EFFECT OF CANNABIS ON MAMMARY EPITHELIAL CELLS AND BREAST MILK

THE EFFECT OF CANNABINOIDS AND CANNABIS ON MAMMARY EPITHELIAL CELL FUNCTION AND BREAST MILK COMPOSITION

BY:

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LAY ABSTRACT

Breast milk is crucial to the nutrition and immunity of the newborn. It is produced by the mammary gland (MG), which is composed of mammary epithelial cells (MEC) that play a key role in producing and secreting proteins and factors into the milk. During pregnancy, the MG undergoes remodeling which is accompanied by differentiation of the MECs (change from unspecialized to a specialized stage that allows the MECs to produce milk proteins and other factors). The disruption in MEC differentiation can result in altered milk production and composition. Cannabis is used by women during pregnancy and breastfeeding. In this study, we investigated the impact of cannabinoids on the MEC differentiation and examined the effect of cannabis use in the perinatal period on the breast milk composition in humans. We reported that cannabinoids reduced the differentiation of MECs and maternal cannabis use during breastfeeding reduced levels of an essential immune factor.

ABSTRACT

Mammary gland (MG) is a dynamic organ that is essential for the production and secretion of breast milk. During pregnancy the MG undergoes a critical phase of remodeling, which is accompanied by the differentiation of mammary epithelial cells (MECs). During lactation, the MG requires high level of energy for proper folding of proteins in the secretory pathway, which takes place in the endoplasmic reticulum (ER). Limited evidence has been reported on the impact of cannabis or its components, delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD), on the differentiation of MECs. However, both THC and CBD have been reported to induce ER stress in various cell types, resulting in impacting cellular function. Furthermore, consequences of cannabis use in the perinatal period on breast milk composition have not been reported. Using the HC11 cell line, we investigated whether THC and CBD evoke ER stress in MECs resulting in the impaired cellular function. We also reported on the effects of cannabis use during pregnancy and lactation on the levels of macronutrients and bioactive factors in the breast milk of cannabis users. Relative to control, 10μM THC and 10μM CBD reduced mRNA levels of milk proteins (*CSN2* and *WAP*) and lipid synthesizing enzymes (*FASN, FABP4, PLIN2* and *LPL*), as well as whey acidic protein and lipid levels. In addition, 10μM THC, 10μM CBD, and the combination of 10μM THC + 10μM CBD significantly induced the expression of ER stress genes in HC11 cells. Furthermore, in the milk of women who used cannabis during pregnancy and lactation, the levels of secretory immunoglobulin A (SIgA) were decreased, relative to non-users. Altogether, our findings indicate that cannabis use in the perinatal period may have implications on breast milk composition and infant health.

PREFACE

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All authors contributed to the study's conception and design. Conceptualization, material preparation, data collection and formal analysis were performed by Chitmandeep Josan. The first draft of the manuscript was written by Chitmandeep Josan. The study was supervised, funded, and conceptualized by Sandeep Raha. The manuscript was reviewed and edited by Sandeep Raha. All authors read and approved the final manuscript.

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Author contributions:

Dr. Sandeep Raha and Dr. Alison Shea conceptualized and designed the study, acquired, and contributed funding for the study, as well as reviewed and revised the manuscript. Chitmandeep Josan obtained ethics approval for the study protocol, acquired breast milk samples, performed instrumental and biochemical analysis of the samples, processed the resulting data, drafted the initial manuscript, and reviewed and revised the manuscript. Dr. Samantha Shiplo facilitated recruitment, attained and analyzed data from the medical charts of subjects at the time of labor and delivery. Dr. Gerhard Fusch coordinated and supervised macronutrient data collection, and critically reviewed the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

DEDICATION

I dedicate this dissertation to my parents. It is for you that I began this journey, and it is for you that I saw it to its completion. You two are the most hardworking, persistent, and dedicated individuals who I have ever met, and you are both my biggest inspiration in life. Although I had many challenges and difficulties in my journey, it is insignificant to the sacrifices you both have made for me to date. I love you both and I hope to keep being inspired by you for the rest of my life.

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LIST OF ABBREVIATIONS AND SYMBOLS

2-AG	2-arachidonoylglycerol
2D	Two-dimensional
3D	Three-dimensional
4-PBA	4-phenylbutyric acid/sodium phenylbutyrate
AEA	Anandamide
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
BMP4	Bone morphogenetic protein 4
BMPR1A	Bone morphogenetic protein receptor-1A
BORN	Better outcomes registry & network
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CBD	Cannabidiol
CBDV	Cannabidivarin
CBG	Cannabigerol
CHOP	C/EBP-homologous protein
CSN2	B-casein
DAGL	Diacyl-glycerol lipase
DEX	Dexamethasone
DHA	Docosahexaenoic acid
DRP1	Dynamin-1-like protein
eIF2α	Eukaryotic Initiation Factor 2 α
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated protein degradation
FABP4	Fatty acid binding protein 4
FASN	Fatty acid synthase
FIS1	Fission, mitochondrial 1
GADD34	Growth arrest and DNA damage-inducible protein 34
GH	Growth hormone
GLUT1	Glucose transporter 1
GLUT2	Glucose transporter 2
GPCR	G-protein coupled receptor
GRP78	G-protein coupled receptor 78
HiREB	Hamilton integrated research ethics board

HK2	Hexokinase 2
IGA	Immunoglobulin A
IGF-1	Insulin-like growth factor-1
IGG	Immunoglobulin G
IGM	Immunoglobulin M
INS	Insulin
IRE1α	Inositol-requiring kinase
JAK2	Janus kinase 2
LALBA	A-lactalbumin
LPL	Lipoprotein lipase
MAGL	Monoacylglycerol lipase
MaSC	Mammary stem cells
MEC	Mammary epithelial cell
MFN1	Mitofusin 1
MFN2	Mitofusin 2
MG	Mammary gland
NRF2	Nuclear factor erythroid 2–related factor 2
PERK	Pancreatic ER eif2α kinase
PI3K	Phosphoinositide 3-kinase
PINK1	PTEN-induced putative kinase 1
PLIN2	Perilipin-2
PRKN	Parkin RBR E3 ubiquitin-protein ligase
PRL	Prolactin
PTHLH	Parathyroid hormone-related protein
RANK	Receptor activator of nuclear factor kappa beta
RANKL	Receptor activator of nuclear factor kappa beta (nfkb ligand)
ROS	Reactive oxygen species
RPL0	Ribosomal protein 0
RPL8	Ribosomal protein 8
SIgA	Secretory immunogolbulin a
SOD2	Superoxide dismutase 2
STAT3	Signal transducer and activator of transcription 3
STAT5	Signal transducer and activator of transcription 5
sXBP1	Spliced-X box-binding protein 1
TDLU	Terminal duct lobular units
TEB	Terminal end bud
TFAM	Transcription factor a

THC	Delta-9-tetrahydrocannabinol
TJ	Tight junction
UPR	Unfolded protein response
WAP	Whey acidic protein
WHO	World health organization

α	Alpha
β	Beta
γ	Gamma
Δ	Delta
κ	Kappa
μ	Micro

DECLARATION OF ACADEMIC ACHIEVEMENT

Chitmandeep Josan and Dr. Sandeep Raha planned and executed this research with guidance from Dr. Alison Holloway and Dr. Alison Shea. All laboratory experiments were performed by Chitmandeep Josan with the assistance of Harmeet Gurm, Sachin Kakar, Kush Patel, Saundarai Bhanot, Mariam Abdel-Kader, and Sanjum Hunjan. Data analysis was performed by Chitmandeep Josan and Dr. Sandeep Raha. This dissertation was written by Chitmandeep Josan with the assistance of Dr. Sandeep Raha.

CHAPTER 1: INTRODUCTION

i. Mammary Gland Development: embryonic, pubertal, and reproductive

The mammary gland (MG) is a sophisticated and highly specialized organ that distinguishes mammals from all other animals [1]. This complex and dynamic organ is responsible for secreting milk for the nourishment and survival of the newborn [1]. The capacity of the MG to synthesize milk depends largely on the number and efficiency of the functional mammary epithelial cells (MECs) [2]. This dynamic organ is composed of two different cell types: basal and luminal [1]. The basal epithelium consists of myoepithelial cells, which generate the gland's outer layer. This outer layer is comprised of a small population of stem cells that supply different cell types such as adipocytes, pre-adipocytes, fibroblasts, and vascular and immune cells [3–5]. The luminal epithelium forms ducts and secretory alveoli, which produce milk. During lactation, the outer myoepithelial cells contract to squeeze milk from the inner alveolar luminal cells [3–5]. Together, the luminal and myoepithelium generate a bi-layered, tubular structure that allows the gland to be formed during lactation (Fig 1) [1]. Lack of demand for milk initiates the involution process, which involves removing the MECs through apoptotic signaling pathways [2]. Overall, the remodeling of the MG at various stages is regulated by numerous signaling pathways that ensure the functionality of the gland and regulate a subpopulation of mammary stem cells, which drive the substantial changes that take place in the MG throughout remodeling.

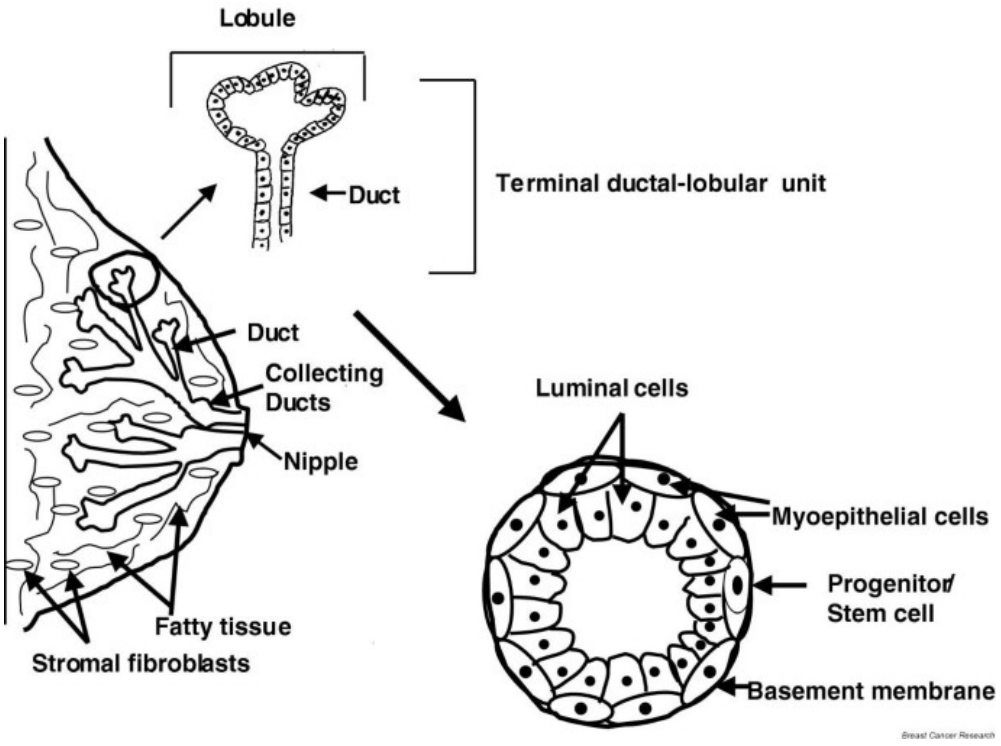

Fig 1 Structure of the mammary gland. The mammary gland is composed of the terminal ductal-lobular units (TDLU) and the stroma, which includes stromal fibroblasts, fatty tissues (adipocytes), and various other cell types. The TDLU is composed of luminal and myoepithelial cells, which ensure the synthesis and secretion of milk components, that are delivered to the newborn during lactation through the ducts and eventually the nipple. This figure has been obtained from the work of Gusterson and Stein [7].

MG development primarily occurs through epithelial changes which begin at the fetal stage, during which time the rudimentary ductal tree forms and grows allometrically with the body post-parturition until puberty [4,6]. In the embryo, the epithelial and the surrounding stromal tissues are derived from ectoderm and mesoderm, respectively. In the mouse, the development of the MG begins at embryonic day (E) 10, which involves the formation of bilateral stripes of multilayered ectoderm on the ventral surface of the embryo [1]. By E11.5, the multilayered ectoderm develops into 5 pairs of placodes, which appear to have several layers of columnar-shaped cells that arise from the migration and aggregation of ectodermal cells into clusters on the surface of the mammary line [1]. In humans, the mammary line forms during the first trimester of gestation and it develops into a single pair of placodes [7]. In mice, at E14, the mammary mesenchyme condenses and forms fibroblastic-rich cell layers that surround the epithelial rudimentary structure of the MG. This is distinct from the fat pad precursor mesenchyme, which arises from subcutaneous mesenchymal cells [1].

The mammary placodes expand and form spheres of cells, which descend into the underlying mesenchyme for the formation of a stalk that connects the mammary bud to the epidermis (E14 in mice). Next, the epithelial cells extend from the mammary bud and grow in the fat pad precursor mesenchyme, which at the embryonic stage is a cluster of preadipocytes (E16 in mice) [1]. In humans, several epithelial sprouts extend from the mammary bud resulting in the formation of multiple mammary trees that unite at the nipple. The tree-like structure of MECs embedded in the fat pad remains in this form until birth. Figure 2 depicts the progression of the MG development from the placode to the ductal tree, which takes place from embryogenesis to birth.

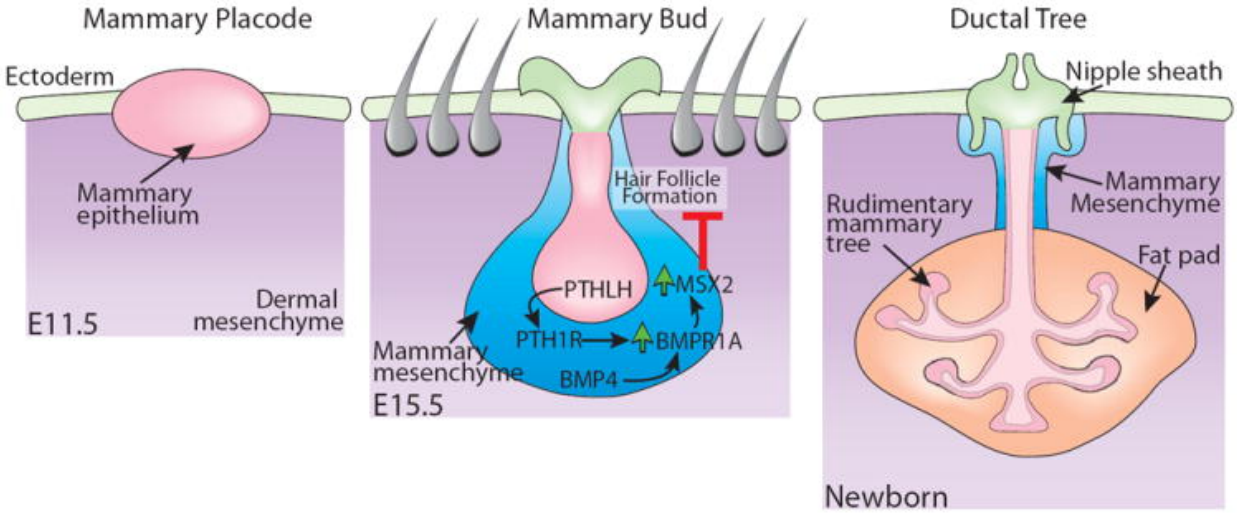

Fig 2 The embryonic mammary gland development. From left to right: The mammary placode expands into a sphere of cells that extend into the mammary epithelial mesenchyme. Signaling in the middle figure: The mesenchyme receives signals from the epithelium through parathyroid hormone-related protein (PTHLH) that causes the expression of bone morphogenetic protein receptor-1A to rise (BMPR1A). Bone morphogenetic protein 4 (BMP4) expressed in the mesenchyme communicates with MSX2 via BMPR1A and prevents the development of hair follicles at the growing nipple sheath. Finally, the mammary epithelium expands and grows in a tree-like fashion into the mammary fat pad. This figure is obtained from the article published by Macias and Hinck [1].

Finally, prior to the conclusion of embryonic development of the MG, three essential morphological processes take place: formation of the ductal lumen, generation of the nipple structure, and in mice (but not in humans), specification of the male versus female MG [1]. Ductal lumen formation involves the development of intracellular spaces within the ducts. A nipple sheath is produced by keratinocytes at the location where the major duct links to the skin's surface during the process of nipple production, which involves changes to the skin that covers the primary mammary mesenchyme. In mice, sexual dimorphism of the MG occurs at this stage as well, however, this process occurs in humans at a later stage [1].

At birth, the MG is a rudimentary ductal organ, which undergoes allometric growth with the rest of the body until puberty [1]. At puberty, an extensive proliferation of MECs takes place, resulting in the filling of the fat pad. The increase of estrogen levels in the female body triggers ductal morphogenesis, which involves the differentiation of ductal progenitors into ductal epithelial cells, while the terminal end buds (TEBs) undergo bifurcation and invade the mammary fat pad. Furthermore, the growth hormone (GH) regulates cellular proliferation by modulating the expression of insulin-like growth factor-1 (IGF1). The TEBs are club-shaped structures at the tips of the growing ducts that penetrate the fat pad, driven by the proliferation of a single layer of cap cells at the tip of the TEB and by the underlying prelumenal epithelium [1]. Cap cells of the TEBs differentiate into myoepithelial cells, forming the outer layer of the tubular ductal bilayer that lines the inner luminal cells. This forms the mammary duct network that spreads throughout the breasts, occupying 60% of the available fatty stroma [5]. The rest of the space (40%) remains available space for filling with differentiated MECs, which only takes place under the influence of hormones during pregnancy [5]. Compared to the adipocyte-rich stroma surrounding the branches of the rodent mammary tree, the human breast contains an intralobular stroma that is considerably more complex. During puberty, the branches of the mammary tree in the human breast give rise to terminal ducts that form terminal ductal lobular units (TDLU), which undergo alveolar differentiation during pregnancy to form acini [7]. These acini remain embedded in the fibroblastic, intralobular stroma and are capable of milk synthesis secretion under the influence of pregnancy hormones [7].

During pregnancy, the MG undergoes a wide range of modifications, including gland maturation and alveologenesis, to prepare for lactation. These changes occur primarily under the control of progesterone (PGR) and prolactin (PRL) [1]. During pregnancy, the concentration of

PGR and PRL increase and bind to PGR receptor-α and the PRL receptor, respectively, on the surface of alveolar progenitors [8,9]. Early in the pregnancy, a remarkable increase occurs in secondary and tertiary ductal branching, resulting in the development of ductal arbours for alveolar development [1]. Proliferating MECs generate alveolar buds that gradually differentiate into distinct alveoli, which later develop into milk-secreting lobules during lactation. This process is accompanied by the disappearance of interstitial adipose tissue, as the proliferating MECs occupy the intraductal area. By mid-pregnancy, increased vascularization surrounds each alveolus by forming a basket-like network of capillaries. By late pregnancy, the alveoli encompass most of the fat pad and are ready for secretion [1].

Histologically, initial changes during pregnancy include increased ductal branching and the formation of alveolar buds [10]. This can be visualized under the phase-contrast microscope using mammary glands that are fixed in formalin, sectioned, and stained with hematoxylin and eosin (H&E staining) [10]. By late pregnancy, the luminal space is evident with the presence of protein substances and large lipid droplets in the cytoplasm of alveolar MECs, as well as to some extent in the luminal space [10].

ii. Progesterone and prolactin signaling are essential for lactation in MECs

PGR is a membrane-soluble ovarian hormone that is responsible for extensive side-branching and alveologenesis to prepare the gland for lactation [11]. The physiological effects of PGR are mediated by the intracellular receptors that belong to the nuclear receptor superfamily of transcription factors [11]. PGR receptors are expressed as two protein isoforms: PGR receptor-A and -B, with the PGR receptor-B isoform claimed to mediate reproductive functions [11]. In combination with PRL, PGR promotes the differentiation of alveoli which synthesize and secrete milk during lactation [11]. Pregnancy-associated ductal proliferation and lobuloalveolar

differentiation of the MG do not occur in PGR receptor knockout mice [11]. Furthermore, PGR acts in a paracrine manner on the MECs. A key paracrine-mediator of PGR-induced cellular proliferation is the tumor necrosis factor ligand superfamily, member 11 (TNFSF11), also known as RANKL (receptor activator of NF-κB ligand) [1]. In addition to regulating osteoclastogenesis and bone-remodelling, RANKL signals through the tumor necrosis factor receptor superfamily, member 11a (TNFRSF11A), also known as RANK, to regulate alveologenesis [1]. In PGR receptor-B knock-out mice, mammary defects are observed resulting from reduced activation of RANKL [11]. Thus, the PGR receptor-B isoform is known as the proliferative stimulus in the MG [11].

The main hormonal contributor to lactational competence is prolactin (PRL). PRL functions both indirectly through its regulation of ovarian PGR production and directly via its effects on MECs [1]. This small polypeptide hormone is produced by the pituitary gland, as well as the mammary epithelium. PRL binds to the PRLR (receptor of a class I cytokine receptor superfamily), resulting in the activation of several signaling pathways, including JAK/STAT, MAPK and PI3K [12]. In PRLR knock-out mice, heterozygous females showed a complete failure of lactation following their first pregnancy, with their MGs being smaller in size due to less developed ductal structures and fewer branch points [13]. In *PRL -/-* mice, PGR treatment restored ductal side branching and rescued infertility in *PRL -/-* mice [1]. Specifically, PGR treatment of *PRL -/-* mice resulted in complete morphological development of the MG, appropriate to the gestational stage [1].

PRL also stimulates the production of RANK, and its ligand RANKL, as well as E74-like factor 5 (ELF5), which are transcription factors, reported to be essential for the modulation of PRL [12]. Furthermore, RANKL stimulates cell proliferation and differentiation through activation of

cyclin D1 [12]. The PRL/PRLR signaling is essential for the activation of the JAK2/STAT5 signaling pathway (Fig 3) [14]. Signaling via the PRLR/JAK2/STAT5 pathway results in the expression of milk protein genes, including β casein (*CSN2*) and whey acidic protein (*WAP*), which contain STAT5 responsive elements in their promoters [14]. Figure 3 depicts the process that involves the binding PRL to its receptor (PRLR), resulting in the activation of receptor-associated tyrosine kinase JAK2 [15]. JAK2 phosphorylates and activates STAT5A and STAT5B. Following activation, STAT5A and STAT5B are transported into the nucleus where they induce the transcription of target genes that promote the proliferation and differentiation of mammary epithelial cells [15].

From mid- to late-pregnancy, the expression of milk proteins, such as caseins, α-lactalbumin, WAP, lactoferrin, secretory immunoglobulin A (sIgA), sIgG, sIgM, and others in the MECs increase up to 20-fold in mice [10]. Milk proteins such as α-lactalbumin (lactose synthesis) and parathyroid hormone-related protein (calcium secretion) switch on secretory processes that are essential for infant health. MECs synthesize lactose from glucose and UDP-galactose [16,17]. This reaction is catalyzed by lactose synthase, which is an enzyme complex made up of β-1,4 galactosyl transferase and α-lactalbumin in the Golgi compartment of MECs [16,17]. Downregulation of α-lactalbumin during pregnancy prevents lactose formation [16,17]. Compared to other cell types, glucose in the milk and the alveolar MECs is present at high concentrations, which decreases in proportion to milk volume at weaning [18]. Glucose transporter 1 (GLUT1) plays an essential role in transporting glucose during milk synthesis and lactation [16]. The MG synthesizes copious amounts of fat during lactation. Fatty acids are transported from the plasma or synthesized *de novo* from glucose in MECs [19]. PRL also upregulates SREBP1 [20], which regulates lipid metabolism genes that encode lipid-synthesizing enzymes [10]. Several key enzymes, including fatty acid

synthase (*FASN*), are responsible for fatty acid synthesis in MECs, all of which are regulated at the mRNA level within the MECs [19].

In the MG of heterozygous PRL knock-out mice females, STAT5 phosphorylation and alveolar development is impaired [13]. Reduced phosphorylation of STAT5 during lactation results in premature involution and weaning [14]. In the MG, two isoforms of STAT5 (STAT5A and STAT5B) have been identified, which are encoded by two separate genes located on chromosome 11 (mouse) and chromosome 17 (human) [14]. While the deficiency of STAT5A (knock-out) inhibits the normal MG development and MEC differentiation during pregnancy, the deletion of STAT5B does not affect MG development [21]. Phosphorylated STAT5 triggers the transcriptional activation of the *AKT1* gene, which encodes for the AKT protein, and of two subunits (p85α and p110α) of phosphatidylinositol-3-kinase (PI3K) in the MG [22]. Overexpression of STAT5 has been demonstrated to increase the expression of total and phosphorylated AKT1 and phosphorylation of downstream mediators of the PI3K/AKT signaling pathway, which delays MG involution. While inhibiting JAK2 or deleting STAT5 leads to reduced expression of AKT1 and PI3K subunits [22]. Reduced expression of AKT has been linked with suppression of MG development [23]. Additionally, the downregulation of AKT has been associated with ER stress-induced autophagy [24], and ER stress in the MG has been associated with reduced milk production [25].

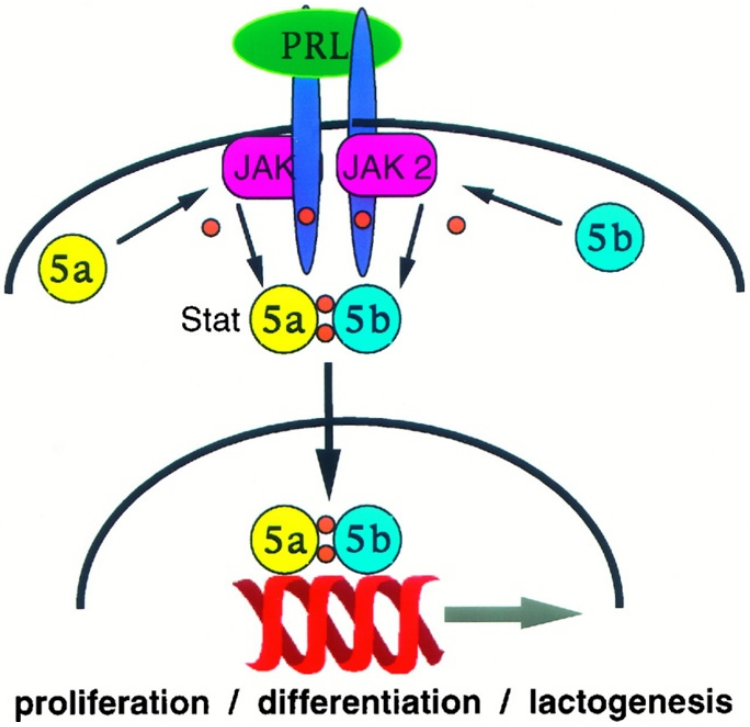

Fig 3 Prolactin (PRL) signaling in the mammary gland leads to the activation of STAT5 and lactogenesis. PRL binds to its receptor PRLR, which activates receptor-associated tyrosine kinase JAK2. JAK2 phosphorylates and activates STAT5a and STAT5b. Activated STAT5A and STAT5B are transported into the nucleus, where they induce the transcription of target genes that promote the proliferation and differentiation of mammary epithelial cells. This results in the initiation of lactogenesis. This figure has been obtained from the work published by Hennighausen et al [15].

iii. Involution results in regression of the lactating MG to a resting stage

The transition from lactating to a non-lactating state is identified as the process of involution. This transition takes place following the lack of demand at weaning or sudden cessation of milk removal due to another reason, resulting in the stagnation of milk in the mammary epithelium [26]. Eventually, this process involves the remodelling of the complex mammary epithelial tree back to simple ductal structures, remodelling of the basement membrane, the collapse of the alveoli, and differentiation of adipocytes [26]. During involution, the secretion volume of the milk significantly decreases and the concentrations of milk components, such as milk proteins, fat, and lactose, also decrease. Interestingly, concentrations of immunoglobulins in

the milk (IgG, IgA, IgM) have been reported to increase in the first few days as the involution progresses and are fully then reduced by 4-5 days of involution [27]. Involution begins with cellular apoptosis, and it is reversible in the first 48 hours of initiation. Following 48 hours, the alveoli begin to collapse, at which point the milk supply is lost and the gland begins to remodel to its virgin state [26]. While STAT5 is downregulated at weaning, activation of STAT3 induces apoptosis during involution and it is essential for involution to proceed [28]. Figure 4 depicts the changes that take place within the MG from embryogenesis, to puberty, pregnancy, lactation, and involution [29].

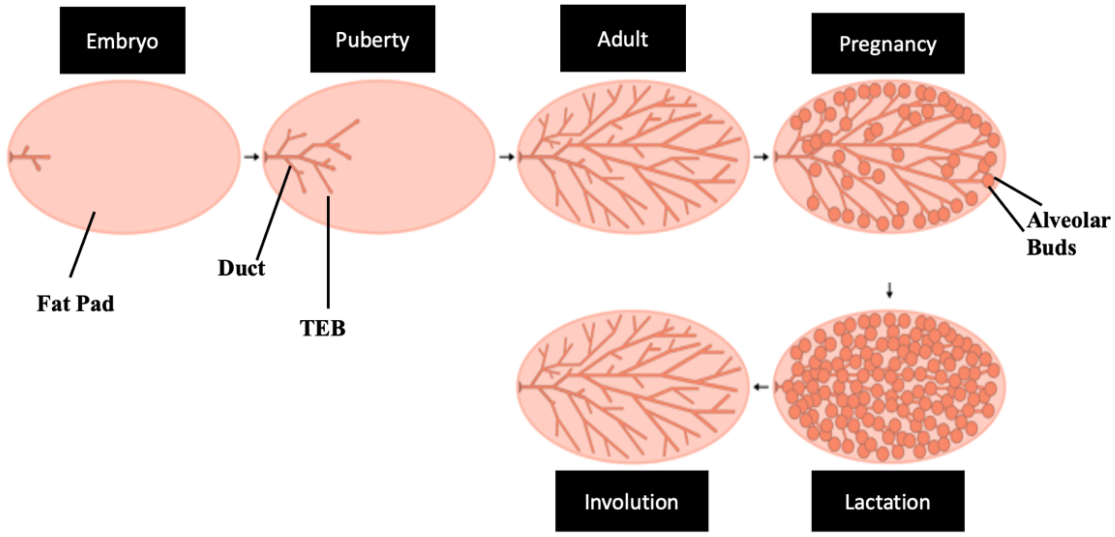

Fig 4 Mammary Gland Development. The development of the mammary gland (MG) primarily occurs through epithelial changes which begin during the fetal stage, during which stage the rudimentary ductal tree forms and grows allometrically with the body post-parturition until puberty. At puberty, the increase of estrogen levels in females triggers ductal morphogenesis, which involves the differentiation of ductal progenitors into ductal epithelial cells, while the terminal end buds (TEBs) undergo bifurcation and invade the mammary fat pad. In the adult MG, increased complexity leads to lateral ductal branching in response to the recurrent estrous cycles. During pregnancy, progesterone stimulates the formation of alveolar clusters, resulting in including gland maturation and alveologenesis. The alveolar clusters function to synthesize and secrete milk during lactation. After weaning, the MG undergoes another round of remodelling which involves its return to the adult MG state. This figure has been adapted from Chen et al [29].

iv. The mammary gland comprises stem cells that support remodelling

Stem cells are unspecialized cells that can differentiate into any cell of the organism and self-renew into daughter cells that hold the capability to produce specialized cells in the future. The mammary epithelium comprises multipotent mammary stem cells (MaSCs) that aid in the ability of the MG to undergo multiple rounds of proliferation, differentiation, and apoptosis with pregnancy. In 2006, self-renewing MaSCs were isolated from the mouse MG, which expressed CD24 (heat stable integrin) and either CD29 (β1-integrin) or CD49f (α6-integrin) [30]. Shackleton et al. demonstrated that a single cell could reconstitute a complete MG, contributing to both the luminal and myoepithelial lineages, and generating functional lobuloalveolar units during pregnancy [30]. Dontu et al. demonstrated that a three-dimensional (3D) culture of human MECs formed spheroids, which are also referred to as mammospheres and they were enriched in early progenitor/stem cells [31]. This scaffold-free model allowed the cultured mammospheres to differentiate into luminal and myoepithelial lineages and form complex 3D structures *in vitro*, which gave rise to physiologically relevant cultures *in vitro.* While two-dimensional (2D) monolayer cultures are convenient, cost-effective, and accessible, 3D culturing of cells allows for the formation of organoids that are physiologically relevant for assessing cellular responses *in vitro.*

v. MG remodeling during pregnancy is essential for breast milk production

Secretory initiation, or Stage I lactogenesis, takes place during the second half of pregnancy when MECs have finished differentiating into milk-producing secretory alveoli [14]. The differentiated stage of MECs is characterized by the expression of key milk proteins (β casein, WAP) and lipogenic markers, the presence of lipid droplets, and tight junction closure to avoid leakage of milk components from the lumen [32]. Impermeability of the tight junctions within the

differentiated MECs is crucial to allow milk components to be stored within the MECs without their leakage from the lumen [32]. Tight junctions control the flow of material along the paracellular pathway by forming a small, continuous seal around each endothelial and epithelial cell at the apical boundary [32]. The build-up of milk within the mammary tissue (milk stasis) or high doses of oxytocin have been found to increase tight junction permeability [32]. Furthermore, mastitis, which is the inflammation of the breast tissue, has been demonstrated to increase the tight junction permeability in MECs [32]. Tight junction permeability is tightly regulated by various hormones and signaling molecules, including PRL, PGR and glucocorticoids. Finally, the rate of milk secretion decreases as tight junction permeability increases [32].

Stage II lactogenesis (secretory activation) starts with milk production following delivery [5]. With the removal of the placenta at delivery and subsequent decrease in PGR, the elevated levels of PRL, cortisol and insulin stimulate the secretion of milk [5]. In addition, oxytocin is another essential hormone that plays a key role during lactation. Oxytocin is released in response to the activation of sensory nerves during labor, breastfeeding, and sexual activity. In response to such sensory stimuli, oxytocin is released within the brain from oxytocinergic nerves emitting from the paraventricular nucleus. Oxytocin is expressed within the myometrium and its levels peak upon labour to promote uterine contractions for labour induction. During the postpartum stage, latching and suckling of the infant on the nipple signal the release of oxytocin from the posterior pituitary gland which stimulates the alveolar-surrounding myoepithelial cells to contract. The pressure produced squeezes the stored milk from the alveolar lumen into the mammary ducts through which it travels to the nipple and transfers to the infant through the milk [5]. Oxytocin has numerous benefits for the mother, including contraction of the mother's uterus after delivery and reduction in bleeding, as well as reduction in stress and calmness psychologically [5].

Human breast milk is typically classified into three groups based on the alteration of milk composition through lactation: colostrum, transitional milk, and mature milk [33]. During the first few days after birth, colostrum milk is produced which is rich in IgA, lactoferrin, leukocytes, and growth factors like the epidermal growth factor [34,35]. Therefore, it is primarily a source of immune factors to ensure that the newborn can combat pathogens and infections before supporting their growth [35]. Because little colostrum milk is produced, lactose levels are also low [34,35]. The lactose levels increase as lactation progresses, which normally occurs 3 to 5 days post-partum, resulting in the transition from colostrum to mature milk [34,35]. Following 4 to 6 weeks after adaptation, the milk reaches the mature stage, which remains uniform unless disturbed by major influencers, such as stressors or drugs [35]. Mature milk is produced in the largest quantity out of all three stages (colostrum, transitional, mature) to satisfy the appetite of the infant [35].

Mouse milk is composed of about 12% proteins (caseins, α-lactalbumin, whey acidic protein (WAP), lactoferrin, secretory immunoglobulin A (sIgA), and others), 30% lipid, and 5% lactose [10]. Human breast milk contains numerous molecules and factors that branch across classes including proteins, non-protein nitrogen, carbohydrates, lipids, water-soluble vitamins, mineral and ionic molecules, and cells [36]. Typically, breast milk comprises 87% water, 3.5 - 3.8% fat, 0.5-1.0% minerals, 3.5% protein, and ~7% sugars (majorly lactose) [37,38]. The fat is secreted in small droplets, with the amount increasing as breastfeeding progresses. Thus, foremilk (secreted at the beginning of the feed) contains less fat than hindmilk (milk secreted towards the end of the feed). Breast milk contains long-chain polyunsaturated fatty acids, such as docosahexaenoic acid or DHA, and arachidonic acid or ARA), which are fatty acids that are essential for the neurological development of the infant. Lipids are the largest source of energy for the infant, contributing approximately 40-55% of the total energy in breast milk, with the 30-50g/L

of fat present in the breast milk [36,39]. Lipids include fat-soluble vitamins, carotenoids, fatty acids, sterols, and triglycerides [36]. Triglycerides are produced by the MG and provide about 97% of fats energy contribution to the infant [36,40]. Free fatty acids, on the other hand, support the infant's retinal and neural tissues, of which linoleic acid, α-linoleic acid, and their derivatives arachidonic acid and docosahexaenoic acid, respectively, are extremely essential [36,41].

Lactose is another major source of energy in breast milk, as it is the most abundant carbohydrate present in breast milk [36,39]. Lactose levels are positively correlated with milk production and are a stable component with little variability shown between and within mothers [36,37]. Carbohydrates generate ~40% of breast milk's total energy, which alone satisfies the high demands of the human brain [37,39]. The milk also contains immunoglobulins, lactoferrin, whey and β-casein proteins, lactose, human milk oligosaccharides (HMOs) and lipids. In milk, whey proteins make up ~60-80% of total protein concentration, while caseins comprise 20-40%, and mucins are fractionally present [42–44]. Major whey proteins include lactoferrin, α-lactalbumin, immunoglobulins and serum albumin, which collectively perform a multitude of functions [45,46]. Lactoferrin is a multifunctional glycoprotein, which binds iron for its delivery to the infant and promotes cellular proliferation and differentiation in the MG [47,48]. It is the second most abundant protein after caseins and mainly functions to transport iron in the blood, however, it also provides antimicrobial, immunomodulatory, and immunostimulatory functions [49,50]. Another whey protein that is of high importance is α-lactalbumin, which plays a central role in milk production as part of the lactose synthase complex that is required for lactose formation [16,51]. Among caseins, β-casein is the most prominent isoform, which is responsible for forming micelles to produce a gastric curd in the infant's stomach that shortens gastric emptying time [52,53]. κ-casein has antioxidant, angiotensin-converting enzyme inhibitory and antibacterial activity [54].

Immunoglobulins and lactoferrin are important immune factors within the protein class present in breast milk. Immunoglobulins are antibodies produced by B-lymphocyte differentiated plasma cells [55,56]. Immunoglobulin (Ig) A (IgA), IgM, and IgG are the three antibodies that pass through the milk to infants to recognize and destroy foreign pathogens while the infants' immune system matures [55,56]. IgA is the predominant form that performs a variety of functions, including blocking bacterial adherence to epithelial cells and neutralizing toxins [55]. IgG primarily functions in tissues by eliminating pathogens [55,57]. Both IgG and IgM activate the complement system to protect the respiratory mucosa and gastrointestinal tract [55,57].

According to the Centers for Disease Control and Prevention, iron, vitamin A, vitamin D, iodine, and folate are among the micronutrients present in breast milk that are needed for an infant's healthy growth [58,59]. Iron assists in the motor and cognitive development of the infant and its insufficiency results in anemia [58,59]. Vitamin A supports the development and function of the retina, immune system, and epithelial surfaces [58,60,61]. In addition to reduced iron levels, low Vitamin A levels also promote anemia [58,60,61]. In addition, deficiency of Vitamin A increases susceptibility to infections and xerophthalmia, which can cause night blindness and keratomalacia [58,59,62]. Vitamin D is important for bone development; iodine supports infant growth and cognition; and folate drives brain and spine formation [63].

The composition of milk is reflective of the transport processes found in MECs [64]. Therefore, understanding the mechanisms underlying mammary epithelial solute transport is essential to appreciating the complexity of the cellular biology and physiology of milk formation. The polarized cells within the MG are joined together by tight junctions, which act as a barrier to help maintain epithelium polarity. Water, solutes, fat, lactose, and various milk proteins are secreted into the milk through various means, including exocytosis mediated by the Golgi, free

crossing of substances across the cellular membrane, paracellular transfer, transcytosis and transport mediated by the transport proteins [64]. Transport proteins are expressed in the apical and basolateral membranes of MECs, which allow transepithelial transport of milk components as well as nutrients and biomolecules [65]. Transport proteins participate in the uptake, re-uptake, or efflux of various proteins, nutrients, and compounds, which are essential to infant nutrition [65]. Transfer of milk components in MECs have been reported to be primarily mediated by the ATP-binding cassette (ABC-) and Solute Carrier (SLC-) transporters [65]. Furthermore, the levels of these transporters in mammary tissues fluctuate based on the lactational status of the MECs. For example, a 4-fold increase in the RNA levels of Organic Cation Transporter 1 (OCT1), Concentrative Nucleoside Transporters 1 (CNT1) and 3 (CNT3), along with 1.5-fold increased mRNA levels of Multidrug Resistance-associated Protein 5 (ABCC5/MRP5) has been reported in lactating MECs, compared to non-lactating MECs [66]. Changes in the expression of transport proteins may alter milk composition, which is of concern to infant health.

vi. ER stress contributes to mammary gland dysfunction

During the synthesis of maternal milk, the MG increases its uptake of amino acids to synthesize structural, catalytic, and secretory proteins. During stage I and stage II of lactogenesis, the MG requires excessive energy (in calories), which often exceeds the amount of caloric intake by the mother. Protein biosynthesis, gluconeogenesis, lipid synthesis, and initial protein maturation, which is crucial for the proper folding of proteins in the secretory pathway, are all processes that take place within the endoplasmic reticulum (ER) [67]. The ER plays an essential role in the creation, folding, classification and distribution of proteins to the correct cellular destination [68]. ER chaperones, such as GPR78, GPR94, and calnexin bind to the hydrophobic

domains of the newly synthesized unfolded proteins, leading to proper protein folding and inhibition of protein aggregation [68].

The ER is the primary intracellular reservoir for Ca^{2+} [68]. During ER stress, Ca^{2+} is released from the ER and it accumulates in the mitochondria, which results in the initiation of cell death [69]. Furthermore, Burgoyne et al. demonstrated that depletion of Ca^{2+} from the ER led to significant inhibition of casein synthesis [70]. Thus, the integrity of the ER structure and function plays an important role in maintaining MG function. ER stress can be induced by exposure to stressful stimuli, hypoxia, nutrient deprivation, and perturbation of redox status [68,71]. Additionally, ER stress can be induced by pharmacological treatments, such as brefeldin A (disruption of ER-Golgi trafficking), thapsigargin (depletion of Ca^{2+} stores), and tunicamycin (inhibition of N-linked glycosylation). In MECs, ER stress has been associated with disruption of lipid production and protein secretion [72,73]. Furthermore, in goats, ER stress has been linked with a decrease in milk fat content, differences in milk composition, induction of permeable tight junctions between MECs, and impaired lactation performance in goats [74].

ER stress has been defined as an imbalance between the folding capacity of the ER and the protein folding load, resulting in the accumulation of unfolded or misfolded proteins in the ER lumen [75,76]. Upon experiencing ER stress, the cell activates unfolded protein response (UPR), which reduces unfolded proteins through several mechanisms such as:

(i) The expansion of the ER membrane,

(ii) the synthesis of key components of the protein folding and quality control machinery

(iii) attenuation of protein influx into the ER

(iv) activation of key signaling pathways.

