‘Investigations of the health benefits of buttermilk fat globule membrane lipid components’

Ph.D. Thesis

by

Anna Kuchta, M.Sc.

Research supervisors: Dr. Rosaleen Devery[1], Dr. Catherine Stanton[2]

[1]National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland;

[2]Biotechnology Centre, Moorepark Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland.

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DECLARATION

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Signed: _____

Student No.: 56121989

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Dedicated to my baby girl, Sophia

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ABSTRACT

The milk fat globule membrane (MFGM) that surrounds fat globules in milk is a natural source of sphingolipids, phoshpholipids and proteins with defined anticancer properties. Dairy processing operations can affect the structure and composition of MFGM, potentially influencing its anticancer activity. The aim of this project was to determine if anticancer activity can be attributed to sweet and fermented buttermilks which contain fragments of MFGM and examine if different milk processing operations (separation, washing, heating and drying) may influence the antiproliferative activity of resultant buttermilks. After 3 days of incubation sweet pasteurized buttermilk at the concentration of 0.38 mg total solids/ml significantly ($P \leq 0.001$) inhibited growth of SW480 human colon cancer cells by 97.5% but had no toxic effect on FHC human normal colon cells as determined by the acid phosphatase cytotoxicity assay. Antiproliferative activity was lost after spray drying of buttermilk but was retained after heat treatment and freeze and spin drying. In contrast to natural buttermilks, fermented buttermilks inhibited growth to a lesser extent. Analysis of mitochondrial permeability and phoshatidylserine exposure in cells using flow cytometry suggest induction of apoptosis as a biological mechanism involved in the inhibitory effect exerted by buttermilk fractions on cancer cell growth.

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ABBREVIATIONS

ACF	aberrant crypt foci,
ADRP	adipocyte differentiation-related protein,
AIF	apoptosis-inducing factor,
Alk-SMase	alkaline sphingomyelinase,
AP	acid phosphatase,
APC	adenomatous polyposis coli,
APC *(filter)*	allophycocyanin *(filter)*,
apoB	apolipoprotein B,
ATP	adenosine triphosphate,
BNIP3	Bcl-2/E1B and 19K-interacting protein 3,
BRCA1	breast cancer type 1 susceptibility protein,
BRCA2	breast cancer type 2 susceptibility protein,
BSA	bovine serum albumin,
BTN	butyrophilin,
C1PP	ceramide-1-phosphate phosphatase,
CC	buttermilk produced from commercial cream,
CCK	cholecystokinin,
CDase	ceramidase,
cDNA	complementary deoxyribonucleic acid,
CDP-choline	cytidine diphosphocholine,
Cer	ceramide,
CEPT	CDP-ethanolamine:1,2 diacylglycerol ethanolaminephosphotransferase,
CHO	chinese hamster ovary,

CK	ceramide kinase,
CLA	conjugated linoleic acid,
CMC	critical micellar concentration,
CMP	cytidine 5'-monophosphate,
COX-2	cyclooxygenase-2,
cPLA2	cytosolic phospholipase A2,
CRS	cerebrosidase,
CS	ceramide synthase,
CSC	buttermilk produced from centrifugally separated cream,
DAG	1,2-diacylglycerol,
DAGK	diacylglycerol kinase,
DES	dihydroceramide desaturase,
DHA	docosahexaenoic acid,
DMEM	Dulbecco's Modified Eagle's medium,
DNA	deoxyribonucleic acid,
DNase	deoxyribonuclease,
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine,
EAE	experimental allergic encephalomyelitis,
ECACC	European Collection of Cell Cultures,
ECT	CTP:phosphoethanolamine cytidylyltransferase,
EK	ethanolamine kinase,
EndoG	endonuclease G,
EPA	eicosapentaenoic acid,
ER	endoplasmic reticulum,
ERK	extracellular signal-regulated kinase,

FA	fatty acid,
FABP	fatty acid binding protein,
FACE	fast activated cell-based ELISA,
FACS	fluorescence-activated cell sorting,
FAP	familial adenomatous polyposis,
FBS	fetal bovine serum,
FHC	human fetal colon,
FITC	fluorescein isothiocyanate,
G4SC	buttermilk produced from gravitationally separated cream at 4°C,
GAIP	Gα-interacting protein,
Gal	galactose,
GC	gas chromatography,
GC	glucosylceramide,
GC–MS	gas chromatography-mass spectrometry,
GCS	glucosylceramide synthase,
GIT	gastrointestinal tract,
Glc	glucose,
GLUT1	facilitated glucose transporter isoform 1,
GSK	glycogen synthase kinase,
GTPases	guanosine triphosphatase,
H&E	haematoxylin and eosin,
haCER2	human alkaline ceramidase 2,
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid,
HPLC	high-performance liquid chromatography,
IFNγ	interferon-γ,

IP_3	inositol 1,4,5-trisphosphate,
JNK	c-Jun N-terminal kinase,
KSR	kinase suppressor of Ras
L-15	Leibovitz-15,
LAB	lactic acid bacteria,
LC	lactosylceramide,
LC3	light chain 3,
LDL	low-density lipoprotein,
MAPK	mitogen-activated protein kinase,
MDPC	Moorepark Dairy Production Centre,
MEKK	MAP/ERK kinase kinase,
MEM	Modified Eagle's medium,
MFG-E8	lactadherin,
MFGM	milk fat globule membrane,
MDF	mucin-depleted foci,
Min mice	multiple intestinal neoplasia mice,
MMP-7	matrix metalloproteinase-7,
mRNA	messenger ribonucleic acid,
MS	multiple sclerosis,
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt,
MUC1	mucin 1,
MW	molecular weight,
NaBut	sodium butyrate,
NF-κB	nuclear factor κB,

NGF nerve growth factor,

NL neutral lipids,

NMR nuclear magnetic resonance,

OD optical density,

PA phosphatidic acid,

PAF platelet activating factor,

PAP phosphatidic acid phosphohydrolase,

PARP poly(ADP-ribose) polymerase,

PAS6/7 lactadherin,

PBS phosphate buffered saline,

PC phosphatidylcholine,

PDK1 3-phosphoinositide-dependent protein kinase 1,

PE phosphatidylethanolamine

PEMT phosphatidylethanolamine *N*-methyltransferase,

PES phenazine ethosulfate,

PGE_2 prostaglandin E2,

pI isoelectric point,

PI phosphatidylinositol,

$PI(3,4,5)P_3$ phosphatidylinositol 3,4,5-trisphosphate,

$PI(4,5)P_2$ phosphatidylinositol-4,5-bisphosphate,

PI3K phosphatidylinositol-3-kinase,

PKB protein kinase B,

PKCα protein kinase C α,

PL polar lipids,

PLA2 phospholipase A2,

PLC	phospholipase C,
PLD	phospholipase D,
PMSF	phenylmethanesulfonyl fluoride,
PP1	protein phosphatase 1,
PP2A	protein phosphatase 2A,
PS	phosphatidylserine,
PTP	permeability transition pore,
PVDF	polyvinylidene difluoride,
RA	rumenic acid,
Rb (RB)	retinoblastoma protein,
RNAi	ribonucleic acid interference,
RNase	ribonuclease,
ROS	reactive oxygen species,
S1P	sphingosine-1-phosphate,
S1PP	sphingosine-1-phosphate phosphatase,
SB	sodium bicarbonate,
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis,
shRNA	short hairpin ribonucleic acid,
SK	sphingosine kinase,
SK	sphingosine kinase,
SM	sphingomyelin,
SMase	sphingomyelinase,
SMS	sphingomyelin synthase,
SMUF	simulated milk ultrafiltrate,
Sph	sphingosine,

SK1-S1P sphingosine kinase 1-sphingosine-1-phosphate,

TBS tris-buffered saline,

TC sodium taurocholate,

TCF T-cell factor,

TEER transendothelial electrical resistance,

TLC thin layer chromatography,

TMRE tetramethylrhodamine ethyl ester perchlorate,

TNFα tumour necrosis factor α,

TS total solids,

VDAC voltage-dependent anion channel,

VEGF vascular endothelial growth factor,

XO xantine oxidase,

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CHAPTER 1

Milk fat globule membrane – a source of polar lipids for colonic health?

The milk fat globule membrane (MFGM) surrounds fat globules, protects it against lipolysis and disperses the milk fat in the milk plasma. Besides their structural and emulsifying role, *in vivo* and *in vitro* studies have demonstrated that phospholipids and sphingolipids of MFGM possess cancer risk-reducing properties. Several reports attribute its chemopreventive activity to products of sphingomyelin hydrolysis, which affect multiple cellular targets that control cell growth, differentiation and apoptosis. With knowledge on the potential health benefits of MFGM lipids and proteins dairy industries could in the future address their research in developing new functional dairy products enriched in beneficial MFGM components.

1.1. Intestinal tract integrity and role of lipids

Maintenance of gastrointestinal tract (GIT) integrity is vital for assimilation of nutrients and elimination of waste. The small intestine in the human adult is approximately 6 m in length and the large intestine (colon) approximately 1.5 m. A monolayer of epithelial cells specialized for absorption of nutrients, water and electrolytes lines the entire length of the intestine (Johnson, 2001). These cells also constitute a mucosal barrier between the body and the lumen affording protection from potentially hostile microorganisms and toxins. The most distinctive feature of the small intestine is the presence of innumerable villi that extend into the lumen as finger-like projections composed of columnar epithelial cells. Between the villi are pit-like crypts that constitute the proliferative zone containing rapidly dividing, undifferentiated epithelial cells that serve to replace cells that are continuously shed into the gut lumen. In contrast, the mucosa of the colon has no villi and is flat. In both small intestine and colon, cell proliferation is at a maximum near the base of the crypt (Johnson, 2001). As cells divide one daughter cell retains its physical location and continues to divide, whilst the other enters a population of dividing transit cells that migrate upward from the crypt base. Crypt-cell replication is balanced by exfoliation and apoptosis. Growth signals, growth inhibiting signals and death signals under the influence of intricate genetic control systems regulate the balance between cell birth and cell death in the intestinal tract. Disturbances in this fine-regulated interaction can lead to tumour formation in the gastrointestinal tract.

As a cell divides in two, it must make sufficient amounts of additional membranes to accommodate the expansion of its surface area and internal organelles. Generation of new cell membranes in the rapidly turning over mucosal cells of the

human intestinal tract is as fundamentally important as protein synthesis or DNA replication. The lipid components of membranes, principally phospholipids, sphingolipids and sterols such as cholesterol, provide the basic structural and physical properties for the new cells. Mucosal cells of the epithelium synthesize as well as import these lipid molecules to varying degrees to form membranes.

Phosphoglycerides and sphingolipids are distributed asymmetrically within the membrane surface of polarized intestinal cells as well as in the membranes of various intracellular organelles (e.g. endoplasmic reticulum, Golgi, lysosome, mitochondria). For instance, the apical membrane of intestinal epithelial cells is more enriched in sphingolipids than the basolateral surface. Glycosphingolipid levels are higher in epithelial cells than in non-epithelial cells of the small intestine and colon. An analysis of sphingolipid profiles in intestinal cells by chromatographic techniques (TLC, GC, and GC-MS) has revealed that sphingolipid biosynthesis and cellular location are intimately linked to mucosal cell differentiation and maturation (reviewed in Duan & Nilsson, 2009). Glycosphingolipid composition changed radically during foetal and neonatal development of the digestive tract and during cell migration from crypt to villus tip (Bouhours and Glickman, 1976). A high content of glucosylceramide, synthesized by glucosylceramide synthase was observed during cell differentiation; GM3, the most abundant ganglioside synthesised in the intestine is located mainly in the apical membrane of villus cells where activity of CMP-sialic acid:lactosylceramide sialyltransferase, the key enzyme regulating ganglioside synthesis is high compared with crypt cells (Bouhours and Glickman, 1976).

Sphingomyelin (SM) and complex glycosphinglipids tend to be imported into intestinal cells much more slowly than sphingosine, their simple sphingoid base.

Instead, they (i.e. dietary sphingolipids) are metabolised in the gut lumen by ectoenzymes expressed on the luminal surface of enterocytes. These include sphingomyelinases which metabolise sphingomyelin to ceramide and ceramidases which metabolise ceramide to sphingosine. Sphingomyelinases also act on endogenous sphingomyelin occurring in the outer leaflet of absorptive cells. Sphingosine is taken up by enterocytes and converted into sphingosine-1-phosphate (S1P) by sphingosine kinase (SK). S1P may be rapidly recycled back to sphingosine by phosphatases or irreversibly degraded by S1P lyase which converts S1P to palmitic aldehyde and ethanolamine phosphate. The metabolites of sphingomyelin serve as intermediates either in the conversion of sphingoid bases to palmitic acid for incorporation into chylomicrons or in sphingolipid synthesis. Additionally, they may serve as bioactive lipids that initiate signalling pathways. Expression levels of sphingolipid metabolic enzymes in the intestinal tract and intracellular compartmentation of ceramide, sphingosine and S1P determine their access to signalling targets and ultimately their biological effects in the gut.

The last two decades have seen an explosion of interest in the regulatory roles of sphingolipids in fundamental biological processes that are integral to cancer pathogenesis. Although the risk of colon cancer is increased by age and certain inherited genetic mutations, external hazards, such as smoking, industrial pollutants, ultraviolet radiation and a diet rich in saturated fat cannot be excluded as risk factors. The term chemoprevention, a strategy designed to block, reverse or delay carcinogenesis prior to tumour invasion applies not only to pharmacological components but also to nutritional agents (Hawk *et al*., 2004). There is an increasing awareness that diet can influence colon cancer development and progression; hence food science and new product development is focused on health aspects of particular

food components. In recent years, dairy food research has yielded much evidence that redresses the negative image of milk as an undesirable dietary source of saturated fat and cholesterol, which were found to be responsible for coronary heart disease (Kannel *et al*., 1971; Parodi, 2003). While much research has focused on the anti-inflammatory, anti-atherogenic and anti-carcinogenic activities of esterified fatty acids such as rumenic acid (RA) in milk fat globules (Parodi, 2003) there is now substantial evidence that the cancer risk-reduction property resides also in the phospholipids, sphingolipids and proteins of the MFGM that surrounds the lipid triglyceride core, protecting it from enzymatic attack by lipases (Spitsberg, 2005; Dewettinck *et al*., 2008). The MFGM is a rich source of phospholipids, sphingolipids and proteins that are known to affect multiple cellular mechanisms, in particular cell proliferation and apoptosis. This review focuses on the impact of lipid components of the milk fat globule membrane on human health, particularly in relation to colorectal cancer.

1.2. Milk fat globule membrane (MFGM)

Milk has evolved as a complete food produced by mammary epithelial cells of lactating female mammals to sustain immunologically immature neonates through a period of intensive growth and development into maturity**.** Being the product of mammalian genes, the rapidly expanding tools of biotechnology enable an understanding of the variation in milk composition as a function of species, time and physiology**.** Bovine milk contains hundreds of diverse components including proteins, peptides, amino acids, triacylglycerides, diacylglycerides, saturated and polyunsaturated fatty acids, phospholipids, lactose, vitamins and minerals (Fox and McSweeney, 1998). Processing strategies are being developed to fractionate bovine milk into constituent molecular components for structure function studies. Increasing knowledge of the molecular structure and natural function of milk's biomolecules has

potential to inform development of novel foods with distinct characteristics that will deliver health and well being to individual consumers.

As discussed above, lipids are a major structural element for cells. Lipid biosynthetic pathways that form the myriad of lipids found in mammalian milks evolved specifically to support growth and survival of neonates. Lipids are 'old' molecules with established roles in storing energy, building membranes, signalling within and between cells, modifying proteins, forming permeability barriers and protecting cells from reactive oxygen species. The new discipline of systems biology aims to define the cellular lipidome, build a comprehensive picture of lipid metabolic pathway interactions, discover new molecular lipid species and predict how lipids modulate biological functions (Brown and Murphy 2009). The approach has generated a lipid renaissance with potential to bring lipidomics into milk processing in order to determine how milk lipids modulate health and disease in humans (Casado *et al.,* 2009).

Triglycerides comprise 98% of milk lipids (Lopez *et al.,* 2011). Containing over 400 different acylchains, milk lipids are the most mixed triglycerides found in nature serving as a remarkable source of energy, fat-soluble nutrients and bioactive lipids for mammals (German and Dillard, 2006). According to Mather and Keenan (1998), MFGM is a tri-layer consisting of proteins and lipids from the endoplasmic reticulum membrane and from the membrane of the mammary epithelial cells which coats lipid droplets during their secretion. As it is derived from the cell membrane, MFGM is naturally rich in important minor lipids (sphingomyelin, phosphatidylserine, phosphatidylcholine) and glycoproteins that are exclusively found in cell membranes of bovine mammary epithelial cells. MFGM shares the physical,

chemical and functional features of the apical surface membrane from which it is secreted, making it suitable as a vehicle for delivery of lipid components to gastrointestinal cells.

1.2.1. Proteins and lipids of MFGM

Extensive proteomic studies have characterized the protein moiety of bovine MFGM (Affolter *et al.,* 2010; Fong *et al.,* 2007; Fong and Norris, 2009; Reinhardt and Lippolis, 2006). Bovine MFGM mainly consists of xanthine oxidase (155 kDa), butyrophilin (67 kDa), glycoprotein B (48 kDa), mucin MUC1 (200 kDa), the redox enzyme, CD36 (77 kDa), the adhesive glycoprotein, MFG-E8 (PAS-6/7), adipocyte differentiation-related protein (ADRP) and fatty-acid binding protein (13 kDa) (Fox and McSweeney, 1998; Mather and Keenan, 1998; reviewed by Dewettinck *et al.,* 2008). Recently, Fong *et al*. (2007) characterized some new minor proteins in bovine MFGM which have not been reported previously: polymeric Ig receptor protein, apolipoproteins E and A1, 71 kDa heat-shock cognate protein, clusterin, lactoperoxidase, Ig heavy chain and peptidylprolyl isomerase A. Some of these MFGM proteins were found to possess beneficial properties on human health, which are briefly reviewed in table 1.1.

Table 1.1. The major proteins of the MFGM and their health benefits (based on Dewettinck *et al*. 2008 and Riccio 2004).

Component	***MW (kDa)***	***pI***	***Health aspects***	***References***
Fatty acid binding protein (FABP)	13	5-5.5	Cell growth inhibitor Anticancer factor (FABP as selenium carrier) Epidermal-FABP contributes to regulation of EAE (experimental allergic encephalomyelitis, disease induced in laboratory animals that displays clinical characteristics similar to human multiple sclerosis, MS)	Spitsberg *et al*. 1995 Bansal *et al*. 1989 Li *et al*. 2009
Breast cancer type 1 susceptibility protein (BRCA1)			Inhibition of breast cancer	Brody and Biesecker 1998
Breast cancer type 2 susceptibility protein (BRCA2)			Inhibition of breast cancer	Vissac *et al*. 2002; Brody and Biesecker 1998

Xantine oxidase (XO)	146 (300)	7.8	Bactericidal agent	Martin *et al*. 2004; Hancock *et al*. 2002; Clare *et al*. 2008
			Anti-inflammatory	Spitsberg 2005; Fong *et al*. 2007
Mucin 1 (MUC1)	160-200	<4.5	Protective effect against rotavirus infection	Kvistgaard *et al*. 2004; Peterson *et al*. 1998
Butyrophilin (BTN)	66-67	5.32	Suppression of multiple sclerosis (MS)	Mana *et al*. 2004
			Induces or modulates experimental allergic encephalomyelitis (EAE)	Johns and Bernard 1999; Stefferl *et al*. 2000
			Influences pathogenesis of autistic behaviour	Vojdani *et al*. 2002
Lactadherin (PAS6/7, MFG-E8)	47	6-6.6	Protective effect against rotavirus infection	Kvistgaard *et al*. 2004; Peterson *et al*. 1998; Newburg *et al*. 1998
			Fusion with antigen promotes antigen-specific immune responses, resulting in delayed tumor growth	Zeelenberg *et al*. 2008; Liu *et al*. 2005
			Lactadherin promotes vascular endothelial growth factor -dependent neovascularization	Silvestre *et al*. 2005

MFGM contains approximately 0.5-1% of the total lipids in milk, of which the majority are sphingolipids and phospholipids (phosphoglycerides) which show polar properties as they are amphiphilic molecules with hydrophobic fatty acyl chains and a hydrophilic organophosphate (choline, serine, ethanolamine or inositol) head group (Dewettinck *et al.,* 2008). Analysis of lipids is traditionally based on chromatographic techniques such as TLC and HPLC with UV detection and on nuclear magnetic resonance spectroscopy. Owing to overlap of individual lipid classes, these techniques provide limited resolution for analysis of complex lipid mixtures as found in MFGM. Mass spectrometry (MS) methods using electrospray ionization in combination with HPLC separation has been successfully used to confirm the lipid composition profile of bovine MFGM as determined by quantitative capillary GC (Fong *et al.,* 2007). Table 1.2 represents the relative lipid composition of bovine MFGM.

Table 1.2. Relative lipid composition of bovine MFGM expressed as a percentage of total lipid extracted (adapted from Fong *et al.* 2007)

Component	***% w/w***
Triglyceride	56
Diglyceride	2.1
Monoglyceride	0.4
Free Fatty Acids	0.9
Phospholipids	40.6
Phosphatidylcholine	31% (w/w) of phospholipids
Phosphatidylserine	5% (w/w) of phospholipids
Phosphatidylinositol	7.1% (w/w) of phospholipids
Phosphatidylethanolamine	30.5% (w/w) of phospholipids
Sphingomyelin	19.9% (w/w) of phospholipids
Lactosylcerebroside	3.4% (w/w) of phospholipids
Glucosylcerebroside	0.3% (w/w) of phospholipids

The fatty acid composition associated with the MFGM triglyceride was found to contain higher proportions of palmitic and stearic acids and fewer unsaturated fatty acids, such as C14:1, C16:1, C18:1 and conjugated C18:2, when compared with the butter fat triglyceride (Fong *et al.,* 2007). Relative phospholipid abundance of MFGM was phosphatidylcholine > phosphatidylethanolamine > sphingomyelin > phosphatidylinositol > phosphatidylserine > lactosylcerebroside > glucosylcerebroside. In general, C18:1 and/or C18:2 were the predominant fatty acyl chains in phospholipids contributing to MFGM fluidity and membrane protein activity. Saturated sphingoid bases were associated with unsaturated or saturated longer chain fatty acids while unsaturated sphingoid bases were associated with saturated shorter chain fatty acids (Fong *et al.,* 2007). In general, the acyl composition of sphingomyelin differed from that of phosphoglycerides in containing significantly higher proportions of long chain fatty acids with carbon chain length greater than 20, particularly C22:0, C23:0 and C24:0. Conjugated C18:2 was observed in sphingomyelin, but not in the phosphoglyceride fractions of MFGM. Dietary supplementation of cow diet with polyunsaturated fatty acids increased the unsaturated FA content and concentration of phospholipids and sphingomyelin in bovine MFGM (Lopez *et al.,* 2008). More recently, MFGM derived from small milk fat globules contained significantly higher amounts of phosphatidylcholine and sphingomyelin than MFGM from large milk fat globules (Lopez *et al.,* 2011). Confocal laser scanning microscopy and fluorescent probes showed that the bilayer of the MFGM was characterized by distinct rigid liquid-ordered microdomains enriched in sphingomyelin that were laterally segregated from liquid-disordered domains enriched in glycerophospholipids in which proteins and glycosylated molecules were dispersed (Lopez *et al.,* 2011). A comprehensive analysis of the anti-cancer and health

beneficial properties of a wide range of MFGM components and other agents in milk can be found in reviews by Spitsberg (2005) and Parodi (2001, 2003). A wide review of the effects of CLA *in vivo* and *in vitro* was published by Wahle *et al*. (2004). The health benefits of sphingolipids and phosphoglycerides, which are the major components of MFGM are discussed further in the text.

1.2.2. Isolation and purification of MFGM

MFGM in milk enables milk fat to remain dispersed throughout the aqueous phase. Ambient and physiological factors such as quality of milk, its age, temperature, bacteriological content as well as stage of lactation and season can influence MFGM structure and composition (Fox and McSweeney, 1998). Dairy processing operations, such as homogenization, heating or drying as well as milk handling and transportation may also affect release of MFGM. Buttermilk, a by-product of butter manufacture is an ideal source of bovine MFGM material for compositional, structural and functional studies **(**Astaire *et al.,* 2003; Corredig *et al.,* 2003; Dewettinck *et al.,* 2008; Jinjarak *et al.,* 2006; Morin *et al.,* 2006, 2007a, 2007b; Rombaut and Dewettinck, 2006; Rombaut *et al*., 2006, Snow *et al.,* 2010). Not only was it a traditional beverage for the Irish population up to the middle of 20th century it was also used for the care of patients with advanced cancer (Grant, 1969)

Traditionally, whole milk was left to sit to allow the cream to separate from skimmed milk. Nowadays separation is quickened by mechanical centrifugation which may cause some damage to MFGM. In traditional buttermilk wild strains of lactic acid bacteria (LAB) acquired from the environment fermented the milk. It lowered the pH of milk and gave a valuable product rich in lactic acid, a by-product naturally produced by LAB while fermenting lactose. In the next step of buttermaking

the cream is washed repeatedly 3-6 times with water. This can cause some damage to the outer layers and also loss of small molecules (Fox and McSweeney, 1998). During churning, as a result of mechanical treatment, the MFGM material is shed and released into the aqueous phase, which is buttermilk. The lipid droplets are then released from the stabilizing polar lipid envelope and aggregate into the fat grains. The cream is split into two phases: a water-in-oil phase and an oil-in-water phase, called buttermilk. In traditional churning, the buttermilk is drained off when fat grains reach a certain size, but in modern buttermaking machines, buttermilk drainage is continuous (Fox and McSweeney, 1998). The bacterial souring of buttermilk makes it even more valuable and "gut-friendly" product, and also tolerable by lactose-intolerant people.

Relative to other dairy products (butter, cheddar cheese, cream) buttermilk has a high polar lipid content (2.03g / 100g dry matter and 33.05g / 100g total lipids) and can be concentrated by removal of water by filtration, evaporation or drying (Rombaut and Dewettinck, 2006). Strategies for purification of polar lipids employ a combination of coagulation, filtration, centrifugation to remove lactose, whey proteins and minerals. A phospholipid-rich retentate fraction was achieved upon cross flow membrane filtration after casein membrane retention was reduced by acid and rennet coagulation (Sachdeva and Buchhein, 1997). A combination of filtration and supercritical CO_2 removed neutral lipids present in microfiltered retentate and yielded a purified polar lipid product that contained 83.15g on a total lipid basis (Astaire *et al.,* 2003). The properties, analysis and purification of milk polar lipids are reviewed extensively (Dewettinck *et al.,* 2008; Rombaut and Dewettinck, 2006). It is apparant that standardisation and optimisation of processes for purification of polar lipids

would improve the quality of MFGM for investigations of its potential health benefits in the colon.

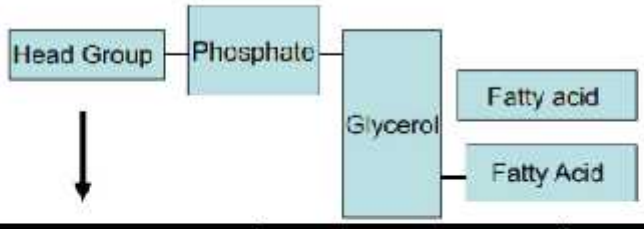
Potential roles for MFGM in the gastrointestinal tract of humans include enhancing binding ability of probiotic bacteria to intestinal cells, inhibition of viral infectivity (Jimenez-Flores and Brisson, 2008) and maintenance of colon health. Recently, MFGM was shown to confer protection against colon carcinogenesis in an animal model of experimental carcinogenesis. Rats fed a diet containing MFGM had significantly fewer aberrant crypt foci compared to rats fed corn oil or anhydrous milkfat (Snow *et al.,* 2010). Sphingomyelin, which accounts for 19% of bovine MFGM polar lipids, provides specific properties to the colon that need to be further investigated. Although the structure-function relationship of individual sphingomyelin species is not known, it has been shown that ceramides containing specific fatty acids are involved in cell death and inhibit cell growth. C16 and C24 ceramide species regulate apoptosis (Osawa *et al*., 2005; Seumois *et al.,* 2007) whereas C18 ceramide inhibits cancer cell growth (Koybasi *et al.,* 2004) Thus, lipidomics approaches to quantification of acyl composition of sphingmyelin will be of great importance in delineating the precise role of bovine MFGM sphingolipids on colon health.

1.3. Profile of polar lipids present in gastrointestinal tract

1.3.1 Phosphoglycerides

Phosphoglycerides (also called glycerophospholipids) are derivatives of *sn*-glycerol 3-phosphate containing two esterified fatty acyl chains and a polar head group esterified to the phosphate (**Fig. 1. 1**). The fatty acids can vary in length and be saturated (no double bonds) or unsaturated (one, two or three double bonds). In

phosphatidylcholine (PC) the head group is choline. **Fig. 1.1** shows the molecules attached to the phosphate group in three other common phosphoglycerides: phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol.



Water	H	Phosphatidic acid
Choline	$CH_2CH_2N^-(CH_3)_3$	Phosphatidylcholine
Serine	$CH_2CH\ (N^+H_3)COO^-$	Phosphatidylserine
Ethanolamine	$CH_2CH_2N^-H_2$	Phosphatidylethanolamine
Inositol	$C_6H_{12}O_5$	Phosphatidylinositol

Fig. 1.1. General structure and major classes of phosphoglycerides.

Lipids, lipid kinases and lipid phosphatases play as important role as proteins in activating and mediating signal transduction pathways in cells (reviewed by Eyster, 2007). When cell-surface receptors are stimulated by extracellular signals, a number of bioactive lipid mediators (e.g. 1,2-diacylglycerol (DAG), phosphatidylinositol-4,5-bisphosphate, phosphatidic acid (PA), lysophospholipids and arachidonic acid) are generated through the action of phospholipases C, D and A2 on specific cellular phospholipids (**Fig. 1.2**). These second messenger products have been shown to function as mediators of numerous cell responses including proliferation, differentiation, migration and apoptosis resistance. Lysophospholipids are products of phospholipase cleavage of phospholipids. Although present in biological membranes in extremely low levels (less than 1% of all phospholipids), they are nevertheless

considered potent endogenous growth factors causing various biological effects, primarily cell proliferation (Rivera and Chun, 2008).

Polyphosphoinositides	PLC	DAG
Phosphatidylcholine	PLD	PA
Phosphatidylcholine	PLA_2	Arachidonic acid + Lysophospholipid

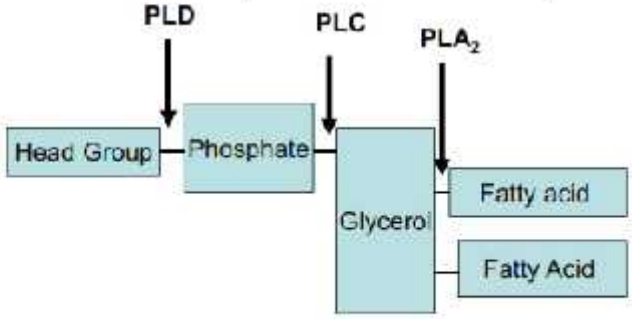


Fig. 1.2. Cleavage products of the action of phospholipases on polyphosphoinositides and phosphatidylcholine. Abbreviations: DAG, diacylglycerol kinase; PA, phosphatidic acid; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D

1.3.1.1. Phosphatidylcholine

Phosphatidylcholine is the most prominent phospholipid in both healthy and colorectal cancer cells (reviewed extensively by Cui and Houweling, 2002) and its biosynthesis is greatly increased in colon cancer tissues, primarily due to decreased activities of phospholipase C and D and enhanced activity of CTP:phosphocholine cytidylyltransferase activity (Dueck *et al*., 1996).

Phosphatidylcholine is synthesized in the intestine by the cytidine diphosphocholine (CDP-choline) pathway. Preformed choline is phosphorylated with ATP by a choline kinase in the cytoplasm of the cell to phosphocholine, which reacts

with cytidine triphosphate to form CDP-choline and pyrophosphate. CDP-choline:1,2-diacylglycerol cholinephosphotransferase, a membrane-bound enzyme in the endoplasmic reticulum catalyses the transfer of phosphocholine moiety of CDP-choline to *sn*-1,2-diacylglycerol to form phosphatidylcholine (**Fig. 1.3**). The CDP-choline to phosphatidylcholine pathway occurs in all cells containing a nucleus; an alternative pathway called the phosphatidylethanolamine methylation pathway occurs only in the liver and accounts for 20-30% of phosphatidylcholine synthesised there. This reaction involves transfer of three methyl groups to the nitrogen of phosphatidylethanolamine by S-adenosyl methionine.

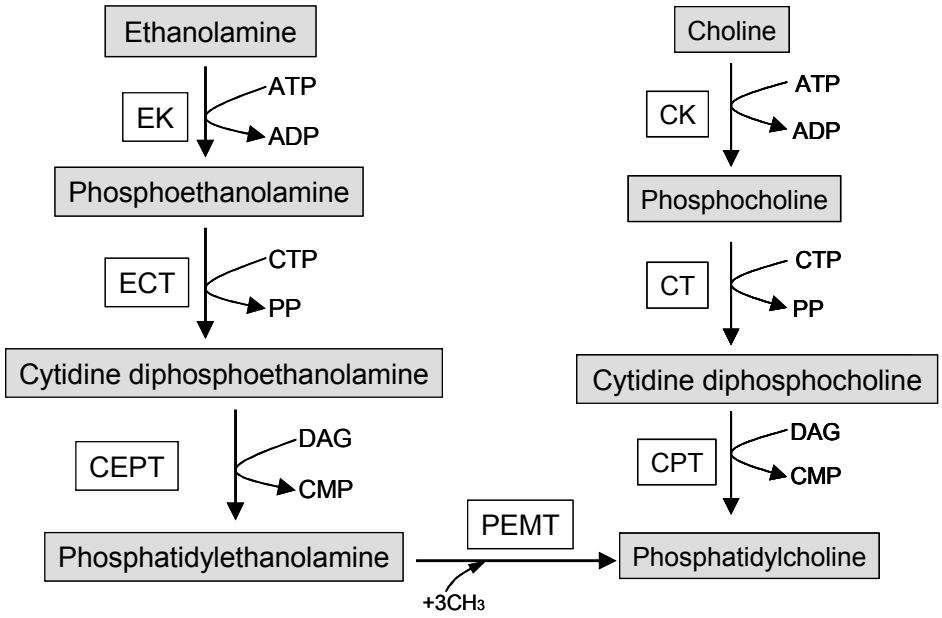


Fig.1.3. Pathways for the synthesis of phosphatidylcholine; it can be formed either from choline (in the intestine) or from ethanolamine followed by methylation of phosphatidylethanolamine (in the liver). Abbreviations: CEPT; CDP-ethanolamine:1,2 diacylglycerol ethanolaminephosphotransferase; CK, choline kinase; CPT, cholinephosphotransferase; CT, CTP:phosphocholine cytidylyltransferase; DAG, diacylglycerol; ECT, CTP:phosphoethanolamine

cytidylyltransferase; EK, ethanolamine kinase; PEMT, phosphatidylethanolamine *N*-methyltransferase.

Increased expression of biosynthetic enzymes provides a pool of phosphatidylcholine for enhanced membrane synthesis that is necessary for rapid growth during tumour development. Microarray transcription profiling revealed overexpression of lysophosphatidylcholine acyltransferase 1, the enzyme that converts lysophosphatidylcholine into phosphatidylcholine in colorectal adenocarcinomas compared to normal mucosas (Mansilla *et al*., 2009).

Phosphatidylcholine is not only a major structural component of cell membranes but is also, along with sphingomyelin, a reservoir of lipid secondary messengers, diacylglycerol and ceramide. Yen *et al*. (1999) reported that deficiency of choline diminished membrane phosphatidylcholine and sphingomyelin levels in PC12 neuronal cells and that this was associated with accumulation of ceramide and diacylglycerol, activation of caspases and induction of apoptosis. Although choline deficiency in humans was associated with increased DNA damage and apoptosis in lymphocytes compared with choline-sufficient humans (da Costa *et al*., 2006), a recent epidemiological study involving 47,302 men showed that dietary intake of choline was not associated with increased colorectal cancer risk (Lee *et al*., 2010).

Nuclear magnetic resonance (NMR)-based metabolomic studies of human tumours identified elevated levels of phosphocholine relative to normal tissues (Yalcin *et al.,* 2010). Phosphocholine is a precursor metabolite synthesized by choline kinase, which provides a ready supply of phosphatidic acid necessary for stimulating cancer survival signalling pathways. Increased levels of choline kinase activity and choline kinase mRNA levels have been found in rat 1,2-dimethylhydrazine-induced

colon cancer and in human colon cancer (Nakagami *et al.,* 1999a and 1999b). Chemical inhibitors that target choline kinase activity and genetic approaches that silence choline kinase by generation of an shRNA against the alpha isoform of choline kinase have proven to be effective anti-tumoural strategies *in vitro* and *in vivo* (Rodriguez-Gonzalez *et al.,* 2004; Sanchez-Martin *et al.,* 2005; Yalcin *et al.,* 2010). The specific depletion of the choline kinase alpha isoform induced apoptosis in several tumour-derived cell lines from breast, bladder, lung and cervix carcinoma tumours, while the viability of normal primary cells was not affected (Banez-Coronel *et al*., 2008).

Signalling by phospholipids is also dependent on fatty acid composition. The major fatty acid incorporated into phosphatidylcholine is arachidonic acid, which is a precursor for prostaglandin formation through activation of COX-2, which leads to cell proliferation and inflammation. The effects of marine phosphatidylcholine and phosphatidylserine have been studied by Hossain *et al*. (2006). They found that phosphatidylcholine and phosphatidylserine highly enriched in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) increased the cytotoxic and pro-apoptotic effect of sodium butyrate on Caco-2 cells.

1.3.1.2. Phosphatidylinositol

Other major membrane phospholipids that cause cell proliferation are phosphatidylinositol and phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$), its phosphorylated derivative. The enzyme phosphatidylinositol-3-kinase (PI3K), when recruited to the membrane by activated receptor tyrosine kinases, generates phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$) (**Fig. 1.4**). The 3-phosphate

added by this enzyme is a binding site for various signal transduction proteins, in particular protein kinase B (PKB) also known as Akt.

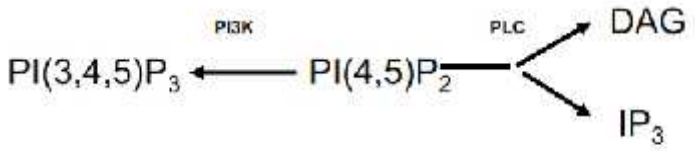


Fig. 1.4. Phosphorylation of PI (4,5)P_2 by PI3K and cleavage products from the action of PLC on PI(4,5)P_2: DAG and IP_3. Abbreviations: DAG, diacylglycerol; IP_3, inositol trisphosphate; PI(3,4,5)P_3, phosphatidylinositol-3,4,5-trisphosphate; PI3K, phosphatidylinositol-3-kinase; PI(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C.

In resting cells, the level of PI-3-phosphates is low. Hormone stimulation results in elevated PI-3-phosphate levels, PKB binding and localisation to the cell surface membrane. Full activation of PKB requires two further site phosphorylations by phosphoinositide-dependent protein kinase 1 (PDK1). Once fully activated, PKB can dissociate from the plasma membrane and phosphorylate pro-apoptotic proteins, such as Bad, thereby preventing activation of an apoptotic pathway leading to cell death. Data accumulated in the last decade have established that this pathway plays a key role in cancer development, progression and resistance to chemotherapy. Therefore, drugs designed to specifically target this pathway are under development to be used as a single agent and in combination to chemotherapy to overcome therapeutic resistance.

1.3.2. Sphingolipids

Sphingolipids represent a broad class of lipids naturally occurring in most cellular membranes. They consist of a sphingoid base, a fatty acid tail and a headgroup (Merrill *et al*., 1993; Merrill *et al*., 1997; Huwiler *et al*., 2000). The sphingoid base on its own, sphingosine ((2S,3R,4E)-2-amino-1,3-dihydroxy-4-octadecene) occurs in most mammalian cells while the most common sphingoid base in yeasts and plants is phytosphingosine. **Fig. 1.5** presents the chemical structure of the most common sphingolipids, of which sphingomyelin, glucosylceramide and lactosylceramide are found in dairy products.

Variations in the type, number and linkage of sugar residues in the oligosaccharide chain give rise to a wide range of naturally occurring glycosphingolipids. Also the lipid moiety can vary in terms of chain length of the fatty acid and in the degree of saturation and hydroxylation of the sphingoid base. Glycosphingolipids form a cell-type specific pattern, which changes with cell growth, differentiation, viral transformation, ontogenesis and oncogenesis (Van Slambrouck and Steelant, 2007; Hakomori, 2003).

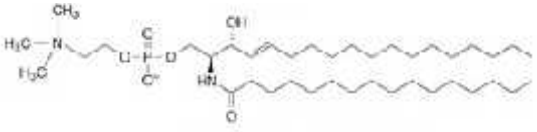
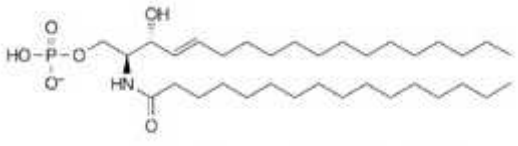
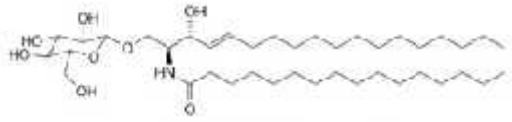
Sphingolipid	*Structure*
D-*erythro*-C18-sphingosine	
D-*erythro*-C16-ceramide	
D-*erythro*-C16-sphingomyelin	
D-*erythro*-C16-ceramide-1-phosphate	
D-*erythro*-C16-sphingosine-1-phosphate	
D-glucosyl-β1-11'-C16-ceramide	

Fig. 1.5. Chemical structure of sphingolipids; sketched with C16 N-acyl chain, commonly found in nature.

Sphingomyelin and glycosphingolipids undergo hydrolysis via the action of mucosal alkaline sphingomyelinase and neutral ceramidase in the epithelial cells of the intestine to form bioactive lipids, such as ceramide, sphingosine, ceramide-1-phosphate or sphingosine-1-phosphate that mediate many signal transduction events necessary for mammalian development. Their importance is evident in the sphingolipidoses, a group of inherited diseases associated with high mortality caused

by defects in lysosomal sphingolipid degradation. Niemann-Pick disease is characterised by defective acid sphingomyelinase activity and excessive storage of sphingomyelin (reviewed in Kolter and Sandhoff, 2006). A defect in acid ceramidase and excessive storage of ceramide leads to Farber disease while Gaucher disease is caused by defect in β-glucocerebrosidase and excessive storage of glucosylceramide (reviewed by Huwiler *et al*., 2000). Both diseases are diagnosed in infants. Symptoms of Farber disease may include moderately impaired mental ability and problems with swallowing. Other symptoms may include vomiting, arthritis, swollen lymph nodes, swollen joints, joint contractures (chronic shortening of muscles or tendons around joints), hoarseness and xanthomas which thicken around joints as the disease progresses. Most children with Farber disease die by age 2, usually from lung disease. Gaucher disease is characterised by enlarged liver and spleen, osteoporosis, yellowish-brown skin pigmentation and the rapid and premature destruction of blood cells, leading to anaemia, neutropenia and thrombocytopenia.

Sphingomyelin hydrolysis starts with the action of sphingomyelinase on phosphorylcholine headgroup (Spence, 1993) and formation of ceramide (**Fig. 1.6**), which is either cleaved into sphingosine and a long-chain fatty acid by ceramidase (Hassler and Bell, 1993) or phosphorylated to ceramide-1-phosphate by ceramide kinase (Sugiura *et al*., 2002). Sphingosine is then phosphorylated at the hydroxyl group by a sphingosine kinase to form sphingosine-1-phosphate. Ceramide can be also glucosylated to form glucocerebrosides, such as glucosylceramide, lactosylceramide and more complex gangliosides. Numerous studies have produced experimental data as proof of their anti-cancer (ceramide, sphingosine), pro-cancer (sphingosine-1-phosphate, ceramide-1-phosphate) or other properties which are described in the following sub-sections. A delicate balance exists between these

metabolites in human cells, the alterations of which may influence cell growth and the process of carcinogenesis.

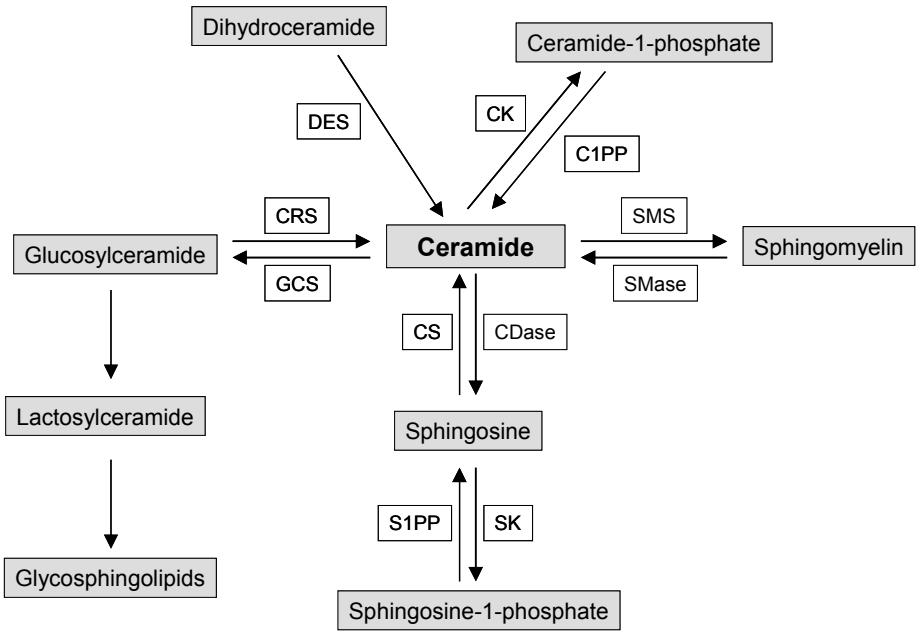


Fig. 1.6. Pathways and products of ceramide metabolism. Abbreviations: C1PP, ceramide-1-phosphate phosphatase; CDase, ceramidase; CRS, cerebrosidase; CK, ceramide kinase; CS, ceramide synthase; DES, dihydroceramide desaturase; GCS, glucosylceramide synthase; S1PP, sphingosine-1-phosphate phosphatase; SMS, sphingomyelin synthase; SMase, sphingomyelinase; SK, sphingosine kinase.

1.3.2.1 Ceramide

Ceramide is composed of an N-acylated (14 to 26 carbons) sphingosine (18 carbons). A *trans* double bond across C4 and C5 of the sphingosine backbone is important for its biological activity, such that dihydroceramide, which lacks this double bond, is mainly biologically inactive (Ogretmen and Hannun, 2004). Ceramide

was first isolated from bovine milk in the free form in 1972 by Fujino and Fujishima. Sixteen component fatty acids were found, among which C23:0 (38.1%), C24:0 (29.5%), C22:0 (17.9%) and C16:0 (7.2%) were predominant. Seven component long-chain bases were detected. The principal bases were C18-sphingosine (35.0%) and C16-sphingosine (31.6%) (Fujino and Fujishima, 1972). Recently, Fong *et al*. (2007) confirmed that in bovine milk the sphingoid base consisted predominantly of C16:1, C17:1 and C18:1 fatty acids.

Ceramide is produced in response to a diverse range of stress stimuli including toxic chemotherapeutic drug treatments, growth factor withdrawal, radiation, hypoxia, hyperthermia and ischemia. Several direct targets of ceramide have been identified *in vitro* and *in vivo* which tie ceramide to regulation of apoptosis, cell cycle arrest and inhibition of proliferation (reviewed by Duan and Nilsson, 2009, Hannun and Obeid, 2008) (**Fig. 1.7**). They include KSR, cRAF, MEKK, cathepsin D and serine/threonine protein phosphatases PP1 (protein phosphatase 1) and PP2A (protein phosphatase 2A) (Wolff *et al*., 1994).

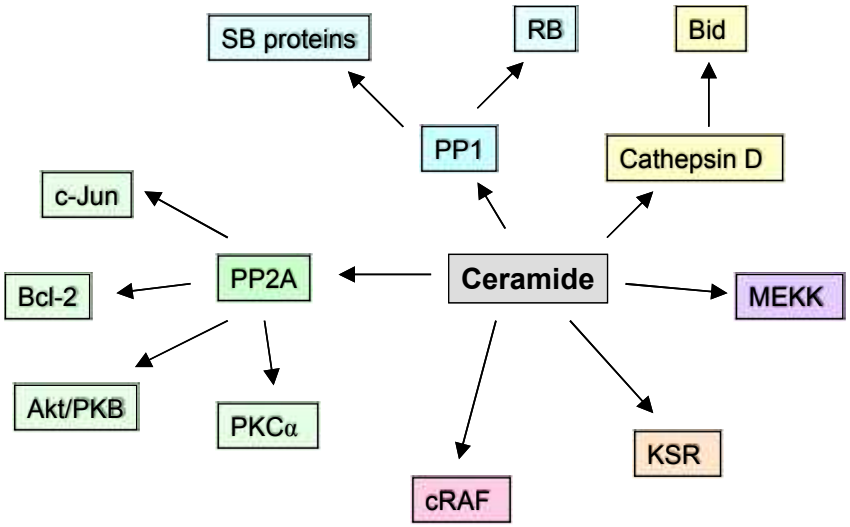


Fig. 1.7. Ceramide-regulated targets. Abbreviations: KSR, kinase suppressor of Ras; MEKK, MAP/ERK kinase kinase; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; RB, retinoblastoma protein.

It is through the protein phosphatases that ceramide can induce apoptosis and inhibit growth. Ceramide-activated protein phosphatase substrates include c-Jun, Bcl-2, Akt/PKB, Rb, PKCα, and SR proteins (Pettus *et al.,* 2002). Reyes and co-workers (1996) showed that treatment with exogenous ceramide or TNFα, a ceramide-generating agonist, led to time-dependent dephosphorylation of c-Jun in A431 cells. Deng and co-workers (2009) reported increased mitochondrial PP2A activity and specific dephosphorylation of mitochondrial Bcl-2 in response to ceramide. PP2A dephosphorylated Bcl-2 leading to inactivation of its anti-apoptotic function (Ruvolo *et al*., 1999; 2002) and it activated pro-apoptotic protein Bad (Chiang *et al*., 2001). Several studies have demonstrated the ability of ceramide to induce dephosphorylation of Akt/PKB with concomitant loss of function. Pre-treatment with okadaic acid, a phosphatase inhibitor blocked the effect, demonstrating a role for PP2A in regulating this activity. Lee and co-workers (2000) reported that PKCα, a Bcl-2 kinase was dephosphorylated in response to exogenous ceramides in cells. Based on inhibitor studies with fuminosin B_1, PP2A was shown to be responsible for the dephosphorylation of PKCα.

PP1 is also a ceramide-activated protein phosphatase enzyme. The dephosphorylation of retinoblastoma protein (Rb) by PP1 following ceramide treatment, which resulted in growth arrest was not affected by okadaic acid at concentrations that specifically inhibit PP2A (reviewed by Pettus *et al.,* 2002; Chao *et al*., 1992). Inhibition of PP1 with phosphatidic acid blocked ceramide-induced dephosphorylation of Rb and inhibited PARP cleavage, thus implicating PP1 in both cell cycle arrest and apoptotic processes, respectively (Kishikawa, 1999).

The dephosphorylation of SR proteins (SRp70, SRp55, SRp40, and SRp30) provides further evidence of the importance of ceramide activation of PP1 in apoptosis. SR proteins owe their nomenclature to the presence of sequences of consecutive serine (S) and arginine (R) dipeptides. They are required for splicing pre-mRNA prior to translation. Dephosphorylation of SR proteins by PP1 promoted the generation of the pro-apoptotic splice variants (i.e. the dominant negative Bcl-X_S and caspase-9) at the expense of the anti-apoptotic splice variants (i.e. Bcl-X_L and caspase-9b) (Chalfant *et al.,* 2002).

The role of ceramide in apoptosis is best illustrated in the case of TNF-induced apoptosis. TNF induces activation of neutral sphingomyelinase and ceramide formation. Ceramide activation of PP2A resulted in dephosphorylation of Bcl-2 as well as the Bcl-2 kinase PKCα in mitochondria (Ruvolo *et al*., 1999; Lee *et al.,* 2000). The net effect was inhibition of the anti-apoptotic actions of Bcl-2. Since Bcl-2 and Bcl-X inhibit mitochondrial cytochrome c release, their dephosphorylation may allow cytochrome c release and downstream caspase-9 activation (Sawada *et al*., 2000).

As mentioned above, ceramide-induced apoptosis, cell cycle arrest and inhibition of proliferation also involve direct activation of protein kinases. These include kinase suppressor of Ras (KSR), cRAF and MEKK, a protein kinase that phosphorylates one or more of the MEK enzymes on Ser or Thr.

KSR is a scaffold protein that regulates the specificity, efficiency and amplitude of the MAP kinase (also referred to as extracellular signal-regulated protein kinase or ERK) signalling cascade. KSR can mediate proliferative responses except when the pro-apoptotic Bcl-2 family member, BAD, is present. In ceramide-treated cells which express dephosphorylated Bad, KSR is involved in converting the

normally proliferative mitogen-activated protein kinase (MAPK) cascade into a pro-apoptotic signalling pathway (Basu *et al.,* 1998)

Cathepsin D is an important target for ceramide generated endosomally by acid sphingomyelinase. The interaction of ceramide with cathepsin D was shown to induce the autocatalytic proteolysis of the 52-kDa pro-enzyme to form the active 48/32-kDa form of cathepsin D. Several studies have shown that activation of the pro-apoptotic protein Bid, cytochrome c release and caspase-3 activation occur upon translocation of cathepsin from the lysosome in response to oxidative stress (Kagedal *et al.,* 2001). Cathepsin D has also been implicated in mediating apoptosis in response to diverse stress stimuli including TNFα, IFNγ, chemotherapeutic agents and serum deprivation (Deiss *et al.,* 1996; Wu *et al.,* 1998; Shibata *et al.,* 1998).

