Role of Polyphenolic Compounds in Chemoprevention of Breast Cancer Stem Cells

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ABSTRACT

In the field of integrative oncology, polyphenols have gained attention for their ability to modulate key signaling pathways involved in breast cancer prevention. One noteworthy product, the Polyphenol-Enriched Blueberry Preparation (PEBP), produced by the fermentation of blueberries by the bacterium *Rouxiella badensis* subsp. *acadiensis* has demonstrated various beneficial properties, including anti-inflammatory effects and the ability to control cancer stem cells.

Cancer Stem Cells (CSCs) are highly tumorigenic cells involved in carcinogenesis and can cause relapses. MicroRNAs (miRNAs) can act as regulators of CSCs, by controlling stemness and invasion.

We postulate that PEBP or its polyphenolic components induce specific epigenetic changes by modulating miRNA networks, reducing CSCs, and preventing breast carcinoma. Thus, the overarching aim of this thesis is to better understand the mechanisms underlying the protective effects of the polyphenol-enriched preparation in mitigating breast cancer.

The objectives are:

1. To investigate the chemopreventive effects of PEBP on breast cancer stem cell development in cell models and *in vivo*, as well as to study the involvement of STAT3 and MAPKs signaling pathways
2. Assess the impact of the polyphenol-enriched blueberry preparation on breast cancer by regulating the expression signatures of miRNA involved with cell proliferation, survival, and CSC self-renewal pathways in in vitro experiments.

3. Characterize PEBP and investigate the effect of a subset of its components on miRNA expression. Furthermore, validate the role of those components in regulating the functional behavior of breast cancer stem cells through experiments using a 4T1 animal model.

The results have shown a decrease in the formation of CSCs by delaying the development of tumors *in vivo*, decreasing metastasis to the lungs, and controlling the PTEN/PI3K/AKT axis, a central node in CSC signaling and homeostasis. In addition, several miRNAs associated with different clinical-pathological characteristics of breast cancer were shown to be differentially expressed in CSCs after exposure to PEBP. Notably, the expression of hypoxamir miR-210, associated with a poor prognosis in breast cancer patients, was downregulated, while tumor suppressor miR-145, which prevents metastasis through FOXO1 was over-expressed. The chemopreventive potential of a polyphenolic mixture containing PCA, gallic acid, and catechin, found in PEBP, was also shown to successfully reduce tumour growth and metastasis in our animal model and decrease the presence of stem-like tumour cells by favouring the upregulation of tumour-suppressor miR-145.

These findings provide novel evidence in translational medicine, highlighting the effectiveness of a natural epigenetic modulator in chemoprevention by specifically targeting CSCs.

RÉSUMÉ

Les polyphénols suscitent l'attention en raison de leur capacité à moduler les voies de signalisation clés impliquées dans la prévention du cancer du sein. La préparation de bleuet enrichie en polyphénols (PEBP), produite par la fermentation de bleuet par la bactérie *Rouxiella badensis* subsp. *acadiensis*, a démontré diverses propriétés bénéfiques, notamment des effets antidiabétiques, neuroprotecteurs et anticancéreux.

Les cellules souches cancéreuses (CSC) sont des cellules hautement tumorigènes impliquées dans le développement du cancer. Les microARN (miARN) peuvent agir en tant que régulateurs des CSC en contrôlant leur caractère souches et leurs capacités d'invasion.

Nous postulons que le PEBP ou ses composants polyphénoliques induisent des changements épigénétiques spécifiques en modulant les réseaux de miARN, réduisant ainsi les CSC et prévenant le carcinome mammaire. Ainsi, l'objectif principal de cette thèse est de mieux comprendre les mécanismes sous-jacents aux effets protecteurs de la préparation enrichie en polyphénols dans la lutte contre le cancer du sein.

Les objectifs de l'étude sont les suivants :

1. Étudier les effets chimiopréventifs du PEBP sur le développement des cellules souches cancéreuses du sein, à la fois dans des modèles cellulaires et in vivo, ainsi que d'étudier l'implication des voies de signalisation STAT3 et MAPK.
2. Évaluer les effets du PEBP sur le cancer du sein en régulant les signatures d'expression des miARN impliqués dans la prolifération cellulaire, la survie et les voies d'autorenouvellement des cellules souches cancéreuses in vitro.