The UPR functions through three ER transmembrane protein sensors: inositol-requiring kinase 1α (IRE1α), pancreatic ER eIF2α kinase (PERK), and activating transcription factor 6 (ATF6) [68]. The luminal domain of each sensor responds to the unfolded/misfolded proteins in the ER. In the case that the cell is unable to resolve the protein-folding defect and homeostasis is not restored, cell-death signaling pathways are activated [71,77].

Upon initiation of ER stress, the chaperone BiP is released from PERK, which results in the activation of PERK [71,77]. Activated PERK phosphorylates Ser51 of the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α), which in turn reduces translation initiation to decrease the protein folding load in the ER. In addition, PERK phosphorylates nuclear factor erythroid 2-related factor 2 (NRF2), a transcription factor involved in redox metabolism. Phosphorylation of eIF2α allows the translation of *ATF4* mRNA, which encodes a transcription factor that modulates the transcription of genes involved in autophagy, apoptosis, amino acid metabolism and antioxidant responses [68]. One of the downstream targets of eIF2α-ATF4 is CCAAT/enhancer-binding protein homologous protein (CHOP) [68]. CHOP is a crucial mediator of ER stress-induced apoptosis. CHOP activates several pro-apoptotic factors, induces oxidative stress, and inhibits anti-apoptotic protein B cell lymphoma (BCL-2) [71,77].

Upon activation, IRE1α undergoes dimerization, which is followed by auto-phosphorylation that triggers its RNase activity [68]. The RNase activity cleaves the unspliced X box-binding protein 1 (XBP1) to an active spliced XBP1. Activated XBP1 modulates genes encoding proteins involved in protein folding, ER-associated degradation (ERAD), protein quality control and phospholipid synthesis. IRE1α also degrades certain mRNAs through regulated IRE1-dependent decay (RIDD) and induces 'alarm stress pathways', including those driven by JUN N-terminal kinase (JNK) and nuclear factor-κB (NF-κB), through binding to adaptor proteins [68].

In stress-free cells, ATF6 has a basic Leu zipper (bZIP) transcription factor in its cytosolic domain, which is localized at the ER. Upon activation, ATF6 is transported to the Golgi apparatus through interaction with the coat protein II (COPII) complex, where it is processed by site 1 protease (S1P) and S2P, releasing its cytosolic domain fragment (ATF6f). ATF6f regulates the upregulation of genes that encode ERAD components, as well as XBP1 [68].

Early UPR is mediated by the activation of PERK, which results in inhibition of gene protein translation, degradation of mRNA encoding for certain ER-located proteins, elimination of damaged ER, and inhibition of translocation of proteins into the ER upon translation [78]. These mechanisms aim to reduce the influx of proteins into the ER to allow for adaptive and repair mechanisms to re-establish homeostasis. These responses are followed by activation of ATF6, XBP1, and ATF4 which regulate the expression of several overlapping target genes and encode proteins involved in modulating adaptation to stress. Furthermore, activation of PERK is essential for adaptation to stress and maintaining cell survival. PERK activates NRF2, which is then located in the nucleus and plays a role in maintaining cell survival [78].

Failure in the mitigation of ER stress results in the induction of apoptosis, which irreversibly eliminates damaged cells. Cell death under ER stress depends on the core mitochondrial apoptosis pathway, which is regulated by the BCL-2 protein family. Chronic or severe ER stress results in oxidative stress [79] and activation of UPR-dependent apoptosis [80], which has been associated with PERK-mediated translation of *ATF4,* and activation of CHOP [77]. CHOP downregulates the expression of the antiapoptotic mediator BCL2 [77], and directly activates the expression of growth arrest and DNA damage-inducible 34 (GADD34) [81]. GADD34 elevates oxidative stress [82], disturbs cellular metabolism, and damages cellular

constituents [83]. Figure 4 depicts the adaptive and pro-apoptotic pathways of ER stress and the essential markers involved in the pathways [84].

The ER interacts with the mitochondria to maintain cellular metabolism and cell survival [68]. Mitochondria are dynamic organelles that contain their own genome and protein synthesis machinery, and are composed of the outer mitochondrial membrane, the intermembrane space, the inner mitochondrial membrane, and the matrix [85]. The mitochondria supply cells with metabolic energy, which is generated by oxidative phosphorylation. Mitochondrial function influences cellular metabolism, cell signaling, cellular proliferation and apoptosis [85]. The dynamic fusion and fission events (fusing and dividing processes) allow constant remodeling of the mitochondria, which keeps it ready to respond to the changing conditions within the cell [85]. Fusion of the mitochondrial outer membrane depends on two dynamin-related GTPases that are known as Mitofusin 1 (MFN1) and MFN2 [85]. MFN2 regulates ER morphology and regulates the transfer of calcium from the ER to the mitochondria. MFN2 has been demonstrated to phosphorylate and upregulate PERK, which results in apoptosis and autophagy during ER stress. Moreover, DRP1 and FIS1 are key mediators of mitochondrial fission [86]. Finally, inner mitochondrial membrane fusion is achieved through the activity of OPA1 (Optic Atrophy 1), which is essential for the maintenance of internal cristae structure. Altogether, changes in mitochondrial morphology have huge implications on cellular survival and apoptosis. During lactation, the alveolar MECs require high oxidative capacity, which is supplied by the mitochondria [87]. Thus, the mitochondria are increased in the MECs during lactation and proper functioning of mitochondria is essential to MEC differentiation and function.

In addition to regulators of fission and fusion proteins, *SOD2* encodes for Superoxide Dismutase 2, which protects the cell against mitochondria-generated ROS toxicity and its

deficiency leads to excessive ROS production [88]. Similarly, *PINK1* encodes for PTEN-induced putative kinase 1 (PINK1), which protects the mitochondria from malfunctioning during cellular stress [89]. PRKN encodes for Parkin RBR E3 ubiquitin-protein ligase, which is regulated by PINK1 to eliminate the dysfunctional mitochondria [89]. Furthermore, *TFAM* encodes for Transcription Factor A, mitochondrial, which is responsible for transcription of the mitochondrial DNA; a process that is downregulated during oxidative stress [90].

During the occurrence of ER stress, Ca^{2+} is released from the ER, crosses the outer mitochondrial membrane, enters the mitochondrial matrix, and through numerous mechanisms, the mitochondria releases Ca^{2+} into the cytosol [91]. Accumulation of Ca^{2+} levels in the mitochondrial matrix triggers proapoptotic signaling via mitochondrial alterations, such as mitochondrial membrane permeabilization, dissipation of electrochemical potential, matrix swelling, and localization of BAX to the mitochondria [91]. Wei et al. demonstrated that BAX is essential for the release of cytochrome c from the mitochondria, which triggers mitochondrial dysfunction and cell death [92]. Impaired signaling of proteins involved in regulating the fission and fusion processes results in fragmented mitochondria within cells. MFN2 has been identified to regulate PERK via phosphorylation and subsequent activation, resulting in the activation of apoptosis during ER stress [86]. Furthermore, DRP1 and FIS1 act in regulating apoptosis following ER stress [86]. Essentially, impaired signaling within the mitochondria and mitophagy result in an inadequate number of mitochondria and/or dysfunction in the electron transport and ATP-synthesis machinery. This leads to the generation of reactive oxygen species (ROS) [80], eventually resulting in cell death [91].

In addition to ROS production, ER stress is strongly associated with the downregulation of tight junction proteins, such as *OCLN* which encodes for Occludin, ZO-1 which encodes for tight

junction protein 1, and claudin-1) [93,94]. Furthermore, both ROS production and ER stress have been linked with tight junction disruption in nasal epithelial cells [95]. Rapid disruption of tight junction permeability in MECs during alveolar MEC differentiation may increase leakage of milk components out of the cells. Altogether, ER stress leads to the activation of UPR, mitochondrial dysfunction, oxidative stress, and tight junction permeability, all of which directly impact cellular differentiation and function.

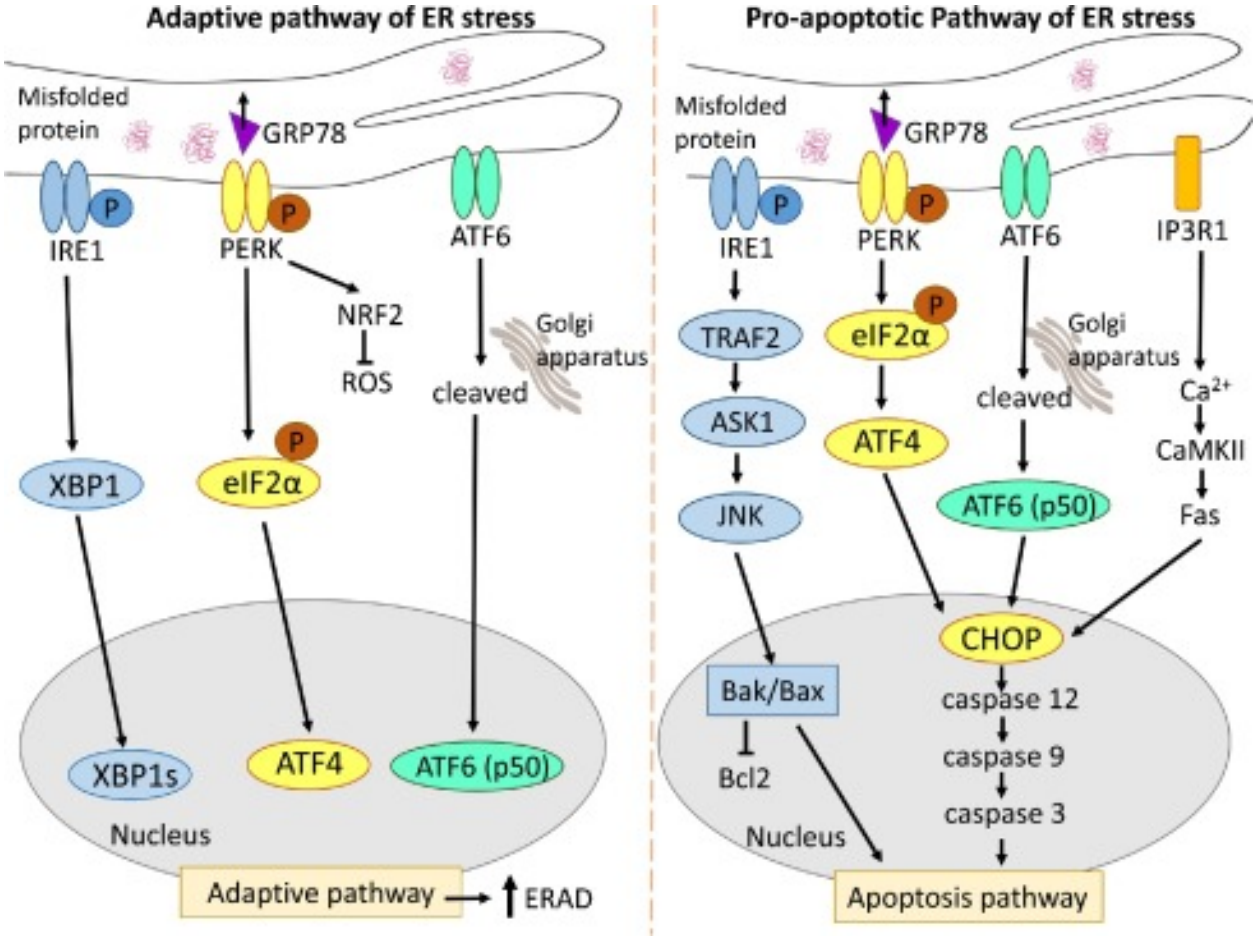

Fig 5 Adaptive and pro-apoptotic pathway of ER stress. Once the cell encounters ER stress, the ER chaperone GPR78 dissociated from the ER transmembrane sensor, resulting in the activation of the unfolded protein response (UPR). The UPR pathway involves the activation of IRE1, PERK, and ATF6, which upon activation induce the expression of the following transcription factors: XBP1s, ATF4, and ATF6 (p50). These transcription factors initiate the adaptive pathway, in addition to the activation of NRF2 by PERK. If the adaptive UPR pathway fails to restore cellular function and chronic ER stress occurs, the apoptotic UPR pathway is

induced. The apoptotic UPR pathway involves the activation of CHOP and downregulation of BCL2. Abbreviations: CHOP, C/EBP-homologous protein; eIF-2α, eukaryotic Initiation factor 2; ERAD, endoplasmic-reticulum-associated protein degradation; eIF2α, eukaryotic translation initiation factor 2α; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; IRE1, inositol-requiring protein 1; JNK, c-Jun N-terminal kinase; NRF2, nuclear erythroid 2 p45-related factor 2; P, phosphorylated; PERK, protein kinase RNA-like endoplasmic. This figure has been obtained from Choy et al [84].

vii. The impact of cannabis use during the perinatal period

Cannabis, also known as marijuana, weed, pot, grass, dope, reefer, ganja, hash, herb and chronic, is a chemically complex and medicinally valuable flower plant species [96]. The cannabis genus includes multiple plant species, such as *Cannabis sativa, Cannabis indica, and Cannabis ruderalis* [96]. *Cannabis sativa L*. has been reported to be one of the more common species and it contains over 421 different chemical compounds [96]. Furthermore, it has a subset of over 70 terpenophenolic phytocannabinoid (cannabinoid) compounds [97]. Cannabinoids are synthesized in plants in carboxylic acid forms, and they form neutral cannabinoids in a non-enzymatic thermal conversion reaction. One of the most recognized conversions is that of delta-9-tetrahydrocannabinol acid (THCA) to the psychoactive delta-9-tetrahydrocannabinol (THC) [98]. THC has been found to produce psychotropic effects that contribute to anxiety and learning and memory deficits [99–101]. CBD is a natural, non-psychoactive constituent of *C. Sativa* that has been reported to be neuroprotective in rats [102], analgesic in humans [103], as well as anti-emetic, anti-spasmodic [104], and anti-inflammatory in mice [105]. While THC and CBD are two of the most widely recognized cannabinoids, cannabis contains numerous other cannabinoids, including cannabinol (CBN), cannabigerol (CBG), and cannabidivarin (CBDV), which are explored in less detail but may have potential effects and pharmacological potential [97].

Substance use during pregnancy and lactation has been associated with significant risks to the unborn child. Despite the knowledge of its potential risks [106], cannabis has been commonly

used by women during pregnancy to alleviate symptoms of morning sickness, i.e., nausea/vomiting, chronic pain, and anxiety [107,108], as well as to manage postpartum stress and fatigue [109]. While reasons to use cannabis during pregnancy have been associated with symptom management, reasons to use cannabis during breastfeeding have been reported to be the same as those expressed prior to pregnancy [110]. The American College of Obstetricians and Gynecologists has recommended that women discontinue cannabis during gestation and while breastfeeding [111]. Employees from 93% of Canadian cannabis dispensaries recommended against cannabis products to treat nausea and vomiting during pregnancy [112]. However, after a closer look at the recommendations provided by employees, it was discovered that while the majority (79.4%) of employees of 714 Canadian cannabis dispensaries recommended against cannabis use during breastfeeding, 20.6% did recommend use [113]. Shockingly, in a study conducted in the United States, Dickson and colleagues reported that 69% (277) of the (400) dispensaries contacted in Colorado recommended cannabis products for the treatment of morning sickness [114]. Only a few dispensaries encouraged discussion with a healthcare provider prior to prompting the use of cannabis products for morning sickness [114]. Thus, a greater degree of awareness around cannabis use during pregnancy is needed so women can make informed decisions.

Most Canadians (73%) have acknowledged either a moderate or significant risk of smoking cannabis during gestation, with 88% accepting that cannabis consumption may be harmful during pregnancy or while breastfeeding [115]. Furthermore, Bartlett and colleagues reported that most women who self-reported using cannabis during pregnancy were aware of its transmission to the fetus during pregnancy and to the infant while breastfeeding (94.3% and 91.2%, respectively) [116]. Despite this awareness, cannabis use among Canadians aged 15 or older has increased from

14% to 20% following legalization [117]. Furthermore, approximately 45% of Canadians of reproductive age (18- to 45 years old) have endorsed cannabis in the past three years months [117].

Based on the Better Outcomes Registry & Network (BORN) Ontario database, which covered live births and stillbirths in Ontario between April 2012 and December 2017, cannabis use during pregnancy was self-reported in about 2% of pregnancies [118]. However, with the legalization of cannabis for recreational use across several countries, there is concern regarding the increase in the prevalence of its usage. A study completed in Hamilton, Ontario, conducted after the legalization of cannabis use in Canada reported that 11% of women used cannabis at some point during pregnancy, with 4.2% of respondents reporting continued use during pregnancy, and 4.8% reporting intention to use cannabis while breastfeeding [116]. In contrast, in a study in Colorado, where recreational cannabis is illegal, a significantly higher rate of cannabis use among women during pregnancy (22.4%) was found upon testing umbilical cord blood, compared to women self-reporting use (2.6%) [119]. Furthermore, a study out of Maryland, Baltimore reported that 34% of women reported using cannabis throughout pregnancy, with an additional 10% of the respondents declaring that they would use cannabis during pregnancy if it were legalized [106].

A high prevalence of cannabis use during gestation has also been reported among indigenous women (9 to 21%) [120–122], and its use has been attributed to socioeconomic disadvantage, marginalization, impacts of colonization and grief, lower levels of education and young childbearing age [120]. Among indigenous and non-indigenous women, cannabis use during gestation has been associated with the co-use of alcohol and tobacco [123]. Altogether, the high prevalence of cannabis use during pregnancy (2-34%) and the increasing rates of use present global clinical and public health concerns. Therefore, it is prudent to understand the impact of cannabis on maternal reproductive organs and its impact on infant health.

The psychoactive component of cannabis, named delta-9-tetrahydrocannabinol (THC), has been reported to cross the placenta [124]. Maternal cannabis use during pregnancy has been linked with decreased fetal growth [125,126], low birth weight [120,127–129], increased risk of premature birth [127], and a higher rate of intrapartum stillbirths [130]. Furthermore, prenatal exposure to cannabis has been reported to alter neurotransmitter and neuroendocrine systems in the offspring of rodents [131] and impact neurobehavioral performance in human infants compared to non-exposed infants [132]. Moreover, *in utero* exposure has been reported to cause frequent startles, alter sleep patterns, and impaired executive functioning [133]. Corsi et al. conveyed that 2.2% of the 508,025 analyzed children exposed to cannabis *in utero* were diagnosed with autism spectrum disorder (ASD), with 1.7% of children experiencing intellectual and learning disorders, and 5.7% of children displaying attention deficit disorder, hyperactivity, and ADHD [134]. Comparatively, these numbers were lower for non-exposed children, which suggests that cannabis has negative neurological implications in infants [134].

Detection of cannabinoids in the milk of users has presented a concern for cannabis use during lactation [135–137]. THC has been detected in the breast milk of mothers up to 6 days after last reported use [136], and up to six weeks in chronic users [138]. Baker et al. have reported that of the maternal dose, 2.5% is transferred to the infant through breastfeeding, which seems insignificant but could be detrimental if maternal consumption is high [135]. Astley and Little explored the relationship between cannabis exposure and infant motor development at one year of age [139]. With 55 1-year-old infants, a 14-point average decrease in the Bayley index of infant motor development was reported for infants exposed to cannabis through breast milk [139]. In rat pups, Scheyer et al. reported that exposure to THC via lactation resulted in delayed GABA maturation [140], which is associated with early-life behavioural aberrations [141]. Results from

Scheyer and colleagues indicate that exposure to a low dose of cannabis through milk can impact infant health. Furthermore, maternal cannabis use during lactation has been reported to alter the lipid composition in the milk of exposed mice dams [142]. Therefore, cannabis use during pregnancy and lactation can negatively impact infant health by exposing the fetus and infants to cannabinoids, as well as by disrupting processes, such as placentation and MG development.

viii. Cannabinoids interact with the endocannabinoid system

The biological effects of cannabinoids are mediated via the endocannabinoid system (ECS), which comprises cannabinoid receptors, endogenous cannabinoids (endocannabinoids), and metabolic enzymes that regulate the levels of endocannabinoids [143]. Endocannabinoids act by binding to the cannabinoid receptors and increasing their activity (i.e., acting as an agonist), decreasing their constitutive activity (i.e., acting as an inverse agonist), or blocking other ligands from accessing the receptor (i.e., acting as an antagonist) (Figure 6). Moreover, agonists shift receptors to the active state and thereby increasing signaling. However, inverse agonists bind to the inactive state of the receptor decreasing the fraction of the active receptor and suppressing basal signaling. A neutral antagonist does not perturb signaling from its basal state; however, an inverse agonist can decrease signaling to less than basal levels [144].

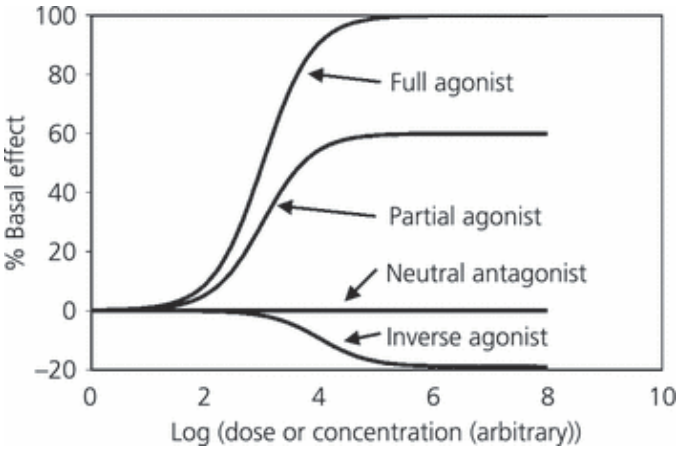

Fig 6 Possible responses of various receptor-ligand interactions. A full agonist stimulates a maximal cellular response. A partial agonist, which has lower intrinsic efficacy, produces an intermediate response. A neutral antagonist has no effect that is detected in the cell. An inverse agonist produces an opposite effect to that of the agonist. This figure has been obtained from Mackie et al. [144]

Cannabinoid receptors are G-protein-coupled-receptors that activate K^+ channels and members of the MAP kinase family to inhibit adenylyl cyclase and voltage-gated Ca^{2+} channels [145]. This results in a decrease in cellular levels of cyclic AMP (cAMP), which leads to alterations in cellular proliferation, differentiation, and function [145]. Cannabinoid receptor 1 (CB1) was discovered in 1988, followed by the discovery of cannabinoid receptor (CB2) in 1993 [146]. The CB1 receptor is one of the most abundant G protein-coupled receptors (GPCRs) in the CNS [147], particularly rich in the basal ganglia, cerebellum, and hippocampus [146], as well as being found in the eye, placenta, the fetal membranes, and myometrium [148–150]. CB1 has been reported to modulate many processes, including mood, appetite, learning, memory, pain signaling, and energy metabolism, as well as being involved in reproductive and cardiovascular system functions [151].

Peripherally, the ECS is involved in bone remodelling and modulating the immune system primarily via the cannabinoid receptor 2 (CB2) [152]. CB2 expression is associated with inflammation, and it is primarily localized to microglia in the CNS, and in peripheral immune cells [152]. CB2 knock-out mice fail to respond to the immunomodulatory effects of cannabinoids, and CB2 ligands have been suggested to have therapeutic applications for the treatment of inflammation and allergy [153]. Moreover, CB2A isoform has been found in the testis and lower brain regions, and CB2 has been identified in the liver, pancreas, gastrointestinal system, skeletal muscle, and adipose tissue [146]. Besides CB1 (encoded by the *CNR1* gene) and CB2 (encoded by the *CNR2* gene), several other receptors involved in the ECS have been discovered, including G-protein coupled receptor (GPR) 18 (GPR18), GPR119, GPR55 and transient receptor potential

cation channel subfamily V member 1 (*TRPV1*) [154]. These receptors have been investigated in much less detail; however, they have been reported to bind endocannabinoids and (phyto)cannabinoids leading to altered physiological processes [154].

The CB1 receptor binds endocannabinoids, as well as (phyto)cannabinoids, and synthetic cannabimimetic compounds such as CP55940, JWH015, and WIN55212-2 [147]. The CB2 receptor also binds endocannabinoids, (phyto)cannabinoids, and synthetic cannabimimetic compounds such as JWH133 [155]. Anandamide (AEA) and 2-Arachidonoylglycerol (2-AG) are two of the most researched endocannabinoids that are produced by the body and regulated by key enzymes [156,157]. AEA is primarily synthesized by phospholipase C (PLC) and N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD). AEA has been characterized as a high-affinity partial agonist of the CB1 receptor and is almost inactive in CB2 [158]. Followed by the uptake of AEA into the cells, its signal is suggested to be terminated and AEA is degraded by fatty acid amide hydrolase (FAAH) [159]. Regulation of AEA levels by FAAH is extremely important, as elevated AEA levels and lower FAAH levels are detrimental in early pregnancy [159].

In comparison to AEA, 2-AG acts as a full agonist at both CB1 and CB2 with moderate to low affinity [158]. 2-AG is synthesized by diacyl-glycerol lipase (DAGL) α and β and is primarily broken down by monoacylglycerol lipase (MAGL), and to a lesser extent by α/β-hydrolase-6 (ABHD6) and α/β-hydrolase--12 (ABHD12) [160]. Like AEA and 2-AG, THC and CBD mediate their effects through the cannabinoid receptors. However, unlike AEA and 2-AG, THC and CBD are not vulnerable to breakdown by the ECS enzymes. THC has been demonstrated to activate both CB1 and CB2 with lower efficacy than certain synthetic CB1/CB2 receptor agonists, resulting in its classification as a partial agonist of CB1 and CB2 [161]. In comparison, CBD has been declared to be capable of antagonizing CB1 and CB2 receptor agonists, resulting in the activity of

CBD being characterized as an inverse agonist at CB1 and CB2 [161]. In the presence of THC, CBD has been found to display high potency as an antagonist of CB1 and CB2 *in vitro* [161]. CBD may modify the effects of THC through various mechanisms. CBD has been reported to be a potent inhibitor of cytochrome P450 (CYP450), which metabolizes THC to 11-OH-THC [162]. Furthermore, the isoforms CYP2D6, CYP2C19, CYP2B6, and CYP2J2 have been reported to be inhibited by THC and CBD, with CYP3A4/5/7 inhibited by CBD. CYP3A4 is responsible for the metabolism of various medicines and reaches high levels in humans at one year of age. Thus, impact of THC and CBD on CYP450 enzymes and the other isoforms may result in altered drug metabolism. As for the impact of the cannabinoids on each other, the downregulation of cannabinoid receptors (in the presence of CBD) may cause THC to produce antagonism rather than agonism [161]. In presence of THC, the antagonistic activity of CBD at CB1 may be of concern. The administration of the CB1 antagonist SR141716A has been reported to cause appetite suppression and weight loss in adult, non-obese rats [163]. In addition to CB1 and CB2, THC and CBD impact cellular processes through various other mechanisms, including interaction with other receptors such as GPR55 and TRPV1 [161].

ix. The role of the ECS in mammary gland development and function

In addition to modulation of the neurological [151], immunological [153] and endocrine systems [164], the ECS plays a role in reproductive processes such as fertilization, implantation, embryonic development, and placental growth [143,165–168]. Remodelling of the MG is a key process that takes place during pregnancy and is crucial for a nutritious and sufficient supply of breast milk for the infant [1]. The role of the ECS in MG development, and the impact of cannabis on this role, is not clear. At parturition, elevated levels of CB1 receptor mRNA in the brains of neonatal mice have been observed, which is accompanied by high concentrations of 2-AG levels

in the maternal milk [169]. Upon injection of CB1 receptor antagonist SR141716A, milk ingestion and subsequent growth were decreased in most pups (75-100%), with death following days after [169]. In rat pups, acute THC administration (0.5 mg/kg BW) to lactating rats has been demonstrated to result in the transient suspension of milk ejections and cause longer intervals between milk ejections, compared to vehicle-treated controls [170]. These effects were reversed upon administration of oxytocin in combination with THC [170]. Furthermore, chronic administration of THC in rats downregulated the expression of oxytocin-neurophysin mRNA and decreased the immunoreactivity of oxytocin in the nucleus accumbens and ventral tegmental area, which are areas involved in the reward and addiction [171]. Thus, exposure to cannabinoids through breast milk may impair CB1 signaling in the infant's brain, resulting in downstream consequences associated with altered CB1 expression.

Synthesis of PRL by lactotrophs in the anterior pituitary gland is extremely important for MG development and milk production [172]. In addition to nipple stimulation, light, olfaction, and stress, the thyrotropin-releasing hormone (TRH), estrogen (pregnancy), and dopamine antagonists (antipsychotics) also stimulate the production of PRL [172]. In the pituitary gland of rats and oophorectomized female and intact male rhesus monkeys [173], the activation of the CB1 receptor has been demonstrated to inhibit PRL secretion. Furthermore, THC administration has been reported to result in the suppression of serum PRL levels in ovariectomized rats[174], and in mice at late pregnancy [175,176] and early lactation [177]. The downregulation of PRL levels may be mediated through various mechanisms, however, one possible explanation is through the activation of the dopamine receptor D2 [178]. Cannabinoids, including THC, induce dopamine release in the human striatum, via CB1 [179,180]. Dopamine (DA) binds D2 receptors expressed on the cell membrane of lactotrophs, reducing PRL exocytosis and gene expression. Therefore, cannabis may

alter levels of key hormones, such as PRL and oxytocin, resulting in altered development of the MG and reduced milk production.

As reviewed above, activation of the PI3K-AKT pathway triggers autocrine-mediated PRL secretion, which indirectly activates the JAK2/STAT5 signaling pathway, which is required for MEC differentiation and milk production. PI3K/AKT signaling pathway has been reported to be downregulated by THC, resulting in reduced cellular differentiation, ER stress, and cellular apoptosis [181]. Hyoda et al. demonstrated that inhibition of PI3K resulted in the induction of ER stress within the mouse fibroblasts [182]. Furthermore, treatment of cells with the ER stress inducers, tunicamycin or thapsigargin, resulted in dephosphorylation of AKT. Overall, the inactivation of AKT and PI3K subunits resulted in the expression of genes involved in ER stress and cellular apoptosis in mouse fibroblasts [182]. Disruption of lipid and protein production and release has been linked to ER stress in MECs [72,73]. In goats, ER stress has been linked with reduced milk fat content, variations in milk composition, the development of permeable tight junctions between MECs, and suboptimal lactation performance [74]. THC and CBD have been reported to induce ER stress in various other cell types. In human trophoblast cells (BeWo), THC (3-30 μM) led to a dose-dependent increase in ER stress markers and CHOP [183]. These effects were blocked with CB1 and CB2 receptor antagonists, as well as with the ER stress inhibitor Tauroursodeoxycholic acid (TUDCA) [183]. In breast cancer cells (MCF7 cells), CBD induced an influx of Ca^{2+} via the TRPV1 receptor, leading to high ROS production and generation of ER stress [184]. Concomitant treatment with CBD and an antioxidant, as well as co-treatment of cells with CBD and a TRPV1 antagonist, resulted in increased cell viability and decreased ER stress [184]. Therefore, THC and CBD may induce ER stress in MECs to disrupt cellular differentiation and function.

The MG goes through a crucial phase of remodeling that is accompanied by alveolar differentiation of the MECs, which is essential for milk production [1]. Among three women who experienced failure of lactation, insufficient glandular tissue and atypical breast changes with pregnancy within the breast were determined to be the underlying cause of lactation failure [185]. Poor or inadequate milk production is one of the most frequently cited reasons for ceasing to exclusively breastfeed and opting to use formula [186]. Although animal and formula milk are commonly used as alternatives to breast milk, several studies have demonstrated that they lack various bioactive and immune factors that are present in human breast milk, which may have implications for the immune system [36,39,187,188]. Breastfeeding strongly influences infant gut bacteria, and the co-occurrence of bacterial species in mothers' milk and infants' stool is reduced when breastmilk is pumped and fed from a bottle [189]. Breastfed infants demonstrated a reduced risk of ear and respiratory infections, diarrhea, and sudden infant death syndrome, as well as decreased odds of diabetes, obesity, and leukemia through adolescence [190]. Due to its vital benefits, the World Health Organization (WHO) has recommended exclusive breastfeeding for the first 6 months of newborn life [188].

Given the importance of a nutritious and sufficient supply of breast milk, an understanding of the impact of cannabis use in the perinatal period on MG development and breast milk composition will be important in informing mothers, developing educational programs, reducing associated harms, and improving infant health outcomes.

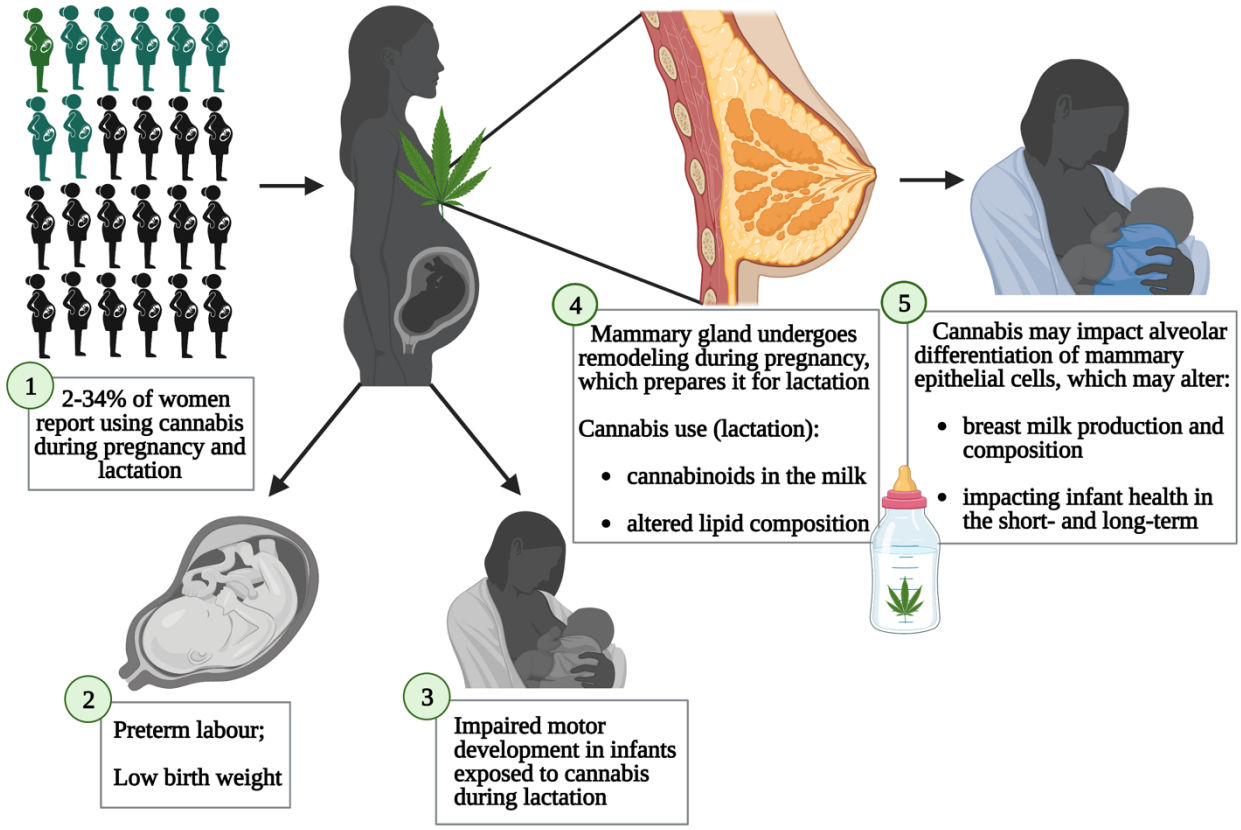

Fig 7 The potential impact of maternal cannabis use in the perinatal period on mammary gland development and breast milk composition. Despite the awareness of its transmission to the fetus during pregnancy and to the infant while breastfeeding, 2 to 34% of women report using cannabis in the perinatal period. Maternal cannabis use during pregnancy has been linked with decreased fetal growth, low birth weight, preterm labour, and increased risk of premature birth. Compared to children not exposed to cannabis in utero, a higher number of children who were exposed to cannabis in utero have intellectual and learning disorders. Cannabis use during lactation has been linked with impaired motor development in humans and altered lipid composition in the milk of mice dams. Furthermore, cannabinoids have been detected in the milk of users up to 6 weeks after the last reported use. Breast milk is crucial for the nourishment of infants and protection against disease while the newborn is developing a mature immune system. Breast milk is produced by the mammary gland (MG), which undergoes a crucial phase of remodelling during pregnancy. MG remodelling is accompanied by alveolar differentiation of mammary epithelial cells (MECs). Impaired MEC differentiation has been linked with abnormal MG development, which has been associated with reduced milk production and altered milk composition. The impact of cannabis on mammary gland development and breast milk composition has not been reported yet. Altered milk composition, low milk production or premature weaning of infants may have consequences on infant health and maternal well-being.

x. Rationale:

a. The MG of mice increases significantly in weight, DNA content, and LPL activity between late pregnancy and early lactation [177]. All three of these increases were inhibited by THC in mice, compared to control mice [177]. Thus, cannabis use in the perinatal period may directly impact MG remodeling.

b. MECs have been reported to express CB2 receptors [191]. CB2 regulates intracellular cAMP levels and increased cAMP levels have been associated with decreased STAT5 phosphorylation and β-casein synthesis. THC and CBD, being an agonist and inverse agonists at CB2, respectively, may alter CB2 signaling in MECs resulting in altered STAT5 phosphorylation and β-casein synthesis.

c. THC increased ER stress markers in human trophoblast cells; an effect that was blocked with CB1 and CB2 receptor antagonists [183]. CBD increased ROS production and induced ER stress in breast cancer cells [184]. Co-treatment of these cells with CBD and a TRPV1 antagonist resulted in increased cell viability and decreased ER stress [184]. THC has been reported to downregulate the AKT signaling pathway, resulting in reduced cellular differentiation, ER stress, and cellular apoptosis [181]. ER stress has been associated with disruption of lipid production and protein secretion in MECs, as well as a decrease in milk fat content, differences in milk composition, induction of permeable tight junctions between MECs, and impaired lactation performance in goats [72,73]**.** Activation of the PI3K-AKT pathway triggers autocrine-mediated PRL secretion, which indirectly activates the JAK2/STAT5 signaling pathway, which is required for MEC differentiation and milk production. Thus, cannabinoids, such as THC and CBD,

may induce ER stress in MECs via cannabinoid receptors, resulting in altered differentiation and function of MECs.

d. THC suppresses serum PRL levels in oophorectomized female and intact male rhesus monkeys [173], in ovariectomized rats[174], and mice at late pregnancy and early lactation [177]. Lower serum PRL levels are correlated with decreased milk volume (mL/day), lactose levels, and oligosaccharide levels in the milk [192]. Furthermore, cannabinoid exposure during lactation has been reported to decrease body weight of pups and alter the lipid composition in the milk of exposed mice dams [142]. Thus, maternal intake of cannabis may impact breast milk production and composition in humans.

xi. **Hypothesis:** Cannabis use will alter breast milk composition in humans, and the components of cannabis, namely THC and CBD, will impact MEC differentiation

xii. **Objectives:**

a. **Objective 1:** Investigate the impact of THC and CBD on the differentiation and function of HC11 cells

b. **Objective 2:** Determine the impact of THC and CBD, separately and in a 1:1 combination, on differentiation, function, ER stress and mitochondrial function of HC11 cells

c. **Objective 3:** Investigate the effect of cannabis use on breast milk composition in humans

CHAPTER 2: EFFECT OF DELTA-9-TETRAHYDROCANNABINOL AND CANNABIDIOL ON MILK PROTEINS AND LIPID LEVELS IN HC11 CELLS

Josan, C., Podinic, T., Pfaff, N., & Raha, S. (2021). Effect of Delta-9-tetrahydrocannabinol and cannabidiol on milk proteins and lipid levels in HC11 cells. *PLoS ONE*, *17*(8). https://doi.org/10.1371/journal.pone.0272819

HC11 Cells:

We utilized the HC11 cell line for conducting the *in vitro* work in this study. HC11 cells originate from midpregnant BALB/c mouse mammary gland (MG) tissue, and upon treatment with prolactin (PRL), dexamethasone (DEX) and insulin (INS), these cells adopt a secretory phenotype that resembles differentiated mammary epithelial cells (MECs) found in alveoli of a lactating MG. To respond efficiently to the lactogenic hormones, HC11 cells must be grown to confluency in the medium that contains epidermal growth factor (EGF) and INS, which promote cellular competency for differentiation. Differentiated HC11 cells produce lipid and milk proteins, including β-casein. This is consistent with the findings reported in the first two chapters of this study.

Full Title: Effect of Delta-9-tetrahydrocannabinol and cannabidiol on milk proteins and lipid levels in HC11 cells

Short Title: THC and CBD affect milk proteins and lipid levels in HC11 cells

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TP and NP contributed to conducting experiments and revising the manuscript

SR contributed to ideation of the study, study design, funding and revised the manuscript

Abstract

Pregnant and lactating women have been discouraged from using cannabis by Health Canada. However, the increasing rate of cannabis use among pregnant women has presented an urgent need to investigate its physiological effects during the perinatal period. During pregnancy, the mammary gland (MG) undergoes remodeling, which involves alveolar differentiation of mammary epithelial cells (MECs), which is essential for breast milk production and secretion. Limited evidence has been reported on the impact of cannabis or its components, delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD), on MG development or MEC differentiation. In this study, we investigated the effects of THC and CBD on the differentiation of MECs by assessing changes in cellular viability, lipid accumulation, and gene and protein expression of major milk protein and lipid synthesizing markers. using the HC11 cells as a model. We hypothesized that THC and CBD will negatively impact the synthesis of milk proteins and lipids, as well as lipid markers in HC11 cells. Our results demonstrated that THC and CBD reduced cellular viability at concentrations above 30μM and 20μM, respectively. Relative to control, 10μM THC and 10μM CBD reduced mRNA levels of milk proteins *(CSN2* and *WAP),* lipid synthesizing and glucose transport markers *(GLUT1, HK2, FASN, FABP4, PLIN2* and *LPL)*, as well as whey acidic protein and lipid levels. In addition, co-treatment of a CB2 antagonist with THC, and a CB2 agonist with CBD, reversed the impact of THC and CBD on the mRNA levels of key markers, respectively. In conclusion, 10μM THC and CBD altered the differentiation of HC11 cells, in part via the CB2 receptor.

Introduction

Cannabis is one of the most common substances used by women during pregnancy for alleviating nausea, vomiting and other symptoms of morning sickness [1,2]. The prevalence of cannabis use has been reported to be high among Canadians of reproductive age, with 44% of individuals aged 16 to 19, 52% aged 20 to 24, and 24% aged 25 years reporting use[3]. Cannabinoids have been reported to cross the placenta[4,5]. The usage of cannabis during pregnancy has been been associated with poor fetal growth [6,7], low birth weight[8–10], smaller head circumference[11], and increased risk of premature birth[9]. These associations have led the Society of Obstetricians and Gynaecologists of Canada to recommend that women should discontinue the use of cannabis during pregnancy[12]. Despite the recommendation, cannabis use by women during pregnancy has been reported to be between 2 and 11.3%[8,13–18], with prevalence among low-income and disadvantaged pregnant women ranging between 14 and 28%[19–21]. Taken together, the high rate of cannabis use among Canadians of reproductive age[3,22] and use during pregnancy has created an urgent need to understand its impact on female reproductive organs and infant health.