1.3.2.2. Sphingosine

Sphingosine is an 18-carbon amino alcohol with an unsaturated hydrocarbon chain (2-amino-4-octadecene-1,3-diol) and is the major digestion product of complex sphingolipids in the gastrointestinal tract (Schmelz *et al.,* 2001). It possesses an effect similar to ceramide on cancer cells and when supplied exogenously can inhibit proliferation and induce apoptosis in many cancer cell types including colon cancer cells (Matsubara and Ozawa, 2001). Schmelz and co-workers (2001) found that sphingosine reduced cytosolic and nuclear β-catenin in SW480 and T84 human colon cancer cells. β-catenin is an essential component of the Wnt signalling pathway which is dysfunctional in colon cancer. Most Wnt-signalling dysfunctions lead to the nuclear accumulation of β-catenin where it associates with transcription factors to drive the transcription of multiple target genes encoding proteins, such as c-myc, cyclin D1, MMP-7, laminin-5γ2 implicated in proliferation, cell-cell attachment, and invasion

(Martensson *et al*., 2007). An important regulator of Wnt signalling pathway is the protein known as adenomatous polyposis coli (APC), the gene product of the tumour suppressor gene *APC*. Together with axin and GSK, it binds to β-catenin targeting it for destruction. Thus, the reduction of cytosolic β-catenin in sphingosine-treated cancer cells maintains Wnt pathway in the OFF position. Defects in the APC/β-catenin pathway are common in up to 60% of sporadic human colon cancers (Powell *et al.,* 1992) and are thought to predispose cells to additional mutations that proceed to neoplasia (Polakis, 1999). It is apparent that dietary sphingolipids, presumably via their digestion products, bypass or correct defects in the APC/β-catenin regulatory pathway.

1.3.2.3. Sphingosine-1-phosphate

The sphingolipid metabolite sphingosine-1-phosphate is a bioactive lipoprotein-borne serum lipid formed by the phosphorylation of sphingosine by sphingosine kinase. It has dual messenger functions acting as a first messenger extracellularly and as a second messenger intracellularly. As a first messenger, sphingosine-1-phosphate binds to a family of five specific G protein-coupled receptors that are differentially expressed in different tissues and which regulate diverse cellular actions, such as growth, differentiation, cell survival, angiogenesis and cell migration (reviewed by Goetzl and An, 1998; Pyne and Pyne, 2000; Spiegel and Milstien, 2003; Hla *et al*., 2001), immune function and lymphocyte trafficking (Jolly *et al*., 2002; Mandala *et al*., 2002; Brinkmann *et al*., 2002). Its binding to $S1P_1$ receptor and subsequent activation of a pertussis toxin-sensitive G_i protein is critical for the formation and extension of new blood vessels. Activation of small GTPases (e.g. Rac and Rho) that are linked to cytoskeletal rearrangements and motility, ERK,

p38 mitogen-activated protein kinases, phospholipase D and Akt are further examples of outside-in signalling conducted by sphingosine-1-phosphate.

Activation of membrane-bound growth factor receptors by platelet-derived growth factor and epidermal growth factor leads to the formation inside the cell of sphingosine-1-phosphate, which as a second messenger regulates target biomolecules including COX-2, ERK, NF-κB, caspase-3 and Bax, vital for inhibition of apoptosis and induction of proliferation.

Immunohistochemical evidence indicated higher levels of sphingosine kinase mRNA and protein in human colon cancer cells when compared with their non-cancerous (normal) cells, indicating a role for sphingosine-1-phosphate in cancer pathogenesis and progression (Kawamori *et al.,* 2009). Adenomas had higher expression of sphingosine kinase 1 compared with normal mucosa, and colon cancers with metastasis had higher expression of sphingosine kinase 1 than those without metastasis. Enzymes that catabolize sphingosine-1-phosphate, such as sphingosine-1-phosphate lyase and sphingosine-1-phosphate phosphatase were observed to be present at higher levels in normal intestinal epithelial cells than in colon cancer cells, suggesting that colon cancer cells manifest a block in sphingosine-1-phosphate catabolism.

The sphingosine kinase 1-sphingosine-1-phosphate (SK1-S1P) pathway exerts significant proliferative activities in cancer cells. Studies in human colon cancer cells have shown that sphingosine-1-phosphate protects colon cancer cells from chemotherapy-induced apoptosis. Silencing sphingosine kinase by RNA interference suppressed cell viability and increased caspase activity and cellular ceramide formation after oxaliplatin treatment (Nemoto *et al.,* 2009). Forced expression of

sphingosine-1-phosphate lyase, the enzyme that degrades sphingosine-1-phosphate, increased apoptosis in DLD1 colon cancer cells both at baseline and in response to treatment with the chemotherapeutic drug, daunorubicin. Several recent studies have identified a novel and specific role for sphingosine kinase 1 in mediating COX-2 expression and PGE_2 production in various cell models (Pettus *et al*., 2003; Pettus *et al*., 2005; Kawamori *et al*., 2005). The arachidonic acid cascade is a major inflammatory pathway involved in colon carcinogenesis. Kawamori *et al*. (2009) reported that downregulation of sphingosine kinase 1 by RNA interference (RNAi) significantly reduced COX-2 expression and PGE_2 production induced by cytokines in HT-29 human colon cancer cells. Enforced sphingosine kinase 1 expression also induced COX-2 protein in normal intestinal epithelial cells. In addition, they found that sphingosine kinase 1 protein and message levels were increased in azoxymethane-induced colon tumours in male F344 rats.

Greenspon and colleagues (2009) found that sphingosine-1-phosphate protected intestinal epithelial cells from apoptosis through the Akt signalling pathway. It increased levels of phosphorylated Akt and increased Akt activity without changing total Akt. The activation of Akt was associated with decreased levels of caspase-3 expression and activity. Moreover, when Akt was inactivated by treatment with a PI3K inhibitor, the protective effect of sphingosine-1-phosphate on apoptosis was abolished.

The opposing effects of ceramide and S1P in signalling pathways underscores the importance of characterising ceramide synthase enzymes with respect to tissue distribution, substrate specificity and inhibition by S1P. A recent study showed that family members of mammalian ceramide synthase (CerS) enzymes have specific

acylchain length preferences. (Laviad *et al*., 2008). For example CerS2 has a remarkable specificity for long acylchain CoA (C20-C26) showing no activity for C16:0-CoA and very low activity for C18:0. Sphingolipidomic analyses of ceramides in cells and tissues using mass spectrometry will be important in providing evidence linking length of fatty acid chain with defined roles in cell physiology.

1.3.2.4. Ceramide-1-phosphate

Ceramide-1-phosphate is another proliferative and proinflammatory factor produced in mammalian cells by the phosphorylation of ceramide by ceramide kinase, an enzyme located on the trans golgi network in many cell types. Marcu and Chalfant (2007) reported that nanomolar concentrations of C16-ceramide-1-phosphate rapidly activated cPLA2, thereby increasing release of arachidonic acid. Mechanistic studies demonstrated that the interaction of ceramide-1-phosphate and cPLA2a was very specific, since closely related lipids and metabolites were unable to activate cPLA2 in cells. It appears to act in concert with sphingosine-1-phosphate to stimulate the formation of PGE_2 and promote inflammation and carcinogenesis (Chalfant and Spiegel, 2005). Other biological responses of ceramide-1-phosphate include a role in membrane fusion, phagocytosis, mobilisation of calcium from the endoplasmic reticulum and cell survival. Possible targets of ceramide-1-phosphate are protein phosphatases PP1 and 2A which, as outlined above, play roles in induction of apoptosis. Gomez-Munoz *et al.* (2005) demonstrated that ceramide-1-phosphate promoted the survival of bone-marrow-derived macrophages by stimulating the PI3K/Akt pathway, activating NF-κB and increasing anti-apoptotic Bcl-X_L levels, which may be indicative of effects of ceramide-1-phosphate on alternative splicing of Bcl-X_L mRNA as a result of PP1 inhibition.

Taken together, there is a lot of evidence that sphingosine-1-phosphate and ceramide-1-phosphate promote significant proliferative and anti-apoptotic activities in cancer cells, leading also to malignant transformation. Enzymes that convert pro-apoptotic sphingosine and ceramide to anti-apoptotic ceramide-1-phosphate and sphingosine-1-phosphate, and vice-versa, play a significant role in regulating the balance between cell death and cell survival in cancer and become a target for pharmaceutical companies.

1.3.3. Glycosphingolipids

Ceramide is also a common precursor for glycosphingolipids. Synthesis of glycosphingolipids starts when glycosyltransferase catalyzes attachment of glucose or galactose to ceramide. Further attachment of galactose residue to glucosylceramide forms lactosylceramide, which is then a mother lipid for all the gangliosides in the cells. Addition of one, two or three sialic acids by membranous glycosyltransferases to lactosylceramide leads to formation of precursors for more complex gangliosides, G_{M3}, G_{D3} and G_{T3}, respectively (Bektas and Spiegel, 2003), which are formed in the lumen of the Golgi apparatus. Milk fat globule membrane (MFGM) is an abundant natural source of glycosylated sphingolipids, in particular lactosylceramide, glucosylceramide and several gangliosides including G_{D3}, G_{D1b1}, G_{M2}, G_{M3} and G_{M1} (Bektas and Spiegel, 2003). Similar to sphingomyelin, glycosylated sphingolipids are mostly involved in membrane structure, signalling and formation of specific detergent-insoluble domains, so-called “lipid rafts”, which are believed to play an additional role in cellular signalling (Parton and Simons, 1995; Hinrichs *et al*., 2005).

1.3.3.1. Glucosylceramide

Glucosylceramide is one of the controversial lipids as it has been shown that it possesses anti-apoptotic properties in cancer cells. The level of glucosylceramidase synthase which converts ceramide to glucosylceramide may determine multidrug resistance in cancer cells. Higher glucosylceramide amounts and overexpression of glucosylceramidase synthase enzyme were observed in multidrug-resistant breast cancer cell line MCF-7-AdrR (Adriamycin-resistant) (Lavie *et al*., 1996). Liu *et al*. (1999a and 1999b) confirmed that the drug sensitive breast cancer cell line transfected with glucosylceramidase synthase gene, MCF-7/GCS was resistant to drug treatment and also to TNF-α induced apoptosis. Van Vlerken *et al*. (2007) reported recently that coadministering ceramide with paclitaxel, a commonly used chemotherapeutic agent, restored apoptotic signalling and overcame multidrug resistance in ovarian cancer *in vitro*. Targeting glucosylceramidase synthase enzyme functions in drug-resistant cancer cells may be an approach for pharmaceutical companies and novel investigations.

1.3.3.2. Lactosylceramide

Lactosylceramide is a member of the glycosphingolipid family. It consists of a non-polar component ceramide, to which glucose and galactose are attached via Galβ1-4Glcβ1-Cer linkages. It is generated predominantly by lactosylceramide synthase, a Golgi localized enzyme that transfers galactose residues from UDP-galactose to glucosylceramide. At least two lactosylceramide synthases occur in mammalian tissues; β1,4-GalT-V is a constitutively expressed lactosylceramide synthase present in almost all human embryonic and adult tissues (Chatterjee *et al*., 2008) while β1,4-GalT-VI is expressed predominantly in human embryonic and adult brain cells. Catabolism of gangliosides, such as GM3 and GD3 and complex

glycosphingolipids, such as globotrosylceramide by sialidases and glycosidases, respectively, can also generate significant amounts of lactosylceramide. Evidence of its potential role in regulating cell function stems from observations that lactosylceramide induced expression of cell adhesion molecules such as intercellular cell adhesion molecule-1, vascular cell adhesion molecular-1 and platelet cell adhesion molecule (Bhunia *et al*., 1998; Gong *et al*., 2004; reviewed in Chatterjee and Pandey, 2008) and that it activated an "oxygen-sensitive" signalling pathway involving superoxides, nitric oxides and PI3K/Akt activation (Rajesh *et al*., 2005), thereby contributing to cell proliferation, adhesion, migration and angiogenesis.

Lactosylceramide is largely found in MFGM, next to sphingomyelin and glucosylceramide. Thin layer chromatographic analysis of glycolipids extracted from colon cancers and adjacent non-cancerous mucosa revealed that colon cancer exhibited a marked accumulation of lactosylceramide (Kakugawa *et al*., 2002). The latter also showed that addition of lactosylceramide to Colo-205, a human colon cancer cell line reduced apoptotic cells during sodium butyrate treatment. The increased expression of the constitutive enzyme, β1,4-GalT-V in endothelial cells from human colon cancer as compared to endothelial cells from normal colon suggests that lactosylceramide metabolism may provide novel targets for development of anticancer therapeutics (Chatterjee and Pandey, 2008).

1.4. Hydrolysis of sphingomyelin

1.4.1. Digestion of dietary sphingomyelin

The main dietary sphingolipids are sphingomyelin and glycosphingolipids. Their amounts in food vary considerably, from dairy products as major sources (1–2

g/kg), followed by meat and fish, eggs and soybeans, while vegetables are poor dietary sources (Vesper *et al*., 1999).

Hydrolysis of dietary sphingolipids takes place in the small intestine as well as in the colon and it is catalyzed by alkaline sphingomyelinase (SMase) (Nilsson, 1968; Schmelz *et al*., 1994; Nyberg *et al*., 1997; reviewed by Duan, 1998). Schmelz *et al*. (1994) and Nyberg *et al*. (1997) found that sphingomyelin digestion is a slow process and not all of the sphingolipids are hydrolyzed and absorbed. They demonstrated that there is a correlation between the amount of sphingolipids that is fed and the amount found in the intestine. It was shown by Nyberg *et al*. (1997) that the level of ceramide in intestine increased with increasing amount of dietary sphingomyelin. Alkaline sphingomyelinase is the first major enzyme responsible for digestion of dietary sphingomyelin (Nyberg *et al*., 1997). It is present in the intestinal tract but is absent from brain, pancreas, kidney, spleen, milk and urine (Nilsson, 1969; Duan *et al*., 1996). A second source of alkaline sphingomyelinase is human bile (Duan *et al*., 1996). Sphingomyelin digestion is under the control of cholecystokinin secreted from endocrine cells in the intestine. Cholecystokinin stimulates release of alkaline sphingomyelinase and bile salts, into the intestinal lumen. Both trihydroxyl and dihydroxyl bile salts stimulate alkaline sphingomyelinase activity up to the critical micellar concentrations and inhibit enzyme activity at concentrations above it (Duan *et al*., 1996). Alkaline sphingomyelinase is also stimulated by the anticancer agent ursulic acid and other pentacyclic triterpenoids widely present in fruits and vegetables (Andersson *et al*., 2006).

An enzyme that cooperates with alkaline sphingomyelinase is neutral ceramidase; its role is to hydrolyze ceramide to sphingosine in the intestinal lumen

(Lundgren *et al*., 2001; Kono *et al*., 2006). Newly formed sphingosine is absorbed by passive diffusion into the enterocytes where it is converted to ceramide. It was shown by Duan *et al*. (2007) that the presence of bile salts is crucial for maintaining high intraluminal concentrations of alkaline sphingomyelinase and neutral ceramidase. A novel human alkaline ceramidase (haCER2) has been identified in gut which regulates cell proliferation and survival by controlling levels of sphingosine and sphingosine-1-phosphate (Xu *et al*., 2006).

Sphingomyelin digestion is a slow process, due to i) complexation of SM with cholesterol (Liu *et al*., 2000 and 2002; Nyberg *et al*., 2000), ii) competition from phosphatidylcholine for the substrate binding side of alkaline sphingomyelinase (Duan and Nilsson, 1997) and iii) inhibitory effect of bile salts (taurocholate and taurochenodeoxycholate) on alkaline sphingomyelinase (Olsson *et al*., 2004). This situation occurs in the upper part of small intestine, where a high bile salts environment is suitable for hydrolysis of fat and phospholipids. When most of the fat has been absorbed, the reduced bile salts concentration in the lower part of the small intestine favours sphingomyelin digestion. Undigested sphingomyelin and unabsorbed ceramide move to the colon where the bile salt concentration is lower and stable alkaline sphingomyelinase and neutral ceramidase are present to permit further absorption (Duan, 2006).

1.4.2. Endogenous sphingomyelin

In addition to digestion of dietary sphingolipids, there is also a source of ceramide from endogenous sphingomyelin hydrolysis. As reviewed by Kolter and Sandhoff (1999), endogenous sphingolipids undergo both lysosomal and

nonlysosomal degradation. The enzymes involved in nonlysosomal degradation of endogenous sphingomyelin are neutral sphingomyelinase and neutral ceramidase. It was first observed by Okazaki *et al*. (1989) that vitamin D_3 induces sphingomyelin hydrolysis in HL-60 cells by increasing neutral SMase activity. Since then, several extracellular agents were shown to stimulate sphingomyelin hydrolysis in various cell types. These agents include: i) tumour necrosis factor α (TNFα), fas ligand, dexamethasone, nitric oxide, staurosporine which induce apoptosis; ii) vitamin D_3, TNFα, NGF, shear stress, retinoic acid, progesterone and serum deprivation which induce differentiation; iii) daunorubicin, vincristine and oxidized LDL which induce DNA damage (Huwiler *et al*., 2000; Kolter and Sandhoff, 1999 and the references within).

In lysosomal degradation, fragments of membrane containing glycosphingolipids and sphingomyelin are first endocytosed and they reach the lysosome as intralysosomal membrane structures. Sphingomyelin is hydrolyzed by acid sphingomyelinase and glycosphingolipids are hydrolyzed by exohydrolases, which cleave sugar residues step-by-step starting from the nonreducing end of glyco-structures to yield ceramide. Ceramide is then deacylated by an acid ceramidase to form sphingosine (Koch *et al*., 1996; Li *et al*., 1998). The cleaved molecules (fatty acids, sphingoid bases or the sugar residues) can then leave the lysosome and be metabolized further, or else re-enter biosynthesis pathways. Lysosomal degradation is normally a non-signalling pathway because ceramide is locked inside the lysosome and not available as a signal transduction messenger, except when ceramide induces apoptosis through activation of cathepsin D.

1.5. Dietary sphingolipids and colon cancer

Sphingolipids are necessary for normal cell function and can be synthesized *de novo* or obtained from the diet. Exogenous sphingolipids of dietary origin were found to be required for the growth of Chinese hamster ovary (CHO) cells which were deficient in serine palmitoyltransferase, the first enzyme in sphingolipid biosynthesis (Hanada *et al*., 1992; Vesper *et al*., 1999). As bioactive sphingolipids have significant roles in modulating colon tumour development and activation of apoptotic pathways in cancer cells, the impact of dietary sphingolipids on different types of colon cancer is of interest to academic and food industry research programmes.

1.5.1. Sphingomyelin digestion and colon cancer

1.5.1.1. Decreased alkaline sphingomyelinase activity in colon cancer

Intestinal mucosal alkaline sphingomyelinase is the enzyme responsible for hydrolysis of sphingomyelin in the intestinal tract. Dudeja *et al*. (1986) first observed an increased level of sphingomyelin in colonic rat mucosa after treating rats with the chemical colonic carcinogen, 1,2-dimethylhydrazine. It has been also shown that the level of sphingomyelin in human colon cancer cells is increased relative to normal cancer cells, which can be a result of either decreased sphingomyelinase activity and/or increased sphingomyelin synthesis (Merchant, 1995). Hertervig *et al*. (1997) measured the activities of all three types of sphingomyelinase in human colorectal carcinomas and compared them with the activities of these enzymes in the surrounding tissues. They showed that alkaline sphingomyelinase activity was predominantly decreased by 75% in colorectal carcinoma in comparison with acid and neutral sphingomyelinase. Campbell *et al*. (1994) and Hertervig *et al*. (1998) showed that in colorectal adenomas, an earlier stage of cancer development, that alkaline

sphingomyelinase activity was reduced by 50%, whereas the acid and neutral sphingomyelinase were unchanged. It has been also shown that, compared with control patients, alkaline sphingomyelinase activity was decreased by 90% in adenomatous polyps in patients with familial adenomatous polyposis (FAP) disease (Hertervig *et al*., 1998). *In vitro* studies have shown that poorly differentiated human colon cancer cells, such as Caco-2 and HT-29, possess a low activity of alkaline sphingomyelinase (Wu *et al*., 2004a). However, unlike Caco-2, the low activity of alkaline sphingomyelinase in HT-29 cells is due to deletion of exon 4 in alkaline sphingomyelinase cDNA. After 21 days of culturing Caco-2 cells under differentiating conditions, activities of alkaline sphingomyelinase and alkaline phosphatase increased 12-fold and 2.4 fold respectively compared to activities found in undifferentiated Caco-2 monolayer cells. No change in activity of these enzymes was observed in HT-29 cells after culturing under the same conditions (Wu *et al*., 2004a). These studies suggest that human alkaline sphingomyelinase, as an ecto-enzyme located on the microvillar surface of epithelial cells shares some similarities with other intestinal hydrolases, which are fully expressed during formation of the brush border surface and differentiation of cells (Duan *et al*., 2003). Wu and co-workers (2004b) suggested that there are at least two forms of alkaline sphingomyelinase: one that is bound to the brush border by a COOH terminal domain and the other that is in free form in the intestinal lumen which has its COOH-terminal anchor cleaved. They showed that trypsin cleaved the alkaline sphingomyelinase at the COOH terminal, releasing it from mucosa and that this action enhanced its activity by approximately 70%. These findings indicated a physiological role for pancreatic trypsin in sphingomyelin digestion. They hypothesize that the membrane-bound alkaline sphingomyelinase may predominantly hydrolyze membrane-bound

sphingomyelin and the free alkaline sphingomyelinase in the intestinal lumen is the major enzyme targeting sphingomyelin in the intestinal content. Alkaline sphingomyelinase may also have phospholipase C activity (Wu *et al*., 2006). They found that alkaline sphingomyelinase cleaved the phosphocholine head group from PAF, a pro-inflammatory phospholipid involved in pathogenesis of inflammatory bowel diseases to generate 1-O-alkyl-2-acetyl-sn-glycerol. The hydrolysis and inactivation of PAF reveals a novel function, by which alkaline sphingomyelinase may counteract the development of intestinal inflammation and colon cancer.

1.5.1.2. Dietary sphingomyelin reduces colon cancer

Dillehay *et al*. (1994) showed that exogenous dietary supplementation with purified bovine milk sphingomyelin reduced the number of aberrant colonic crypt foci (an early marker of colonic carcinogenesis) by ~70% in the CF1 mouse model of chemical carcinogenesis. In subsequent investigations, a significant shift in tumour type from adenocarcinomas to the more benign adenomas was observed following supplementation with milk sphingomyelin (Schmelz *et al*., 1996) and with milk glycosphingolipids (glucosylceramide, lactosylceramide and gangliosides) (Schmelz *et al*., 2000). Similar observations were made when CF1 mice and multiple intestinal neoplasia (Min) mice, which develop intestinal tumour spontaneously, were fed with soy glucosylceramide (Symolon *et al*., 2004). Tumour reduction was reported by Lemonnier *et al*. (2003) when mice were fed sphingomyelin before and after tumour initiation, indicating both chemopreventive and chemotherapeutic effects of sphingolipids. Simon *et al*. (2009) observed that dietary sphingomyelin downregulated β-catenin in carcinogen-treated CF1 mice. These changes suggest that dietary sphingomyelin may reduce the risk of colon cancer and inhibit development to malignant type.

Studies have also highlighted a potential benefit of exogenous sphingomyelin to propagate apoptotic signals in response to a variety of cytotoxic agents. Modrak *et al*. (2002) provided evidence that sphingomyelin enhanced the efficacy of 5-fluorouracil and irinotecan for the treatment of HT-29, HCT15 and GW-39 human colonic tumour xenografts. Van Vlerken *et al.* (2007) showed that co-administration of ceramide with paclitaxel, a commonly used chemotherapeutic agent restored apoptotic signalling in a drug resistant human ovarian cancer cell line SKOV3. Studies in pancreatic cancer have provided further evidence that targeting sphingolipid metabolism is a means of enhancing the efficacy of chemotherapeutic agents (Modrak *et al.,* 2004 and 2009)

1.6. Summary and perspectives for future research

Cancer and cardiovascular disease are leading causes of morbidity and mortality in Western societies. To address this problem, research institutes together with pharmaceutical companies aim to discover drugs for cancer and other lethal diseases. At the same time increasing consumer interest in the health improving role of bioactive food components has enhanced demand for rapid advances in food science and technology and development of new functional foods with health-promoting or disease-preventing properties. Knowing potent biological activities of sphingolipids, it is likely that they can be categorized as functional components of food with regards to human health, especially for prevention of colon cancer as well as being a cholesterol-lowering agent (Eckhardt *et al*., 2002; Noh and Koo, 2004) and protectant against bacterial infections in the gut (Pfeuffer and Schrezenmeir, 2001). However, currently there are no known nutritional requirements for their daily intake.

Occurrence of sphingolipids and phospholipids in food is wide-spread, from lower amounts in fruits and vegetables to rich sources, such as MFGM in dairy products. The idea of functional food development inclines towards introducing dairy products enriched in MFGM as a rich source of sphingolipids and phospholipids, as well as proteins with beneficial properties. Additionally, MFGM is also a source of antioxidants, such as vitamin E (Jensen and Nielsen, 1996) and riboflavin/vitamin B_2 (Kanno, 1990). Investigation of the influence of enhancing cows' fodder in polyunsaturated fatty acids like omega-3 (DHA and EPA) on bovine milk composition may be worthy, as it was shown that beside their wide range of beneficial properties (Freeman *et al*., 2006; Mozaffarian and Rimm, 2006; Serini *et al*., 2009), they also possess ability to activate the neutral sphingomyelinase-mediated pathway of apoptosis in breast cancer cells (Siddiqui *et al*., 2005; Wu *et al*., 2005; Paranavitana *et al*., 2006).

Expanding studies on enriching dairy products with probiotics may be of interest because of their gut health and digestion properties. Additionally, Di Marzio *et al*. (2001) showed that apoptotic effect of selected strains of lactic acid bacteria (*Streptococcus thermophilus*) on Jurkat cells and colon cancer cells is also connected to the sphingomyelin cycle. Soo *et al*. (2008) found that VSL#3 probiotic product which consisted of eight different strains of bacteria (*Bifidobacterium longum*, *Bifidobacterium infantis*, *Bifidobacterium breve*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus bulgaricus*, *Lactobacillus plantarum*, and *Streptococcus salivarius* subspecies *thermophilus*) upregulated mucosal alkaline sphingomyelinase activity both in mouse models with colitis and humans with ulcerative colitis, a form of inflammatory bowel disease. Duan (2007) confirmed that LAB, such as *Streptococcus thermophilus* and *Lactobacillus acidophilus* possess the

ability to hydrolyse sphingomyelin (Duan 2007). Duan *et al*. (1996) suggested that alkaline sphingomyelinase is not derived from intestinal bacteria as they found high alkaline sphingomyelinase activity in germ-free mice. However, Cheng *et al*. (2004) fed mice with a diet including psyllium, which is a water-soluble fibre that acts as a food source for colonic bacteria and observed 57% increase in alkaline sphingomyelinase activity over the control. They suggested that this was a result of the influence of psyllium on luminal bacteria growth (Cheng *et al*., 2004), however the mechanism remains unclear. The importance of LAB as an alternative source of alkaline sphingomyelinase in digestion of dietary sphingomyelin in colon cancer cells and production of highly bioactive ceramide and sphingosine requires further investigations. Nonetheless, in order to produce functional foods that could improve consumer's health and potentially prevent cancer and other diseases, as well as maintain a healthy gut, there is a substantial necessity for further studies on the influence of sphingolipids on human health and their mode of action at the cellular level.

1.7. Thesis objectives

- To validate a rapid *in vitro* colorimetric assay for screening antiproliferative activity of buttermilk samples
- To investigate the effects of a range of processing variables (cream separation methods, washing, heating) and inoculation with microbial starter cultures on antiproliferative activity of resultant buttermilks in the SW480 human colon cancer cell line and FHC human normal colon epithelial cell line.

- To examine the antiproliferative activity of feed, retentate and permeate samples of buttermilk produced at pilot scale from unwashed and washed cream on human colon cancer cell lines.
- To investigate the influence of drying (including spray, freeze and spin drying) on antiproliferative activity of buttermilk on colon cancer cells.
- To investigate effects of buttermilk on biomarkers of apoptosis (ie morphology of apoptotic cells, cleavage of caspases, translocation of phosphatidylserine, mitochondrial outer membrane depolarization, translocation of apoptosis inducing factor and endonuclease G to cells' nuclei), and proliferation (i.e. β-catenin, phosphorylated Akt and proteins involved in MAPK signalling pathway: ERK1/2, p38 protein and JNK).

CHAPTER 2

Selection of colorimetric assay and processing method for studying antiproliferative activity of buttermilk samples *in vitro*.

Milk is well recognised as a source of lipid components with anti-carcinogenic properties, such as rumenic acid and sphingolipids; however little data is available on antiproliferative activity of dairy products, such as buttermilk. The aim of this study was to set up a reliable and fast colorimetric method for preliminary screening of potential antiproliferative activity of buttermilk samples, which would be produced in large amount during process optimization in subsequent steps of the project. We also attempted to separate buttermilk samples into lipid classes and deliver them to the SW480 colon cancer cells in the form of bile salt micelles. The acid phosphatase (AP) activity assay was selected for the study of antiproliferative activity of buttermilk because it exhibited a better linear response than MTS to cell number in SW480, Caco-2 and FHC cells. Our model was validated using commercial lipids, spingosine, ceramide and lactosylceramide, which inhibited growth of SW480 colon cancer cells by 50% at concentrations 6, 14 and 9 μM, respectively after 3 days of treatment using acid phosphatase assay. Unfortunately, after separation of buttermilk fat into lipid classes, neutral lipid fractions did not form clear micellar solutions and the results could not be compared with other fractions. Although fatty acid and polar lipid fractions of buttermilks showed antiproliferative activity on SW480 colon cancer cells (up to about 60% cell growth inhibition after 3 days of treatment), it was decided that this method was not optimal for studying antiproliferative activity of buttermilks on colon cancer cells because disruption of MFGM material during fractionation may adversely affect the bioactivity of its components and would not be representative of MFGM when presented *in viv*o as a whole beverage.

2.1. Introduction

To date, numerous biologically active food components, such as sphingolipids, flavonoids, isoflavonoids, vitamins, minerals, and omega-3 fatty acids have been reported to exhibit anticancer effects based on *in vitro* or *in vivo* data (Milner, 2006, Stan *et al*., 2008, Nobili *et al*., 2009). Of these, sphingolipids are found almost exclusively in animal products with a level of approximately several millimoles/kg in dairy products (Vesper *et al*., 1999). Intake of sphingolipids in the normal diet is ~ 0.3-0.4 g/day (Vesper *et al*., 1999). Sphingolipids are polar lipids found predominantly in the disrupted milk fat globule membrane (MFGM) material of dairy products, especially buttermilk (Rombaut *et al*., 2006). Buttermilk is a by-product of the butter-making process and the composition and characteristics of MFGM depend on the production technology and the quality of milk and cream used. Buttermilk consists of approximately 91% water and contains about 9% total solids present in milk.

The nutritional and techno-functional properties of buttermilk have been investigated in recent years. Compositional analysis of MFGM isolated from buttermilk has revealed that it is a rich source of valued bioactive components with potential to deliver health benefits as functional food ingredients. These bioactive components include sphingolipids, phospholipids, fatty acid binding protein, breast cancer type 1 and 2 susceptibility proteins, xantine oxidase, mucin 1, butyrophilin and lactadherin (Spitsberg *et al*., 1995, Li *et al*., 2009, Brody and Biesecker, 1998, Vissac *et al*., 2002, Mana *et al*. 2004, Johns and Bernard, 1999, Stefferl *et al*., 2000, Zeelenberg *et al*., 2008, Liu *et al*., 2005).

In order to optimise technology for producing buttermilk with anticancer activity, it is essential to set up a reliable and fast method for preliminary

screening of large amounts of samples produced under different processing conditions. In 1991 rapid colorimetric assay method for initial stage screening of cytotoxic components was developed (Barltrop *et al*., 1991, Cory *et al*., 1991). Using this method cell proliferation and viability can be quantified based on reduction by live cells of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) in the presence of the electron coupling reagent phenazine ethosulfate, PES. This tetrazolium salt forms a soluble purple formazan product under the action of a mitochondrial dehydrogenase in active cells. Amongst other miniaturized *in vitro* colorimetric assays, such as staining with a protein-binding dye, sulforhodamine B; lysosome-accomulated dye, neutral red or protein-binding dye, crystal violet (Skehan *et al*., 1990, Monks *et al*., 1991, Borenfreund and Puerner, 1985, Kueng *et al*., 1989) there is also acid phosphatase (AP) assay developed by Martin and Clynes (1991) and based on the ability of cytosolic and lysosomal AP enzyme of cells to hydrolyze *p*-nitrophenylphosphate yielding a *p*-nitrophenyl chromophore (O'Connor *et al*., 1998), which has shown higher sensitivity than MTT in determining cell numbers (Martin and Clynes, 1993, O'Connor *et al*., 1998).

Formation of bile salt micelles is essential for optimal absorption of dietary fat and fat-soluble vitamins *in vivo*. At a critical micellar concentration (CMC), bile salts formed in the liver and secreted by the gall bladder form micelles with fatty acids, which then are transported to the intestinal brush border membrane. This allows complex lipids (e.g. lecithin) and lipid soluble vitamins (A, D, E and K) to overcome the diffusion barrier and be absorbed by cells of the small intestine. In order to optimise technology for producing buttermilk fractions with anticancer activity, it was necessary to fractionate the lipid components of

buttermilk and to incorporate them into micelles to mimic physiological absorption in cell culture.

The overall aim of the work presented in this chapter was to validate a rapid *in vitro* colorimetric assay for screening antiproliferative activity of buttermilk samples produced under different processing conditions. The specific objective of this study was to determine the suitability of the MTS and AP assays to study growth inhibition of SW480 and Caco-2 cell lines after treatment with commercially available pure MFGM lipids (phosphatidylcholine, lactosylceramide, sphingomyelin and its hydrolysis products, sphingosine and ceramide). We also fractionated buttermilk samples produced under different processing conditions into three lipid classes: fatty acids, polar lipids and neutral lipids and compared the effects of micellar lipid fractions on growth of SW480 human colon cancer cells.

An investigation of the health benefits of buttermilk's MFGM may greatly impact the dairy industry and may trigger conversion of buttermilk from a waste product of butter-making process into one with added value. However, significant scientific agreement on evidence provided by all preclinical and clinical studies is required before a chemopreventive claim can be assigned to buttermilk's MFGM. Strength of evidence will be based on the quality of all the laboratory data involving *in vitro* and *in vivo* animal and human studies. Because of the difficulty in obtaining primary human gastrointestinal tissues and cells, human cancer cell lines of intestinal origin that exhibit relatively stable behaviour, morphology and growth were our first choice for beginning a study of the chemopreventive potential of buttermilk.

2.2. Materials and Methods

2.2.1. Materials

Chemicals and cell culture media and supplements were purchased from Sigma-Aldrich Ltd. (Ireland) unless otherwise stated. C8-lactosylceramide and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were supplied by Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA).

2.2.2. Preparation of buttermilk samples for antiproliferative studies

2.2.2.1. Cream preparation

Buttermilk samples were generated by a project team member. Raw whole milk was collected from the Moorepark Dairy Production Centre (MDPC) farm (Fermoy, Co. Cork, Ireland) and immediately subjected to pasteurization at 72°C for 15s in a thermostatically controlled waterbath and cooled to approximately 20°C in an ice bath. Raw milk was then separated into cream and skim milk using variety of methods. One batch of cream was produced from raw milk separated at 45°C using an Armfield disc bowl centrifuge (Armfield, Ringwood, UK) at max rpm (buttermilk 2). Raw milk was also allowed to separate naturally by gravitational force at 4°C (buttermilk 3) and 20°C (buttermilk 4) over a period of 24h. One batch of raw cream was subjected to heat treatment at 95°C for 3s prior separation (buttermilk 5). All creams were then standardised as required using their corresponding skim milk to approximately 40% fat.

2.2.2.2. Buttermilk preparation

The experimentally-prepared creams were held at 4°C overnight prior to high-speed mixing (setting 5) to simulate churning in a Kenwood Chef food mixer (Model KM220, Harvant, Hants, UK) until the cream emulsion inverted and buttermilk was generated. Mixing then continued at a slower rate (setting 1) until the butter grains had aggregated. Buttermilk was obtained after filtering through a cheesecloth to remove butter grains and collected and stored at – 30°C for further analytical and antiproliferative analyses. As reference, a buttermilk sample was also produced from commercial cream purchased in a local shop (buttermilk 1).

2.2.3. Compositional analyses of buttermilk samples

All compositional analyses of buttermilk samples were prepared by a project team member. Protein content was determined using the macro-Kjeldahl procedure for nitrogen determination and a conversion factor of 6.38 (International Dairy Federation, 1993). All measurements were made in duplicate. Total solids (TS) were determined using a CEM Smart System 5 (CEM Incorporated, Matthews, North Carolina, USA) set at maximum power. All measurements were made in duplicate. Fat content was determined gravimetrically using the Röse Gottlieb extraction method which uses ammonia to break down any lipoprotein bonds in milk allowing all the fat to be dissolved by ether (International Dairy Federation, 1987 a, b) and Gerber fat analysis for cream which is based on separation of milk fat from proteins by adding sulfuric acid. All measurements were made in duplicate.

2.2.4. Cell culture

SW480, HT-29 and Caco-2 human colon cancer cell lines, HepG2 human hepatocellular cancer cell line and FHC normal human foetal colon epithelial cell line were obtained from European Collection of Cell Cultures, ECACC. Culture media and supplements were purchased from Sigma-Aldrich Ireland, Ltd. The HepG2 and Caco-2 cells were maintained in modified Eagle's medium (MEM). The SW480 and HT-29 cells were grown in Leibovitz-15 and McCoy's 5A medium, respectively. All media were supplemented with L-glutamine (4 mM), fetal bovine serum (FBS) (10%, v/v) and penicillin/streptomycin (1 U/ml). The FHC cells were grown in DMEM/Ham's Nutrient Mixture F-12 supplemented with L-glutamine (2.5 mM), sodium bicarbonate (1.2 g/L), HEPES (25 mM), sodium puryvate (0.5 mM), FBS (10%, v/v), cholera toxin (10 ng/ml), insulin (0.005 mg/ml), transferrin (0.005 mg/ml), hydrocortisone (100 ng/ml) and penicillin/streptomycin (1 U/ml). Cells were grown at 37°C, with 5% CO_2 access, except when grown in Leibovitz-15 medium which does not require CO_2 (flask's caps were fully closed).

2.2.5. Optimization of cell growth of different cell lines

To optimize seeding density SW480 human colon cancer cells were seeded in 96-well plates at densities ranging 1-10x10^3 cells/well. All the other cell lines (HT-29, Caco-2, HepG2 and FHC) were seeded in the same way but in the range 0.5-5x10^3 cells/well. To allow the cells to attach, they were cultured for 24 h at 37°C without 5% CO_2 (SW480 cells) or with 5% CO_2 access (HT-29, Caco-2, HepG2 and FHC cells). After 3 days of incubation at 37°C, cell survival was measured by an indirect colorimetric endpoint assay for acid phosphatase activity

(O'Connor *et al*., 1998). The basis of the assay is the ability of the acid phosphatase enzyme present in cell lysosomes to hydrolyze *p*-nitrophenyl phosphate yielding formation of *p*-nitrophenyl chromophore and development of a yellow colour. For this purpose, after 3-day incubation, the medium was removed and wells were rinsed with 100 µl PBS. Next, 100 µl of freshly prepared 10 mM *p*-nitrophenyl phosphate in 0.1 mol/L sodium acetate buffer (pH 5.5) containing 0.1% Triton X-100, was added to each well and incubated at 37°C for 2 h. The reaction was terminated by the addition of 50 µl 1M NaOH. The OD measurements at 405 nm were then recorded on Multi-Detection Microplate Reader Synergy™ HT (supplied by BioTek® Instruments, Inc).

Cell growth at different seeding densities was also determined using MTS assay for SW480 and Caco-2 cell lines. Briefly, after 3-day incubation MTS reagent (Promega Corporation, Madison, WI, USA) was added to the wells. After 2 h of incubation at 37°C OD at 490 nm was recorded on Multi-Detection Microplate Reader Synergy™ HT (supplied by BioTek® Instruments, Inc).

2.2.6. Antiproliferative activity of commercial lipids: sphingosine, ceramide, lactosylceramide, sphingomyelin and phosphatidylcholine (DOPC)

Commercial lipids, C2-ceramide (N-acetyl-D-sphingosine), D-sphingosine and C8-lactosylceramide were dissolved in 100% ethanol to a final stock concentration of 5 mg/ml. Sphingomyelin and phosphatidylcholine (1,2-dioleoyl-sn-glycero-3-phosphocholine, DOPC) were dissolved in 100% ethanol to a final concentration of 100 mg/ml. SW480 cells were cultured at a density of $3.5x10^3$ cells/well in 96-well plates at 37°C without CO_2 access for 24 h allowing cells to attach. Next, cells were incubated for further 3 days in Leibovitz-15 medium

supplemented with commercial lipids at varying concentrations between 0-20 μM for ceramide and sphingosine; 0-35.7 μM for lactosylceramide; 0-1 mM for sphingomyelin and 0-0.9 mM for phosphatidylcholine (DOPC). As controls for lipid treatments, cells were also incubated with pure ethanol at the same ethanol concentrations which were used for commercial lipids (0-0.5%, v/v). Cells were treated with sodium butyrate (5 mM), a growth inhibitory fatty acid (McBain *et al*., 1997) which served as biological control for each experiment. After 3 days of incubation at 37°C, cell survival was measured by an indirect colorimetric endpoint assay for acid phosphatase activity and/or MTS assay, as described.

2.2.7. Lipid extraction and fractionation of buttermilk lipid classes

To extract lipid classes from buttermilk samples, a modified version of the extraction method of Folch *et al*., (1957) and Bligh and Dyer (1959) was used. Briefly, 3.75 ml of chloroform:methanol (2:1, v:v) was added to 500 μl of each buttermilk sample and vortexed for 3 min. Next, 1.25 ml of chloroform was added, vortexed and centrifuged at 2000 rpm for 8 min. The lower organic phase was collected and all steps above were repeated to obtain better phase separation. The lower organic phase was then dried under nitrogen. Fractionation of buttermilk lipid classes was performed according to the method of Kaluzny *et al*., (1985). Sep-Pak Vac 3cc (500 mg) NH_2 cartridges (Bond Elut columns) were placed in the Vac Elut apparatus and washed twice with 2 ml aliquots of hexane. Collection tubes for eluates were placed in the Vac Elut and buttermilk lipids dissolved in chloroform were applied to the column without the vacuum to let the lipid mixture stay on the column and chloroform was collected in collection tubes. Next, the columns were eluted with 4 ml of different solvents to separate different

lipid classes: chloroform:2-propanol (2:1, v/v) for separation of neutral lipids, 2% acetic acid in diethyl ether for separation of fatty acids, methanol for separation of phospholipids. Lipid film was then mixed in sodium taurocholate solution as described below.

Immediately after lipid separation, 100 µl of each lipid fraction was collected into Eppendorf tubes and frozen in -20ºC.

2.2.8. Preparation of micellar solutions

Micellar solutions of bile salt sodium taurocholate were prepared prior to cell treatment. Fatty acid (FA), polar lipid (PL) and neutral lipid (NL) fractions were dissolved in chloroform:methanol 2:1 (v/v) and mixed with phosphatidylcholine (DOPC) and sodium taurocholate in molar ratio of DOPC:NaTC of 1:1. The concentration of sodium taurocholate added to form micelles was 0.36 mM for fatty acid and polar lipid fractions and 1.2 mM for the neutral lipid fraction. The organic solvent was removed under N_2 and the resulting lipid film on the bottom of the tube was dispersed in ethanol under gentle heat and mixed with serum-free Leibovitz-15 medium, followed by bath sonication with ultrasound at 37°C until optically clear.

2.2.9. Antiproliferative activity of buttermilk fractions

To assess the antiproliferative activity of buttermilk samples, SW480 cells were cultured at a density of $3x10^3$ cells/well (for 5 and 7 days of treatment) and $5x10^3$ cells/well (for 3 days of treatment) in 96-well plates. Cells were cultured for 24 h to allow the cells to attach. Next, fatty acid, polar and neutral lipid micellar

solutions from 5 different buttermilk samples were diluted in serum-free feeding media and were added to the wells containing the cells. Appropriate concentrations of sodium taurocholate and phosphatidylcholine (DOPC) were added to control wells. All the buttermilk samples were sterilized prior to treatment by incubation at 72°C for 15s. The wells with the SW480 cells were covered with parafilm to prevent CO_2 access to the cells. Four replicates of each buttermilk concentration were performed. After 3, 5 or 7 days of incubation at 37°C, cell survival was measured by an indirect colorimetric endpoint assay for acid phosphatase activity, as described above.

2.2.10. Statistical analysis

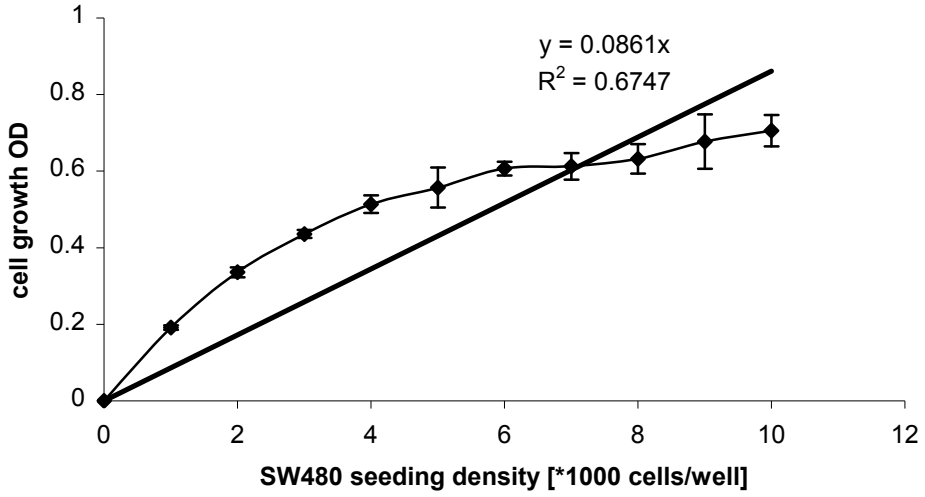
Three independent experiments were performed in quadruplicate and the Student's *t*-test was used to determine significant differences between treatments and controls. Statistically significant differences were represented as follows: * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$.

2.3. Results

2.3.1. Optimization of 96-well plate cytotoxicity assays (MTS vs. acid phosphatase) for evaluating effects of buttermilk on cell growth

To select a sensitive and efficient method for cytotoxicity testing we examined the relationship between seeding density and growth of SW480 cells using two indirect growth assays (MTS and AP) (**Fig. 2.1 a, b**). Both assays showed high repeatability within three different experiments. However, better linearity of cell growth against cell seeding density was observed using AP assay; hence this assay was used to determine optimal seeding density for other cell lines planned for use in further studies: HT-29 colon cancer, HepG2 hepatocellular cancer and FHC normal colon epithelial cells (**Fig. 2.2 a, b, c**). All three cell lines showed good linear increase in growth from initial seeding density. For further studies on the antiproliferative effect of buttermilk after 3 days of treatment, cells were seeded at following densities: HT-29, HepG2 and FHC at $3.5x10^3$ cells/well; SW480 at $5x10^3$ cells/well. Caco-2 cells were seeded in the range of $0.5\text{-}5x10^3$ cells/well and cell growth was determined using MTS and acid phosphatase assays (**Fig. 2.3 a, b**).

a)



b)

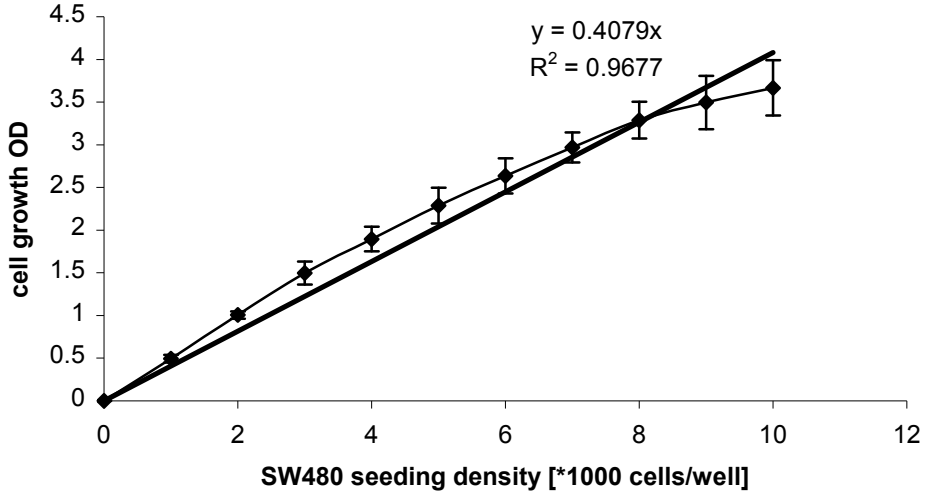
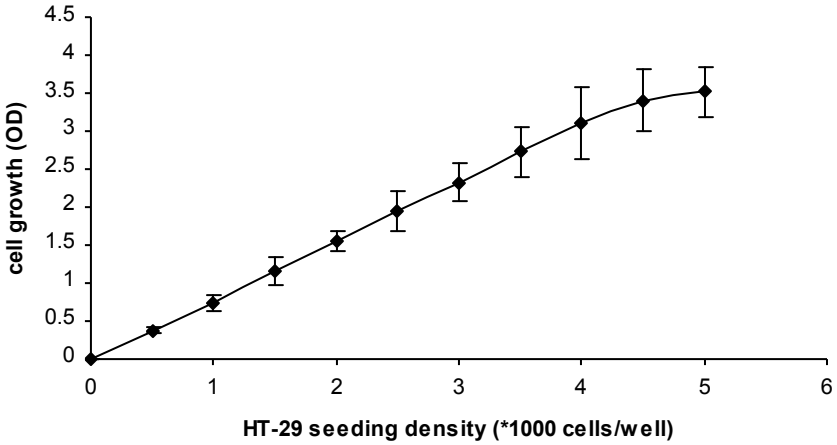


Fig. 2.1. Comparison of two assays for measuring cell growth: MTS (a) and acid phosphatase AP (b) as a function of cell seeding density. The SW480 cells were grown in Leibovitz-15 medium. Data refer to mean ± SD of three independent experiments.

a)



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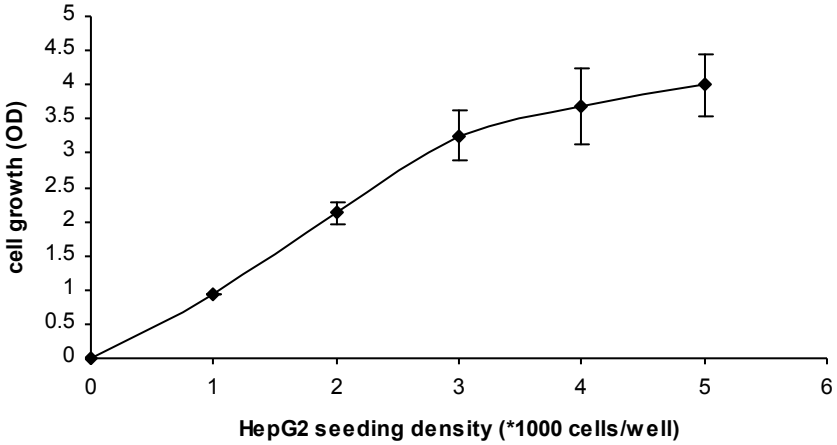


c)

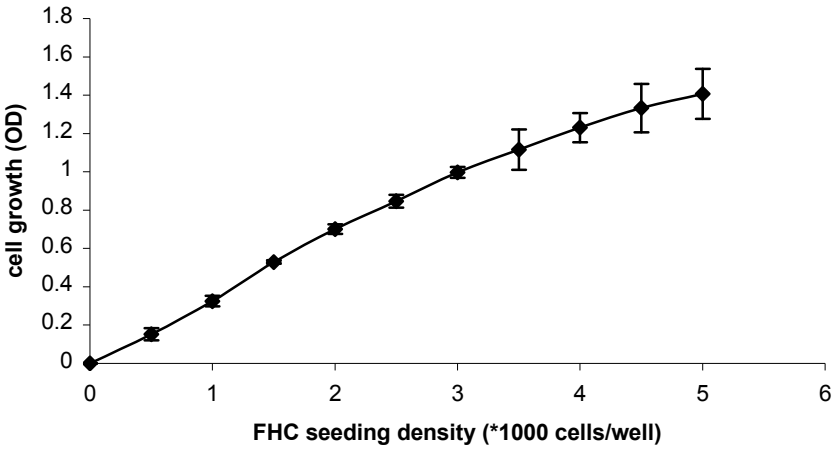
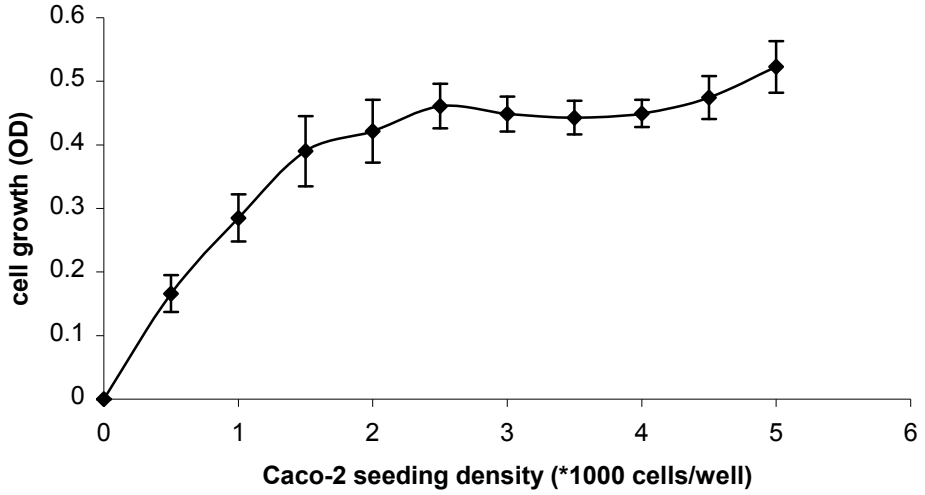


Fig. 2.2. HT-29 (a), HepG2 (b) and FHC (c) cell growth, obtained using AP assay after 4 days from seeding. The HT-29, HepG2 and FHC cells were grown in McCoy's 5A, MEM and DMEM/Ham's Nutrient Mixture F-12 media, respectively. Data refer to mean ± SD of three independent experiments.

a)



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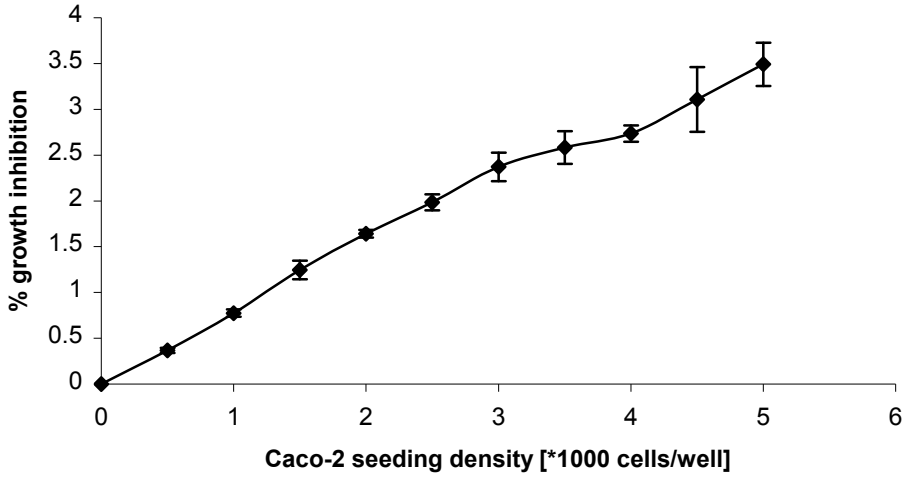


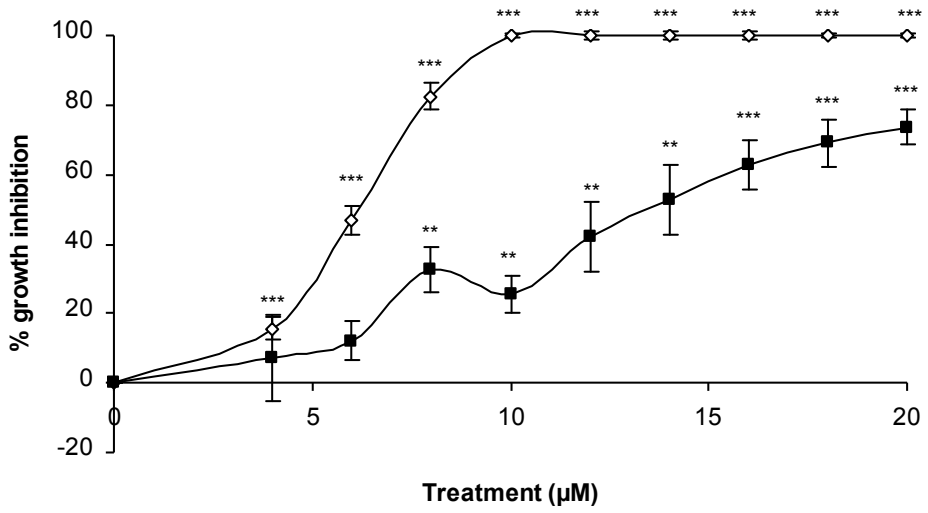
Fig. 2.3. Caco-2 cell growth, obtained using MTS (a) and AP (b) assays after 4 days from seeding. The Caco-2 cells were grown in MEM medium. Data refer to mean ± SD of three independent experiments.