3. Caractériser PEBP et étudier l'effet d'un sous-ensemble de ses composants sur l'expression des miARN. De plus, valider le rôle de ces composants dans la régulation du comportement fonctionnel des cellules souches du cancer du sein grâce à des expériences utilisant un modèle animal 4T1.

Les résultats ont montré une diminution de la formation des CSC en retardant le développement des tumeurs in vivo, en réduisant les métastases pulmonaires et en contrôlant l'axe PTEN/PI3K/AKT, important pour les CSCs. De plus, plusieurs miARN associés à différentes caractéristiques clinico-pathologiques du cancer du sein ont été différemment exprimés dans les CSC après exposition au PEBP. Notamment, l'expression de l'hypoxamiR miR-210, associé à un mauvais pronostic chez les patientes atteintes d'un cancer du sein, a été régulée à la baisse, tandis que le miR-145, qui prévient les métastases par le biais de FOXO1, a été surexprimé. Le potentiel chimiopréventif d'un mélange polyphénolique contenant du PCA, de l'acide gallique et de la catéchine, présents dans le PEBP, a également été démontré en réduisant efficacement la croissance des tumeurs et les métastases dans notre modèle animal, tout en diminuant la présence de cellules tumorales semblables à des cellules souches en favorisant la surexpression du miR-145 suppresseur de tumeur.

Ces résultats fournissent des preuves novatrices en médecine translationnelle, mettant en évidence l'efficacité d'un modulateur épigénétique naturelle dans la chimioprévention en ciblant spécifiquement les CSC.

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LIST OF ABBREVIATIONS

Akt ..... protein kinase B
ALDH ..... Aldehyde dehydrogenases
Atg7 ..... Autophagy related 7
ATM ..... Ataxia-telangiectasia mutated kinase
CDH1 ..... E-cadherin
CDK ..... Cyclin dependent kinase
CHEK2 ..... Checkpoint kinase 2
CSC ..... Cancer Stem-like Cells
CTLA-4 ..... cytotoxic T-lymphocyte-associated protein 4
EMT ..... epithelial-to-mesenchymal transition
ER ..... estrogen receptor
FOXO ..... Forkhead box protein O
GAE ..... Gallic Acid Equivalent
HER2 ..... human epidermal growth factor receptor 2
HIF1α ..... Hypoxia-inducible factor 1-alpha
HR ..... hormone receptor positive
IDC ..... invasive ductal carcinoma
IGF-I ..... Insulin like growth factor I
IL-6 ..... interleukin 6
ILC ..... invasive lobular carcinoma
Jak ..... Janus kinase
MAPK ..... Mitogen-activated protein kinase
MDM2 ..... Mouse double minute 2 homolog
mTor ..... mammalian target of rapamycin
NFκB ..... Nuclear factor kappa-light-chain-enhancer of activated B cells
N-RAS ..... Neuroblastoma RAS viral oncogene homolog
OCT4 ..... octamer-binding transcription factor 4
PARP ..... poly ADP ribose polymerase
PD-L1 ..... Programmed death-ligand 1
PEBP ..... Polyphenol Enriched Blueberry Preparation
PI3K phosphatidylinositol-3-kinase
PIK3CA ..... PI3K p110α subunit
PIP3 ..... phosphatidylinositol (3,4,5)-trisphosphate
PLK1 ..... Polo like kinase 1
PR ..... progesterone receptor
PTEN ..... Phosphatase and tensin homolog
RAF ..... Rapidly Accelerated Fibrosarcoma kinase
SGK ..... Serum and glucocorticoid-inducible kinase
SIRT2 ..... NAD-dependent deacetylase sirtuin 2
TLRs ..... Toll-like receptor
TNBC ..... Triple negative breast cancer
TP53 ..... Tumor protein P53

TP53RK..... TP53-regulating kinase
Twist..... Twist-related protein 1
ZEB1 .....Zinc finger E-box-binding homeobox 1