Cannabis contains over 400 chemical components, including the psychoactive component delta-9-tetrahydrocannabinol (THC) and the antiemetic component cannabidiol (CBD)[23]. THC and CBD, bind to and differentially activate cannabinoid receptor 1 (CB1) and CB2[24], which are key receptors of the endocannabinoid system (ECS). In addition to modulating pain and neurodevelopment, the ECS has been reported to impact implantation and placentation[25]. Dysregulation of ECS signaling by THC and CBD may impact critical processes, resulting in altered cell proliferation, differentiation and function[25,26]. THC treatment has been found to reduce proliferation and invasion of human extravillous trophoblast cells[27], which are outcomes

linked to poor placentation[28]. In trophoblasts cells, CBD treatment caused loss of cell viability, disruption in cell cycle progression and apotosis through a mitochondrial pathway[29]. Altogether, both THC and CBD have been demonstrated to impact cellular differentiation and function, however their impact on the differentiation of mammary epithelial cells (MECs) remains unclear.

Alveolar differentiation of MECs takes place within the mammary gland (MG) during pregnancy to prepare the gland for milk production[30]. Differentiated MECs are capable of synthesizing and secreting lactose[31], fat[32], and milk proteins, such as β-casein and whey acidic protein (WAP)[33–35]. Improper differentiation of secretory MECs is associated with fewer alveoli, decreased milk production, and altered milk composition[36]. Between late pregnancy and early lactation, the MG of control mice showed significant increase in weight, DNA content and levels of lipoprotein lipase (LPL)[37]. The increase in MG weight, DNA content and LPL activity were inhibited in THC-treated mice between late pregnancy and early lactation[37].

No study has evaluated the effect of THC or CBD on alveolar differentiation of MECs. We hypothesized that THC and CBD will impact MEC differentiation and milk protein expression. Furthermore, these effects may be mediated through CB2, as its presence has been previously reported in MECs[38]. Herein, we reported that 10μM THC and 10μM CBD reduced mRNA levels of milk proteins, lactose and lipid synthesizing enzymes, as well as WAP and lipid levels. Furthermore, our results suggested that the impact of THC and CBD on changes in mRNA levels of *CSN2, HK2* and *FABP4* may be mediated, in part, via CB2.

Materials and methods

Cell culture and differentiation

All work with cells was approved by the McMaster Biosafety committee under Bio Utilization protocol BUP023. As these cells were commercially purchased under a Materials Transfer

agreement between McMaster University and the American Type Culture Collection patient consent was not required. HC11 cells were obtained from American Type Culture Collection (CRL-3062) and cultured in RPMI 1640 (Corning, 10-041-CV), supplemented with 10% Fetal Bovine Serum (FBS) (Hyclone, SH30396.03), 1% L-glutamine (Gibco, 25030081) and 1% penicillin-streptomycin (Gibco, 15140122), which formed the base RPMI media. Cells were routinely incubated at 5% CO_2 and 37°C. For experiments, HC11 cells were seeded at 100,000 cells/cm^2 in base RPMI media for 24 hours, then supplemented with 10ng/mL epidermal growth factor (EGF) (Thermo Scientific, PMG8041) and 5µg/mL insulin (INS) (Sigma-Aldrich, I0516), for 3 days. The cells were then differentiated with base RPMI media, supplemented with 5µg/mL PRL (PepProTech, 315-16), 1µM dexamethasone (DEX) (Sigma-Aldrich, D4902) and 5µg/mL INS over 4 days. Media was changed every 48 hours, supplemented with appropriate factors at each stage.

Treatment with THC (Sigma-Aldrich, T4764) or CBD (Sigma-Aldrich, C6395) was carried out by supplementing HC11 cells at differentiation with the appropriate concentration of each drug or 0.1% methanol (MeOH) as the vehicle control. For experiments that involved the administration of CB2 agonist, JWH133 (Cayman Chemical, 10005428) or CB2 antagonist, AM630 (Sigma-Aldrich, SML0327), HC11 cells were pre-treated with the agonist/antagonist at differentiation for half an hour followed by treatment with appropriate differentiation factors and drugs in the RPMI base media. Both AM630 and JWH133 were solubilized in dimethyl sulfoxide (DMSO). Therefore, cells were separately treated with 0.1% DMSO at differentiation in the RPMI base media as vehicle control. All experiments were conducted with four to six distinct replicates, which were performed at a different point in time.

Cytotoxicity and cell proliferation assays

Changes in cellular viability were investigated 4 days post-differentiation, using the CyQUANT lactate dehydrogenase (LDH) Cytotoxicity Assay (LDH assay) (Thermo Fisher, C20300). Furthermore, changes in cellular proliferation were investigated 4 days post-differentiation, using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS assay) (Promega, G5421). Both assays were conducted according to the manufacturer's instructions.

Oil Red O assay

Lipid levels in differentiated HC11 cells were visualized and quantified using an Oil Red O (ORO) assay, a method that has been well characterized for staining and quantifying lipids in fat cells[39]. HC11 cells were washed with Dulbecco's Phosphate-Buffered Saline (DPBS) (Corning, 20-030) and then fixed with 4% paraformaldehyde (Sigma-Aldrich**,** P6148-500g) for 45 minutes. Once fixed, the cells were incubated in 60% isopropanol (Sigma-Aldrich, 67-63-0) for 5 minutes and stained with the ORO solution (Sigma-Aldrich, O1391) for 20 minutes. The dye was extracted using 100% butanol (Sigma-Aldrich, B7906) and quantified using a spectrophotometer (Multiskan Spectrum, Thermo Electron Corporation) at 510 nm.

RNA harvest, cDNA preparation, and RT-qPCR

Harvested cells were resuspended in Trizol (Thermo Fisher Scientific, 15596026), which was extracted with chloroform and eluted through spin columns using the Direct-zol RNA MiniPrep Kit (Cedarlane Labs, R2050). Complementary DNA (cDNA) was prepared using 1μg of total RNA, using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368813). Real time quantitative polymerase chain reaction (RT-qPCR) was performed at the following conditions: polymerase activation (95^{o}C for 10 minutes), 39 cycles of denaturing (95^{o}C

for 10 seconds), denaturing (60ºC for 10 seconds), elongation (72ºC for 15 seconds) (Bio-Rad C1000 Touch Thermal Cycler, CFX384 Real-time System). Relative gene expression changes were calculated using the ΔΔCT method, referenced to Ribosomal Protein L0 *(RPL0)* and Ribosomal Protein L8 *(RPL8)* as housekeeping genes. Primer sequences of all the genes investigated in this study, including the housekeeping genes, have been outlined in Table 1.

Primer Name	Forward Sequence	Reverse Sequence
B4GALT1	TCCCTTTACTCTTGGCAGATTAG	TCTTGTCTCTTGAATGCCGGA
CNR2	TGATTGGTGTCAGCTCTCAGT	TTGGTCACTTCTGTCTCCCG
CSN2	CCTTCATGGCTGTCAAGTCCT	GTTGTGAGCTGAAGACCACG
FAAH	GCTGTGCTCTTTACCTACCTG	GAAGCATTCCTTGAGGCTCAC
FABP4	ATTTCCTTCAAACTGGGCGTG	CTTTCCATCCCACTTCTGCAC
FASN	CACGAGTGAGTGTACGGGAG	GATCGGAGCATCTCTGGTGG
GLUT1	TACACCCCAGAACCAATGGC	CCCGTAGCTCAGATCGTCAC
HK2	CATCACGCCAGTGACTCTGATA	AGGTACCCTAAGTCTCACTCCT
LALBA	AGCTGGGTCATGTGTGGAAT	GTAATGCAGACACCGCTCAC
LPL	ATGGCAAGCAACACAACCAG	AGCAGTTCTCCGATGTCCAC
NAPEPLD	TTCTTTGCTGGGGATACTGG	GCAAGGTCAAAAGGACCAAA
PLIN2	TAAACGTCTGTCTGGACCGAAT	AAGGAAAAACCTCACCTCAAGC
RPL0	CCAGCAGGTGTTTGACAACG	TCCAGAAAGCGAGAGTGCAG
RPL8	ACGTGAAGACC GTAAGGG	GATGCCTTTAATGTAGCCGTGT
WAP	CAGCCCAAGCCTATACAGCA	TTGCCACCCTGGAGATCCTA

Table 1. Primer sequences of all the genes investigated in this study. *B4GALT1*: β-1,4-galactosyl transferase; *CNR2:* cannabinoid receptor 2; *CSN2:* β-casein; *FAAH:* fatty acid amide hydrolase; *FABP4:* fatty acid binding protein 4; *FASN:* fatty acid synthase; *GLUT1:* glucose transporter 1; *HK2:* hexokinase 2; *LALBA:* α-lactalbumin; *LPL:* lipoprotein lipase; *NAPEPLD:* N-acyl phosphatidylethanolamine-specific phospholipase D; *PLIN2:* perilipin 2; *RPL0:* ribosomal protein 0; *RPL8:* ribosomal protein 8; *WAP:* whey acidic protein.

Western blotting

Cells were washed with cold DPBS, scraped off in cold Radioimmunoprecipitation assay (RIPA) buffer, and sonicated for 10-15 seconds. Protein concentrations were measured by using

the Pierce BCA Protein Assay (Thermo Fisher Scientific, 23227)[40]. 15µg of protein was separated by SDS-PAGE on 10-well, 12% Mini-Protean TGX Stain-free protein gels (Bio-Rad, 4568043), at constant current (0.03 Amps). 10µL of Precision Plus Protein™ All Blue Prestained Protein Standard (Bio-Rad, 1610373) was loaded onto to the gel. Gels were transferred to a Trans-blot turbo Mini 0.2µm polyvinylidene difluoride (PVDF) membrane (Bio-Rad, 1704156), using the transblot apparatus (Bio-Rad), at the following conditions: 21 volts, 1.0 amps, 7 minutes. PVDF membranes were blocked with 5% bovine serum albumin (BSA) (Sigma-Aldrich, A-6003) for 2 hours at room temperature on a shaker, followed by incubation in primary antibody dissolved in 5% BSA, overnight at 4°C with gentle rocking. Following the overnight incubation in the primary antibody, blots were incubated in the appropriate secondary antibody, dissolved in 1.5% BSA, for 2 hours at room temperature. For protein detection, the PVDF membranes were incubated in Clarity Max Western Enhanced Chemiluminescence (ECL) Substrate (Bio-Rad, 1705062) for 3 minutes, and imaged on the ChemiDoc™ Imaging System (Bio-Rad). The full list of concentrations and manufacturers of the primary and secondary antibodies have been outlined in Table 2.

Antibody Name	Company Name	Catalog Number	Working Dilution
Anti-β-casein mAb	Santa Cruz Biotechnology	sc-166684	1:1000
Anti-WAP mAb	Santa Cruz Biotechnology	sc-374648	1:200
Anti-CB2 pAb	Cayman Chemical Company	101550	1:200
β-Actin Rabbit mAb	Cell Signaling Technology	4970S	1:10,000
Goat Anti-Mouse IgG	Miltenyi Biotec, Inc.	130-048-401	1:5000
Donkey Anti-rabbit IgG	Cytiva	NA9340-1ML	1:5000

Table 2. List of primary and secondary antibodies used in this study. The list includes name of the antibody and its manufacturer, catalogue number and concentrations used in this study. CB2: cannabinoid receptor 2; WAP: whey acidic protein; mAb: monoclonal antibody; pAb: polyclonal antibody.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism software (Prism 9 for macOS, Version 9.1.2 [225]). Data points were expressed as mean ± SEM, and analysed using either a t-test (for <3 test groups), or one-way analysis of variance (ANOVA) (for >3 test groups) with Tukey's post-test. Results represent data collected from experiments conducted 4 to 6 times. Data points with $p \leq 0.05$ will be considered significant and statistical changes were denoted by differing letters on data figures. Different letters (a, b, etc.) indicate a statistically significant difference between two data points. A mix of letters indicate a statistically significant difference between two data points that have distinct set of letters (i.e., ab and cd are statistically different). Meanwhile a mix of letters that have at least one same letter in the combination indicate no significant difference between data points (i.e., ab and b).

Results

THC and CBD alter markers of HC11 cell differentiation

Prior to treating cells with THC or CBD, we characterized HC11 differentiation by monitoring changes in gene and protein expression changes of key markers of differentiation, as well as lipid levels. Relative to undifferentiated HC11 cells, differentiated cells displayed significantly higher mRNA levels of *CSN2* and *WAP* (Fig 1A-B), and β-casein expression (Fig 1C). HC11 cells differentiated in the presence 0.1% MeOH (vehicle control to THC and CBD) did not display any differences in mRNA levels of milk proteins and β-casein expression, relative to HC11 cells differentiated without MeOH (Fig 1A-C). EGF and INS, alone, did not result in HC11 cell differentiation, and cells treated with these compounds displayed similar gene and protein levels of milk proteins as undifferentiated cells (Fig 1A-C). Furthermore, relative to

undifferentiated HC11 cells (UD) (Fig 1D), differentiated cells (DF) (Fig 1E) displayed significantly higher lipid accumulation (p-value <0.0001) (Fig 1F).

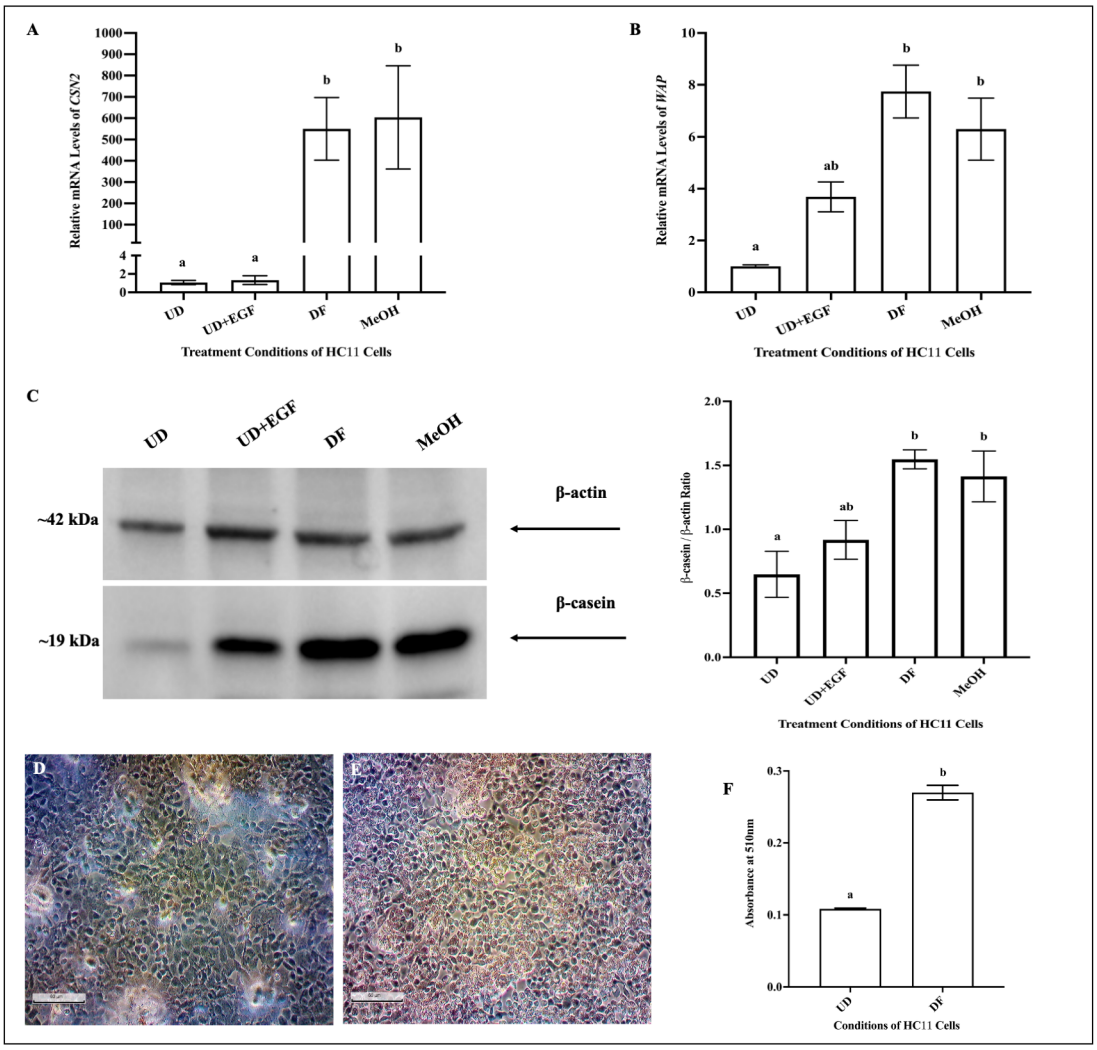

Fig 1. Differentiated HC11 cells displayed increased milk proteins and lipid levels. HC11 cells were seeded in base media for 24 hours (UD). HC11 cells were seeded and supplemented with EGF and INS for 3 days (UD+EGF). Finally, HC11 cells were differentiated in base media containing PRL, INS and DEX, and allowed to proceed for 4 days in the absence (DF) or presence of the vehicle (MeOH). Cells from all conditions were processed for total RNA or protein isolation, as described in methods. The relative (compared to *RPL0 and RPL8*) mRNA levels of *CSN2* (A)

and *WAP* (B) were determined. Gene expression analysis was repeated with 6 distinct replicates. Protein expression of β-casein was assessed, as described in methods, using 15μg of total protein for each sample. (C) A representative blot of β-casein expression is shown along with a bar graph quantifying its expression, normalized to β-actin, for undifferentiated (UD), EGF supplemented (UD+EGF), differentiated (DF) and differentiated in the presence of vehicle (MeOH), based on 4 replicates. Lipid levels were assessed in undifferentiated cells (UD) and differentiated cells (DF), by conducting an Oil Red O (ORO) assay, as described in methods. Representative images of ORO-stained undifferentiated HC11 cells (UD) and differentiated cells (DF), taken with a phase-contrast microscope (20x magnification). (F) Lipid levels were quantified in cells, as described in the methods, from 6 distinct replicates of undifferentiated HC11 cells (UD) and differentiated cells (DF). Results were plotted as mean ± SEM and compared using either a student's t-test (for groups ≤ 2), or one-way ANOVA (for groups ≥ 3). Statistically significant changes were represented by distinct letters on bar graphs.

Relative to undifferentiated HC11 cells (UD), differentiated cells (DF) demonstrated increased mRNA levels of genes that encode proteins and enzymes responsible for fat synthesis: fatty acid synthase *(FASN)*[41], fat transport: fatty acid binding protein 4 *(FABP4),* coating lipids: perilipin 2 *(PLIN2)*[42], triglyceride lysis: lipoprotein lipase *(LPL)*[37], and markers that aid in supplying glucose for lactose and lipid synthesis: glucose transporter 1 *(GLUT1)* and hexokinase 2 *(HK2)*[43] (Fig 2A-F). As for changes in expression of genes that encode major ECS markers, differentiated cells (DF) displayed increased levels of *CNR2* (the gene that encodes CB2) and *N*-acyl phosphatidylethanolamine-phospholipase D *(NAPEPLD)*, no change in fatty acid amide hydrolase (*FAAH)* levels, relative to undifferentiated cells (UD) (Fig 2G-I).

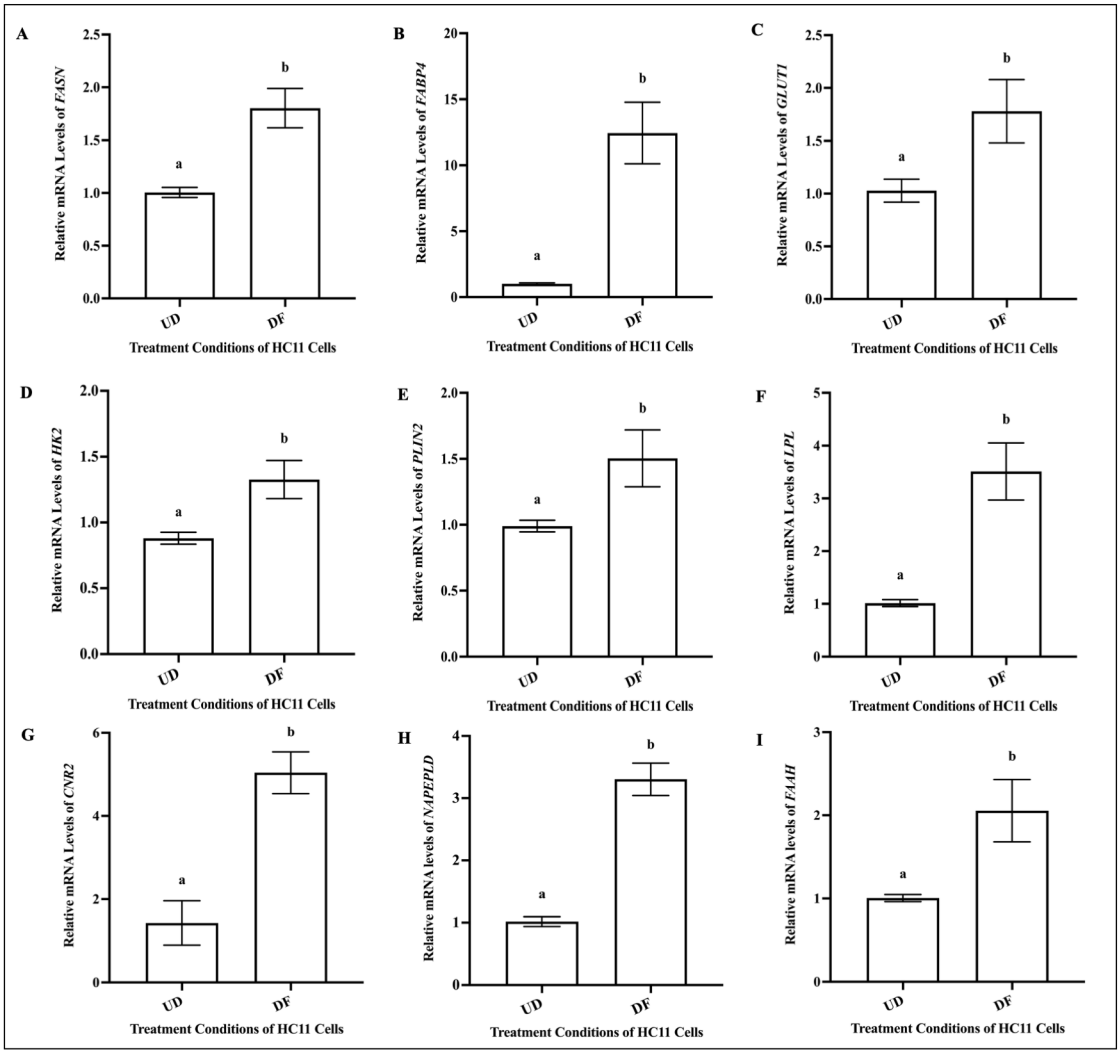

Fig 2. Differentiation increases the expression of mRNA for markers of lipid synthesis and alters mRNA levels of key ECS markers in HC11 cells. HC11 cells were seeded in base media for 24 hours (UD) and collected for RNA isolation. HC11 cells were seeded in base media for 24 hours, supplemented with EGF and INS for 3 days, and differentiated in base media containing PRL, INS and DEX 4 days (DF). Undifferentiated and differentiated cells were processed for total RNA, as described in methods. The relative mRNA levels of *FASN* (A), *FABP4* (B), *GLUT1* (C), *HK2* (D), *PLIN2* (E), *LPL* (F), *CNR2* (G), *NAPE-PLD* (H*)* and *FAAH* (I) were determined. Gene expression analysis was repeated with 6 distinct replicates. Results were represented as mean ±

SEM and compared using a t-test. Statistically significant changes were represented by distinct letters on the bar graphs.

High concentrations of THC and CBD reduce HC11 cell proliferation and viability

The impact of various concentrations of THC and CBD (0.1, 0.1, 1, 10, 20, 30 and 100 μM), independently, was investigated on cytotoxicity and proliferation of HC11 cells during differentiation (Fig 3). Relative to vehicle control (MeOH), 30μM and 100μM THC (Fig 3A), as well as well as 20μM, 30μM and 100μM CBD (Fig 3B), significantly reduced HC11 cell proliferation. Following the MTS assay, absorbance values were recorded, normalized to the vehicle control, and represented as a percentage in the bar graphs (Fig 3A-B). In addition, relative to vehicle control (MeOH), 30μM and 100μM THC (Fig 3C), as well as well as 20μM, 30μM and 100μM CBD (Fig 3D), significantly induced cellular toxicity in HC11 cells. Cellular toxicity was measured using a LDH assay and calculated according to the manufacturer's directions. Based on these results, all following experiments were conducted using 10μM THC and 10μM CBD.

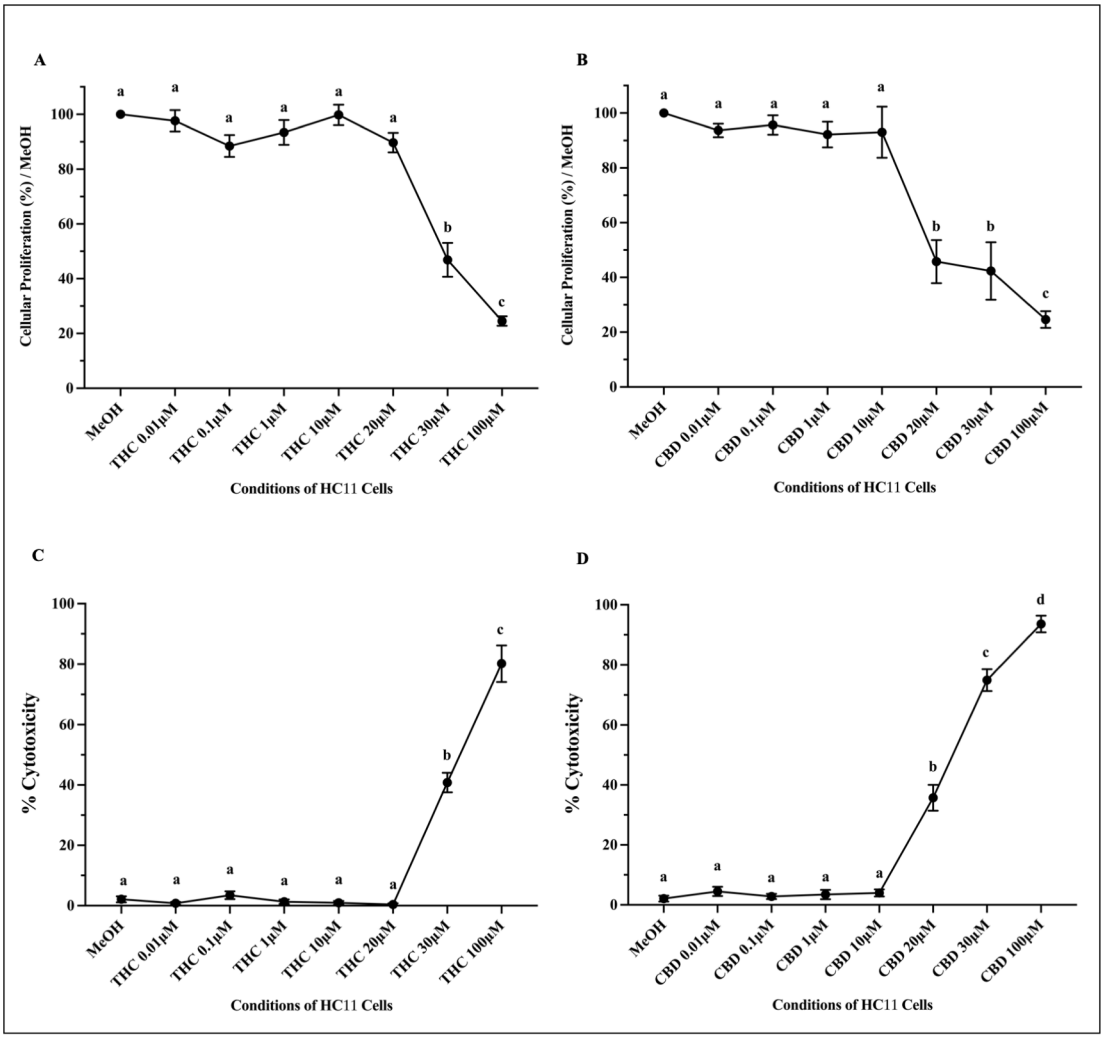

Fig 3. High concentrations of THC and CBD reduced proliferation and viability of HC11 cells. HC11 cells were seeded in base media for 24 hours, supplemented with EGF and INS for 3 days, and differentiated in base media containing PRL, INS and DEX, as described in methods, and treated with vehicle control (MeOH), or 0.01, 0.1, 1, 10, 20, 30, 100µM THC (A and C) or CBD (B and D) for 4 days. Changes in cellular proliferation and toxicity were evaluated using a MTS assay and LDH assay, respectively, as described in methods. (A-B) Changes in absorbances were recorded following a MTS assay and results were normalized to vehicle control (MeOH) as a percentage. (C-D) The relative cytotoxicity of THC and CBD was assessed using a LDH assay

and percent cytotoxicity (compared to the vehicle control) was plotted. All experiments were repeated with 6 distinct replicates. Results were represented as mean ± SEM and compared using one-way ANOVA. Statistically significant changes were indicated by distinct letters.

THC and CBD exhibit a differential effect on β-casein protein levels

Relative to vehicle control, 10μM THC and 10μM CBD significantly downregulated the mRNA levels of *CSN2* and *WAP* (Fig 4A-B). The mRNA levels of *CSN2* and *WAP* in cells treated with 10μM THC and CBD were similar to levels seen in undifferentiated cells (UD) (Fig 4A-B). To validate the changes observed in the mRNA encoding milk proteins upon THC and CBD treatment, protein expression of β-casein and WAP was quantified in HC11 cells and normalized to β-actin. Relative to vehicle control, THC-treated cells did not display any changes in β-casein expression (Fig 4C) but displayed significantly lower WAP expression (Fig 4D). HC11 cells treated with CBD displayed decreased expression of both β-casein (Fig 4C) and WAP (Fig 4D), relative to vehicle control cells. Compared to THC, CBD-treated cells demonstrated a significant decrease in β-casein expression (Fig 4C), but no change in WAP expression (Fig 4D).

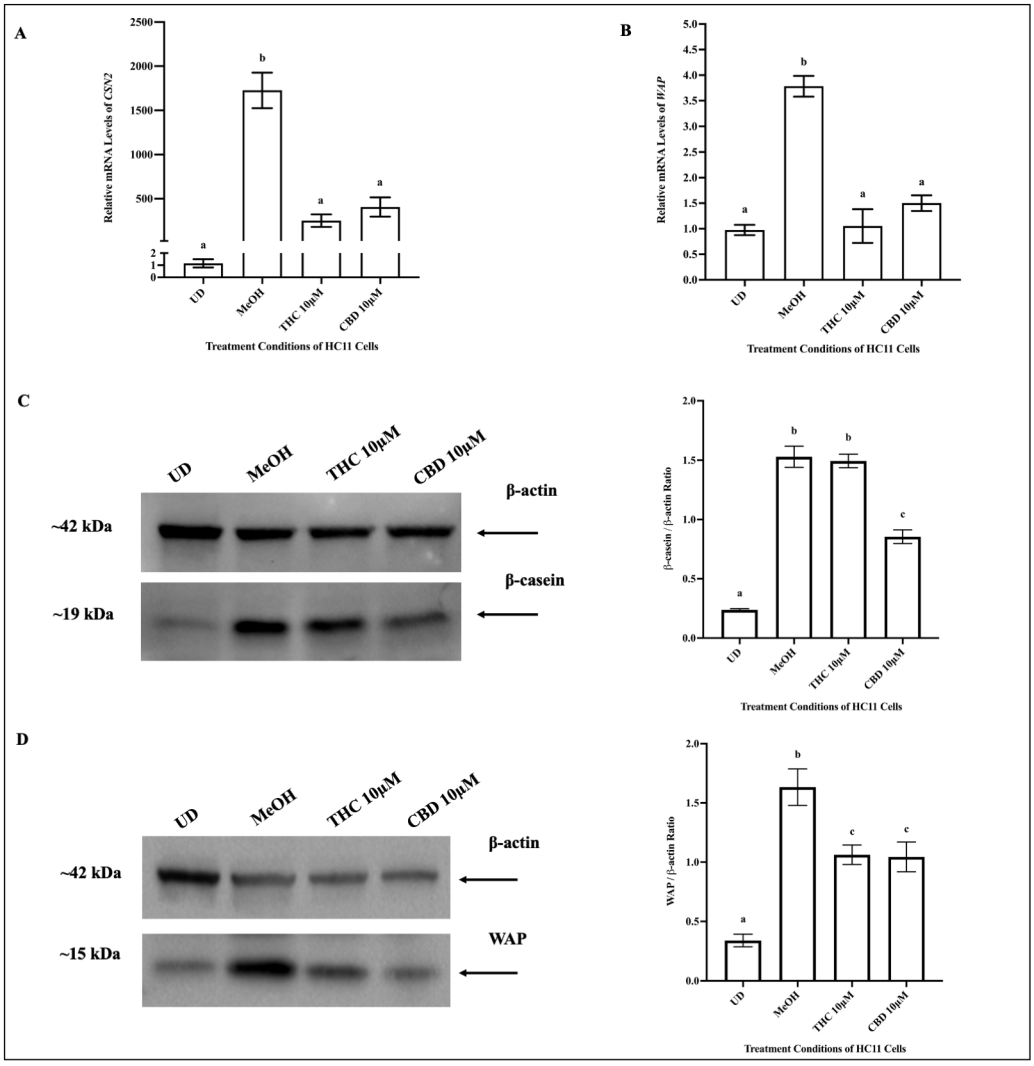

Fig 4. 10μM THC and 10μM CBD reduced levels of milk proteins in HC11 cells. HC11 cells were seeded in base media for 24 hours (UD) and processed for total RNA or protein, as described in the methods. HC11 cells were differentiated in base media containing PRL, INS and DEX, as described in methods, and treated with vehicle control (MeOH), 10μM THC or 10μM CBD for 4 days, and processed for total RNA or protein. The relative mRNA levels of *CSN2* (A) and *WAP* (B) were determined. Gene expression analysis was repeated with 6 distinct replicates. Milk protein expression was assessed, as described in the methods, using 15μg of total protein for each sample. Representative blots of β-casein (C) and WAP (B) are shown along with a bar graph

quantifying the protein expression, normalized to β-actin, for undifferentiated cells (UD), vehicle control (MeOH), 10μM THC and 10μM CBD treated HC11 cells, based on 4 replicates. Results were plotted as mean ± SEM and compared using one-way ANOVA. Statistically significant changes were represented by distinct letters.

THC and CBD alter the gene expression of lactose and lipid synthesizing enzymes, and reduce lipid levels in HC11 cells

Relative to vehicle control, 10μM THC and 10μM CBD significantly decreased mRNA levels of *FASN, FABP4, GLUT1, HK2, PLIN2* and *LPL* (Fig 5 A-F). THC decreased the mRNA levels of *FABP4* and *HK2* to a greater extent than CBD (Fig 5B, Fig 5D). In contrast, CBD downregulated *FASN* gene expression more profoundly than THC (Fig 5A). Relative to undifferentiated HC11 cells (Fig 5G) and differentiated vehicle control cells (Fig 5H), the cells treated with 10μM THC (Fig 5I) and 10μM CBD (Fig 5J) demonstrated significantly lower lipid levels after 4 days of differentiation (Fig 5K).

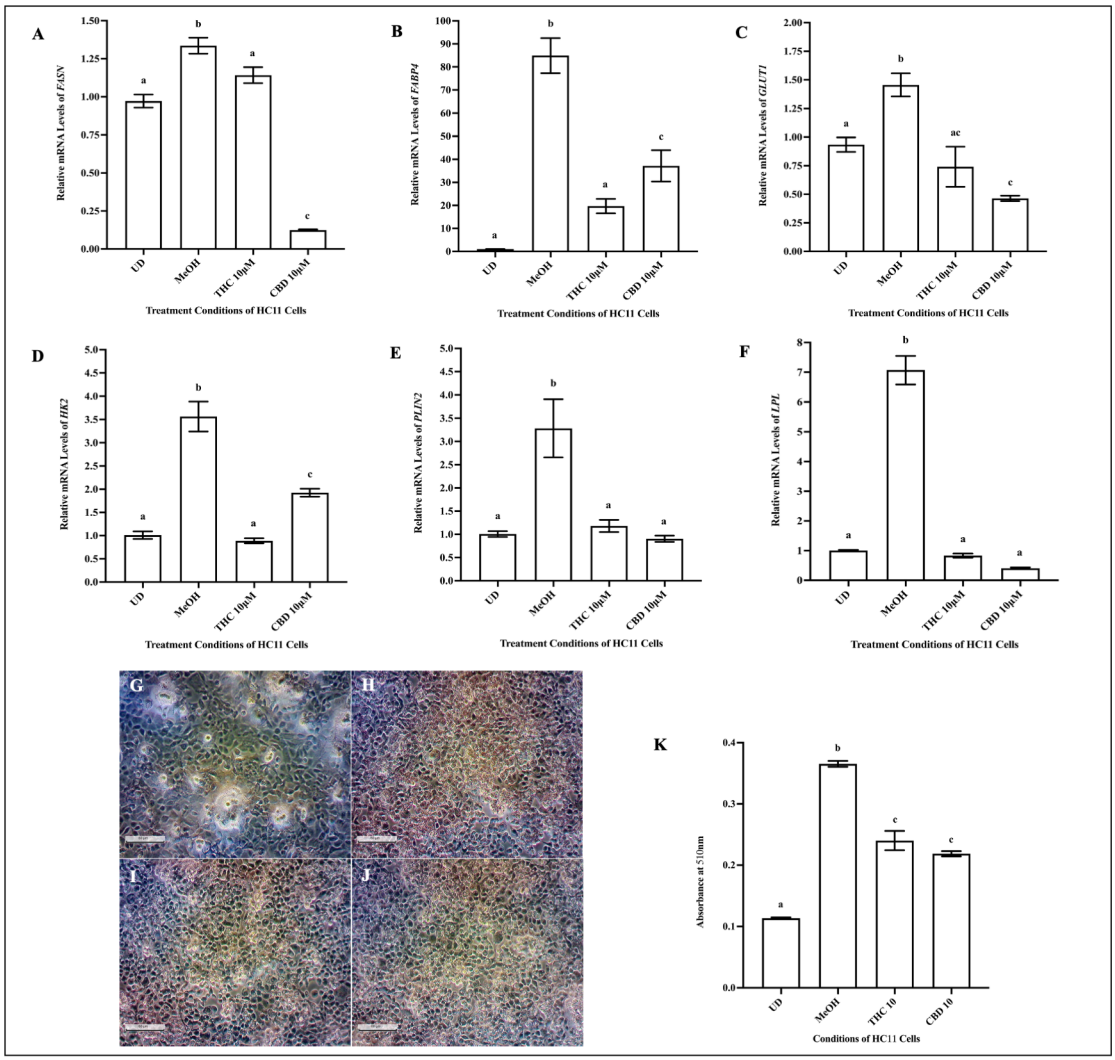

Fig 5. 10µM THC and 10µM CBD reduced lipid levels and mRNA levels of associated markers in HC11 cells. HC11 cells were seeded in base media for 24 hours (UD) and processed for total RNA isolation, as described in the methods. HC11 cells were differentiated in base media containing PRL, INS and DEX, as described in methods, and treated with vehicle control (MeOH), 10µM THC or 10µM CBD for 4 days, and processed for total RNA isolation. The relative mRNA levels of *FASN* (A), *FABP4* (B), *GLUT1* (C), *HK2* (D), *PLIN2* (E) and *LPL* (F) were determined. Gene expression analysis was repeated with 6 distinct replicates. Lipid levels in cells were quantified by conducting an Oil Red O (ORO) assay, as described in methods. Representative

images of ORO-stained undifferentiated (UD) cells (G), and cells differentiated with vehicle control (MeOH) (H), 10μM THC (I), and 10μM CBD (J), were taken using a phase-contrast microscope (20x magnification). (K) Lipid levels were quantified in undifferentiated (UD) cells, and cells treated with vehicle control (MeOH), 10μM THC or 10μM CBD, from 6 distinct replicates. Results were plotted as mean ± SEM and compared using one-way ANOVA. Statistically significant changes were represented by distinct letters on bar graphs.

THC and CBD treatment results in altered expression of CB2 and endocannabinoid metabolizing mRNA markers in HC11 cells

Relative to vehicle control, 10μM THC significantly decreased mRNA levels of *CNR2* (Fig 6A), did not change *NAPEPLD* mRNA levels (Fig 6B), and significantly increased *FAAH* mRNA levels in HC11 cells (Fig 6C) in HC11 cells. In contrast, 10μM CBD significantly downregulated the gene expression of *CNR2* and *NAPEPLD* (Fig 6A-B) and upregulated the gene expression of *FAAH* (Fig 6C), compared to vehicle control. Changes in the mRNA levels of *CNR2* were further validated by conducting protein expression analysis. Relative to undifferentiated cells (UD), cells differentiated in the presence of the vehicle control (MeOH) demonstrated significant upregulation in CB2 expression (Fig 6D). Compared to vehicle control (MeOH), THC treatment did not alter CB2 expression, while CBD significantly downregulated the levels of CB2 in HC11 cells (Fig 6D).

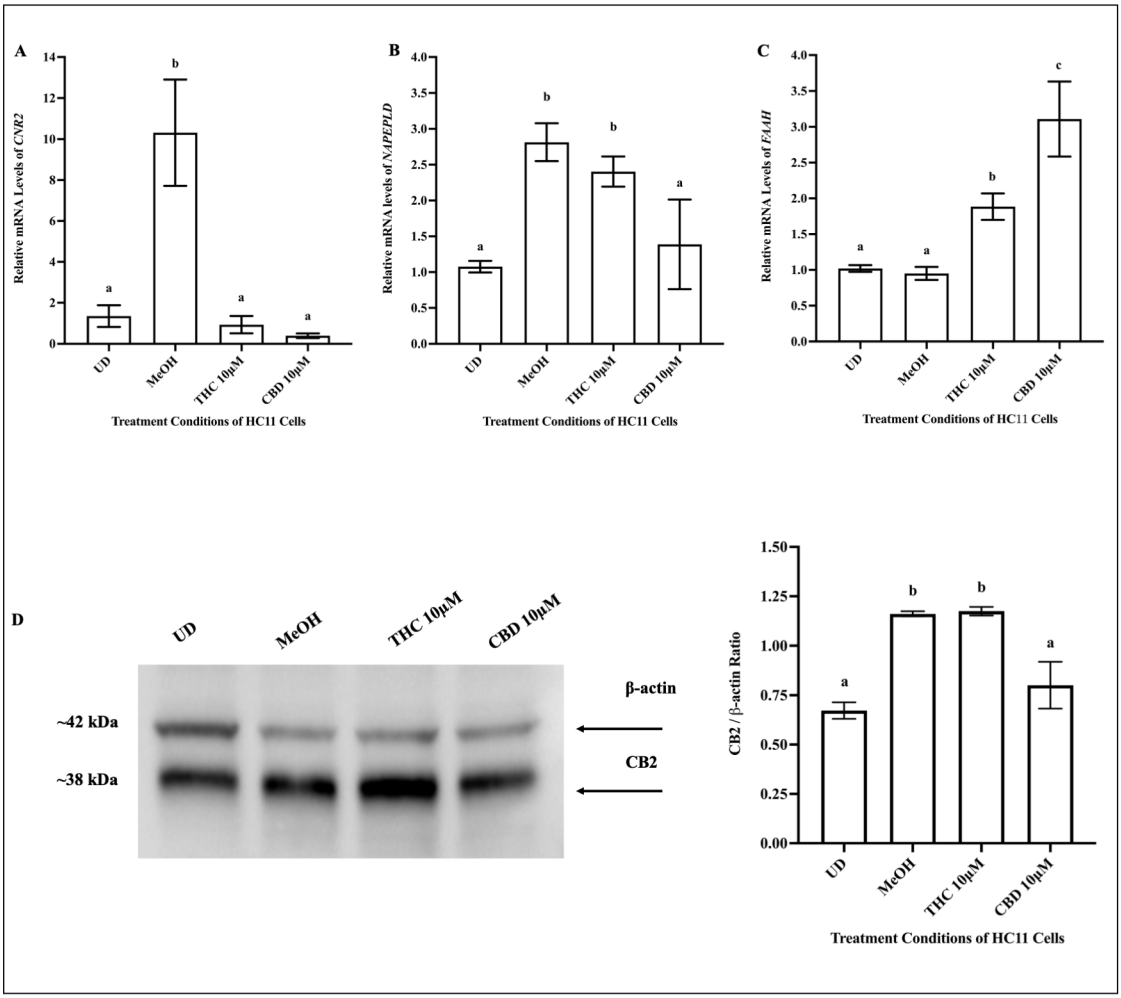

Fig 6. 10μM THC and 10μM CBD altered mRNA levels of ECS markers in HC11 cells. HC11 cells were seeded in base media for 24 hours (UD) and processed for total RNA or protein isolation, as described in the methods. HC11 cells were differentiated in base media containing PRL, INS and DEX, and treated with vehicle control (MeOH), 10μM THC or 10μM CBD for 4 days. Treated cells were processed for total RNA or protein isolation, as described in methods. The relative mRNA levels of *CNR2* (A), *NAPEPLD* (B) and *FAAH* (C) were determined. Gene expression analysis was repeated with 6 distinct replicates. Milk protein expression was assessed, as described in methods, using 15μg of total protein for each sample. (D) A representative blot

of CB2 expression is shown along with a bar graph quantifying its expression, normalized to β-actin, for undifferentiated (UD), and differentiated cells in the presence of vehicle (MeOH), 10μM THC, or 10μM CBD, based on 4 replicates. Results were plotted as mean ± SEM and compared using one-way ANOVA. Statistically significant changes were represented by distinct letters on bar graphs.