2.3.2. Comparative analysis of effects of sphingosine and ceramide on SW480 cell growth as determined by MTS and AP assays

Fig. 2.4 demonstrates that sphingosine inhibited growth of SW480 cells to a greater extent than C2-ceramide (N-acetyl-D-sphingosine). The IC50 for sphingosine was 2.3-fold lower (6 μM) than IC50 for ceramide (14 μM) using the

AP assay (**Fig. 2.4 a**). The corresponding IC50 values for sphingosine (11.5 μM) was 1.9-fold higher using MTS and the IC50 value for ceramide was the same (14 μM) using MTS (**Fig. 2.4 b**).

a)



b)

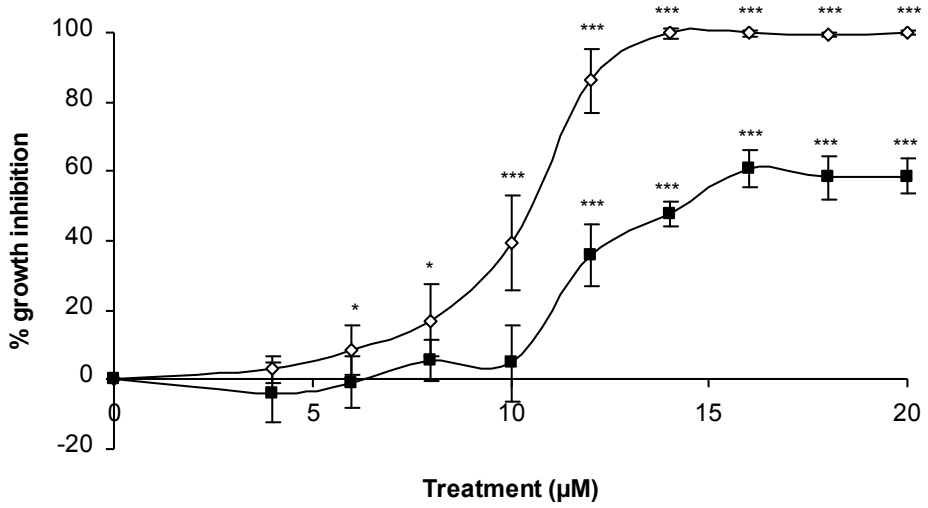


Fig. 2.4. Growth inhibition in SW480 human colon cancer cell line incubated with C2-ceramide (■) and sphingosine (◊) (0-20 μM) for 3 days assessed using AP (a) and MTS (b) assays. The SW480 cells were grown in Leibovitz-15 medium. Growth inhibition was expressed as a percentage relative to control which was taken to be 0%. Data refer to mean ± SD of three independent experiments.

Growth inhibition was considered significantly different than control when: * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

To exclude possible antiproliferative effect of lipid solvent, cells were treated with ethanol in the range used in lipid treatment (**Fig. 2.5**). Ethanol in the range 0-0.5% (v/v) did not exhibit antiproliferative activity on SW480 colon cancer cells. Minor cell growth progression was observed, however it was not constant between each experiments, resulting in relatively large error bars.

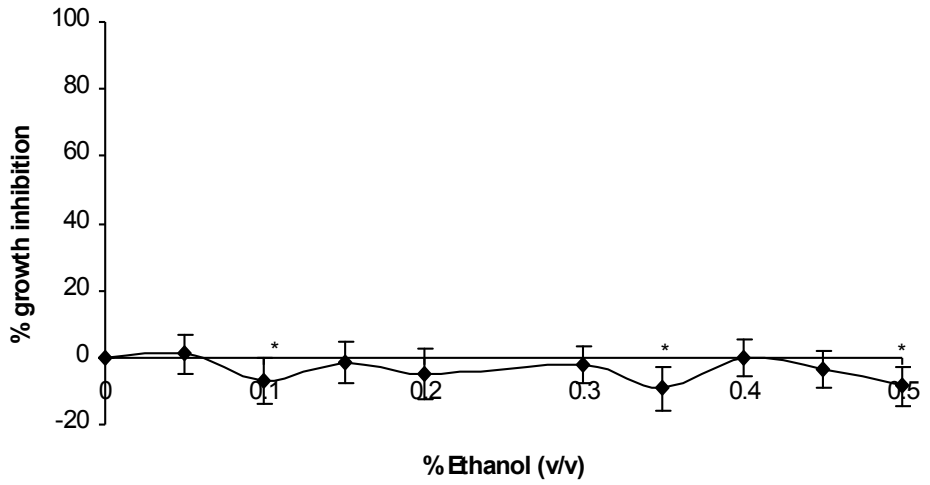


Fig. 2.5. Effect of ethanol (v/v) on SW480 colon cancer cell growth assessed using AP assay. Growth inhibition was expressed as a percentage relative to control which was taken to be 0%. Data refer to mean ± SD of three independent experiments. Growth inhibition compared to control without treatment was considered significant when: * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

2.3.3. Effect of commercial lipids: sphingomyelin, lactosylceramide and phosphatidylcholine (DOPC) on growth of SW480 colon cancer cells

Commercial lipids: sphingomyelin, lactosylceramide and phosphatidylcholine (DOPC) were screened for antiproliferative activity using SW480 colon cancer cells. Treatment of cells with sphingomyelin in the range 0-1 mM inhibited cell growth by up to 40% (**Fig. 2.6**). Treatment with lactosylceramide in ethanol inhibited cell growth by up to 100% (**Fig. 2.7**). IC50 values were approximately 9 μM and 10 μM after 3 and 7 days, respectively, indicating a possible cytostatic effect after 3 days. Lactosylceramide, when presented to cells in micellar form inhibited cell growth to a lower extent - IC50 values were 20 and 25 μM after 3 days and 7 days, respectively. Treatment of cells with phosphatidylcholine (DOPC) in the range 0-0.9 mM stimulated growth by up to 25% while less growth was observed following 3 days of treatment for 7 days (**Fig. 2.8).**

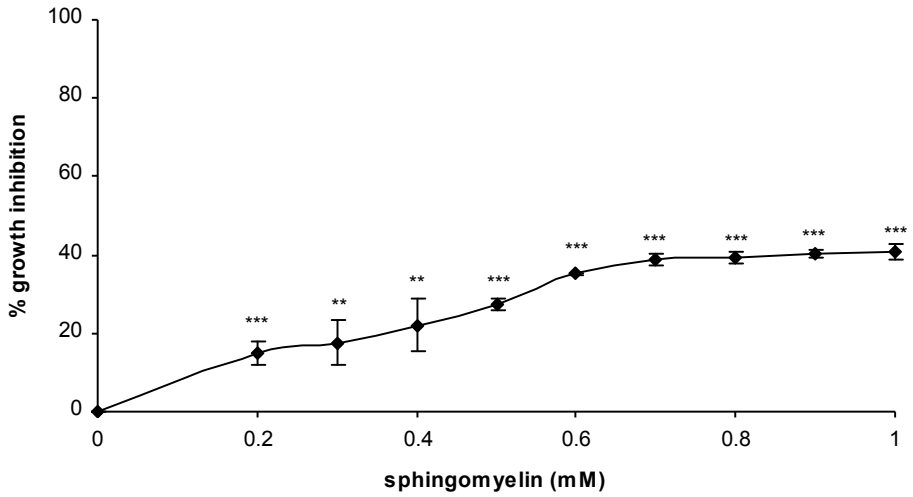


Fig. 2.6. Growth inhibition in SW480 human colon cancer cell line incubated with sphingomyelin assessed using acid phosphatase (AP) assay after 3 days of

treatment. The SW480 cells were maintained in Leibovitz-15 medium. Growth inhibition was expressed as a percentage relative to control which was taken to be 0%. Data refer to mean ± SD of three independent experiments. Growth inhibition compared to control without treatment was considered significant when: * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

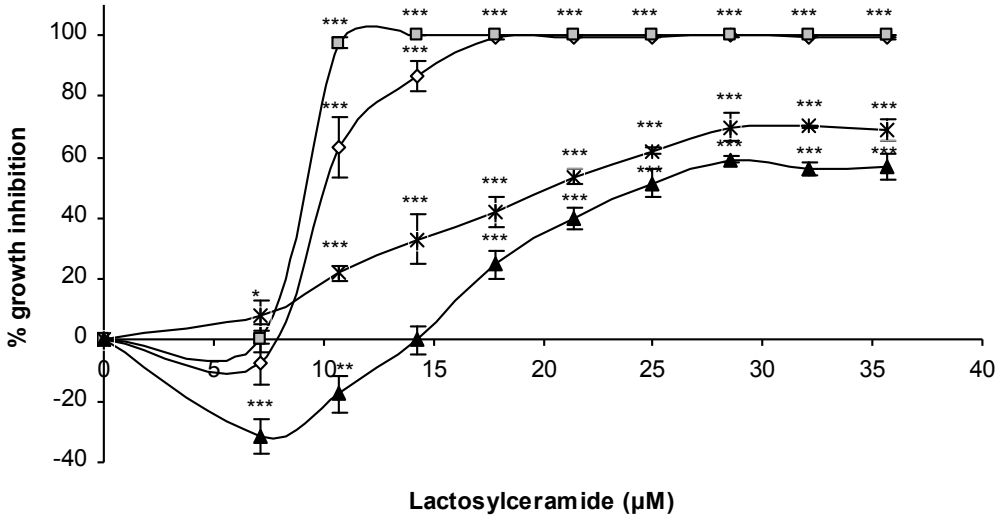


Fig. 2.7. Growth inhibition in SW480 human colon cancer cell line incubated with: LacCer dissolved in EtOH for 3 (◊) and 7 (◘) days; LacCer in form of micelles formed with sodium taurocholate incubated for 3 (▲) and 7 (✱) days. Results were assessed using acid phosphatase (AP) assay. The SW480 cells were maintained in Leibovitz-15 medium. Growth inhibition was expressed as a percentage relative to control which was taken to be 0%. Data refer to mean ± SD of three independent experiments. Growth inhibition compared to control without treatment was considered significant when: * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

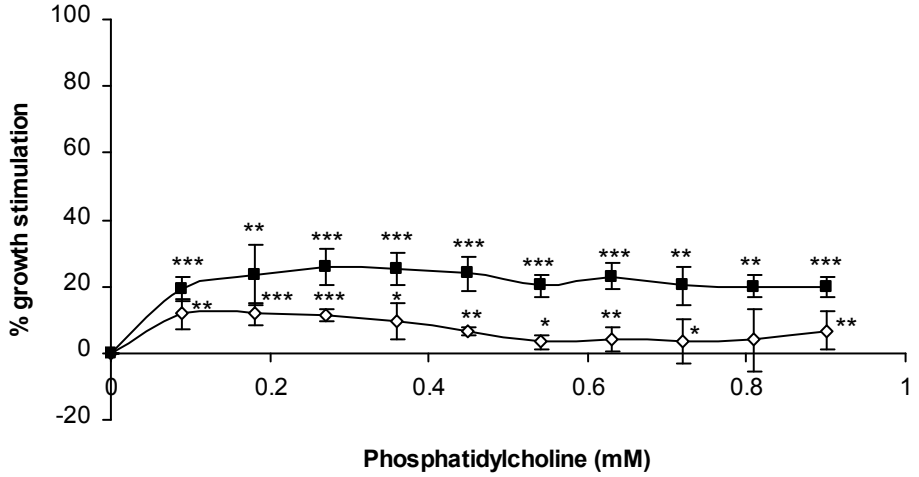


Fig. 2.8. Growth stimulation in SW480 human colon cancer cell line incubated with phosphatidylcholine (DOPC) for 3 (◊) and 7 (■) days assessed using acid phosphatase (AP) assay. The SW480 cells were maintained in Leibovitz-15 medium. Growth stimulation was expressed as a percentage relative to control which was taken to be 0%. Data refer to mean ± SD of three independent experiments. Growth inhibition compared to control without treatment was considered significant when: * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

The inhibitory effects of sphingosine, ceramide, sphingomyelin and lactosylceramide relative to sodium butyrate (5 mM) are shown in **Fig. 2.9**. Butyrate inhibited SW480 cell growth by 85 % after 3 days of exposure. It is apparent that hydrolytic products of sphingomyelin at concentrations as low as 20 μM were more potent than sphingomyelin (1 mM) and were as potent as sodium butyrate (**Fig. 2.9**). Sphingomyelin, by contrast was less potent than sodium butyrate. Lactosylceramide (17.8 μM) was a more potent inhibitor of cell growth than ceramide alone (**Fig. 2.9**).

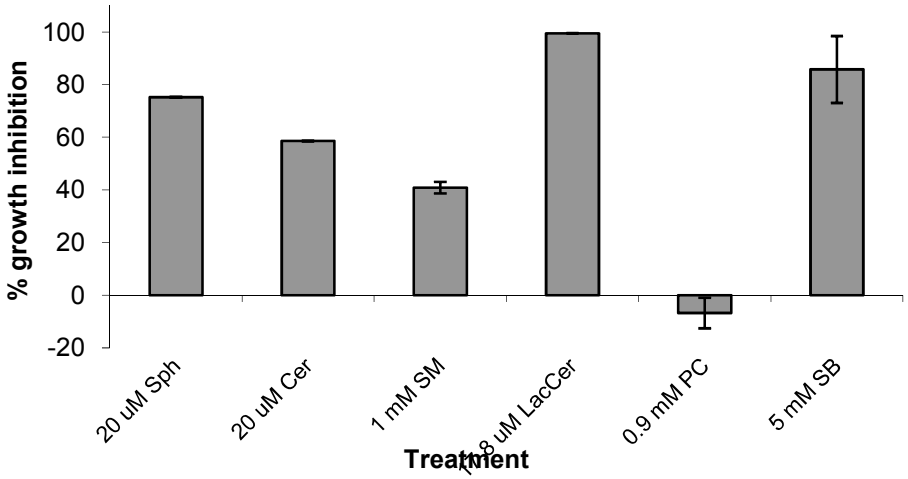


Fig. 2.9. Comparison of antiproliferative activity of different commercial lipids with 5 mM sodium butyrate assessed using AP assay after 3 days of treatment in SW480 cells. The SW480 cells were maintained in Leibovitz-15 medium. Growth inhibition was expressed as a percentage relative to control which was taken to be 0%. Data refer to mean ± SD of three independent experiments.

2.3.4. Compositional analyses of buttermilk samples

Table 2.1 shows compositional analysis of buttermilk samples. The highest amount of total phospholipids was found in buttermilk sample produced from centrifugally separated commercial cream (0.31%), following sample produced from gravitationally separated cream at 4°C (0.13%). However, the latter showed significantly ($P \leq 0.001$) higher contest of total solids (32.98%) and fat (29.2%) than other samples, which comprise total solids and fat in the ranges: 2-6.7% and 1.7-6.4%, respectively).

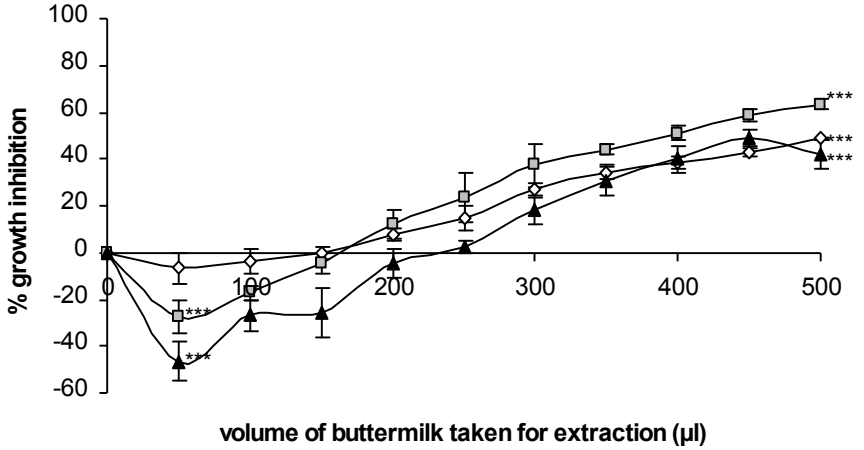
Table 2.1. Compositional analysis of buttermilk samples prepared from: commercial cream centrifugally separated (1), cream from raw milk centrifugally separated (2), cream from raw milk separated naturally by gravitational force at 4°C (3) and 20°C (4), cream from raw milk heat-treated at 95°C for 3s prior centrifugal separation (5). Data obtained by a project team member. All values are expressed as mean %.

Sample	Total solids (%)	Protein (%)	Fat (%)	Phospholipid as % of:		
				Total weight	Fat	Total solids
1	3.02	0.28	1.7	0.31	1.84	1.03
2	6.71	0.30	6.4	0.05	0.74	0.71
3	32.98	0.43	29.2	0.13	0.46	0.40
4	2.03	0.21	1.7	0.05	2.98	2.49
5	4.24	0.32	1.7	0.07	3.81	1.57

2.3.5. Fatty acids

Fatty acid (FA) fractions from each buttermilk sample were prepared as described previously. Micellar solution with sodium taurocholate was incubated with SW480 colon cancer cells for 3, 5 and 7 days. Due to insufficient volume of buttermilk samples received, fatty acid fractions collected were not quantified; hence final results were expressed as volume of buttermilk (µl) subjected to lipid extraction and separation. After 3 days of incubation at lower concentrations (100 and 150 µl buttermilk) FA micellar solutions showed cancer promoting effect, however at 500 µl of buttermilk taken for extraction, FA micellar solutions from all buttermilk samples, except sample 4 (buttermilk produced from cream separated by gravitational force at 20°C) significantly ($P \leq 0.001$) inhibited growth of SW480 colon cancer cells (**Fig. 2.10 a, b, c, d, e**).

a)


100
80
60
40
20
0
-20
-40
-60
0
100
200
300
400
500
% growth inhibition
volume of buttermilk taken for extraction (µl)
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***
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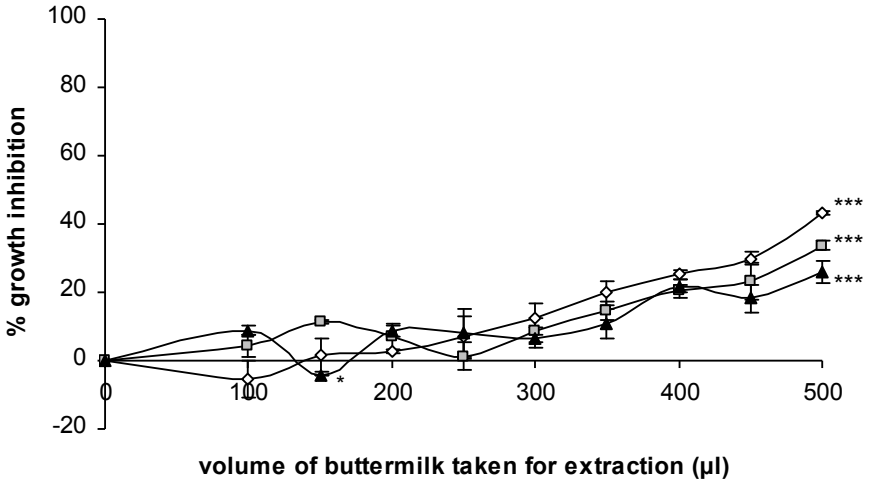
b)


100
80
60
40
20
0
-20
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100
200
300
400
500
% growth inhibition
volume of buttermilk taken for extraction (µl)
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***
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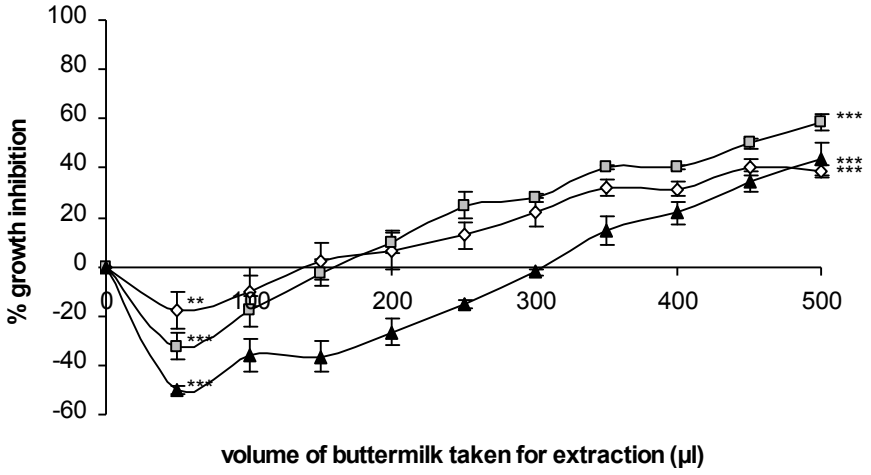
*

c)


100
80
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20
0
-20
-40
-60
0
100
200
300
400
500
% growth inhibition
volume of buttermilk taken for extraction (µl)
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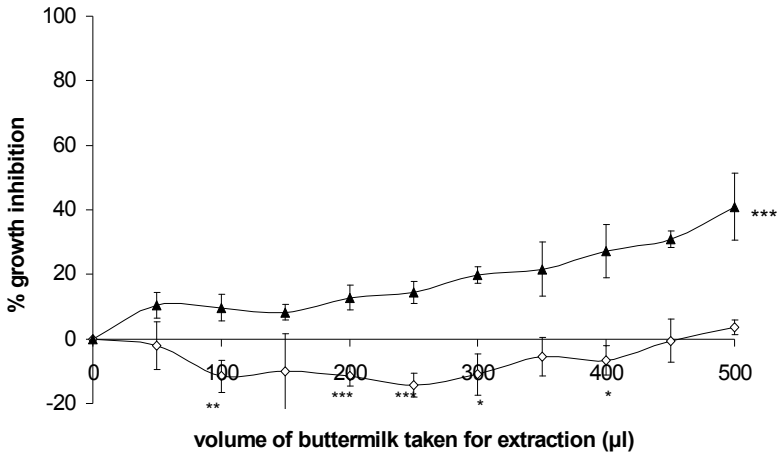
d)



e)

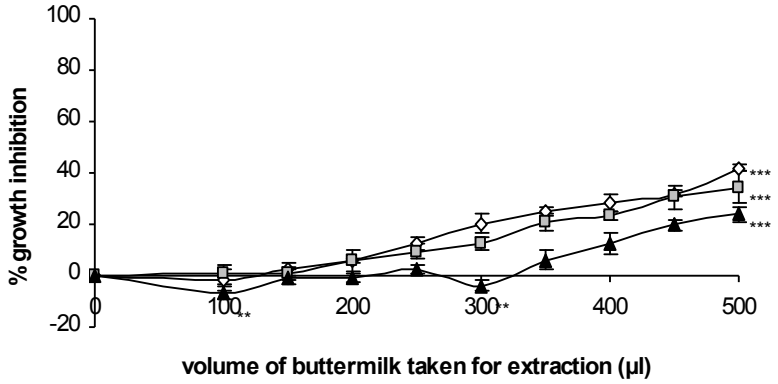


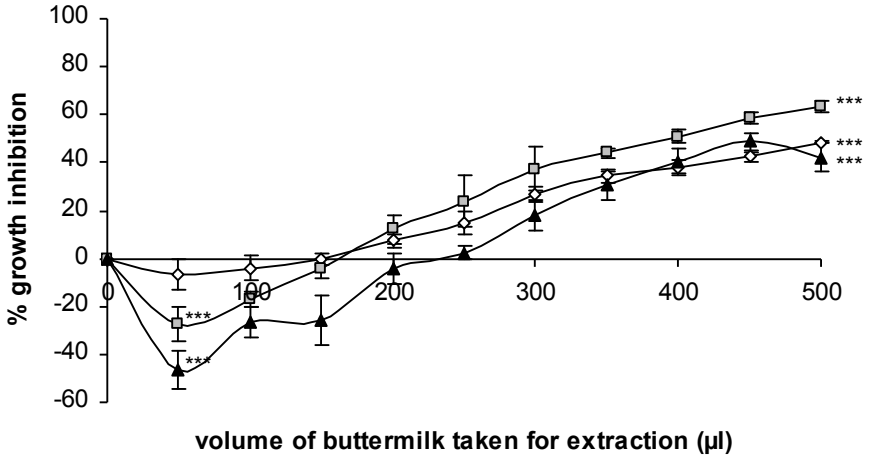
Fig. 2.10. Growth inhibition of SW480 colon cancer cells incubated with micellar solution of fatty acid fractions from buttermilks produced from: commercial cream centrifugally separated (a), raw milk cream centrifugally separated (b), raw milk cream separated by gravitational force at 4°C (c) and 20°C (d), raw milk cream heat-treated at 95°C for 3s prior centrifugal separation (e) assessed using AP assay after 3 (◊), 5 (◘) and 7 (▲) days of treatment. The SW480 cells were maintained in Leibovitz-15 medium. Growth inhibition was expressed as a percentage relative to control which was taken to be 0%. Growth inhibition compared to control without treatment was considered significant when: * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$. Significance was calculated only for

minimum (100 µl) and maximum (500 µl) treatment, as well as for treatment points exhibiting cancer cell growth stimulation.

2.3.6. Polar lipid fraction

Polar lipid (PL) fractions from each buttermilk sample were collected. Micellar solution with sodium taurocholate was incubated with SW480 colon cancer cells. Due to insufficient volume of buttermilk samples received, polar lipid fractions collected were not quantified; hence final results were expressed as volume of buttermilk (µl) subjected to lipid extraction and separation. It could be observed that after 3 days of incubation, at 500 µl of buttermilk taken for extraction, PL micellar solution from buttermilks produced from: commercial cream (1), centrifugal separated cream (2) and gravitational separated cream at 4°C (3) showed similar antiproliferative activity but higher than PL solution from buttermilks produced from: gravitational separated cream at 20°C (4) and heat-treated cream (5) (**Fig. 2.11 a, b, c, d, e**). After 7 days of incubation, at 500 µl of buttermilk taken for extraction, PL micellar solution from buttermilk 1 showed the highest antiproliferative activity, inhibiting SW480 colon cancer cell growth by 71.6%, followed by buttermilks 3 and 5, which inhibited cell growth by 58.2% and 55.5%, respectively. The lowest antiproliferative activity after 7 days of incubation was observed for PL solution from buttermilk 4, which inhibited cancer cells growth by 28.8% (**Fig. 2.11 a, b, c, d, e**).

a)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% growth inhibition
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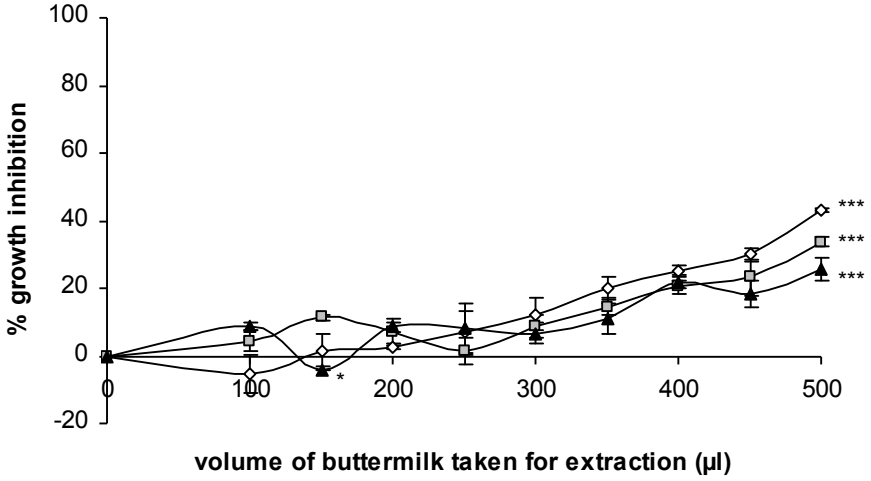
volume of buttermilk taken for extraction (µl)

b)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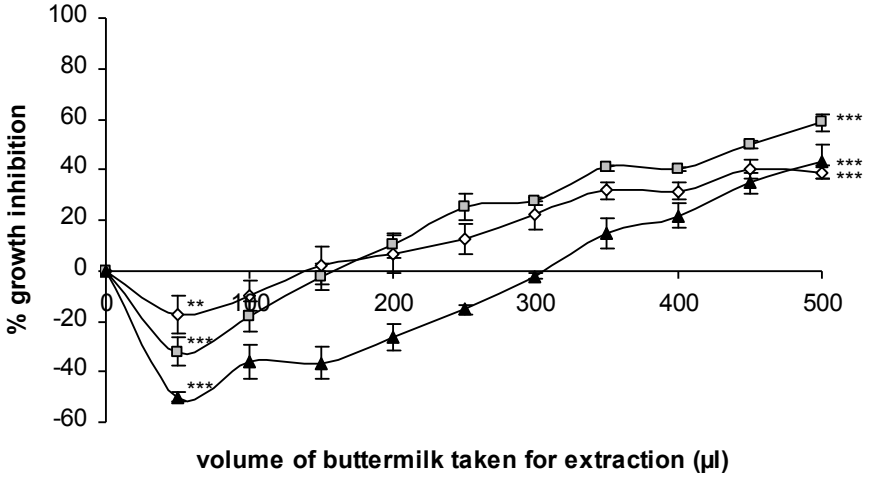
volume of buttermilk taken for extraction (µl)

c)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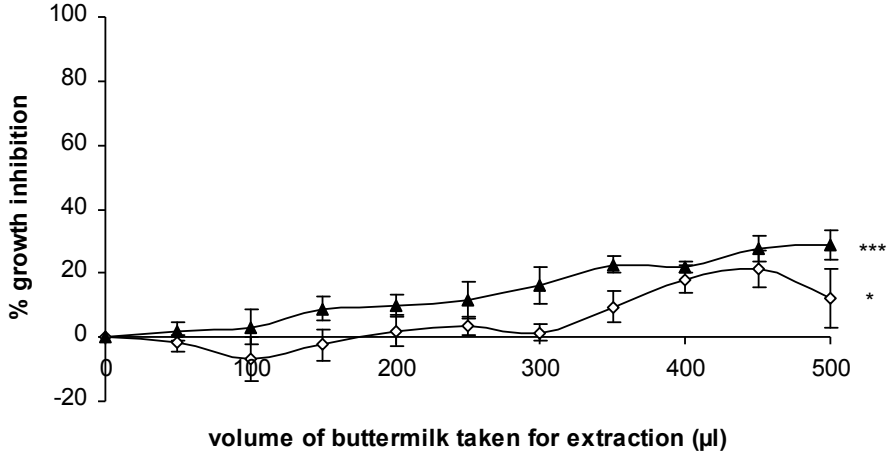
volume of buttermilk taken for extraction (µl)

d)



e)

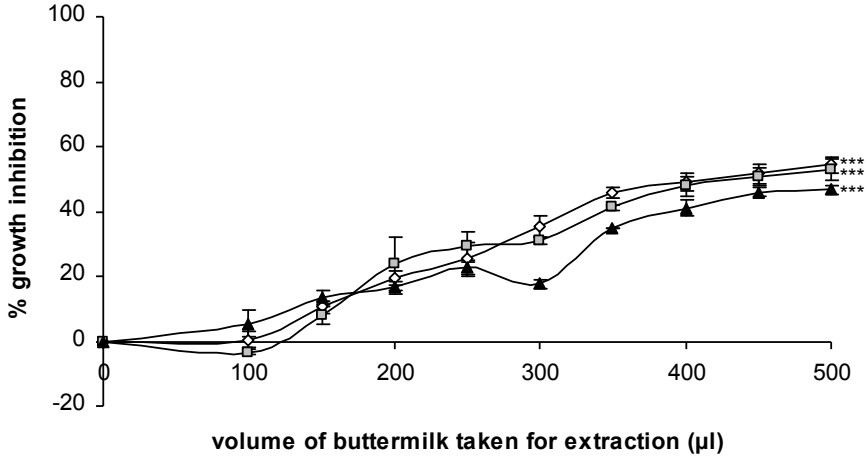
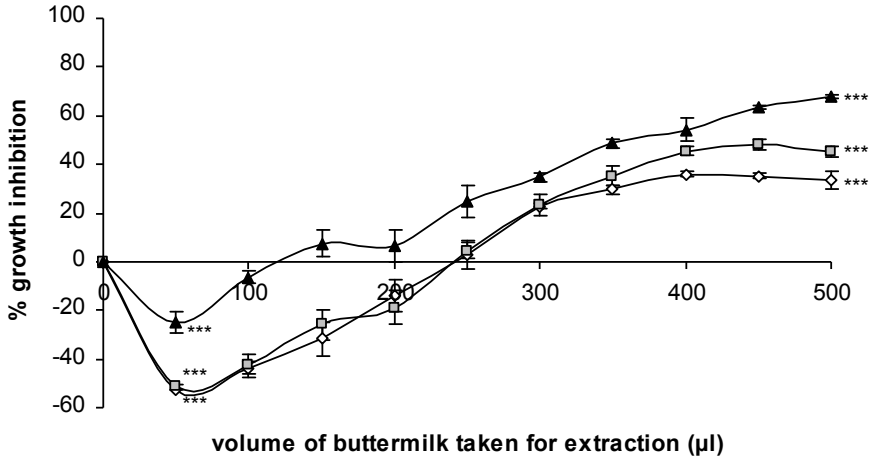


Fig. 2.11. Growth inhibition of SW480 colon cancer cells incubated with micellar solution of polar lipid fractions from buttermilks produced from: commercial cream centrifugally separated (a), cream from raw milk centrifugally separated (b), cream from raw milk separated naturally by gravitational force at 4°C (c) and 20°C (d), cream from raw milk heat-treated at 95°C for 3s prior centrifugal separation (e) assessed using AP assay after 3 (◊), 5(□) and 7 (▲) days of treatment. The SW480 cells were maintained in Leibovitz-15 medium. Growth inhibition was expressed as a percentage relative to control which was taken to be 0%. Growth inhibition compared to control without treatment was considered significant when: * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$. Significance was calculated only for minimum (100 µl) and maximum (500 µl) treatment.

2.3.7. Neutral lipid fraction

Neutral lipid (NL) fractions from each buttermilk sample were collected, as described above. Micellar solution with sodium taurocholate was incubated with SW480 colon cancer cells. Due to insufficient volume of buttermilk samples received, neutral lipid fractions collected were not quantified; hence final results were expressed as volume of buttermilk (µl) subjected to lipid extraction and separation. Micellar solutions of neutral lipids were not optically clear after sonication, even at 50ºC. However, cells were treated with unclear solutions to test possible influence of the unclear NL micellar solutions on SW480 colon cancer cells. At lower concentrations (up to 150 µl of buttermilk taken for extraction), after 3 days of incubation only NL micellar solutions from buttermilks produced from centrifugal separated cream (2) and heat-treated cream (5) showed antiproliferative activity against SW480 colon cancer cells (**Fig. 2.12 a, b, c, d, e**). Furthermore, NL solutions from buttermilks produced from commercial cream (1), gravitational separated creams at 4°C (3) and at 20°C (4) significantly ($P \leq 0.01$ after 3-day incubation and $P \leq 0.001$ after 5 and 7-day incubation) promoted cancer cell growth when compared to control with sodium taurocholate only. However, after 3 days of incubation, at 500 µl of buttermilk taken for extraction, NL solutions from all buttermilks inhibited growth of SW480 colon cancer cells, with buttermilks 2 and 5 being the most potent. At 500 µl taken for extraction, NL solutions from all buttermilks maintained antiproliferative activity (**Fig. 2.12 a, b, c, d, e**).

a)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% growth inhibition
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volume of buttermilk taken for extraction (µl)
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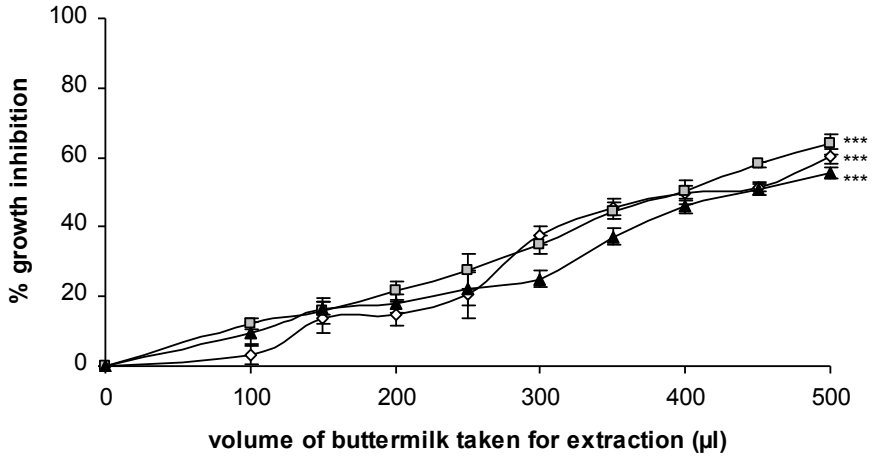
b)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volume of buttermilk taken for extraction (µl)
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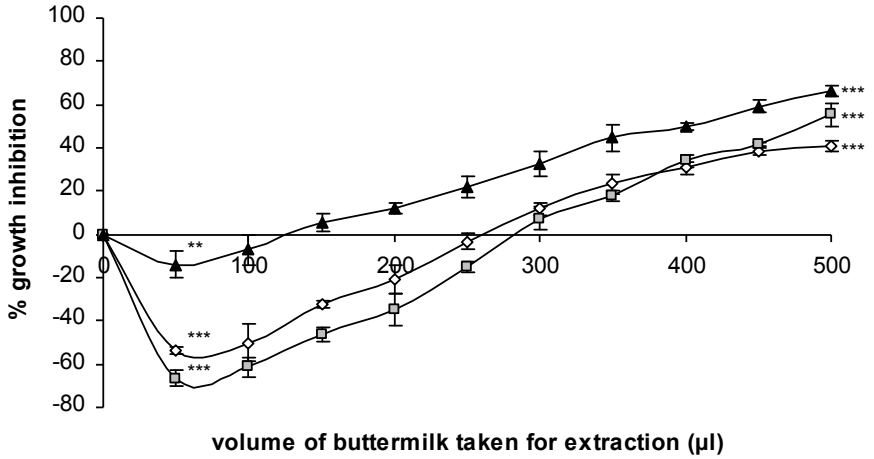
c)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volume of buttermilk taken for extraction (µl)
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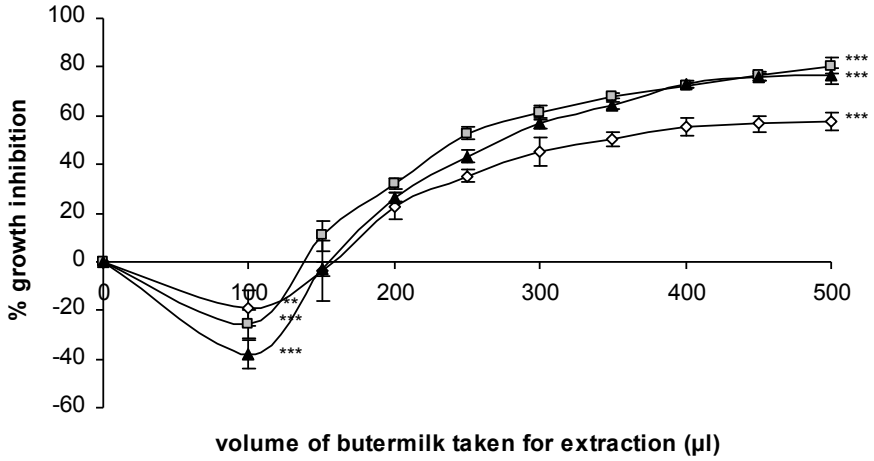


e)

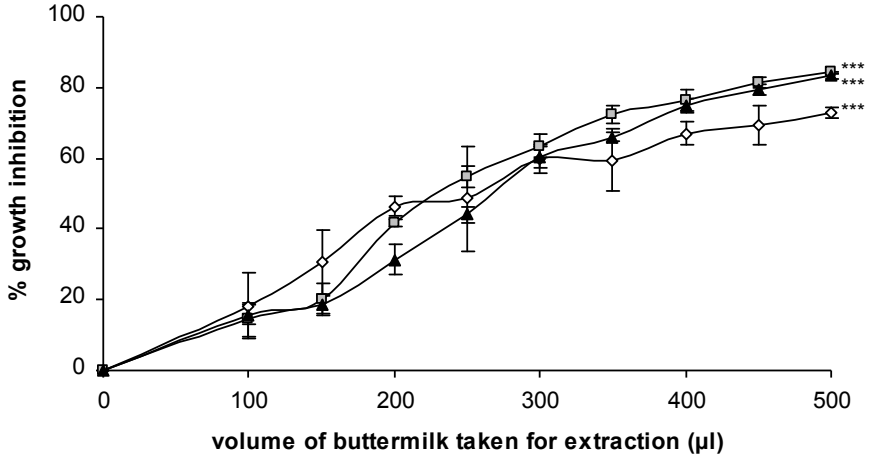
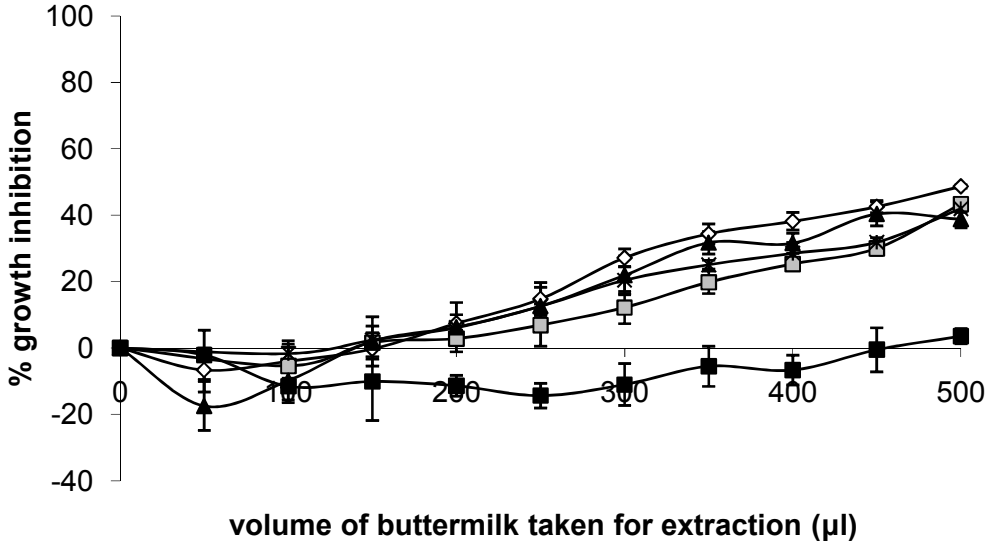


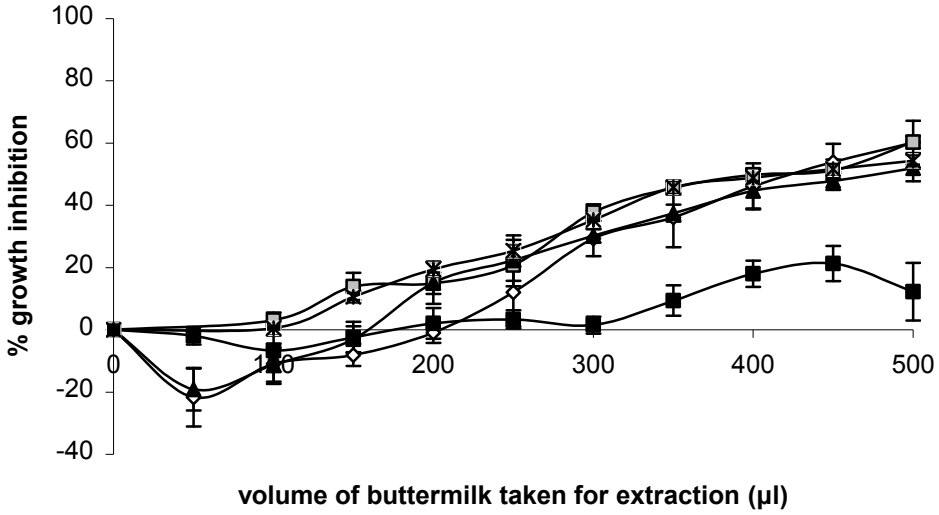
Fig. 2.12. Growth inhibition of SW480 colon cancer cells incubated with micellar solution of neutral lipid fractions from buttermilks produced from: commercial cream centrifugally separated (a), cream from raw milk centrifugally separated (b), cream from raw milk separated naturally by gravitational force at 4°C (c) and 20°C (d), cream from raw milk heat-treated at 95°C for 3s prior centrifugal separation (e) assessed using AP assay after 3 (◊), 5 (◘) and 7 (▲) days of treatment. The SW480 cells were maintained in Leibovitz-15 medium. Growth inhibition was expressed as a percentage relative to control which was taken to be 0%. Growth inhibition compared to control without treatment was considered significant when: * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$. Significance was calculated only for minimum (100 µl) and maximum (500 µl) treatment.

For the comparative purposes, fatty acid, polar lipid and neutral lipid fractions of all buttermilks after 3 days of incubation are shown on **Figure 2.13** (**a**, **b**, **c**, respectively). **Figure 2.14** shows effect of sodium taurocholate on SW480 colon cancer cells. At the concentration of 0.36 mM, which was used for the formation of fatty acid and polar lipid micelles sodium taurocholate did not significantly ($P > 0.05$) inhibit growth of SW480 colon cancer cell (about 5% of growth inhibition). At concentration 1.2 mM NaTC, which was used to form micelles with neutral lipids sodium taurocholate showed high antiproliferative activity (about 35%) against SW480 colon cancer cells ($P \leq 0.001$).

a)



b)



c)

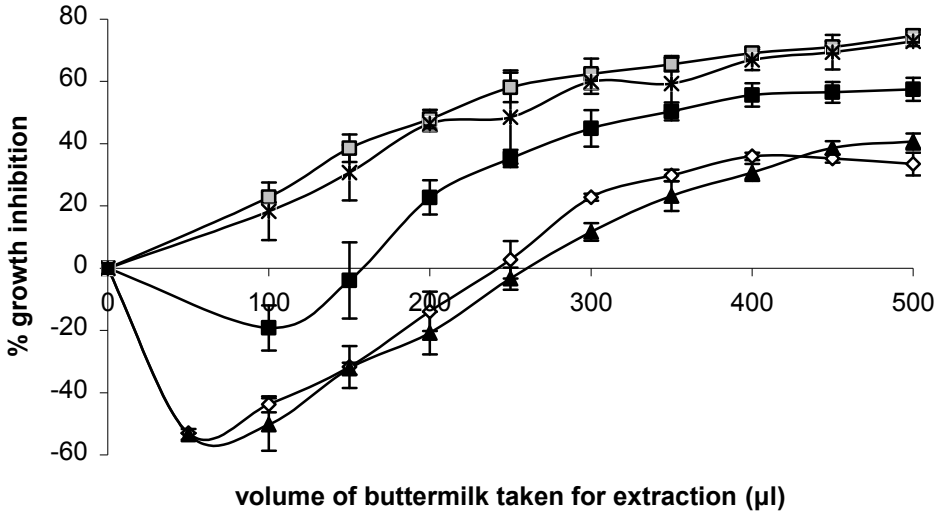


Fig. 2.13. Comparison of antiproliferative activity of fatty acid (a), polar lipid (b) and neutral lipid (c) fractions of buttermilks produced from: commercial cream centrifugally separated (◊), cream from raw milk centrifugally separated (□), cream from raw milk separated naturally by gravitational force at 4°C (▲) and 20°C (■), cream from raw milk heat-treated at 95°C for 3s prior centrifugal separation (✱). Results were assessed after 3 days of incubation in SW480 cells using AP assay. The SW480 cells were maintained in Leibovitz-15 medium.

Growth inhibition was expressed as a percentage relative to control which was taken to be 0%.