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1 INTRODUCTION

1.1 BREAST CANCER

In Canada, breast cancer is the most frequently diagnosed cancer in women and the second most frequent in the entire population (Brenner et al., 2022). Women in Canada have an 88% chance of surviving 5 years after their initial diagnosis (Canadian Cancer Advisory Committee, 2021). The high survival rate can be attributed to a robust and well-established treatment model that includes chemotherapy, radiotherapy, immunotherapy, endocrine therapy, and mastectomy (Smith and Prewett, 2017). Despite the significant advances made in breast cancer treatment, the side effects of these therapies continue to adversely affect patients' quality of life. While efforts to mitigate these side effects have been made, they have not been entirely successful. There is hope that a combination of exercise (Ligibel et al., 2019; Wilson, 2017) and good nutrition (De Cicco et al., 2019) can result in less recurrence and fewer side effects, but a clear and effective method has not yet been established. Although diet has been proven effective in alleviating symptoms and decreasing recurrence in breast cancer patients, dietary interventions have also been extensively documented as a primary prevention strategy for breast cancer (Glade, 1999). Nonetheless, some dietary recommendations show promising results in reducing the risk of cancer, such as the ones proposed by the World Cancer Research Fund and the American Institute for Cancer Research (Lavalette et al., 2018) and the Prevención con Dieta Mediterránea study (PREDIMED), a Mediterranean Diet that includes extra virgin olive oil (Kargin et al., 2019). Notably, these dietary recommendations have been proven to be particularly effective in reducing the risk of both breast cancer and colorectal cancer, regardless of non-modifiable risk factor status (Catsburg et al., 2014; Nomura et al., 2016; Toledo et al., 2015).

1.2 MUTATIONS IN BREAST CANCER

The incidence of breast cancer is influenced by many lifestyle choices and environmental factors. There is evidence that a higher body mass index, the use of hormonal contraceptives, hormonal replacement therapy, age at the first pregnancy over 30 years old, and not breastfeeding, all increase the chances of developing breast cancer (Anand et al., 2008). Of all the risk factors for breast cancer, age and having a family history of breast cancer have the highest impact on the likelihood of developing the disease. A study using data from the Swedish Family-Cancer Database showed that 73% of breast cancer cases had a familial link, which goes up to 96% when the patient developed cancer before the age of 40 (Couto and Hemminki, 2007). This can be explained partly by hereditary factors, such as early first menstruation, late menopause (Surakasula et al., 2014), and breast density on mammograms (Evans et al., 2020). Although the influence of family on the risk of breast cancer has been known since the 1940s (Smithers, 1948), it was not until DNA sequencing became available that it was understood that specific genetic mutations are responsible for the increased risk of breast cancer in families with a history of the disease.

1.2.1 BRCA

BRCA1 and *BRCA2* genes encode caretaker proteins known as Breast cancer type 1 susceptibility protein and Breast cancer type 2 susceptibility protein that play a vital role in DNA repair. Identified in the 1990s, mutations in these genes prevent effective DNA repair, leading to even more mutations and an increased risk of breast and ovarian cancer (Hall et al., 1992, 1990; Miki et al., 1994; Wooster et al., 1995, 1994). Although they share a similar name and are both important in DNA double-strand breaks (DSBs) or replication fork collapse repair, BRCA1 and BRCA2 are not related to each other and serve two different functions in

the same pathway (Roy et al., 2011). A set of autosomal dominant gene mutations are highly penetrant and carry a stunning 70% risk of developing breast cancer and a 45% risk of ovarian cancer for certain mutations (Hartmann and Lindor, 2016). Although these germline mutations significantly increase the probability of developing breast cancer, they are found in only 5% to 10% of breast cancer patients (Rhei et al., 1998) and cannot account for all cases of familial breast cancer. However, these mutations are more common in certain populations, such as Ashkenazi Jewish where the frequency is up to 1 in 40, compared to 1 person in 500 in the general population, due to founder mutations (Friedman et al., 1995; Neuhausen et al., 1996; Simard et al., 1994). A similar founder effect has been observed in French Canadian breast cancer incidence, where 10% to 13% of early-onset cases can be related to less than 10 specific variants of BRCA1 and BRCA2 (Behl et al., 2020; Tonin et al., 2001). While many other genes, such as CDH1, PTEN, and CHEK2, have been identified that increase the risk of breast cancer, none have the same high occurrence and penetrance as BRCA1 and BRCA2 in the general population.