CB2 receptor contributes to effects of THC and CBD on milk protein and lipid synthesis

Next, we assessed whether co-treatment of HC11 cells with THC AM630, a synthetic antagonist of CB2[44], would reverse the impact of THC on mRNA levels of *CSN2, HK2* and *FABP4* in HC11 cells (Fig 7A-C). In addition, we investigated whether co-treatment of HC11 cells with CBD and JWH133, a potent selective CB2 agonist[45], will reverse the impact of CBD on mRNA levels of *CSN2, HK2* and *FABP4* (Fig 7D-F). As DMSO is the vehicle for AM630 and JWH133, a set of HC11 cells were differentiated with 0.1% DMSO, as an additional vehicle control. Relative to undifferentiated cells (UD), MeOH- and DMSO-treated differentiated controls displayed significantly increased *CSN2, HK2* and *FABP4* mRNA levels (Fig 7). While THC reduced the mRNA levels of *CSN2, HK2* and *FABP4* relative to differentiated controls (MeOH, DMSO), AM630 co-treated with THC reversed the effects of THC to levels comparable to those seen in vehicle treated cells (MeOH, DMSO) (Fig 7A-C). Similarly, CBD downregulated the gene expression of *CSN2, HK2* and *FABP4,* relative to differentiated control cells (MeOH, DMSO). Co-treatment of HC11 cells with JWH133 and CBD reversed the effects of CBD, on all tested genes, to levels comparable to those seen in vehicle treated cells (MeOH, DMSO) (Fig 7D-F).

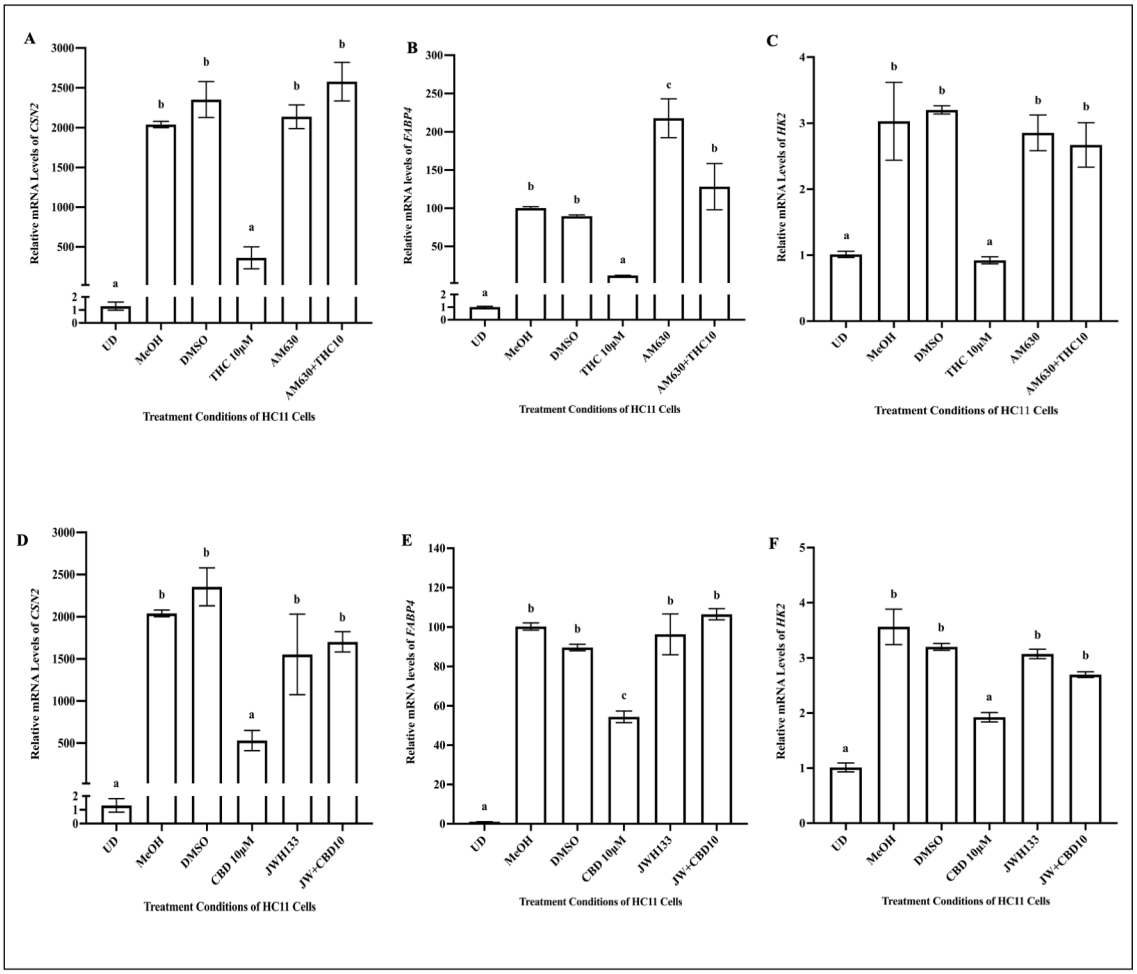

Fig 7. AM630 and JWH133 rescued the effect of THC and CBD, respectively, in HC11 cells. HC11 cells were seeded in base media for 24 hours (UD) and processed for total RNA isolation, as described in the methods. HC11 cells were differentiated in base media containing PRL, INS and DEX, as described in methods, and treated with vehicle controls (MeOH, DMSO), 10μM THC, 1μM AM630, and 1μM AM630 + 10μM THC for 4 days, during cellular differentiation and processed for total RNA isolation. The relative mRNA levels of *CSN2* (A), *HK2* (B) and *FABP4* (C) were determined. Next, the HC11 cells were differentiated in base media containing PRL, INS and DEX, and treated with vehicle controls (MeOH, DMSO), 10μM CBD, 10μM JWH133, and

10μM JWH133 + 10μM CBD for 4 days, and processed for total RNA isolation. The relative mRNA levels of *CSN2* (D), *HK*2 (E) and *FABP4* (F) were quantified. Gene expression analysis was repeated with 6 distinct replicates, as described in methods. Results were represented as mean ± SEM, and compared using one-way ANOVA. Statistically significant changes were indicated by distinct letters on the bar graphs.

Discussion

While the concentrations of THC and CBD used in this study are higher than those reported in human plasma[46,47] and maternal hair[48], they remain on the lower end of concentrations used *in-vitro*. However, the average THC content in cannabis has increased from 4% to 12% over the past two decades[49]. In fact, levels as high as 30% of THC have been reported in legal cannabis grown for recreational use[50]. In rats, daily administration of THC resulted in ten times greater concentrations of THC in fat, compared to any other tissues investigated, and the drug persisted in fat for 2 weeks[51]. Furthermore, Rawitch et al demonstrated that while THC was detected at 200 ng/g, 100 ng/g, and 30 ng/g in the liver, lung and brain of mice, respectively, the levels of THC in the gonadal fat organ were detected between 2000 and 3000 ng/g of tissue[52]. As MECs synthesize and store lipid[53], these cells may sequester, and be exposed to, higher concentrations of cannabinoids than those reported in plasma. *In vitro* studies have used concentrations between 10μM and 40μM to explore the impact of THC[54–56] and CBD[29,57] on proliferation and differentiation in various tissue types. In our study, the HC11 cells were treated with 10μM THC and 10μM CBD.

HC11 cells originate from midpregnant BALB/c mouse MG tissue, and upon treatment with PRL, DEX and INS, these cells adopt a secretory phenotype that resembles differentiated

MECs found in alveoli of a lactating MG [35,58,59]. 10μM THC and CBD reduced *CSN2* mRNA levels, relative to vehicle control treated HC11 cells. *CSN2* encodes for β-casein, which is an extremely important milk protein for infant health due to its ability to aid in digestion and absorption of macronutrients and micronutrients[60]. β-casein is digested to phosphopeptides, which have antithrombotic, antihypertensive, and opioid activities that contribute to maintaining proper sleep behaviour and decreasing risk of hypertension in infants[61]. Decreased *CSN2* levels in mammary tissue of mice have been associated with morphological changes in the MG during peak lactation[62]. Distorted MG architecture and less dense alveoli have been accompanied with decreased β-casein and WAP levels in the milk of mice[63]. Decreased expression of *CSN2* in MECs has been demonstrated to be consistent with altered differentiation of MECs[64].

WAP is the principal whey protein in rodent milk, its mRNA levels in mice have been reported to increase by 30 to 50-fold over gestation[65], and its presence in MECs is indicative of terminal differentiation[59]. Overexpression of WAP transgene impaired lobulo-alveolar development in the MG of mice[66], and its forced expression in HC11 cells inhibited MEC proliferation[67]. WAP has been reported to regulate short- and long-term food intake by inducing satiety[68], have potent antimicrobial and protease inhibitory activity[69], and is being investigated for its key roles in immunity[70]. In this study, reduction in gene and protein expression of WAP suggests that THC and CBD may negatively impact terminal differentiation of MECs, and potentially result in lower WAP levels in the milk of those exposed to THC or CBD.

The decrease in mRNA levels of markers involved in lipid and glucose, caused by THC and CBD, was accompanied by reduced lipid levels in HC11 cells treated with 10μM THC and 10μM CBD. In milk, lipids are the primary triglycerides that contribute a sizable percentage of calories, essential fatty acids, and bioactive components, which are required for neonatal growth

and development[71]. Decreased expression of markers responsible for glucose uptake *(GLUT1),* fat transport *(FABP4)* and fatty acid synthesis *(FASN)*, accompanied with reduced lipid levels may lead to lower lipid synthesis and secretion by MECs. Altogether, our results demonstrate that 10μM THC and 10μM CBD impact HC11 cell differentiation, determined by reduced gene expression of milk proteins and lipid synthesizing markers, decreased milk protein (WAP) and lipid levels. However, the effect of cannabis or its metabolites on mammary gland remodeling during pregnancy and on breast milk composition needs to be confirmed by *in vivo* work using animal models and clinical studies in women who use cannabis during the entire perinatal period vs just during lactation.

Exogenous cannabinoids, such as THC and CBD, impact the growth and function of cells through various mechanisms, including via cannabinoid receptors (CBs). Previous studies exploring CB distribution in peripheral cells have reported the presence of CB2 in MECs[38,72]. CB2 is a G protein-coupled receptor (GPCR), and its activation or inhibition results in alterations to cellular physiology, including changes in synaptic function, proliferation, and differentiation[73]. Relative to undifferentiated cells, differentiated HC11 cells displayed increased *CNR2* and *NAPEPLD* levels (the gene that encodes the enzyme that synthesizes AEA). AEA is a weak agonist of CB2 and an important intermediate in lipid metabolism[25]. Therefore, upregulation of *CNR2* and *NAPEPLD* levels may be required in alveolar differentiation for AEA uptake in MECs.

Similar to AEA, THC and CBD are stored in fat depots and activate CB receptors, but neither THC nor CBD are vulnerable to degradation by FAAH[25]. THC has been reported to be a partial agonist of CB2 and is known to compete with endogenous ligands, such as AEA[74]. THC treatment of HC11 cells, reduced the expression of *CNR2*, but the protein expression of the

CB2 receptor was unaltered during 4 days of cannabinoid treatment. It is possible that CB2 expression changes may be transient or take longer to manifest, which should be investigated in the future. Moreover, our results demonstrated that the impact of THC on HC11 cell differentiation may be mediated via CB2. When co-treated with THC, AM630 (a selective antagonist of CB2[75]) restored the levels of *CSN2*, *HK2* and *FABP4,* which had decreased upon THC treatment. In contrast to THC, CBD has been reported to be a potent inverse agonist of CB2[76]. An inverse agonist competes with an agonist for the orthosteric binding site on a receptor[76]. CBD may compete with endogenous ligands of CB2, resulting in reduced activity of the CB2 receptor and reduction in its gene and protein expression. Moreover, the selective CB2 agonist, JWH133[77], rescued the effects of CBD on *CSN2, HK2* and *FABP4* mRNA levels, when co-treated with CBD.

In addition, THC upregulated mRNA levels of *FAAH,* suggesting that THC may be resulting in breakdown of AEA. CBD downregulated *NAPEPLD* levels and upregulated mRNA levels of *FAAH*, which suggests that treatment of HC11 cells with CBD may result in decreased synthesis and increased breakdown of AEA in HC11 cells. Altogether, the evidence suggests that THC and CBD alter canonical ECS signaling within HC11 cells. Furthermore, reversal of the effects of THC and CBD following co-treatment with a CB2 receptor antagonist and agonist, respectively, indicates that THC and CBD may be impacting HC11 cell differentiation, in part through CB2. However more detailed investigation is required to further elucidate these findings.

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CHAPTER 3: DELTA-9-TETRAHYDROCANNABINOL AND CANNABIDIOL EVOKE ER STRESS AND IMPACT MAMMARY EPITHELIAL CELL FUNCTION

Delta-9-tetrahydrocannabinol and cannabidiol evoke ER stress and impact mammary epithelial cell function

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KEYWORDS

Δ9-Tetrahydrocannabinol (THC), Cannabidiol (CBD), Endoplasmic Reticulum (ER) stress; Tight Junction (TJ); Mammary Gland (MG); Mammary Epithelial Cells (MEC); Differentiation; Milk Proteins; Milk Fat; Tunicamycin (Tu); sodium phenylbutyrate (4-PBA)

STATEMENT AND DECLARATIONS

The authors have declared that no competing interests exist.

AUTHOR CONTRIBUTIONS

All authors contributed to the study's conception and design. Conceptualization, material preparation, data collection and formal analysis were performed by Chitmandeep Josan. The first draft of the manuscript was written by Chitmandeep Josan. The study was supervised, funded, and conceptualized by Sandeep Raha. The manuscript was reviewed and edited by Sandeep Raha. All authors read and approved the final manuscript.

ABSTRACT

While Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are detected in the milk of cannabis users, the impact of these components and their mechanism of action on the mammary gland (MG) development remain elusive. Altered differentiation of mammary epithelial cells (MECs) results in abnormal MG development, which can highly impact the production and composition of breast milk. THC and CBD negatively affect cellular differentiation and cause endoplasmic reticulum (ER) stress in other cell types. ER stress has been associated with compromised MG development and function. Thus, we investigated the impact of THC and CBD on HC11 MECs. We reported that 10 mM THC and 10 mM CBD separately and in combination (10μM THC + 10μM CBD), significantly decreased mRNA levels of differentiation, milk protein, and lipogenic markers, reduced lipid levels and induced the expression of ER stress genes. The effects of 10mM THC, 10mM CBD and the combination of 10mM THC and 10mM CBD on HC11 MECs were ameliorated by 1mM sodium phenylbutyrate, which is known to inhibit ER stress. Collectively, these findings have indicated that THC and CBD, separately and in combination, directly induce ER stress in HC11 MECs, resulting in reduced lipid levels and lower mRNA levels of milk protein markers. Furthermore, compared to the control, the combination of THC and CBD significantly reduced the expression of genes that encode for proteins essential in mitochondrial oxidative stress response, mitochondrial dynamics and tight junction formation, and also exhibited increased reactive oxygen species production and higher levels of lactose release from HC11 cells.

INTRODUCTION

Breast milk is crucial for the nourishment of infants and protection against disease while the newborn is developing a mature immune system[1]. Breast milk is produced by the mammary gland (MG), which undergoes a crucial phase of remodeling during pregnancy to prepare itself for lactation[2]. MG remodeling is accompanied by alveolar differentiation of mammary epithelial cells (MECs), which has been characterized by the ability of the cell to synthesize and secrete milk proteins and milk fat[2]. Delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) have been reported to reduce the gene expression of milk proteins *(CSN2, WAP)* and lipid synthesizing markers *(FASN, FABP4, GLUT1, HK2, PLIN2, LPL)*, and decrease milk protein and lipid levels in HC11 MECs[3]. Impaired MEC differentiation has been linked with abnormal MG development, reduced milk production, and altered milk composition[2,4].

In mice, exposure to THC, CBD and the combination of THC and CBD during lactation has been associated with altered lipid composition in the milk of exposed dams[5]. In humans, the milk of cannabis users contained reduced levels of secretory immunoglobulin A (SIgA), compared to non-users [6]. Altered milk composition, low milk production or premature weaning of infants may have consequences on infant health and maternal well-being[7]. In humans, cannabis use during breastfeeding has been linked with decreased motor development in infants at one year of age[8]. Although the literature suggests negative consequences of cannabis use on MG development and MEC differentiation[3,9], there is a lack of evidence on the mechanism of action of THC and/or CBD on MEC differentiation. Given the high prevalence of cannabis use during pregnancy (2 and 34%)[10–13] and lactation (~5%)[11], a mechanistic understanding of the action of cannabinoids on MEC differentiation and function is important for getting an insight into the effect of cannabis use during pregnancy and lactation on MG development and function.

Differentiation of MECs is driven by transcriptional changes mediated by the upregulation of prolactin (PRL)[14], which induces the activation of STAT5 to stimulate the expression of milk protein *(CSN2, WAP)* and lipid synthesizing genes *(FASN, GLUT1, SREBP1c)*[15]. Phosphorylated STAT5 also triggers transcriptional activation of *AKT1*[15], which encodes for AKT that is responsible for the autocrine induction of PRL in mammary epithelium, and aids in the functional differentiation of MECs[16]. Furthermore, PRL stimulates the production of Receptor of Activated NF-κB (RANK), and its ligand RANKL, as well as E74-like factor 5 (ELF5)[17]. Alveolar differentiation of MECs during pregnancy demands high energy for protein biosynthesis, gluconeogenesis, lipid synthesis, and initial protein maturation, which are key processes that take place within the endoplasmic reticulum (ER)[18]. The ER plays an essential role in the creation, folding, classification and distribution of proteins to the correct cellular destination[19], and is the primary intracellular reservoir for Ca^{2+}[20]. Burgoyne et al. demonstrated that depletion of Ca^{2+} from the ER resulted in the inhibition of casein synthesis [21]. Thus, the integrity of the ER is essential for MG function, and ER stress may be underlying the negative effects of THC and CBD on the HC11 MEC function reported in Josan et al[3].

ER stress has been defined as an imbalance between the folding capacity of the ER and the protein folding load, resulting in the accumulation of unfolded/misfolded proteins in the ER lumen[22,23]. ER stress triggers the upregulation of genes in the unfolded protein response (UPR) pathway[24], which includes three major ER stress transducers: inositol requiring 1 α (IRE1α), PKR-like ER kinase (PERK), and activating transcription factor 6α (ATF6α)[25]. PERK is essential for cell survival as it activates the nuclear factor erythroid 2–related factor 2 (NRF2)[26]. IRE1α activation causes splicing and translation of the x-box binding protein 1 (*XBP1*) mRNA[22]. Both XBP1 and ATF6α stimulate the expansion of the ER membrane[22]. Severe ER

stress results in the activation of UPR-dependent apoptosis[27], which has been associated with PERK-mediated translation of *ATF4,* and activation of the C/EBP-homologous protein (CHOP)[28]. CHOP downregulates the expression of the antiapoptotic mediator BCL2[28], and directly activates *GADD34*[29], which elevates oxidative stress[30], disturbs cellular metabolism, and damages cellular constituents[31].

During ER stress, Ca^{2+} released from the ER accumulates in the mitochondrial matrix[20], which triggers proapoptotic signaling via mitochondrial alterations[32]. Impaired signaling of proteins involved in regulating mitochondrial fusion and fission results in the fragmentation of the mitochondria. These markers include Mitofusin 1 (MFN1) and MFN2, which are key mediators of fusion[33], and Dynamin-related protein 1 (DRP1) and mitochondrial fission 1 protein (FIS1), which are key mediators of fission[33]. Compromised mitochondrial signaling and mitophagy result in the generation of reactive oxygen species (ROS) [27], eventually leading to cell death [32]. Moreover, ER stress has been reported to increase cell membrane permeability by downregulation of tight junction proteins[34–36]. In the MG, tight junctions of the alveolar MECs ensure the permeability of the cell to minimize the chance of leakage of milk components from the lumen[37]. Reduced expression, translocation or disassembly of tight junctions results in increased permeability, which has been accompanied by a decrease in the rate of milk secretion[37].

Commercial cannabis products often used by consumers contain a mixture of THC and CBD[38]. In fact, cannabis use by women during pregnancy and lactation has been accompanied by the presence of THC and CBD in their milk[6,39,40], which suggests that these products contain a mixture of cannabinoids. Thus, using the HC11 cell line as a model of alveolar differentiation of MECs, we hypothesized that 10μM THC and 10μM CBD, independently and in combination (10μM THC + 10μM CBD) will directly induce ER stress within HC11 cells resulting in the

downregulation of gene expression of differentiation, milk protein and lipogenic markers, and decreasing milk protein and lipid production. Furthermore, we hypothesized that alleviation of ER stress in HC11 cells will ameliorate the effects of THC and CBD in HC11 MECs.

MATERIALS & METHODS

Cell culture

Cell culture-based experiments were approved by the McMaster Biosafety committee under Bio Utilization protocol BUP023. HC11 cells were purchased from the American Type Culture Collection (ATCC) (CRL-3062), under a Materials Transfer agreement between McMaster University. HC11 cells were cultured in RPMI 1640 (Corning, 10-041-CV), and supplemented with 10% Fetal Bovine Serum (FBS) (Hyclone, SH30396.03), 1% L-glutamine (Gibco, 25030081), and 1% penicillin-streptomycin (Gibco, 15140122), which formed the base RPMI media. Cells were routinely incubated at 5% CO_2 and 37°C. For experiments, HC11 cells were seeded at 100,000 cells/cm^2 in base RPMI media for 24 hours, then supplemented with 10ng/mL epidermal growth factor (EGF) (Thermo Scientific, PMG8041) and 5µg/mL insulin (INS) (Sigma-Aldrich, I0516) for 3 days. Then, the cells were differentiated in base RPMI media, supplemented with 5µg/mL PRL (PepProTech, 315-16), 1µM dexamethasone (DEX) (Sigma-Aldrich, D4902), and 5µg/mL INS over 4 days. Media was changed every 48 hours, supplemented with appropriate factors.

Treatment of HC11 cells with cannabinoids and ER stress promoters and inhibitors

Treatment with THC (Sigma-Aldrich, T4764) or CBD (Sigma-Aldrich, C6395) was carried out by supplementing HC11 cells at differentiation with the appropriate concentration of each drug or 0.1% methanol (MeOH) as the vehicle control. For HC11 cells that were treated with both THC and CBD, the cells were administered with an equal concentration of THC and CBD (i.e., 10µM

THC + 10μM CBD) at differentiation. For experiments that involved the administration of 4-PBA HC11 cells were supplemented with 1mM 4-PBA 1 hour following differentiation and then incubated at 5% CO2 and 37°C. 4 hours later, the cells were supplemented with the appropriate concentration of Tunicamycin (Tu), THC, CBD, or both THC and CBD and maintained over 4 days of differentiation. Tu and 4-PBA were solubilized in DMSO and serum-free RPMI 1640 media, respectively. For experiments with Tu, one set of HC11 cells was treated with 0.1% DMSO and that set was used as vehicle control for these experiments.

Assessment of cellular function: LDH, ORO, DCFDA, and lactose assays

HC11 cells were treated with various concentrations of THC or CBD (0.1μM to 50μM), or 0.1% MeOH (vehicle) over 4 days of differentiation. Changes in cell viability were investigated, using the CyQUANT lactate dehydrogenase (LDH) Cytotoxicity Assay (ThermoFisher, C20300). Lipid levels in differentiated HC11 cells were quantified using the Oil Red O (ORO) assay, as performed by Josan et al[3]. The DCFDA Cellular ROS Assay Kit (Abcam, 113851) was used to quantify reactive oxygen species (ROS), according to the manufacturer's instructions, and as performed by Walker et al[41]. Lactose levels were quantified in the media, which was collected from treated HC11 cells at the end of each experiment, using the Lactose Assay Kit (Abcam, ab83384) and according to the manufacturer's instructions.

Isolation of total RNA and RT-qPCR

Harvested cells were resuspended in Trizol (Thermo Fisher Scientific, 15596026). From these cells, RNA was extracted with chloroform and eluted through spin columns using the Direct-zol RNA Miniprep Kit (Cedarlane Labs, R2050). Complementary DNA (cDNA) was prepared from 1μg of total RNA, using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368813). Using the cDNA, a real-time quantitative polymerase chain reaction was

performed as described by Josan et al[3]. Relative gene expression changes were calculated using the ΔΔCT method[42], referenced to Ribosomal Protein L0 *(RPL0)* and Ribosomal Protein L8 *(RPL8)*. Primer sequences have been outlined in Table 1.

Primer Name	Forward Sequence	Reverse Sequence
ATF4	CAACCTATAAAGGCTTGC GGC	CACTGCTGCTGGATTTCGTG
ATF6	ACCTGTTCTTCCTCTGAAATCCAA	AGGACAGAGAAACAAGCTCGG
AKT	AATGTGGGCTCATGGGTCTG	AGAGGGAGAGGGCCAGTTAG
BCL2	TGAAGCGGTCCGGTGGATA	CAGCATTTGCAGAAGTCCTGTGA
CSN2	CCTTCATGGCTGTCAAGTCCT	GTTGTGAGCTGAAGACCACG
CHOP	CTGAAAGCAGAGCCTGATCC	GTCCTCATACCAGGCTTCCA
DRP1	GCCTCAGATCGTCGTAGTGG	TTTTCCATGTGGCAGGGTCA
ELF5	TTCTACAGTCCGCTGGTGC	TACGGAGTCCAACATCACCG
FABP4	ATTTCCTTCAAACTGGGCGTG	CTTTCCATCCCACTTCTGCAC
FASN	CACGAGTGAGTGTACGGGAG	GATCGGAGCATCTCTGGTGG
FIS1	GCCGTTACT CCT TCT ACC CC	ACACGCAACACTGTAACCCT
GADD34	AGGAGATAGAAGTTGTGGGCG	GACTGTTTCCCAGAGCGACT
GLUT1	TACACCCCAGAACCAATGGC	CCCGTAGCTCAGATCGTCAC
IRE1α	ACTTTGTGGTTGCCCAGACT	ACTTCCGTCCCAGGTAGACA
LPL	ATGGCAAGCAACACAACCAG	AGCAGTTCTCCGATGTCCAC
MFN1	GCTGTCAGAGCCCATCTTTC	CAGCCCACTGTTTTCCAAAT
MFN2	TCACACGGGCGTTTCCC	CCTGTCCAAGCTTCTTGAATGG
NRF2	CAGCATAGAGCAGGACATGGAG	GAACAGCGGTAGTATCAGCCAG
OCLN	TATCTTGGGAGCCTGGACATTT	AAAAAGAGTACGCTGGCTGAGA
PERK	ATGGACGAATCGCTGCACT	AGCTGTAGGTTGGTTTCGGAT
PINK1	GGGACTCAGATGGCTGTCC	GCTGTAGTCAATTACCGCACTG
PLIN2	TAAACGTCTGTCTGGACCGAAT	AAGGAAAAACCTCACCTCAAGC
PRKN	AGCCAGAGGTCCAGTTAAACC	GAGGGTTGCTTGTTTGCAGG
RANK	ACACAGAGACTCCTTTGCGG	ACCCCTGGGTATGGAGTGAA
RANKL	CATCGGGAAGCGTACCTACA	CGACCAGTTTTTCGTGCTCC
RPL0	CCAGCAGGTGTTTGACAACG	TCCAGAAAGCGAGAGTGCAG
RPL8	ACGTGAAGACC GTAAGGG	GATGCCTTTAATGTAGCCGTGT
SOD2	AGGAGAGTTGCTGGAGGCTA	TCTGTAAGCGACCTTGCTCC
STAT5A	CGTTCATCATCGAGAAGCAGC	GGGAAGGGAAAGACACACTCA
STAT5B	GAA GAC CCA GAC CAA GTT TGC	AAG GAA GAG TGG CAA GTA GGG
TFAM	ATGTGGAGCGTGCTAAAAGC	TGGGTAGCTGTTCTGTGGAAA
WAP	CAGCCCAAGCCTATACAGCA	TTGCCACCCTGGAGATCCTA
XBP1	TGGGCATCTCAAACCTGCTT	CCTCCCAGGAGTGGTCTGTA
ZO-1	AGGTGAAACTCTGCTGAGCC	GCAAAAGACCAACCGTCAGG
ZO-2	GCTGTCGGGCTGGCA	ACGATTGACGTCTCCCCATTT

Table 1 Primer sequences of all the genes investigated in this study.

Western blotting

Cells were harvested in Radioimmunoprecipitation assay (RIPA) buffer and sonicated for 10-15 seconds. Protein concentrations were measured by using the Pierce BCA Protein Assay (Thermo Fisher Scientific, 23227)[43]. 20µg of protein was separated by SDS-PAGE on 10-well, 12% Mini-Protean TGX Stain-free protein gels (Bio-Rad, 4568043), at a constant current (0.03 Amps). 10µL of Precision Plus Protein™ All Blue Prestained Protein Standard (Bio-Rad, 1610373) was loaded onto the gel. Gels were transferred to a Trans-blot turbo Mini 0.2µm polyvinylidene difluoride (PVDF) membrane (Bio-Rad, 1704156) under the following conditions: 21 volts, 1.0 amps, 7 minutes, as described by Josan et al[3]. PVDF membranes were blocked with 5% bovine serum albumin (BSA) (Sigma-Aldrich, A-6003) for 2 hours at room temperature (RT), followed by incubation in primary antibody overnight at 4ºC. Next, the membranes were incubated in the secondary antibody for 2 hours at RT. For protein detection, the membranes were incubated in Clarity Max Western Enhanced Chemiluminescence Substrate (Bio-Rad, 1705062) and imaged on the ChemiDoc™ Imaging System (Bio-Rad). The full list of concentrations and manufacturers of the antibodies has been outlined in Table 2.

Antibody Name	Company Name	Catalog Number	Working Dilution
Anti-β-casein mAb	Santa Cruz Biotechnology	sc-166684	1:1000
Anti-WAP mAB	Santa Cruz Biotechnology	sc-374648	1:200
Anti-β-Actin	Cell Signaling Technology	4970S	1:10,000
Goat Anti-Mouse IgG	Miltenyi Biotec, Inc.	130-048-401	1:5000
Donkey Anti-rabbit IgG	Cyvita	NA9340-1ML	1:5000

Table 2 List of primary and secondary antibodies used in this study. The list includes the name of the antibody and its manufacturer, catalogue number and concentrations used in this study. WAP: whey acidic protein; mAb: monoclonal antibody; pAb: polyclonal antibody.

Statistical analysis

All experiments were conducted with 6 experimental replicates. Each experiment was conducted at a different time point. Statistical analyses were performed using GraphPad Prism

software (Prism 9 for macOS, Version 9.1.2 [225]). Data points were expressed as mean ± SEM and analyzed using one-way analysis of variance (ANOVA) with Tukey's post-test. Data points with $p \leq 0.05$ were considered significant and statistical changes were denoted by differing letters on data figures. Different letters (a, b, etc.) indicate a statistically significant difference between the two data points. A mix of letters implies a statistically significant difference between two data points that have a distinct set of letters (i.e., ab and cd are statistically different). Meanwhile, a mix of letters that have at least one same letter in the combination indicates no significant difference between data points (i.e., ab and b).

RESULTS

10 mM THC, 10 mM CBD, and the combination of 10mM THC and 10 mM CBD reduced expression of the mRNA encoding milk proteins and lipid levels in HC11 cells

We evaluated the impact of THC, CBD, and the equimolar combination of these two compounds on HC11 cell viability. Over 4 days of differentiation, significant increases in THC-mediated cytotoxicity were observed at concentrations of 30mM and above (SI 1). CBD and the equimolar combination of THC and CBD demonstrated significant increases in cytotoxicity at a concentration of 20mM (SI 1). Following the cytotoxicity assay, the mRNA levels of milk protein and lipogenic markers were assessed. Relative to control, 10μM THC, 10μM CBD, and the combination of 10μM THC and 10μM CBD reduced mRNA levels of *CSN2* by almost 2-fold (Fig 1a) and *WAP* by almost 2.5-fold (Fig 1b). *CSN2* and *WAP* encode two major milk proteins: *β-casein* and whey acidic protein (WAP), respectively[44]. Compared to the control, the protein expression of *β-casein* (Fig 1c) and WAP (Fig 1d) was significantly reduced by 10μM CBD, and the mixture of 10 mM THC and 10mM CBD, but not by treatment with THC alone.

Next, the impact of cannabinoids was assessed on the mRNA levels of lipid synthesizing (lipogenic) markers (*LPL, FABP4*, and *PLIN2)*. *LPL* encodes for lipoprotein lipase (LPL), which promotes lipid uptake by MECs[45]; *FABP4* encodes for Fatty Binding Protein 4 (FABP4), which is responsible for fatty acid transport into the MECs[46]; and PLIN2 encodes perilipin 2, which surrounds lipid droplets and is accountable for the stability of lipid droplets[47]. 10μM THC, 10μM CBD, and the combination of 10μM THC and 10μM CBD significantly downregulated the mRNA levels of *LPL* (Fig 1e)*, FABP4* (Fig 1f)*,* and *PLIN2* (Fig 1g) in HC11 cells, compared to control. The decrease in mRNA levels of these lipogenic markers was accompanied by lower lipid levels in HC11 cells. Relative to control, lipid levels in HC11 cells were significantly decreased upon treatment with 10μM THC (mean difference: 0.02275, p-value < 0.0001), 10μM CBD (mean difference: 0.02340, p-value < 0.0001), and the mixture of THC and CBD (mean difference: 0.02150, p-value < 0.0001) (Fig 1h).

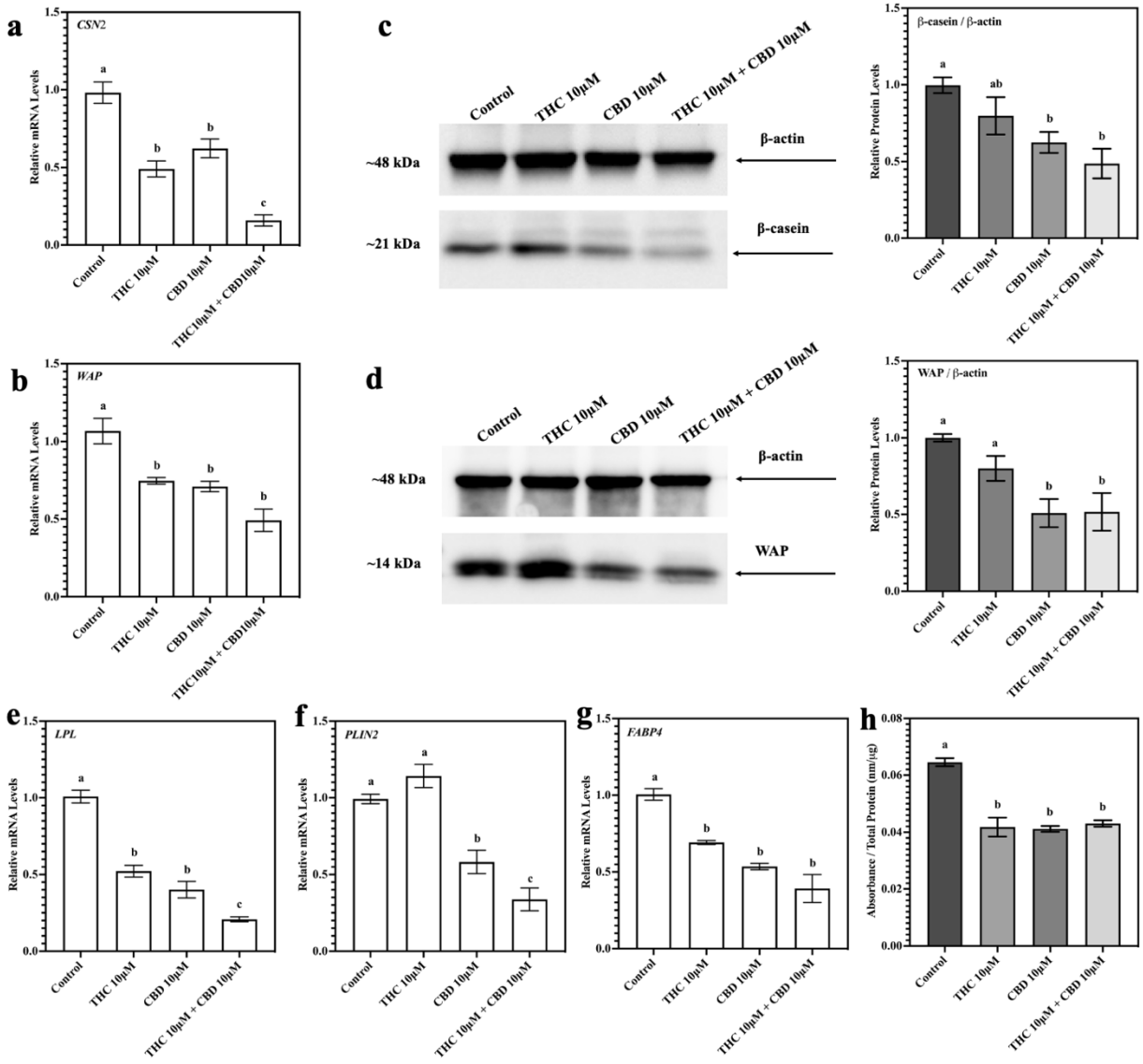


Fig. 1 10μM THC and 10μM CBD, independently and in a mixture (10μM THC + 10μM CBD), significantly reduced mRNA levels of milk proteins and lipogenic markers, and decreased lipid levels in HC11 cells. HC11 cells were differentiated in base RPMI 1640 media, supplemented with PRL, INS, and DEX for 4 days, as described in the methods. Throughout differentiation, the HC11 cells were treated with vehicle control (0.1% methanol), 10μM THC, 10μM CBD, or a mixture of 10μM THC and 10μM CBD. Cells from all conditions were processed for total RNA or protein isolation, as described in the methods. The relative mRNA levels of *CSN2* (a), *WAP* (b), *LPL* (e), *PLIN2* (f), and *FABP4* (g) were determined. Milk protein expression was assessed using 20μg of total protein for each sample. Representative blots of β-casein (c) and WAP (d) have been represented along with bar graphs quantifying their protein expression. Results from 6 replicates were referenced to *RPL0* and *RPL8* for mRNA analysis, and to β-actin for protein analysis. As described in the methods, lipid levels were quantified in cells from 6 replicates and normalized to total protein levels for each sample (h). All results were plotted as mean ± SEM and compared using one-way ANOVA. Each experimental replicate was

performed with cells from a different passage and statistically significant changes were indicated by distinct letters on the bar graphs.

10μM THC, 10μM CBD, and the mixture of 10μM THC and 10μM CBD decreased mRNA levels of differentiation markers in HC11 cells

The impact of cannabinoids on the mRNA levels of key transcription factors involved in the differentiation of MECs was determined. Relative to control, 10μM THC, 10μM CBD, and the combination of 10μM THC and 10μM CBD, significantly reduced gene expression of *RANK* (Fig 2a), *RANKL* (Fig 2b), *ELF5* (Fig 2c), *AKT* (Fig 2d) and *STAT5A* (Fig 2e). Relative to cells treated with 10μM THC and 10μM CBD separately, HC11 cells treated with the combination of 10μM THC and 10μM CBD demonstrated significantly lower mRNA levels of *ELF5* (Fig 2c) and *STAT5B* (Fig 2f). Compared to control cells, the average gene expression level of *STAT5B* was lower in cells treated individually with 10μM THC, and 10μM CBD, however, the changes were not statistically significant (Fig 2f).

In addition to testing the impact of 10μM THC and 10μM CBD on the mRNA levels of milk protein, lipogenic and differentiation genes in HC11 cells, we also investigated the impact of 20μM THC and 20μM CBD on those markers to understand the impact of 20μM cannabinoid concentration on HC11 cells. Relative to control, 20μM THC and 20μM CBD significantly downregulated the gene expression of *STAT5A* (Fig SI 2a), *WAP* (Fig SI 2b), and *FABP4* (Fig SI 2c). Furthermore, compared to the control, 20μM CBD significantly decreased the gene expression of *GLUT1* (Fig SI 2d), which encodes glucose transporter protein type 1 (GLUT1)[48]. Relative to cells treated with the combination of 10μM THC and 10μM CBD, 20μM CBD significantly decreased the mRNA levels of *STAT5A, WAP, FABP4,* and *GLUT1* (Fig SI 2).