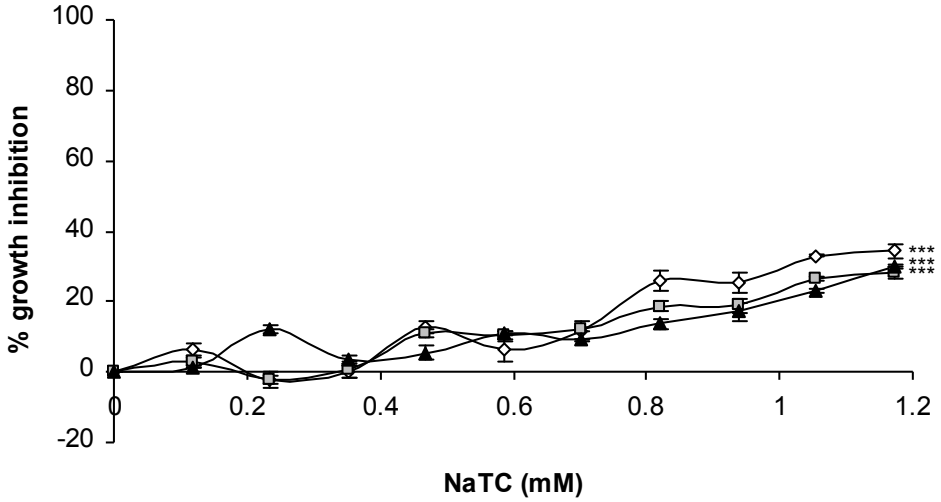


Fig. 2.14. Growth inhibition of SW480 colon cancer cells incubated with sodium taurocholate (NaTC) solution. Results were assessed after 3 (◊), 5 (■) and 7 (▲) days of incubation using AP assay. The SW480 cells were maintained in Leibovitz-15 medium. Growth inhibition was expressed as a percentage relative to control which was taken to be 0%. Growth inhibition compared to control without treatment was considered significant when: * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

2.4. Discussion

The overall aim of this project was to optimize technology for production of buttermilk that is enriched in biologically active components of MFGM. Initially, it was necessary to establish a robust, efficient and economically attractive method for evaluating cytotoxicity of buttermilk samples prepared using different processing methods and for selecting a buttermilk product for further studies. Selection of an appropriate cell based *in vitro* assay for screening buttermilks during the production process is important as it will be used in the post-production phase to monitor the desired effectivity and safety of the product. In choosing between MTS and acid phosphatase (AP) for *in vitro* cytotoxicity testing two decisive factors were considered. The first critical issue was the sensitivity and accuracy of the cytotoxicity assay. Both the MTS and AP assays were comparable with respect to variation but SW480 cell growth in the range up to 10^3 cells was better detected using AP assay than MTS assay, as judged by the linearity of the standard curve. This confirms a previous report that acid phosphatase activity assay demonstrated a higher sensitivity than MTS in determining cell number (Martin and Clynes, 1993, O'Connor *et al*., 1998). Furthermore, complex dietary sphingolipids, such as sphingomyelin and glycosphingolipids have been reported to inhibit the development of colon cancer *in vivo* (Dillehay *et al*., 1994, Schmelz *et al*., 1996, Schmelz *et al*., 2000, Symolon *et al*., 2004). This protective role may be the result of the conversion of complex sphingolipids to bioactive metabolites including sphingosine and ceramide which were reported to inhibit proliferation and stimulate apoptosis also in colon cancer cell culture models, such as HT-29 and HCT-116 (Ahn and Schroeder, 2002, Ahn and Schroeder, 2010), as well as SW480 and T84 cells (Schmelz *et al*., 2001). In our study, we used commercial sphingosine and ceramide to evaluate the suitability and

the difference between MTS and AP assay. In both cases, we found that sphingosine and ceramide showed antiproliferative activity against SW480 colon cancer cells indicating both assays as potentially adequate in our further studies. Sphingolipids which are present in MFGM, such as sphingomyelin and lactosylceramide were also screened for antiproliferative activity. We found that sphingomyelin and lactosylceramide inhibited growth of SW480 colon cancer cells, which confirmed previous findings; hence validated our model for antiproliferative studies. The second factor taken into consideration in choosing a cytotoxicity method for further studies was the economical aspect, by which we included a costing of the reagents for performing assays in large quantities. Due to the large number of buttermilk samples planned for screening in this project, it was decided to use the more sensitive and economically more efficient AP assay in place of the MTS assay.

Development of functional foods designed to combat diseases, such as coronary heart disease, diabetes, hypertension, and cancer is hugely dependent on effective delivery systems to encapsulate, protect and release bioactive lipophilic components, such as sphingolipids, omega-3 fatty acids, conjugated linoleic acid, tributyrin, vitamins, antioxidants, carotenoids, and phytosterols. Dietary lipids must be hydrolysed by pancreatic lipase to fatty acids and monoacylglycerol or by sphingomyelinase to sphingosine and ceramide before they can cross the apical membrane of enterocytes. Although passive diffusion of lipids, such as free fatty acids can take place across the apical brush-border membrane, it is understood that physiological absorption of polar lipolytic products takes place from a micellar phase made up of bile salts (taurocholate) and lecithin (phosphatidylcholine). This chapter reports on the relative effects of exposing colon cancer cells to pure sphingolipid preparations and to lipid fractions of various processed buttermilks. The former were

presented to cells in the form of ethanolic solutions; the latter were presented in micellar form.

Delivery of lipid mixtures in the form of micellar solutions formed with bile salts (e.g. taurocholate) is increasingly being used in *in vitro* studies (Jackson *et al*., 2009, Ho and Storch, 2001, Trotter *et al*., 1996). Monoglycerides and fatty acids can be solubilized to form a colloidal dispersion within bile salt-lecithin mixed micelles (Shiau, 1987). Bile salt micelles solubilize the hydrophobic lipids before they are transported across the unstirred water layer to the intestinal brush border membrane. Ranheim *et al*., (1994) showed that human intestinal cell line Caco-2 can assimilate fatty acids four times faster from a micellar solution than from albumin-bound fatty acids *in vitro* and can incorporate them into triacylglycerols that are subsequently secreted. In recognition that most lipid fractions have a low water solubility and must be incorporated into some kind of delivery system to make them readily taken up by cells in culture, mixed micelles made from taurocholate and phosphatidylcholine (DOPC) were used to deliver fatty acids, polar lipids and neutral lipid fractions of buttermilk to SW480 cells in culture. The concentration of sodium taurocholate (0.36 mM) used for the formation of fatty acid and polar lipid micelles did not significantly ($P > 0.05$) affect the antiproliferative activity of treatments (about 5% cytotoxicity). However, 1.2 mM NaTC, which was used to form micelles with neutral lipids showed high antiproliferative activity (about 35%) against SW480 colon cancer cells. This and the fact that the neutral lipids fractions were not clear when treating cells (indicating that micelles were not formed) suggest that this method of delivering neutral lipids to cells was not successful. Hence, the antiproliferative activity of the neutral lipid fraction from buttermilk was not compared with fatty acid and polar lipid fractions.

In our studies, the polar lipid fraction of buttermilks showed relatively greater anticancer activity in comparison with the free fatty acid fraction (*P* not significantly different for buttermilk sample 4; $P \leq 0.05$ for buttermilk 1; $P \leq 0.001$ for buttermilks 2, 3 and 5). The polar lipid fraction from each buttermilk (except buttermilk 4, which was produced from cream separated at 20ºC using gravitational force) reached about 50-60% cell growth inhibition when 500 µl buttermilk was taken for extraction and micellar solution was incubated with cells for 3, 5 and 7 days. The antiproliferative activity may be associated with sphingolipids in MFGM membrane, which were shown to possess anticancer activity (Simon *et al*., 2009, Schmelz, 2004, Lemonnier *et al*., 2003) or with phosphoglyceride-containing polyunsaturated fatty acids or conjugated linoleic acid present in bovine milk. Fong *et al*. (2007) have studied composition of fatty acids incorporated into bovine MFGM phospholipids using capillary GC. According to Fong *et al*. (2007), the major fatty acids associated with MFGM phospholipids were palmitic, stearic, oleic and linoleic acid. They also observed that SM contained significantly higher proportions of fatty acids with carbon chain length greater than 20. Numerous *in vitro*, *in vivo* and epidemiology studies show that polyunsaturated fatty acids and cis9, trans11-conjugated linoleic acid (CLA) but not saturated, monounsaturated and dienoic fatty acids, have an inhibitory effect on cell proliferation/viability (Nano *et al*., 2003, Igarashi and Miyazawa, 2000, O'Shea *et al*., 2000). It is likely that the presence of unesterified CLA and/or omega-3 fatty acids accounts for the inhibitory effect on cell proliferation of the fractions reported in this study.

In our study, processing of buttermilk did influence the antiproliferative activity of buttermilk fractions in SW480 cells. Irrespective of the type of lipid in each fraction, buttermilks produced from centrifugally separated creams were more potent

than buttermilks produced from gravity-separated creams maintained at 4ºC or 20ºC. Cream that was heated to 95ºC for 3 s prior to centrifugal separation also yielded a greater potency than unheated gravity-separated cream.

Although mixed micelles are commonly used in food and pharmaceutical research, we did not find them suitable for delivering different lipid classes of buttermilk in our *in vitro* studies. It was particularly difficult to evaluate the growth modulatory properties of neutral lipids because the final micellar solution was not optically clear, even at high concentrations of sodium taurocholate (up to 1.2 mM) and when sonication at 50ºC was used. A possible solution to address this difficulty may have been to subject the neutral lipid fraction to *in vitro* digestion by pancreatic lipase and entrap the resulting lipolytic product in liposomes comprising phosphatidylcholine so as to form a stable emulsion for delivery to cells. Microfluidization is increasingly being used to form liposomes for encapsulation of hydrophobic and hydrophilic compounds. This technique has the advantage over traditional techniques in that liposomes are formed by high pressure homogenisation without the use of sonication, detergents, solvents, or alcohols. Microfluidised liposomes prepared from MFGM phospholipid fraction were shown to possess significantly higher entrapment efficiency for hydrophobic β-carotene than liposomes formed from soybean phospholipids (Thompson *et al*., 2009), a property that was attributed to differences in the composition of phospholipid material used.

Given that whole buttermilk contains disrupted MFGM and that the objective of the study was to examine the antiproliferative potential of buttermilk samples containing MFGM material, it was considered that testing whole buttermilks would provide more information than just testing lipid fractions. MFGM is a complex formation of proteins, glycoproteins, lipids (including fatty acids, as well as polar and

neutral lipids) and enzymes (e.g. oxidoreductases and transferases). Some of the MFGM proteins were found to possess beneficial properties on human health, such as fatty acid binding protein (FABP) (Spitsberg *et al*., 1995, Li *et al*., 2009), breast cancer type 1 and 2 susceptibility protein (BRCA1, BRCA2) (Brody and Biesecker, 1998, Vissac *et al*., 2002), butyrophilin (Mana *et al*. 2004, Johns and Bernard, 1999, Stefferl *et al*., 2000), and lactadherin (PAS6/7, MFG-E8) (Zeelenberg *et al*., 2008, Liu *et al*., 2005). Fractionation of this material may adversely affect the bioactivity of its components and would not be representative of MFGM when presented *in vivo* as a whole beverage. Therefore, we decided that further studies on the effect of processed buttermilk samples on growth of colon cancer cells would be conducted using whole buttermilk samples in preference to mixed micelles of its lipid components.

2.5. Conclusions

The data show that acid phosphatase assay is a reliable colorimetric method for screening antiproliferative activity of buttermilk fractions *in vitro*. However, separation of buttermilks into lipid classes and delivery of them into the cells in the form of bile salt micellar solutions does not yield reliable results. In particular, there was difficulty in formation of micellar solution with neutral lipid fractions, even at high concentrations of sodium taurocholate (up to 1.2 mM), which itself was toxic to colon cancer cells. Furthermore, given that whole buttermilk contains disrupted MFGM and that the objective of the study was to examine the antiproliferative potential of buttermilk samples containing MFGM material, it was considered that testing whole buttermilks would provide more information than just testing lipid fractions.

CHAPTER 3

Effect of sweet and fermented buttermilk samples on growth of SW480 human colon cancer cells and FHC normal colon epithelial cells.

The milk fat globule membrane (MFGM) that surrounds fat globules in milk is a natural source of sphingolipids, phoshpholipids and proteins with defined anticancer properties. Dairy processing operations can affect the structure and composition of MFGM, potentially influencing its anticancer activity. The aim of this study was to determine if anticancer activity can be attributed to unfermented and fermented buttermilks which contain fragments of MFGM and examine if different milk processing operations (mode of separation, washing, heating) may influence the antiproliferative activity of resultant buttermilks. After 3 days of incubation of SW480 cells with buttermilk produced from centrifugally separated cream (0.38 mg total solids/ml) there was significant ($P \leq 0.001$) inhibition of growth of SW480 human colon cancer cells by 97.5%, but no toxic effect on FHC human normal colon cells was found, as determined by the acid phosphatase cytotoxicity assay. Corresponding buttermilk produced from washed cream (0.062 mg total solids/ml) inhibited SW480 cell growth by 96.2%. The anticancer activity of buttermilks was retained after heat treatment but was lost after spray drying. Fermented buttermilks inhibited SW480 cell growth to a lesser extent; the highest cytotoxicity was obtained for buttermilk fermented with *Streptococcus thermophilus* HDPC4694, which inhibited SW480 cell growth by 46.6% at concentration of 0.94 mg total solids/ml.

3.1. Introduction

A functional food/beverage is a conventional food/beverage that is consumed as part of a usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions, i.e. they contain bioactive compound. The potential of buttermilk to serve as a food ingredient and as a functional beverage is high due to the beneficial properties of milk fat globule membrane (MFGM) constituents: phospholipids, sphingolipids and membrane proteins. The approximate ratio of proteins, lipids, and carbohydrates within the membrane is 4:3:1 (Spence *et al*., 2009). The major PL fractions consist of phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), phosphatidylinositol (PI) and phosphatidylserine (PS) (Barenholz and Thompson, 1999). Much research work has focused on sphingolipids present in MFGM, which are known to play roles in differentiation, proliferation, immune recognition, as well as transmembrane signalling and cell-to-cell interactions (Okazaki *et al*., 1989, Kim *et al*., 1991, Huwiler *et al*., 2000, Deguchi, 2004, Colombaioni and Garcia-Gil, 2004, Duan and Nilsson, 2009). Because of antiproliferative activity of sphingomyelin break-down products, such as ceramide and sphingosine (Ogretmen and Hannun, 2004), buttermilk has the potential to become a functional beverage assisting in chemopreventive therapies (Schmelz *et al*., 1996, Spitsberg, 2005, Snow *et al*., 2010).

Modern dairy processing operations, such as homogenization, heating, aeration, agitation or drying as well as milk handling and transportation affect MFGM structure and composition (Fox & McSweeney, 1998). Churning of cream during buttermaking results in disruption of MFGM from lipid droplets and the release of fragments into the aqueous phase (buttermilk), a byproduct of butter production. Hence, buttermilk is naturally rich in disrupted MFGM material and its polar lipid

content is relatively high compared with most other dairy products. According to Rombaut *et al*. (2006) when butter is made, up to 42.4% of the membrane material is recovered in the aqueous fraction.

Dairy operations produce buttermilk for commercial use by inoculating milk with starter and probiotic cultures. For example *Lactococcus lactis* is used to stimulate lactic acid production by fermentation of lactose, the primary sugar found in milk. However, unlike buttermilk produced in the traditional fashion, most of the fat in cultured buttermilk is skimmed, leaving only traces of MFGM. Advances in dairy technology, such as membrane filtration have the potential to produce MFGM enriched fractions from buttermilk, thereby converting a low value by-product into a new high value functional beverage.

To address whether a specific functionality could be attributed to a by-product originating from the dairy industry, we prepared a variety of buttermilks and examined the effects of cream separation methods, washing, heating and inoculation with different probiotic cultures on the antiproliferative activity of resultant buttermilks in the SW480 human colon cancer cell line and the FHC human normal colon epithelial cell line. Unfermented buttermilk selectively inhibited growth of SW480 cells. In general, samples heated to 95ºC maintained the growth inhibitory effect towards cancer cells; washing of cream before churning increased anticancer activity of buttermilk. Spray-dried buttermilk powder had negligible activity on growth of cells. Fermented buttermilks were in general less inhibitory comparing to unfermented buttermilks. To our knowledge this is the first *in vitro* study to demonstrate bioactivity of dairy liquid products enriched in MFGM.

3.2. Materials and methods

3.2.1. Preparation of buttermilk samples for antiproliferative studies

3.2.1.1. Cream preparation

Buttermilk samples were generated by a member of the project team. Raw whole milk was collected from the Moorepark Dairy Production Centre (MDPC) farm (Fermoy, Co. Cork, Ireland) and immediately subjected to pasteurization at 72°C for 15s in a thermostatically controlled waterbath and cooled to approximately 20°C in an ice bath. Raw milk was then separated into cream and skim milk using variety of methods. One batch of cream was produced from raw milk separated at 45°C using an Armfield disc bowl centrifuge (Armfield, Ringwood, UK) at max rpm. Raw milk was also allowed to separate naturally by gravitational force at 4°C over a period of 24h. One batch of raw cream was subjected to heat treatment at 95°C for 3s prior centrifugal separation. All creams were then standardized as required using their corresponding skim milk to approximately 40% fat. In order to determine the influence of cream composition on that of the buttermilks subsequently produced, a cream-washing option was included at this stage. An aliquot (500 ml) of each cream sample was washed with simulated milk ultrafiltrate (SMUF, Jenness and Koops, 1962). The cream was suspended in 5 volumes of SMUF and held at 37 °C for 1 h. This mixture was then separated (Armfield disc bowl centrifuge) and cream collected. Washing in SMUF was repeated twice more. Buttermilk samples were prepared from each of the unwashed and washed creams.

3.2.1.2. Buttermilk preparation

The experimentally-prepared creams were churned until the cream emulsion inverted and buttermilk was generated by a project team member, as described in Chapter 2, subsection 2.2.2.1. As a reference a buttermilk sample was also produced from commercial cream purchased in a local shop.

Fermented buttermilks, skim milk and buttermilk powder after spray drying (inlet 180°C, outlet 85°C) were also assayed for anticancer activity. Skim milk was purchased from a local supermarket.

3.2.1.3. Fermentation studies

Buttermilk samples were fermented at 37°C for 12h using individual lactic strains e.g. *Lactococcus lactis* DPC 4268, *Lactobacillus helveticus* DPC TH3, *Streptococcus thermophilus* DPC 4694 and *Lactococcus lactis diacetylactis* DPC 911, which are commonly used in dairy industry as starter cultures. The buttermilks were thermised (65°C, 30 min) prior to inoculation. Bacterial inactivation on completion of incubation was accomplished by heating to 72°C for 15 min before freezing of samples.

3.2.2. Compositional analysis of buttermilk samples

All compositional analysis of buttermilk samples were performed by a project team member, as described in Chapter 2, subsection 2.2.3.

3.2.2.1. Characterisation of MFGM phospho- and sphingolipid components

Extraction and analysis of phospho- and sphingolipids in natural and washed cream buttermilk samples was carried out using HPLC according to the method of Rombaut *et al*. (2005). Briefly, 5 g of sample was diluted with deionised water to 20 ml and mixed thoroughly. Phospho- and sphingolipids were then extracted by addition of 80 ml 2:1 (v/v) chloroform:methanol, followed by mixing. The mixture was allowed to stand and the lower chloroform layer was collected. This procedure was repeated twice using 40 ml of 20:1 (v/v) chloroform:methanol which was added to the upper phase. In the final step 40 ml of 86:14:1 (v/v/v) chloroform:methanol:water containing 0.9 % NaCl (pH 4.5) was used. Each of the lower phases were pooled and evaporated using a rotary vacuum evaporator at 50°C. The lipid extracts were redissolved in 10 ml 2:1 (v/v) chloroform:methanol and stored at –30°C until HPLC analysis.

Lipid extracts were analyzed using a Waters HPLC system (Milford, MA, USA) comprising of a 2695 separations module, column heater and Empower Pro software, which was coupled with an ESA Corona charged aerosol detector (ESA Ltd., Chelmsford, MA, USA). The nebulising gas was N_2, which was supplied at a pressure of 35 psi (2.4 bar). A 150 x 3.2 mm Prevail silica column with 3 µm particle diameter (Alltech, Lokeren, Belgium) was used in addition to a pre and post in line filters with the same packing. The elution programme was a linear gradient with 87.5:12:0.5 (v/v/v) chloroform:methanol:1M formic acid (pH 3, triethylamine) at t = 0 min to 28:60:12 (v/v/v) at t = 16 min. the mobile phase was brought back to the initial conditions at t = 17 min and the column was allowed to equilibrate for the next

4 min (t = 21 min). The flow rate was 0.5 ml/min and injection volume was 20 µl. The samples and column were equilibrated and held at 40°C throughout. Standards used for calibration were phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), sphingomyelin (SM), lactosylceramide (LC) and glucosylceramide (GC).

3.2.3. Cell culture

SW480 human colon cancer cell line and FHC human normal colon cell line were obtained from European Collection of Cell Cultures, ECACC. Culture media and supplements were purchased from Sigma-Aldrich Ireland, Ltd. The SW480 cells were maintained in Leibovitz-15 medium supplemented with L-glutamine (4 mM), foetal bovine serum (FBS) (10%, v/v) and penicillin/streptomycin (1 U/ml). The FHC cells were grown in DMEM/Ham's Nutrient Mixture F-12 supplemented with L-glutamine (2.5 mM), sodium bicarbonate (1.2 g/l), HEPES (25 mM), sodium pyruvate (0.5 mM), FBS (10%, v/v), cholera toxin (10 ng/ml), insulin (0.005 mg/ml), transferrin (0.005 mg/ml), hydrocortisone (100 ng/ml) and penicillin/streptomycin (1 U/ml). Both cell lines were grown at 37°C, with 5% CO_2 for the FHC and in non-CO_2 conditions for the SW480.

3.2.4. Acid phosphatase activity assay

To assess the antiproliferative activity of buttermilk samples, SW480 and the FHC cells were seeded in 96-well plates at densities of $5x10^3$/well and $3.5x10^3$/well,

respectively. Cells were cultured for 24 h to allow the cells to attach and then sweet or fermented buttermilks were diluted in feeding media and were added to the wells with the cells. Cells were also incubated in the presence buttermilk samples which were high-heat treated (95°C, 30 min) prior incubation with cells, skim milk and reconstituted buttermilk powder after spray drying. Controls contained only media. Sodium butyrate (5 mM), a potent apoptotic fatty acid (McBain *et al*., 1997) served as a positive control. Different buttermilk samples were taken for the experiments in the range of concentrations between 0-0.94 mg total solids/ml. All the buttermilk samples were pasteurized prior to treatment by incubation at 72°C for 15 s. The wells with the SW480 cells were covered with parafilm to prevent CO_2 access to the cells. Four replicates of each buttermilk concentration were performed. After 3 days of incubation at 37°C, cell survival was measured by an indirect colorimetric end point assay for acid phosphatase activity (O'Connor *et al*., 1998), as described in Chapter 2, subsection 2.2.5.

3.2.5. Statistical analysis

3.2.5.1. Compositional analyses of buttermilks

Results were analyzed statistically using Graph Pad Prism 3.03 for Windows software package (GraphPad Software, San Diego, California, USA). The ANOVA procedures were performed and mean comparisons were carried out using Tukey's test. Results were considered significantly different at $P \leq 0.05$.

3.2.5.2. Cytotoxicity assays

Three independent experiments were performed in quadruplicate and the Student's *t*-test was used to determine significant difference between treatments and the controls. Results were considered significantly different when: * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

3.3. Results

3.3.1. Compositional analysis of buttermilks

Tables 3.1-3.2 show compositional analysis of buttermilk samples produced from unwashed and washed creams. It was found that buttermilks showed significant ($P \leq 0.05$) differences between their respective total solids, fat, protein and phospholipid contents (**Table 3.1**). Washing of cream leads to decrease in total solids of the resultant buttermilks; hence buttermilks produced from unwashed cream possess significantly ($P \leq 0.05$) higher total solids contents, ranging from 8.87% to 10.61%, when compared to buttermilks produced from washed cream which range from 2.43 to 3.45% (**Table 3.1**). Washing of cream before churning also leads to decrease in serum protein; hence to a higher proportion of MFGM protein material. We also found a decrease in PE, PI, PS, PC and LC in buttermilk produced from washed gravitational separated cream comparing to its corresponding unwashed sample (**Table 3.2**). However, PE, PI and LC contents increased in buttermilk produced from washed centrifugally separated cream comparing to unwashed sample.

Table 3.1. Compositional analysis of unwashed and washed buttermilk samples; data obtained by a project team member. All values are expressed as mean %. [b] Values for phospholipid expressed as a % of fat and total solids. [c, d] Significant ($P \leq 0.05$) difference between buttermilks produced from unwashed and washed cream. Abbreviations: CC, buttermilk produced from commercial cream; CSC, buttermilk produced from centrifugally separated cream; G4SC, buttermilk produced from gravitational separated cream at 4°C.

Sample	Total solids (%)	Protein (%)	MFGM proteins (%)	Lipids (%)	Percentage of phospholipids in:		
					Sample (%)	Total fat (%)	Total solids (%)
Unwashed							
CC	9.36±0.05[c]	3.06±0.23[c]	48.11	0.80±0.00[c]	0.05±0.00[c]	8.05±0.04 [b]	0.56±0.00 [b]
CSC	10.61±0.06[c]	3.82±0.00[c]	46.92	1.85±0.05[c]	0.08±0.00[c]	4.53±0.00 [b]	0.79±0.00 [b]
G4SC	8.87±0.04[c]	3.90±0.02[c]	50.74	1.00±0.00[c]	0.04±0.00[c]	3.94±0.01 [b]	0.44±0.00 [b]
Washed							
CC	3.45±0.02[d]	0.41±0.02[d]	61.67	1.70±0.10[d]	0.03±0.00[d]	1.84±0.00 [b]	1.03±0.00 [b]
CSC	2.43±0.01[d]	0.29±0.00[d]	64.75	0.45±0.05[d]	0.04±0.00[d]	8.22±0.07 [b]	1.52±0.00 [b]
G4SC	2.98±0.01[d]	0.49±0.01[d]	60.55	2.20±0.10[d]	0.03±0.00[d]	1.52±0.00 [b]	1.12±0.00 [b]

Table 3.2. Phospho- and sphingolipid compositions of unwashed and washed buttermilk samples; data obtained by a project team member. All values are expressed as mean %. Abbreviations: CC, buttermilk produced from commercial cream; CSC, buttermilk produced from centrifugally separated cream; G4SC, buttermilk produced from gravitationally separated cream at 4°C; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; GC, glucosylceramide; LC, lactosylceramide; SM, sphingomyelin.

Buttermilk sample	PE (%)	PI (%)	PS (%)	PC (%)	GC (%)	LC (%)	SM (%)
Unwashed							
CC	30.44±0.01	2.21±0.04	8.64±0.11	35.47±0.11	ND	4.42±0.18	18.18±0.06
CSC	34.34±0.04	4.01±0.02	8.49±0.01	33.36±0.03	ND	3.61±0.01	16.19±0.03
G4SC	32.46±0.06	5.90±0.01	12.46±0.07	35.16±0.03	ND	3.27±0.10	10.76±0.08
Washed							
CC	27.05±0.04	8.81±0.06	10.84±0.04	32.79±0.05	ND	1.15±0.02	19.36±0.00
CSC	35.68±0.08	13.62±0.11	5.19±0.08	25.61±0.10	ND	9.51±0.16	10.40±0.20
G4SC	26.80±0.00	2.86±0.01	9.34±0.00	28.99±0.00	ND	3.19±0.04	27.37±0.05

3.3.2. Cytotoxicity

3.3.2.1. Optimization of incubation time and concentration of buttermilk for cytotoxicity assay using SW480 human colon cancer cells

Growth inhibition was expressed as a percentage relative to control which was set at 0%. Data represent the mean ± standard error of the mean of three independent experiments performed in quadruplicate. Buttermilk produced from unwashed centrifugally separated cream was incubated with SW480 colon cancer cells at different concentrations and different time points. The antiproliferative activity of buttermilk (in the concentration range: 0.19-0.94 mg total solids/ml) was assessed after 1, 2 and 3 days of incubation and it was proportional to incubation time and concentration of buttermilk solids used in the experiment (**Fig. 3.1**). At concentration of 0.38 mg total solids/ml buttermilk reduced cell growth by 22.2%, 61.4% and 97.1% after 1, 2 and 3 days of incubation, respectively. Furthermore, after 1 day of incubation with buttermilk solids in the range 0.19 to 0.94 mg/ml, growth inhibition did not exceed 25%; after 2 days growth inhibition was 78.1% at a concentration of 0.94 mg total solids/ml.

3.3.2.2. Comparison of antiproliferative activity of buttermilk samples produced from unwashed and washed creams

Reduced levels of protein in serum during cream washing contribute to an increase relative proportion of MFGM in total solids in the resulting buttermilk samples (Morin *et al*., 2007 a). Hence, buttermilk samples generated from washed and unwashed creams were screened for antiproliferative activity. Washed buttermilks inhibited growth of SW480 colon cancer cells with a greater potency than

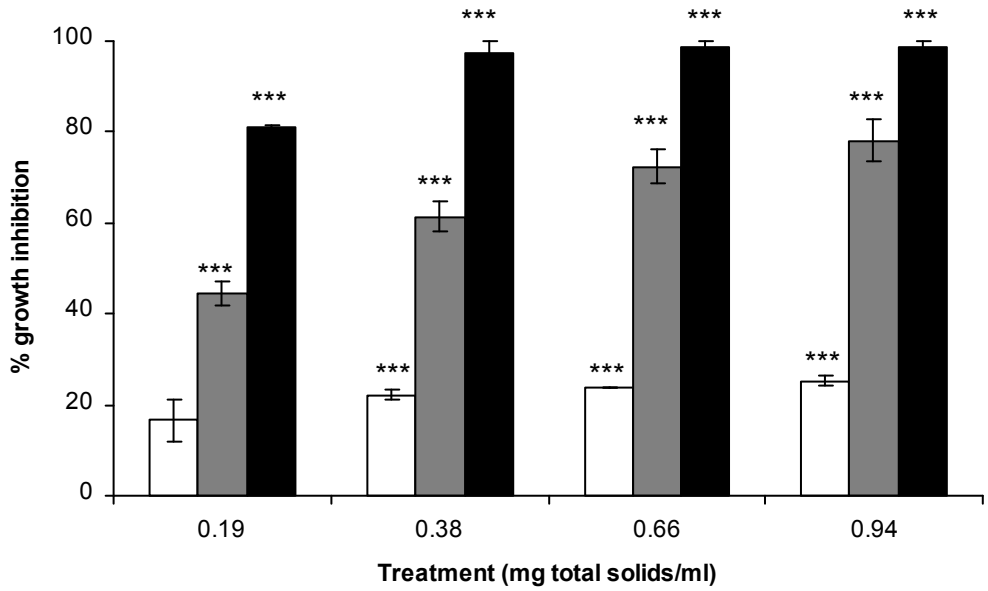


Fig. 3.1. Growth inhibition in SW480 colon cancer cells incubated with buttermilk produced from unwashed centrifugally separated cream for 1 (□), 2 (▣), 3 (■) days assessed using AP assay. The SW480 cells were grown in Leibovitz-15 medium. Data refer to mean ± SD of three independent experiments. Growth inhibition was considered significantly different than control without treatment when: * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$.

their corresponding unwashed samples (**Fig. 3.2**). Buttermilks produced from unwashed: commercial cream, centrifugally separated cream and gravitational separated cream inhibited cell growth by 97.3%, 98.2% and 29.5% at concentrations: 0.4, 0.47 and 0.87 mg total solids/ml, respectively. Their corresponding washed samples were more potent, inhibiting cell growth by 98.5%, 96.2% and 97.8% at concentrations: 0.27, 0.062 and 0.074 mg total solids/ml, respectively. It was also found that pasteurisation of cream before churning at 72°C for 15s did not influence antiproliferative activity of buttermilk on SW480 colon cancer cells.

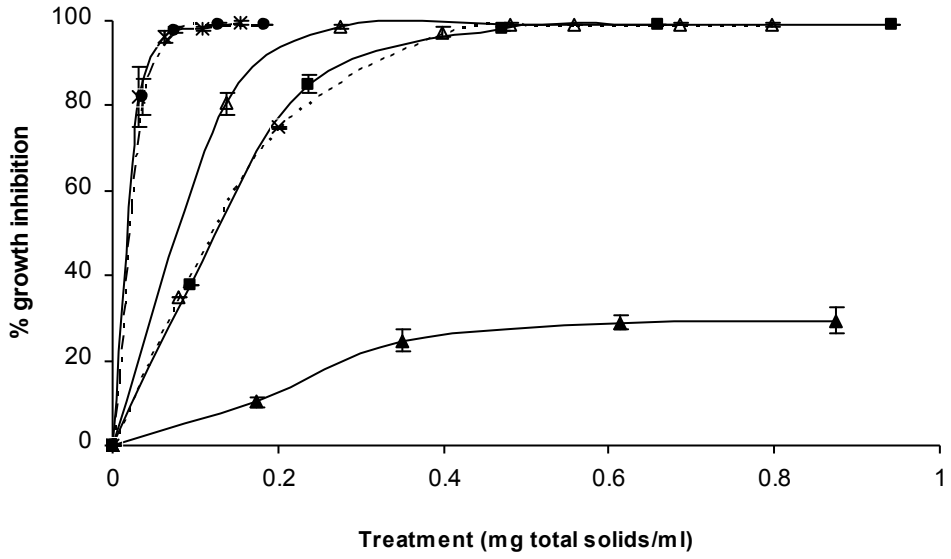


Fig. 3.2. Growth inhibition in SW480 colon cancer cells incubated with buttermilk produced from: unwashed commercial cream (△ with dashed line), unwashed centrifugally separated cream (■), unwashed gravitationally separated cream (▲), washed commercial cream (△ with continuous line), washed centrifugally separated cream (×) and washed gravitationally separated cream (● with dashed line) for 3 days assessed using AP assay. The SW480 cells were grown in Leibovitz-15 medium. Data refer to mean ± SD of three independent experiments. The same volume of different buttermilk samples was taken for the experiments and the concentrations of buttermilks were calculated as mg of total solids in 1 ml of buttermilk taken.

3.3.2.3. Comparison of influence of buttermilk on growth of SW480 colon cancer cells and FHC normal colon epithelial cells

To investigate selectivity of buttermilk's cytotoxic activity on colon cancer cells, buttermilk was also incubated with FHC normal colon epithelial cells. It was

found that the growth inhibitory effect of buttermilk produced from centrifugally separated cream was selective to SW480, as growth inhibition on FHC was only 3.4 ± 3.1% (**Fig. 3.3**). It was also apparent that sodium butyrate inhibited growth to a greater extent in SW480 cells (96.9 ± 1.1%) compared to FHC cells (53.3 ± 0.5%) (**Fig. 3.3**).

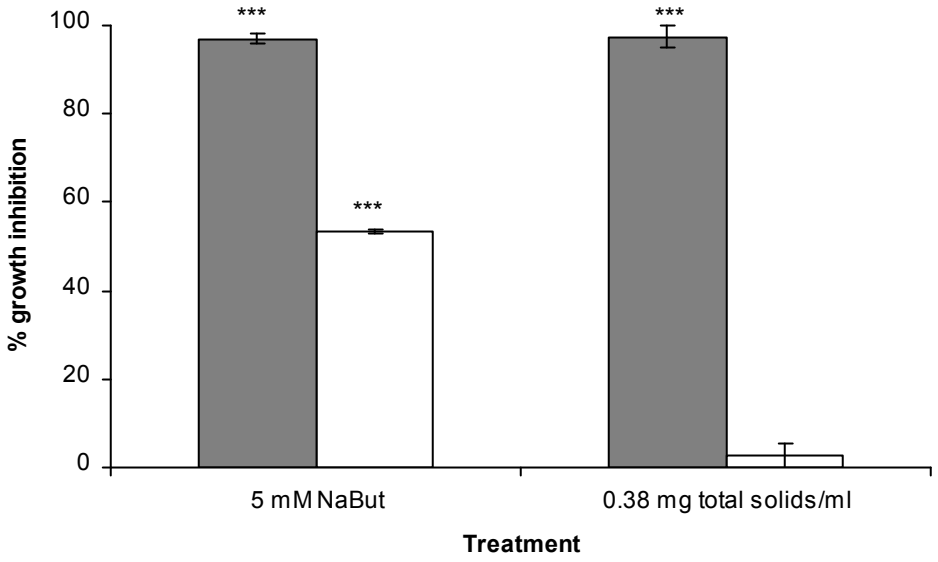


Fig. 3.3. Comparison of growth inhibition in SW480 colon cancer cells (■) and FHC normal colon epithelial cells (□) incubated with sodium butyrate (5 mM) and buttermilk produced from unwashed centrifugally separated cream (0.38 mg total solids/ml) for 3 days assessed using AP assay. Data refer to mean ± SD of three independent experiments. Growth inhibition compared to control without treatment was considered significant when: * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

3.3.2.4. Evaluation of influence of high heat treatment and spray drying on antiproliferative activity of buttermilk

It was found that incubation of buttermilk at 95°C for 30 min prior to treatment with SW480 colon cancer cells did not alter the antiproliferative activity of buttermilk (**Fig. 3.4)**. However, it was found that buttermilk which was spray-dried and then reconstituted to initial volume in water lost all antiproliferative activity. No activity was also observed when incubating SW480 cells with skim milk (**Fig. 3.4**).

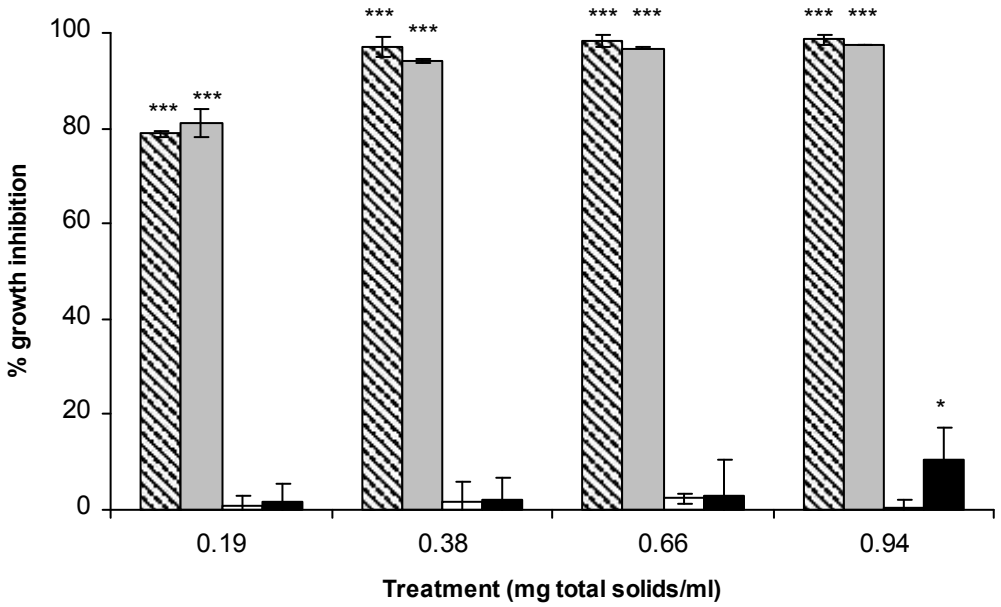


Fig. 3.4. Comparison of growth inhibition in SW480 colon cancer cell line incubated with unheated buttermilk produced from unwashed centrifugally separated cream (▨), high temperature (95°C, 30 min) treated buttermilk produced from unwashed centrifugally separated cream (▣), reconstituted buttermilk after spray drying (■) and skim milk (□) for 3 days assessed using AP assay. The Sw480 and FHC cells were grown in Leibovitz-15 and DMEM/Ham's Nutrient Mixture F-12 media, respectively. Data refer to mean ± SD of three independent experiments. Growth inhibition

compared to control without treatment was considered significant when: * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

3.3.2.5. Antiproliferative activity of fermented buttermilk samples

Buttermilks were also fermented with lactic acid bacteria strains in order to obtain a product beneficial for digestion and balance of intestinal bacteria. For this purpose the following strains were used for fermentations of buttermilks: *Lactococcus lactis* DPC 4268, *Lactobacillus helveticus* DPC TH3, *Streptococcus thermophilus* DPC 4694 and *Lactococcus lactis diacetylactis* DPC 911. It was found that antiproliferative activity of fermented buttermilks was significantly lower ($P \leq 0.001$) compared to unfermented buttermilk produced from centrifugally separated cream (**Fig. 3.5**). Fermented buttermilks inhibited SW480 colon cancer cell growth by 16.8%, 24.8%, 38.2% and 46.6% at buttermilk concentrations: 0.84, 0.78, 0.73 and 0.94 mg total solids/ml, for buttermilks fermented with *Lactococcus lactis* DPC 4268, *Lactobacillus helveticus* DPC TH3, *Lactococcus lactis diacetylactis* DPC 911 and *Streptococcus thermophilus* DPC 4694, respectively. To eliminate a negative influence of low pH of fermented buttermilks on viability of cancer cells, pH of fermented buttermilk was adjusted to 6.5. At this pH, the precipitated casein was reconstituted in the buttermilk solution. Colon cancer cells were treated with the above fermented buttermilk (pH 6.5) as previously described. It was found that pH adjustment to 6.5 did not alter toxicity of fermented buttermilks on SW480 colon cancer cells (**Fig. 3.6**).

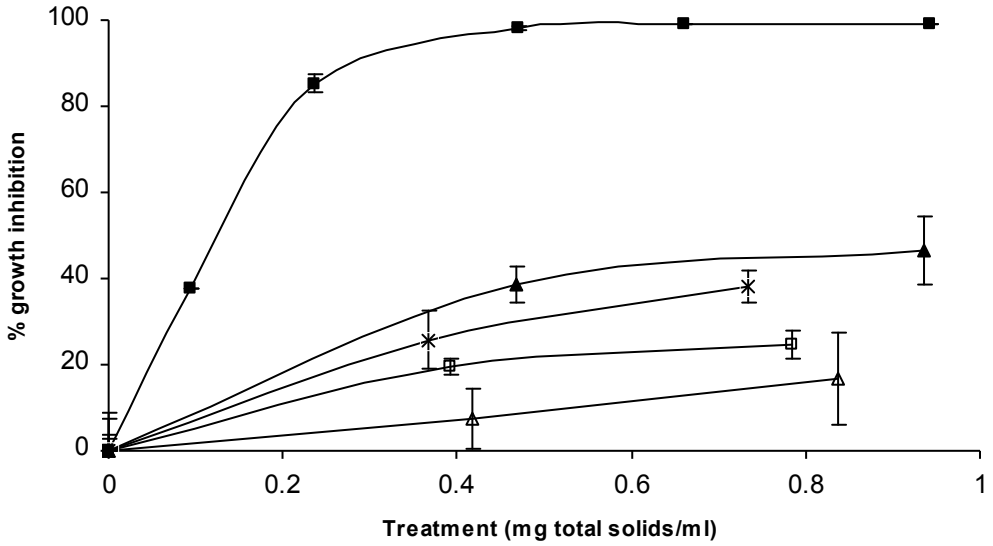


Fig. 3.5. Comparison of growth inhibition in SW480 colon cancer cells incubated with unfermented buttermilk produced from unwashed centrifugally separated cream (■) with buttermilks fermented with strains: *Lactococcus lactis* DPC 4268 (△), *Lactobacillus helveticus* DPC TH3 (□), *Streptococcus thermophilus* DPC 4694 (▲) and *Lactococcus lactis diacetylactis* DPC 911 (×) assessed using AP assay. The same volume of different buttermilk samples was taken for the experiments and the concentrations of buttermilks were calculated as mg of total solids in 1 ml of buttermilk taken. The SW480 cells were maintained in Leibovitz-15 medium. Data refer to mean ± SD of three independent experiments.

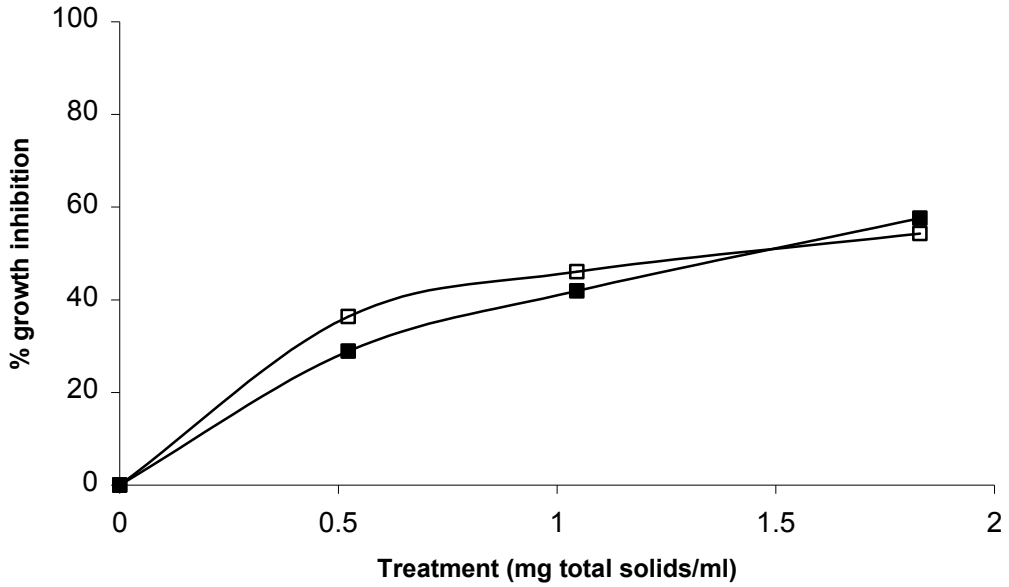


Fig. 3.6. Comparison of growth inhibition in SW480 colon cancer cells incubated with fermented buttermilk at pH 3.7 (■) and fermented buttermilk with pH adjusted to 6.5 (□) assessed using AP assay. The SW480 cells were maintained in Leibovitz-15 medium. Data refer to mean ± SD of three independent experiments. Statistical difference between treatments was not significant ($P > 0.05$).

3.4. Discussion

In this study, we have described the use of a human colon cancer cell line, SW480 to study the effect of processed buttermilks on growth of cancer cells. Using an indirect cytotoxicity assay, it was possible to show that cell growth was inhibited to varying extents depending on the method of cream separation and heat treatments used. Interestingly, fermented buttermilks were relatively less potent in inhibiting cell growth than buttermilks produced from unfermented creams. Demonstration of an antiproliferative activity that was selective to colon cancer cells by buttermilks is novel and is in accordance with recent papers showing that colon cancer cell lines represent a useful model system to investigate the effects of dairy products at the cellular level.

Previously reported studies have demonstrated that several steps in the processing of buttermilk affect the composition and microstructure of buttermilk and MFGM isolates (Morin *et al*., 2007 a). Processing of buttermilk is therefore of particular importance considering the inhibition of tumour growth observed by MFGM lipids in an animal model of colon carcinogenesis (Schmelz *et al*., 2001). We have initiated a study into the positive health benefits of buttermilks produced by modern and traditional cream separation techniques. Washing of cream produced buttermilks that were depleted in casein and whey (Morin *et al*., 2007 b) while centrifugal separation of cream was more effective than gravitational separation in producing buttermilks having higher contents of MFGM proteins and phospholipids (Table 3.1). However, no work to date has evaluated the relative effects of modern cream separation techniques, washing and heating on the antiproliferative effects of buttermilks. To this end, we compared the effects of buttermilk produced from commercial cream, gravitationally and centrifugally separated creams with and

without cream washing on acid phosphatase activity, an enzymatic marker of SW480 cell growth.

Buttermilk produced from unwashed gravitationally separated cream had a significantly lower ($P \leq 0.05$) antiproliferative activity than buttermilks produced from commercial cream and from centrifugally separated cream (Table 3.1; Fig. 3.2). The latter however contained higher phospholipids content and lower MFGM protein content compared with buttermilk produced from gravitationally separated cream, suggesting that antiproliferative activity may be more associated with polar lipid components of the buttermilks than with protein components.

The highest antiproliferative activity was observed for buttermilks produced from washed centrifugally separated cream and washed gravitationally separated cream. Washing of the cream before churning decreased total solids, casein and whey proteins. Similar observations of the effect of washing were reported by Morin *et al*. (2007 b). Analysis of the lipid content (Table 3.2) showed that washing decreased the level of SM in centrifugally separated cream but increased SM in buttermilks produced from commercial cream and gravitationally separated cream. It is apparent therefore that the greater cytotoxicity observed for buttermilk produced from washed centrifugally separated cream compared to other buttermilk samples could not be attributed solely due to SM content. Many studies now suggest that glycosylated sphingolipids are an important defence against tumour growth (Bektas & Spiegel, 2003) and that MFGM is an abundant natural source of glycosylated sphingolipids, in particular lactosylceramide, glucosylceramide and several gangliosides including G_{D3}, G_{D1b1}, G_{M2}, G_{M3} and G_{M1} (Vesper *et al*., 1999). Interestingly, LC was observed to increase by a factor of 2.6 in buttermilk produced from washed centrifugally separated cream and may contribute to the potent cytotoxicity of this sample. It is apparent from

a previous study (Schmelz *et al*., 2001) that a complex mixture of glycosylated sphingolipids, which included 20% LC and 67% SM was more potent than ceramide alone in suppressing tumour formation in colon. Another lipid factor that may have contributed to the greater antiproliferative activity of washed buttermilk samples was a relative decrease in PC that was observed in all washed samples. PC is an important membrane source of cellular arachidonic acid metabolites that play a key role in cancer cell survival and proliferation.

Other phospholipids present in buttermilk samples were PE, PS and PI. PE was the predominant phospholipid and was present in highest amounts in buttermilk produced from washed centrifugally separated cream (Table 3.2). Exposure of colon cancer cells to exogenous buttermilk sources enriched in PS and PE may trigger early steps in the apoptotic cascade and may account for cytotoxicity of buttermilk samples in this study. A recent study showed that exogenous exposure of HepG2 cells to PE, a neutral phospholipid normally present in the inner leaflet of the plasma membrane in mammalian cells induced apoptosis via the Bcl-2/Bax pathway (Yao *et al*., 2009). The normally pro-survival PI was the relatively least abundant of the phosphoglycerolipids in buttermilk samples tested but it did increase in buttermilks produced from washed commercial cream and centrifugally separated cream, suggesting that epidermal growth factor receptor-phosphatidylinositol 3-kinase signaling may have been antagonised by PE and PS or by MFGM protein components, known to possess anticancer activity such as breast cancer type 1 and type 2 susceptibility proteins (Brody & Biesecker, 1998) and fatty acid binding protein (Bansal *et al*., 1989).

Heat-treated buttermilk produced from unwashed centrifugally separated cream showed the same antiproliferative activity as unheated buttermilk. This

suggests that β-lactoglobulin interactions with MFGM as a result of heat treatment had negligible effects on the cellular signalling circuitry that regulates cell growth and death. This is in accordance with an earlier study of the comparative cytotoxic activity of dairy derived ingredients in human cancer cells which demonstrated that β-lactoglobulin had only mild cytotoxic activity (Chan-Remillard & Ozimek, 2008). However, we did observe loss of anticancer activity in the case of reconstituted spray-dried buttermilk powder. Morin *et al*. (2007 a) reported significant loss in phospholipids content in buttermilk after spray drying, mostly from the inner leaflet of the membrane: PE, PS and PI. However, they observed that the relative distribution of PC and SM increased. They suggested that heat combined with rapid water removal and increased ionic strength created by spray drying could lead to formation of complexes within MFGM proteins and phospholipids; hence modifying their solubility in the extraction solvent. In our studies, we have observed total loss of antiproliferative activity of spray-dried buttermilk and we propose that it might be caused by changes within MFGM as suggested first by Morin *et al*. (2007 a). Changes in the structure within and between the membrane components after spray drying and its influence on cytotoxicity require further investigation.

A decrease in anticancer activity was also observed when SW480 cells were incubated with fermented buttermilks, which were pasteurized prior to testing (72ºC, 30s). Of the four fermented buttermilks that were tested, anticancer activity of DPC4694 and DPC911 was greater than that of DPC4268 and DPCTH3. Compositional analysis revealed no significant difference between sweet and fermented buttermilks in MFGM protein content (unpublished data). However, there was a decrease in total phospholipid content in fermentations DPC4694 and DPC911

compared with DPC4268 and DPCTH3 suggesting that phospholipid content is an influencing factor in growth.

To our knowledge, it has not been examined if there are any changes in the structure of MFGM components that might account for the lower anticancer activity of fermented and unfermented buttermilks enriched in MFGM. A comparative examination of the arrangement of protein and lipid complexes within the MFGM of buttermilks including an investigation of the extent to which non MFGM proteins adsorb to MFGM components in fermented buttermilks and the influence of bacterial enzymes (lipases, phospholipases, proteinases or glycosidic hydrolases) on MFGM components may provide an understanding of why fermented buttermilks show lower anticancer activity than unfermented samples. The choice of bacterial strain in DPC4694 (*Streptococcus thermophilus*) and DPC911 (*Lactococcus lactis diacetyllactis)* in fermentations may be a factor in the relative production of bioactives that reduced growth of SW480 cells. Baricault *et al*. (1995) reported that milk fermented with *L. helveticus* and *Bifidobacterium* had a role not only in inhibiting colon cancer cell growth but also in promoting cell differentiation. Casein peptides generated by dairy starter cultures and yogurts fermented with *Streptococcus thermophilus* and *Lactobacillus bulgaricus* are other examples of dairy products that have been shown to exert antiproliferative properties in human colon cancer Caco-2 cells (Macdonald *et al*., 1994, Ganjam *et al*., 1997). Dairy propionibacteria are known to induce apoptosis in colon cancer cells via production of short-chain fatty acids that activate the mitochondrial death pathway (Jan *et al*., 2002). Apoptotic activity in HT-29 and Caco-2 cells was attributed to non-heated propionibacteria strains as heated or lyophilised strains had no antiproliferative effect. It is of interest that in our studies, the fermented buttermilks used for cytotoxicity assays were heat-treated before

incubating with cells, hence the probiotics were rendered inactive prior to investigating the effects of fermented dairy products at the cellular level. Together, these studies suggest that the antiproliferative activity of fermented dairy products is dependent on the presence of live bacteria and may involve activity of bacterial enzymes. It is of interest that some colon cancer cells, like HT-29, lack alkaline sphingomyelinase, the enzyme responsible for intestinal SM digestion. A deletion of exon 4 in alkaline sphingomyelinase cDNA caused loss of enzyme function in HT-29 cells (Wu *et al*., 2004). Hence, the formation of bioactive hydrolytic components, ceramide and sphingosine was significantly reduced. Duan (2007) has shown that lactic acid bacteria used in production of yogurt, in particular *Streptococcus thermophilus* and *Lactobacillus acidophilus* have high levels of alkaline sphingomyelinase and thus the ability to hydrolyse sphingomyelin. It remains to be determined if bacterial strains used in this study also have sphingomyelinase activity which may affect cell growth via affecting levels of bioactive ceramide in SW480 colon cancer cells.