1.2.2 Epithelial cadherin

The *CDH1* gene provides instructions for making a protein called epithelial cadherin or E-cadherin. This protein is found within the membrane that surrounds epithelial cells and contributes to cell adhesion with other members of the cadherin family of proteins (Gamble et al., 2021). Mutation in the gene is associated with tumour proliferation, invasion, migration, and metastases (Shenoy, 2019).

Inherited mutations in the CDH1 gene increase a woman's risk of developing a form of breast cancer that begins in the milk-producing glands. In many cases, this increased risk occurs as part of an inherited cancer disorder called hereditary diffuse gastric cancer (Hansford

et al., 2015). Inherited mutations in the *CDH1* gene are thought to account for only a small fraction of all breast cancer cases (Corso et al., 2020).

1.2.3 Checkpoint kinase 2

CHEK2 encodes a kinase that is involved in the DNA damage response and has established roles in cell cycle arrest and triggering apoptosis when double-strand breaks are found in DNA (Mustofa et al., 2020). CHEK2 works through an assorted group of proteins that include cyclin-dependent kinases and p53 to prevent cell proliferation (Smith et al., 2020). Germline mutations in CHEK2 can predispose individuals to a wide range of cancers, including breast cancer. Although CHEK2 mutations are only found in 2% of breast cancer patients, those with the mutation tend to develop higher-grade cancers and are more likely to have secondary cancers (Kleiblová et al., 2019).

1.2.4 Tumour suppressor p53

Tumour protein p53 acts as a tumour suppressor by halting the cell cycle and activating DNA repair upon the detection of DNA damage. If repairs fail, p53 pushes the cell toward apoptosis. Normally, p53 is bound to Mouse double minute 2 homolog (MDM2). MDM2 plays a critical role in this context by obstructing the transactivation domain of p53, effectively inhibiting its functions. Additionally, MDM2 tags p53 for degradation through ubiquitination. MDM2 also possesses a nuclear export signal, which facilitates the translocation of the MDM2/p53 complex to the cytosol, where p53 is subsequently degraded by the proteasome (Chène, 2003). However, when activated by stressors, such as through JNK1-3, ERK1-2, and p38 MAPK activation, or DNA damage, through ATM, CHK1, CHK2, and TP53RK activation, p53 separates from MDM2 and accumulates in the cell. The free p53 can then interact with coactivators to activate a long list of genes involved in cell repair, survival, or

programmed cell death (Nakamura, 2004; Surget et al., 2013). With such a central role in DNA repair and apoptosis, somatic mutations in *TP53* are found in most cancers and it is believed to be the most common loss-of-function mutation in all cancers (Olivier et al., 2010). It has been found that p53 has multiple splice variants and mutations that can affect many of those variants. The loss of the canonical p53 function is also associated with the gain of function of these variants, contributing to the tumourigenicity of the mutations (Surget et al., 2013). Surprisingly, mutated p53 accumulates in the cells but does not induce apoptosis, unlike the accumulation of wild-type p53. This can be partially explained by the loss of the MDM2 binding region, which can no longer mark it for destruction, and the previously mentioned gain of functions of the mutant proteins (Yue et al., 2017).

Although p53 mutations are rare in breast cancer compared to other cancers (Pharoah et al., 1999), certain mutations are associated with a poorer prognosis (Bertheau et al., 2008). Counter-intuitively, not all somatic mutations or deletion of p53 in breast cancer affect survival and some can even have protective effects (Bourdon et al., 2011; Laptenko and Prives, 2006). Women who carry germline mutations in the TP53 gene have a very high likelihood of developing breast cancer (85% by age 60 years), which even surpasses the risk for patients carrying pathogenic mutations in *BRCA1* and *BRCA2*. Strikingly, they develop breast cancer at a very young age, with a median age of 34 years old (Schon and Tischkowitz, 2018).