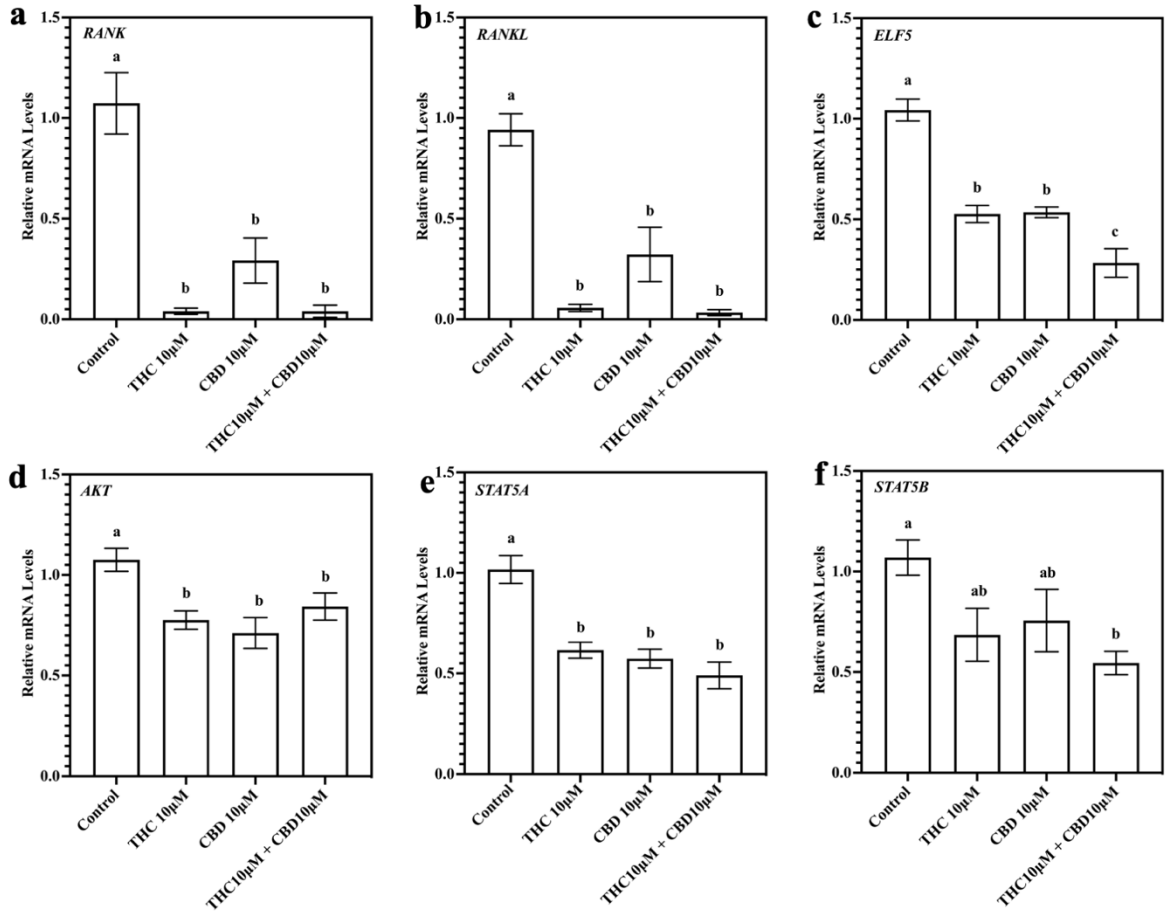


Fig. 2 10μM THC and 10μM CBD, independently and in a mixture (10μM THC + 10μM CBD), decreased mRNA levels of differentiation markers in HC11 cells. HC11 cells were differentiated in base RPMI 1640 media, supplemented with PRL, INS, and DEX for 4 days, as described in the methods. Throughout differentiation, the HC11 cells were treated with vehicle control (0.1% methanol), 10μM THC, 10μM CBD, or a mixture of 10μM THC and 10μM CBD. Cells from all conditions were processed for total RNA isolation, as described in the methods. The relative mRNA levels of *RANK* (a), *RANKL* (b), *ELF5* (c), *AKT* (d), *STAT5A* (e), and *STAT5B* (f) were determined. Results from 6 replicates were referenced to *RPL0* and *RPL8* for mRNA analysis. All results were plotted as mean ± SEM and compared using one-way ANOVA. Each experimental replicate was grown from cells of a different passage and statistically significant changes were indicated by distinct letters on the bar graphs.

10µm THC and 10µm CBD, independently and in a mixture (10µm THC + 10µm CBD), altered the expression of the mRNA encoded by ER stress genes in HC11 cells

The impact of cannabinoids was assessed on key genes involved in the UPR pathway. Relative to control, 10µM THC significantly increased mRNA levels of *IRE1α* (Fig 3a), *ATF6α* (Fig 3b), and *PERK* (Fig 3c) in HC11 cells. Furthermore, HC11 cells treated with THC (10µM), CBD (10µM), or the combination of 10µM THC and 10µM CBD did not significantly impact the mRNA levels of *NRF2* as compared to control cells (Fig 3e). Relative to control, 10µM THC, 10µM CBD, and the combination of 10µM THC and 10µM CBD significantly upregulated mRNA levels of *XBP1* (Fig 3d), *ATF4* (3f), *CHOP* (Fig 3g), and *GADD34* (Fig 3h), and decreased the mRNA levels of *BCL2* (Fig 3i). Compared to HC11 cells treated with 10µM THC and 10µM CBD (individually), the cells treated with the combination of 10µM THC and 10µM CBD demonstrated significantly increased mRNA levels of *ATF4, CHOP,* and *GADD34*, and significantly decreased mRNA levels of *BCL2*.

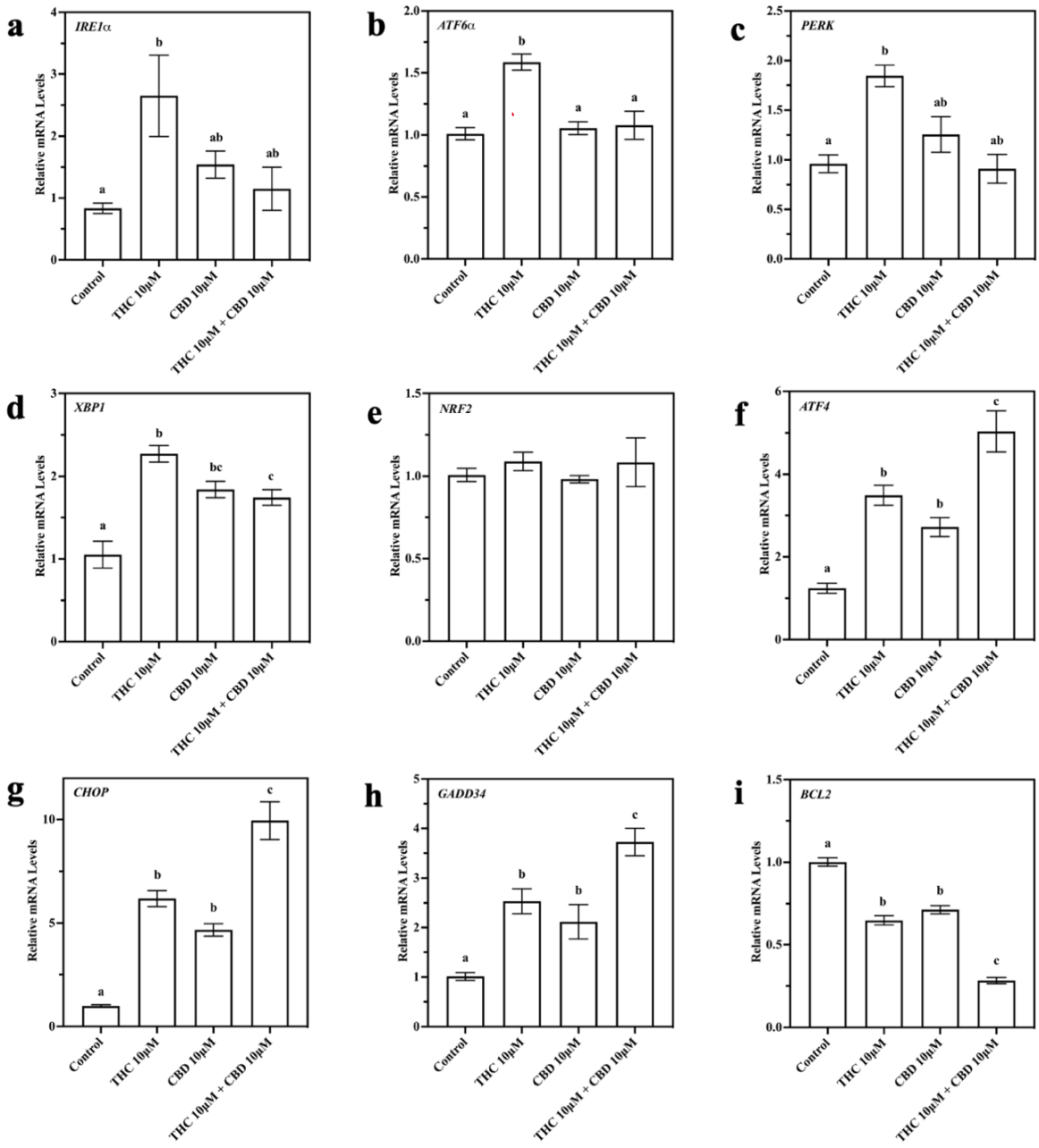


Fig. 3 10μM THC, 10μM CBD, and the mixture of 10μM THC and 10μM CBD significantly altered mRNA levels of markers involved in the unfolded protein response pathway in HC11 cells. HC11 cells were differentiated in base RPMI 1640 media, supplemented with PRL, INS, and DEX for 4 days, as described in the methods. Throughout differentiation, the HC11 cells were treated with vehicle control (0.1% methanol), 10μM THC, 10μM CBD, or a mixture of 10μM THC and 10μM CBD. Cells from all conditions were processed for total RNA isolation, as described in the methods. The relative mRNA levels of *IRE1α* (a), *ATF6α* (b), *PERK* (c), *XBP1* (d), and *NRF2* (e), *ATF4* (f), *CHOP* (g), *GADD34* (h), and *BCL2* (i) were determined. Results from 6 replicates were referenced to *RPL0* and *RPL8* for mRNA analysis. All results were plotted as mean ± SEM and compared using one-way ANOVA. Each experimental replicate was performed using cells of

a different passage and statistically significant changes were indicated by distinct letters on the bar graphs.

The combination of 10µm THC And 10µm CBD reduced the expression of the mRNA encoding markers of mitochondrial dynamics and elevated cellular ROS in HC11 cells

ER stress has been associated with mitochondrial dysfunction and increased ROS levels [24]. There we assessed changes in the mRNA levels of genes that encode for mitochondrial fission and fusion proteins (*MFN1*, *MFN2*, *DRP1,* and *FIS1* [49]) upon treatment of HC11 cells with THC and CBD. In addition, the expression of the following mitochondrial genes was assessed: *PINK1* encodes for PTEN-induced putative kinase 1, which protects the mitochondria from malfunctioning during cellular stress[50]; PRKN encodes for Parkin RBR E3 ubiquitin-protein ligase, which is regulated by PINK1 to eliminate dysfunctional mitochondria[50]; *SOD2* encodes for Superoxide Dismutase 2, which protects the cell against mitochondria-generated ROS toxicity and its deficiency leads to excessive ROS production[51]; *TFAM* encodes for Transcription Factor A, mitochondrial, which is responsible for transcription of mitochondrial DNA[41].

Relative to control, the combination of 10µM THC and 10µM CBD significantly decreased mRNA levels of *PINK1* (Fig 4a), *PRKN* (Fig 4b), *SOD2* (Fig 4c), *TFAM* (Fig 4d), *MFN1* (Fig 4e), *MFN2* (Fig 4f), *DRP1* (Fig 4g) and *FIS 1* (Fig 4h). Furthermore, the combination of 10µM THC and 10µM CBD significantly increased ROS levels in HC11 cells (mean difference: 0.3311, p-value = 0.0140) (Fig 4i), compared to control. Relative to control, THC downregulated the mRNA levels of *TFAM* (Fig 4d) and upregulated the mRNA levels of *DRP1* (Fig 4f), and CBD decreased the gene expression of *TFAM* (Fig 4d). Although CBD increased the mean ROS levels in HC11 cells, compared to control, the changes were not statistically significant (p-value = 0.0888).

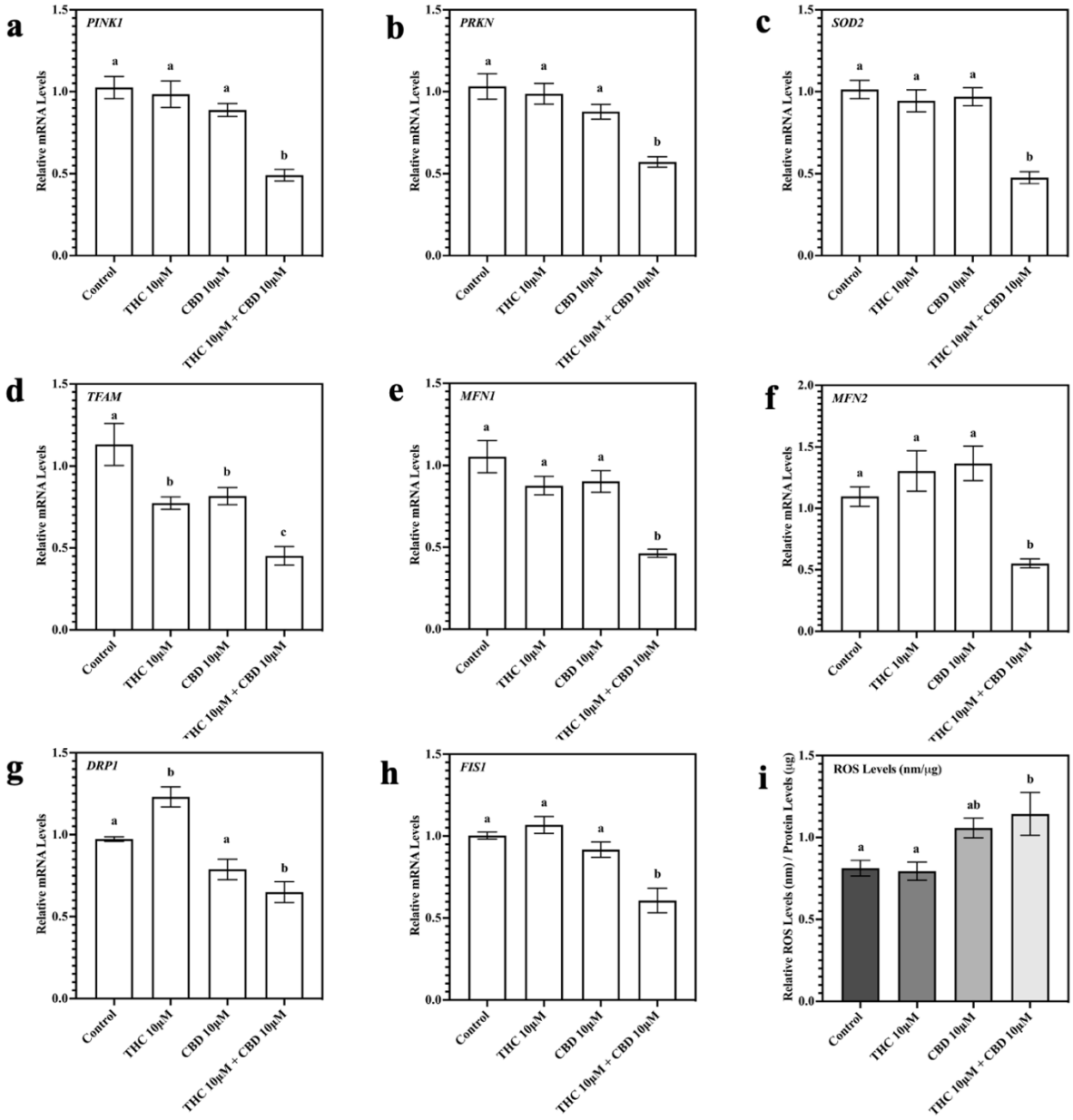


Fig. 4 The combination of 10µM THC and 10µM CBD reduced mRNA levels of key mitochondrial markers and increased ROS levels in HC11 cells. HC11 cells were differentiated in base RPMI 1640 media, supplemented with PRL, INS, and DEX for 4 days, as described in the methods. Throughout differentiation, the HC11 cells were treated with vehicle control (0.1% methanol), 10µM THC, 10µM CBD, or a mixture of 10µM THC and 10µM CBD. Cells from all conditions were processed for total RNA isolation, as described in the methods. The relative mRNA levels of *PINK1* (a)*, PRKN* (b)*, SOD2* (c), *TFAM* (d)*, MFN1* (e), *MFN2* (f), *DRP1* (g)*,* and *FIS1* (h) were determined. Results from 6 replicates were referenced to *RPL0* and *RPL8* for mRNA analysis. Levels of Reactive Oxygen Species (ROS) were quantified in HC11 cells using the 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) assay kit, as described in the methods (k). Results from the DCFDA assay were normalized to total protein levels in each condition (k). All results were plotted as mean ± SEM and compared using one-way ANOVA. Each experimental replicate

was performed using cells of a different passage and statistically significant changes were indicated by distinct letters on the bar graphs.

The mixture of 10μM THC and 10μM CBD downregulated the expression of tight junction mRNA and increased lactose release from HC11 cells

Differentiation of HC11 cells with PRL, DEX and INS has been demonstrated to result in increased tight junction permeability[52]. However, ER stress has been associated with permeable tight junctions and decreased expression of tight junction markers[35,37]. We assessed the impact of cannabinoids on the mRNA levels of *ZO-1*, *ZO-2*, and *OCLN,* which encode for tight junction protein 1, tight junction protein 2, and occludin, respectively[37]. Compared to control, HC11 cells treated with 10μM CBD and the mixture of 10μM THC and 10μM CBD significantly downregulated the gene expression of *ZO-1* (Fig 5a), *ZO-2* (Fig 5b), and *OCLN* (Fig 5c). 10μM THC decreased the mRNA levels of *ZO-2* (Fig 5b) and *OCLN* (Fig 5c), relative to the control. Permeable tight junctions in the MG have been associated with increased release of lactose into the plasma and decreased levels of lactose in the milk[53]. To assess whether the altered expression of tight junction markers in HC11 cells was linked with changes in lactose release from HC11 cells into the media, we quantified the amount of lactose in the media of HC11 cells. The media of HC11 cells treated with the mixture of 10μM THC and 10μM CBD over 4 days of differentiation displayed significantly higher levels of lactose (mean difference: 0.4913, p-value = 0.0093), compared to control cells (Fig 5d).

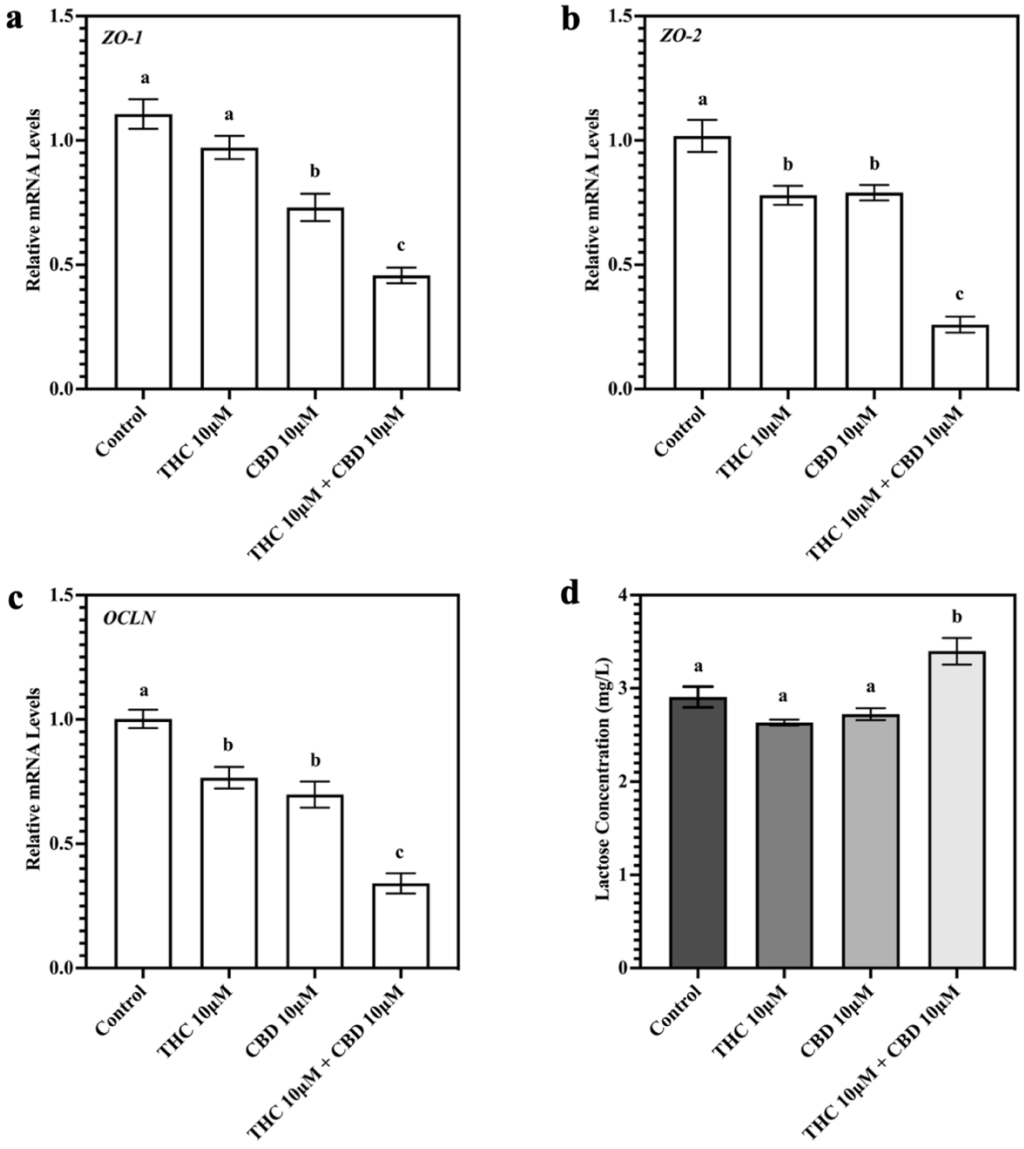


Fig. 5 The mixture of 10μM THC and 10μM CBD significantly reduced gene expression of tight junction markers and resulted in high levels of lactose in the media of HC11 cells. HC11 cells were differentiated in base RPMI 1640 media, supplemented with PRL, INS, and DEX for 4 days, as described in the methods. Throughout differentiation, the HC11 cells were treated with vehicle control (0.1% methanol), 10μM THC, 10μM CBD, or a mixture of 10μM THC and 10μM CBD. Cells from all conditions were processed for total RNA isolation, as described in the methods. The relative mRNA levels of *ZO-1* (a), *ZO-2* (b), and *OCLN* (c) were determined. Results from 6 experimental replicates were referenced to *RPL0* and *RPL8* for mRNA analysis (a-c). Lactose levels were measured in the media collected from HC11 cells that were treated with either the vehicle control (0.1% MeOH), 10μM THC, 10μM CBD, or 10μM THC and 10μM CBD over 4 days of differentiation (d). Results were plotted as mean ± SEM and compared using one-way ANOVA. Each experimental replicate was performed using cells of a different passage and statistically significant changes were indicated by distinct letters on the bar graphs.

Co-treatment with 1mM 4-PBA reversed the effect of 0.5μM Tu on milk protein, lipogenic, and expression of the mRNA encoded by ER stress genes in HC11 cells

To investigate whether ER stress is underlying the functional changes found in HC11 cells treated with 10μM THC, 10μM CBD, and the combination of 10μM THC and 10μM CBD, we hypothesized that an ER stress inhibitor (sodium phenylbutyrate [4-PBA]) will reverse the effect of these cannabinoids on HC11 cells[54]. This was tested by treating HC11 cells with a known ER Stress inducer, 0.5μM Tunicamycin (Tu)[55], and investigating whether its effects on HC11 cells were reversed by 1mM 4-PBA. One set of HC11 cells was supplemented with 0.1% DMSO, which was the vehicle control to Tu and referred to as the DMSO control (DMSO Ctrl).

Relative to DMSO Ctrl, HC11 cells treated with 0.5μM Tu displayed a significant downregulation in the mRNA levels of *CSN2* (Fig 6a), *WAP* (Fig 6b), *LPL* (Fig 6c), *FABP4* (Fig 6d), *TFAM* (Fig 6e), *ZO-1* (Fig 6f), and *BCL2* (Fig 6k). Furthermore, 0.5μM Tu significantly upregulated the mRNA levels of *XBP1* (Fig 6g), *PERK* (Fig 6h), *ATF4 (*Fig 6i), and *CHOP* (Fig 6j) in HC11 cells, relative to control. Compared to the DMSO Ctrl, cells treated with 1mM 4-PBA did not display any changes in mRNA levels of the tested genes. Upon co-treatment of HC11 cells with 0.5μM Tu and 1mM 4-PBA, HC11 cells displayed no change in the mRNA levels of *CSN2* (Fig 6a), *WAP* (Fig 6b), *LPL* (Fig 6c), *FABP4* (Fig 6d), *TFAM* (Fig 6e), *ZO-1* (Fig 6f), *XBP1* (Fig 6g), *PERK* (Fig 6h), A*TF4* (Fig 6i), *CHOP* (Fig 6j), and *BCL2* (Fig 6k), compared to DMSO Ctrl, as well as HC11 cells treated with 1mM 4-PBA.

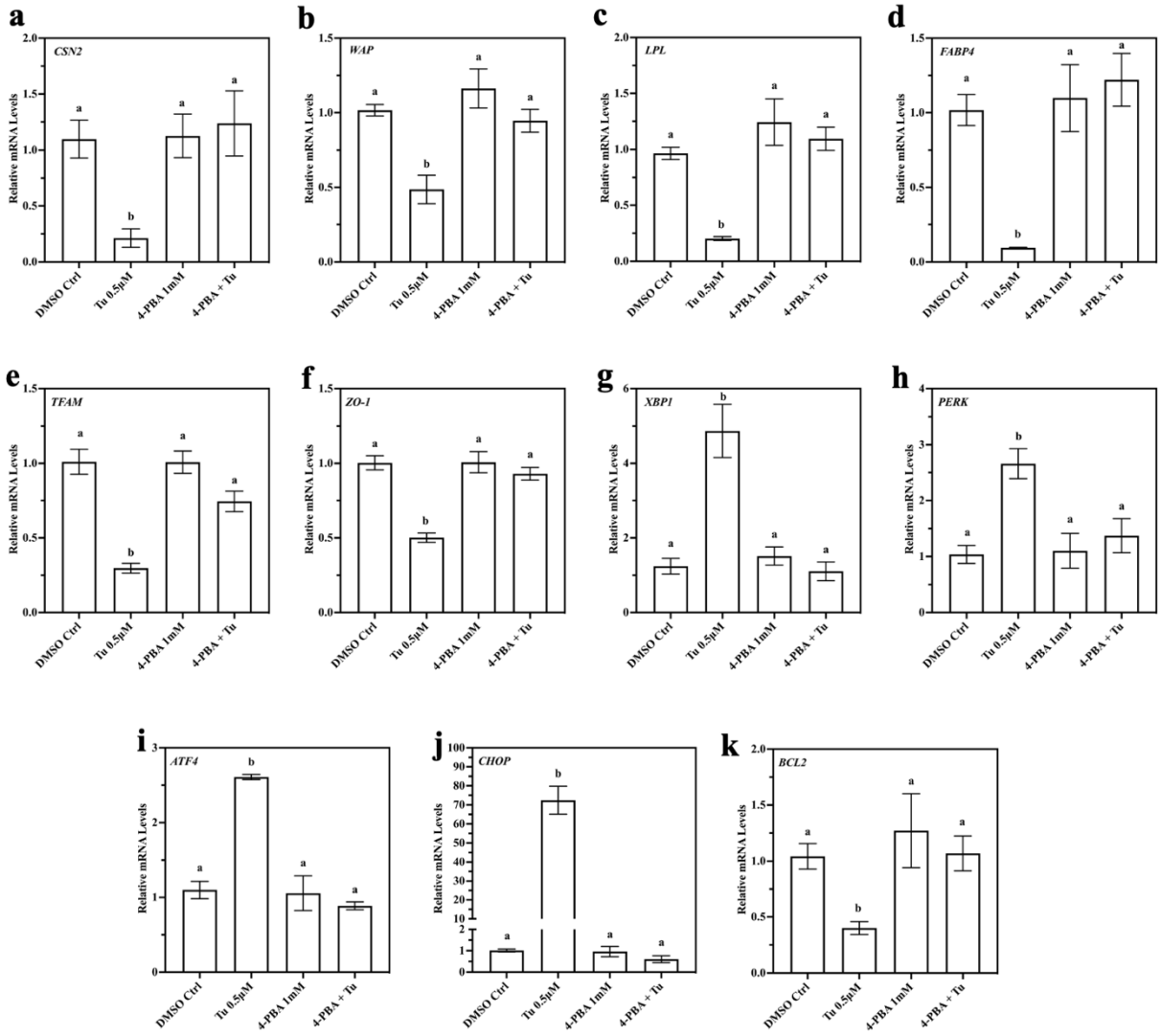


Fig. 6 The effects of Tunicamycin induced ER-Stress on the mRNA levels of milk protein and lipogenic markers, are reversed by 1mM 4-PBA. HC11 cells were differentiated in base RPMI 1640 media, supplemented with PRL, INS, and DEX for 4 days, as described in the methods. Throughout differentiation, the HC11 cells were treated with vehicle control (0.1% DMSO), 0.5μM Tunicamycin (Tu), 1mM sodium phenylbutyrate (4-PBA), or a combination of 0.5μM Tu and 1mM 4-PBA. The relative mRNA levels of *CSN2* (a), *WAP* (b), *LPL* (c), *FABP4* (d), *TFAM* (e), *ZO-1* (f), *XBP1* (g), *PERK* (h), *ATF4* (i), *CHOP* (j), and *BCL2* (k) were determined. Results from 6 experimental replicates were referenced to *RPL0* and *RPL8* for mRNA analysis. Results were plotted as mean ± SEM and compared using one-way ANOVA. Each experimental replicate was grown from cells of a different passage and statistically significant changes were indicated by distinct letters on the bar graphs.

The effect of 10μM THC, 10μM CBD, and the combination of 10μM THC and 10μM CBD on the mRNA levels of key biomarkers for milk proteins, lipogenesis, and ER-Stress were reversed upon co-treatment with 1mM 4-PBA

Relative to control, the mRNA levels of milk proteins (*CSN2* and *WAP*), lipogenic (*LPL* and *FABP4*), and ER stress genes (*ATF4* and *CHOP*) were significantly altered in HC11 cells upon treatment with 10μM THC, 10μM CBD, and the mixture of 10μM THC and 10μM CBD. The attenuation in the levels of *CSN2* (Fig 7a), *WAP* (Fig 7b), *LPL* (Fig 7c), and *FABP4* (Fig 7d) mRNA observed following treatment with THC, CBD, and the mixture of THC and CBD, were normalized upon co-treatment with 1mM 4-PBA, when compared to control. Similarly, the increase in the mRNA levels of, *ATF4* (Fig 7e), and *CHOP* (Fig 7f) following treatment with THC, CBD, and the mixture of THC and CBD, were reversed upon co-treatment with 1mM 4-PBA.

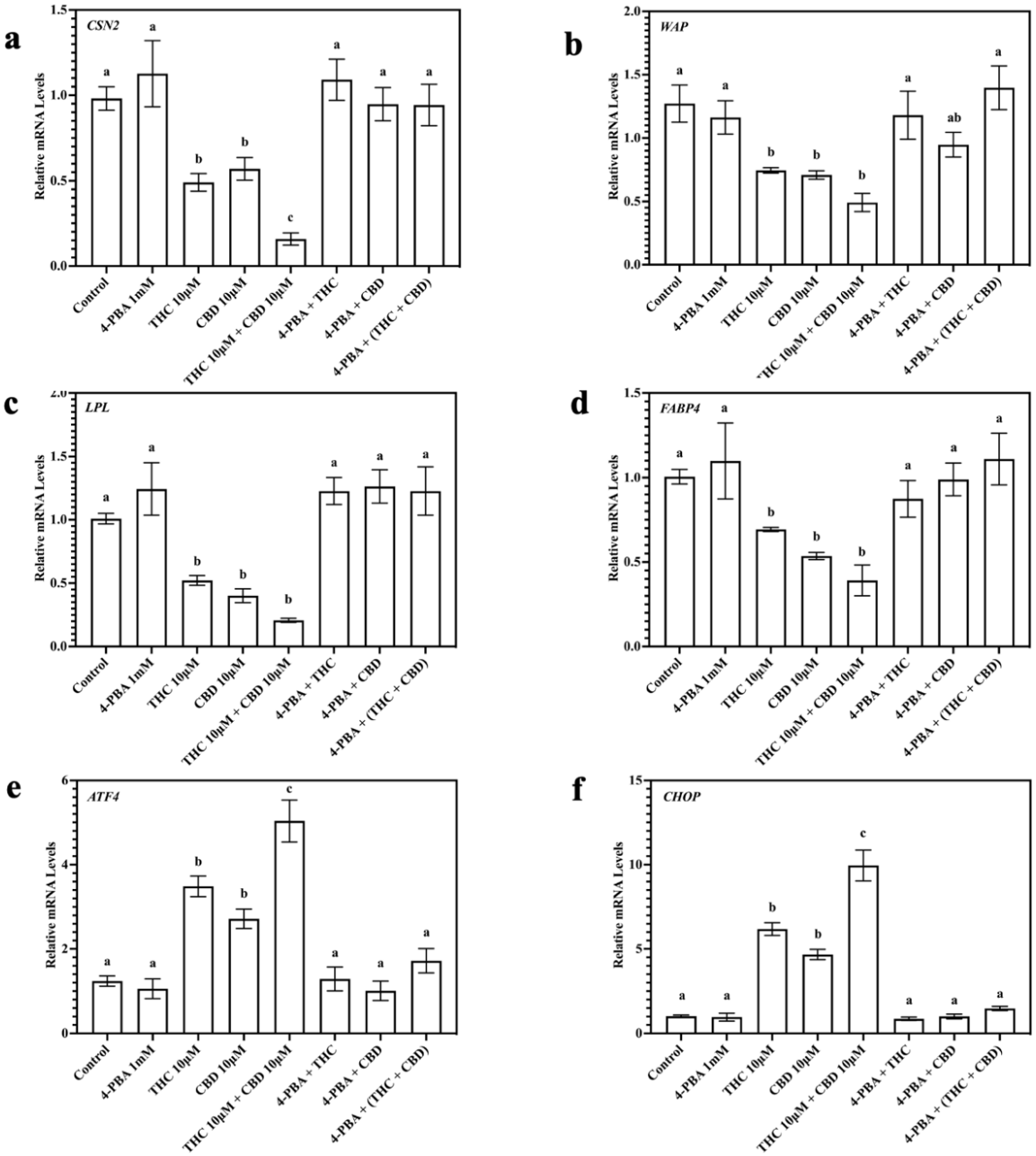


Fig. 7 Effect of 10μM THC, 10μM CBD, and the mixture of 10μM THC and 10μM CBD, on the mRNA levels of milk protein, lipogenic, and ER stress markers was reversed by 1mM 4-PBA. HC11 cells were differentiated in base RPMI 1640 media, supplemented with PRL, INS, and DEX for 4 days, as described in the methods. Throughout differentiation, the HC11 cells were treated with either vehicle control (0.1% methanol), 1mM sodium phenyl-butyrate (4-PBA), 10μM THC, 10μM CBD, the combination of 10μM THC and 10μM CBD, and the mixture of 10μM THC and 1mM 4-PBA, 10μM CBD and 1mM 4-PBA, and 10μM THC + 10μM CBD and 1mM 4-PBA. The relative mRNA levels of *CSN2* (a)*, WAP* (b)*, LPL* (c), FABP4 (d), *ATF4* (e)*,* and *CHOP* (f) were assessed, as described in the methods. Results from 6 experimental replicates were referenced to *RPL0* and *RPL8* for mRNA analysis (a-f). Results were plotted as mean ± SEM and compared

using one-way ANOVA. Each experimental replicate was performed using cells of a different passage and statistically significant changes were indicated by distinct letters on the bar graphs.

DISCUSSION

THC, CBD, impact HC11 differentiation and function

Despite the concern regarding cannabis use during pregnancy, not much attention has been focused on exploring the impact of cannabis on MG development and function. Impaired MEC differentiation has been demonstrated to result in delayed and abnormal MG development, which is accompanied by decreased expression of transcription factors, such as AKT1, STAT5, and milk proteins, including β-casein and WAP[56]. Compromised development of the MG results in reduced milk yield and modified milk composition[57]. Milk insufficiency has been cited as the most common reason underlying the low percentage of women breastfeeding[58]. 10μM THC and 10μM CBD have been demonstrated to downregulate the markers that represent the alveolar differentiation of HC11 MECs[3]. In addition, cannabis use during lactation has been linked with decreased levels of SIgA in the milk of users, compared to non-users[6].

In this study, we investigated whether ER stress is underlying the negative effects of THC and CBD on the mRNA levels of biomarkers responsible for encoding milk proteins, lipid synthesizing enzymes and transcription factors in HC11 cells. Furthermore, the 1:1 mixture of THC and CBD has been demonstrated to provide protection against nausea and vomiting[59]. Pregnant women may choose cannabis products that contain mixtures of THC and CBD, particularly ones with a 1:1 mixture, to treat the symptoms of morning sickness. Thus, along with testing the effect of 10μM THC and 10μM CBD (separately), we explored the impact of the combination of 10μM THC and 10μM CBD on HC11 cells. Results from this study have provided insight into the mechanism of action of cannabinoids on MEC differentiation and function, which

may aid in understanding the effect of maternal cannabis use during pregnancy and lactation on mammary gland development and breast milk production.

Our results demonstrated that 10μM THC and 10μM CBD, separately and in a 1:1 mixture (10μM THC + 10μM CBD) significantly decreased the mRNA levels of key transcription factors, including *RANK, RANKL, ELF5, AKT, STAT5A,* and *STAT5B.* During gestation, RANK/RANKL signaling ensures ductal branching and alveologenesis by inducing MEC proliferation[60]. The absence of RANK or RANKL has been demonstrated to result in marked attenuation of alveologenesis in the MG and impaired lactation at parturition[61], which resembles the morphology of the MG in PRL receptor knockout mice[62]. The transcription factor ELF-5 is essential for the alveolar differentiation of MECs [63]. *ELF-5* null mice display disorganized MG morphology, failed differentiation of MEC during pregnancy[64] and decreased phosphorylated STAT5 staining and mRNA levels of *CSN2, WAP,* and *STAT5A*[63]. A 50% decrease in the mRNA level of *STAT5A* was accompanied by a complete lack of nuclear staining of phosphorylated STAT5[63]. Thus, we monitored the mRNA levels of *STAT5A* and *STAT5B*, along with other transcription factors, to understand the effect of THC and CBD on HC11 cell differentiation. *STAT5A* and *STAT5B* are products of 2 different genes, with *STAT5A* being crucial for the lactogenic differentiation of MECs[65]. Finally, the downregulation of AKT has also been linked with the suppression of MG development[66]. Altogether, in addition to the effect of THC and CBD on HC11 MEC function[3], results from this study suggest that THC and CBD directly impact HC11 cell differentiation by reducing the gene expression of key transcription factors.

Differentiation of MECs has been characterized by the synthesis of milk fat, and the expression of milk protein genes, such as β-casein and WAP, which are target genes of STAT5[67,68]. 10μM THC and 10μM CBD, separately and in a 1:1 mixture significantly

decreased the gene expression of key milk proteins (*CSN2* and *WAP*) in HC11 cells. CSN2 and WAP mRNA changes have been positively correlated with β-casein and WAP expression[69,70]. 10μM THC did not alter β-casein and WAP expression in HC11 cells. A possible explanation is that the HC11 cells require a longer incubation time with 10μM THC for the gene expression changes to manifest into protein expression changes. In contrast, 10μM CBD, and the mixture of THC and CBD, significantly reduced the expression of β-casein and WAP in HC11 cells.

10μM THC, 10μM CBD, and the combination of 10μM THC and 10μM CBD significantly decreased the gene expression of lipogenic markers and reduced lipid levels in HC11 cells. A strong correlation has been reported between the expression of genes involved in the *de novo* synthesis of milk fat and milk yield in buffalos[71]. Thus, the negative impact of THC and CBD, separately and in combination, on the mRNA levels of lipogenic markers and lipid levels in HC11 cells present a concern for optimal milk yield and milk composition upon cannabis consumption during pregnancy. Altogether, the decreased mRNA levels of milk protein and lipogenic markers, along with reduced lipid levels, indicate that 10μM THC, 10μM CBD, and the mixture of 10μM THC and 10μM CBD adversely affect the functioning of HC11 cells.

THC and CBD upregulate mRNA levels of ER stress markers in HC11 cells

In this study, THC, CBD, and the combination of THC and CBD increased the mRNA levels of *XBP1, ATF4, CHOP,* and *GADD34,* and decreased the mRNA levels of *BCL2.* THC and CBD have been reported to induce mitochondrial dysfunction[72,73] and ER stress in various cell types[74]. Chronic or severe ER stress has been associated with oxidative stress[75] and activation of UPR-dependent apoptosis[27]. UPR-dependent apoptosis involves the upregulation of CHOP[28]and GADD34[28], and the downregulation of BCL2, which is an anti-apoptotic protein[76]. The overexpression of BCL2 allows resistance to cell death in cancer cells and its

downregulation has been associated with apoptosis[76]. Both THC and CBD have been reported to stimulate cellular apoptosis in various cell types in a cannabinoid receptor-dependent and receptor-independent manner[77–80]. In this study, THC, CBD, and the combination of THC and CBD induced ER stress and apoptotic signaling in HC11 cells, however not to a degree that was sufficient to cause significant cytotoxicity over 4 days of differentiation (SI 1).

4-PBA reversed the effects of THC and CBD (separately and in combination) on HC11 cells

To determine whether 4-PBA can relieve the effect of a known ER stress inducer, HC11 cells were treated with Tu, and then co-treated with Tu and 4-PBA. The effect of 0.5μM Tu on the mRNA levels of milk protein, mitochondrial, tight junction and ER stress markers was completely reversed upon co-treatment with 1mM 4-PBA. Similarly, the effects of THC, CBD, and the combination of THC and CBD on the mRNA levels of key milk protein, lipogenic, and ER stress genes were reversed by 1mM 4-PBA. Results from this study suggest that the impact of THC and CBD (separately and in combination) on the gene expression of key markers that characterize HC11 MEC differentiation and function was induced by ER stress.

The combination of THC and CBD elevated ROS levels and increase leakiness of lactose into the media in HC11 cells

In this study, the combination of THC and CBD increased the gene expression of *CHOP* and *GADD34* to a greater degree than THC and CBD (individually) in HC11 cells, compared to control cells. Furthermore, the combination of THC and CBD caused a marked reduction in the mRNA levels of markers crucial for mitochondrial function, fission, and fusion. These striking changes were accompanied by elevated levels of ROS in HC11 cells treated with the combination of THC and CBD, compared to the control. The presence of ER stress markers in the MECs has been accompanied by the generation of ROS[81], which has been associated with mitochondrial

dysfunction[82]. In bovine MECs, ER stress induced by Zearalenone resulted in cellular apoptosis, increased ROS content, decreased mitochondrial membrane potential and increased expression of ATF6 and CHOP[83]. The induction of ER stress and increased ROS levels in cows has been linked with reduced milk yield[84].

Furthermore, ER stress has been associated with tight junction permeability, which can be rescued through the alleviation of ER stress[35]. Leading up to lactation, the tight junctions of the alveolar MECs become impermeable to allow secure storage of milk components without leakage, particularly lactose[37]. In this study, THC, CBD, and the combination of THC and CBD significantly reduced the gene expression of *ZO-1, ZO-2,* and *OCLN*. Downregulation of the gene and protein expression of tight junction markers results in the permeability of tight junctions, resulting in increased levels of lactose in the plasma and lower levels of lactose in milk[53]. In this study, the combination of THC and CBD resulted in the leaking of lactose into the medium, relative to control, THC and CBD (alone). This may be attributed to the increased gene expression of ER stress markers, elevated ROS levels, and decreased level of the mRNA encoding proteins involved in forming tight junctions in MECs treated with the combination of THC and CBD to a greater degree than the treatment of the cells with THC and CBD alone, compared to control.

In the MG of goats, ER stress was accompanied by reduced milk fat content, altered milk composition, induction of permeable tight junctions between MECs, and impaired lactation performance[85]. Furthermore, the detection of ER stress markers in the milk of goats was accompanied by premature cessation of lactation[85]. Therefore, the induction of ER stress genes, increase in ROS levels, and indication of increased tight junction permeability in HC11 MECs upon the treatment of THC and CBD (in combination) presents potential risk of cannabis use during pregnancy and lactation for milk yield, milk composition and premature weaning.