Although the growth of SW480 cells was impaired by sodium butyrate, a short chain fatty acid that induces apoptosis and terminal differentiation in a variety of human colon cancer cell lines, there was a greater level of protection in FHC cells following exposure to butyrate. Comalada *et al*. (2006) reported that the growth-inhibitory effects of butyrate in colonic epithelia depended on cellular phenotype; the greater the level of cell confluence and differentiation the more protection from butyrate.

3.5. Conclusions

The data show that using acid phosphatase activity assay unfermented buttermilk samples within the concentration range 0.19-0.94 mg total solids/ml significantly ($P \leq 0.001$) inhibited growth of SW480 colon cancer cells up to 99% and had no toxic effect on FHC normal colon cells. The cytotoxic activity of buttermilk was found to be similar to that of sodium butyrate, a key apoptotic fatty acid in colon cancer cells. Centrifugal separation of cream and washing of cream before churning increased anticancer activity of buttermilk. It was also found that pasteurisation of cream (72°C, 15s) before churning and high heat treatment of buttermilk (95°C, 30 min) did not change antiproliferative properties of buttermilk. However, the anticancer activity of buttermilk was lost after spray drying suggesting that the phase state of buttermilk may influence the functional activity of its components. Fermented buttermilks demonstrated lower antiproliferative activity compared with unfermented samples. As yet, the reasons for the decrease in anticancer activity in fermented buttermilks remain unclear and need to be further investigated.

CHAPTER 4

Effect of buttermilk samples produced at pilot scale on growth of SW480, HT-29 and Caco-2 human colon cancer cell lines and HepG2 human hepatocellular cancer cell line.

Buttermilk is a rich source of milk fat globule membrane (MFGM), which is naturally formed during lactation by phospholipids, sphingolipids and proteins from mammary cellular membrane. A wide range of health benefits has been attributed to MFGM components, including anti-viral, anti-inflammatory, anti-bacterial and chemopreventive properties mediated through effects on cell growth, transmembrane signalling and cell-to-cell interactions. The aim of this study was to determine if the buttermilks produced in pilot scale and subjected to fractionation using microfiltration exhibit antiproliferative activity *in vitro*. It was found that at the concentration of 0.3 mg total solids/ml feed, retentate and permeate inhibited growth of SW480 and HT-29 colon cancer cells and HepG2 hepatocellular cancer cells by approximately 92-99%, but had no effect on differentiated Caco-2 cells representing normal intestinal enterocytes. Furthermore, buttermilk inhibited growth of cancer cells to a greater extent in cells cultured in medium containing galactose than in media with glucose suggesting that buttermilk components may be selectively toxic to mitochondria.

4.1. Introduction

For centuries churning of butter has been widespread in different populations of the world, however different sources for butter manufacturing had been used, such as whole milk, cream or yoghurt. In United States butter is also made from whey cream (Spence *et al*., 2009; Tunick, 2009). The most popular ethnic method in conventional butter production was to allow whole milk to naturally (gravitationally) separate into cream and skimmed milk. In the mean time, cream was fermented by wild strains of lactic acid bacteria (LAB) acquired from the environment. Butter was then manually churned. Nowadays, to fasten butter-making process and to improve recovery of milk elements, cream is washed repeatedly three to six times with water before churning. During churning, as a result of mechanical treatment, the membrane that surrounds the milk fat droplets, i.e. the milk fat globule membrane (MFGM) is shed and released into buttermilk, whereas the lipid droplets aggregate into butter (Fox and McSweeney, 1998). Technological processes that improve the recovery of health beneficial sphingolipid- rich MFGM in buttermilk are critical for successful development of possible functional beverage. Some researchers have attempted to use different methods for fractionating polar lipid content in buttermilk. Rombaut *et al*., (2006) used microfiltration to separate MFGM from butter serum upon addition of sodium citrate for casein micelle disassociation. Morin *et al*. (2007 a) showed that washing of cream before churning removes casein and whey proteins from buttermilk, yielding a higher proportion of MFGM components in the final product. Moreover, microfiltration combined with supercritical fluid extraction (SFE) (Astaire *et al*., 2003, Spence *et al*., 2009) can selectively remove triglycerides while concentrating polar lipids of the MFGM in buttermilk.

Cancer cells differ from normal cells in that they avidly convert glucose to lactate, even when oxygen is adequate for oxidative phosphorylation. Known as the Warburg effect (Warburg, 1930), this altered pattern of metabolism allows cancer cells to sustain higher proliferative rates and resist cell death signals, particularly those mediated by increased oxidative damage. Glycolytic rates that are about 200 times higher (regardless of aerobic or anaerobic conditions) than in normal cells have been documented (reviewed by Jezek *et al*., 2010, Heiden *et al*., 2009). The high glycolytic phenotype of most cancer cells is triggered by activation of oncogenes, such as *MYC, Ras, Akt* and *PI3K* genes or loss of tumour suppressor genes, such as *p53* which induce transcriptional and post-transcriptional effects in glycolytic enzymes. The large increase in glucose requirement of glycolytically poised cancer cells is met by an increased expression of glucose transporter genes, which encode proteins that facilitate diffusion of glucose and galactose into epithelial cells. GLUT1 glucose transporter, normally restricted to red blood cells and blood-tissue barriers is expressed in many epithelial malignancies. Haber *et al*. (1998) provided evidence that increased GLUT1 expression in tumours from colorectal cancer patients has prognostic significance. Oxidative phosphorylation operates at low capacity in cancer cells leading to a higher than normal level of ROS generation which promotes genetic instability of tumour cells and favours growth.

Furthermore, interest in cancer cell metabolism has re-emerged recently following reports that metabolism may be engineered to scale up production of novel therapeutics (Wlaschin and Hu, 2007). Additionally, it was found that growing cancer cells in DMEM containing galactose instead of glucose is a better cell model for drug safety testing as it forced them to shift from a reliance on

aerobic glycolysis to oxidative phosphorylation for energy production (Marroquin *et al*., 2007). For instance, glucose-grown HepG2 cells were resistant to a wide range of classical mitochondrial toxicants and a selection of drugs having documented mitochondrial toxic activity, whereas galactose-grown cells exhibited an increased rate of oxygen consumption and were more susceptible to mitochondrial toxins. In addition to respiration, biosynthesis of pyrimidines, lipids, hemes and fatty acid oxidation are essential and fundamental metabolic pathways carried out in the mitochondria that ensure cancer cell survival. Many of these pathways provide druggable targets for cancer therapy (reviewed in Tennant *et al*., 2010).

The objective of the present study was to examine the antiproliferative activity of feed, retentate and permeate samples of buttermilk produced at pilot scale from unwashed and washed cream. For this purpose, SW480 and HT-29 colon cancer cell lines were examined in addition to Caco-2 colon cancer cell line, which also served as normal colon epithelial cells when grown in polarizing conditions. Antiproliferative activity of buttermilk samples were also studied using HepG2 hepatocellular carcinoma cells, which is a major organ for lipid metabolism. A comparative analysis of the effect of buttermilk samples on growth of cells relying on oxidative phospohorylation or on aerobic glycolysis was conducted to determine if buttermilks specifically targeted mitochondria.

4.2. Materials and methods

4.2.1. Buttermilk production

Buttermilk samples were generated by a member of the project team from raw whole milk collected from the Moorepark Dairy Production Centre (MDPC) farm, Fermoy, Co. Cork, Ireland. Briefly, in the first pilot plant trial cream was separated from skim milk in separator at 45-50°C. Cream was pasteurized at 72°C for 15sec, and cooled down to 10°C. During churning, buttermilk was separated from butter. Buttermilk (feed) was also microfiltered through an 0.8 μm ceramic membrane at 50°C giving retentate (fraction of buttermilk that did not cross the membrane) and permeate (fraction of buttermilk that crossed the membrane). In the second pilot plant trial, cream was washed before churning. Briefly, cream sample was suspended in 5 volumes of Simulated Milk Ultra-Filtrate (SMUF), held at 37°C for 1h and separated using an Armfield disc bowl centrifuge (Armfield, Ringwood, USA) at max rpm. Washing in SMUF was repeated twice more. Feed, retentate and permeate samples were collected and stored at -30 for further analytical and antiproliferative analyses. Compositional analysis of buttermilk samples was performed by a project team member as described in Chapter 2, subsection 2.2.3. Analysis of MFGM phospho- and sphingolipid components was also performed by a project team member as described in Chapter 3, subsection 3.2.2.1.

4.2.2. Cell culture

SW480, HT-29 and Caco-2 human colon cancer cell lines and HepG2 human hepatocellular cancer cell line were obtained from European Collection of

Cell Cultures, ECACC. Culture media and supplements were purchased from Sigma-Aldrich Ireland, Ltd. The HepG2 and Caco-2 cells were maintained in modified Eagle's medium (MEM). The SW480 and HT-29 cells were grown in Dulbecco's modified Eagle's medium (DMEM) and McCoy's 5A medium, respectively. The HepG2, SW480 and HT-29 cells were also grown in Leibovitz-15 medium. All media were supplemented with L-glutamine (4 mM), fetal bovine serum (FBS) (10%, v/v) and penicillin/streptomycin (1 U/ml). Cells were grown at 37°C, with 5% CO_2 access, except when grown in Leibovitz-15 medium which does not require CO_2.

4.2.3. Acid phosphatase activity assay

To assess antiproliferative activity of buttermilk samples, the cells were grown in parallel in two different media to eliminate possibility of the influence of media components on the antiproliferative activity of buttermilk samples. SW480 cells were seeded in 96-well plates at density of $5x10^3$/well both in Leibovitz-15 and in DMEM medium. HT-29 and HepG2 cells were seeded at density $7x10^3$ cells/well in Leibovitz-15 medium and at density $3.5x10^3$cells/well in McCoy's 5A and MEM media, respectively. To allow the cells to attach, they were cultured for 24 h at 37°C without 5% CO_2 (Leibovitz-15 medium) or with 5% CO_2 access (all the other media). Next, buttermilk samples from both pilot plant trials (feed, retentate and permeate) were diluted in feeding media and were added to the wells with the cells in the range of concentrations between 0-1 mg total solids/ml. Only media was added to the control wells. All the buttermilk samples were sterilized prior to treatment by incubation at 72°C for 15s. The wells with the cells grown in Leibovitz-15 media were covered with parafilm to prevent CO_2 access to the cells.

Four replicates of each buttermilk concentration were performed. After 3 days of incubation at 37°C, cell survival was measured by an indirect colorimetric endpoint assay for acid phosphatase activity (O'Connor *et al*., 1998), as described in Chapter 2, subsection 2.2.5. To perform comparative studies and eliminate influence of media components on buttermilk's cytotoxicity the same growing media was used for all cell lines, Leibovitz-15 supplemented with 4 mM L-glutamine, 10% fetal bovine serum (FBS) and 1 U/ml penicillin/streptomycin (37ºC, without CO_2 access). Cancer cell growth inhibition was expressed as a percentage relative to control which was taken to be 0%. Data represent the mean ± standard error of the mean of three independent experiments performed in quadruplicate.

4.2.4. Preparation of Caco-2 cells monolayer

Caco-2 cells were grown and maintained in supplemented MEM in T-75 flasks at 37°C with 5% CO_2. At about 75% confluency, cells were trypsinized and seeded at density $3.5x10^3$ cells/well in presoaked Millicell hanging cell culture inserts with pore size 3 μm (Millipore Corporation, MA, USA), which were placed in a 12-well plate and incubated at 37°C with 5% CO_2. Medium was replaced carefully every other day. The transendothelial electrical resistance (TEER) values were taken to measure monolayer integrity formation. After 21 days of incubation wells showing the same TEER values were taken for experiment. Differentiated Caco-2 monolayers as well as undifferentiated Caco-2 cells were treated with 1 mg total solids/ml of buttermilk and 5 mM sodium butyrate, which served as positive control. After 3 days of incubation at 37°C with 5% CO_2 cells were washed twice with PBS carefully. Cell viability was

determined using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium) reagent (Promega Corporation, Madison, WI, USA). After 2 h of incubation at 37°C, OD was read at 490nm on Multi-Detection Microplate Reader Synergy™ HT (supplied by BioTek® Instruments, Inc).

4.2.5. Preparation of DMEM media for metabolism study

Dulbecco's Modified Eagle's Medium base was purchased from Sigma Aldrich, Ireland. It was supplemented with 4 mM L-glutamine, 10% fetal bovine serum (FBS) and 1 U/ml penicillin/streptomycin. Media then was divided into three separate batches containing: 4.5 g/l glucose and 3.7 g/l sodium bicarbonate; only 0.9 g/l galactose and 0.9 g/l galactose with 3.7 g/l sodium bicarbonate. Phenol red was added to all media to final concentration of 0.015 g/l, pH was adjusted to 7 and media was sterile filtered.

4.2.6. Statistical analysis

Three independent experiments were performed in quadruplicate for each treatment and the Student's *t*-test (Microsoft Office Excel) was used to determine significant difference between treatments or comparing to the control. Statistical analysis of antiproliferative activity of retentate produced from unwashed cream on SW480 colon cancer cells grown in DMEM media supplemented with different sugar sources (Fig. 4.9) was analyzed by the analysis of variance (ANOVA) coupled with Tukey's post-hoc analysis (PASW statistics 17.0).

4.3. Results

4.3.1. Compositional analysis of buttermilks

Tables 4.1-4.4 show compositional analysis of buttermilk samples produced from unwashed and washed creams, provided by project team member . Comparative analysis of phospholipid distribution of feed from washed cream showed a greater concentration of PE and PS that averaged 26.34 % and 14.47% respectively compared with 18.42% and 5.26%, respectively in feed from unwashed cream. Washing had little effect on sphingomyelin content of feed. However, feed from washed cream was depleted of PC and PI by 33% and 50%, respectively. Retentate from washed cream was depleted of SM, PC, PS, PI and GC by 60%, 75%, 30%, 80% and 50% and was enriched in both LC (44.26% relative to 1.55%) and PE (21% relative to 12.93%) compared to retentate from unwashed cream. Unfortunately, analytical composition of permeate fraction was not supplied, hence it is impossible to perform analysis on possible components associated with lower antiproliferative activity of permeate compared to feed and retentate fractions.

Table 4.1. Compositional analyses of feed, retentate and permeate produced from unwashed cream, obtained by a project team member. All values are expressed as mean %.

Sample	Total solids (%)	Protein (%)	MFGM proteins (%)	Lipids (%)	Percentage of phospholipids in:			Lactose (%)
					Sample	Total fat	Total solids	
Feed	9.07	3.18	0.93	1.15	0.14	12.65	1.60	4.03
Retentate	10.35	3.47	2.49	2.78	0.20	7.16	1.93	3.33
Permeate	8.00	3.04	1.13	0.64	0.11	17.01	1.05	3.66

Table 4.2. Phospho- and sphingolipid composition of feed, retentate and permeate produced from unwashed cream, obtained by a project team member. All values are expressed as mean %. Abbreviations: CC, buttermilk produced from commercial cream; CSC, buttermilk produced from centrifugally separated cream; G4SC, buttermilk produced from gravitationally separated cream at 4°C; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; GC, glucosylceramide; LC, lactosylceramide; SM, sphingomyelin.

Sample	GC (%)	LC (%)	PE (%)	PI (%)	PS (%)	PC (%)	SM (%)
Feed	0	0	18.42	17.41	5.26	33.21	25.7
Retentate	1.01	1.55	12.93	17.19	6.22	31.57	29.52
Permeate	0.58	1.20	15.92	19.15	5.77	35.8	21.58

Table 4.3. Compositional analysis of feed, retentate, permeate produced from washed cream, obtained by a project team member. All values are expressed as mean %.

Sample	Total solids (%)	Protein (%)	MFGM proteins (%)	Lipids (%)	Percentage of phospholipids in:			Lactose (%)
					Sample	Total fat	Total solids	
Feed	8.19	0.43	0.43	5.55	0.098	1.76	1.19	2.18
Retentate	12.38	0.19	0.19	11.25	0.088	0.78	0.71	0.89

Table 4.4. Phospho- and sphingolipid composition of feed, retentate and permeate produced from washed cream, obtained by a project team member. All values are expressed as mean %. Abbreviations: CC, buttermilk produced from commercial cream; CSC, buttermilk produced from centrifugally separated cream; G4SC, buttermilk produced from gravitationally separated cream at 4°C; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; GC, glucosylceramide; LC, lactosylceramide; SM, sphingomyelin.

Sample	GC (%)	LC (%)	PE (%)	PI (%)	PS (%)	PC (%)	SM (%)
Feed	0	0	26.34	9.03	14.47	23.68	26.48
Retentate	0.59	44.26	21.00	3.83	9.87	8.57	11.87

4.3.2. Cytotoxicity

4.3.2.1. Antiproliferative activity of feed, retentate and permeate samples produced from unwashed cream

Three fractions of buttermilk filtrates feed, retentate and permeate produced from unwashed cream were incubated with SW480 and HT-29 colon cancer cells and HepG2 hepatocellular cancer cells in Leibovitz-15 medium at different concentrations ranging from 0-0.3 mg total solids/ml. Buttermilk samples at concentrations ranging from 0-1 mg total solids/ml were also incubated with SW480 and HT-29 colon cancer cells and HepG2 hepatocellular cancer cells grown in DMEM, McCoy's 5A and MEM medium, respectively. The cytotoxicity was assessed after 3 days of incubation. There was a dose-dependent effect of the feed, retentate and permeate samples on antiproliferative activity of SW480, HT-29 and HepG2 cancer cells.

At a concentration of 0.3 mg total solids/ml, all buttermilk fractions produced from unwashed cream reached about 95-99% cell growth inhibition in all three cancer cell lines grown in Leibovitz-15 medium (**Fig. 4.1**). However, at lower concentrations it was observed that feed fraction was less potent in inhibiting SW480, HT-29 and HepG2 cell growth compared to retentate and permeate fractions. In Leibovitz-15 medium SW480, HT-29 and HepG2 cell growth was inhibited by feed by 15.5, 26.2 and 33.2%, respectively; by retentate by 44.3, 69.7 and 64.5%, respectively and by permeate by 55.6, 88.2 and 91.4%, respectively at concentration of 0.03 mg total solids/ml (**Fig. 4.1**). At a concentration of 0.06 mg total solids/ml, cell growth inhibition by retentate and permeate was significantly higher ($P \leq 0.01$ and $P \leq 0.001$, respectively) than by

feed in all three cell lines. SW480, HT-29 and HepG2 cell growth was inhibited by feed by 34.1, 55 and 56.7%, respectively; by retentate by 73.2, 90.6 and 93.6%, respectively and by permeate by 82.8, 94.6 and 97.1%, respectively. These results correlate with our previously reported, obtained from buttermilk produced in laboratory scale. Production of feed fraction of buttermilk produced from unwashed cream yielded a level of antiproliferative activity comparable to buttermilk produced from pasteurized cream (72ºC, 15 sec) in laboratory scale (**Fig. 4.3**).

When growing and treating cells in their assigned media, buttermilk feed, retentate and permeate fractions inhibited cell growth in all three cell lines with significantly lower potency than when the same cell lines were grown in Leibovitz-15 medium (**Fig. 4.2**). Maximum buttermilk concentration used for cell treatment when grown in their assigned media was 1 mg total solids/ml and even then, there was a high cell survival observed. At concentration 1 mg total solids/ml feed fraction inhibited SW480, HT-29 and HepG2 cell growth by 31.6, 38.6 and 71.2%, respectively; retentate fraction by 57.4, 54.6 and 83.1%, respectively and permeate by 48.1, 52.1 and 91.6%, respectively. SW480 and HT-29 colon cancer cells responded similarily to feed, retentate and permeate fractions. However, all three buttermilk fractions were relatively more potent in inhibiting growth of HepG2 liver cancer cells.

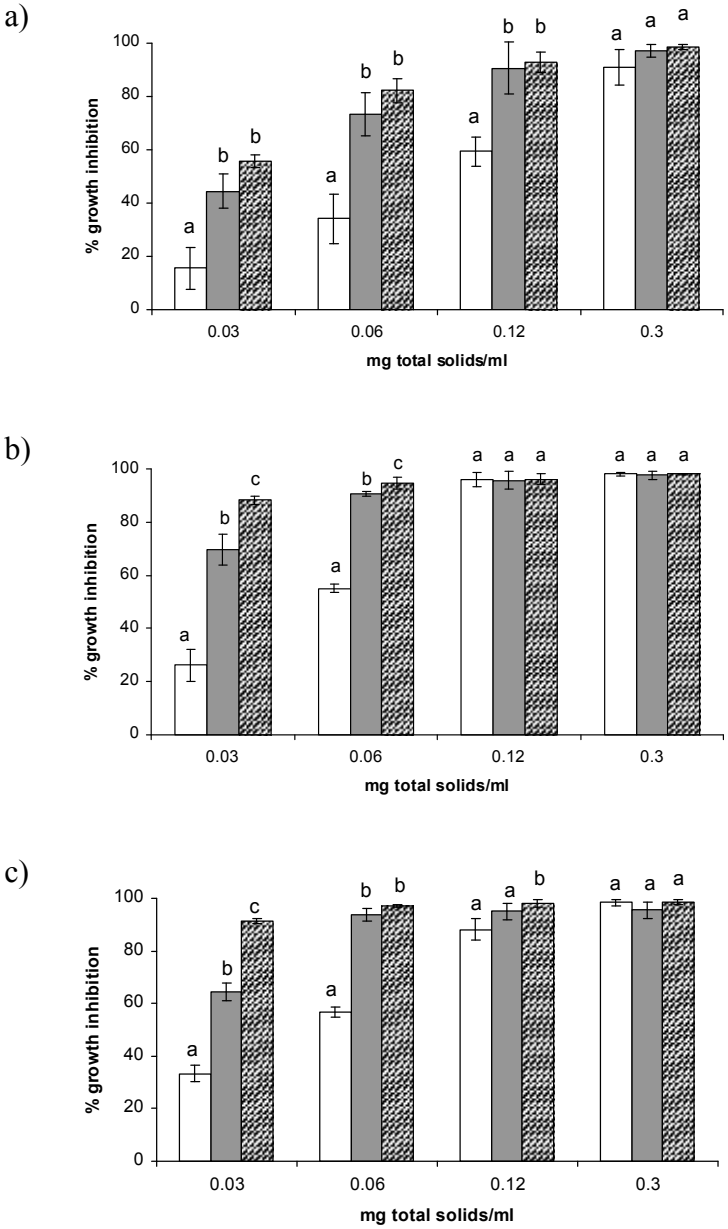
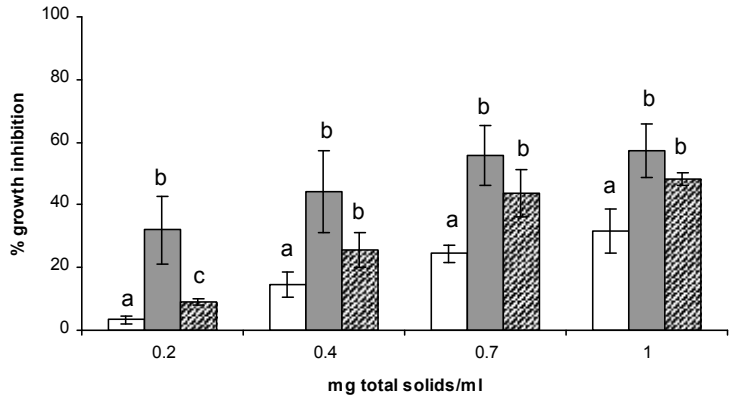


Fig. 4.1. Antiproliferative activity of feed (□), retentate (■) and permeate (▩) fractions produced from unwashed cream on growth of a) SW480, b) HT-29 and c) HepG2 cancer cells assessed using AP assay. Cells were grown and treated in galactose-containing Leibovitz-15 medium. Data refer to mean ± SD of three independent experiments. ^{a,b,c} values without a common subscript are significantly different ($P \leq 0.05$) at given concentration.

a)



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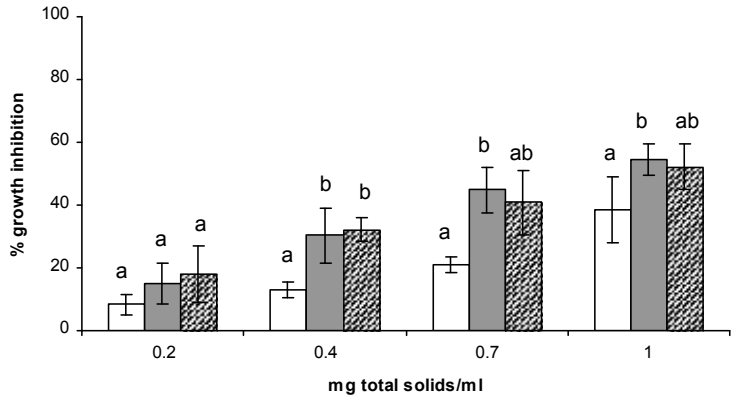


c)

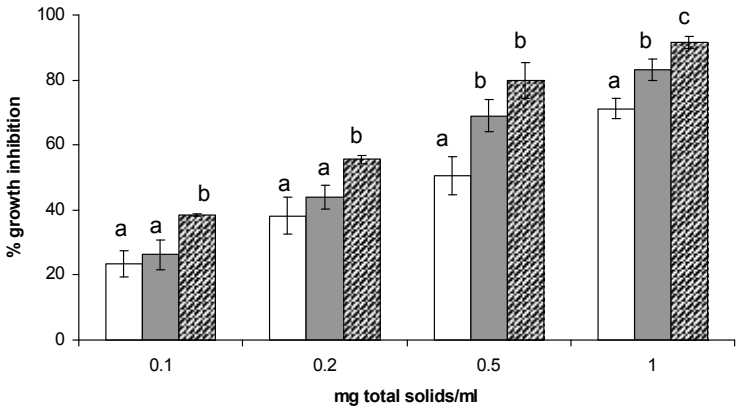


Fig. 4.2. Antiproliferative activity of feed (□), retentate (■) and permeate (▨) fractions produced from unwashed cream on growth of a) SW480, b) HT-29 and c) HepG2 cancer cells maintained and treated in their corresponding media, contaning glucose; assessed using AP assay. Data refer to mean ± SD of three independent experiments. ^{a,b,c} values without a common subscript are significantly different ($P \leq 0.05$) at given concentration.

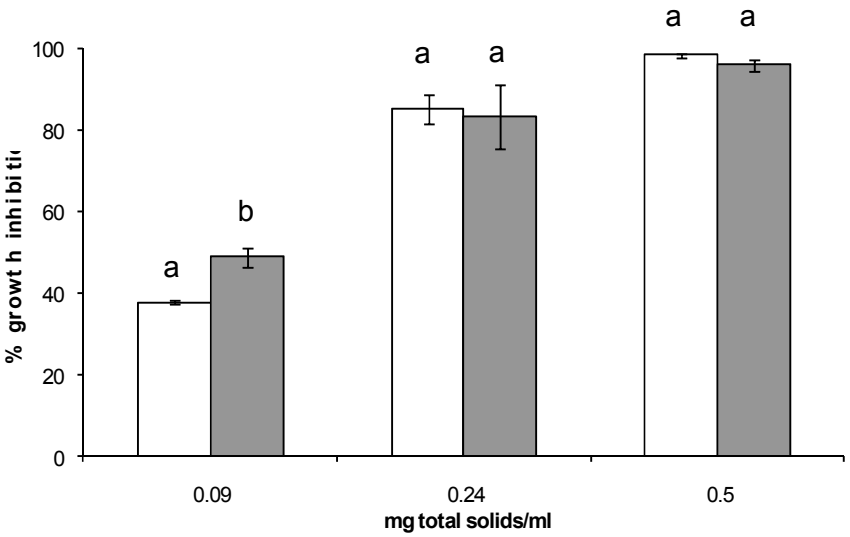


Fig. 4.3. Comparison of growth inhibition of SW480 colon cancer cells incubated with feed produced from unwashed cream in pilot plant (■) to buttermilk produced in laboratory scale (□) at given concentrations; assessed using AP assay. The SW480 cells were grown in Leibovitz-15 medium. Data refer to mean ± SD of three independent experiments. [a,b] values without a common subscript are significantly different ($P \leq 0.05$) at given concentration.

4.3.2.2. Antiproliferative activity of feed, retentate and permeate samples produced from washed cream

Different tendency could be observed in cell growth treated with feed, retentate and permeate buttermilk samples produced from washed cream (**Fig. 4.4**). Buttermilk fractions were incubated with SW480, HT-29 and HepG2 cells grown in Leibovitz-15 medium as described in Material and Methods. All three fractions inhibited SW480 cell growth at lower potency than HT-29 and HepG2 cell lines. At concentrations 0.06-0.12 mg total solids/ml permeate was

significantly less potent in inhibiting HT-29 and HepG2 cells growth than feed ($P \leq 0.01$ and $P \leq 0.05$, respectively) and retentate ($P \leq 0.05$ for both cell lines). Moreover, permeate produced from washed cream was significantly ($P \leq 0.001$) less potent in inhibiting cancer cell growth than permeate fraction produced from unwashed cream at concentrations 0.03-0.12 mg total solids/ml. However, in HepG2 hepatocellular cancer cells retentate at concentrations 0.03-0.06 mg total solids/ml showed significantly higher ability in inhibiting cell growth comparing to feed ($P \leq 0.01$) and permeate ($P \leq 0.05$). At concentration 0.03 mg total solids/ml none of the buttermilk fractions produced from washed cream reached IC50 in inhibiting cell growth. SW480, HT-29 and HepG2 cell growth was inhibited by feed by 9.8, 37.7 and 13.4%, respectively; by retentate by 19.9, 43.6 and 39.1%, respectively and by permeate by 16.7, 35.2 and 15.3%, respectively at concentration of 0.03 mg total solids/ml. At concentration of 0.06 mg total solids/ml cell growth of SW480, HT-29 and HepG2 cells was inhibited by feed by 38.3, 77 and 42%, respectively; by retentate by 48.3, 72.5 and 76.1%, respectively and by permeate by 31.5, 59.6 and 33.8%, respectively.

Feed, retentate and permeate fractions produced from washed cream inhibited growth of SW480, HT-29 and HepG2 cell lines grown in their assigned media with lower potency than when growing cells in Leibovitz-15 medium (**Fig. 4.5**). At concentration 1 mg total solids/ml feed fraction inhibited SW480, HT-29 and HepG2 cell growth by 30.5, 30.4 and 56.7%, respectively; retentate fraction by 37.8, 31.7 and 56.1%, respectively and permeate by 20.5, 28.1 and 54.6%, respectively. The highest cell growth inhibition was observed for HepG2 liver cancer cells and it was similar for all three buttermilk fractions.

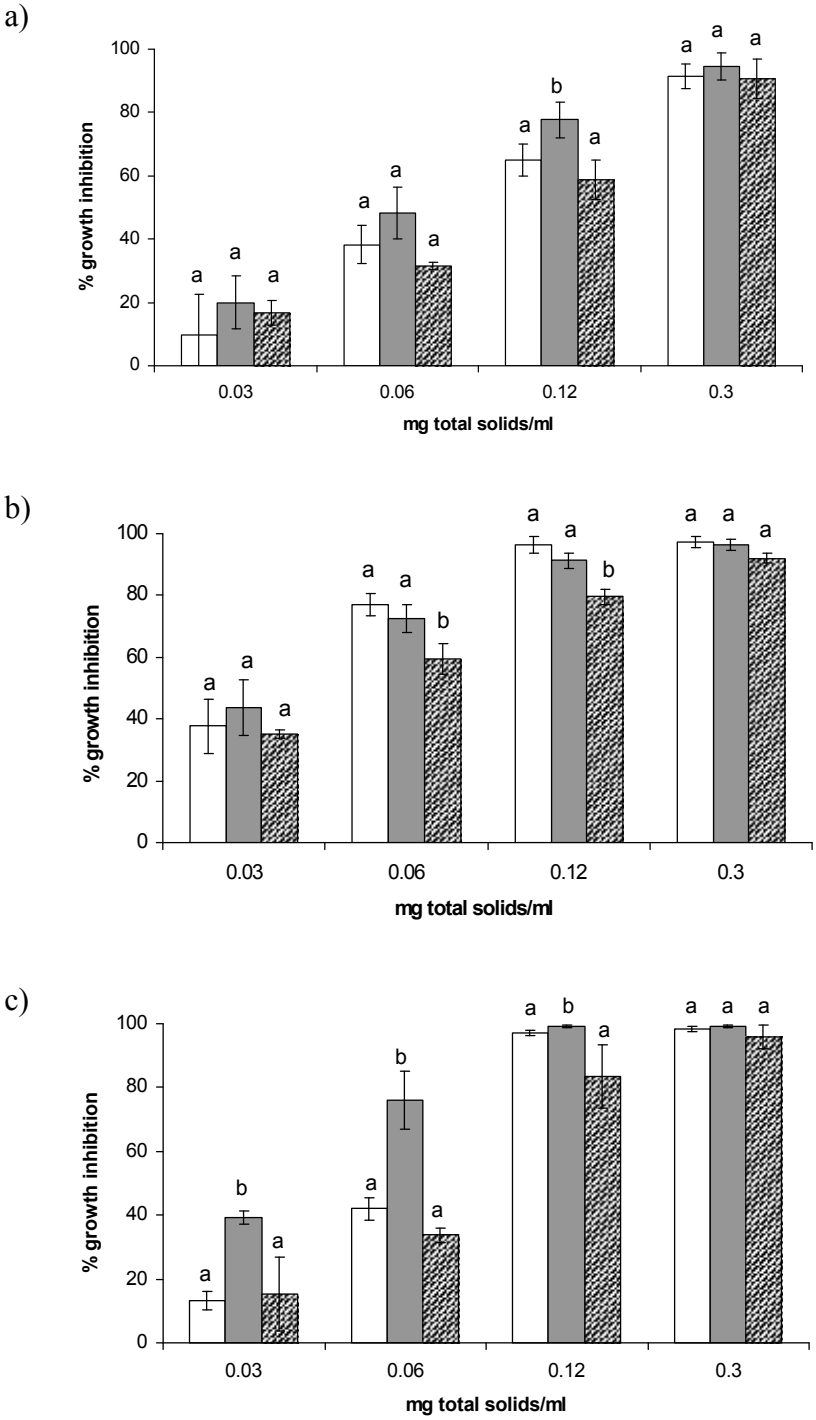


Fig. 4.4. Antiproliferative activity of feed (□), retentate (■) and permeate (▨) fractions produced from washed cream on growth of a) SW480, b) HT-29 and c) HepG2 cancer cells; assessed using AP assay. Cells were grown and treated in galactose-containing Leibovitz-15 medium. Data refer to mean ± SD of three independent experiments. ^{a,b,c} values without a common subscript are significantly different ($P \leq 0.05$) at given concentration.

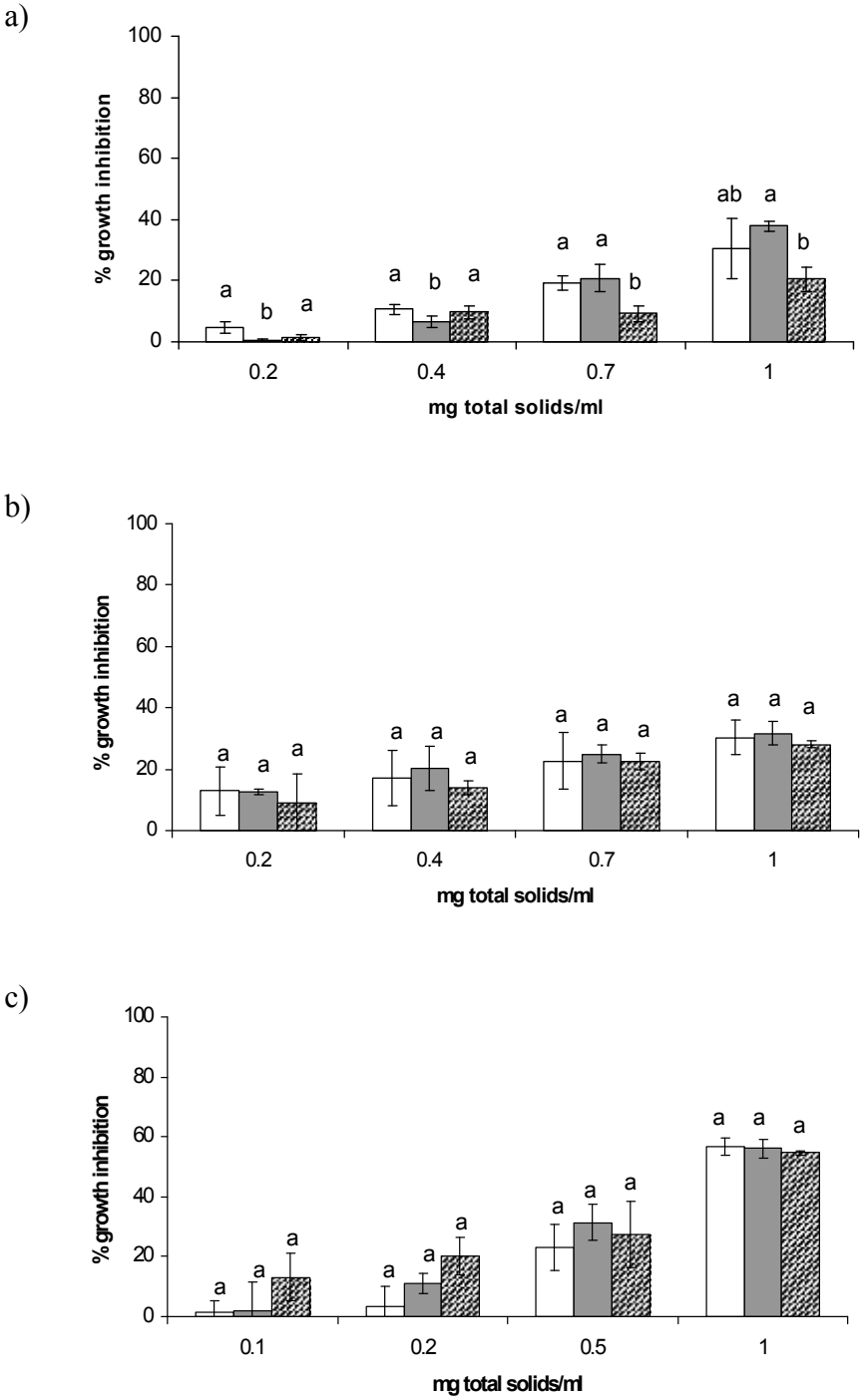


Fig. 4.5. Antiproliferative activity of feed (□), retentate (■) and permeate (▨) fractions produced from washed cream on growth of a) SW480, b) HT-29 and c) HepG2 cancer cells maintained and treated in their corresponding media containing glucose; assessed using AP assay. Data refer to mean ± SD of three independent experiments. ^{a,b,c} values without a common subscript are significantly different ($P \leq 0.05$) at given concentration.

4.3.2.3. Evaluation of buttermilk treatment on growth of differentiated and non differentiated Caco-2 cells

Due to economical reasons in the evaluation of antiproliferative activity of buttermilk on proliferating and non-proliferating Caco-2 cells only one representative buttermilk was used, retentate produced from unwashed cream. At high concentration of 1 mg total solids/ml it inhibited proliferating Caco-2 cell growth by 56.2% and differentiating Caco-2 cells by only 6.6% (**Fig. 4.6**).

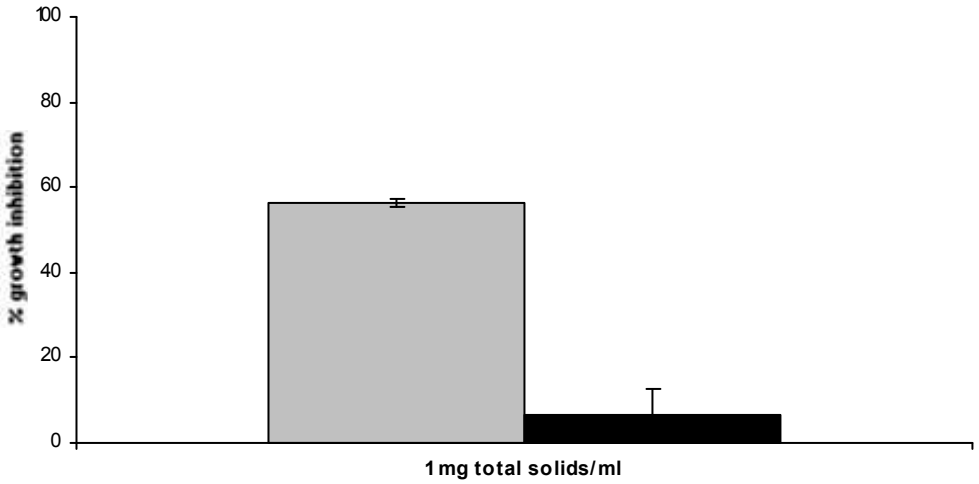


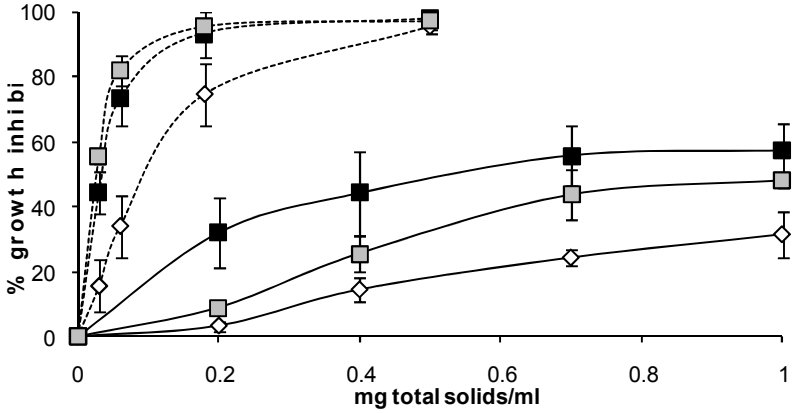
Fig. 4.6. Influence of buttermilk (retentate produced from unwashed cream) on growth of proliferating (■) and non-proliferating polarized (🔲) Caco-2 cells. Data was assessed using MTS assay. Data refer to mean ± SD of three independent experiments.

4.3.3. Analysis of influence of media components (glucose vs galactose) on buttermilk's antiproliferative activity

To examine the response of SW480 and HT-29 colon cancer and HepG2 hepatocellular cancer cells to buttermilk's components when treated in aerobic

and anaerobic conditions, cells were grown in media, which differed in energy sources: glucose versus galactose. It was apparent that when cells were grown in high-glucose media, they were more resistant to buttermilk and their survival was higher (**Fig. 4.7 a-f**). In the next step 0.9 g/l galactose was added to DMEM media already containing 4.5 g/l glucose but this did not alter buttermilk's cytotoxicity in initial high-glucose DMEM media. However, after adding 4.5 g/l glucose to Leibovitz-15 medium, antiproliferative activity of buttermilk was reduced but not as much as in cells grown with high-glucose media (**Fig. 4.8**). To eliminate influence of other media components, DMEM powder media (glucose-deprived) was supplemented according to Materials and Methods and results are shown on **Fig. 4.9**. Buttermilk in DMEM supplemented with galactose but without sodium bicarbonate inhibited growth of SW480 cell line with the same potency as buttermilk in Leibovitz-15 medium indicating that differences in essential amino-acids, vitamins and other media components have no effect of buttermilk's cytotoxicity on colon and hepatocellular cancer cells used in experiment. After adding sodium bicarbonate to DMEM medium supplemented with galactose, the antiproliferative effect of buttermilk decreased, resulting in 63.6% growth inhibition at concentration 0.3 mg total solids/ml indicating that addition of sodium salt decreases buttermilk's antiproliferative activity ($P \leq 0.001$). Moreover, cells grown in DMEM media with 4.5 g/l glucose showed much higher ($P \leq 0.001$) resistance to buttermilk treatment with 79.1% survival.

a)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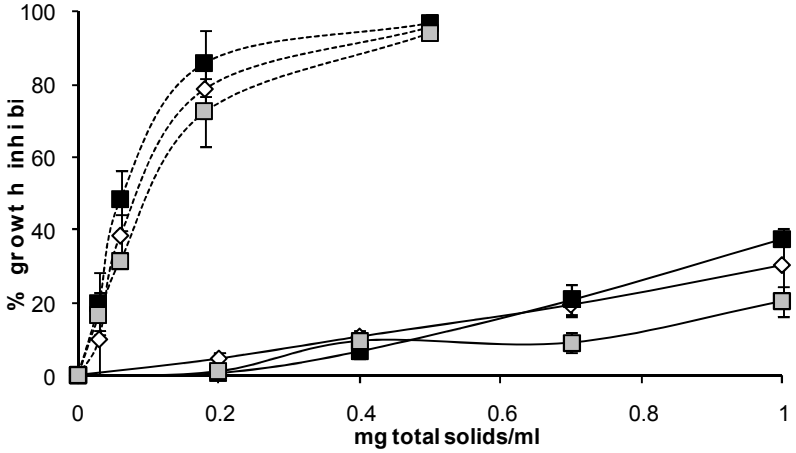
b)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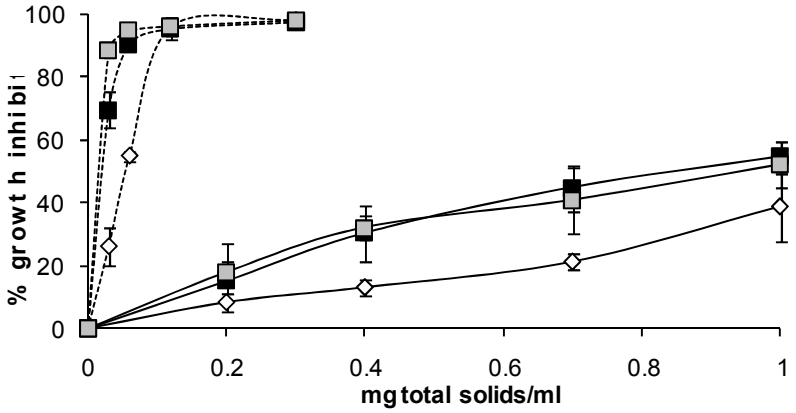
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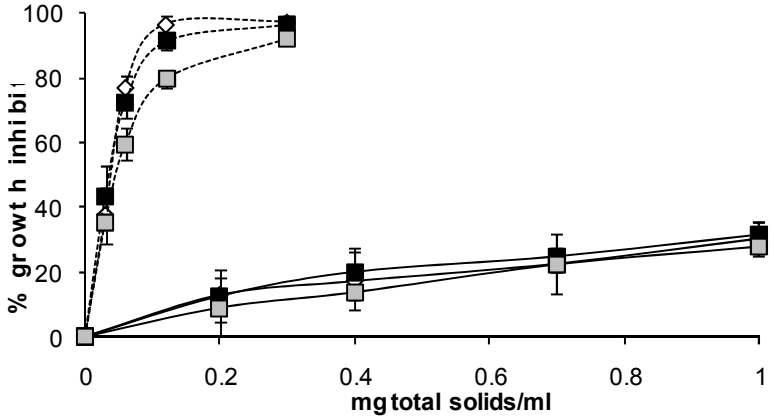
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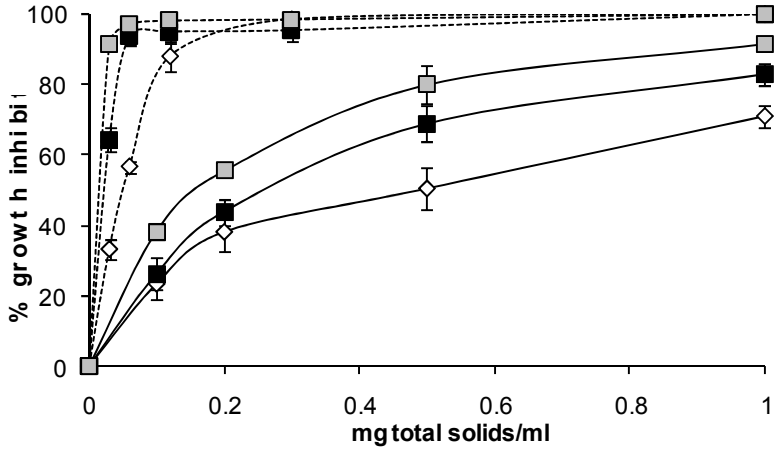
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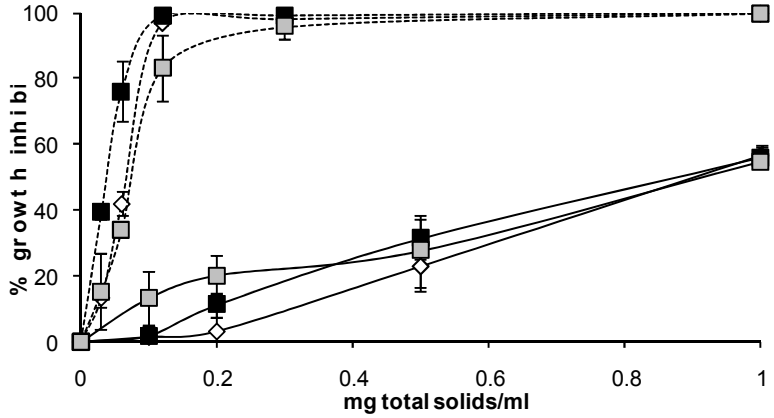
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mg total solids/ml

Fig. 4.7. Antiproliferative activity of feed (◊), retentate (■) and permeate (▣) produced from unwashed cream (a, c, e) and washed cream (b, d, f) on SW480 (a, b), HT-29 (c, d) and HepG2 (e, f) cancer cells. Cells were grown either in Leibovitz-15 (*dashed lines*), a galactose-containing medium or in DMEM, McCoy's 5A and MEM glucose-containing media, respectively (*continuous lines*). Data was assessed using AP assay. Data refer to mean ± SD of three independent experiments.

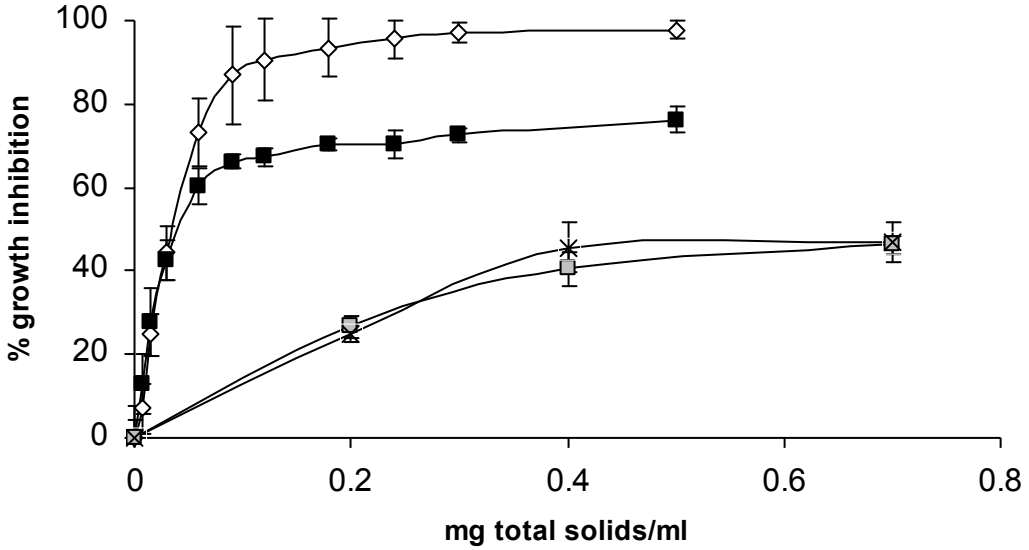


Fig. 4.8. Antiproliferative activity of retentate produced from unwashed cream on SW480 colon cancer cells, assessed using AP assay. Cells were grown in Leibovitz-15 (◊), in Leibovitz-15 medium with addition of 4.5 g/l glucose (■), in

DMEM medium (□) and in DMEM medium with addition of 0.9 g/l galactose (✱).Data refer to mean ± SD of three independent experiments.

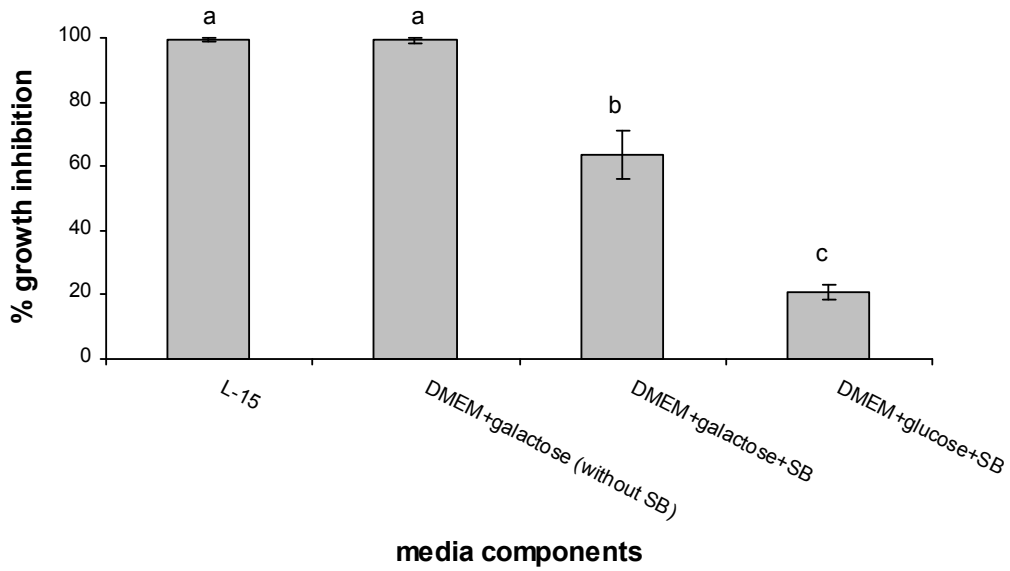


Fig. 4.9. Antiproliferative activity of retentate produced from unwashed cream on SW480 colon cancer cells grown in sugar-free DMEM media supplemented with different sugar sources compared to SW480 cells grown in Leibovitz-15 medium. Data was assessed using AP assay. Data refer to mean ± SD of three independent experiments. Abbreviations: L-15, Leibovitz-15 medium; DMEM, Dulbecco's Minimum Essential medium; SB, sodium bicarbonate. ^{a,b,c} values without a common subscript are significantly different ($P \leq 0.001$), as analyzed using ANOVA.