1.2.5 Phosphatase and tensin homolog and phosphatidylinositol-3-kinase

PTEN is a phosphatase that plays a crucial role in tumour suppression by inhibiting the activity of the oncogenic phosphatidylinositol-3-kinase (PI3K) / protein kinase B (Akt) pathway. Specifically, PTEN acts by dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate (PIP3), a key signalling molecule produced by PI3K activation (Figure 1). By

reducing PIP3 levels, PTEN effectively inhibits the downstream activation of the Akt pathway, which is responsible for promoting cell growth, survival, and migration. Thus, loss of PTEN expression or function can lead to uncontrolled PI3K/Akt pathway activity and contribute to tumourigenesis. (Carracedo and Pandolfi, 2008).

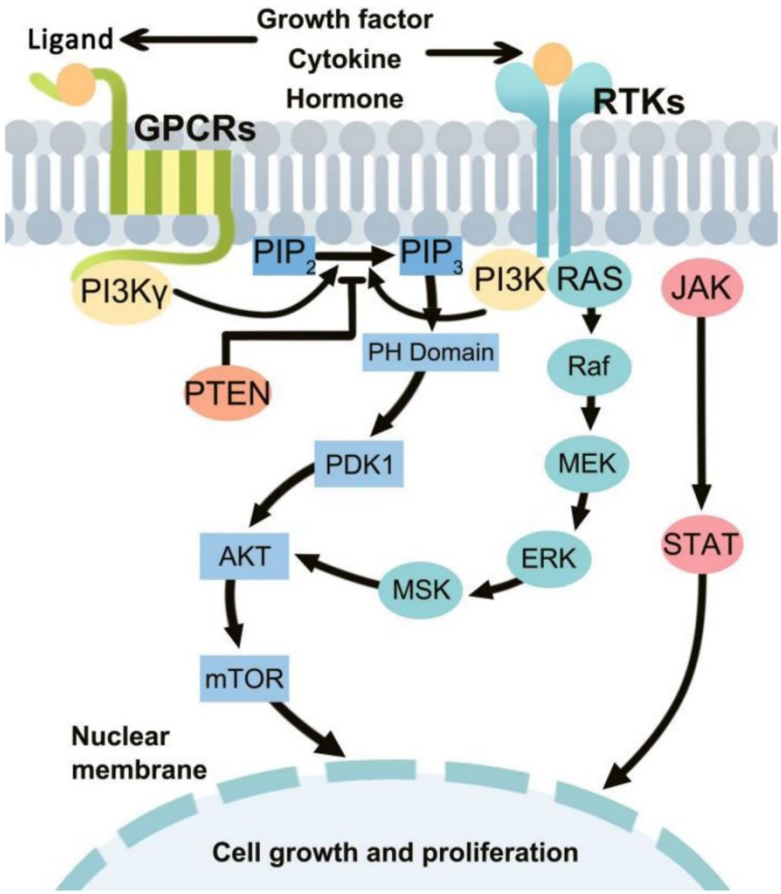

Figure 1: PTEN/PI3K/AKT pathway (Yang et al., 2019).

The loss of PTEN expression is observed in about 40% of all breast cancers and is often associated with a concurrent loss of estrogen receptors (Zhang et al., 2013). The PI3K pathway involves multiple proteins in each step toward activating the mammalian target of rapamycin (mTOR), a key regulator of cell growth and metabolism. Mutations have been identified at all stages of this activation process, particularly in the p110α subunit (PIK3CA), which has been

found in approximately 40% of advanced breast cancer tumours (Guerrero-Zotano et al., 2016).