Distinct effects of THC and CBD on mRNA levels of key markers tested

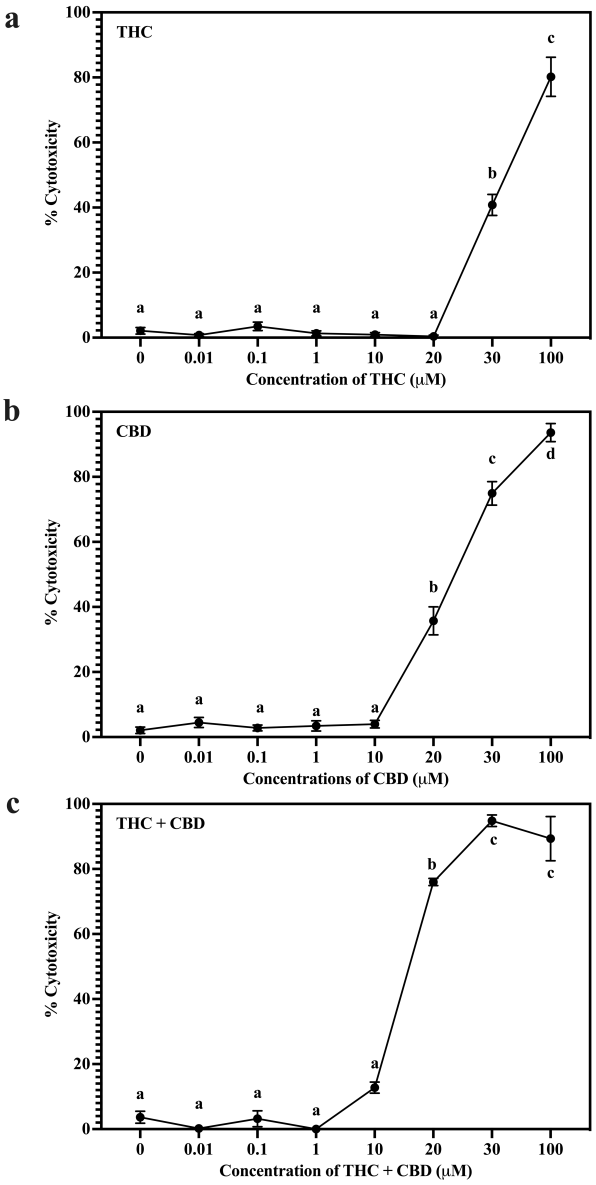
In this study, the combination of THC and CBD induced distinct effects on the mRNA levels of milk protein, lipogenic, differentiation, ER stress, mitochondrial, and tight junction markers (*CSN2, WAP, LPL, PLIN2, ELF5, ATF4, CHOP, GADD34, BCL2, CASP12, CASP3, CASP9, PINK1, PRKN, SOD2, TFAM, MFN1, FIS1, ZO-1, ZO-2, OCLN),* compared to the impact of THC and CBD (separately) on these markers in HC11 cells. We propose that the effects of THC and CBD (in combination) on HC11 cells are different from THC and CBD treatment alone, rather than simply being additive due to the addition of two compounds of equal concentration. This was confirmed by testing the impact of 20μM THC and 20μM CBD on the mRNA levels of key functional markers in HC11 cells (SI 2). Compared to the combination of 10μM THC and 10μM CBD, 20μM CBD significantly reduced levels of *STAT5A* (SI 2a)*, WAP* (SI 2b)*, FABP4* (SI 2c)*,* and *GLUT1* (SI 2d). However, the combination of THC and CBD does not act in a distinct manner on HC11 cells for all functional outcomes. The decrease in β-casein and WAP expression in HC11 cells treated with the combination of THC and CBD was not significantly different from cells treated with CBD only.

The unique effect of the combination of THC and CBD on HC11 cells, as observed for some of the biomarkers, may be a result of the different mechanisms of action of the mixture on the cells. In the presence of THC, CBD has been found to display high potency as an antagonist of CB1 and CB2 *in vitro* [86]. CBD may modify the effects of THC through various mechanisms. CBD has been reported to be a potent inhibitor of cytochrome P450 (CYP450), which metabolizes THC to 11-OH-THC [87]. Furthermore, the downregulation of cannabinoid receptors (in the presence of CBD) may cause THC to produce antagonism rather than agonism [86]. Similar results have been reported in the literature. Samarut et al. demonstrated a synergistic effect of a 1:1

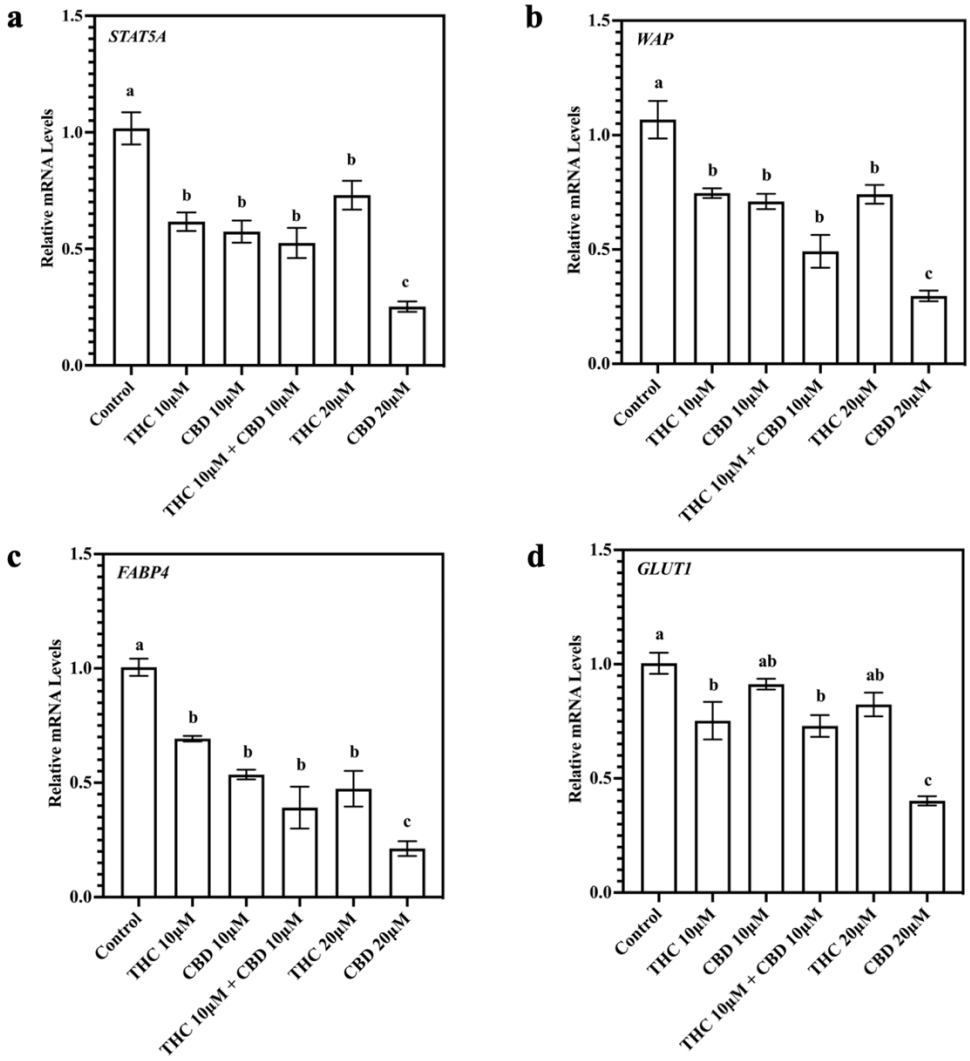
combination of THC and CBD at inducing neuro-hyperactivity in Zebrafish models[88] Furthermore, Jamontt et al. attributed an additive effect of the combination of THC and CBD in the model of colitis for the therapeutic benefit[89].

Altogether, this study supports that 10μM THC, 10μM CBD and the combination of 10μM THC and 10μM CBD induced ER stress in HC11 cells, resulting in the downregulation of the mRNA encoding biomarkers of cell differentiation, milk proteins, MEC tight junction formation, mitochondrial dynamics, oxidative stress, and ER stress. Furthermore, in combination, 10μM THC and 10μM CBD appear to act in a distinct manner on HC11 cells, resulting in increased ROS production and higher lactose release from the cells. The reduced mRNA levels of milk protein markers and induction of ER stress in HC11 cells upon THC and CBD treatment present a concern for cannabis use during gestation and lactation. This study helps elucidate a potential mechanism of action of the effect of THC and CBD on HC11 cells. Future studies must investigate the effect of THC and CBD on the ER structure and function in primary MECs and explore the effect of maternal cannabis use during pregnancy and lactation on the levels of milk proteins, lactose, immune factors, and other components that are synthesized and processed by the ER.

SUPPLEMENTARY FIGURES



SI 1 High concentrations of THC, CBD and the combination of THC and CBD reduced the viability of HC11 cells. HC11 cells were differentiated in base RPMI 1640 media, supplemented with PRL, INS, and DEX for 4 days, as described in the methods. Throughout differentiation, the HC11 cells were treated with vehicle control (0.1% methanol), 0.01, 0.1, 1, 10, 20, 30, or 100μM THC (a), 0.01, 0.1, 1, 10, 20, 30, or 100μM CBD (b), or 0.01, 0.1, 1, 10, 20, 30, or 100μM THC with equal amount of CBD in a 1:1 mixture (c). Changes in cellular toxicity were evaluated using the LDH assay and percent cytotoxicity (compared to the vehicle control: 0) was plotted, as described in the methods. All results were plotted as mean ± SEM and compared using one-way ANOVA. All experiments were repeated with 6 distinct replicates. Each experimental replicate was grown with cells from a different passage and statistically significant changes were indicated by distinct letters on the bar graphs.



SI 2 10μM THC, 10μM CBD, the combination of 10μM THC and 10μM CBD, 20μM THC, and 20μM CBD decreased the mRNA levels of *STAT5A, WAP, FABP4,* and *GLUT1* in HC11 cells. HC11 cells were differentiated in base RPMI 1640 media, supplemented with PRL, INS, and DEX for 4 days, as described in the methods. Throughout differentiation, the HC11 cells were treated with vehicle control (0.1% methanol), 10μM THC, 10μM CBD, or the mixture of 10μM THC and 10μM CBD, 20μM THC, or 20μM CBD for 4 days during differentiation. Cells from all conditions were processed for total RNA isolation, as described in the methods. The relative mRNA levels of *STAT5A* (a), *WAP* (b), *FABP4* (c), and *GLUT1* (d) were determined. Results from 6 replicates were referenced to *RPL0* and *RPL8* for mRNA analysis. All results were plotted as mean ± SEM and compared using one-way ANOVA. Each experimental replicate was grown with cells from a different passage and statistically significant changes were indicated by distinct letters on the bar graphs.

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DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST STATEMENT

The authors of this article declare no conflict of interest in relation to this article.

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CHAPTER 4: CANNABIS USE DURING LACTATION MAY ALTER THE COMPOSITION OF HUMAN BREAST MILK

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TITLE: Cannabis use during lactation may alter the composition of human breast milk

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IMPACT STATEMENT

- Metabolites of cannabis are found in breast milk and can accumulate in higher concentrations with ongoing consumption, which is concerning for potential exposure among infants born to mothers who consume cannabis.
- This work reports that lactose levels are increased and SIgA levels are decreased in the breast milk of cannabis users, relative to the milk of non-users.
- Change in levels of lactose and SIgA in the milk of cannabis users may have significant implications on infant health, which must be investigated in the future to better inform mothers.

ABSTRACT

Background: Cannabis is often used by women to manage symptoms of morning sickness during pregnancy, and post-partum stress and anxiety. While exclusive breastfeeding has been recommended for the first six months of an infant's life, presence of cannabinoids in the milk of cannabis users complicates this recommendation. The objective of this study was to investigate the effect of maternal cannabis use on changes in the levels of macronutrients and bioactive factors in breast milk. **Methods:** Milk was collected from women who were 6-8 weeks postpartum and were either using cannabis post-delivery, had used cannabis during pregnancy, or were non-users. Levels of cannabinoids, macronutrients, lactose, and SIgA were assessed in the milk of all subjects. **Results:** THC was detected in the milk of women who reported cannabis use during lactation (n=13, median: 22 ng/mL). Carboxy-THC, 11-hydroxy-THC, CBD and CBN were also detected in the milk of women who used cannabis post-partum. Relative to non-users (n=17), lactose levels were higher and SIgA levels were significantly lower in the milk of subjects who used cannabis during lactation (n=14). **Conclusions:** The presence of cannabinoids, along with altered lactose and SIgA levels in the milk of cannabis users may have implications for infant health.

INTRODUCTION

Cannabis has existed for over 6000 years[1], and has been used by women during pregnancy and lactation to manage nausea, vomiting, chronic pain, anxiety, as well as post-partum stress and fatigue[2]. Cannabis use during pregnancy has been found to negatively influence fetal growth[3,4], and postnatal exposure to cannabis through breastmilk has been associated with decreased infant motor development at one year of age[5]. Cannabinoids in maternal blood have been reported to transit the placenta[6], and into breast milk[7–9]. In a recent survey of Canadian pregnant women, most understood that cannabis could transfer to the fetus during pregnancy (94.3%), or to the infant while breastfeeding (91.2%)[10]. The American College of Obstetricians and Gynecologists has recommended women to discontinue the use of cannabis during pregnancy and lactation[11]. Despite the knowledge of its potential harm, cannabis use during pregnancy among women in their reproductive years has been increasing in recent years[12]. The self-reported prevalence of cannabis use during pregnancy are found to be between 2 and 11.3%[10,13–18], with rates higher among younger women and those with lower household incomes (14 to 28%)[19–21]. However, self-reports may underestimate actual use. In one study, the incidence of cannabis use was much higher when umbilical cord tissue samples were tested (22.4%) versus the self-reported rate of 6%[22]. Underreporting may arise from the threat of stigma, feelings of guilt or negative consequences of use during pregnancy and while breast feeding[23].

Metabolites of cannabis accumulate in lipid filled tissues at higher concentrations than other organs, such as the liver, brain and lungs[24]. The mammary gland is responsible for the production of breast milk, and it contains fat stores which have been suggested to allow long-term storage and slow release of cannabinoids into the breast milk during lactation[25]. In a population of women who used cannabis exclusively during breastfeeding, Δ^9-tetrahydrocannabinol (THC) was

detectable in the milk after 20 minutes, with maximum concentration of 94ng/mL THC in the breast milk at 1 hour post-use[7]. Furthermore, THC can remain in breast milk for up to 6 days after last reported use, with its levels being positively correlated with frequency of use[8]. Among 25 women with prenatal cannabis use, 12 reported abstinence. However, THC was detected in the milk of all women throughout the 6-week study period[9]. In addition to THC, levels of CBD (median: 4.99 ng/mL[8]; 1.2 ng/mL[9]), and 11-OH-THC (median: 2.38 ng/mL)[8] have been collected in the breast milk. Levels of other known cannabinoids, such as CBN, or other metabolites of THC, such as 11-nor-9-carboxy-THC (carboxy-THC) have not been reported in breast milk yet.

One study reported that from the 0.1 g of cannabis consumed by mothers, an estimated amount of 8 μg/kg/day may be transferred to the infant through the milk[7]. While exclusive breastfeeding has been recommended by the World Health Organization (WHO) for the first six month of an infant's life[26], presence of cannabinoids in the milk of users complicates this recommendation. Furthermore, the impact of maternal cannabis use breast milk composition has not been reported thus far. Breast milk has several important bioactive components which are associated with physiological functions for the infant. Lipids are the largest source of energy for the infant, contributing approximately 40-55% of the total energy in the breast milk[27,28]. Secretory immunoglobulin A (SIgA) is the predominant immunoglobulin found in human colostrum and mature milk (88-90%)[29], which performs a variety of functions, including obstructing bacterial adherence to epithelial cells and neutralizing toxins[30]. Lactose levels are positively correlated with milk production and is a stable component with little variability between mothers[28,31]. The objective of this study was to investigate the changes in lipid, protein, carbohydrate, lactose and SIgA levels in the milk of cannabis users and non-users. We hypothesized that macronutrient levels and bioactive components would be altered in the milk of cannabis users compared to non-users.

METHODS

Subject enrollment

This research protocol (Project number: 8152) was approved by the Hamilton Integrated Research Ethics Board (HiREB). Pregnant and recently postpartum women, who were either using cannabis or not using any substances (control group), were recruited out of pre-natal and ante-natal OB-GYN clinics and birthing units in the Hamilton area (Birthing Unit at St. Joseph's Healthcare Hamilton, 2 Fontbonne Obstetrics and Gynecology Clinic at St. Joseph's Healthcare Hamilton, and the Labour and Delivery Unit at McMaster University Medical Centre). All participants provided written and verbal consent and completed a questionnaire administered through the REDCAP system. Medical information from the time of delivery was collected from patient charts, and milk samples were obtained from participants between 6 to 8 weeks post-partum. Subjects were asked to provide us with 2 ounces (oz) of sample from their daily or weekly pumped milk, using a manual or electric pump. Subjects were presented with $25 for participation and milk samples were stored at -80^0C at McMaster University.

Cannabinoid analysis

To quantify levels of cannabinoids in the milk samples that were collected for this study, protocols from relevant studies were reviewed[32,33]. 11-nor-9-carboxy-THC (carboxy-THC), 11-hydroxy-THC (11-OH-THC), cannabidiol (CBD), cannabinol (CBN), and THC standards were purchased from Sigma Aldrich Canada. The analysis method for cannabinoids was developed and validated by the Centre for Microbial Chemical Biology (CMCB), McMaster University, Hamilton. In summary the milk samples that were collected for this study were extracted by a protein precipitation method (cold acetonitrile containing 1% (v/v) formic acid), followed by a lipid removal step (Agilent Captiva EMR – Lipid cartridge (6mL, 600mg). Samples were injected

into an Agilent 1290 Infinity II HPLC coupled to an Agilent 6495C iFunnel QQQ mass spectrometer (Agilent, Santa Clara, CA). Quantitation of each cannabinoid was based on the peak area measurement. Calibration curves were generated using an unweighted linear regression analysis of the standards and the concentration of each cannabinoid was calculated from the calibration curve. Quality control samples were processed and analyzed alongside samples, with one control solvent blank and one control blank breast milk sample were analyzed for any background. Control samples were fortified at two levels (low: 5ng/mL and high: 400ng/mL). All recoveries were within the acceptable mean range of 70-120% and a standard deviation of <20%. Method validation was performed at three levels, low (2ng/mL), mid (40ng/mL) and high 400ng/mL), each with three replicates, except the lowest level which was conducted with eight replicates to calculate the method detection limit (MDL) of 0.25 ng/mL for 11-OH-THC, CBD and CBN and 0.5 ng/mL for Carboxy-THC and THC respectively, The limit of quantification (LOQ) was defined as LOQ = 3 x MDL.

Macronutrient Analysis

Miris Human Milk Analyzer™ (Miris HMA™) was used to measure energy, fat, carbohydrates, and protein content in human milk samples. While true protein is a measure of only the proteins in milk, crude protein is a measure of all sources of nitrogen and includes non-protein nitrogen. Miris HMA™ was warmed to 40^0C, adjusted for zero-setting with *Miris Check* solution, and calibrated using *Miris Calibration Control 1* solution and *Miris Calibration Control 2* solution. Thawed breast milk samples were homogenized for 1.5 seconds/mL using the Miris ultrasonic processor. 1.5mL of each sample was then injected into the machine, in replicates of 2, for macronutrient measurement. The measured replicates were averaged for each sample, and the averaged values were plotted in bar graphs.

Human SIgA, Lactose and Protein Analysis

For analysis of SIgA and lactose levels, milk samples were de-fatted by centrifugation at 1000xg for 15 minutes, and re-centrifugation of the aqueous fraction at 3000xg for 15 minutes. Using the de-fatted milk samples, SIgA concentrations were measured using a Human IgA ELISA Kit (Abcam, Canada), and lactose levels were measured using the Lactose Assay (Abcam, Canada), according to the manufacturer's instructions.

Statistical Analysis

Statistical analyses were conducted using the GraphPad Prism Software (Prism 9 for macOS, Version 9.3.1) and the SPSS software (IBM, SPSS Statistics, Version 28.0.1.0). A student's t-test and descriptive statistics were used to compare the mean of conditions between non-users and cannabis users. One-way Analysis of Variance (ANOVA) was used to determine statistical differences between three or more groups, with Tukey's multiple comparisons test, using GraphPad. To determine the correlation between two variables, a simple linear regression analysis was conducted using GraphPad. Covariates, including Body Mass Index (BMI) and age were used to conduct Analysis of Covariance (ANCOVA) on the SPSS Software. The $p \leq 0.05$ significance level was used for statistical analysis.

RESULTS

A total of 78 women provided consent to be contacted for the study. Of these women, 50 were not using any substances and 28 had used cannabis either during pregnancy or in the early postpartum period (Table 1). Among the 32 non-users that were not enrolled, we were unable to reach 22 subjects after the initial meeting. Other subjects provided a variety of reasons for not participating in the study, which are listed in Table 1. Among the 18 non-users that were enrolled, 94% (n=17) completed the entire study, and one subject only completed the survey (Table 1). 28

cannabis users were recruited for this study, among which only 6 were not enrolled. Reasons provided by subjects for not continuing with the study have been outlined in Table 1. Among the 22 cannabis users, 86% (n=19) completed the entire study, and 3 users only completed the survey (Table 1). Little to no milk supply was one of the major reasons for not finishing the entire study.

The median age was 32 years (range: 23-43) among the 18 non-users, and 28.5 (range: 21-38) among the 22 cannabis users (Table 2). The mean age of cannabis users was significantly lower than non-users (95% CI, -3.34 ± 1.50, p-value = 0.03). Maternal education and marital status have been summarized in Table 2. 100% (n=18) of non-users and 86% (n=19) of cannabis users reported that they breastfed their infants and similar numbers of non-users and cannabis users were recommended to supplement their feed with formula in the first two weeks (Table 2). The average amounts of milk pumped by subjects have been summarized in Table 2. In the 6 months before pregnancy, 91% (n=20) of cannabis users reported using cannabis, compared to 22% (n=4) of non-users (Table 3). Among cannabis users, 82% (n=18) reported using it during pregnancy and 55% (n=12) reported use post-delivery. Among those who reported using cannabis during pregnancy, 94% (n=17) reported its use during the first trimester, 67% (n=12) used in the second trimester, and 56% (n=10) used in the third trimester. 44% (n=8) reported using cannabis in the entire pregnancy. During pregnancy, 94% (n=17) cannabis users reported use by smoking, and 67% (n=12) used it daily. Similarly, subjects who used cannabis post-delivery, 83% (n=10) consumed it via smoking and 67% (n=8) used it daily (Table 3). Concurrent use of cannabis with alcohol and cigarettes during pregnancy and/or post-delivery has been summarized in Table 3. In addition to self-use, most cannabis users reported cannabis usage by their partner or roommate, compared to fewer non-users reporting the same (Table 4).

Milk samples of all subjects were assessed for levels of carboxy-THC, 11-hydroxy-THC, CBD, CBN and THC. Cannabinoids were not detected in the milk samples collected from non-users or women who used cannabis during pregnancy. 14 milk samples collected from 13 cannabis users presented detectable levels of cannabinoids (Table 5). The 13 milk samples were collected from 12 women who reported cannabis use post-delivery, and one sample was collected from a woman who did not indicate period of cannabis usage. Among the 14 samples, 13 milk samples had detectable levels of THC (median = 22 ng/mL), 5 milk samples displayed carboxy-THC levels (median = 2.6 ng/mL), 3 milk samples showed 11-OH-THC levels (median = 6 ng/mL), 2 milk samples presented CBN levels (median = 7.15 ng/mL), and only 1 milk sample displayed 9.3 ng/mL of CBD. Mean, median, maximum and range of concentrations of all cannabinoids have been detailed in Table 5. The mean THC concentration was higher among subjects who reported daily use (117.7 ng/mL, n=6), compared to those who used it occasionally post-delivery (9.8 ng/mL, n=4). The milk sample that presented the highest level of THC (503 ng/mL), belonged to a user who reported daily consumption. It was also the only sample with detectable levels of CBD (9.3 ng/mL) and had presence of all cannabinoids tested (4.9 ng/mL carboxy-THC; 22 ng/mL 11-OH-THC; and 11 ng/mL CBN).

Only the milk samples of cannabis users that presented levels of cannabinoids were used in the following macronutrient analysis. These samples comprised the group defined as cannabis users. The levels of fat, energy, total solids, carbohydrates, crude protein, and true protein were not significantly different among milk samples of non-users and cannabis users (Figure 1). Lactose levels were significantly higher in the milk of cannabis users compared to the milk of non-users (95% CI, 1.18 ± 0.52, p-value = 0.04) (Figure 2). In contrast, relative to non-users, the milk of cannabis users displayed significantly lower levels of SIgA (95% CI, -0.17 ± 0.08, p-value = 0.04)

(Figure 2). The levels of carboxy-THC were positively correlated with crude protein (95% CI, p-value = 0.04) and true protein (95% CI, p-value = 0.03) (Figure 3). Similarly, 11-OH-THC levels were negatively correlated with carbohydrate levels (95% CI, p-value = 0.01) and SIgA levels (95% CI, p-value = 0.04) (Figure 3). As this is a pilot study on a topic that has not been investigated before, the sufficiency of the samples size was determined based on results from the IgA assay. The power calculation was conducted with alpha of 0.05, and beta (power) of 80%. The sample size of 13 was calculated. The macronutrient analysis was conducted in this study with 17 milk samples collected from non-users, and 14 milk samples collected from 13 cannabis users that presented detectable levels of cannabinoids.

Moreover, using BMI and age as covariates, we assessed whether macronutrient, lactose and SIgA levels differed among subjects. There were no differences between non-users and cannabis users, controlling for BMI. In comparison, greater lactose levels were found in the milk of cannabis users compared to non-users (95% CI, -1.39 ± 0.38, p-value = 0.01), adjusting for maternal age (Table 6). To assess the effect of cigarette smoking co-used with cannabis during lactation on milk composition, macronutrient levels in the milk of non-users were compared to the milk of subjects who used cannabis only and subjects who used cannabis and cigarettes. Compared to non-users, subjects who co-used cannabis and cigarettes showed lower carbohydrate levels (95% CI, 1.23 ± 0.475, p-value = 0.03), as well as greater crude protein (95% CI, -0.68 ± 0.22, p-value = 0.01), and true protein levels (95% CI, -0.59 ± 0.19, p-value = 0.01) (Figure 4). There was no effect of alcohol co-use on any of our outcome variables. Finally, compared to non-users, there were no differences in lactose and SIgA levels in the milk of subjects who used cannabis during pregnancy only (Figure 5). Table 7 reports the birth weight of infants of mothers who did not use cannabis during pregnancy (non-users, n=15), and of mothers who used cannabis during pregnancy

(cannabis users, n=8), adjusted with the mother's gestational age. The adjusted mean birth weights of infants born to mothers using cannabis were lower than birth weights of infants born to non-users by 155.573 grams, however this difference was not statistically significant (Table 7).

DISCUSSION

In this pilot study, we presented the impact of maternal cannabis use on the macronutrient composition of breast milk. On average, cannabis users were younger than non-users, which is similar to the trend reported by Wymore et al[9]. Majority of cannabis users and non-users enrolled in this study were well educated (i.e., attended college/university). Among the 22 cannabis users, only 3 subjects were unable to complete the entire study (i.e., finished survey by unable to donate milk). Not breastfeeding due to limited milk supply was reported as the primary reason by the 3 subjects for not finishing the study. A higher percentage of non-users (100%) reported breastfeeding their infants, relative to cannabis users (86.4%). In fact, cannabis-using mothers reported lower levels of milk production in the first, second, fourth- and sixth-week post-partum, compared to non-users. The differences in milk supply between cannabis users and non-users could be due to lower prolactin (PRL) levels. Frequent cannabis users have been reported to display lower baseline plasma PRL levels, relative to healthy controls[34], and lower serum PRL levels are correlated with decreased milk volume (mL/day)[35]. However, only 5 non-users and 8 cannabis users reported the volumes of their pumped milk in the survey. Thus, the data collected in this study is not sufficient to make conclusions regarding changes in milk volume. It can be hypothesized that cannabis intake may reduce milk supply by decreasing PRL levels.

Among the women who disclosed cannabis use, 91% indicated that they used it in the 6 months before pregnancy, suggesting a continuum of use despite gestation status. The pattern of cannabis use among users in this study varied during all trimesters of pregnancy, which was

consistent with other studies that have reported highest cannabis use in the first trimester, followed by lower rates of use in the second, and lowest use in the third trimester[10,36]. Majority of the cannabis users in this study admitted to smoking the cannabis and using it daily, which are trends reported among other pregnant women[8], and the general population in Canada[37].

Rates of concurrent use of cigarettes and cannabis in women during pregnancy have been reported between 45%[38] and 84.5%[39]. Cigarette smoking has been linked with reduced production of milk and shorter lactation periods[40–42]. Carbohydrate levels are correlated with lower milk volume[43], which may result in shorter lactation periods[44]. Thus, decreased carbohydrate levels in the milk of subjects who co-used cannabis and cigarettes (n=4) in this study may be partially due to smoking cigarettes. Maternal cigarette smoking has also been associated with lower protein levels in milk[45]. In contrast, in this study, co-use of cannabis and cigarettes (n=4) augmented protein levels in the milk. Protein levels are reported to decrease from colostrum to mature milk[46]. It can be hypothesized that the co-use of cannabis with cigarette smoking disrupts the process that decreases protein levels from colostrum to mature milk. Understanding the influence of co-use of cigarettes and cannabis has considerable implications for infant health.

Median THC concentrations reported in the milk samples of users enrolled in this study were comparable to the levels reported in other studies[7–9,47]. In this study, cannabinoids and the metabolites of THC were found in the milk of women who reported using cannabis post-delivery. However, the amounts and frequency of cannabis used per day were not recorded, and the milk samples were only collected at a single time point. In comparison, Moss et al. collected milk from subjects who used cannabis in the last 48 hours, as well as at 2 weeks and 2 months postpartum[47]. They reported that most women increased their weekly use of cannabis during lactation, which directly impacted the accumulation of THC in their milk as the median breast milk THC

concentration was higher from visit 1 to visit 2 by 30.2 ng/mL[47]. Although women are recommended to avoid cannabis use during lactation, it has been suggested that women should be strongly advised to avoid breastfeeding within 1 hour of inhaled use to decrease infant exposure to peak cannabinoid levels in breast milk[48]. This has been supported by results from a study conducted by Baker et al., which reported maximum THC concentration in the milk of the subjects at 1 hour following consumption of 0.1 g of cannabis, compared to later time points[7]. Therefore, knowledge of quantity and frequency of cannabis used, as well as collection of milk at multiple time points may provide further insight into the accumulation and effect of cannabis on the milk.

In this study, lactose levels were quantified to be higher in the milk of subjects who used cannabis during lactation. While this finding was interesting, it was unsettling as there were no significant differences in the levels of carbohydrates in the milk of cannabis users, relative to non-users. Because lactose has been identified as the most stable[49] and abundant carbohydrate in human milk (90-95%)[49,50], changes in its levels are expected to be reflected in the carbohydrate levels tested. A higher sample size and further investigation is required to make conclusions on the effect of maternal cannabis use on lactose levels in the milk.

Compared to non-users, decreased SIgA levels were detected in the milk of cannabis users. Though there is a high, naturally occurring variability in protein concentration, we speculate that the low SIgA concentrations could impact the immune profile of the milk. Furthermore, SIgA levels were significantly lower in the milk of cannabis users who did not report cigarette smoking compared to non-users (95% CI, 0.24 ± 0.09, p value = 0.01), which rules out the possibility of cigarette smoking in combination with cannabis use impacting the SIgA levels. However, postpartum-specific stress has been linked to reduced SIgA concentration in breast milk[51]. Stress relief is a major reason for cannabis use reported by women post-delivery[52]. Therefore, further

investigation is required to understand whether decreased SIgA levels in the milk of subjects using cannabis are directly associated with postpartum stress or cannabis use, or both. This is important because the presence of IgA in the milk is an essential source of immunity for the newborn[53], as the mucosal immune system is poorly developed at birth[54].

Finally, we reported that the adjusted mean birth weight of the infants born to cannabis users (using during pregnancy) were slightly lower than non-users (~213 grams), however the changes were not statistically significant. Evidence supports that cannabis use during gestation is linked to reduced birthweight[55]. However, there is limited information on the impact of cannabis use during lactation on infant health. In rat dams, THC exposure during the first 10 days of postnatal development resulted in neurobehavioral deficits in the pups[56]. Furthermore, birth weight in pups exposed to THC through breast milk was decreased by 9.62 grams ($p<0.0001$), compared to control pups, at postnatal day 10[56]. Future studies should investigate the impact of cannabis use on infant health outcomes, specifically neurodevelopmental and behavioral outcomes, as well as body weight and growth of infants exposed to cannabis through breast milk.

Although, a few authors have reported on the presence of cannabinoids in the milk of cannabis users, there are no studies reporting the effect of maternal cannabis use on breast milk composition[7,8,47]. We report that maternal cannabis consumption during lactation is associated with presence of several cannabinoids, including metabolites of THC, and attenuated IgA levels in the milk of users. This indicates that maternal cannabis consumption may impact infant's health due to direct cannabinoid exposure and altered milk composition. Further studies are needed to increase the body of evidence and to better inform mothers and the general population.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Competing interests: The authors have no conflicts of interest relevant to this article to disclose.

Consent statements: All subjects provided consent to participate in this study.

Ethics: This research protocol (Project number: 8152) was approved by the Hamilton Integrated Research Ethics Board (HiREB). HiREB represents the institutions of Hamilton Health Sciences, St. Joseph's Healthcare Hamilton, Research St. Joseph's-Hamilton, and the Faculty of Health Sciences at McMaster University and operates in compliance with and is constituted in accordance with the requirements of: The Tri-Council Policy Statement on Ethical Conduct of Research Involving Humans; The International Conference on Harmonization of Good Clinical Practices; Part C Division 5 of the Food and Drug Regulations of Health Canada, and the provisions of the Ontario Personal Health Information Protection Act 2004 and its applicable Regulations; For studies conducted at St. Joseph's Healthcare Hamilton, HiREB complies with the Health Ethics Guide of the Catholic Alliance of Canada.

FIGURES

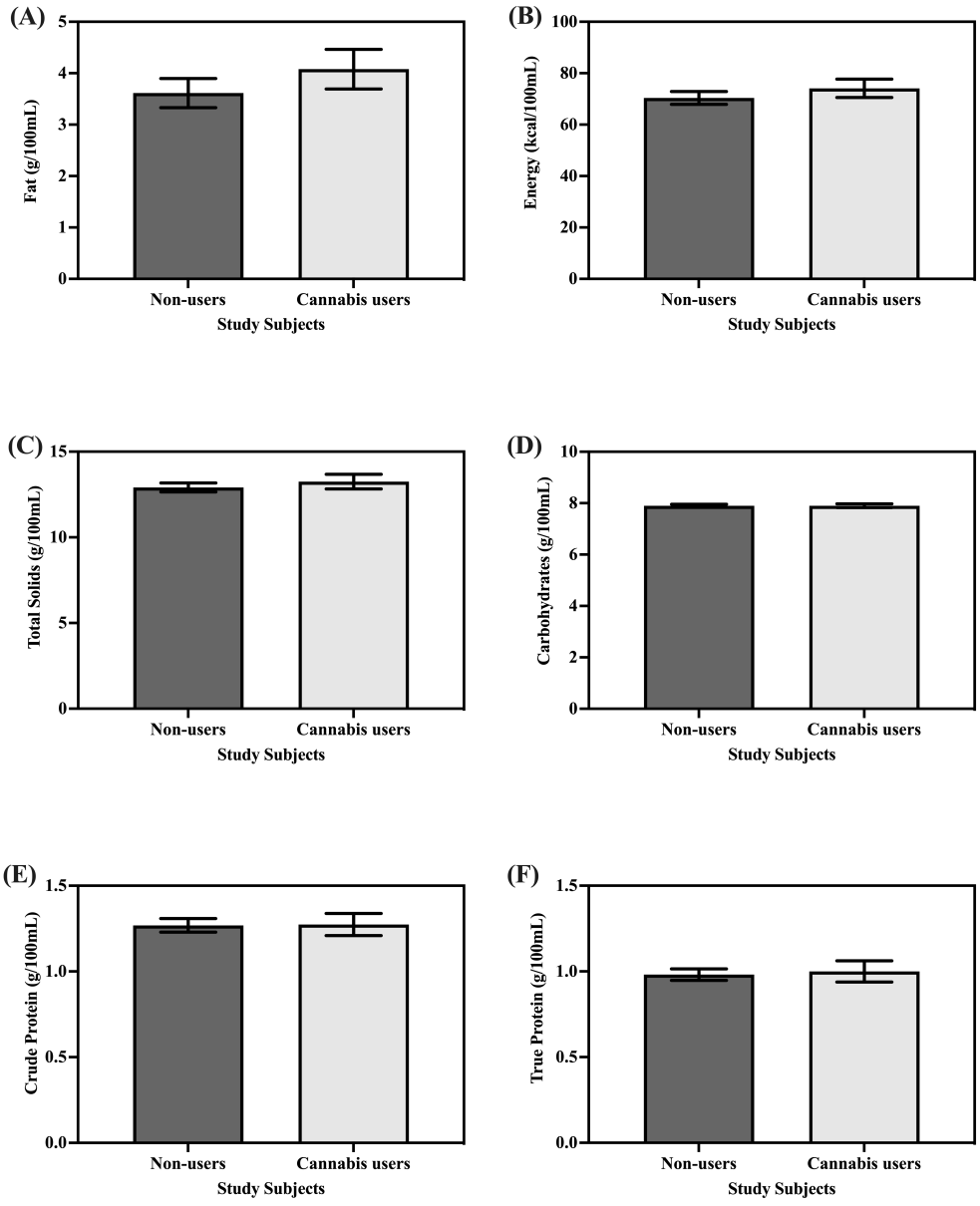

Figure 1. Macronutrient levels in the milk of cannabis users were not different from the milk of non-users. (A-F) Levels of fat, energy, total solids, carbohydrates, crude, and true protein were analyzed in the milk of non-users (n=17) and cannabis users (n=14), using the Miris Human Milk Analyzer™. Results were plotted as mean ± SEM, compared using a Student's T-test, with $p \leq 0.05$ used to assess significance.

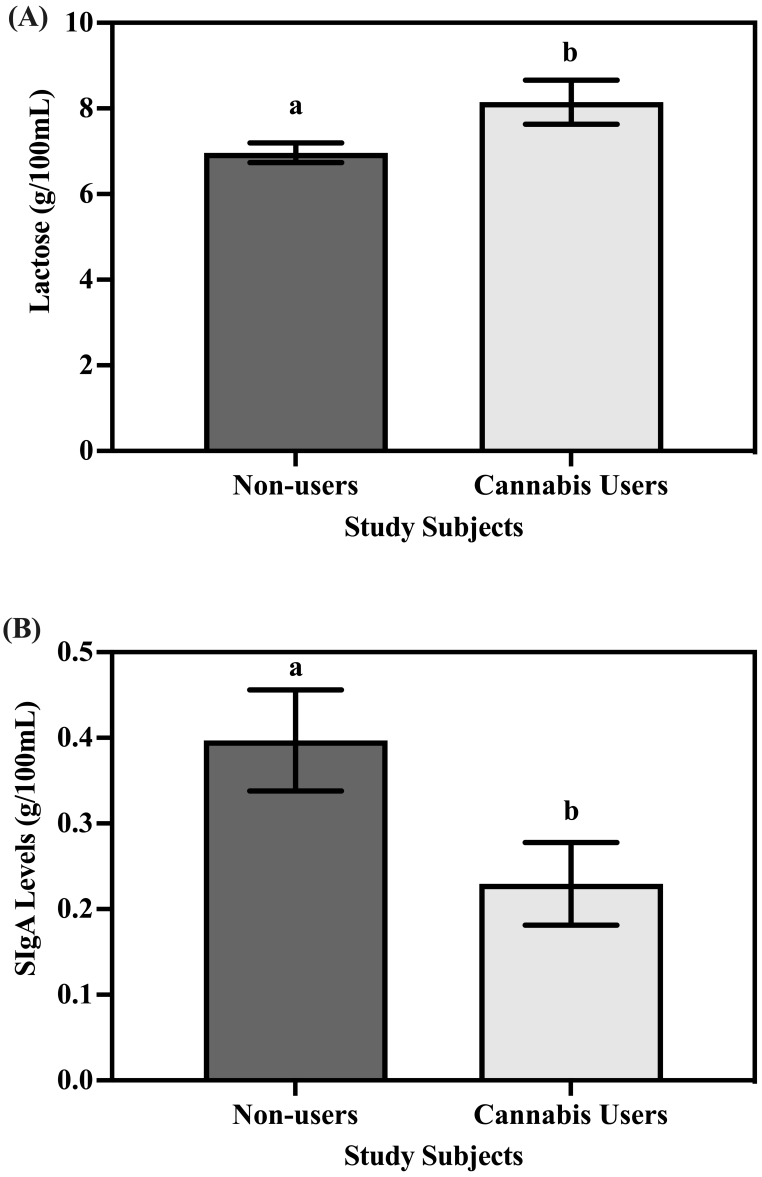

Figure 2. Lactose levels were higher and SIgA levels were lower in the milk of cannabis users relative to non-users. (A) Higher levels of lactose (p-value = 0.0136), and (B) lower levels of SIgA (p-value = 0.0427) in the milk samples of cannabis users (n=14), relative to non-users (n=17), were plotted as mean ± SEM. A Student's T-test was used to perform statistical analysis, with $p \leq 0.05$ used to assess significance. Statistically significant differences were represented by distinct letters on the bar graphs.