4.4. Discussion

We have previously shown that buttermilk produced from whole milk in the laboratory scale significantly ($P \leq 0.001$) inhibited SW480 colon cancer cell growth and had no toxic influence on growth of FHC normal foetal epithelial cells (Chapter 3). In this study, we examined if buttermilk produced in pilot plant scale also exhibited antiproliferative activity against human colon cancer cells. To address this question three colon cancer cell lines were used: SW480, HT-29, Caco-2 and also the hepatocellular carcinoma cell line HepG2.

Feed, retentate and permeate fractions of buttermilk produced from unwashed cream inhibited growth in a dose dependent manner in all cell lines (Fig. 4.1). Of the three fractions that were analyzed it was apparent that the feed fraction was less potent in inhibiting cancer cell growth than retentate and permeate. At the concentration 0.5 mg total solids/ml the level of growth inhibition (96%) by feed produced from unwashed cream in pilot plant corresponded with our previously reported findings (98%) obtained from buttermilk sample produced in laboratory scale from unwashed centrifugally separated cream. This indicates that scaling up production maintained buttermilk's biological activity.

Compositional analysis of buttermilks showed that retentate and permeate fractions produced from unwashed cream had higher MFGM protein content than feed fraction, suggesting that their antiproliferative activity might be associated with MFGM proteins. MFGM proteins that inhibit growth of cancer cells include breast cancer type 1 and type 2 susceptibility proteins (Brody and Biesecker, 1998, Vissac *et al*., 2002) and fatty acid binding protein (Bansal *et al*., 1989). The former are ubiquitously expressed tumour suppressor proteins whose activities are

not restricted to breast cancers (Tannenbaum *et al*., 2007). Marot *et al*. (2006) reported a pronounced *in vitro* antiproliferative effect on H1299 lung and HT-29 colon cells when treated with a recombinant adenovirus expressing the wt*BRCA1* gene. Growth of HT-29 tumours in nude mice was also significantly reduced when BRCA1 was overexpressed. FABP are a family of proteins that have been reported to alter growth and differentiation of various cell types, including embryonic stem cells, hepatocytes, prostate cancer cells, oral squamous carcinoma cells and melanoma breast cancer cells. Liver fatty acid binding protein and intestinal fatty acid binding protein are two such forms found in colon epithelial cells. A comparative proteome analysis and histochemical analysis identified consistent loss of liver fatty acid binding proteins in colorectal cancer compared with normal colon (Lawrie *et al*., 2004, Carroll *et al*., 1990 and Davidson *et al*., 1993).

Other potential growth inhibitory components present in buttermilk fractions include sphingomyelin, lactosylceramide and glucosylceramide. Sphingomyelin was shown to have chemopreventive and chemotherapeutic properties (Schmelz *et al*., 1996, Lemonnier *et al*., 2003). Glucosylceramide and lactosylceramide, major glycosphingolipids in milk, also inhibited early stages of colon cancer to a similar extent as sphingomyelin, as manifested by decrease in appearance of aberrant crypt foci (ACF) (Schmelz *et al*., 2000, 2001).

In our studies, washing of cream prior to fractionation significantly reduced the sensitivity of SW480, HT-29 and HepG2 cells to feed, permeate and retentate when presented to cells at 0.03 mg total solids/ml. At higher concentrations cells responded to washed fractions in a manner similar to unwashed samples. Washing of cream before churning is expected to reduce the

casein content of buttermilk so as to increase the phospholipids to protein ratio. Lamothe *et al*. (2008) reported that washing bovine cream and caprine cream before churning to make buttermilk and butter had no effect on phospholipid profiles of the creams but improved the phospholipids/protein ratio in respective buttermilks and butter sera. Comparative analysis of feed and retentate fractions obtained in our study revealed a 4 to 5 fold higher lipids content in washed fractions compared to unwashed fractions (Tables 4.1 and 4.3). This suggests that buttermilk fractions from washed cream may offer potential for the production of phospholipids concentrates that have antiproliferative activity against cancer cells. Elucidation of which phospholipid component(s) may be responsible is not possible as each fraction differed in phospholipid distribution and yet all fractions were equally inhibitory at concentration 0.3 mg total solids/ml. For instance, feed from washed cream was enriched in PE and PS; retentate from washed cream was enriched in LC and PS.

Feed, retentate and permeate produced from washed cream also showed a dose-dependent inhibition in SW480, HT-29 and HepG2 cells with maximum inhibition observed at 0.3 mg total solids/ml. No significant difference was apparent between the fractions in colon cancer cell lines; however at concentrations less than 0.12 mg total solids/ml, the potency of retentate to inhibit growth of HepG2 cells was greater than feed and permeate (Fig. 4.4 c). It is apparent from Tables 4.3 and 4.4 that retentate and feed differed substantially in composition analysis. Retentate from washed cream had relatively lower levels of not only MFGM protein and SM but also growth proliferative phospholipids, in particular PC and PI. By contrast, it had higher levels of glycosphingolipids. The biological significance of such differences in composition cannot be explained as

growth in colon cancer cells was similar following treatment with each fraction. It is apparent that decreases in the level of PC and PI more than compensated for the decrease in MFGM protein and SM and may account for the relatively greater growth inhibition of the retentate in HepG2 cells. The presence of elevated levels of glucosylceramide and lactosylceramide in retentate compared with feed had apparently negligible effect on colon cancer cells.

To study if buttermilk targets mitochondria of cancer cells, we have grown cells in media containing glucose and/or galactose. Leibovitz-15 medium, as used in our study, is a bicarbonate-free medium which is buffered by its complement of salts, basic amino acids (arginine, cysteine and histidine) and galactose. The use of galactose instead of glucose as a carbohydrate source is expected to reduce the amount of lactic acid produced by cells and hence reduces the need for buffering. It is apparent from Fig. 4.2, 4.5 and 4.7 that SW480, HT-29 and HepG2 cells were resistant to buttermilk fractions when grown in DMEM, MEM and McCoy's 5A medium, respectively, due to high glycolytic capacity. When grown in L-15 media, cells maintained ATP levels by increasing respiration rates. Our studies showing that buttermilk inhibited growth of SW480, HT-29 and HepG2 cells to a greater extent in cells cultured in L-15 medium than in media containing glucose (Fig. 4.2, 4.5 and 4.7) suggest that buttermilk components may be selectively toxic to mitochondria.

However, addition of 25 mM glucose to L-15 medium enhanced the survival of SW480 cells treated with the retentate buttermilk fraction (Fig. 4.8). This suggests that growing SW480 cells in L-15 medium containing supraphysiological glucose allowed them to up-regulate GLUT1 carriers to

achieve an increased intracellular glucose level that enabled cells to switch phenotype and derive energy from glycolysis rather than from mitochondrial oxidative phosphorylation. Furthermore, addition of physiological level of galactose to DMEM had no apparent effect on phenotype as the level of growth in the presence of buttermilk over the concentration range 0 to 0.8 mg total solids/ml was similar to that observed in DMEM. It was apparent from Fig. 4.9 that removing glucose from bicarbonate-free DMEM and substituting galactose as the sole source of carbohydrate enhanced the susceptibility of SW480 cells to buttermilk to a level similar to what was achieved in bicarbonate free Leibovitz-15 medium. Adding bicarbonate to DMEM media containing galactose enhanced the survival of cells exposed to buttermilk. A role for exogenous bicarbonate in regulating mitochondrial ATP production was reported by Acin-Perez *et al*. (2009). HCO_3^{2-} may ensure cell survival by initiating a mitochondrial signalling cascade that activates protein kinase A. The latter phosphorylates mitochondrial proteins regulating ATP production, ensuring cell survival. Even in the presence of bicarbonate, buttermilk targeted oxidative phosphorylation-poised SW480 cells to a greater extent than glycolytically-poised cells indicating that cells growing in galactose are a better cell model for understanding underlying mechanisms by which buttermilks impair cell viability.

In this study we have also shown that buttermilk was not only cytotoxic for cancer and hepatocellular carcinoma cells but also it did not possess cytotoxic activity towards normal intestinal cells, which were represented by Caco-2 cells grown in polarized conditions. Caco-2 cells were previously used as human intestinal cell line to study absorbtion of fatty acids and other dietary components

from the lumen into polarized enterocytes that line the small intestine (Ranheim *et al*., 1994, Cogburn *et al*., 1991). When grown in culture, Caco-2 cells spontaneously develop many functions characteristic of mature villus cells of the small intestinal epithelium, including tight junction formation, lipoprotein secretion across the basolateral membrane, and the expression of L- and I-fatty acid binding protein (Darimont *et al*., 1998). However, Caco-2 cells were found to have few different features from native enterocytes, including the synthesis of apoB-100 rather than apoB-48 (Traber *et al*., 1987). It was also found that Caco-2 cells still possess some characteristics similar to foetal cells or crypt cells of the colon (Pinto *et al*., 1983). Despite their limitations, Caco-2 cells allow the study of a polarized enterocyte monolayer, provide separate access to apical and basolateral surfaces and are a commonly used model system for studies of intestinal lipid absorption and metabolism (Field *et al*., 2002, Rong *et al*., 2002, Ho *et al*., 2002, Chateau *et al*., 2005, Murota and Storch, 2005) as well as studying the ways food modulate intestinal functions and promote gut health via molecular interactions (Shimizu, 2010).

4.5. Conclusions

The data show that using acid phosphatase activity assay buttermilk samples produced from unwashed and washed creams and followed by microfiltration significantly ($P \leq 0.001$) inhibited growth of SW480 and HT-29 colon cancer cells and HepG2 hepatocellular cancer cells at the concentration 0.3 mg total solids/ml. However, buttermilk did not show toxic effect on differentiated Caco-2 cells representing normal intestinal enterocytes. It is not yet established which buttermilk components play crucial roles in its antiproliferative activity; however they possibly control functions of mitochondria in cancer cells. Evaluating compositional analysis of buttermilk we could not point definite bioactive ingredient(s) responsible for buttermilks' anticancer activity, as yet. With the object of production of functional beverage, further investigations are required to understand accurate mechanism of action of buttermilk's bioactive component(s).

CHAPTER 5

Effect of spray, freeze and spin drying of buttermilk on its antiproliferative activity against SW480 human colon cancer cells.

Buttermilk, a by-product from butter production, is naturally enriched in MFGM components with anticancer properties (Dewettinck *et al*., 2008, Vesper *et al*., 1999), which gives opportunity for dairy industry to develop potential functional beverage. However, large scale processing operations may lead to changes in the bioactivity of some buttermilk's components. In industry food ingredients are dried to the form of powder for storage, preservation and transport. In this study we investigated influence of different drying methods, such as spray, freeze and spin drying on antiproliferative activity of buttermilk on SW480 colon cancer cells, using acid phosphatase activity assay. We found that spray drying, but not freeze and spin drying, significantly reduced anticancer activity of buttermilk fractions, suggesting that harsh conditions of drying (hot air stream) might negatively affect active components in buttermilk. Therefore, the form of drying and its parameters should be considered in buttermilk production in large commercial scale.

5.1. Introduction

Drying is a method of preservation, by which products are changed to a form which is light and capable of being stored at room temperature. It is widely used in the dairy industry in the manufacture of skimmed milk powder (Bank, 1993). Dried milk products play a significant part in storage since their biological value can be retained for long periods of time under relatively simple storage conditions. To avoid deterioration during storage careful drying is necessary (Labuza and Tannenbaum, 1972). Spray drying is a commonly used method in the dairy industry for drying raw milk into milk powders because of rapid moisture removal. By spraying into a stream of hot air, liquid products can be dried within a few seconds. Spray drying involves atomizing concentrated milk into a hot air stream (180-200°C). By controlling the size of the droplets, the air temperature, and the airflow, it is possible to evaporate almost all the moisture while exposing the solids to relatively low temperatures (Bank, 1993).

Another method of drying is freeze drying, which refers to drying of a product in the frozen state (Mellor and Bell, 2003). Ice evaporates without passing through the liquid state in a process known as sublimation (Pikal *et al*., 1983). Products to be dried almost always contain substances in solution, which lower the freezing point of the pure solvent. The most important advantage of freeze drying is the greatest possible degree of preservation of the structure, the colour, the vitamin and amino acid content, flavour and odour of the freeze dried product (Fennema, 1976). The sublimation of the ice in the frozen material leads to a product with a highly porous structure which is generally easily reconstituted in water and which has good instant properties. Due to higher capital and running costs than other drying methods, freeze drying is recommended for valuable products such as coffee, tea, egg and milk products.

In spin (centrifugal) drying samples are spun in a centrifuge and the g-force applied prevents the solvent from evaporating or ejecting material in an uncontrolled way. As atmospheric pressure decreases, the boiling point of solvent reduces. When pressure is low enough, solvent boils and is removed by vacuum applied. Evaporation under lower pressure is the main process involved in spin drying (Bouman and Waalewijn, 1994).

The aim of this project is to produce buttermilk with high concentration of MFGM components which have been shown to possess anticancer properties (Dewettinck *et al*., 2008, Vesper *et al*., 1999). However, methods for concentrating valuable components in buttermilk may become ineffective if other processing operations adversely affect bioactivity. In large scale production, spray drying is one of the most convenient methods for producing milk powders and for stabilizing milk constituents. However, spray drying leads to undesirable changes and/or losses of variety of nutrients, such as proteins, fats, vitamins, flavours and others due to high temperature and pressure used (Indyk *et al*., 1996).

To address our previous findings of spray drying dramatically influencing buttermilk's antiproliferative activity (chapter 3), we also examined influence on possible changes in activity of powdered buttermilks following other methods for drying, such as freeze drying and spin drying. In general, we found that freeze and spin drying, as opposed to spray drying, maintained antiproliferative activity of buttermilk.

5.2. Materials and methods

5.2.1. Buttermilk production

Pilot plant buttermilk samples were generated by a member of the project team, as described in Chapter 4, subsection 4.2.1. Buttermilk samples (feed, retentate and permeate) were collected, concentrated by water evaporation and spray dried (inlet 180°C, outlet 85°C). Liquid samples, as well as evaporated and spray dried samples were collected and stored at -30 for further analytical and antiproliferative analyses. Compositional analysis of buttermilk samples was performed by a project team member as described in Chapter 2, subsection 2.2.3. Analysis of MFGM phospho- and sphingolipid components was also performed by a project team member as described in Chapter 3, subsection 3.2.2.1.

Buttermilk samples (feed, retentate and permeate) were also subjected to freeze and spin drying. Freeze drying was performed using the AdVantage Freeze Dryer (VirTis, NY, USA). Spin drying was performed using miVac Quattro Concentrator with a miVac SpeedTrap refrigerated condenser from Genevac Technology.

5.2.2. Cell culture

SW480 human colon cancer cell line was obtained from European Collection of Cell Cultures, ECACC. Culture media and supplements were purchased from Sigma-Aldrich Ireland, Ltd. The SW480 cells were grown in Leibovitz-15 medium. All media were supplemented with L-glutamine (4 mM), fetal bovine serum (FBS) (10%, v/v) and penicillin/streptomycin (1 U/ml). Cells were grown at 37°C, without 5% CO_2 access.

5.2.3. Acid phosphatase activity assay

To assess antiproliferative activity of buttermilk samples, the cells were grown in parallel in two different media to eliminate possibility of the influence of media components on the antiproliferative activity of buttermilk samples. SW480 cells were seeded in 96-well plates at density of $5x10^3$/well. To allow the cells to attach, they were cultured for 24 h at 37°C without 5% CO_2. Next, concentrated and powdered buttermilk samples from both pilot plant trials (feed, retentate and permeate) were reconstituted to former concentration, diluted in feeding media and added to the wells with the cells in the range of concentrations between 0-1 mg total solids/ml. Only media was added to the control wells. All the buttermilk samples were sterilized prior to treatment by incubation at 72°C for 15s. The wells with the SW480 cells were covered with parafilm to prevent CO_2 access to the cells. Four replicates of each buttermilk concentration were performed. After 3 days of incubation at 37°C, cell survival was measured by an indirect colorimetric endpoint assay for acid phosphatase activity (O'Connor *et al*., 1998), as described in Chapter 2, subsection 2.2.5.

5.2.4. Statistical analysis

Three independent experiments were performed in quadruplicate for each treatment and the Student's *t*-test (Microsoft Office Excel) was used to determine significant difference between treatments or comparing to the control.

5.3. Results

5.3.1. Compositional analysis of buttermilks

Tables 5.1-5.4 show analytical analysis of buttermilk samples. Comparative analysis of phospholipid distribution of concentrated feed, retentate and permeate produced from unwashed cream showed about 3-fold greater concentration of PI comparing to concentrated feed, retentate and permeate from washed cream. Washing had little effect on sphingomyelin content of concentrated feed and retentate; however concentrated permeate from washed cream was enriched in sphingomyelin comparing to permeate produced from unwashed cream (38% relative to 22%). It was also observed that concentrated retentate and permeate from washed cream were about 2-fold enriched in PS and about 2-fold depleted in PC. Moreover, compositional analysis of spray-dried powders from both washed and unwashed creams showed altered profile of phospholipids. Levels of PI, PC, GC and LC were reduced by ~60-100% while PE and PS were increased. Unfortunately, only retentate from unwashed cream was spray-dried; hence it could not be compared with retentate from washed cream.

Table 5.1. Compositional analysis of liquid, concentrated and spray-dried feed, retentate and permeate produced from unwashed cream; data obtained by a project team member. All values are expressed as mean %.

Sample	Total solids (%)	Protein (%)	MFGM proteins (%)	Lipids (%)	Percentage of phospholipids in:			Lactose (%)
					Sample	Total fat	Total solids	
Feed	9.07	3.18	0.93	1.15	0.14	12.65	1.60	4.03
Retentate	10.35	3.47	2.49	2.78	0.20	7.16	1.93	3.33
Permeate	8.00	3.04	1.13	0.64	0.11	17.01	1.05	3.66
Feed CONC	46.64	16.75	12.74	4.33	0.64	14.80	1.37	21.86
Retentate CONC	38.13	12.49	8.44	10.43	1.06	10.13	2.77	12.75
Permeate CONC	51.04	17.75	5.09	3.51	0.71	20.18	1.39	25.88
Retentate SPRAY DRIED	96.80	31.54	23.58	23.91	1.45	6.09	1.50	35.60
Permeate SPRAY DRIED	96.73	34.45	8.23	5.72	0.99	17.27	1.02	49.06

Table 5.2. Phospho- and sphingolipid composition of feed, retentate and permeate produced from unwashed cream; data obtained by a project team member. All values are expressed as %. Abbreviations: CC, buttermilk produced from commercial cream; CSC, buttermilk produced from centrifugally separated cream; G4SC, buttermilk produced from gravitationally separated cream at 4°C; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; GC, glucosylceramide; LC, lactosylceramide; SM, sphingomyelin.

Sample	GC (%)	LC (%)	PE (%)	PI (%)	PS (%)	PC (%)	SM (%)
Feed	0	0	18.42	17.41	5.26	33.21	25.70
Retentate	1.01	1.55	12.93	17.19	6.22	31.57	29.52
Permeate	0.58	1.20	15.92	19.15	5.77	35.80	21.58
Feed CONC	0.11	0.35	20.81	15.96	12.52	24.50	25.75
Retentate CONC	0.57	0.17	14.83	15.80	6.31	31.11	31.22
Permeate CONC	1.74	0.41	17.00	13.34	5.44	40.01	22.07
Retentate SPRAY DRIED	0	0.05	14.39	12.26	6.28	37.94	29.08
Permeate SPRAY DRIED	0	0.16	24.40	10.93	8.33	33.75	22.44

Table 5.3. Compositional analysis of liquid, concentrated and spray-dried feed, retentate, permeate produced from washed cream; data obtained by a project team member. All values are expressed as mean %.

Sample	Total solids (%)	Protein (%)	MFGM proteins (%)	Lipids (%)	Percentage of phospholipids in:			Lactose (%)
					Sample	Total fat	Total solids	
Feed	8.19	0.43	0.43	5.55	0.10	1.76	1.19	2.18
Retentate	12.38	0.19	0.19	11.25	0.09	0.78	0.71	0.89
Feed CONC	60.80	2.21	2.21	41.58	0.23	0.57	0.39	16.84
Retentate CONC	28.87	0.76	0.76	26.39	0.23	0.87	0.80	1.59
Permeate CONC	9.27	3.21	3.21	3.56	0.49	13.85	5.32	2.01
Permeate SPRAY DRIED	96.73	33.01	32.22	34.26	2.89	8.42	2.98	23.84

Table 5.4. Phospho- and sphingolipid composition of feed, retentate and permeate produced from washed cream; data obtained by a project team member. All values are expressed as mean %. Abbreviations: CC, buttermilk produced from commercial cream; CSC, buttermilk produced from centrifugally separated cream; G4SC, buttermilk produced from gravitationally separated cream at 4°C; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; GC, glucosylceramide; LC, lactosylceramide; SM, sphingomyelin.

Sample	GC (%)	LC (%)	PE (%)	PI (%)	PS (%)	PC (%)	SM (%)
Feed	0	0	26.34	9.03	14.47	23.68	26.48
Retentate	0.59	44.26	21.00	3.83	9.87	8.57	11.87
Feed CONC	0	6.98	23.96	5.38	14.20	19.57	29.91
Retentate CONC	2.47	16.01	18.06	6.25	13.47	15.64	28.10
Permeate CONC	0.65	4.85	19.59	4.19	13.85	19.22	37.66
Permeate SPRAY DRIED	0	13.13	16.52	6.32	12.26	16.84	34.94

5.3.2. Cytotoxicity

5.3.2.1. Antiproliferative activity of concentrated buttermilk fractions produced from unwashed and washed cream

From the industrial point of view it was important to examine if antiproliferative activity of buttermilk was unaffected following spray drying - the most common drying method used in industry. All buttermilk fractions were concentrated prior to drying and samples were collected for cytotoxicity studies. The antiproliferative activity was assessed after 3 days of incubation. At the concentration of 0.5 mg total solids/ml concentrated feed, retentate and permeate produced from unwashed cream inhibited SW480 colon cancer cell growth by 93.8, 93.3 and 88.7%, respectively. However, at lower concentrations the difference in colon cancer cell growth inhibition was greater. At the concentration of 0.18 mg total solids/ml concentrated feed, retentate and permeate produced from unwashed cream inhibited SW480 colon cancer cell growth by 68.0, 71.9 and 64.4%, respectively (**Fig. 5.1**).

There could be observed a significant difference in SW480 colon cancer growth inhibition in the case of concentrated feed and retentate produced from washed cream. At the concentration of 0.5 mg total solids/ml concentrated feed and retentate inhibited SW480 colon cancer cell growth by 6.7 and 14.6%, respectively indicating loss of antiproliferative activity. Nevertheless, permeate produced from washed cream was more potent in inhibiting colon cancer cell growth after concentration than before concentration. At concentrations of 0.09, 0.18 and 0.5 mg total solids/ml concentrated permeate inhibited SW480 colon cancer cell growth by 83.5, 97.6 and 99.1%, respectively and liquid permeate inhibited cancer cell growth by 45.4, 72.5 and 94.3%, respectively (**Fig. 5.2**).

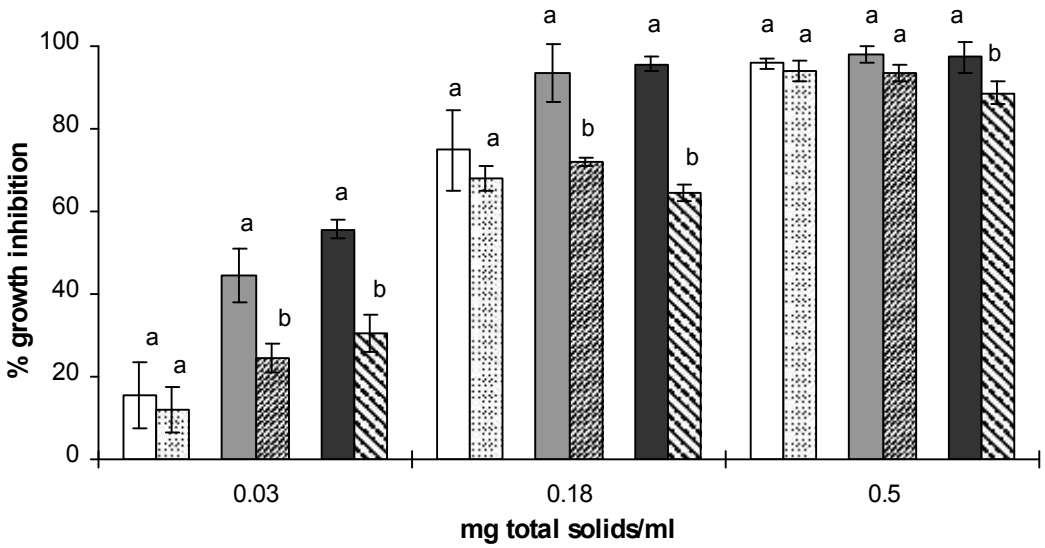


Fig. 5.1. Antiproliferative activity of concentrated feed (▨), retentate (▨) and permeate (▨) produced from unwashed cream compared to their former samples: liquid feed (□), liquid retentate (■) and liquid permeate (■) on growth of SW480 colon cancer cells. Cells were maintained and treated in Leibovitz-15 medium. The level of significance was established between liquid sample and its corresponding concentrate. Data was assessed using AP assay. Data refer to mean ± SD of three independent experiments. ^{a,b,c} values without a common subscript are significantly different ($P \leq 0.05$) at given concentration.

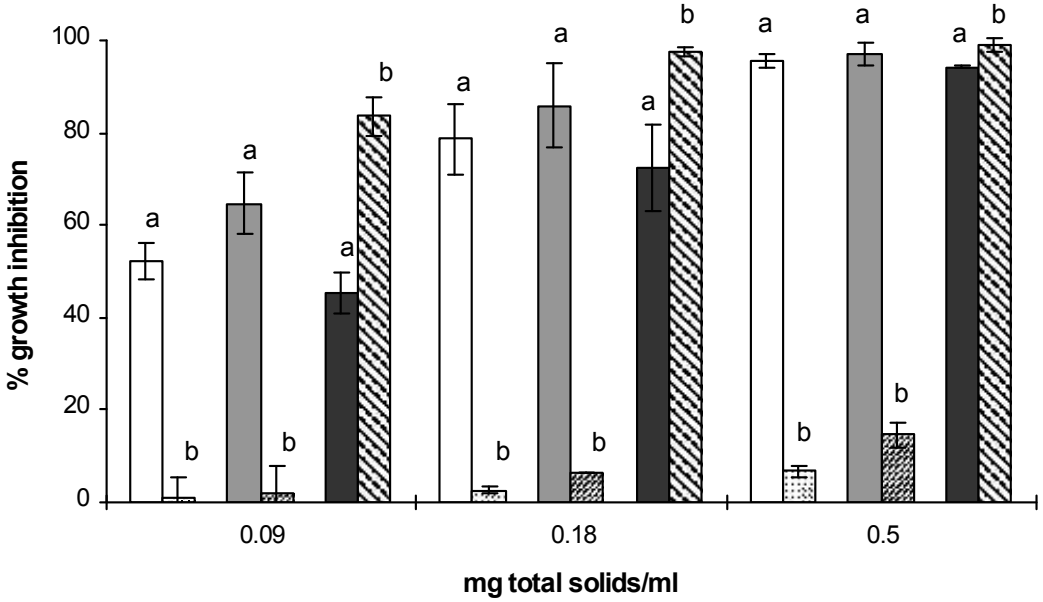


Fig. 5.2. Antiproliferative activity of concentrated feed (▨), retentate (▨) and permeate (▨) produced from washed cream compared to their former samples: liquid feed (□), liquid retentate (■) and liquid permeate (■) on growth of SW480 colon cancer cells. Cells were maintained and treated in Leibovitz-15 medium. The level of significance was established between liquid sample and its corresponding concentrate. Data was assessed using AP assay. Data refer to mean ± SD of three independent experiments. ^{a,b,c} values without a common subscript are significantly different ($P \leq 0.05$) at given concentration.

5.3.2.2. Antiproliferative activity of buttermilk samples after spray, freeze and spin drying

Buttermilk samples produced from washed and unwashed cream were also subjected to freeze, spray and spin drying. The resultant powders were reconstituted back in distilled water and incubated with cell lines to assess their antiproliferative activity. It was observed that after spray drying buttermilk fractions lost the anticancer activity. Among the three spray-dried buttermilk samples used for experiment (unwashed retentate and permeate and washed permeate) the highest activity was observed for washed permeate, which inhibited SW480 colon cancer cell growth by 37.7% at concentration 0.3 mg total solids/ml (**Fig. 5.3**). However, after freeze drying and spin drying, the activity remained almost the same. At concentration 0.12 mg total solids/ml unwashed feed, retentate and permeate after freeze drying inhibited SW480 colon cancer cell growth by 61.2, 84.1 and 95%, respectively (**Fig. 5.4 a**). At the same concentration washed feed, retentate and permeate after freeze drying inhibited SW480 cell growth by 64.4, 77.8 and 55.3%, respectively (**Fig. 5.4 b**). At concentration 0.12 mg total solids/ml unwashed feed, retentate and permeate after spin drying inhibited SW480 colon cancer cell growth by 14.7, 86.1 and 98.2%, respectively (**Fig. 5.5 a**). At the same concentration washed feed, retentate and permeate after spin drying inhibited SW480 cell growth by 76.1, 82.6 and 74.1%, respectively (**Fig. 5.5 b**).

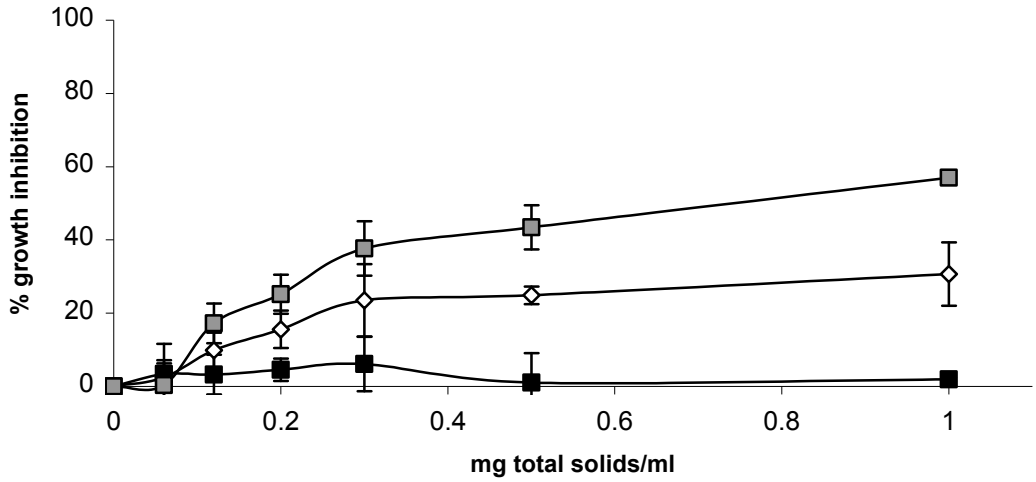
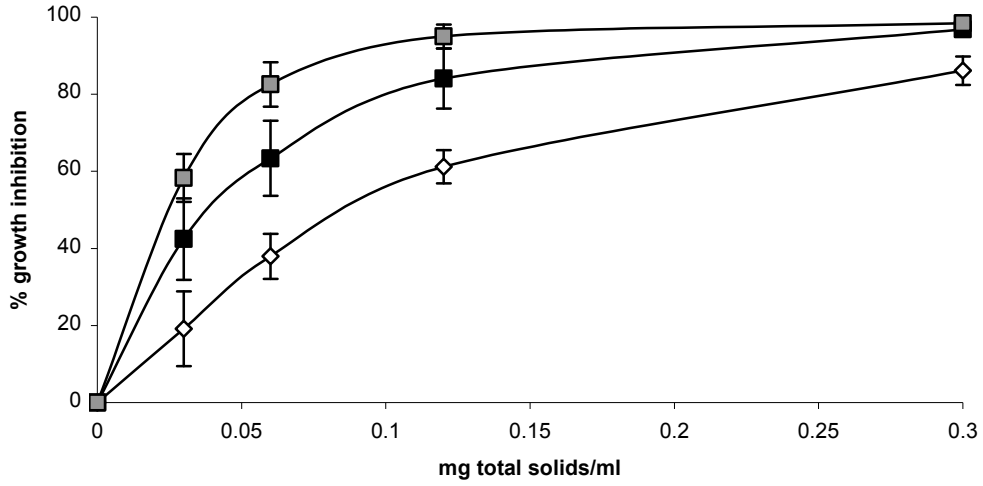


Fig. 5.3. Antiproliferative activity of buttermilk samples: retentate produced from unwashed cream (◊), permeate produced from unwashed cream (■) and permeate produced from washed cream (◘) following spray drying on SW480 colon cancer cells. Cells were maintained and treated in Leibovitz-15 medium. Data was assessed using AP assay. Data refer to mean ± SD of three independent experiments. At concentration 0.3 mg total solids/ml of buttermilk showed significantly ($P \leq 0.001$) lower SW480 cell growth inhibition after spray drying comparing to their former liquid samples.

a)



b)

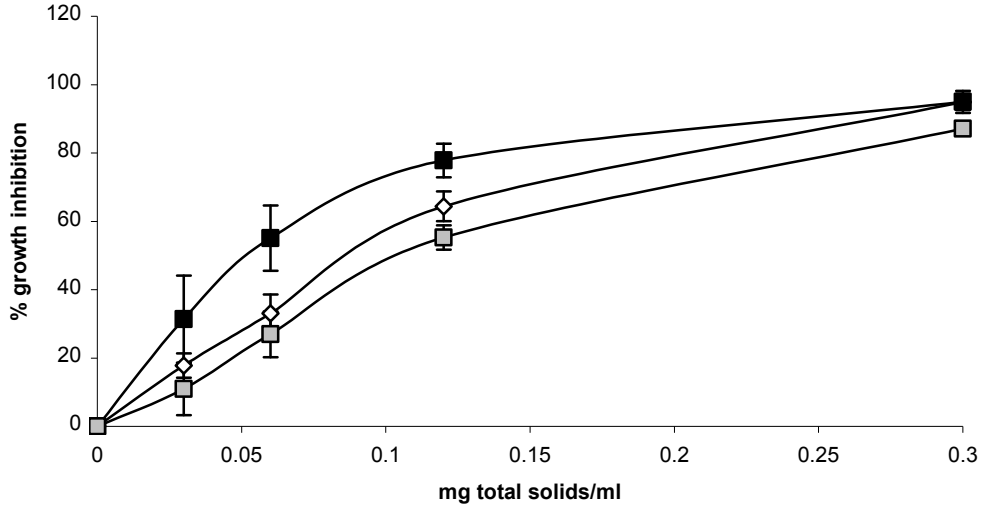
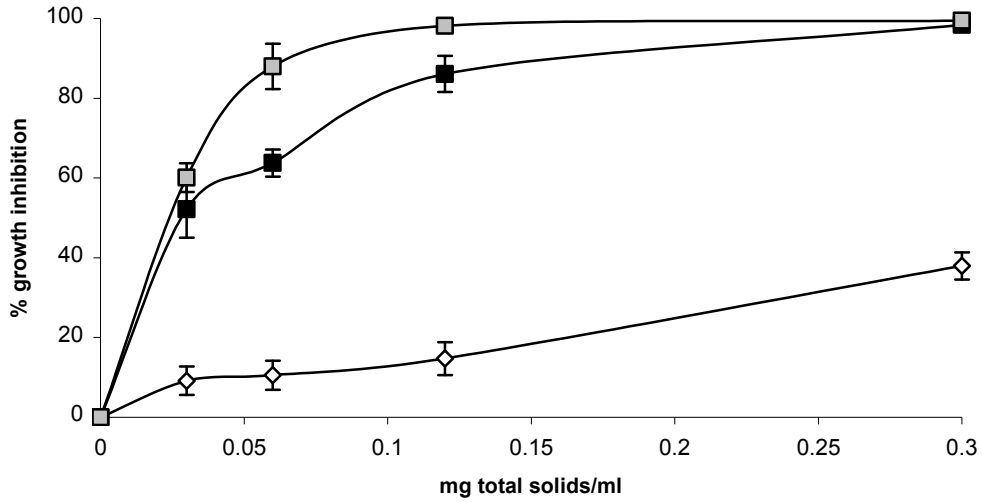


Fig. 5.4. Antiproliferative activity of buttermilk samples produced from a) unwashed and b) washed cream: feed (◊), retentate (■) and permeate (🔲) following freeze drying on SW480 colon cancer cells. Cells were maintained and treated in Leibovitz-15 medium. Data was assessed using AP assay. Data refer to mean ± SD of three independent experiments.

a)



b)

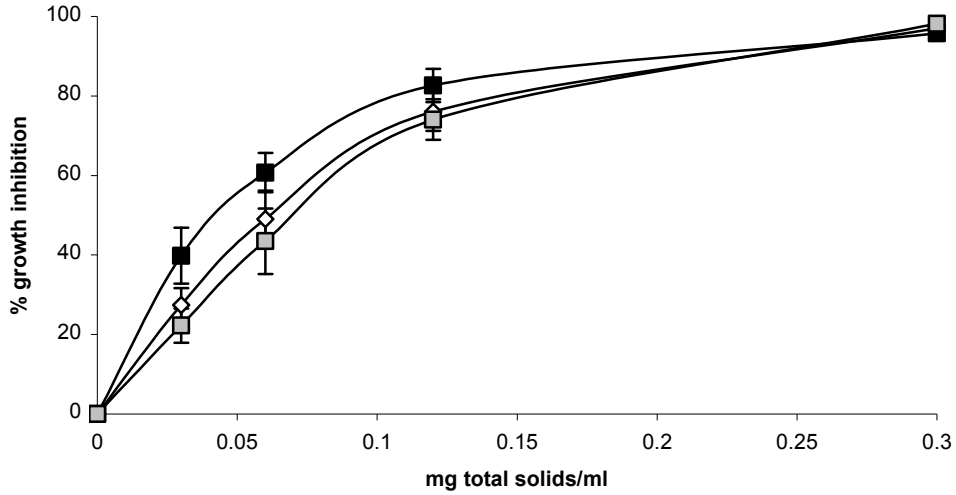


Fig. 5.5. Antiproliferative activity of buttermilk samples produced from a) unwashed and b) washed cream: feed (◊), retentate (■) and permeate (◘) following spin drying on SW480 colon cancer cells. Cells were maintained and treated in Leibovitz-15 medium. Data was assessed using AP assay. Data refer to mean ± SD of three independent experiments.

5.4. Discussion

As described in Chapter 1, the MFGM comprises mainly membrane-specific glycoproteins and polar lipids including phospho- and sphingolipids. Many of these components are biologically active and have potentially important modulatory effects on cancer cell biology. Development of new functional beverage/food containing MFGM material demands that methods used in production minimize loss of MFGM, so that the end material ingredient is representative of the natural MFGM in terms of composition, technical properties and bioactivity. Objective of the work described in this chapter was to evaluate the growth modulatory effects of buttermilk fractions obtained in pilot scale and subjected to water evaporation and drying.

As described in Chapter 4, the enhanced antiproliferative activity of retentate and/or permeate fractions over feed demonstrated the advantage of fractionation of buttermilk. However, for production of a long term stable ingredient with antiproliferative activity it was necessary to investigate the effects of concentration (water evaporation) and drying on a colon cancer cell line. SW480 was chosen, as it was a responsive cell line to each of the buttermilk fractions. Evaporation of water from fractions of both washed and unwashed creams concentrated MFGM proteins, lipids and lactose. Evaporation of water from feed fraction from unwashed cream had negligible effects on sphingomyelin and phospholipids, except for phosphatidylserine which was increased by a factor of 2.2 (Table 5.2). Concentration of feed did not adversely effect the antiproliferative activity of feed sample in SW480 cells. Unlike unwashed sample, concentrated feed from washed cream had an opposite effect; cell growth was promoted in the range 0.09 to 0.5 mg total solids/ml, suggesting that concentration may have removed bioactive lipid or protein component responsible for growth inhibition from the washed feed. A possible candidate for promoting growth

of washed feed fraction may be lactosylceramide, which was present at 7%. Although Schmelz *et al*. (2001) revealed an inhibory effect of lactosylceramide on a key marker of cell migration (β-catenin), another study by Kakugawa *et al*. (2002) showed that lactosylceramide had an anti-apoptotic effect on a colon cancer cell line and that accumulation of lactosylceramide was prevalent in colon cancer tissue relative to adjacent normal tissue. Concentrated retentate and permeate fractions from unwashed cream inhibited growth in a dose dependent manner, similar to fractions prior to evaporation. Concentrated permeate, but not retentate, had a potent antiproliferative effect. Analysis of composition of concentrated permeate suggest the antiproliferative activity may be attributed to the effects of a relative enrichment of sphingomyelin. Changes in antiproliferative activity suggest that water evaporation led to physical changes within MFGM protein/lipid active complexes. No similar studies could be found on this subject; hence it needs further investigations.

Snow *et al*. (2010) used in their studies spray-dried buttermilk, which showed chemopreventive properties in Fischer-344 rats treated with carcinogen. Earlier on Schmelz *et al*. (1996) also showed that sphingomyelin purified from spray-dried buttermilk showed antiproliferative activity in CF1 mice. Moreover, spray drying is commonly used as method for encapsulation of bioactive molecules as oils and flavours (Jafari *et al*., 2008) and bioactive living cells (probiotics) in dairy products, however with lower survival rates than other microencapsulation methods (de Vos *et al*. 2010). Some recent data show that parameters used for spray drying might influence storage and functionality of spray dried powders. Gaiani *et al*. (2010) claim that functional properties of powders during storage can be related to temperature of spray drying, as it influences dairy powder surface composition. They found that surface active components, as proteins and phospholipids were over-expressed at the

surface when drying at low temperatures (Gaiani *et al*., 2010, symposium abstract). In our study, spray-dried concentrated retentate and permeate fractions yielded buttermilk powders containing 96% total solids and fat content between 5 and 34%. Retentate and permeate samples after spray drying showed much lower ($P \leq 0.001$) antiproliferative activity than their corresponding liquid samples. Spray-dried permeate produced from washed cream and retentate produced from unwashed cream inhibited growth of SW480 cells by 57% and 30.7%, respectively, while spray-dried permeate produced from unwashed cream showed no antiproliferative activity in SW480 colon cancer cells (1.9%), suggesting temperature and/or pressure-induced impairment of bioactive components responsible for antiproliferative activity inhibition. Compositional analysis of spray-dried powders from both washed and unwashed creams showed altered profile of phospholipids. Levels of PI, PC, GC and LC were reduced by ~60-100% while PE and PS were increased. Loss of nutrients, such as proteins, fats, vitamins, flavours as a result of the high temperature and pressure of spray drying has been previously documented (Indyk *et al*., 1996). Temperature is the single most important factor affecting the drying of buttermilk and its MFGM. Morin *et al*., (2007 b) showed that spray drying affected the composition of buttermilk and its MFGM, in particular, it significantly lowered phospholipid content when compared with initial buttermilks. A reduction in phospholipid content was also observed in MFGM isolates following spray drying. Accordingly, non-thermal treatments including freeze drying may be more effective in overcoming the loss of nutrients during spray drying process (Kim *et al*. 2010). Furthermore, heat occurred during spray drying can be crucial to initiate and/or increase the lipase activity in milk (Hwang *et al*., 2007).

However, freeze drying of feed, retentate and permeate fractions maintained their antiproliferative activity (Fig. 5.4). It is apparent that freeze-dried retentate and permeate fractions from unwashed cream were more potent than freeze-dried feed fraction, as had been observed prior to freeze drying (Fig. 4.1, Chapter 4). Similarly, freeze-dried retentate fraction from washed cream was more potent than freeze-dried feed and permeate, as had been observed prior to freeze drying (Fig. 4.4, Chapter 4).

Like freeze drying, spin drying of feed, retentate and permeate fractions maintained their antiproliferative activity (Fig. 5.5). It was apparent that spin-dried retentate and permeate fractions from unwashed cream were more potent than spin-dried feed fraction, as had been observed prior to spin drying (Fig. 4.1, Chapter 4). Similarly, spin-dried retentate fraction from washed cream was also more potent than spin-dried feed and permeate, as had been observed prior to spin drying (Fig. 4.4, Chapter 4).

5.5. Conclusions

It was found that spray drying, but not freeze and spin drying. significantly ($P \leq 0.001$) reduced antiproliferative activity of buttermilk samples produced in pilot scale from unwashed and washed creams followed by microfiltration. Antiproliferative activity that was maintained by buttermilk samples after freeze and spin drying suggest that harsh conditions used during spray drying (pressure and/or temperature) may permanently deactivate buttermilk's bioactive component(s). However, because spray drying is commonly used in dairy industry as the cheapest and the most efficient method of drying powders, further studies are necessary to establish possible spray drying conditions that would maintain bioactivity of buttermilk.

CHAPTER 6

Analysis of antiproliferative mode of action of buttermilk on SW480 colon cancer cells.

Knowing that buttermilk is a rich source of sphingolipids with antiproliferative activity, we examined possible biochemical pathways known to be triggered by pure sphingolipids in colon cancer. The aim of this study was to analyze key signalling molecules in cell growth and cell death pathways, such as hallmarks of apoptosis (phosphatidylserine translocation, mitochondrial outer membrane depolarization, cleavage of caspase-3, caspase-9 and poly(ADP-ribose) polymerase (PARP), as well as translocation of apoptosis-inducing factor, AIF and endonuclease G to cells' nuclei), tumour biomarkers (Akt, β-catenin), as well as key proteins of the mitogen-activated protein kinase (MAPK) pathways, which lead to a wide range of cellular responses, including growth, differentiation, inflammation and apoptosis. Using a variety of fluorescence methods (fluorescence-activated cell sorting, fluorescence microscopy, confocal microscopy), cytological staining and immunoblotting we observed that buttermilk induced apoptosis in SW480 colon cancer cells (morphological changes, membrane alteration and mitochondria depolarization); however we could not detect other hallmarks of apoptosis, such as cleavage of caspases or PARP, which indicated that cell death may be caspase-independent or induced by autophagy. Immunofluorescent labelling of AIF and endonuclease G showed their translocation from mitochondria to cells' nuclei, signifying caspase-independent apoptosis as a possible pathway involved in colon cancer cells death. Buttermilk also downregulated levels of colon cancer biomarkers: β-catenin and phosphorylated Akt, as well as MAPK pathways proteins: ERK1/2 and c-myc. However, it did not alter levels of other MAPK pathway signalling components, such as phosphorylated p38 and phosphorylated JNK.

6.1. Introduction

Phospholipids and sphingolipids of milk fat globule membrane (MFGM) are known to possess cancer risk reduction properties *in vivo* and *in vitro* (Segui *et al*., 2006, Ogretmen, 2006, Schmelz, 2004). Recent reports attribute their chemopreventive activity to products of sphingomyelin hydrolysis, in particular ceramide, sphingosine, and glycosphingolipids, which affect multiple cellular targets that control cell growth, differentiation and apoptosis (Huwiler *et al*., 2000, Merill *et al*., 1997). Knowing the potential health benefits of MFGM lipids and proteins and the influence of a well-balanced diet on colon cancer prevention dairy industries address their research in developing new functional dairy products enriched in beneficial MFGM components, i.e. buttermilk.

Chapters 3-5 demonstrated anticancer activity of buttermilk samples across model colorectal cancer cell lines. Moreover, washing of cream before churning into butter and microfiltration of buttermilk through 0.8 μm ceramic membrane at 50°C allowed us to obtain a final product, retentate, enriched in MFGM components (Chapter 4). The aim of this study was to examine the mode of action of resultant product (retentate produced from washed cream) on SW480 colon cancer cells.

Two basic types of cell death exist: apoptosis, referred to as programmed cell death and necrosis (Kerr *et al*., 1972, Saikumar *et al*., 1999, Adams, 2003). Cytotoxic compounds that exert anti-tumour effects by induction of programmed cell death afford greater potential for prevention of colon cancer than those which cause damaging inflammation, characteristic of necrosis. Identification of biomarkers from within the apoptotic machinery of colon cancer cells that are responsive to buttermilk would be the first critical aspect in predicting buttermilk's suitability as a functional dairy beverage. However, to make a claim at a scientific level further steps are

required in design and development of functional food/beverage. They include demonstration of efficacy, determination of the intake level necessary to achieve the desired effect; demonstration of safety of the component at efficacious levels and development of a suitable food vehicle for the component. Independent peer review of scientific evidence for efficacy and health claim accuracy is also warranted.

In this study, we set out to identify biochemical and morphological changes that serve as hallmarks of the cell death process. Biochemical changes during the early stages of apoptosis include activation of caspase enzymes and disruption of active mitochondria, leading to changes in mitochondrial membrane potential (Kluck *et al*., 1997). Biochemical changes during the middle to late stage of the cell death process include the externalisation of phosphatidylserine on cell membrane (Fadok *et al*., 1992). Several different approaches were used in this study to examine the effect of buttermilk on cell death. Haematoxylin and eosin staining was used to distinguish live cells from early and late apoptotic cells. Flow cytometry and fluorescence microscopy were used to demonstrate externalisation of phosphatidylserine, FACS was used to quantify apoptosis and cell cycle distribution using propidium iodine, a water-soluble DNA intercalator dye. Mitochondrial dysfunction was assessed by measuring loss of retention of TMRE, a highly fluorescent cationic lipophilic dye whose retention by mitochondria is dependent on mitochondrial transmembrane potential.

Molecular markers of apoptosis, such as cleaved caspase-3, caspase-9, cleaved PARP, AIF and Endo G were measured using a variety of immunochemical methods, such as immunoblotting and immunofluorescence. Proteins involved in cell survival (Akt, β-catenin, ERK1/2, c-myc, p-p38, p-JNK) were studied using In-Cell Westerns, also referred to as In-Cell ELISA.

6.2. Materials and Methods

6.2.1. Materials

Antibodies: AIF, c-Myc (D84C12) XP^{TM}, cleaved caspase-3 (Asp175), cleaved PARP, cleaved caspase-9 (Asp330) and anti-rabbit secondary antibody were purchased from Cell Signaling Technology, Inc. (Danvers, Massachusetss, USA). β-catenin and FITC-conjugated anti-rabbit were purchased from Sigma-Aldrich, Ireland. ENDOG (FL-297) (endonuclease G), p-JNK (G-7), p-p38 (Thr180/Tyr182), ERK2 (D-2), p-ERK1/2 (Thr177/Thr160) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA). All other reagents were purchased from Sigma-Aldrich (Ireland) or as stated in text.

Buttermilk sample was provided by a project team member and prepared from raw whole milk collected from the Moorepark Dairy Production Centre (MDPC) farm, Fermoy, Co. Cork, Ireland. Briefly, cream was separated from skim milk in separator at 45-50°C. Cream was pasteurized at 72°C for 15sec, and cooled down to 10°C. Next, during churning buttermilk was separated from butter. Buttermilk sample was microfiltered through 0.8 μm ceramic membranes at 50°C giving retentate and permeate samples. Retentate sample was used to determine mode of action in the whole study presented.

6.2.2. Cell culture

SW480 human colon cancer cell line was obtained from European Collection of Cell Cultures, ECACC. Culture media and supplements were purchased from Sigma-Aldrich, Ireland. The SW480 cells were grown in Leibovitz-15 medium supplemented with L-glutamine (4 mM), fetal bovine serum (FBS) (10% , v/v) and

penicillin/streptomycin (1 U/ml). Cells were grown at 37°C, without 5% CO_2 access (flask's caps were fully closed and culture plates were sealed with parafilm).

6.2.3. Haematoxylin and eosin staining of cultured cells

Haematoxylin and eosin stains were used to display cytoplsmic and nuclear features of cells treated with buttermilk. SW480 cells were seeded in T-25 flasks at density $1x10^6$ cells/flask. After 24h they were treated with buttermilk at concentration 0.4 mg total solids/ml and incubated at 37°C for another 24h. Suspension and attached cells were harvested separately, centrifuged at 1000 rpm for 5 min, supernatant was discarded and cell pellets were resuspended in PBS. A drop of cell solution was then added to a cytospin slide (Andreas Hettich GmbH & Co., Germany) and spun at 500 rpm for 3 min using Hettich centrifuge with Hettich cyto-system (Andreas Hettich GmbH & Co., Germany). To fix the cells slides were put into 3.7% paraformaldehyde (Sigma-Aldrich, Ireland) for 10 minutes at room temperature and rinsed with PBS. Next, slides were put into 100% methanol for 5 minutes followed by 40 seconds in 0.5% eosin (Sigma-Aldrich, Ireland). Next, the slides were washed in running tap water and put into haematoxylin (Sigma-Aldrich, Ireland) for 5 seconds. The slides were washed in tap water and allowed to dry at room temperature. About 5 µl of DPX Mountant (Sigma-Aldrich, Ireland) for histology was put onto each slide and covered with cover slips. Microscopy was done using fluorescent microscope Olympus BX51 (Olympus America Inc, Center Valley, Pennsylvania, USA) at a magnification of 400X.