1.3 BREAST CANCER SUB-TYPE

Breast cancer is often used as a catch-all term for all cancers originating in the breast. Nevertheless, with an understanding of the complex nature of the disease, an accurate classification of breast cancer subtypes is essential for developing personalized treatment plans that can improve patient outcomes and quality of life. With this in mind, it is important to note that the vast majority of breast cancers are carcinomas that originate from epithelial cells lining the breast ducts and glands. Carcinomas can be further separated by their supposed area of origin; invasive ductal carcinoma (IDC) which begins from the epithelial cells lining the milk ducts accounts for 65-85% of all breast cancers, followed by invasive lobular carcinoma (ILC), which originates in the milk-producing glands and accounts for 5-15% of cases (Dossus and Benusiglio, 2015; Zhao, 2021). Another subtype, invasive ductal and lobular carcinoma (IDLC), which combines characteristics of both IDC and ILC, is rarer and found in less than 5% of patients. In contrast, breast sarcomas, which are tumours that originate from the connective tissue, are extremely rare and make up less than 1% of all breast cancers. The remaining cases comprise a diverse group of even rarer types (Matsumoto et al., 2018; Sinn and Kreipe, 2013).

Breast cancer can be further classified into various subtypes based on their morphological and histological features, each with different prognoses and requiring distinct treatment approaches (Sinn and Kreipe, 2013; Zheng et al., 2018). Histological markers such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor

receptor 2 (HER2) are used to guide treatment decisions. The most common subtypes express either estrogen or progesterone receptors, or both, and are identified as hormone receptor-positive (HR+). HR+ tumours account for around 70% of breast cancer patients (Howlader et al., 2014). Treatment generally involves hormonal therapy drugs that lower estrogen levels or block estrogen receptors (Waks and Winer, 2019). For example, tamoxifen acts as an antagonist by binding to the estrogen receptors in the breast competitively and preventing estrogen from interacting with its receptor (Early Breast Cancer Trialists' Collaborative Group, 1998; Shagufta and Ahmad, 2018), while aromatase inhibitors such as anastrozole, exemestane, and letrozole are used in postmenopausal patients to inhibit the aromatase enzyme, which converts androgens to estrogen, effectively reducing estrogen levels (Kharb et al., 2020). Aromatase inhibitors are generally not effective in premenopausal women who have functioning ovaries, as their ovaries produce a large amount of aromatase, and they will respond to lowered estrogen levels by producing even more aromatase (Fabian, 2007). Both therapies slow the growth of estrogen-dependent tumours, however, over time, the cancer cells can bypass the need for hormones by upregulating the PI3K/AKT/mTOR pathway and the Cyclin D/cyclin-dependent kinase (CDK) 4/6 pathway (Hanamura and Hayashi, 2018; Mills et al., 2018). When this occurs, the patients typically need chemotherapy to treat the tumour.

The second group is classified by their expression of HER2. Approximately 15–20% of breast cancer patients are diagnosed with $HER2^+$ tumours (Howlader et al., 2014). Patients with breast cancers that express a high level of HER2 have a worse prognosis compared to those who express a low level to no HER2 (McCann et al., 1991). This effect is even seen in HR+ breast cancer patients, where tumours that also express HER2 have been shown to have a significantly worse prognosis, likely due to interactions between HER2 and estrogen receptor

(ER) signalling (Ding et al., 2019). The use of monoclonal antibodies targeting different domains of HER2 in addition to adjuvant chemotherapy significantly improved the survival of patients with HER2+ tumours (Waks and Winer, 2019).

Lastly, triple-negative breast cancer (TNBC) is a subtype of breast cancer that does not express HR and HER2. It is the rarest of the main subtypes, accounting for 10–20% of newly diagnosed breast cancer cases (Foulkes et al., 2010; Howlader et al., 2014). TNBC is the hardest to treat due to the lack of receptors on the surface of the cells, which makes it less responsive to hormonal therapies and targeted treatments (Bergin and Loi, 2019). For a long time, chemotherapy has been the only treatment option for TNBC, but unfortunately, the disease often develops resistance to this approach (Lyons, 2019). They are often diagnosed at a younger age and are possibly associated with BRCA1 (Kirk, 2010; Musolino et al., 2007). On top of that, TNBC has the highest early relapse risks of the three major subtypes of breast cancer but long-time survival might be better than HR+ breast cancers (Reddy et al., 2018). Recently, two new therapies have shown promise in a broad group of cancers, including TNBC. The first is poly ADP ribose polymerase (PARP) inhibitors, which prevent single-strand breaks from being repaired by PARP, causing major DNA damage, especially in cells lacking other DNA-repair mechanisms like those having BRCA1/2 mutations and PTEN mutations (Slade, 2020). This leads to cell death by mitotic catastrophe (Colicchia et al., 2017). The second group is composed of immune checkpoint blockers, such as monoclonal antibodies targeting programmed cell death protein-1 (PD-1) (Yi et al., 2021), programmed death-ligand 1 (PD-L1) (Schmid et al., 2018), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (Lens et al., 2008). Although anti-CTLA-4 has seen a diminution in its use because of the higher side effects and lower efficacy than alternative inhibitors, others, like PD-L1 show some