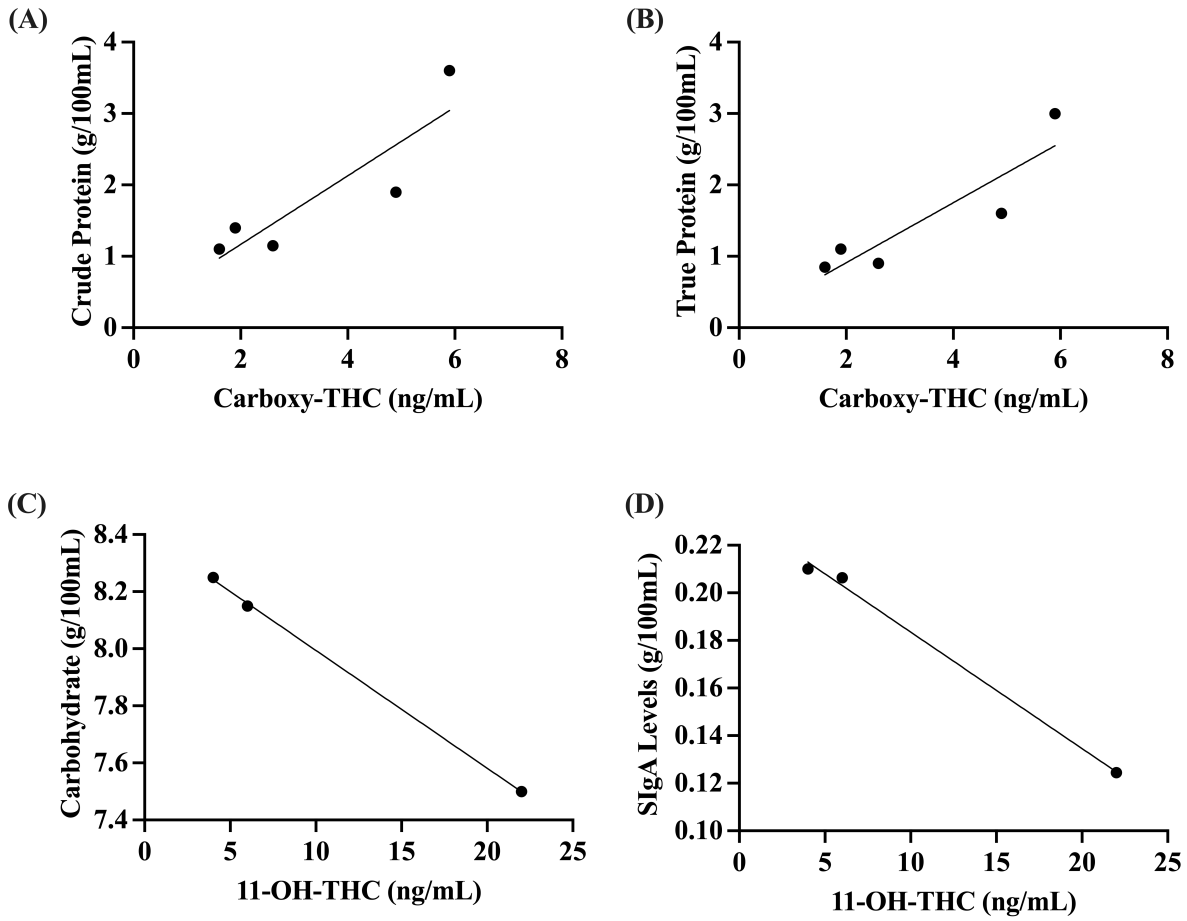

Figure 3. Correlation of Carboxy-THC and 11-OH-THC with protein and SIgA levels in the milk of cannabis users. (A-B) Increasing levels of Carboxy-THC levels (n=5) were positively correlated with crude protein (p-value = 0.0460) and true protein (p-value = 0.0382). (C-D) Increasing levels of 11-OH-THC (n=3) were negatively correlated with carbohydrate (p-value = 0.0137) and SIgA levels (p-value = 0.0408). Correlation was assessed by conducting simple linear regression analysis, with $p \leq 0.05$ used to assess significance.

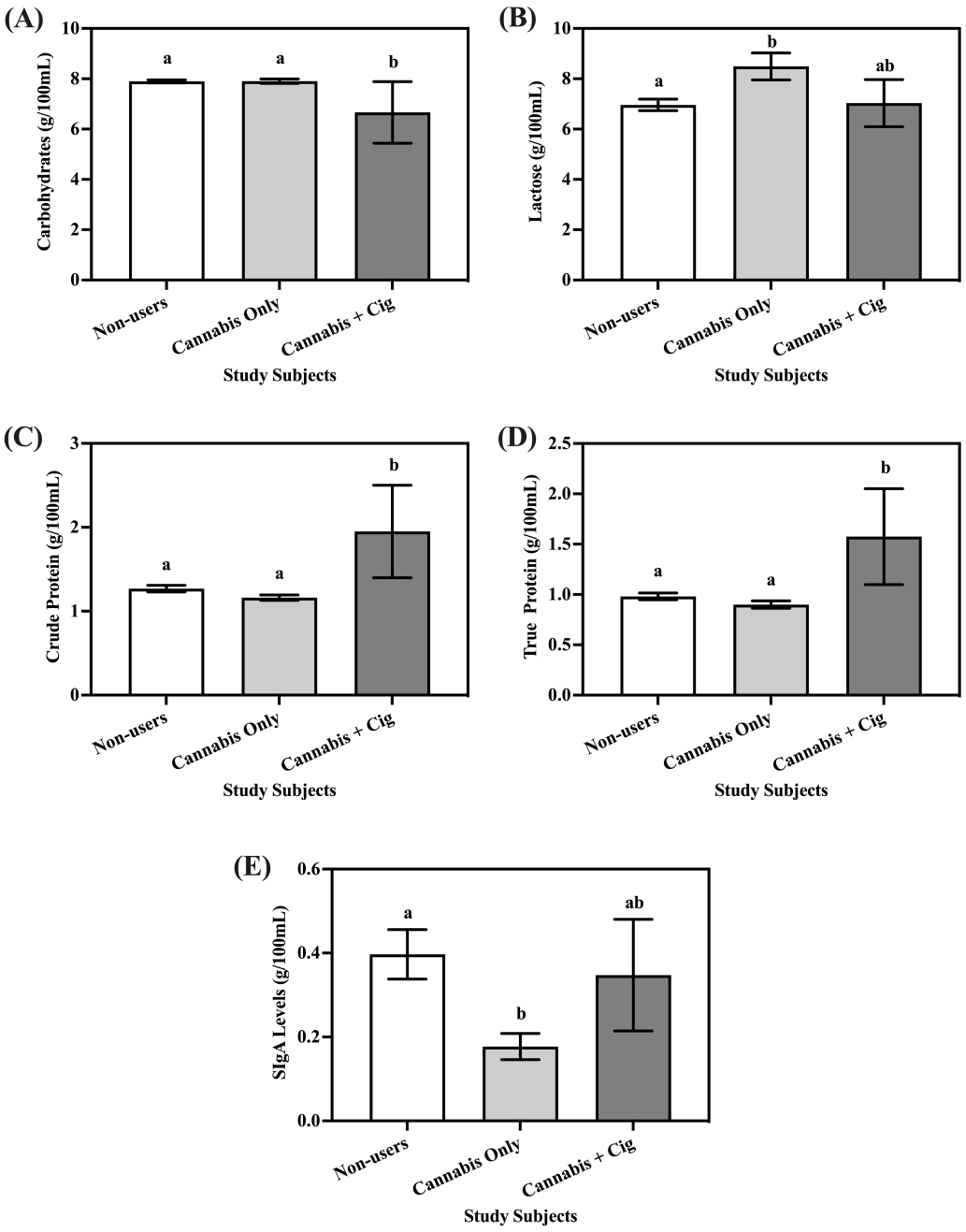

Figure 4. Macronutrient, lactose and SIgA levels in the milk of cannabis and cigarette co-users relative to non-users and cannabis users. (A-E) Levels of carbohydrates, lactose, crude protein, true protein and SIgA in the milk of non-users (n=17), cannabis (only) users (n=10), and co-users of cannabis and cigarettes (n=4) were plotted as mean ± SEM. One-way ANOVA was

conducted to determine statistical changes, with $p \leq 0.05$ used to assess significance. Statistically significant differences were represented by distinct letters on the bar graphs.

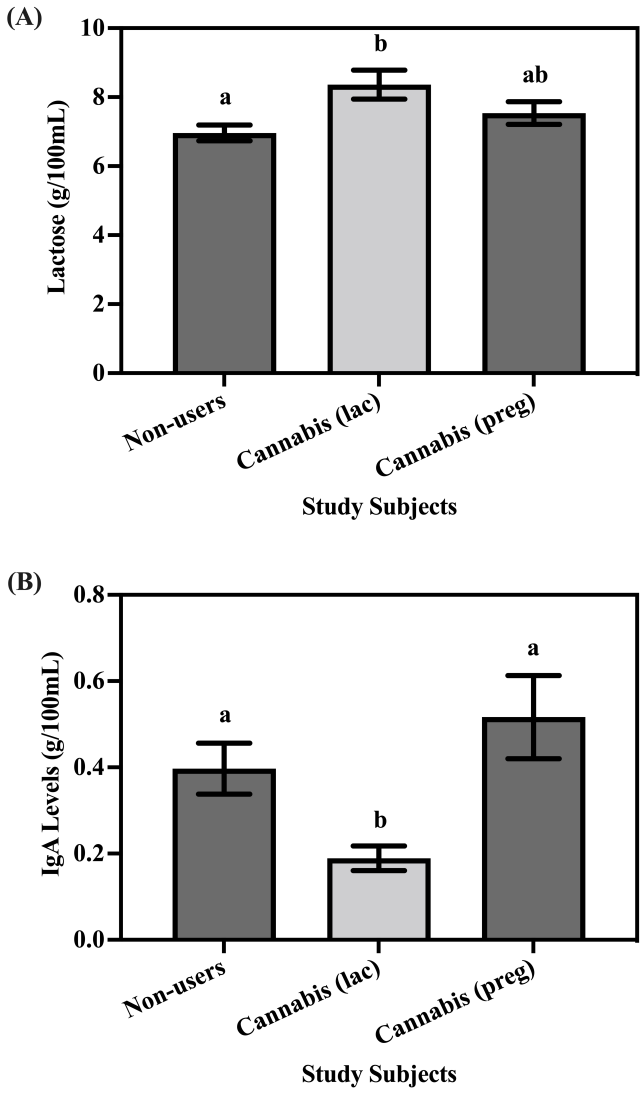

Figure 5. Lactose and SIgA levels in the milk of non-users and cannabis users. Lactose and SIgA levels in the milk of non-users (n=17), subjects who used cannabis during pregnancy "cannabis (preg)" (n=7), and subjects who used cannabis during lactation "cannabis (lac)" (n=14) were plotted as mean ± SEM. One-way ANOVA was conducted to assess statistical differences, with $p \leq 0.05$ used to determine significance. Statistically significant differences were represented by distinct letters on the bar graphs.

TABLES

Table 1. Number of recruited and enrolled non-users and cannabis users in the study

		Non-users	Cannabis users
Recruited subjects		50	28
No of subjects recruited but not enrolled in the study		32	6
Reasons for not participating in the study	Could not produce milk/low milk supply	2	-
	Was not breastfeeding	1	1
	Unable to get in touch	22	4
	Does not pump	2	-
	Busy	1	-
	Not interested in the study	1	1
	Past 9 weeks	3	-
	Did not show up to their 6-week visit	-	-
Enrolled subjects		18	22
	Finished the entire study	17	19
	Finished survey only	1	3

Table 2. Age, education, marital status and breastfeeding practices of subjects in the study

	Non-users (N=18); N (%)	**Cannabis users (N=22); N (%)**
Age (years)		
18-24	1 (6%)	4 (18%)
25-31	5 (28%)	12 (55%)
32-45	10 (56%)	6 (27%)
Unidentified	2 (11%)	0
Highest level of education completed		
High school	2 (11%)	9 (41%)
College/University	10 (56%)	12 (54.5%)
Graduate/professional school	6 (33%)	1 (4.5%)
Marital Status		
Single	1 (5%)	5 (23%)
Separated/Divorced	0	1 (4.5%)
Common Law	3 (17%)	7 (32%)
Married	12 (67%)	3 (13.6%)
Unidentified	2 (11%)	6 (27%)
Breastfeeding	18 (100%)	19 (86.4%)
Recommended to supplement with formula in the first 2 weeks	7 (39%)	7 (32%)
Recommended to use an electric pump to pump milk	6 (33.33%)	10 (45.5%)
The average amount of milk pumped in first week, second week, 6th week at one time? (mL per pump)		
First Week:	10-150mL	30-60mL
Second Week:	20-240mL	60-120mL
Fourth Week:	40-240mL	60-120mL
Sixth Week:	80-900mL	90-150mL

Table 3. Usage patterns of cannabis, cigarettes, and alcohol among subjects in the study

		Non-users (N=18); N (%)	**Cannabis users (N=22); N (%)**
Cannabis use in the six months before pregnancy		**4 (22%)**	**20 (91%)**
Cannabis use during pregnancy		**0**	**18 (82%)**
	1st Trimester	-	17 (94%)
	2nd Trimester	-	12 (66.7%)
	3rd Trimester	-	10 (55.6%)
	Entire Pregnancy	-	8 (44%)
	Type of cannabis used during pregnancy		
	Smoking flower/joints/pipe/bong	-	17 (94%)
	Smoking oil/shatter/concentrate/vaporizing	-	4 (22%)
	Edibles	-	3 (16.7%)
	Frequency of cannabis used during pregnancy		
	Daily	-	12 (66.7%)
	Occasionally	-	2 (11%)
	A few times per week	-	3 (16.7%)
Cannabis use post-delivery		**0**	**12 (55%)**
	Type of cannabis used post-delivery		
	Smoking flower/joints/pipe/bong	-	10 (83%)
	Smoking oil/shatter/concentrate/vaporizing	-	2 (16.7%)
	Edibles	-	3 (25%)
	Frequency of cannabis used post-delivery		
	Daily	-	8 (66.7%)
	Occasionally	-	2 (16.7%)
	A few times per week	-	1 (8%)
Cigarette use before the knowledge of pregnancy		**0**	**2 (9%)**
Cigarette use during pregnancy		**0**	**9 (41%)**
Cigarette use post-delivery		**0**	**8 (36.4%)**
Cigarette use during pregnancy and post-delivery		**0**	**7 (32%)**
Alcohol consumption before the knowledge of pregnancy		**2 (11%)**	**4 (20%)**
Alcohol consumption during pregnancy		**0**	**0**
Alcohol consumption post-delivery		**9 (50%)**	**13 (59%)**
Alcohol consumption during pregnancy and post-delivery		**0**	**0**
Cannabis + cigarette use during pregnancy		**0**	**7 (32%)**
Cannabis + cigarette use during post-delivery		**0**	**3 (14%)**
Cannabis + cigarette + alcohol use post-delivery		**0**	**3 (14%)**

Table 4. Cannabis usage patterns by partner or roommate of subjects in the study

	Non-users (N=18); N (%)	Cannabis users (N=22); N (%)
Cannabis use by partner (smoking/vaping)		
Yes, in the house/around me	0	5 (22.7%)
Yes, but not in the house/around me	6 (33.33%)	10 (45.5%)
No	12 (6.66%)	7 (31.8%)
Cigarette use by partner		
Yes, in the house/around me	0	1 (4.5%)
Yes, but not in the house/around me	1 (5.6%)	8 (36.4%)
No	17 (94.4%)	13 (59.1%)
Cannabis use by anyone else in the household (smoking/vaping)		
Yes, in the house/around me	0	2 (9%)
Yes, but not in the house/around me	0	3 (13.6%)
Frequency of cannabis user by partner or roommate		
Daily	0	6 (27%)
Occasionally	2 (11.11%)	1 (4.5%)
A few times per week	1 (5.6%)	0
No one smokes or vapes around me	15 (83.3%)	15 (68.2%)

Table 5. Cannabinoid levels in the milk of women who used cannabis during lactation

	THC	Carboxy-THC	11-OH-THC	CBD	CBN
Number of samples with detectable level of each cannabinoid	13	5	3	1	2
Minimum (ng/mL)	0.625	1.6	4	9.3	3.3
Median (ng/mL)	22	2.6	6	9.3	7.15
Maximum (ng/mL)	503	5.9	22	9.3	11
Range (ng/mL)	502.4	4.3	18	-	7.7
Mean (ng/mL)	60.03	3.38	10.67	9.3	7.15

Table 6. Covariate analysis of lactose levels in non-users and cannabis users

Lactose Levels			95% Confidence Interval		
	Mean	**Std. Error**	**Lower Bound**	**Upper Bound**	**Sig. (p-value)**
NON-USERS	6.788	.371	6.025	7.551	.017
CANNABIS USERS	8.181	.385	7.390	8.972	
Covariates appearing in the model are evaluated at the following values: Age = 30.5517					

Table 7. Birth weights of infants of non-using and cannabis using mothers

	Non-users (N=16)	**Cannabis users (pregnancy, N=9)**
Mean (± STD) (grams)	3400 ± 604.2	3187 ± 430
Mean (± SEM) (grams)	3400 ± 151.1	3187 ± 143.3
Minimum (grams)	2460	2300
Maximum (grams)	4400	3660
Range (grams)	1940	1360

CHAPTER 5: CONCLUSION

The mammary gland (MG) is a dynamic organ that produces breast milk for the nourishment and survival of the newborn [193]. Among all mammals, the MG's structure, development, and breast milk composition (i.e., carbohydrates, fat globules, casein micelles, and milk proteins) are highly conserved [193]. Exclusive breastfeeding has been recommended by the World Health Organization (WHO) for the first 6 months of an infant's life [194]. Major developmental changes take place in an infant's life in the first 6 months, including the growth of the infant's brain from ~350g at birth to 925g at 1 year [195]. Docosahexaenoic acid (DHA), an omega-3 fatty acid found in breast milk, is an essential fatty acid that plays a significant role in infant neurodevelopment [195]. The consumption of substances, such as nicotine (humans) [40] and cannabis (mice dams) [196] by lactating mothers, has been demonstrated to influence the lipid composition of the milk. The impact of cannabis consumption during pregnancy and lactation on breast milk composition and infant health in humans has not been deeply explored yet.

Cannabis users have been reported to exhibit decreased baseline plasma prolactin (PRL) levels, relative to healthy controls [197]. During pregnancy, PRL induces the differentiation of mammary epithelial cells (MECs) into specialized alveolar structures, which synthesize and secrete milk during lactation [193]. Thus, cannabis use during pregnancy and lactation may impact MG development and MEC differentiation. In 1971, Borgen and colleagues reported that long-term (days 1-20 of gestation) administration of 50 mg/kg THC in pregnant rats caused postnatal mortality of pups due to insufficient lactation [198]. Then, in 1978, Raine et al. reported that the MG of control mice increased significantly in weight between late pregnancy (day 17) and early lactation (day 2), and again between early (day 2) and peak lactation (day 12) [177]. However, both increases were inhibited in mice treated each day with 25mg/kg THC [177]. Furthermore,

LPL activity increased in control mice between late pregnancy and early lactation, however, no significant increase was observed in the MG of mice treated with THC [177]. LPL activity in the MG has been reported to be promoted by PRL and has been strongly correlated with lactation [199]. Raine et al. proposed that decreased growth of the MG and lower LPL activity may be attributed to decreased PRL levels (50%) in THC-treated mice in early lactation compared to control mice [177].

Although the studies conducted by Borgen et al. and Raine et al. involved the treatment of mice with a very high dosage of THC, both studies produced enticing evidence implicating the negative impact of THC on MG development and breast milk production. No study since then has further explored the effects of cannabis on MG development and MEC differentiation. The MG development that takes place during pregnancy for successful lactation is accompanied by alveolar differentiation of MECs [200]. Compromised development of the MG has been demonstrated to result in reduced milk yield and modified milk composition [201]. Thus, a stronger understanding of the mechanistic pathways in MECs which are impacted by cannabinoids will provide insight into changes that may be taking place in breast milk production and composition due to cannabis consumption during pregnancy and lactation.

i. The effect of THC and CBD on HC11 mammary epithelial cell function

The major aim of our *in vitro* work was to understand the effects of THC and CBD and their mechanism of action on the alveolar differentiation of MECs. Using the HC11 cell line, which is a well-characterized model of alveolar MECs [202–204], we investigated the impact of THC and CBD on HC11 cell differentiation and function. Our results demonstrated that THC and CBD (independently) decreased the mRNA levels of milk protein and lipogenic genes and reduced β-casein expression and lipid levels in HC11 cells (Chapter 2). These functional outcomes have been

used to characterize the alveolar differentiation of MECs [10]. Changes in lipid levels within MECs and milk protein expression (gene and protein), upon treatment with THC and CBD, provide evidence of the negative impact of cannabinoids on MEC function and possibly MEC differentiation. One of the primary reasons for weaning off infants prior to 6 months has been cited to be "not producing enough milk" [205]. Decreased levels of lipids and milk proteins synthesized by MECs upon cannabis consumption may result in an insufficient milk supply. In mice, decreased expression of milk protein genes has been linked with failed secretory activation, which is defined as the onset of milk secretion [206]. Therefore, we hypothesize that cannabis use in the perinatal period may be linked to insufficient milk supply, and possibly premature weaning.

Next, we reported that HC11 cells presented the gene and protein expression of cannabinoid receptor 2 (CB2). This is consistent with the presence of CB2 reported in the murine 4T1 and human MCF7 mammary carcinoma cell lines *in vitro* [207]. Based on this discovery, we hypothesized that the effects of THC and CBD on HC11 cells may be mediated via the CB2 receptor. This research question was investigated by co-treatment of HC11 cells with THC and AM630 (a selective antagonist of CB2 [208]), and co-treatment of HC11 cells with CBD and the selective CB2 agonist, JWH133 [155]. THC has been characterized as a partial agonist of CB2 [161], and CBD has been characterized as an inverse agonist of CB2 [161]. Agonists act by binding to the cannabinoid receptors, increasing their activity, and blocking other ligands from accessing the receptor [144]. Conversely, inverse agonists bind to an alternative site on the receptor, decreasing the active receptor's fraction and suppressing basal signalling [144]. While a neutral antagonist does not perturb signalling from its basal state, an inverse agonist can lower the signal beyond basal levels [144]. Hence, the HC11 cells were treated with a selective antagonist of CB2 (AM630) to block THC from accessing CB2. Conversely, HC11 cells were treated with a CB2

agonist (JWH133) to increase the activity of CB2, which may be getting reduced by the inverse agonistic activity of CBD at CB2. We reported that AM630 and JWH133 restored the mRNA levels of key genes (*CSN2*, *HK2* and *FABP4*), which were reduced by THC and CBD (Chapter 2). However, the decrease in mRNA levels of *WAP,* which encodes for the whey acidic protein, upon THC and CBD treatment was not fully recovered by AM630 or JWH133, respectively (Appendix A, Fig A1). Thus, the activity of THC and CBD on HC11 MECs may be partially mediated through CB2, and possibly through a variety of other mechanisms as well.

In addition to CB2, THC and CBD have been reported to interact with various other cannabinoid receptors, including CB1, G-protein coupled receptor 55 (GPR55), and TRPV1 [144,146]. Treatment of human trophoblast cells (BeWo) with increasing doses of THC (3-30μM) for 24 hours resulted in the activation of the unfolded protein response (UPR) pathway [183]. The effects of THC on the endoplasmic reticulum (ER) stress markers were reversed by the treatment of BeWo cells with SR141716 (CB1 receptor antagonist) and SR144528 (CB2 receptor antagonist) [183]. These findings indicate that THC acts through CB1, along with CB2, on human trophoblast cells (BeWo). Furthermore, this study also highlighted that THC has the capability to induce ER stress in human trophoblast cells (BeWo) leading to cellular dysfunction. Likewise, CBD has been reported to induce an influx of Ca^{2+} via the TRPV1 receptor, resulting in increased ROS production and initiation of ER stress in MCF7 breast cancer cells [184]. Co-treatment of the MCF7 cells with CBD and a TRPV1 antagonist resulted in increased cell viability and decreased ER stress [184]. These findings suggest that (1) THC and CBD act through various cannabinoid receptors to mediate their effects on the cells, and (2) THC and CBD cause cellular stress, specifically ER stress, in various cell types, resulting in cellular dysfunction and apoptosis. Future steps can involve investigating the effects of THC and CBD on other cannabinoid receptors, such as CB1,

CB2, GPR55 and TRPV1, and exploring whether normalizing the levels of these receptors with the appropriate agonists/antagonists block the effects of THC and CBD on HC11 MECs. Overall, understanding the impact of THC and CBD on the endocannabinoid system (ECS) signalling in the MECs will delineate mechanistic pathways that may be induced/altered by THC and CBD, resulting in their effects on MEC differentiation and function.

ii. The effect of THC, CBD, and the combination of THC and CBD on HC11 cell differentiation and ER function

In Chapter 2, we reported that THC and CBD, reduce the gene expression of milk proteins and lipogenic markers, as well as decreased lipid levels in HC11 cells. Milk protein expression and lipid accumulation are functional outcomes of MEC (alveolar) differentiation [193]. Thus, in Chapter 3 we investigated whether THC and CBD impact the HC11 cellular differentiation. Furthermore, both THC and CBD have been demonstrated to evoke ER stress and oxidative stress in various cell types [183,184]. CBD-induced ER stress has been demonstrated to result in pro-apoptotic signalling and cellular death in hepatic stellate cells [209]. Similarly, THC has been reported to cause ER stress-induced cell death in pancreatic cancer cells [210]. Therefore, we also assessed the impact of THC and CBD on the ER function in HC11 cells. Finally, the cannabinoids detected in the milk of cannabis-using women are often a combination of THC and CBD [211,212]. Hence, in Chapter 3, we investigated the effect of THC and CBD, separately and in a 1:1 combination, on MEC differentiation, and function, and we examined whether these cannabinoids stress cellular organelles, such as the ER and the mitochondria, resulting in the impaired differentiation and function of HC11 MECs.

We reported that THC and CBD, separately and in a 1:1 combination, reduced the mRNA levels of genes responsible for encoding differentiation markers, key transcription factors, and

milk protein and lipogenic markers (Chapter 3). These changes were accompanied by reduced lipid levels in HC11 cells treated with the cannabinoids, relative to control. Impaired MEC differentiation and function have been reported to result in delayed and abnormal MG development, which is accompanied by decreased expression of transcription factors, such as AKT1, STAT5, and milk proteins (β-casein and WAP) [213]. The negative impact of THC and CBD on differentiation markers in HC11 cells suggests that cannabis use may lead to disruptions in MG development. A future study that utilizes *ex vivo* explants of the MG, or an *in vivo* study, must be conducted to confirm the impact of cannabinoids on MG development (structurally).

Stages I and stage II lactogenesis, which take place to synthesize and secrete milk components, require high levels of energy from the MG [67]. This energy is used for protein biosynthesis, gluconeogenesis, lipid synthesis, and initial protein maturation [67]. Most of these processes, specifically the creation, folding, classification and distribution of proteins to the correct cellular destination in the MECs take place within the ER [68]. Disruption in the activity of the ER activity leads to ER stress, which initiates the UPR to mediate the stress. Stimulation of ER stress leads to the release of Ca^{2+} from the ER into the mitochondria, which results in mitochondrial dysfunction and initiation of cell death [69]. In bovine MECs, Zearalenone-induced ER stress caused cell death, increased ROS levels, decreased mitochondrial membrane potential, and increased the expression of ER stress markers (ATF6 and CHOP) [214]. Our results demonstrated that THC and CBD, separately and in a 1:1 combination, induced the gene expression of UPR markers, altered the mRNA levels of markers involved in mitochondrial dynamics, and decreased the gene expression of tight junction proteins in HC11 cells. Finally, the decrease in the mRNA levels of milk protein and lipogenic genes was recovered upon co-treatment of the cannabinoids with an ER stress inhibitor (4-PBA) (Chapter 3). Altogether, these results indicated that THC and

CBD, individually and in a 1:1 combination, induced ER stress within HC11 MECs, resulting in the altered differentiation and function of these cells.

Although 10μM THC, 10μM CBD, and the combination of 10μM THC and 10μM CBD, did not induce significant cell death in HC11 cells, the cannabinoids at this concentration induced pro-apoptotic signalling at the mRNA level (i.e., ATF4 and CHOP) in HC11 cells (Chapter 3). During gestation, overexpression of ATF4 has been associated with reduced proliferation and differentiation of the mammary alveolar epithelium in mice, accompanied by decreased expression of α-lactalbumin and WAP, and STAT5A phosphorylation [215]. These findings indicate that increased expression of ATF4 during pregnancy leads to impaired MG development [215]. CHOP has been reported to be the first apoptotic protein induced by ER stress and its expression is induced downstream of the PERK-ATF4 and ATF6 pathways [81,82]. Following induction, CHOP activates various other markers that are functionally associated with apoptosis [67]. The gene expression of *CHOP* in bovine MECs has been negatively correlated with milk yield [56].

Since milk yield is an outcome of functional MECs, the decrease in MECs due to cellular apoptosis and altered signaling has the potential to negatively impact milk volume [216]. In fact, the detection of ER stress markers (ATF6 and caspase 12) in the milk of goats has been accompanied by premature cessation of lactation [74]. Therefore, results from the first two chapters of this study provide evidence to hypothesize that cannabis use in the perinatal period may induce ER stress in the MECs, resulting in abnormal MG development, altered breast milk composition (due to impaired capacity of the ER to synthesize lipid and milk proteins) and reduced breast milk production (due to MEC apoptosis). This is a significant issue that must be further investigated because milk insufficiency has been cited as the most common reason underlying the low percentage of women breastfeeding past the first 6 months of an infant's life [217].

iii. Cannabis use during lactation reduces SIgA levels in the milk of users

50% of milk lipids are synthesized in the MG, triacylglycerols are synthesized in the ER [218], and milk protein folding occurs in the ER [219]. In this study, we have reported that THC and CBD induced ER stress in HC11 MECs, resulting in the downregulation of MEC differentiation and function (reduced lipid levels) [220]. Thus, cannabis use in the perinatal period may be linked with altered milk production (lower yield) and composition (altered levels of milk proteins, lipids and bioactive factors). We conducted a pilot study with human subjects to investigate the effect of maternal cannabis use during pregnancy and lactation on milk production and composition. Human milk is the primary source of nutrition and immune factors for the infant at birth [221]. Thus, we assessed the changes in the levels of macronutrients and one immune factor, namely secretory immunoglobulin A (SIgA), in the milk of all subjects. In addition to collecting milk, we collected data from subjects regarding their age, marital status, and usage.

Literature has reported that younger age, being unmarried, and having lower levels of education are significantly associated with cannabis use during pregnancy [222,223]. Consistent with these findings, we found that the median age of non-users (n=17) was higher than cannabis users (women who reported using cannabis during pregnancy and/or lactation) (n=22) (Chapter 4). Most non-users (67%) were married compared to only 3 (~14%) cannabis users being married. In contrast, most cannabis users and non-users attended college/university in our study. The major difference in the educational background within the two groups was that compared to 6 non-users who attended graduate school, only one cannabis user attended graduate/professional school.

We also found that 41% of cannabis users in our study reported smoking cigarettes during pregnancy, 36% used cigarettes post-delivery, and 32% smoked during pregnancy and postpartum (Chapter 4). Compared to the milk of non-smokers, the breast milk of mothers who smoked

cigarettes displayed higher concentrations of monounsaturated and polyunsaturated fatty acids [40]. Furthermore, maternal cigarette smoking in early pregnancy has been associated with lower levels of DHA in the milk at 1 month postpartum [41]. Since cannabis has been reported to be co-used with cigarettes, future studies should involve the investigation of the impact of concurrent use of cannabis and cigarettes on breast milk composition. Since cigarette smoking has been associated with reduced production of milk and shorter lactation periods [224], cigarette-smoking mothers may not have enough supply for donation to research studies. This is an important consideration because this analysis will require milk from cigarette-smoking mothers as a control, in addition to non-users. Collection of milk at an earlier time point (i.e., 1-2 weeks postpartum) may increase the chances of obtaining these rare samples.

Upon determining the impact of cannabis use on milk composition, we observed that among the 14 milk samples of women who reported cannabis use during lactation, 13 milk samples had detectable levels of THC (median = 22 ng/mL), 5 milk samples displayed carboxy-THC levels (median = 2.6 ng/mL), 3 milk samples displayed 11-OH-THC levels (median = 6 ng/mL), 2 milk samples presented CBN levels (median = 7.15 ng/mL), and 1 milk sample showed 9.3 ng/mL of CBD (Chapter 4). This is consistent with what has been reported in the literature [138,211,212], however, the presence of cannabinol or carboxy-THC has not been reported in the milk of cannabis users in any other studies. In addition, one subject in our study admitted to using cannabis during pregnancy and donated a milk sample that was collected at 6 weeks postpartum, with no use of cannabis during the 6 weeks of lactation. The same subject donated another milk sample at 6.5-week postpartum following cannabis use (right before collection). We found that the first milk sample contained no cannabinoids in the milk, while the second milk sample presented detectable levels of THC in the milk. Although this result is not conclusive, as it is obtained from one subject,

it suggests that the accumulation of cannabinoids in the milk of users is limited to a time point of fewer than 6 weeks. Future studies should involve the collection of milk from women at 4 and 8 weeks postpartum. The separate time points will provide insight into the accumulation of cannabis used over time in the milk.

The milk samples that presented detectable levels of cannabinoids presented lower levels of SIgA compared to the milk of non-users (Chapter 4). These milk samples belonged to women who reported using cannabis during lactation, as well as during pregnancy and lactation. We suspect that decreased SIgA levels in the milk of cannabis users (use during lactation) may impact the immune health of the infant. SIgA in the milk is an essential source of immunity for the newborn [225], as the mucosal immune system is poorly developed at birth [226]. However, changes in the immunity of children exposed to the milk of mothers who used cannabis during pregnancy and lactation have not been reported. Future studies on that topic could confirm such speculations. The levels of SIgA and other immune factors can be tested in the saliva of the newborns of cannabis smoking and non-user mothers, using the protocol described by Sandin et al. [227], to understand whether changes in the milk are correlated with changes in the infants. Furthermore, the incidence of illness in the first year postpartum of infants born to cannabis-using mothers (pregnancy and lactation) should be monitored to determine whether changes in immunity due to lower SIgA levels in the milk lead to sickness in the infants.

Next, our results demonstrated that an increase in the 11-OH-THC levels, which is a primary active metabolite of THC [228], was significantly correlated with decreasing carbohydrate levels in the milk of cannabis users. Carbohydrate levels have been positively correlated with milk volume [229]. Perhaps the decreasing levels of carbohydrates due to increasing 11-OH-THC levels (or increasing THC consumption) may be linked with decreasing milk yield. In fact, there were 3

cannabis users who were recruited for this study, but they were unable to provide milk. The reason cited was "little to no milk supply". Additionally, cannabis-using mothers reported lower levels of milk production in the first, second, fourth- and sixth-week post-partum, relative to non-users. The changes in milk yield between cannabis users and non-users could be due to lower prolactin (PRL) levels. Cannabis users have been reported to exhibit decreased baseline plasma PRL levels, relative to healthy controls [197]. Lower serum PRL levels are correlated with decreased milk volume (mL/day) [192]. Future work should involve the collection of blood samples from subjects at the later stage of pregnancy (third trimester) and during lactation to confirm the impact of cannabis use on PRL levels in pregnant and breastfeeding mothers.

Along with the data collected in our study, evidence suggests that cannabis use by breastfeeding mothers is associated with the presence of cannabinoids in the milk [135,138,211], as well as reduced SIgA levels [230]. Women are strongly recommended to avoid cannabis use during pregnancy and lactation. Since women continue to use cannabis during pregnancy and lactation, one study has provided advice to avoid breastfeeding within 1 hour of inhaled use to decrease infant exposure to the highest cannabinoid levels in the milk [231]. However, our work offers insight that cannabis use is not only linked with the presence of cannabinoids in the milk but also it is associated with lower SIgA levels in the milk. Thus, the usage of cannabis during pregnancy and lactation can impact infant health as a result of exposure to cannabinoids through breast milk and exposure to cannabinoids and altered breast milk. Altogether, this work should provide stronger support backing the recommendation against cannabis use during pregnancy and lactation.

iv. Limitations of the studies presented in this dissertation

a. Usage of an immortalized cell line for the *in vitro* work

Immortalized cells have been used in place of primary cells to investigate the impact of a specific drug or molecule on cellular differentiation and function [232]. Immortalized cell lines are cost-effective, possess the ability to divide indefinitely, and provide a homogenous population of cells that provide a consistent sample for reproducible results [232]. However, these cell lines also carry the potential of expressing unique gene expression patterns, which might not be found in that cell type *in vivo.* Thus, the usage of the HC11 cell line in this study may be considered a major limitation while interpreting the results obtained in the first two chapters of this study.

Various cell lines derived from the human and mouse MG have been used to study MG development and function *in vitro.* The MCF10A cell line has been derived from the human MG and it expresses basal and luminal markers of MECs [233]. However, the MCF10A cells display negative staining of β-casein (CSN2) and α-lactalbumin (LALBA) [233], which are milk protein markers expressed in differentiated MECs [234]. Because the MCF10A cells do not express markers of alveolar differentiation, they may not be suitable for studying the effect of drugs on the milk protein production of MECs, which was one of the key foci of this study. In comparison, the HC11 cells, which originate from midpregnant BALB/c mouse mammary gland (MG) tissue, adopt a secretory phenotype upon treatment with prolactin (PRL), dexamethasone (DEX) and insulin (INS). Differentiated HC11 cells produce lipid and milk proteins, including β-casein [235], resembling differentiated mammary epithelial cells (MECs) found in the alveoli of a lactating MG [202–204]. The functional features of the differentiated HC11 cells in our study resembled the characteristics of MECs obtained from the pregnant or lactating MG of mice [10,236,237].

Besides immortalized cell lines, primary MECs isolated from human or mouse MG has gained attention for being a tissue-specific model of the MG [238]. However, primary cells have short life spans among various other limitations, such as the requirement of feeder cells or extracellular matrix (ECM) proteins for the culturing and growth of primary cells [239]. Although these processes result in the cultivation of MECs that express basal and luminal phenotypes, no study has reported the production of milk proteins by primary MECs [238]. Human milk is another source of pure human MECs, which are characterized as differentiated luminal MECs. However, huge quantities of milk are required to isolate the MECs, and these cells only proliferate for 1-2 passages [240], which can produce various limitations for studies that require larger cell batches for multiple experiments. Induced pluripotent stem cells (iPSCs) can be used the assess the impact of drugs, such as cannabinoids, on MEC differentiation and function [241]. The iPSCs can be generated directly from terminally differentiated MECs (humans or mice) and they allow for personalized or patient-specific modelling, along with bypassing the need for embryos [242]. Thus, future studies can utilize iPSCs obtained from terminally differentiated mice or human MG tissue to understand the impact of cannabinoids on MECs in a more physiologically relevant manner.

b. Treatment of HC11 cells with 10μM THC and 10μM CBD

Using the 10μM THC and 10μM CBD concentration may be considered high for testing the impact of cannabinoids on cellular function. *In vitro* studies have used concentrations between 10μM and 40μM to explore the impact of THC [183,243,244] and CBD [245,246] on proliferation and differentiation in various tissue types. Peak plasma concentration (N=6) of THC at 9 minutes during smoking of a single cigarette containing 3.55% THC has been reported to be 162.2 ng/mL (range 76-267 ng/mL) or about 0.5μM [247]. Similar mean THC concentrations have been reported in other human and animal studies, ranging between 0.04 and 0.7μM [248,249]. Besides

THC, some cannabis-based medicine extracts and clinical-grade cannabis contains high quantities of CBD [250]. Levels ranging from 3 to 17.8 ng/mL CBD (N=3, mean 10.2 ng/mL) were reported 1 hour after smoking a cigarette containing 19.2 mg of deuterium-labelled CBD [251], while 1.1 to 11 ng/mL CBD was reported after 1 hour of oral intake of 40 mg CBD in the plasma of subjects.

Following inhalation, cannabis metabolites are rapidly absorbed into the bloodstream from where they can diffuse into various tissues, including the adipose tissue, brain, and liver [135]. Rawitch et al. have demonstrated that while THC was detected at 200 ng/g, 100 ng/g, and 30 ng/g in the liver, lung, and brain of mice, respectively, the levels of THC in the gonadal fat organ were detected between 2000 and 3000 ng/g of tissue [252]. These results indicate that cannabinoids may be stored in fat at concentrations that are ten times greater than concentrations of cannabinoids reported in blood or plasma. In addition, THC is found to persist in fat for 2 weeks [253]. The storage of THC in adipose tissues within the body for lengthy periods of time contributes to the half-life of THC of 20 to 36 hours, which is equivalent to an elimination rate of 3% per hour, and a THC detection time of up to 6 days after administration [135,254,255]. The MG and breast milk have elevated levels of fat, which may be the reason underlying the presence of THC in the milk of cannabis users. The average concentration of THC in the milk of chronic cannabis users (cannabis smoked seven times per day) was reported to be 340 ng/mL (~1μM), compared to an average concentration of 105 ng/mL (~0.3μM) THC in women who used cannabis once daily [136], indicating that usage determines the concentration of cannabinoids found in milk.

Moreover, cannabis products continue to be prepared with higher concentrations of THC and CBD [256]. With significant profits at stake, the legal cannabis market has been implementing selective growing methods to boost psychoactive potency. Over the last two decades, the average THC content of cannabis has increased from 4 to 12% [256], with documentation of levels as high

as 30% in legal cannabis grown for recreational use [257]. Finally, women use cannabis use to alleviate symptoms of morning sickness, i.e., nausea/vomiting, chronic pain, and anxiety during pregnancy [107,108], and to manage postpartum stress and fatigue [109]. Women using cannabis to help alleviate such symptoms may be using it at a range of distinct concentrations, which may not be declared to their healthcare professionals due to the fear of stigmatism. Such reasons make it difficult to estimate a clinically appropriate dose. After consideration of all factors, as well as closely examining the cytotoxicity profile of THC and CBD in HC11 cells, the cells in this study were treated with 10μM THC and 10μM CBD. Future studies can test the effect of a range of concentrations of THC and CBD on the alveolar function of MECs to understand the impact of these cannabinoids at the different levels of the drug treatment.

c. Lack of data on the impact of cannabis on the structure of the MG

While *in vitro* studies have major advantages, such as the ability to understand drug interactions and mechanistic underpinning in an isolated environment at low costs, these models do not fully recapitulate the biology of the MG *in vivo. In vivo,* luminal MECs interact with myoepithelial cells, and together these cells interact with the stroma that is composed of the mesenchymal stem cells, immune cells, fibroblasts, and adipocytes [10,65,258]. While two-dimensional (2D) cell culture is routinely used to conduct mechanistic studies, it fails to recapitulate the structural characteristics of tissues found in the body, which may limit our understanding of regulatory pathways. *In vivo* models can be used to answer complex questions prior to conducting human trials, however, animal studies are also accompanied by higher demand for resources, cost, and ethical considerations. Thus, when it is not feasible to conduct an *in vivo* study, unique and novel methods must be used to answer intricate structural and mechanistic questions while keeping the system physiologically relevant. Three-dimensional (3D) models have

been used to fill the gap between *in vitro* and *in vivo* systems [259,260]. 3D models can be developed to investigate regulatory pathways and high-throughput drug screening in a cost-effective manner. 3D models allow for the generation of different proliferation areas (lumen vs. periphery), enhanced cell-cell interaction and crosstalk, and the generation of a complex microenvironment and functional differentiation at a higher level, which is often not seen on the two-dimensional (2D) plastic cell surface [261,262]. Finally, MG explants have been well characterized as physiologically relevant models of MG *in vitro* [263–265]. The explants can be cultured *in vitro* and used to study the impact of drugs on the structural and biochemical aspects of the MG. Thus, 3D models, MG explants and *in vivo* studies can be utilized to understand the impact of cannabis on the structure and function of MG development and MEC differentiation in a physiologically relevant manner.

d. Limitations associated with clinical studies that involve human subjects

Clinical studies conducted with human subjects provide highly valuable data sets. However, the hesitancy of human subjects to participate in cannabis studies, providing inaccurate answers in questionnaires, and co-using other substances with cannabis are some of the major limitations of human studies. Larger sample sizes may allow for a lower probability of negative findings and more accuracy with the results gathered [266]. However, the high cost related to a larger clinical study requires strong evidence at the studies' *in vitro* and *in vivo* levels. One of the biggest challenges in the clinical trial reported in our clinical study was recruiting a high number of subjects. Lower sample size is a major limitation in interpreting results from a clinical study. We suggest providing a higher monetary incentive to subjects for participation, such that subjects are paid $50-$100 for participation, as numerous subjects in our study communicated that $25 compensation was low. Moreover, we recommend that some information about their milk (i.e., the

content of fat, protein and carbohydrates) could be conveyed to the subjects as an incentive to participate in the study. This may increase the interest level of potential participants. Most women who participated in our study were recruited from the Obstetrics and gynecology clinic at the St. Joseph Hospital and the majority requested for their milk to be collected from their homes. Thus, keeping those considerations may help with planning the logistics of future work. Finally, some women presented hesitance to report cannabis use during pregnancy and lactation, possibly due to the fear of stigma or exposure of their usage to their healthcare provider. It should be conveyed to the participants clearly that although women who are pregnant or breastfeeding are recommended against cannabis use, adults over the age of 18 years or older are legally able to possess up to 30 grams of legal cannabis in Canada [267]. Furthermore, it should be emphasized to cannabis-using mothers that the data collected by researchers is anonymized and will not be shared with their healthcare providers. Such information may reduce the hesitancy associated with participating in such studies and essentially reduce the limitations associated with a low sample size.

v. Direction for future research

a. Utilizing 3D models to address the gap between *in vitro* and *in vivo* studies

The MG is a complex organ, comprised of multiple cell types and surrounded by a proteinaceous extracellular matrix (ECM) [258]. Furthermore, the adult human MG is a tubule-alveolar structure, which is composed of milk-secreting polarized epithelial cells that are surrounded by myoepithelial cells and a basement membrane surrounds the two-layered tissue organization. Acini is the structure that is composed of spherical mammary epithelial cells (luminal) and lined with myoepithelial cells, with a lumen in the centre where milk components (proteins and lipids) are released to be transported to the infant through the mammary ducts and nipples [1,7,268]. While animal models provide invaluable information in understanding complex

scientific questions, these models require immense resources. The luminal structure of MECs can be formed *in vitro* by using a spheroid culture of luminal MECs, followed by the co-culturing of these cells with basal MECs. The inner layer of luminal and the outer layer of myoepithelial cells form a dynamic bi-layered structure in the MG, which involves continuous changes in cellular proliferation, migration, polarity and cell-cell signaling with stromal cells and the proteins of the ECM [269]. Due to this dynamic nature of the MG, the model can be further advanced by integrating microengineering and tissue engineering to develop an organ-on-a-chip model for reconstituting the physiological features of human organs [270]. Overall, 3D cultures have been recognized to provide better physiological relevance than 2D cultures, by mimicking the *in vivo* conditions of tissues and organs [271].

