaZulu-Natal Centres

Biomedical Resource Unit (Westville)

☒

School of Life Sciences (SLS) (P) Animal House

☐

Ukulunga Farm (P)

☐

Other (specify)

4.2 Non-University of KwaZulu-Natal Centres*

Please specify

☐

*N.B. If ALL your work involving animals is performed at a Non-University of KwaZulu-Natal Centre, you need not complete the rest of this form, but you HAVE TO attach a letter of ethical approval obtained from the relevant authority at the Non-University of KwaZulu-Natal Centre.

5. CHANGES IN RESEARCH DIRECTION SINCE PREVIOUS APPLICATION

5.1 Are there any changes in the Sections "background, objectives and potential benefits of the project" and "description of workplan" outlined in your previous application?

Yes

No NO

5.2 If your answer in 5.1 is Yes, please indicate briefly what these changes are. Use additional sheets if necessary

Please note that IRRESPECTIVE of your answer in 5.1, you need to complete Sections 6 and 7.

SURNAME AND INITIALS OF APPLICANT:

Animal Ethics Research Renewal (AERR) 2013 p3

<< Odun-Ayo F. O.

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6. BRIEF REPORT ON ANIMAL USAGE AND MAIN FINDINGS

Please provide a brief report on your research activities involving animals in the past year (numbers of animals used, any deviations from planned activities/numbers of animal main findings/progress). Please do not exceed one page).

In this research study, AOM-induced colon cancer animal model was set up to test the therapeutic effect of a special probiotic in the treatment and prevention of colon cancer. However, according to the objectives of this study, a preliminary dose and time response study was conducted determining the optimization of AOM-induced colon cancer in mice (n=36). Based on these preliminary, the optimal dose concentration and time response effect to the colon carcinogen has been established following histopathological and immunohistochemical analysis. Optimization of the microencapsulated probiotic uptake following oral consumption in mice (n=9) was also determined using microbiological analysis of faecal bacterial from the animal. Currently, the effect of the microencapsulated probiotic on colon tumour growth in mice (n=40) will be investigated using histological evaluation and immunochemical analysis. All description of work plan remains intact.

7. INDEX OF PROCEDURES

Consult the Approved Standard Procedures Booklet (Research office website).

7.1 Experimental procedures (other than antibody production-see Table 7.2) included in the Approved Standard Procedures Booklet: Using the *Approved Standard Procedures Booklet*, note by title and code the protocols to be used for each of the experimental procedures.

Species ¹	Balb/C mice				
Age/weight	6 weeks old				
Sex	Male				
Number of animals	N= 40				
Code	MH				
Procedure	MIIP&MIIG				
Pain level	Moderate				
Euthanasia (code)	MEOD				
Name of anaesthetic/ analgesic	Halothane				

¹ In the case of amphibians and reptiles indicate genus and fish genus or other convenient grouping

SURNAME AND INITIALS OF APPLICANT:

Animal Ethics Research Renewal (AERR) 2013 p4

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7.2 Antibody production:

Antibody production follows the general format of animal handling, immunisation, bleeding and eventually euthanasia, with each researcher using a number of unique schedules. To expedite review, use the *Approved Standard Procedures Booklet* code numbers and simply indicate the species, route of injection, total number of injections, type of adjuvant, method of bleeding, including volume and frequency, and method of euthanasia.

Species					
Strain					
Age/weight					
Sex					
Number of animals/immunogen					
Handling (code)					
Injection (code)					
Total number of immunisations					
Adjuvants (CFA, etc.)					
Bleeding route (code)					
Blood volume					
Bleeding frequency					
Euthanasia (code)					

List immunogens to be used:

Will non-physiological, unusually painful, or harmful material be injected? If so, explain and justify (see pain categories).

7.3 Experimental procedures **NOT** included in the *Approved Standard Procedures Booklet*. Please give details of all procedures using the same format as that in the *Booklet*. Please use additional sheets if necessary. **NONE**

SURNAME AND INITIALS OF APPLICANT:

Animal Ethics Research Renewal (AERR) 2013 p5

<< Odun-Ayo F. O.