promise and are actively tested on new cancer types (El Osta et al., 2017). PD-L1, in particular, shows promise for TNBC, as it is expressed in 20% - 50% of tumours and correlates with the evasion of the immune system (Mittendorf et al., 2014; Xiao et al., 2020). Blocking its effect can reactivate the T-cell-mediated tumour cell death process (Killock, 2021). Interestingly, the treatment also works in some PD-L1-negative tumours (Shen and Zhao, 2018), although the exact reason is not fully understood yet (Chen et al., 2018; Zaslavsky et al., 2020).

1.4 THE CANCER STEM-LIKE CELLS (CSCS)

Although there have been significant advancements in our understanding of tumourigenesis and neoplasia, there are still some divergences in the theory of the cancer-initiating cell/the cell-of-origin. For cancer to develop, cells must not only multiply uncontrollably but also evade the immune response. This requires the accumulation of a certain number of mutations or epigenetic changes. For instance, in breast cancer, Wood et al. found mutations in 15 candidate cancer genes and 70 mutations in other genes (Wood et al., 2007). While there is little resemblance between different cancers, they tend to have mutations in genes that serve similar functions (Huang et al., 2018). Similar numbers of mutations have also been reported for colon cancer (The Cancer Genome Atlas Network, 2012). However, most cells do not live long enough to accumulate all these mutations.

One hypothesis suggests that the presence of cells with characteristics similar to stem cells could explain many tumour characteristics. This concept, known as the Cancer Stem Cells (CSCs) theory, proposes that tumours are driven by a subpopulation of highly tumourigenic cells (Sansone et al., 2007). They are called cancer stem cells because they possess similar characteristics to those found in stem cells, such as the ability to self-renew and differentiate

into all types of cells that compose the tumour (Duru et al., 2012). They possess several markers, including CD133, CD44, CD24, and ALDH (Karsten and Goletz, 2013). When breast cancer cell lines are cultured in a low attachment environment, cells form spheroids called mammospheres with a $CD44^{+}/CD24^{-/low}$ phenotype (Sansone et al., 2007).

While the concept that a cancer stem cell is the cell of origin of a tumour is still controversial, the tumourigenicity of CSCs is well established. For instance, injecting mice with only 100 potential cancer stem cells was enough to induce tumour formation (Al-Hajj et al., 2003), in contrast to the 10,000 non-stem cancer cells, suggesting that not all cancer cells have the same tumourigenic potential. The large number of cells required to initiate a tumour was previously attributed to technical errors, such as the loss of cancer cells' tumour-initiating potential after harvest. Additionally, it was believed that the microenvironment of the tumour played a crucial role in sustaining cell proliferation, whereas the microenvironment in which the cells were injected did not promote tumour formation (Fokas et al., 2012; Swartz et al., 2012). However, with the emerging evidence for CSCs, many researchers now propose that only a small population of cells possess stemness, which explains why a large number of cells need to be transferred to provide enough tumour-initiating cells.

Inflammation is linked clinically and epidemiologically to cancer (Coussens and Werb, 2002). While inflammation provides an environment that promotes metastasis and recurrence, it is now well established that inflammation also favours the development of CSC. Pro-inflammatory cytokines, such as interleukin 6 (IL-6), play a crucial role in inflammation and are closely linked to the development of epithelial tumours like breast and lung cancer (Sansone et al., 2007). IL-6, a potent pleiotropic cytokine, mediates a plethora of physiological functions including cell proliferation, survival, and resistance to chemotherapy.