As the first validation point towards creating a 3D organoid model of the MG, the luminal MECs can be cultured as spheroids and their function can be characterized. Although primary cells must be used to create a physiologically relevant 3D structure of the MG, the initial experiments can be conducted using an immortalized cell line, such as the HC11 cells. We cultured and differentiated HC11 cells in ULA plates and characterized the gene expression of key functional markers within the cells (Appendix B, Fig B1). HC11 cells formed spheroids within 24 hours of seeding and displayed round spheroids with smooth edges throughout the 6 days post-seeding (Fig B1). The viability of HC11 spheroids was consistent with cells cultured in 2D conditions (Appendix B, Fig B1). We reported that compared to 2D cells, HC11 spheroids expressed significantly higher mRNA levels of milk proteins (*CSN2* and *WAP)* following 3 and 4 days of differentiation (Appendix B, Fig B2). Our results indicated that the increase in milk protein expression may have been an outcome of the increased mRNA level of mammary stem and progenitor markers (*Vimentin, ALDH1A3,* and *SOX2)* that give rise to alveolar structures in the

MG [105]. This is consistent with the findings reported by Qu et al., who cultured MCF10A cells (human MECs) in 96-well ULA plates and reported that MCF10A spheroids presented stem/progenitor markers, as well as *CK18, CK7, CSN2* and *LALBA*, which are undetectable in 2D culture in MCF10A cells [233]. The phenomenon of upregulated signaling in 3D models, compared to 2D models, has been demonstrated in other cell lines as well. Wong et al., cultured HTR8/SVneo extravillous trophoblast (EVT) cells in ULA plates and reported the formation of spheroids, significant upregulations in canonical pathways and biological processes such as immune response, angiogenesis, wound healing, and others[273]. This is aligned with the explanation of 3D cultures preserving morphological characteristics of the organs and allowing for greater cell-cell interactions, resulting in the upregulation of signaling pathways involved in cellular differentiation and function [271].

To further advance 3D culture models, mammary organoids can be formed from primary MECs that are freshly isolated from murine or human mammary epithelial fragments. Freshly isolated MECs have been claimed to preserve cellular, structural, and microenvironmental features of the MG function *in vivo* [274]. Furthermore, the organoids can be embedded in an ECM because the MG is embedded in the stroma, which is composed of various cells (i.e., fibroblasts, adipocytes and immune cells) and an ECM [274]. These primary mammary epithelial fragments are often mechanically or enzymatically dissected and embedded in a reconstituted ECM [269,275,276]. Utilizing these innovative models, future work can involve the culturing of MECs in spheroids for the purposes of testing the transfer of cannabinoids across the spheroids, as well as monitoring changes in tight junctions and leakage of lactose into the media upon cannabinoid treatment.

In addition to organoids, MG explants have been well characterized as physiologically relevant models of MG *in vitro* [263–265]. The MG explant culture contains fat cells, as well as

immune cells, and therefore, the response of cells within the explant to any added drug (e.g., cannabinoids) is physiologically relevant as the culture contains the presence of cells from the entire gland rather than being an isolated cell line. The secretory activity of MGs isolated from pregnant mice can be maintained with PRL, INS and a corticosterone [277]. It is recommended that for explant culture of the MG, the gland must be isolated from mid-pregnant mice, as that time-point shows good preservation of histological structure in media [277]. Based on the protocol of Mills and Topper, the MG was isolated from 11–12-day pregnant mice, followed by incubation in media containing penicillin, INS, hydrocortisone and PRL for 96 hours (4 days). This was followed by cutting these glands into 4 pieces, incubation in a gel to polymerize for 48 hours and then sectioning for study under electron and light microscopy [277]. Sections of the MG prepared from mouse explants can be used for immunofluorescent staining of milk proteins and other markers of alveolar differentiation. Additionally, sections from explant culture can also be utilized to view secondary and tertiary ducts, as well as the alveoli [277]. Therefore, MG explants provide a physiologically relevant model that can be used to investigate the impact of cannabis on the milk-producing function of MECs, as well as to determine structural changes in the MG. Altogether, the formation of a robust and physiologically relevant model will allow for accuracy in reporting the effects of drugs on MG function.

b. Studying the effect of cannabis on MG and infant health in rodents

In addition to unique and cost-effective *in vitro* models, future works should involve the utilization of *in vivo* models to understand the impact of cannabis exposure on MG development and function. The majority of cannabis users report smoking as the principal route of administration [115]. Smoking provides a rapid and efficient method of drug delivery from the lungs to the brain, contributing to its immediate psychoactive potential [115]. The bioavailability

of THC following smoking has been reported as 2-56%, due to intra- and inter-subject variability and altered dose delivery [247]. Thus, exposing rodents to cannabis through a smoke exposure technique would better represent the use of cannabis by women during pregnancy and lactation [115]. Based on Dr. Hirota's work, we suggest exposing mice to cannabis cigarettes containing 14% THC and 0-2% CBD via smoke exposure for a duration of 45 minutes [278]. This group reported 150ng/mL (~5μM) THC in the blood collected within 15 minutes after exposure [278]. Since women consume cannabis via smoking and the THC reported in the plasma cannabis users is about 5μM [247], Dr. Hirota's mice model is clinically relevant for exploring our research questions. Furthermore, a mice model can be used to understand the effect of cannabis exposure at separate time points in the perinatal period. For example, a subset of mice can be exposed to cannabis during pregnancy, while a second subset can be exposed to cannabis during pregnancy and lactation, and finally, the third subset can be exposed to cannabis during lactation only. Recruiting a reasonable sample size of human subjects at all these time points can be challenging. Exposure to cannabis smoke at each different time point (pregnancy, pregnancy and lactation, lactation) in a controlled manner will provide insight into the impact of cannabis on MG development, breast milk production and composition specific to each window of exposure. In addition, pups of dams exposed to cannabis during pregnancy can be cross-fostered with wild-type dams to understand whether their health is impacted if they were exposed to cannabis during gestation but received milk from unexposed mothers. Finally, it would be useful to understand the impact of cannabis exposure during early pregnancy on MG development and function because many mothers claim only use cannabis during the first trimester of pregnancy to manage symptoms of morning sickness or by mistake due to not being aware of their pregnancy [110].

Since current studies lack evidence on a clear representation of the endocannabinoid system (ECS) in the MG, the *in vivo* study could be used to understand the changes in key components of the ECS (endogenous ligands, cannabinoids receptors, and key enzymes that synthesize and degrade the endocannabinoids) throughout pregnancy, lactation, and involution. Next, the impact of cannabis on the components of the ECS can be determined to better understand the changes in the ECS dynamics with exposure to cannabis. At parturition, high levels of 2-AG have been reported in the milk of mice dams, which are accompanied by elevated levels of CB1 receptor mRNA in the brain of pups [169]. CB1 has been reported to modulate many processes, including mood, appetite, learning, memory, pain signaling, and energy metabolism[151]. Thus, examining the impact of cannabis exposure on the 2-AG levels in the milk of dams, as well as CB1 levels in the brain of mice, can provide insight into the impact of cannabis on infant health. Assessment of size, sex, and weight of litters at PND1 (birth), PND21 (weaning), and PND105 (young adult) can provide information regarding the effect of cannabis exposure on pup health and survival. To understand the impact of cannabis on breast milk composition, milk from exposed dams can be collected, using well-established methods [279], and changes in the milk composition can be assessed using highly sensitive ELISA kits.

THC administration has been reported to result in the suppression of serum PRL levels in ovariectomized rats[174], and in mice at late pregnancy [175,176] and early lactation [177], however, the direct effect of cannabis exposure during pregnancy and/or lactation on PRL levels has not been reported. Furthermore, PRL receptor (PRLR) knock-out mice showed a complete failure of lactation following their first pregnancy [13]. Thus, future work should investigate the influence of cannabis exposure on the levels of key hormones that are essential to lactation (i.e., prolactin) in the blood collected from exposed dams. In addition, assessment of cannabis exposure

on the levels of PRLR in the MG of exposed mice can provide mechanistic direction. PRL has been reported to signal through the PRLR to activate the JAK2/STAT5 pathway resulting in the expression of milk protein genes, including β casein (*CSN2*) and whey acidic protein (*WAP*) [14]. Furthermore, phosphorylated STAT5 has been suggested to regulate milk protein and lipid production in MECs by activating the AKT-PI3K pathway [22]. Thus, future work should include an investigation of the impact of cannabis on the JAK2/STAT5 and AKT/PI3K pathways to delineate the mechanisms of action of cannabis. This work can be conducted by monitoring changes in the gene and protein expression profiles from minced MGs (exposed vs control), as described by Kobayashi et al [280].

During pregnancy, the epithelial structures in the mammary gland undergo extensive branching [281]. Differences in the branching can be observed at mid-gestation (12-15 days pregnant) and at parturition [282]. The structural changes in the MG of dams exposed to cannabis can be assessed by whole mount analysis. This involves sacrificing the exposed pregnant female mice, isolating the mammary gland, mounting the gland onto an adherent microscopy slide, and staining the epithelial structures of the gland with Carmine alum, as described by Tolg et al [282]. Branching in the stained glands can be observed under a phase-contrast microscope, and the number of branches and branch length can be counted to determine changes in the MG structure upon cannabis exposure, compared to wild-type dams [282]. The MG of litters exposed to cannabis during gestation (pups sacrificed at PND1) and lactation (pups sacrificed at PND21) can be assessed by whole mount analysis to investigate the changes in the rudimentary ductal tree and terminal end buds (TEBs), respectively. The TEBs form at the tips of the ducts of the rudimentary ductal tree. An altered form of the TEBs, including changes in the number of TEBs, has been correlated with breast cancer [283]. Furthermore, the majority of breast cancers arise from the

dysfunction of MECs in the mammary ducts (50-70%) [284]. Since THC has been identified to pass through the placenta [285] and the breast milk [135–137] to the fetus and the infant, respectively, monitoring the MG structure of pups will be important to detect possible abnormalities at an earlier time point in the pup's life.

In addition, immunohistochemical studies can be conducted with stained slices of exposed MGs. These studies can allow for confirmation of the milk production ability of the MG (or the lack of it), by assessing changes in milk protein levels (beta-casein) and changes in levels of perilipin-2/adipophilin (a major protein coating cellular lipid droplet [280]. Finally, tight junction permeability can be assessed by immunostaining for claudin 3, occluding, or tight junction protein 1 and/or 2 [280]. The tight junctions of alveolar MECs are impermeable during lactation to allow the storage of milk components in the lumen [32]. Our results have demonstrated that THC and CBD induce ER stress in MECs, which is associated with tight junction permeability [94]. If tight junction permeability is impacted in the MG of mice, then changes in the lactose levels of the milk of dams can be quantified to further understand compositional changes caused by cannabis usage.

c. Future direction for the clinical study

Based on the study conducted by Moss et al. [212], we recommend that future work should involve the collection of milk from subjects at multiple points postpartum. Specifically, we recommend that milk is collected between 2 to 4 weeks, as well as 6 to 8 weeks postpartum. This allows for the collection of milk at two-time points to understand the changes in milk composition over first 2 months of usage (post-partum). It also gives the subjects a break between the collections, as it can be stressful to participate in the study as a new mother. Furthermore, we suggest that cannabis is used within 48 hours of milk collection and that the amount, frequency, and strain of cannabis used to be meticulously recorded in the questionnaire by all subjects. The

changes in the levels of macronutrients can be adjusted to the values of frequency and amounts of cannabis consumed. In addition to macronutrients, the collected milk could be analyzed for changes in specific immune factors and proteins, such as other immunoglobulins and milk proteins (i.e., casein, lactoferrin). Finally, we recommend that 3 mL of blood is donated by women at the two-time points as that blood samples will allow for analysis of various proteins and factors in the plasma of subjects [212]. The levels of PRL, oxytocin, cortisol and other relevant hormones can be tested in the plasma of all subjects to understand whether cannabis use is associated with changes in hormones that play a key role in MG function during lactation.

In this study, the adjusted mean birth weight of the infants born to women who used cannabis during pregnancy was slightly lower than non-users (~213 grams), however, the changes were not statistically significant. Evidence supports that cannabis use during gestation is linked to reduced birth weight [286]. This indicates that the effects normally seen in infants exposed to cannabis during gestation are not clear in this study; demanding a high sample size to conclude its effects. In addition to birth weight, we recommend that future studies involve the collection of fecal matter of infants (meconium and fecal matter of infants at the time of milk collection) to understand the passage of cannabinoids through the placenta and breastmilk to the infants. Gray et al. provide methodology surrounding the collection of meconium and analysis of cannabinoids in the samples [287]. Finally, exposure to cannabinoids through breast milk may impact various neurodevelopmental outcomes in infants. Treatment of rat dams with 2mg/kg/day (subcutaneous) THC during the first 10 days of postnatal development resulted in delayed GABA development in pups [140]. Delayed GABA development has been linked to disorders, such as fragile X syndrome, early-life epilepsies, and autism [288–290]. Furthermore, pups from dams treated with THC exhibited slower growth, which was quantified by lower average weights from P07 to P10 [140].

Thus, future human studies investigating the impact of maternal cannabis use during lactation on infant health should investigate changes in infant weight over the first two months postpartum (measured at 4 and 6 weeks postpartum). In addition, the effect of exposure to cannabinoids through the placenta and breast milk on the neurodevelopment of infants can be tested using various developmental screening tests at 9, 18 and 30 months after birth [291].

vi. Significance of the work

Cannabis has been used for centuries for recreational purposes as well as for its anti-inflammatory and medicinal benefits [109,292]. The legalization of cannabis and wider recognition of its beneficial properties may be contributing to the rise in the availability and usage of cannabis [293–295]. However, much is yet to be discovered about the impact of cannabis and its components on the human body, when they are taken alone or in combination – with other smaller compounds, known as terpenes or terpenoids. While THC is used for its psychoactive properties, CBD is considered as the "safer" cannabinoid that produces anti-anxiety effects that generate calming and relaxing feelings upon use [296]. However, our work reports that both THC and CBD treatments decrease alveolar differentiation of MECs and alter its lipid synthesizing and milk protein producing function. These are fundamental processes during pregnancy, which prepare the MG for milk production at parturition for infant nutrition and survival. Although it was not investigated in this study, we hypothesize that THC acts as an agonist on the CB2 receptor [161], resulting in the inhibition of adenylate cyclase and resulting in the downregulation of cAMP levels. Reduced cAMP levels have been associated with reduction in the activity of protein kinase A (PKA), resulting in reduced cellular differentiation and function [297]. In comparison to THC, we hypothesize that CBD acts as an inverse agonist on the CB2 receptor [161], which reduces the activity of the CB2 receptor resulting in downregulation of cellular differentiation as function.

Furthermore, reduced CB2 receptor levels result in increased cAMP levels, which results in increased activity of PKA [297]. Increased activation of PKA leads to phosphorylation and inhibition of mTORC1, which plays an essential role in milk protein and lipid synthesis in mammary epithelial cells [298].

Anecdotal evidence suggests that compounds in cannabis, when taken together, produce a better effect relative to when taken alone. Although taking cannabinoids together in a mix may prove to be more beneficial for pain, anxiety, inflammation or infections, their impact on reproductive organs, such as the MG may be extremely harmful to the mother and the infant. Our results demonstrate that the treatment of HC11 cells with a mixture of THC and CBD decreased the gene and protein expression of milk proteins and lipid levels to the same or higher degree, compared to THC and CBD treatment of the cells independently. Furthermore, the combination of THC and CBD elevated the levels of reactive oxygen species in HC11 cells; a finding that was not observed with the treatment of HC11 cells with THC and CBD alone. Overall, our group was the first to publish on the impact of THC and CBD on MEC differentiation and function. These findings produce evidence for further investigation into the effects of cannabis use on MG development and function. As for use of cannabis during lactation, our results demonstrate that cannabinoids pass through the milk to the infant, which may have major implications for the growth and development of the infant. Furthermore, we provide evidence that cannabis use during lactation is associated with decreased levels of an immune factor (SIgA). This work provides evidence to further investigate the effect of cannabis use during pregnancy and lactation on breast milk composition. The legalization of cannabis and its use over many centuries may have dampened the perception of its harmful impacts. However, our work demonstrates that infants born to mothers who are using cannabis during lactation are exposed to cannabinoids through

breast milk and these infants are potentially exposed to an altered composition of the milk as it contains lower SIgA levels. As summarized in Figure 1, we hypothesize that the effect of THC and CBD on MECs may be underlying the effect of cannabis use found on milk produced by cannabis using mothers. Future work must be conducted to confirm this hypothesis.

We propose that future studies investigating the impact of cannabinoids on MECs should be investigated using an advanced *in vitro* model, such as a 3D model, or by using an explant of the MG to understand the effects of cannabinoids in a physiologically relevant manner. In addition, an animal study must be conducted by exposing mice to cannabis smoke during distinct windows of exposure (pregnancy, pregnancy and lactation, lactation) to understand the effect of cannabis on MG development and function, as well as on pup health in a clinically relevant manner (Figure 2). In the clinical study, we propose that other essential milk components, such as milk proteins and essential fatty acids, may be tested in the milk of cannabis users and non-users. Overall, research must continue on this topic to deeply explore the effects of cannabis on the maternal gland that produces milk, as well as on the infants exposed to cannabinoids through the milk. The accumulation of scientific evidence on this topic will better inform new and expecting parents regarding cannabis use in the perinatal period.

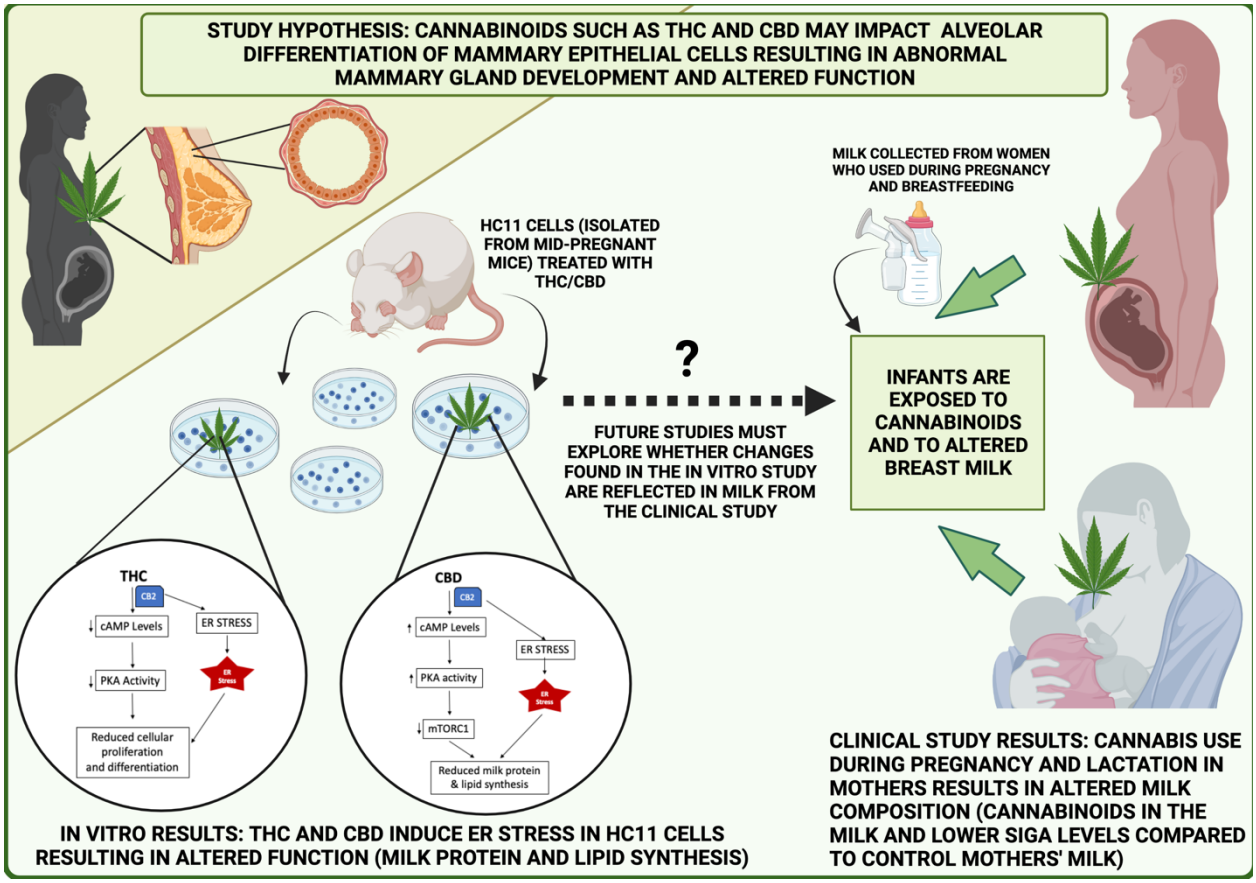

Fig 1. The hypothesized mechanism of action of THC and CBD on mammary epithelial cells (MECs). THC acts as an agonist on the CB2 receptor, resulting in the inhibition of adenylate cyclase and resulting in the downregulation of cAMP levels. Reduced cAMP levels have been associated with reduction in the secretion of beta-casein. In comparison to THC, we hypothesize that CBD acts as an inverse agonist on the CB2 receptor, which reduces the activity of the CB2 receptor resulting in downregulation of cellular differentiation as function. Both THC and CBD have been demonstrated to cause ER stress resulting in altered cellular function. It is hypothesized that changes in MECs upon THC and CBD administration may relate to altered milk composition found in women using cannabis during pregnancy and breastfeeding. Cannabinoids pass through the milk to the infant, which may have major implications for the growth and development of the infant. Cannabis use during lactation is associated with decreased levels of an immune factor (SIgA). Infants of mothers who are using cannabis during lactation are exposed to cannabinoids through breast milk and these infants are potentially exposed to an altered composition of the milk.

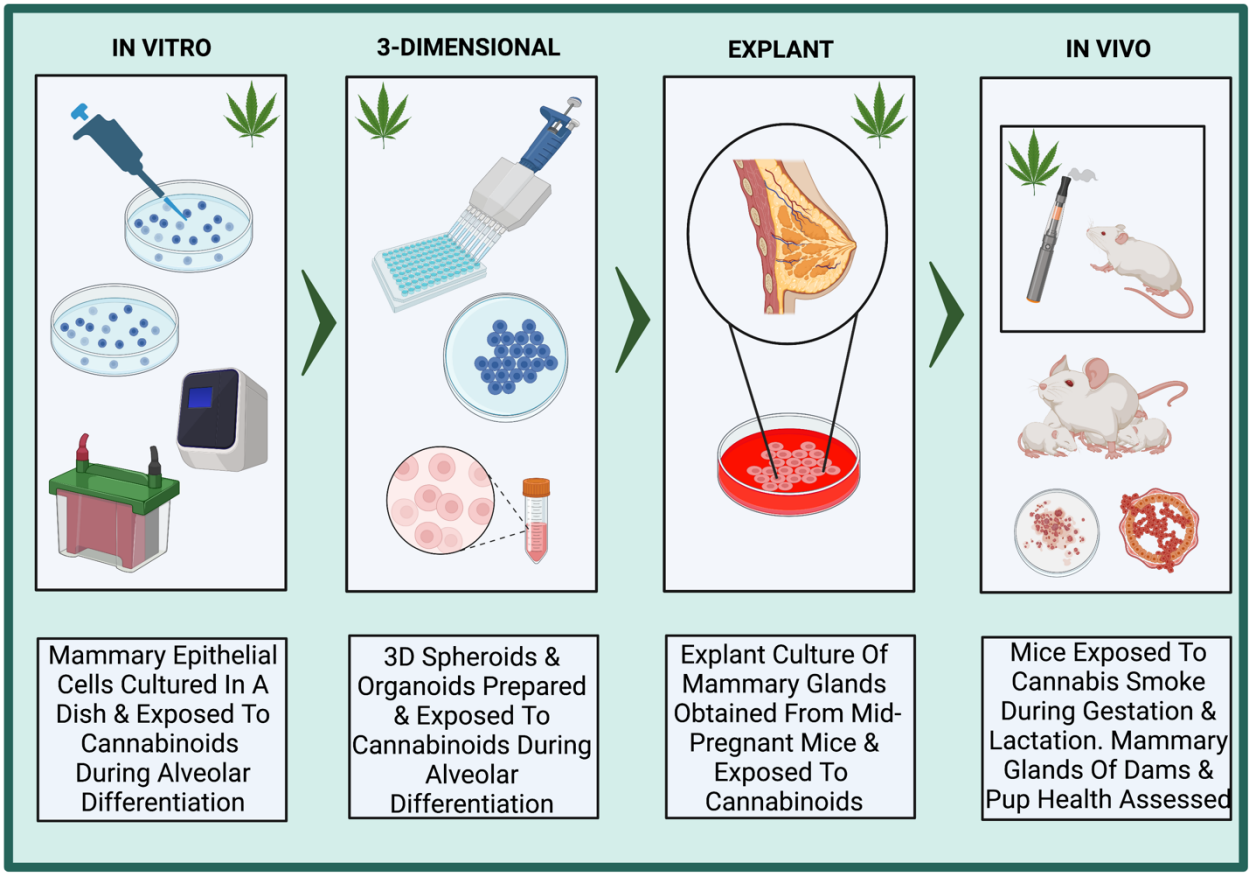

Fig 2. A variety of models can be used to assess the impact of cannabinoids on the mammary epithelial cells and the mammary gland. Immortalized cell lines are cost-effective, possess the ability to divide indefinitely, and provide a homogenous population of cells that provide a consistent sample for reproducible results. Three-dimensional (3D) models have been used to fill the gap between *in vitro* and *in vivo* systems. 3D models allow for the generation of different proliferation areas (lumen vs. periphery), enhanced cell-cell interaction and crosstalk, and the generation of a complex microenvironment and functional differentiation at a higher level. Mammary gland explants can be utilized to understand the response of mammary epithelial cells within the explant to any added drug (e.g., cannabinoids) is physiologically relevant manner because the explants contain a variety of cells (i.e., fat cells, immune cells) that are present in the mammary gland. Finally, utilization of *in vivo* models allows for investigation of the impact of cannabis exposure on the mammary gland development and function in a clinically relevant manner. Exposing mice to cannabis smoke better represent the use of cannabis by women during pregnancy and lactation.

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APPENDICES

APPENDIX A

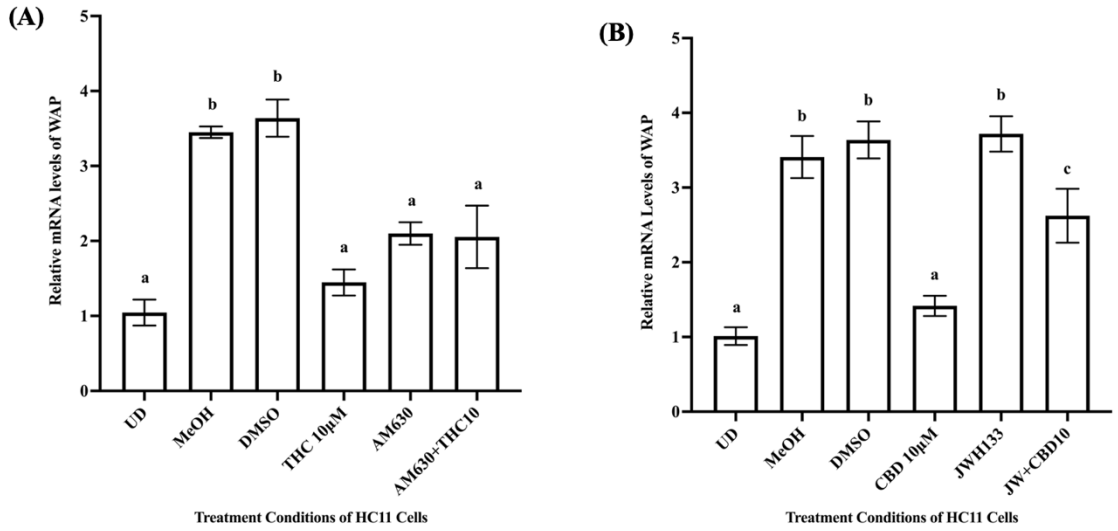

Fig A1 AM630 rescues the effect of THC and JWH133 rescues the effect of CBD on the mRNA levels of *WAP* and in HC11 cells. (A) The mRNA levels of *WAP* were assessed in undifferentiated (UD) HC11 cells, as well as in differentiated cells treated with vehicle controls (MeOH, DMSO), 10μM THC, 1μM AM630, and 1μM AM630 + 10μM THC, over 4 days of differentiation. (B) The mRNA levels of *WAP* were assessed in undifferentiated (UD) HC11 cells, as well as in differentiated cells treated with vehicle (MeOH, DMSO), 10μM CBD, 10μM JWH133, and 10μM JWH133 + 10μM CBD, over 4 days. Results from 4 biological replicates were referenced to *RPL0* and *RPL8* for mRNA analysis, represented as mean ± SEM, and compared using one-way ANOVA. Experimental replicates were separated by cell passage and statistically significant changes were indicated by distinct letters on the bar graphs.

APPENDIX B

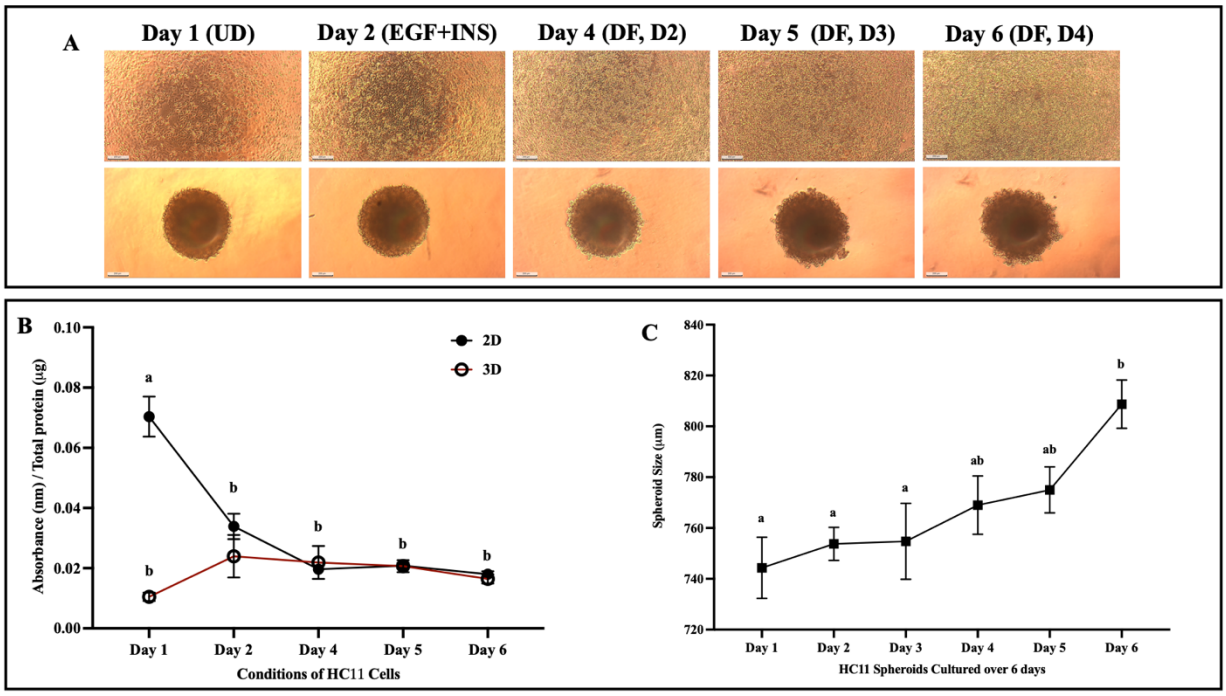

Fig B1. Proliferation of HC11 cells and spheroids throughout differentiation. HC11 cells were seeded in base media for 24 hours (day 1), followed by supplementation with EGF and INS for 24 hours (day 2). Then, HC11 cells were differentiated in base media containing PRL, INS and DEX, and allowed to proceed for 4 days (day 6). HC11 cell spheroids were assessed for changes in cellular proliferation using the Alamar Blue Assay, and the results were normalized to protein content. The spheroid size was measured using Image J. Results from 4 experimental replicates were plotted as mean ± SEM and compared using one-way ANOVA. Statistical analyses were performed using GraphPad Prism software (Prism 9 for macOS, Version 9.1.2 [225]). Statistically significant changes were indicated by distinct letters on the line graphs.

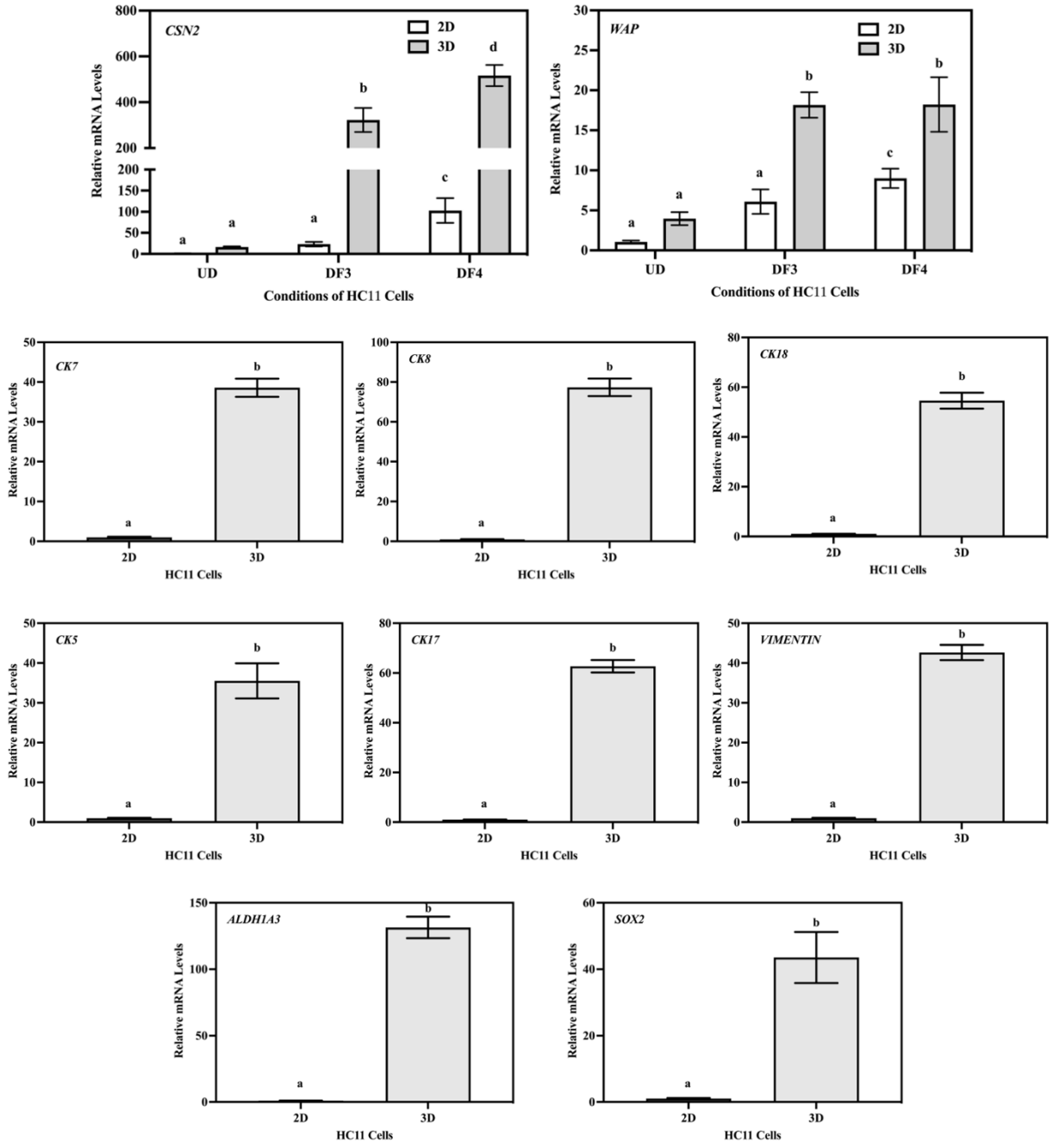

Fig B2. Differentiated HC11 spheroids present increased mRNA levels of milk proteins, cytokeratins and stem and progenitor markers compared to 2D cultures of HC11 cells. HC11 cells were seeded in base media for 24 hours, followed by supplementation with EGF and INS for 24 hours. Then, HC11 cells were differentiated in base media containing PRL, INS and DEX, and allowed to proceed for 4 days. The relative (compared to *RPL0* and *RPL8*) mRNA levels of *CSN2, WAP, CK5, CK7, CK8, CK18, CK17, VIMENTIN, ALDH1A3,* and *SOX2* were determined. Results from 4 replicates were referenced to *RPL0* and *RPL8* for mRNA analysis. All results were plotted as mean ± SEM and compared using one-way ANOVA (n=4). Statistical analyses were performed using GraphPad Prism software (Prism 9 for macOS, Version 9.1.2 [225]). Each experimental replicate was grown from cells of a different passage and statistically significant changes were indicated by distinct letters on the bar graphs.

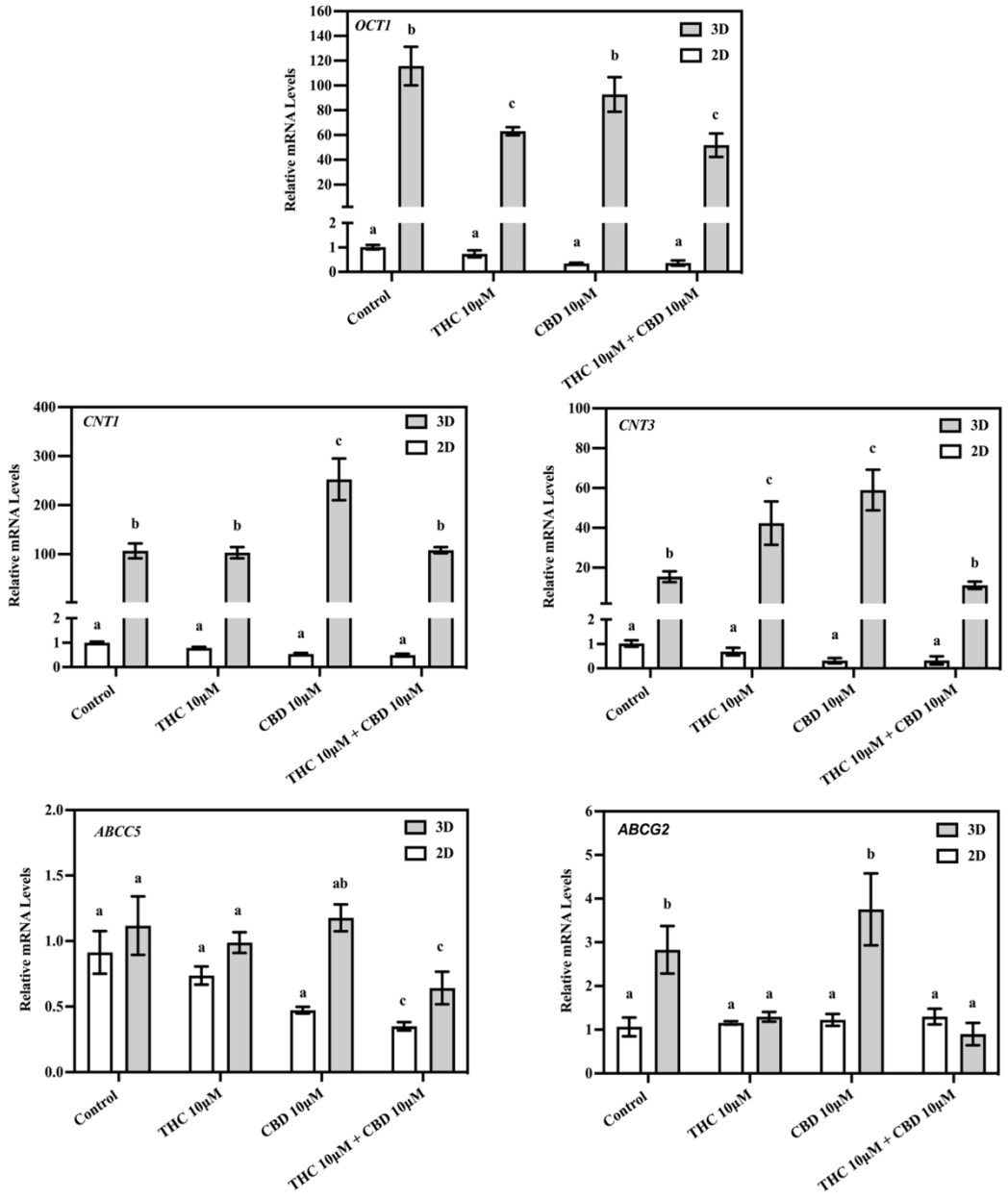

Fig B3. Differentiated HC11 spheroids present altered mRNA levels of transporters compared to 2D cultures of HC11 cells. HC11 cells were seeded in base media for 24 hours, followed by supplementation with EGF and INS for 24 hours. Then, HC11 cells were differentiated in base media containing PRL, INS and DEX, for 4 days. Throughout differentiation, the HC11 cells were treated with vehicle control (0.1% methanol), 10μM THC, 10μM CBD, or a mixture of 10μM THC and 10μM CBD. Cells were processed for total RNA isolation. The relative (compared to *RPL0* and *RPL8*) mRNA levels of *OCT1, CNT1, CNT3, ABCC5* and *ABCG2* were determined. Statistical analyses were performed using GraphPad Prism software (Prism 9 for macOS, Version 9.1.2 [225]). All results were plotted as mean ± SEM and compared using one-way ANOVA (n=4). Each experimental replicate was grown from cells of a different passage and statistically significant changes were indicated by distinct letters on the bar graphs.