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8. DECLARATION BY THE APPLICANT AND HEAD OF SCHOOL


I have considered the feasibility of achieving the purpose of the project by means other than those using animals and, in my opinion, no such alternatives would achieve the objectives of this project. I agree to follow the Approved Standard Protocols and any delineated modifications as approved by the Animal Ethics Sub-committee. I will also supervise and assure compliance and training by my co-workers and students as listed above.


SIGNATURE APPLICANT

22-07-2014
DATE


INITIALS AND SURNAME SUPERVISOR

(Please complete - required for mailing copy of approval letter)


SIGNATURE SUPERVISOR*

22/01/14
DATE

S. SINGH
INITIALS AND SURNAME HEAD OF SCHOOL

(Please complete - required for mailing copy of approval letter)


SIGNATURE HEAD OF SCHOOL

22/01/2014
DATE

*Signature of supervisor required if application submitted by research student

FOR ANIMAL ETHICS COMMITTEE USE

REFERENCE NUMBER

AEC REVIEW/APPROVAL DATE

APPROVAL AEC

SIGNATURE

REFERRED BACK TO APPLICANT
FOR REASONS SHOWN

29 January 2014

Reference: 046/14/Animal

Mr F Odun-Ayo
Dept of Biotechnology & Food
Technology
Durban University of Technology
P O Box 1334
DURBAN
4000

Dear Mr Odun-Ayo

RENEWAL: Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Research Ethics Committee has granted ethical approval for **2014** on the following project:

"Inhibition of colon cancer in mice by probiotics."

Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Research Ethics Committee

Cc Registrar – Mr C Baloyi
Research Office – Dr N Singh
Supervisor – Dr L Reddy
Co-Supervisor – Dr J Mellem
BRU – Dr S Singh

Animal Ethics Committee
Professor Theresa HT Coetzer (Chair)
Postal Address: Room 105, John Bews Building, Private Bag X01, Pietermaritzburg, 3201, South Africa
Phone: +27 (0)33 260 5463/35 Facsimile: +27 (0)33 260 5105 Email: animalethics@ukzn.ac.za Website: www.ukzn.ac.za
Learning Campuses: ■ Edgewood ■ Howard College ■ Medical School ■ Pietermaritzburg ■ Westville

INSPIRING GREATNESS



Appendix 5: Statistical analysis of immunoexpression data

Table A5: Immunoexpression analysis of gal-3 and VEGF markers in AOM treated Balb/c mice

	Gal-3 (crypt)			Gal-3 (endothelial)			VEGF		
	9 weeks	12 weeks	16 weeks	9 weeks	12 weeks	16 weeks	9 weeks	12 weeks	16 weeks
10 mg/kg/ 2 weeks	+	-	+	+	+	+	+	++	++
10 mg/kg/ 4 weeks	++	-	++	-	-	+	-	+	++
15 mg/kg/ 2 weeks	+	+	++	+	+	+	+	+	+
15 mg/kg/ 4 weeks	+	+	++	+	+	+	++	++	++
Normal control/ 2 weeks	-	-	-	-	-	-	-	-	-
Normal control/ 4 weeks	-	-	-	+	+	-	+	-	+

0-2 = negative (-), 3-4 = weakly positive (+) and 5-6 = strongly positive (++)

Table A6: Statistical analysis of gal-3 and VEGF immunoexpression in AOM treated Balb/c mice model