6.2.4. Fluorescence methods for detection of apoptosis

6.2.4.1. Tetramethylrhodamine ethyl ester perchlorate (TMRE) staining of cultured cells

Mitochondrial function in buttermilk-treated cells was analysed by staining with TMRE. This is a cell-permeant cationic red-orange fluorescent dye that is readily sequestered by active mitochondria. SW480 cells were seeded in 24-well plate at density $6x10^4$ cells/well. After 24h they were treated with buttermilk at concentrations between 0.1-0.6 mg total solids/ml and incubated at 37°C for another 24h or 48h. Media was removed, cells were washed with PBS and incubated with 0.1 μM TMRE solution (Sigma-Aldrich, Ireland) for 30 minutes at 37°C, cells were harvested by scraping and analyzed on flow cytometer at excitation 540 nm and max. emission 595 nm (BD FACSCanto™ II, BD Biosciences, San Jose, California, USA).

6.2.4.2. Apoptosis and cell cycle analysis using propidium iodide by flow cytometry

DNA fragmentation is a hallmark of apoptosis and provides a basis for the development of flow cytometry assays to identify apoptotic cells. Low molecular weight DNA is extracted prior to staining and measurement of cellular DNA. Flow cytometry was used to analyse DNA content of cells in the presence and absence of buttermilk. In this method, ethanol was used to fix and permeabilise SW480 cells aiding access of propidium iodide to nuclear DNA. Propidium iodide is a DNA-fluorochrome used to stain DNA in fixed cells. Because it reacts with both DNA and RNA, the staining procedure requires incubation with RNAse. SW480 cells were seeded in T-25 flasks at density $1x10^6$ cells/flask. After 24h they were treated with

buttermilk at concentration of 0.4 mg total solids/ml or 5 mM sodium butyrate as a positive control. After 48h attached and detached cells were collected together and counted, $1x10^6$ cells were pelleted and resuspended in 1 ml cold PBS. Next, 3 ml of cold (-20°C) 100% ethanol was gently added while gently swirling tube with cells. The cells were then frozen at -20°C for few days. On the day of analysis cells were thawed at room temperature and centrifuged at 3000 rpm for 5 min. Supernatant was discarded and the cell pellets were washed in 1 ml of PBS following centrifugation at 2000 rpm for 5 min. Supernatant was discarded and cell pellets were resuspended in 100 µl of 200 µg/ml DNAse free RNAse A solution and incubated at 37°C. After 30 min propidium iodine solution was added to cells (part of Annexin-V-FLUOS Staining Kit, Roche Diagnostics GmbH, Germany) at concentration 15 µl per 1 ml of cell suspension, incubated at room temperature for 15-20 min. Cell fluorescence was measured on flow cytometer (BD FACSCanto™ II, BD Biosciences, San Jose, California, USA).

6.2.4.3. Apoptosis detection using Annexin-V-FLUOS staining

Externalisation of phosphatidylserine was determined by staining with Annexin-V-FLUOS. SW480 cells were seeded in T-25 flasks at density $1x10^6$ cells/flask. After 24h they were treated with buttermilk at concentration 0.4 mg total solids/ml or 5 mM sodium butyrate as a positive control. After 48h attached and detached cells were collected separately and $1x10^6$ cells were counted, washed with PBS and centrifuged at 1000 rpm for 5 min. The cell pellet was resuspended in 100 µl of Annexin-V-FLUOS labeling solution (Roche Diagnostics GmbH, Germany), containing 20 µl/ml of Annexin-V-Fluorescein labelling reagent and 20 µl/ml of propidium iodide solution. Cells in labelling solution were incubated for 15 min at

room temperature in the dark and analysed on flow cytometer (BD FACSCanto™ II, BD Biosciences, San Jose, California, USA) at excitation 488 nm and max. emission 519 nm for Annexin-V- Fluorescein and exc. 532 nm and max. emission 578 nm for propidium iodide.

Samples were also analysed under a fluorescence microscope. For microscopy analysis SW480 cells were seeded in Lab-Tek Chamber Slides (Sigma-Aldrich, Ireland) at density $0.2x10^6$ cells/chamber. After 0.4 mg total solids/ml buttermilk incubation medium, chambers and silicon borders were removed from chamber slides and cells were covered with Annexin-V-FLUOS labeling solution, incubated for 15 min at room temperature in the dark and analyzed by fluorescence microscopy (Olympus BX51; Olympus America Inc, Center Valley, Pennsylvania, USA) using 488nm excitation wavelength and green channel for detection.

6.2.5. Determination of Akt using fast activated cell-based ELISA (FACE)

Determination of phosphorylated and total Akt in SW480 human colon cancer cells before and after buttermilk treatment was performed using colorimetric Fast Activated Cell-based ELISA (FACE) Kit (Active Motif, Belgium) according to manufacture's instruction manual. In short, SW480 cells were seeded in 96-well plates at density $8x10^3$ cells/well. After 48h they were treated with buttermilk at concentration between 0-1 mg total solids/ml and incubated at 37°C. After 24h media was removed and cells were fixed with 4% formaldehyde in PBS and incubated at room temperature. After 20 min cells were washed 3 times with wash buffer (0.1% Triton X-100 in PBS) and incubated for another 20 min with quenching buffer (10% H_2O_2 and 10% sodium azide in wash buffer) to inactivate cells' endogenous

peroxidase activity. Cells were then washed 2 times with wash buffer, incubated for 1h at room temperature with antibody blocking buffer and washed 2 times with wash buffer. Next, cells were incubated overnight at 4°C with primary antibody (against phosphorylated Akt or total Akt) diluted in antibody dilution buffer. Next day primary antibody was removed, cells were washed with wash buffer and incubated with secondary antibody diluted in antibody dilution buffer for 1h at room temperature. Cells were then washed 3 times with wash buffer and 5 times with PBS and incubated with developing solution for 10-20 min at room temperature protected from direct light. After the blue colour being developed reaction was stopped with stop solution and absorbance was read on Multi-Detection Microplate Reader Synergy™ HT (supplied by BioTek® Instruments, Inc) at 450nm. To normalize readings to cell number cells were washed with wash buffer and with PBS, followed by incubation with Crystal Violet for 30 min at room temperature. Cells were then washed with wash buffer and incubated with 1% SDS solution for 1 h on shaker. Absorbance was read at 595 nm. The measured OD_{450} readings were corrected for cell number by dividing the OD_{450} reading for a given well by the OD_{595} reading for that well.

6.2.6. Analysis of β-catenin, ERK2, phosphorylated-ERK 1/2, c-myc, phosphorylated p38 and phosphorylated JNK using In-Cell Western Assay

In-Cell Western Assay was performed using LI-COR Biosciences In-Cell Western™ Assay Kit I containing IRDye 800CW-labeled anti-rabbit and anti-mouse antibodies, Odyssey Blocking Buffer and DRAQ5™ and Sapphire700™ reagents for cell number normalization. Assay was performed according to manufacturer's instruction; briefly SW480 cells were seeded in 96-well plates at density $8x10^3$ cells/well. After 48h they were treated with buttermilk at concentration ranging

between 0-1 mg total solids/ml and incubated for another 24h or 48h. Cells were then fixed with 3.7% paraformaldehyde in PBS for 20 min at room temperature. To allow permeabilization, cells were washed 5 times with wash buffer (0.1% Triton X-100 in PBS). Next, 150 µl of Odyssey Blocking Buffer was carefully added to each well and incubated for 1.5h at room temperature with moderate shaking. Blocking buffer was removed and cells were incubated with primary antibody, diluted in Odyssey Blocking Buffer overnight at 4ºC with gentle shaking. Next day, plates with cells were washed 5 times with 0.1% Tween-20 wash buffer and incubated with fluorescently labeled secondary antibody (1:800), DRAQ5™ (1:2000) and Sapphire700™ (1:1000) solution diluted in Odyssey Blocking Buffer and incubated in dark for 1h at room temperature with gentle shaking. Next, cells were washed 5 times with 0.1% Tween-20 wash buffer and read on the Odyssey Imager using 800 nm channel for IRDye 800CW detection and 700 nm channel for normalization stains. Data was processed using Odyssey Application Software version 3.0.

6.2.7. Western Blot analysis of caspase-9 and caspase-3

6.2.7.1 Harvesting the cells

After 48h following buttermilk treatment SW480 human colon cancer cells in T-25 flasks were placed on ice, washed with cold PBS, scraped and centrifuged at 1000 rpm for 5 min. Supernatant was then removed, the pellets were resuspended in 0.5 ml ice-cold PBS, transferred into eppendorf tubes and centrifuged at 1000 rpm for 5 min. Supernatant was then removed and the appropriate volume of lysis buffer was added. Lysis buffer contained the following protease inhibitors: 2 µg/ml aprotinin, 1 µg/ml leupeptin, 1 mM PMSF and phosphatase inhibitors: 5 mM NaF and 1 mM

Na_3VO_4 (all supplemented by Sigma-Aldrich, Ireland). The cells were then sonicated (3 times for 5 sec) on MSE Soniprep150 sonicator (MSE (UK) Ltd, Wolf Laboratories, London, UK) and spun at 14 000 rpm for 15 min at 4°C. The supernatant was transferred into new tubes and stored at -70°C until analyzed.

6.2.7.2. Protein estimation

To estimate the protein concentration in each sample the Bicinchoninic Acid Protein assay (Sigma-Aldrich, Ireland) was carried out. Standards for the standard curve were prepared using bovine serum albumin (BSA) solutions at concentration range 0-2 mg/ml in lysis buffer. The volume required to load 30 µg protein was calculated before loading the SDS-PAGE gel.

6.2.7.3. SDS-PAGE

Acrylamide gel (12%) for electrophoresis was prepared for protein separation. The prestained protein marker (Cell Signaling Technology, Inc; Danvers, Massachusetss, USA) and the samples mixed with 4X sample buffer containing β-mercatoethanol were put into the boiling water for 5 minutes. The samples and the markers were loaded onto gels and run in a chamber with running buffer at 120V for about 100 min.

6.2.7.4. Blotting and detection

After electrophoresis, each gel was placed in a cassette along with PVDF membrane, between 4 sheets of filter paper and 2 sponges. Transfer of the proteins to the membrane was carried out in the chamber with the transfer buffer for 45 minutes

at 100V. The membranes were then washed using TBS-Tween and the non-specific binding sites were blocked with 5% milk-TBS-Tween solution for 1 hour at room temperature with gentle shaking. Primary antibodies were then diluted in 5% milk-TBS-Tween solution and incubated overnight at 4°C with gentle shaking. Next, the membranes were washed 4 times in TBS, for 7 minutes each time. Next, the membranes were incubated with secondary antibody in 3% milk-TBS-Tween for 1 hour and washed in TBS, 3 times for 10 minutes each time. The membranes were incubated in Pierce® ECL detection solution (Thermo Scientific, Rockford, Illinois, USA) for 5 minutes, exposed to autoradiography film (Kodak™, Sigma-Aldrich, Ireland) and developed in D-19 Developer (Kodak™, Sigma-Aldrich, Ireland).

6.2.8. Immunofluorescence analysis of cleaved PARP, apoptosis-inducing factor (AIF) and endonuclease G

SW480 cells were seeded on Lab-Tek Chamber Slides (Sigma-Aldrich, Ireland) at density of $0.2x10^6$ cells/chamber. After 24h they were treated with buttermilk at concentration 0.4 mg total solids/ml and incubated at 37°C for 48h. Media was then removed and cells were fixed with 3.7% paraformaldehyde in PBS and left for 15 minutes at room temperature. Next, fixative was removed and following washing cells three times in PBS for 5 min each, with gentle shaking, they were blocked in Blocking Buffer (PBS containing 5% normal goat serum and 0.3 % Triton X-100). After 1 h blocking cells were incubated overnight at 4°C with primary antibody diluted in Antibody Dilution Buffer (PBS containing 1% BSA and 0.3 % Triton X-100). Next day cells were rinsed three times in PBS for 5 minutes each, with gentle shaking and incubated in FITC-conjugated anti-rabbit IgG developed in goat (Sigma-Aldich, Ireland) and DRAQ5™ (LI-COR Biosciences; Lincoln, Nebraska

USA) diluted in Antibody Dilution Buffer for 2 hours in dark at room temperature, with gentle shaking. Cells were then rinsed three times in PBS for 5 minutes each and examined under Leica DM TCS SP5 confocal scanning laser microscope (Leica Microsystems, Wetzler, Germany) and using Leica LAS AF Lite program.

6.2.9. Statistical analysis

Three independent experiments were performed in quadruplicate for each treatment and the Student's *t*-test (Microsoft Office Excel) was used to determine significant difference between treatments or comparing to the control.

6.3. Results

6.3.1. Haematoxylin and eosin staining of cultured cells

Haematoxylin and eosin staining, method commonly used in histology and cytology, was carried out to determine if cells undergo apoptosis after buttermilk treatment. (**Fig. 6.1 a-f**). This stain can display a broad range of cytoplasmic, nuclear and extracellular matrix features. Haematoxylin stained nuclear chromatin deep blue, whereas eosin stained cytoplasmic structures and extracellular matrix with varying degrees of pink. Compared to untreated healthy cells (**Fig. 6.1 a**) we observed condensation of chromatin and shrinkage of nuclei of some cells (**Fig. 6.1 d-f**) after buttermilk treatment indicating activation of apoptosis (black arrows). However, we could not detect characteristic apoptotic bodies after buttermilk treatment and necrotic cells were also observed.

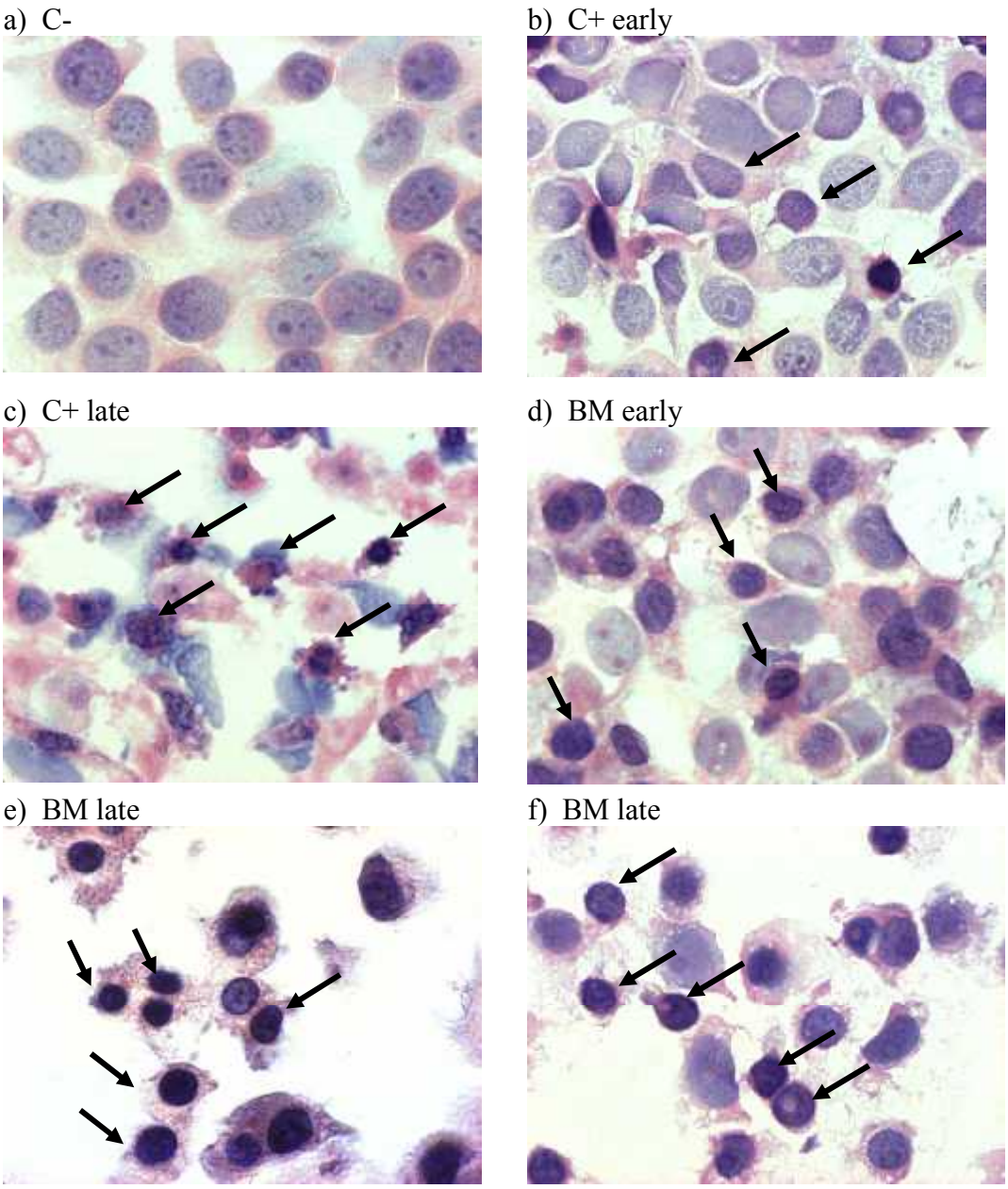


Fig. 6.1. Light microscopy images (400X) following haematoxylin and eosin staining of SW480 cells in Leibovitz-15 medium: a) negative control, C- b) positive control (sodium butyrate), C+, early stages of apoptosis, c) positive control (sodium butyrate), C+, late stages of apoptosis, d) cells after buttermilk treatment (BM), early stages of apoptosis, e) & f) cells after buttermilk treatment (BM), late stages of apoptosis. The arrows indicate the apoptotic cells.

6.3.2. Apoptosis detection using Annexin-V-FLUOS by FACS

We also investigated other apoptotic biomarkers in colon cancer cells after buttermilk treatment. In all stages of apoptosis phosphatidylserine is translocated from the inner part of the membrane to the outer layer. Flow cytometry is a technique for counting and examining microscopic particles by suspending them in a stream of fluid and passing them by an electronic detection apparatus. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of up to thousands of particles per second. Fluorescence-activated cell sorting (FACS) is a specialized type of flow cytometry. It provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. It is a useful scientific instrument, as it provides fast, objective and quantitative recording of fluorescent signals from individual cells as well as physical separation of cells of particular interest. The acronym FACS is trademarked and owned by Becton, Dickinson and Company (BD). Annexin-V-Fluorescein binds in a Ca^{2+}-dependent manner to negatively charged phospholipid surfaces and shows high specificity to phosphatidylserine. It does not bind normal intact cells. This protein can be used as a sensitive probe for phosphatidylserine exposure upon the outer leaflet of the cell membrane and is therefore suited to detect apoptotic cells in cell populations. Since necrotic cells also expose phosphatidylserine as they lose membrane integrity, propidium iodide is used as a DNA stain to distinguish between necrotic cells and Annexin-V labelled cells. Excitation and emission wavelengths for Annexin-V-Fluorescein detection were 488 nm and 518 nm, respectively; hence FITC filter was used. For identification of propidium iodide we used APC filter, which detects fluorescence signal at excitation 595 nm and emission 660 nm, which was the closest

to fluorescence characteristics of this dye. Using FACS method we observed an increase in % of green staining suggesting that phosphatidylserine was externalised in buttermilk-treated cells (**Fig. 6.2 d**). Analysis showed that 91.1% of control cells were viable and that the remainder (8.9%) were apoptotic (**Fig. 6.2 a**). Following treatment with buttermilk 57.5% of cells were viable and 42.5% of cells were apoptotic (**Fig. 6.2 d**). Treatment with the positive control (5 mM sodium butyrate) yielded 62.9% viable cells and 36.6% apoptotic cells (**Fig. 6.2 c**).

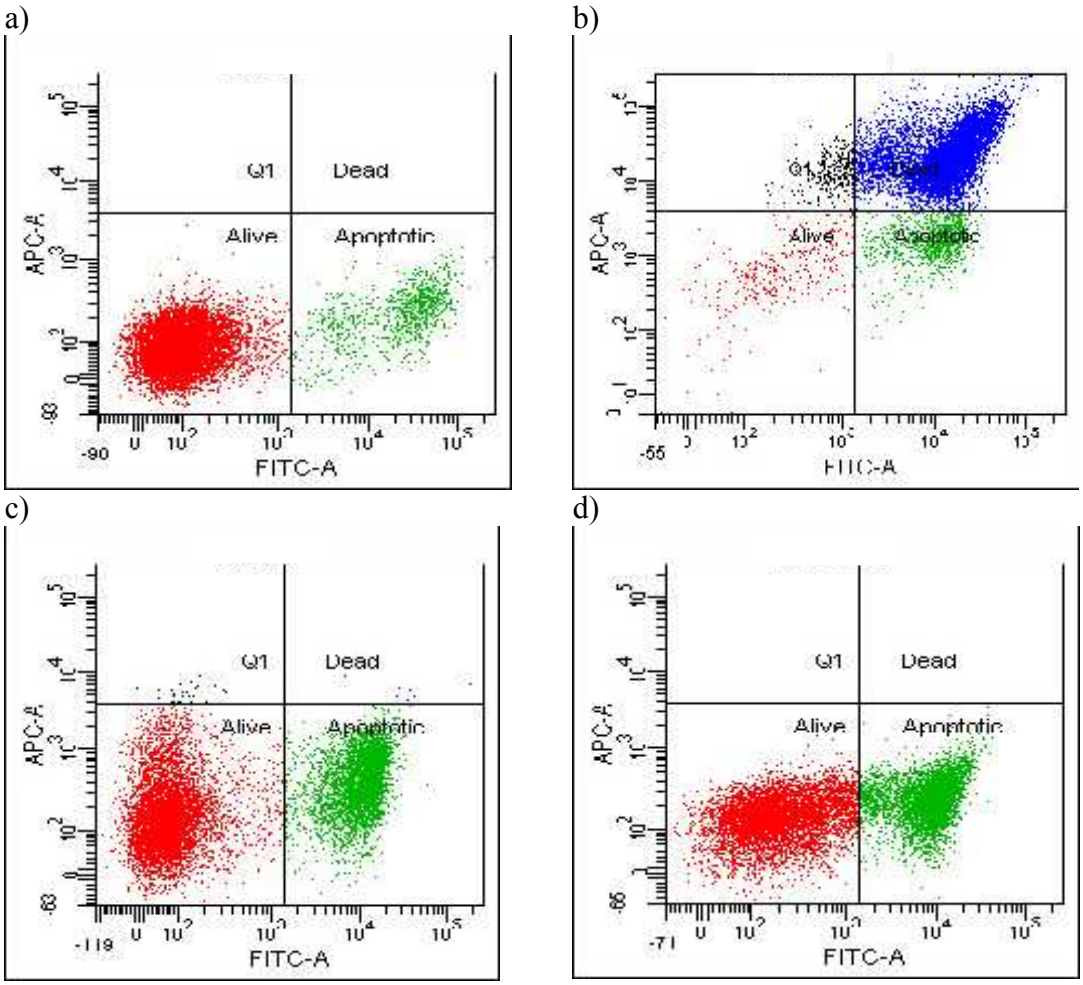


Fig. 6.2. FACS examination of phosphatidylserine exposure to the outer membrane with Annexin-V-FITC and counterstaining with propidium iodide, performed 48h after exposure of the SW480 cells in Leibovitz-15 medium to buttermilk in order to detect early apoptotic cells (green population) and late apoptotic/dead cells (blue population), respectively. A) control with untreated, healthy cells, b) dead, necrotic cells, c) positive control (5 mM NaBut), d) cells after 48h buttermilk treatment.

6.3.3. Apoptosis detection using Anexin-V-FLUOS by fluorescence microscopy

Fluorescent imaging using the same Annexin-V-FLUOS dyes and green filter (excitation wavelength in the range 450-500 nm, emission wavelength in the range 515-565 nm) confirmed results from flow cytometry (**Fig. 6.3 a-e**). Apoptotic cells bind to fluorescein, hence are visible in green and can be differentiated from necrotic cells because they exclude propidium iodide. Necrotic cells took up propidium iodide and stained orange/green while apoptotic cells stained green only.

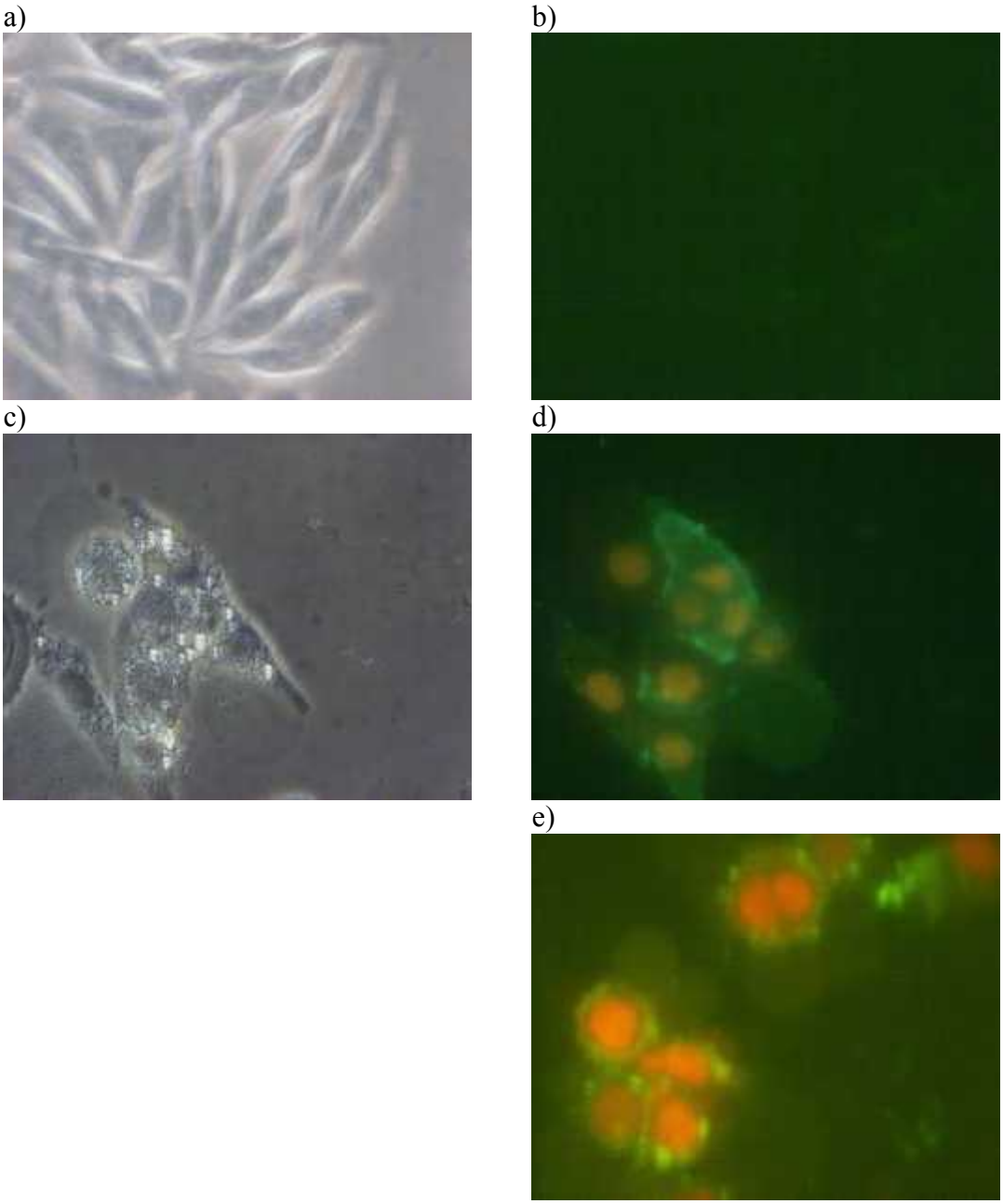


Fig. 6.3. Fluorescent microscopic examination of phosphatidylserine exposure to the outer membrane with Annexin-V-FITC and counterstaining with propidium iodide, performed 48h after exposure of the SW480 cells in Leibovitz-15 medium to buttermilk. Light (a) and fluorescent (b) microscopic images of untreated, healthy cells; light (c) and fluorescent (d, e) microscopic images of cells after 48 h buttermilk treatment.

6.3.4. Apoptosis and cell cycle analysis by FACS

DNA analysis is, after immunofluorescence, the second most important application of flow cytometry. Propidium iodide is used to bind to DNA. The amount of propidium iodide bound correlates with the content of DNA within a given cell. Once cells are stained they may be analysed on a flow cytometer. Apoptotic cells are detected as cells having degraded DNA (hypodiploid DNA) content due to the fact that cells loose DNA by shedding apoptotic bodies. Additionally, rinsing and staining extracts DNA resulting in apoptotic cells manifesting reduced DNA content. They are recognised following staining of cellular DNA as cells with decreased stainability (sub-G1 cells) located to left of a peak representing G1 cells on DNA content frequency histograms. Our studies using FACS showed apoptotic cells with fractional DNA content after buttermilk treatment (**Fig. 6.4 c**). The number of apoptotic SW480 cells was found to be significantly greater ($P \leq 0.001$) when cells were treated with sodium butyrate (36.8%) or with buttermilk (34.85%) compared to control (9.15%). The cell cycle position of non-apoptotic cells was also estimated. Percentage of G1/G0 non-apoptotic cells was 50.2, 44.4 and 30.55% in control, sodium butyrate and buttermilk treatments, respectively. Together the data suggest that buttermilk triggered apoptosis in SW480 cells and affected progression of non-apoptotic cells through the cell cycle.

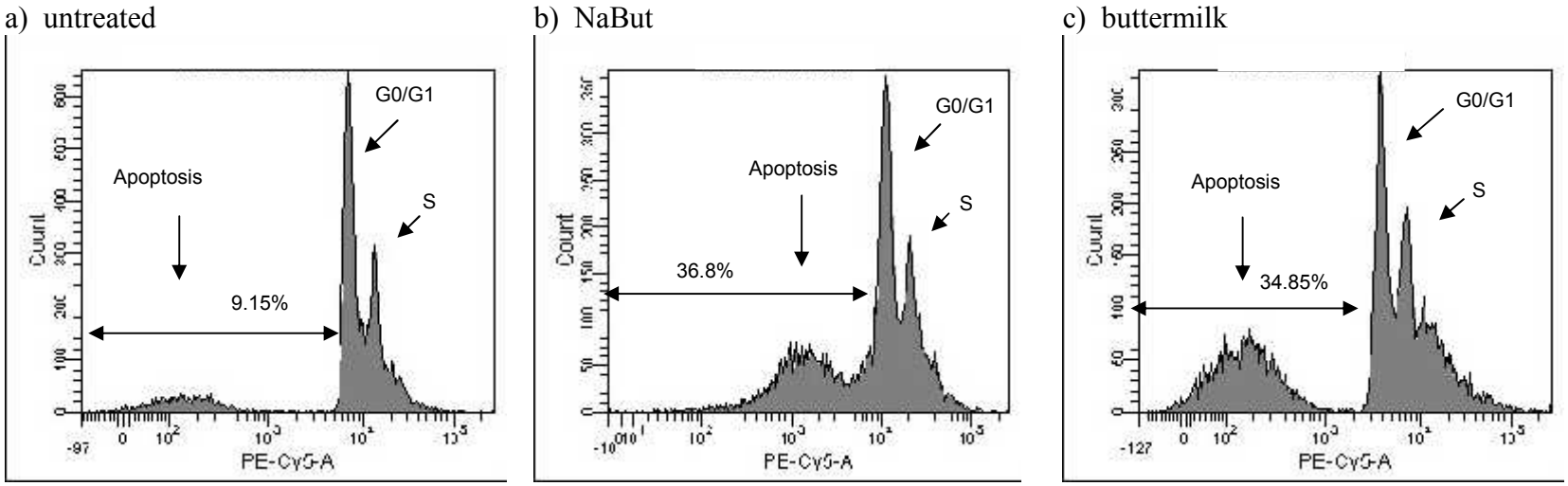


Fig. 6.4. Induction of apoptosis in SW480 cells by buttermilk treatment. SW480 cells in Leibovitz-15 medium were treated with buttermilk (0.4 mg total solids/ml) and NaBut (5mM) for 48 h. Subsequently, nuclei were isolated by hypotonic lysis, stained with propidium iodide and analysed by flow cytometry for hypodiploid DNA content as indicator of apoptosis. Figure shows representative histograms of: a) untreated cells, b) 5 mM NaBut (positive control), c) cells after 48h of buttermilk treatment. A total of 10 000 nuclei were counted per data point. The rate of nuclei with hypodiploid DNA content is expressed as % total nuclei. The values represent the mean of triplicate experiments.

6.3.5. Analysis of mitochondrial membrane depolarization

Dissipation of mitochondrial membrane potential (Δ_ω) is a critical event in the process of cell death. To examine whether antiproliferative activity of buttermilk in SW480 cells was associated with changes in Δ_ω, we measured uptake and retention by mitochondria of tetramethylrhodamine ethyl ester perchlorate (TMRE). This is a fluorescent probe whose accumulation and subsequent retention in healthy non-apoptotic cells is dependent on Δ_ω. In apoptotic cells, a trans-membrane electrical potential gradient collapses and TMRE dye no longer accumulates inside the mitochondria and becomes more evenly distributed throughout the cytosol; hence overall cellular fluorescence levels drop. **Fig 6.5 a-f** shows representative histograms from cells treated with varying concentrations of buttermilk solids and subjected to FACS analysis. Beyond an arbitrary threshold of $5x10^3$, buttermilk decreased TMRE fluorescence in a concentration-dependent manner and shifted the distribution curve leftward, indicating depolarisation of Δ_ω relative to untreated cells. Approximately 76.6% of control cells retained high levels of fluorescence as compared to 16.8% of cells treated with buttermilk (0.15 mg total solids/ml) for 48h ($P \leq 0.001$).

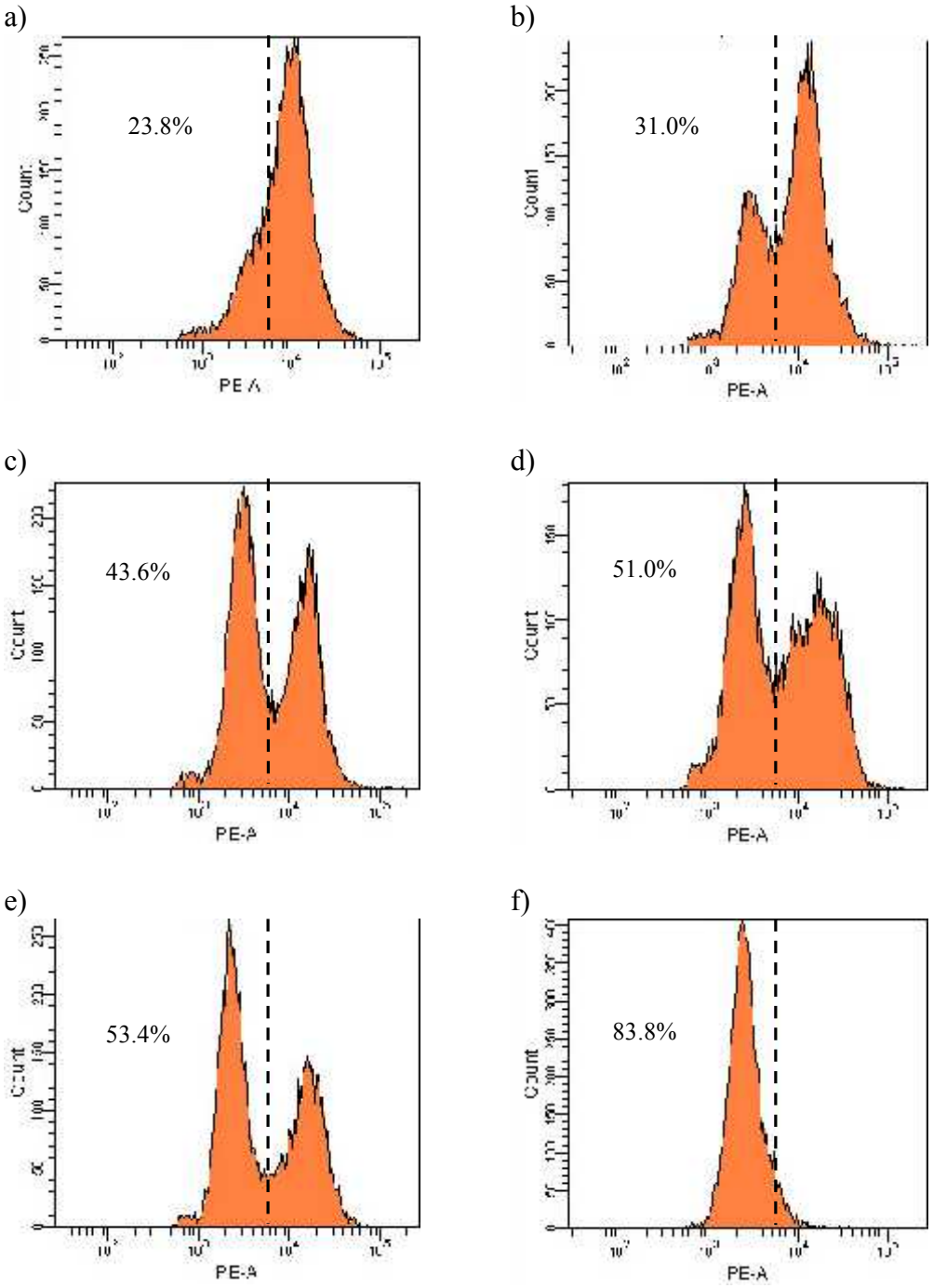


Fig.6.5. Loss of the mitochondrial membrane potential Δ_{ω}. The histograms are representative of experiments with SW480 cells in Leibovitz-15 medium that were either left untreated (a) or treated with buttermilk at concentrations: 0.1 mg total solids/ml for 24h (b); 0.15 mg total solids/ml for 24h (c); 0.2 mg total solids/ml for 24h (d); 0.25 mg total solids/ml for 24h (e); 0.15 mg total solids/ml for 48h (f). The mitochondrial membrane potential was determined by staining of

cells with the fluorescent dye, TMRE (tetramethylrhodamine ethyl ester perchlorate), which migrates and accumulates inside mitochondria in healthy cells. In apoptotic cells, a trans-membrane electrical potential gradient collapses and TMRE dye becomes more evenly distributed throughout the cytosol, hence shifting the distribution curve leftward. The control values were obtained with untreated SW480 cells. The rate of cells with collapsed mitochondrial membrane potential is expressed as % of total cell counts. The values represent the mean of triplicate experiments.

6.3.6. Analysis of cleavage of apoptotic biomarkers: caspase-3 , caspase-9 and PARP

Apoptotic cell death is orchestrated by activation of death proteases known as caspases which target an array of cellular proteins for degradation, resulting in controlled disassembly of the cell. Caspases are present in cells as inactive precursors that require limited proteolysis to become activated. The activation status of an initiator caspase (caspase-9) and an executioner caspase (caspase-3) was analysed by SDS-PAGE/immunoblotting. Immunoblotting with antibodies raised against caspase-9 (**Fig. 6.6 a**) and caspase-3 (**Fig. 6.6 b**) revealed appearance of processed subunits (MW 17kDa) of these enzymes in cells treated with 5 mM sodium butyrate (positive control). However processed forms of these caspases were not detected in cells treated with buttermilk (**Fig. 6.6 a** and **b**). Western Blot analysis (**Fig. 6.6 a**) showed a very weak band (MW 17kDa) of cleaved caspase-9 in detached/dead (S) cells after 48h of buttermilk treatment at concentration of 0.3 mg total solids/ml. However, no band was observed in

attached (A) cells from the same experimental flask, which was a predictable result, as some small population of cells is highly likely to enter apoptosis pathway while dying. Other methods, such as In-Cell Western analysis of cleaved caspase-3 in cells treated with increasing amounts of buttermilk (**Fig. 6.7**), incubation of treated cells with Rhodamine 110-based derivative of bis-L-aspartic acid amide (Molecular Probes, Inc; Eugene, Oregon, USA) (data not shown) and ApoToxGlo™ Triplex Assay (Promega Corporation, Madison, WI, USA) (data not shown) also did not show cleavage of caspases, whereas cleaved caspase was observed in the positive control, (cells treated with 5 mM sodium butyrate) (**Fig. 6.7**).

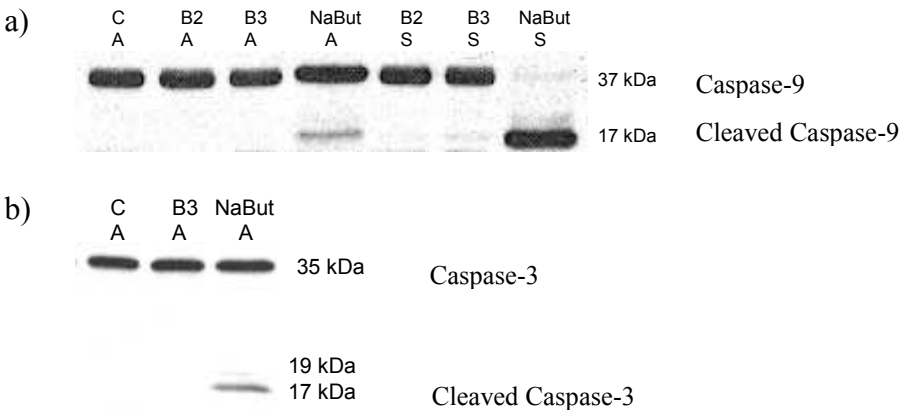


Fig. 6.6. Western blot analysis for the status of caspase-9 and caspase-3. SW480 cells in Leibovitz-15 medium were treated with buttermilk for 48 h. Then, cell lysates were prepared and subjected to immunoblot analysis. Abbreviations: A, cells attached to the bottom of flask during harvesting cells for analysis; S, cells detached (in suspension) from the bottom of the flask during harvesting cells for analysis; C, negative control; NaBut, cells treated with 5 mM sodium butyrate, positive control; B2, cells treated with buttermilk at concentration 0.2 mg total solids/ml; B3, cells treated with buttermilk at concentration 0.3 mg total solids/ml.

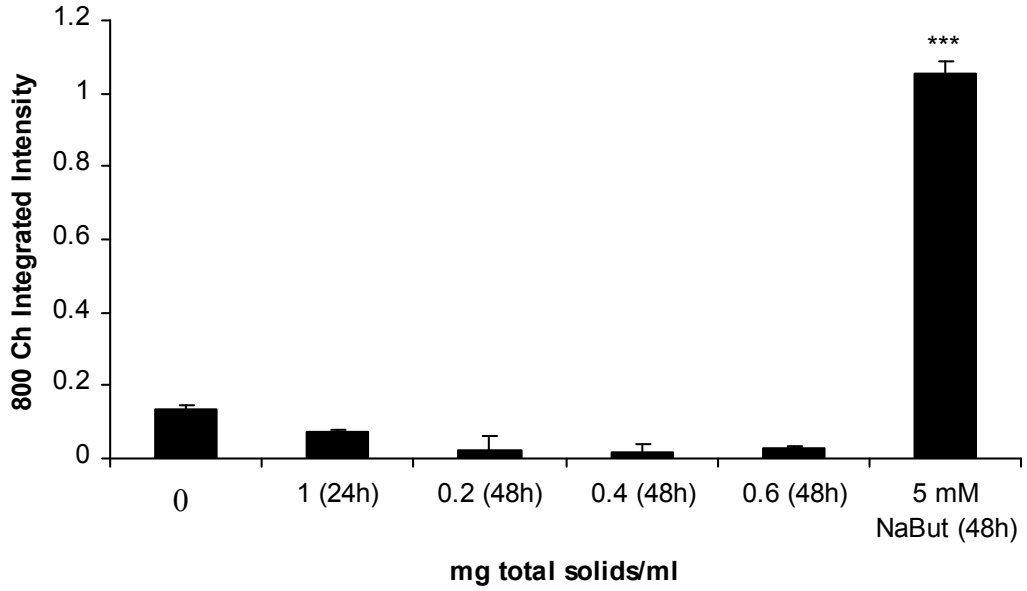


Fig. 6.7. Expression of cleaved caspase-3. SW480 cells in Leibovitz-15 medium were either left untreated or exposed to buttermilk at indicated times and concentrations. SW480 cells were additionally treated with 5 mM NaBut, which served as positive control. After the indicated times, treatment was removed, cells were washed and subjected to In-Cell Western analysis using specific antibody against cleaved caspase-3. Results are shown as mean ± S.D. of three independent experiments. Changes in caspase-3 were considered significantly different than control when: * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

Immunostaining for cleaved PARP was carried out as an alternative indirect method for detecting caspase activity. PARP is a 116kDa nuclear poly (ADP-ribose) polymerase that is involved in DNA repair. Cleavage of PARP by caspase-3 into 89 kDa and 24 kDa fragments facilitates cellular disassembly and is a valuable marker of apoptosis. The effect of buttermilk on PARP cleavage was examined by immunofluorescence analysis of cells using a cleaved PARP

antibody that recognises the large fragment (89 kDa) of mouse PARP1. It does not recognise full length PARP1 or other isoforms. Apoptosis evaluation induced *in vitro* by sodium butyrate in SW480 cells yielded green-labeled cleaved PARP positive cells (**Fig. 6.8**). Neither control cells nor buttermilk-treated cells yielded green-labelled cells, indicating that cleavage of PARP did not contribute to the commitment to apoptosis by buttermilk (**Fig. 6.8**).

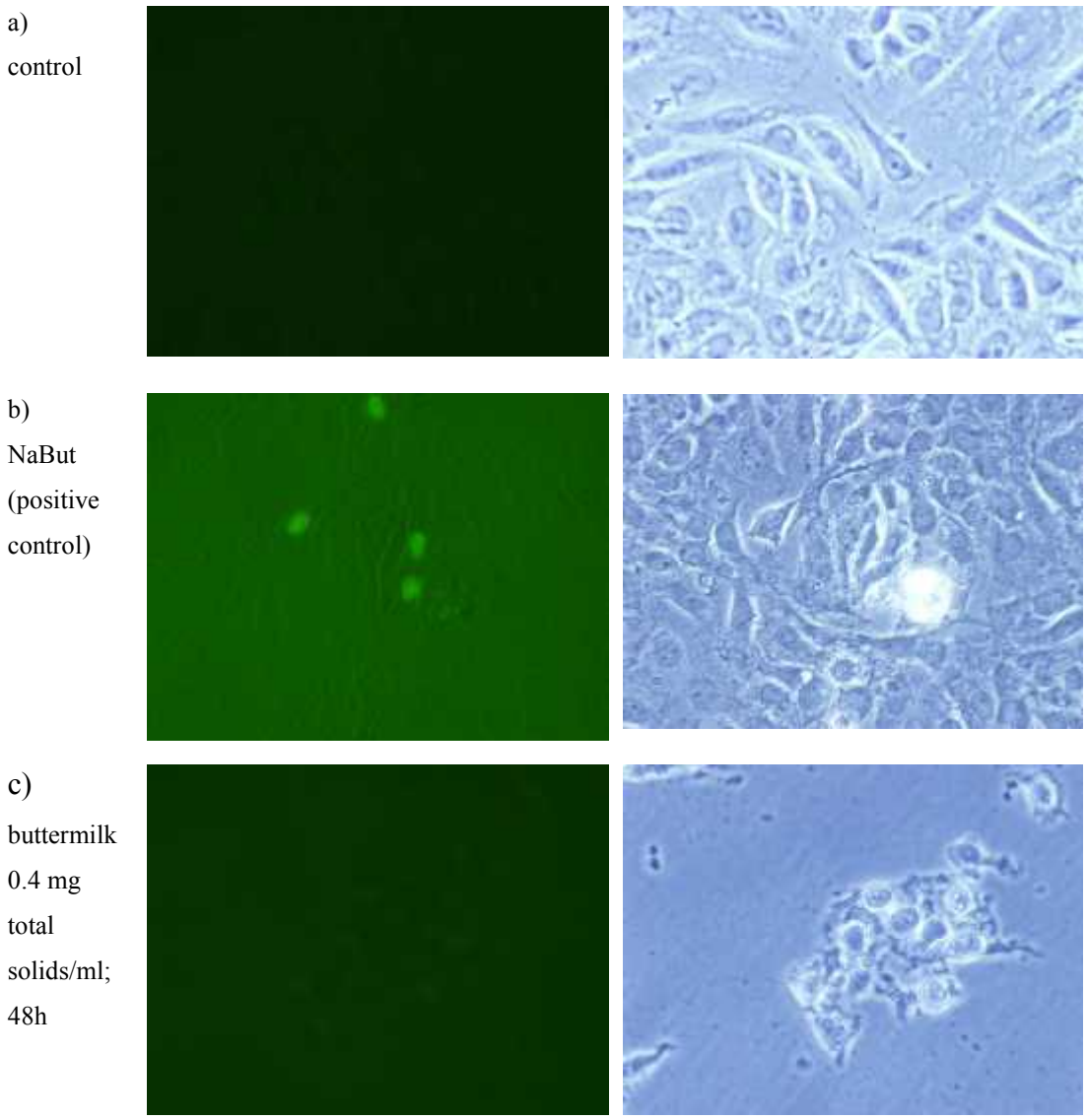


Fig. 6.8. Immunofluorescent detection of cleaved PARP. SW480 cells in Leibovitz-15 medium were either untreated (a) or treated with buttermilk at concentration 0.4 mg total solids/ml (c). SW480 cells were also treated with 5mM

NaBut, which served as positive control (b). After 48h cells were labelled with antibody against cleaved PARP and examined under immunofluorescent and light microscope. Green signal of cleaved PARP was observed only in positive control.

6.3.7. Efect of buttermilk on signalling molecules: Akt, ERK 1/2, β-catenin and c-myc

Protein phosphorylation is the principal regulatory mechanism of cell signalling pathways. Protein kinases including Akt and ERK1/2 become activated by phosphorylation before activating nuclear targets including c-myc transcription factor. Fast activated cell-based ELISA (also termed In-Cell Western) methodology was used to evaluate the activation state of signalling pathways in SW480 cells after treatment with buttermilk. The relative amounts of phosphorylated Akt and ERK1/2 were determined using antibodies to phosphorylated and native proteins. We detected a decrease in phosphorylated Akt protein in SW480 cells after 24h of buttermilk treatment, while total Akt remained unchanged compared to the control (**Fig. 6.9**). Interestingly, at very low buttermilk concentration, 0.1 mg total solids/ml, we could observe an increase in phosphorylated Akt, as well as total Akt ($P \leq 0.01$). A dose-dependent decrease in β-catenin protein levels, which is overexpressed in colon cancer and plays a significant role in Wnt signalling pathway, was also seen after 24 h of buttermilk treatment (**Fig. 6.10**). Furthermore, we also observed a time-dependent decrease in ERK2 (**Fig. 6.11**) and a dose-dependent decrease in phosphorylated ERK 1/2 (**Fig. 6.12**) proteins, as well as c-myc (**Fig. 6.13**) protein; however using the same

method, we could not see any changes in other MAPK proteins, such as phosphorylated p38 (**Fig. 6.14**) and phosphorylated JNK (**Fig. 6.15**).

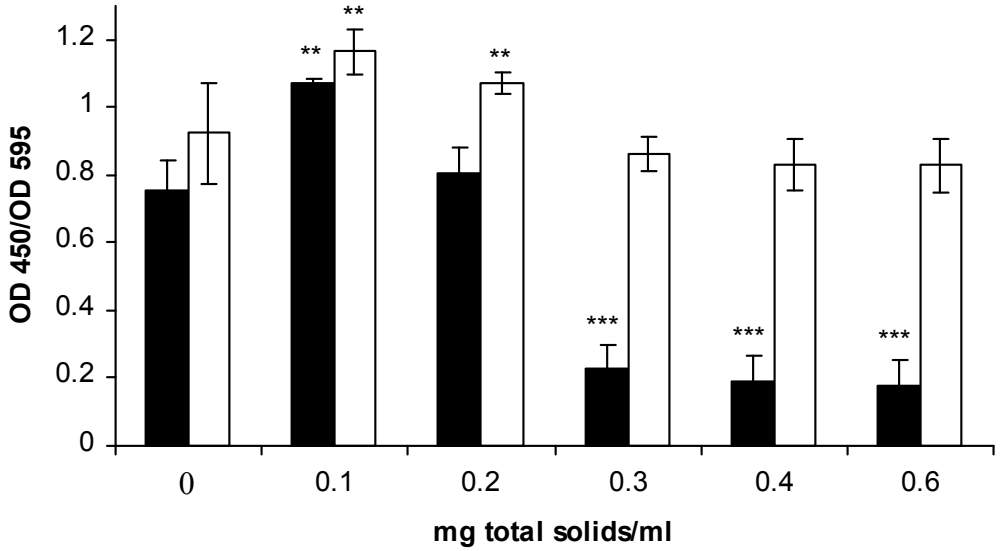


Fig. 6.9. Changes in total Akt (*white bars*) and phosphorylated Akt (*black bars*) in SW480 cells in Leibovitz-15 medium after 24h of buttermilk treatment. SW480 cells were either left untreated or exposed to buttermilk at indicated concentrations. After 24h buttermilk was removed and cells were subjected to colorimetric Fast Activated Cell-based ELISA assay based on total Akt or phosphorylated Akt as primary antibodies. Results are shown as mean ± S.D. of three independent experiments. Changes in Akt and phosphorylated Akt were considered significantly different than their controls when: * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

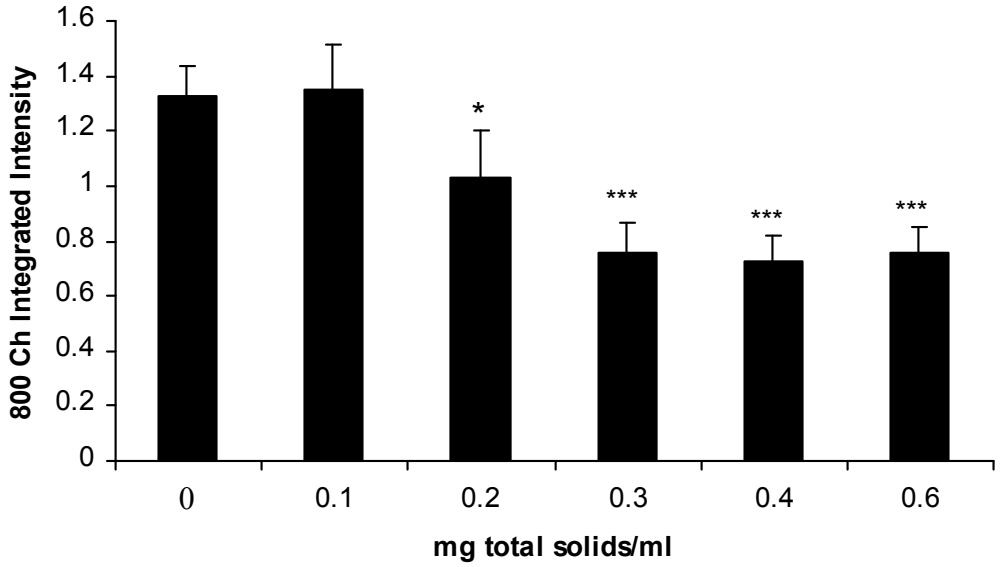


Fig. 6.10. Changes in β-catenin protein in SW480 colon cancer cells after 24h of buttermilk treatment. SW480 cells in Leibovitz-15 medium were either left untreated or exposed to buttermilk at indicated concentrations. After 24h treatment was removed, cells were washed and subjected to In-Cell Western analysis using specific antibody against β-catenin. Results are shown as mean ± S.D. of three independent experiments. Changes in β-catenin were considered significantly different than control when: * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

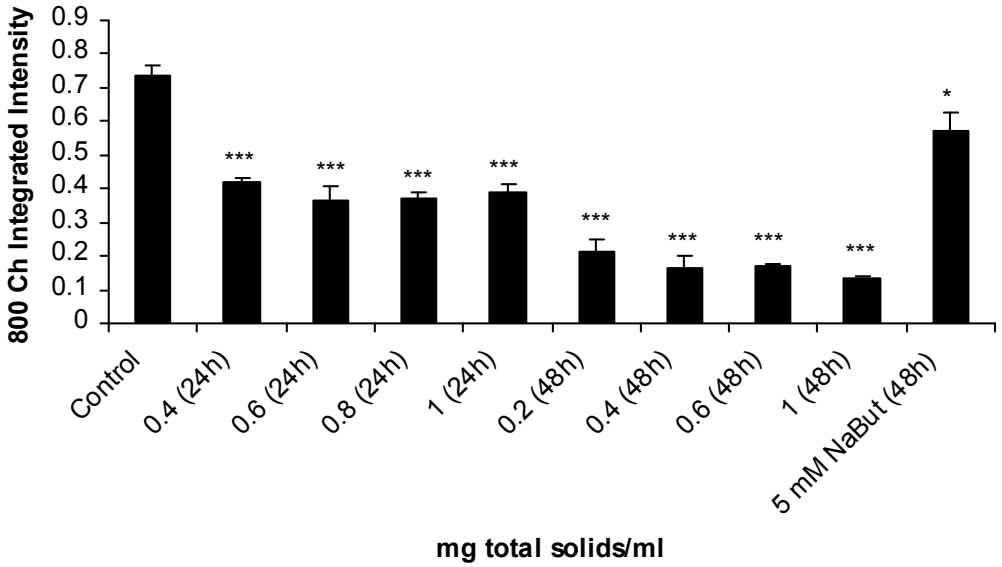


Fig. 6.11. Changes in ERK2 protein in SW480 colon cancer cells after 24h and 48h (as indicated) of buttermilk. SW480 cells in Leibovitz-15 medium were either left untreated or exposed to buttermilk at indicated times and concentrations. SW480 cells were additionally treated with 5 mM NaBut, which served as positive control. After the indicated times, treatment was removed, cells were washed and subjected to In-Cell Western analysis using specific antibody against ERK2 protein. Results are shown as mean ± S.D. of three independent experiments. Changes in ERK2 were considered significantly different than control when: * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

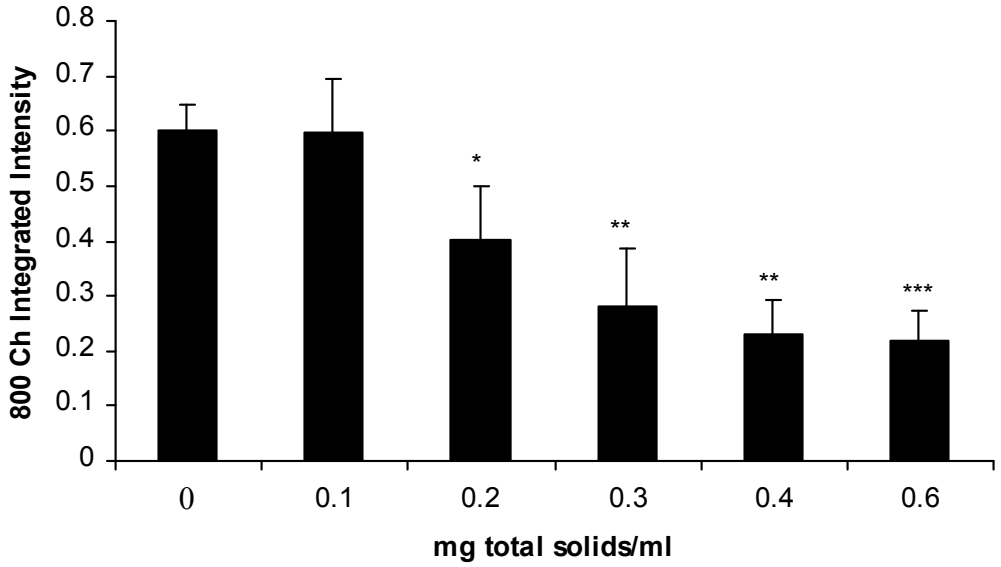


Fig. 6.12. Changes in phosphorylated ERK1/2 protein in SW480 colon cancer cells in Leibovitz-15 medium after 24h of buttermilk treatment at different concentrations. SW480 cells were either left untreated or exposed to buttermilk at indicated times and concentrations. After 24h treatment was removed, cells were washed and subjected to In-Cell Western analysis using specific antibody against phosphorylated ERK1/2 protein. Results are shown as mean ± S.D. of three independent experiments. Changes in phosphorylated ERK1/2 were considered significantly different than control when: * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

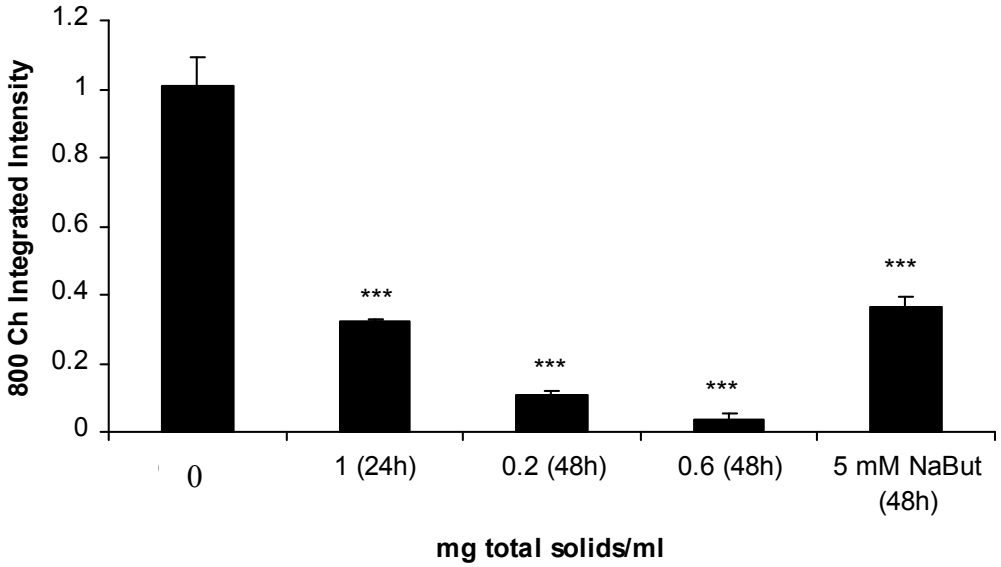


Fig. 6.13. Changes in c-myc protein in SW480 colon cancer cells after 24h and 48h (as indicated) of buttermilk treatment at different concentrations. SW480 cells in Leibovitz-15 medium were either left untreated or exposed to buttermilk at indicated times and concentrations. SW480 cells were additionally treated with 5 mM NaBut, which served as positive control. After the indicated times, treatment was removed, cells were washed and subjected to In-Cell Western analysis using specific antibody against c-myc protein. Results are shown as mean ± S.D. of three independent experiments. Changes in c-myc protein were considered significantly different than control when: * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

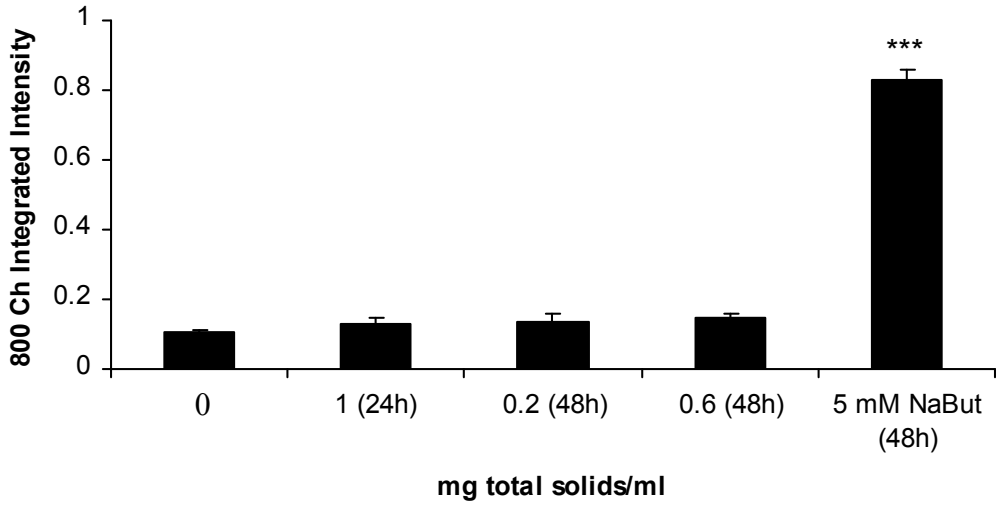


Fig. 6.14. Expression of phosphorylated p38 protein in SW480 colon cancer cells after 24h and 48h (as indicated) of buttermilk treatment at different concentrations. SW480 cells in Leibovitz-15 medium were either left untreated or exposed to buttermilk at indicated times and concentrations. SW480 cells were additionally treated with 5 mM NaBut, which served as positive control. After the indicated times, treatment was removed, cells were washed and subjected to In-Cell Western analysis using specific antibody against phosphorylated p38 protein. Results are shown as mean ± S.D. of three independent experiments. Changes in phosphorylated p38 protein were considered significantly different than control when: * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

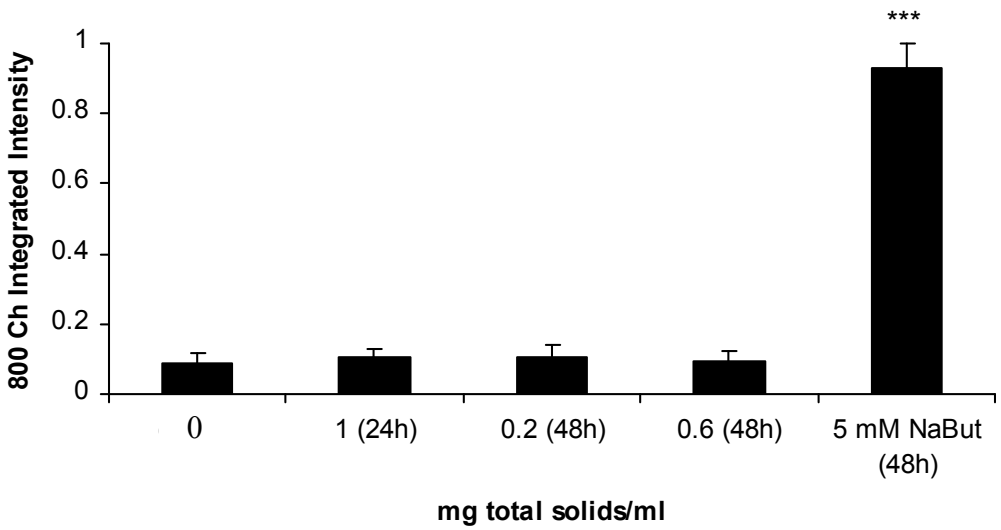


Fig. 6.15. Expression of phosphorylated JNK protein in SW480 colon cancer cells after 24h and 48h (as indicated) of buttermilk treatment at different concentrations. SW480 cells in Leibovitz-15 medium were either left untreated or exposed to buttermilk at indicated times and concentrations. SW480 cells were additionally treated with 5 mM NaBut, which served as positive control. After the indicated times, treatment was removed, cells were washed and subjected to In-Cell Western analysis using specific antibody against phosphorylated JNK protein. Results are shown as mean ± S.D. of three independent experiments. Changes in phosphorylated JNK protein were considered significantly different than control when: * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

6.3.8. Analysis of endonuclease G and AIF by immunofluorescence

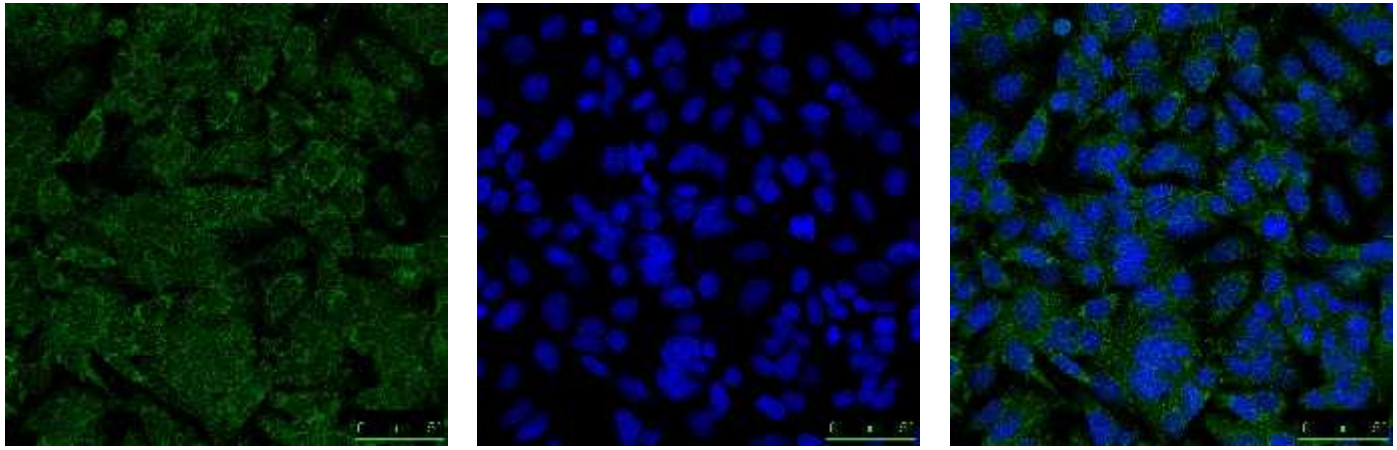
Knowing that buttermilk induced apoptosis in SW480 colon cancer cells, but did not trigger cleavage of either caspases or PARP protein, we investigated further if it led to release of AIF and endonuclease G from depolarized mitochondria, as these molecules can lead to suicidal cell death in a caspase-

independent manner. Endonuclease G is a mitochondrial specific nuclease that translocates to the nucleus and cleaves chromatin DNA during apoptosis. Using immunofluorescence, we observed that endonuclease G was prominent after treatment with buttermilk and sodium butyrate but was not detected in control cells. It relocated to the nucleus upon treatment with buttermilk for 48h and with sodium butyrate (positive control) (**Fig. 6.16**). Similar results were observed in cells labelled with AIF antibody (**Fig. 6.17**), however more cells showed AIF signal from the inside of cells nuclei than in the case of endonuclease G. Staining with DRAQ5 dye, which stains nuclei of cells, showed that nuclei of buttermilk-treated cells were more condensed than those of healthy cells; however, as with H&E staining, apoptotic bodies could not be observed.

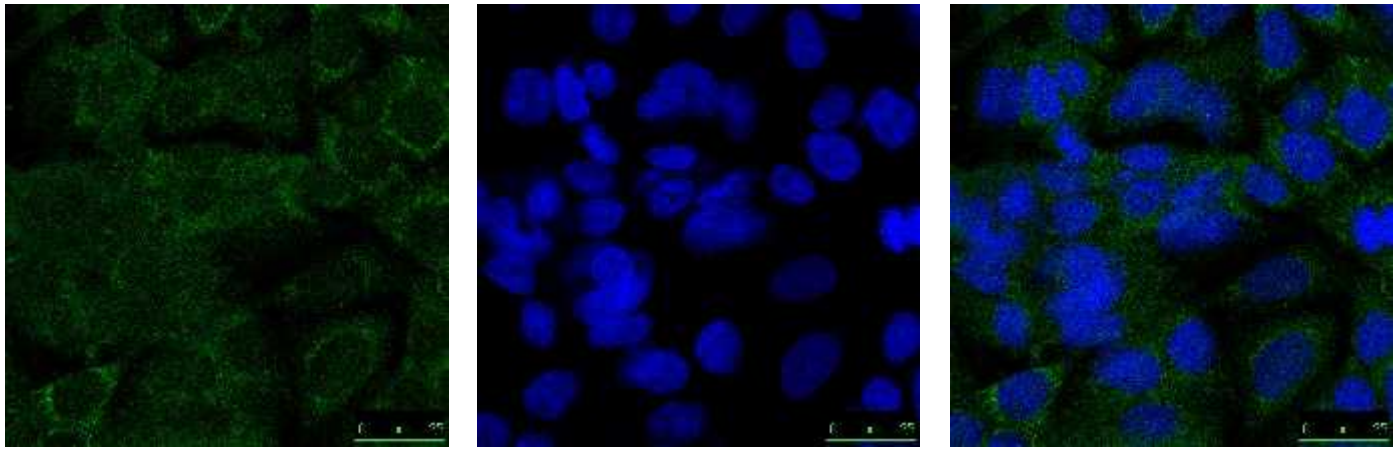
a) Endonuclease G

b) Nuclei (DRAQ5)

c) Merged



Control (C-)

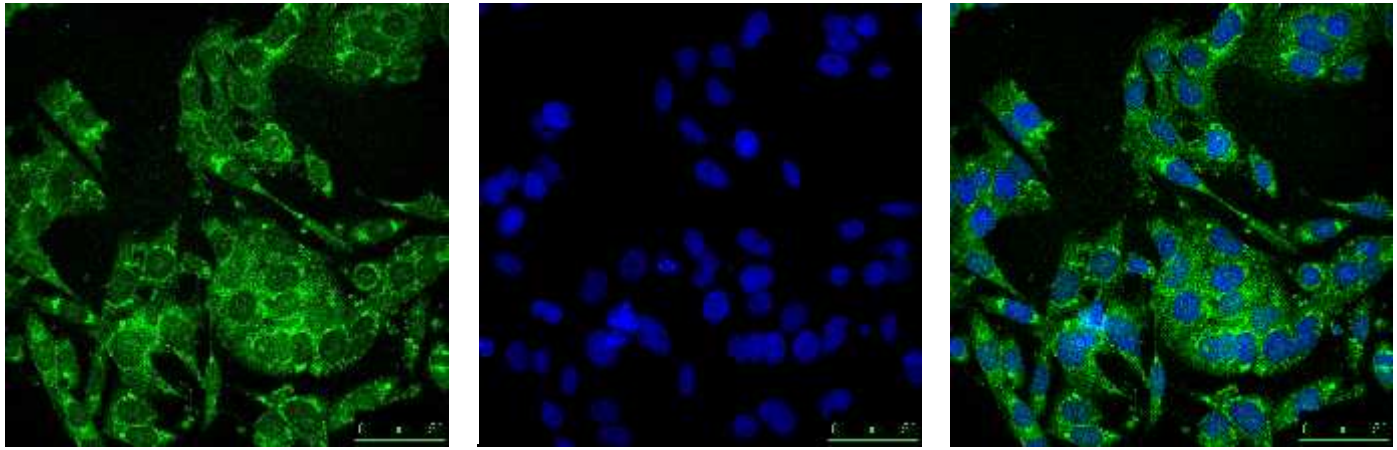


Control (C-), magnified

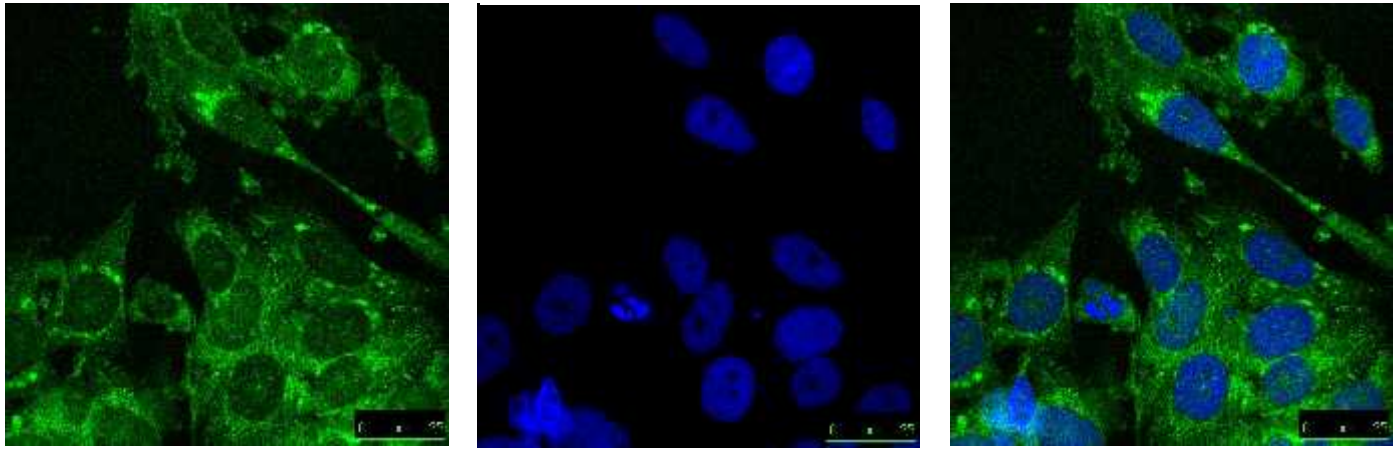
a) Endonuclease G

b) Nuclei (DRAQ5)

c) Merged



NaBut (C+)

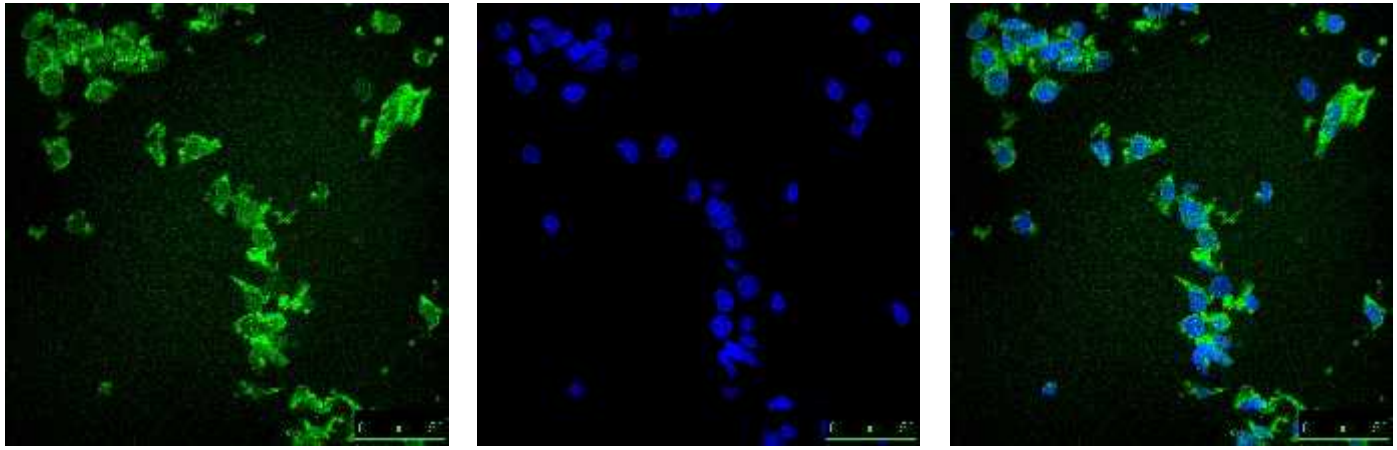


NaBut (C+), magnified

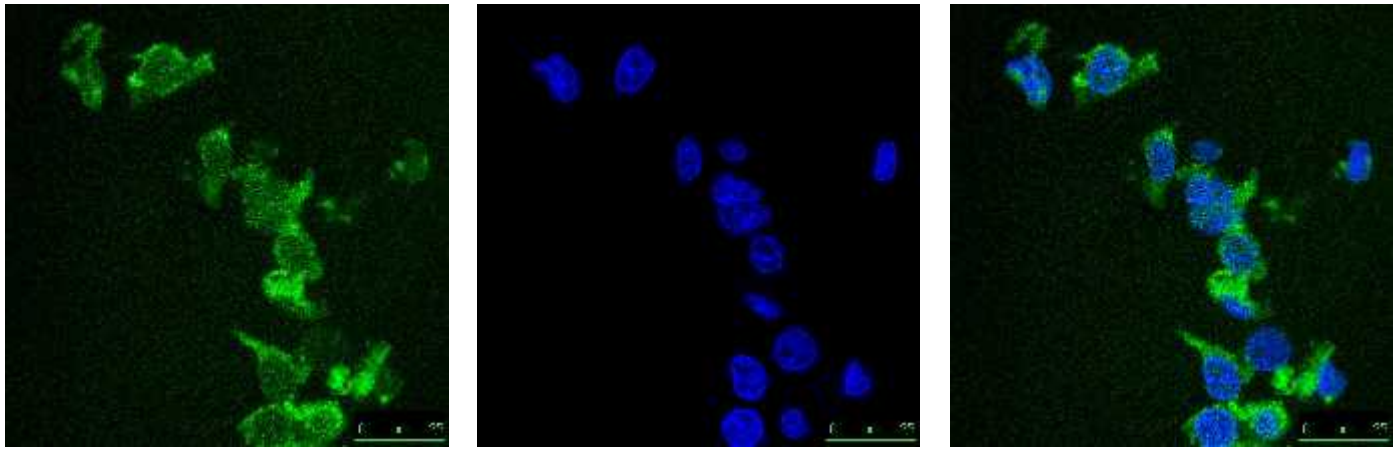
a) Endonuclease G

b) Nuclei (DRAQ5)

c) Merged



B2



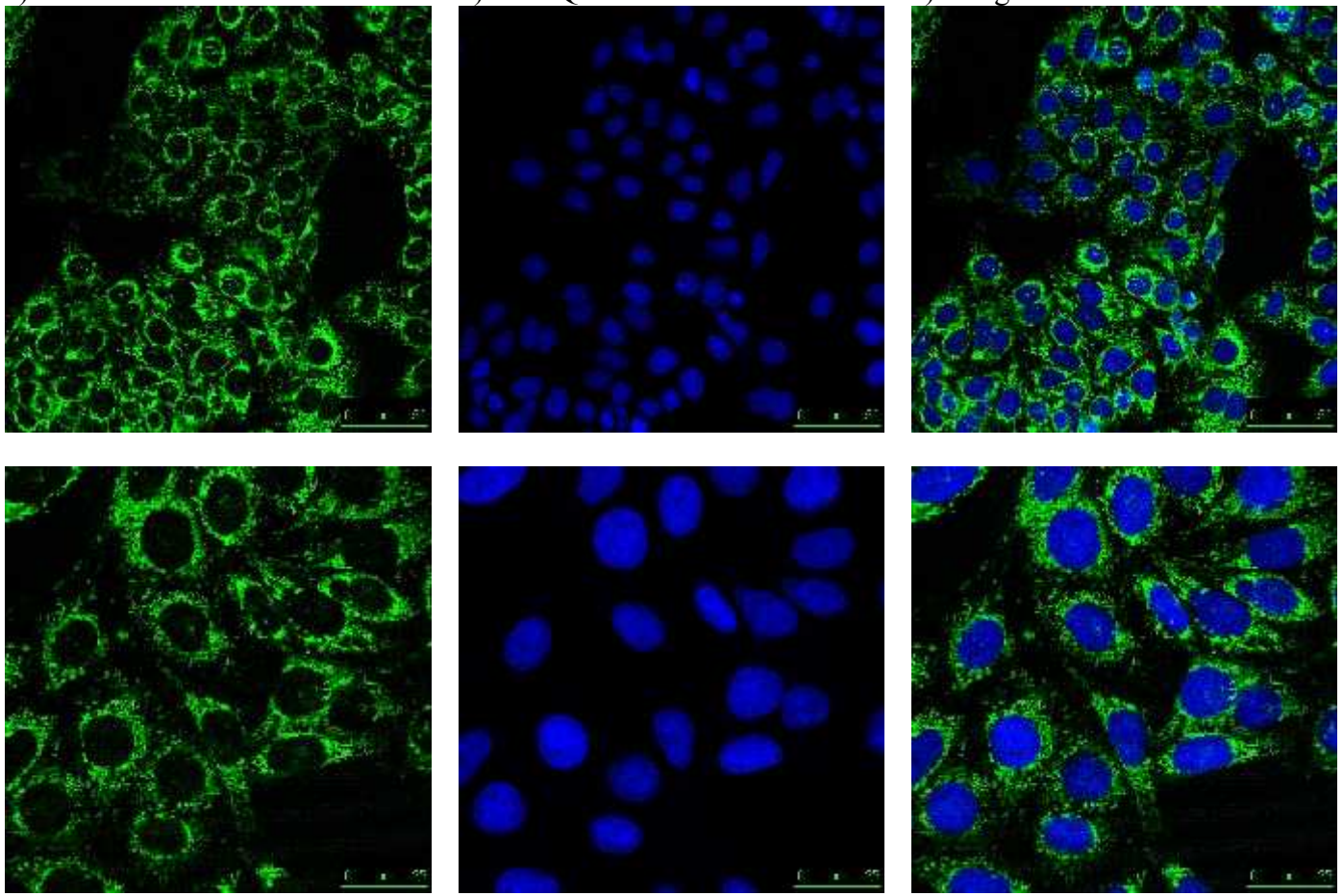
B2, magnified

Fig. 6.16. Confocal immunofluorescent examination of endonuclease G in SW480 cells. SW480 cells were either untreated (control, C-) and treated with 0.2 mg total solids/ml (B2). SW480 cells in Leibovitz-15 medium were also treated with 5 mM sodium butyrate (NaBut, C+), which served as positive control. After 48h cells were washed and labelled with endonuclease G antibody (*green*) (a). SW480 cells were also counterstained with DRAG5 (b), which is fluorescent DNA dye to detect cells' nuclei (*blue*). Merged images (c) show nuclei of SW480 cells (*blue*) and endonuclease G (*green*). Figure shows representative images of duplicate experiments.

a) AIF

b) DRAQ5

c) Merged



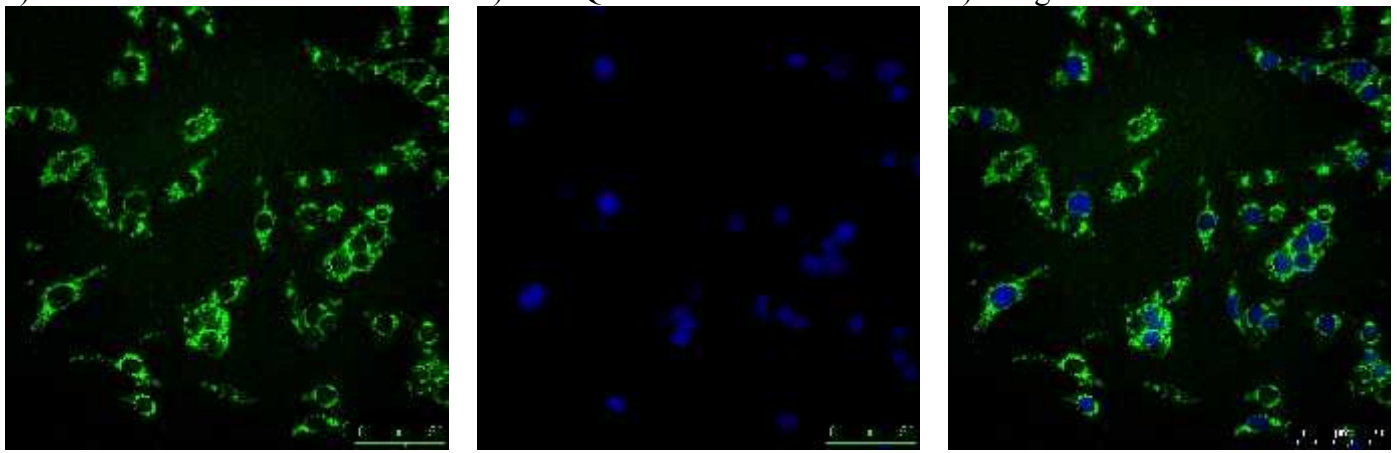
Control (C-)

Control (C-), magnified

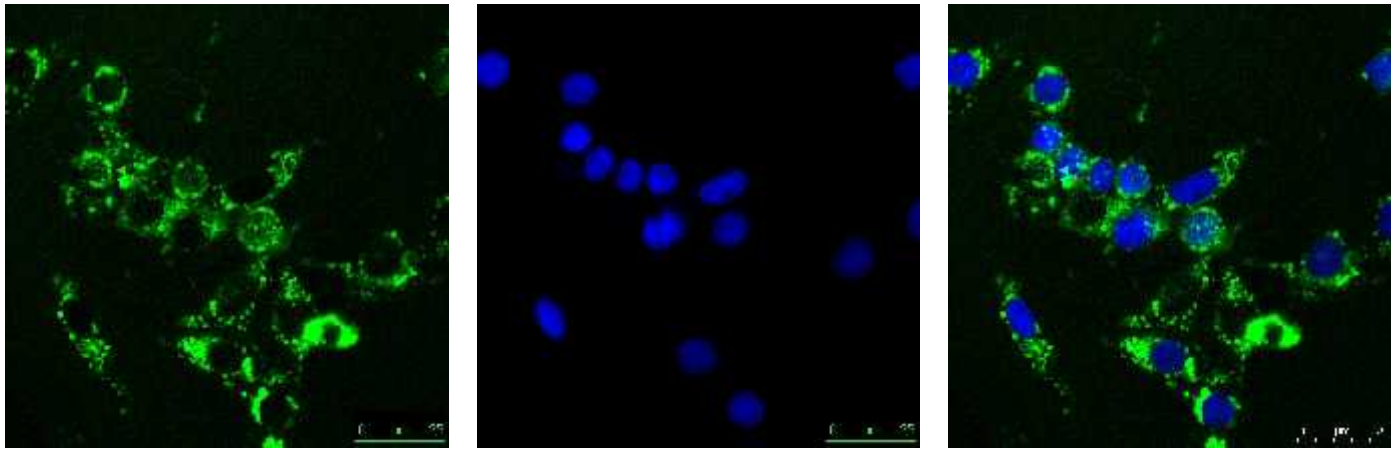
a) AIF

b) DRAQ5

c) Merged



B2



B2, magnified

Fig. 6.17. Confocal immunofluorescent examination of AIF in SW480 cells. SW480 cells were either untreated (control, C-) and treated with 0.2 mg total solids/ml (B2). SW480 cells in Leibovitz-15 medium were also treated with 5 mM sodium butyrate (NaBut, C+), which served as positive control. After 48h cells were washed and labelled with AIF antibody (*green*) (a). SW480 cells were also counterstained with DRAG5 (b), which is fluorescent DNA dye to detect cells' nuclei (*blue*). Merged images (c) show nuclei of SW480 cells (*blue*) and AIF (*green*). Figure shows representative images of duplicate experiments.

6.4. Discussion

To determine the mode of action of buttermilk's components on SW480 colon cancer cells we investigated different key components of cell survival and death pathways using variety of biochemical techniques. First of all, we observed a decrease in phosphorylated (active) Akt in SW480 colon cancer cells after buttermilk treatment (Fig. 6.10). We hypothesise that the sensitivity of SW480 cell growth to buttermilk derives in part from down regulation of an Akt-induced bioenergetics program in these cells, possibly by disrupting mitochondrial integrity. Enzyme activity of phosphorylated Akt is essential for cell survival. Its contribution to cellular bioenergetics appears to be a key factor in cell survival. Elstrom *et al*. (2004) observed that Akt stimulated glucose consumption in Akt-transfected blood cancer cells without affecting the rate of oxidative phosphorylation. Akt stimulates glycolysis and increases protein and lipid synthesis while blocking β-oxidation and autophagy, an evolutionarily conserved response to starvation by which cells catabolise their components to create an internal supply of essential nutrients. Such metabolic changes render cells with constitutively active Akt unable to activate quiescence. The role of Akt in cancer cell bioenergetics may account for the effect of culture conditions in determining the cellular response to buttermilk as reported in Chapter 4. Growing Akt expressing SW480 cells in Leibovitz-15 medium containing galactose may have rendered them more susceptible to mitochondrial-mediated cell death by buttermilk than growing cells in DMEM medium containing glucose. Two Akt isoforms (Akt1 and Akt2) were reported to be upregulated in human colon cancers compared with normal mucosa (Bellacosa *et al*., 2005). It is tempting to speculate that suppression of Akt expression by buttermilk components would render Akt and apoptosis likely targets for chemosensitisation.

FACS analysis of TMRE loading in buttermilk-treated cells revealed poor mitochondrial retention of TMRE and provided evidence that buttermilk decreased the mitochondrial membrane potential of SW480 cells in a dose and time-dependent manner. Mitochondrial membrane potential (Δ_ω) is a measure of cellular viability, as it reflects the pumping of protons across the inner membrane during electron transport and oxidative phosphorylation, the driving force behind ATP production. Permeabilization of outer mitochondrial membrane by buttermilk may allow release of proteins such as the apoptosome and possibly endonuclease G and AIF may account for the presence of apoptotic cells as shown by flow cytometry in Fig. 6.5.

Apart from Akt, the MAPK signaling pathway is a second critical pathway involved in cell survival, proliferation, differentiation and tumor growth (Wang *et al*., 2010) Three major subfamilies of enzymes make up MAPK. They are extracellular signal-regulated protein kinase 1/2 (ERK1/2), C-Jun N-terminal kinase (JNK) and p38 MAPK (p38). The former, ERK MAPK is one of the most important enzyme families driving cell proliferation in human colorectal cancer. It was found that buttermilk treatment significantly ($P \leq 0.001$) reduced activation levels of ERK1/2 in SW480 cells compared with untreated control cells, which suggests that buttermilk is an important source of bioactive components that can potentially disrupt transduction of growth stimulatory signals in colorectal cancer. ERK enzymes phosphorylate cytoplasmic targets or migrate to the nucleus where they phosphorylate transcription factors that upregulate proliferation genes and protect cell against apoptosis. Inhibition of ERK1/2 phosphorylation is sufficient to induce apoptotic cell death in human colon cancer cells (Rice *et al*., 2003). The other MAPK subfamilies, JNK and p38 are involved in growth arrest and apoptosis (Fang and Richardson, 2005). However, neither of these signalling molecules were targeted by buttermilk in our

studies. Butyrate may have induced apoptosis through JNK and p38 activation in colon cancer cells (Zhang *et al*., 2010). Although buttermilk treatment failed to activate JNK and p38 in SW480 cells, other studies have shown that pure sphingolipids including sphinganine and ceramide are capable of activating JNK/p38 MAPK and play critical roles in triggering apoptosis in HT-29 human colon cancer cells (Ahn and Schroeder, 2006).

We also investigated the effect of buttermilk on β-catenin, a key component of Wnt signalling pathway in SW480 colon cancer cells. In our studies, we found using the In-Cell Western method, that buttermilk treatment decreased the level of β-catenin and its target, c-myc after 24 ($P \leq 0.001$) and 48h ($P \leq 0.001$), respectively in SW480 colon cancer cells. β-catenin is an essential component of the Wnt signalling pathway which is dysfunctional in up to 60% of sporadic human colon cancers (Powell *et al.*, 1992) and is thought to predispose cells to additional mutations that proceed to colon cancer (Polakis, 1999). Most Wnt signalling dysfunctions lead to the nuclear accumulation of β-catenin where it associates with transcription factors to drive the transcription of multiple target genes, such as *c-myc* implicated in proliferation (Martensson *et al*., 2007). Our findings, that buttermilk treatment reduced β-catenin, corroborates similar observations by other workers studying the apoptotic effects of sphingolipids. Schmelz and co-workers (2001) found that sphingosine reduced cytosolic and nuclear β-catenin in SW480 and T84 human colon cancer cells. Sphingolipid components appear to regulate β-catenin levels at the post transcriptional level rather than at transcription level, possibly at level of protein degradation or reduced stabilization. Simon *et al*., (2009) found that SM-rich diet did not significantly alter mRNA levels of β-catenin and connexin-43 (member of the gap junction proteins) in CF1 mice, but it downregulated levels of protein β-catenin and

increased protein levels of connexin-43, which are both early biomarkers of colon cancer development.

Using H&E staining, Annexin-V-FLUOS and PI staining, FACS analysis and cell cycle analysis, we determined that buttermilk induced apoptosis in SW480 colon cancer cells (Fig. 6.1-6.5). However, we did not detect typical biomarkers of apoptosis, such as cleavage of caspases and PARP. Buttermilk components triggered mitochondria membrane depolarization in colon cancer cells. The important role of mitochondria in promoting caspase activation has been a major focus of apoptosis research; however, mitochondria may also contribute to cell death by a caspase-independent mechanism (Pradelli *et al*., 2010). One of the possibilities is that pro-apoptotic Bcl-2 family members, such as Bax and Bak permeabilize the mitochondrial outer membrane allowing release of apoptotic molecules, such as cytochrome c or apoptosis-inducing factor (Lindsten *et al*., 2000); however, the exact mechanism is not known. Other models of mitochondrial membrane permeabilization (which leads to its depolarization) include opening of the voltage-dependent anion channel (VDAC), opening of the mitochondrial permeability transition pore (PTP), opening of ceramide channels, swelling of matrix and disruption of the inner membrane or formation of pores in the outer membrane (Pradelli *et al*., 2010, Kroemer *et al*., 2007, Roy *et al*., 2009). Evidence for caspase-independent cell death has come from *in vivo* and *in vitro* studies in which cell death occurred following mitochondrial outer membrane permeabilization but without caspase activation (Kroemer *et al*., 2007). Among the cytochrome c and other proteins, which are released from mitochondria, endonuclease G (EndoG) was identified as a mitochondrial endonuclease that could relocate to the nucleus and induce caspase-independent nucleosomal DNA fragmentation (Li *et al*., 2001). Another protein released from the mitochondrial inner

membrane is AIF, a flavoprotein, which translocates to the nucleus where it cooperates with EndoG and/or cyclophilin A in DNA degradation complex (Niikura *et al*., 2007, Cande *et al*., 2004). In our studies we could see labelled EndoG and AIF in some of the nuclei of colon cancer cells after buttermilk treatment, indicating that in some cells these molecules translocate from mitochondria to nuclei.

In addition to caspase-independent cell death, autophagic cell death has emerged as another mechanism of non-apoptotic cell death. Autophagy occurs at basal levels in most tissues, contributing to the routine turnover of cytoplasmic components and promoting cell adaptation and survival during stress, such as starvation. It is downregulated in most cancer cells. Oncogenic signalling molecules (e.g. class 1 PI3K/Akt and mTor) suppress autophagy and tumour suppressors (e.g. PTEN , p53, beclin 1) stimulate autophagy. An increasing number of studies propose that activation of autophagy is how cancer cells resist cytotoxic drug treatments because it allows cancer cells to recoup essential building blocks when they are exposed to chemotherapy agents or irradiation (Bijnsdorp *et al*., 2010). The balance between survival-related autophagy and death-related autophagy appears to be critically dependent on the type of cellular stress and particular cellular settings. A growing number of studies now suggest that in certain conditions, aberrant ERK activation can promote cell death (reviewed in Cagnol and Chambard, 2010). Sustained ERK activity during amino acid depletion or following treatment of HT-29 and HCT15 colon cancer cells with aurintricarboxylic acid or soyasaponins signalled autophagic cell death (Ogier-Denis *et al.,* 2000, Pattingre *et al.,* 2003 and Ellington *et al.,* 2006). Reactive oxygen species have been identified as the critical mediators of ERK-induced cell death (Cagnol and Chambard, 2010), promoting sustained ERK activation and inhibiting ERK phosphatases. Morphological characteristics of

autophagy, as detected using transmission electron microscopy and immunoelectron microscopy include sequestration of cytoplasm and organelles such as mitochondria and endoplasmic reticulum in double or multiple-membraned vesicles (Blank and Shiloh, 2007). Biochemically, autophagy is associated with induction of microtubule-associated protein 1 light chain 3 (LC3) and conversion of LC3-I to LC3-II, induction of beclin 1 (Cheng *et al*., 2008), BNIP-3 (An *et al*., 2006) and phosphorylation of Gα-interacting protein (GAIP) (Ogier-Denis *et al*., 2000) and p53 (Cheng *et al*., 2008). Other biochemical markers include cytosolic sequestration of lactate dehydrogenase activity (Blank and Shiloh, 2007). An investigation of the effect of buttermilk on specific autophagic markers is required to determine if caspase-independent cell death might also be accompanied by autophagic activity.

6.5. Conclusions

Summarizing our observations, buttermilk's components initiate suicidal death of SW480 colon cancer cells; however the whole pathway could not be established. Using immunofluorescence and flow cytometry, we could detect some of the apoptotic hallmarks, such as phosphatidylserine translocation to the outer membrane, mitochondrial outer membrane depolarization, condensation of chromatin (observed by flow cytometry analysis of cell cycle, haematoxylin and eosin staining of cells and DRAQ5 staining with confocal microscopy detection), but we could not detect apoptotic bodies as well as biochemical markers of caspase-dependent apoptosis, such as cleavage of caspases and PARP. However, downregulation of Akt and ERK 1/2 in SW480 colon cancer cells after buttermilk treatment indicated possible activation of autophagy. Not all details are yet fully understood in different types of suicidal cell deaths; however, to farther investigate buttermilk components' mode of action on colon cancer cells, it is necessary to take in the consideration wider range of cell signalling pathways. High levels of sphingolipids present in buttermilk indicate a potential role for sphingolipids in mediating anticancer activity of buttermilk; thus far, pure sphingosine and C2 ceramide have been shown to trigger cancer cell death; it remains unclear if fatty acyl chain length modulates anticancer activity of ceramide. It is also unclear whether other buttermilk components may modulate the activity of sphingolipids. While it could not be established which buttermilk components possess antiproliferative activity, our results indicate that buttermilk, a by-product in buttermaking process possesses anticancer activity against a colon cancer cell line. A combination of lipidomics and phosphoproteomic analyses of cancer cells will be required to identify a) biomarkers of exposure to buttermilks enriched in unique sphingolipids and b) molecular targets responsible for anticancer activity. Their

validation in appropriate *in vitro* and *in vivo* models will be critical to determining whether buttermilk holds promise to be a novel functional beverage with health-promoting properties for the human gastrointestinal tract.

Final discussion

This project investigated the anticancer activity of traditional buttermilk, a dairy-based beverage which formed the main part of the staple diet in Ireland up to the middle of the last century. It is naturally rich in disrupted MFGM material and its polar lipid content is relatively high compared with most other dairy products. Sphingolipids and phospholipids which comprise the bulk of polar lipids in MFGM are of interest because they define the structural properties of biological membranes and they also function as intracellular signalling molecules regulating cell growth, development, adhesion and cell death.

No work to date has evaluated the relative effects of modern cream separation techniques, washing or heating on the antiproliferative effects of buttermilk. In this project we evaluated the potential health benefits of buttermilks produced under different technological conditions. Using an indirect cytotoxicity assay, based on activity of acid phosphatase, we found that buttermilk produced in laboratory scale significantly ($P \leq 0.001$) inhibited growth of SW480 colon cancer cells, but not FHC normal human epithelial cells, indicating that it targeted antiproliferative pathways in cancer cells, which was also confirmed using Caco-2 cells. After scaling up buttermilk production, it was apparent that it did not lose antiproliferative activity; moreover, it significantly ($P \leq 0.001$) inhibited growth of another colon cancer cell line, HT-29 as well as the hepatocellular carcinoma cell line HepG2.

We also studied influence of cream separation techniques, gravitational versus centrifugal, on antiproliferative activity of buttermilk. Buttermilk produced from gravitationally separated cream had a significantly lower ($P \leq 0.05$) antiproliferative activity and also higher MFGM protein content than buttermilks produced from commercial cream and from centrifugally separated cream. The latter contained higher phospholipids content compared with buttermilk produced from gravitationally

separated cream, suggesting that antiproliferative activity may be more associated with polar lipid components of the buttermilks than with protein components. Moreover, we also found that high heat (95°C, 30 min) treatment did not alter antiproliferative activity of buttermilk. This suggests that β-lactoglobulin interactions with MFGM as a result of heat treatment had negligible effects on the cellular signalling circuitry that regulates cell growth and death. This is in accordance with an earlier study of the comparative cytotoxic activity of dairy derived ingredients in human cancer cells which demonstrated that β-lactoglobulin had only mild cytotoxic activity (Chan-Remillard & Ozimek, 2008).

We also found that at concentration 0.3 mg total solids/ml buttermilks produced from washed creams showed the same antiproliferative activity against colon cancer cells as buttermilks produced from unwashed creams. Washing of cream before churning showed higher lipids content in washed fractions compared to unwashed fractions, as expected (Lamothe *et al*., 2008, Morin *et al*., 2007). This suggests that buttermilk fractions from washed cream may offer potential for the production of phospholipids concentrates that have antiproliferative activity against cancer cells.

Spray-dried retentate and permeate samples showed much lower antiproliferative activity than their corresponding liquid samples, suggesting temperature and/or pressure-induced impairment of bioactive components responsible for antiproliferative activity. Morin *et al*., (2007) showed that spray drying affected the composition of buttermilk and its MFGM, in particular, it significantly lowered phospholipid content when compared with initial buttermilks. Gaiani *et al*. (2010) also found that spray drying influenced dairy powder surface composition. According to Kim *et al*., (2010) non-thermal treatments including freeze drying may be more

effective at overcoming the loss of nutrients during spray drying process. In our studies, we found that freeze and spin drying of feed, retentate and permeate fractions maintained their antiproliferative activity.

Dairy products fermented with lactic acid bacteria have been shown to inhibit colon cancer cell growth (Baricault *et al*.,1995, Macdonald *et al*., 1994, Ganjam *et al*., 1997, Jan *et al*., 2002, Kumar *et al*., 2010). In this study, fermented buttermilks also inhibited colon cancer cell growth, though to a lesser extent than buttermilks produced from unfermented creams.

We also studied hallmarks of selected signalling pathways inside cancer cells to better understand biochemical mechanisms underlying buttermilk's antiproliferative activity. Using immunofluorescence and flow cytometry, we could detect some apoptotic hallmarks, such as phosphatidylserine translocation to the outer membrane, mitochondrial outer membrane depolarization, condensation of chromatin (observed by flow cytometry analysis of cell cycle, haematoxylin and eosin staining of cells and DRAQ5 staining with confocal microscopy detection), but we did not detect apoptotic bodies nor biochemical markers of caspase-dependent apoptosis, such as cleavage of caspases and PARP. Downregulation of Akt and ERK 1/2 in SW480 colon cancer cells after buttermilk treatment may underlie the antiproliferative activity of buttermilk. Not all details of different types of suicidal cell deaths are yet fully understood; however, to further investigate buttermilk components' mode of action on colon cancer cells, it is necessary to take into consideration a wider range of cell death pathways.

The use of *in vitro* models to study hydrolysis of exogenous sources of sphingolipids, i.e. buttermilk, is limited to cell lines that express alkaline sphingomyelinase. By contrast an *in vivo* model would permit evaluation of

hydrolysis by endogenous and microflora-derived alkaline sphingomyelinase which is specifically stimulated by bile salts. Recently, MFGM was shown to confer protection against colon carcinogenesis in an animal model of experimental carcinogenesis. Rats fed a diet containing MFGM had significantly fewer aberrant crypt foci (ACF) compared to rats fed corn oil or anhydrous milkfat (Snow *et al*., 2010). Further studies should be conducted *in vivo* to establish the modulatory effect of attainable dietary buttermilk treatments in the azomethane-treated rats and to identify the number of mucin-depleted foci (MDF). The latter are postulated to be a subgroup of ACF that predict tumour outcome better than ACF as their number and multiplicity correlated with tumour number and incidence (Caderni, 2007, Femia and Caderni, 2008). MDF were shown to be decreased by dietary treatments with synbiotics comprising a prebiotic derivative of inulin and two probiotics, lactobacilli and bifidobacteria and increased by a high corn oil cancer-promoting diet. Its usefulness as a biomarker of an early pathological alteration in human colon was recently confirmed when they identified MDF-like lesions in familial adenomatous polyposis (FAP) subjects who are at high risk of developing colorectal cancer. Research is required to address whether ceramide and sphingosine metabolites formed from buttermilk sphingolipids by the combined action of alkaline sphingomyelinase and ceramidase influence the cellular kinetics of the gut epithelium under normal and tumourigenic conditions. This would involve determining effects of buttermilk samples on proliferation, apoptosis, autophagy and differentiation of crypt cells at an early step in the process of carcinogenesis. An investigation of chemopreventive activity of buttermilk in a rodent model can significantly improve our knowledge about its mode of action and provide an opportunity for development of a new functional beverage with widely demanded chemopreventive activity.

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