Tukey's multiple comparison test										
	Gal-3 (crypt)				Gal-3 (endothelial)				VEGF	
	9 weeks	12 weeks	16 weeks	9 weeks	12 weeks	16 weeks	9 weeks	12 weeks	16 weeks	16 weeks
10 mg/kg/ 2 wks vs. 10 mg/kg/ 4 wks	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
10 mg/kg/ 2 wks vs. 15 mg/kg/ 2 wks	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
10 mg/kg/ 2 wks vs. 15 mg/kg/ 4 wks	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
10 mg/kg/ 2 wks vs. N. control/ 2 wks	ns	ns	*	*	*	*	ns	*	*	*
10 mg/kg/ 4 wks vs. 15 mg/kg/ 2 wks	ns	*	ns	ns	ns	ns	ns	ns	ns	ns
10 mg/kg/ 4 wks vs. 15 mg/kg/ 4 wks	ns	ns	ns	ns	ns	ns	*	ns	ns	ns
10 mg/kg/ 4 wks vs. N. control/ 4 wks	*	ns	ns	ns	ns	ns	ns	ns	ns	ns
15 mg/kg/ 2 wks vs. 15 mg/kg/ 4 wks	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
15 mg/kg/ 2 wks vs. N. control/ 2 wks	ns	**	*	*	ns	*	ns	ns	ns	ns
15 mg/kg/ 4 wks vs. N. control/ 4 wks	ns	*	*	*	ns	*	*	*	*	*

VEGF	10 mg/kg/ 2 wks	10 mg/kg/ 4 wks	15 mg/kg/ 2 wks	15 mg/kg/ 4 wks	Normal controls
9 weeks vs 12 weeks	ns	ns	ns	ns	ns
9 weeks vs 16 weeks	ns	*	ns	ns	ns
12 weeks vs 16 weeks	ns	ns	ns	ns	ns

Gal-3 (crypt)	10 mg/kg/ 2 wks	10 mg/kg/ 4 wks	15 mg/kg/ 2 wks	15 mg/kg/ 4 wks	Normal controls
9 weeks vs 12 weeks	ns	**	ns	ns	ns
9 weeks vs 16 weeks	ns	ns	ns	ns	ns
12 weeks vs 16 weeks	ns	*	ns	ns	ns
Gal-3 (endothelial)	10 mg/kg/ 2 wks	10 mg/kg/ 4 wks	15 mg/kg/ 2 wks	15 mg/kg/ 4 wks	Normal controls
9 weeks vs 12 weeks	ns	ns	ns	ns	ns
9 weeks vs 16 weeks	ns	ns	ns	ns	ns
12 weeks vs 16 weeks	ns	ns	ns	ns	ns

ns = not significant ($p > 0.05$), * = $p < 0.05$ (significant), ** = $p < 0.01$ (very significant), *** = $p < 0.001$ (extremely significant), **** = $p < 0.0001$ (extremely significant).

Table A7: Post treatment Immunoexpression analysis of galectin-3 (gal-3) and vascular endothelial growth factor (VEGF) markers in colon cancer-induced Balb/c mice

	Gal-3 (C)(A+B)	Gal-3 (E) (A+B)	VEGF (A+B)
MCPA	-	-	-
AP	-	+	-
MCP	-	+	-
control	++	++	+

(A) % - percentage of positive cells, (B) SI- Staining intensity: A+B - 0-2 = negative (-), 3-4 = weakly positive (+) and 5-6 = strongly positive (++)

Table A8: Statistical analysis of post treatment immunoexpressions of galectin-3 (gal-3) and vascular endothelial growth factor (VEGF) markers

	MCPA	AP	MCP	Control
Gal-3 (C) vs Gal-3 (E)	ns	ns	ns	ns
Gal-3 (C) vs VEGF	ns	ns	ns	ns
Gal-3 (E) vs VEGF	ns	ns	ns	ns

Tukey's multiple comparison test			
	Gal-3 (C)	Gal-3 (E)	VEGF
MCPA vs AP	ns	ns	ns
MCPA vs MCP	ns	ns	ns
MCPA vs control	****	****	*
AP vs MCP	ns	ns	ns
AP vs control	****	***	**
MCP vs control	****	***	***

ns = not significant ($p > 0.05$), * = $p < 0.05$ (significant), ** = $p < 0.01$ (very significant), *** = $p < 0.001$ (extremely significant), **** = $p < 0.0001$ (extremely significant).