The CSC theory stipulates that conventional cancer treatments such as chemotherapy and radiotherapy target most of the cells or non-CSC cancer cells composing the tumour but fail to eliminate the CSC. CSCs have been identified in numerous types of cancer (Dave et al., 2012), including less malignant forms, where they can lie dormant during the treatment. However, when many cells are killed, CSCs can re-emerge, repair the damage, and begin to divide, leading to a relapse that is often more aggressive than the original tumour (**Figure 2**) (Ayob and Ramasamy, 2018).

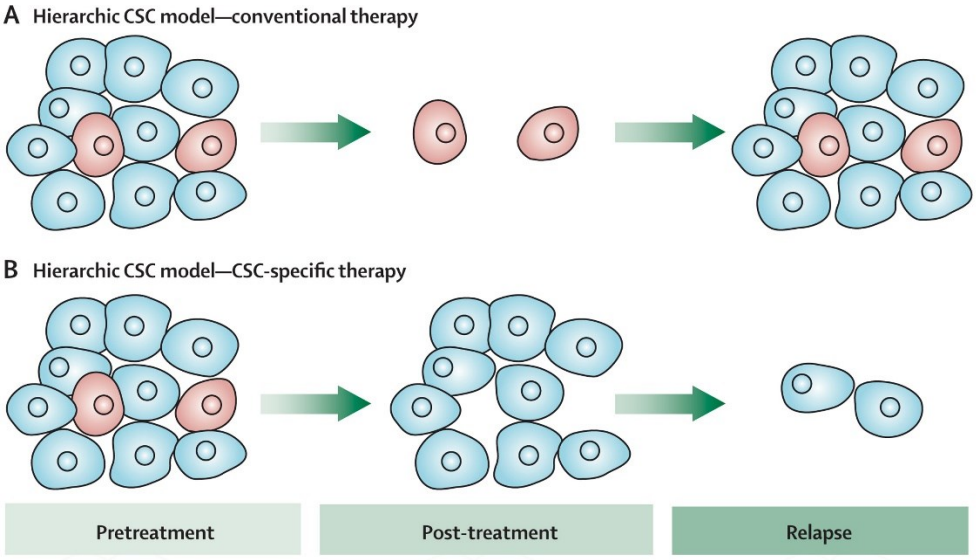

Figure 2: Comparison of conventional and CSC-specific therapy (Vermeulen et al., 2012).

CSCs possess several advantageous characteristics that allow them to resist treatment more effectively than other cancer cells. They often over-express multi-drug efflux pumps and reactive oxygen species scavengers, which can prevent medications from causing DNA damage. They also have a low rate of multiplication, efficient DNA repair systems, and a greater chance of escaping mitotic catastrophe (Rajaraman et al., 2006). Additionally, they have epithelial-mesenchymal transition (EMT) regulators and the potential to multiply

indefinitely, allowing them to reform a tumour at the original and distant sites of the original tumour (Najafi et al., 2019). Moreover, the treatments may even target some CSCs and only leave the most resistant ones, further increasing the tumorigenicity and resistance potential of the tumour. Furthermore, CSCs exploit the same pathways that normal stem cells use to evade destruction, just like how tumours use PD-L1 to evade the immune system (Han et al., 2020). For example, the OX-2 membrane glycoprotein, also known as CD200, protects a variety of cells from macrophages and has been observed to be over-expressed by CSCs in carcinoma and leukemia (Herbrich et al., 2021; Jung et al., 2015).

The origin of the CSC is still controversial. It is believed that they may arise from less differentiated cells that still possess stem or progenitor characteristics (Atashzar et al., 2020; Blaas et al., 2016). Alternatively, changes in the microenvironment of the tumour may cause differentiated cells to revert to a less differentiated, stem-like state (Nakano et al., 2019). This shift to an undifferentiated or stem-like state has been linked to the EMT (Guttilla et al., 2012; Lambert et al., 2017). The process, coopted by cancer, is necessary for embryonic development and wound healing by reducing cell-to-cell adhesion and increasing cell motility, allowing cells to enter specific tissues (Savagner, 2010). However, in the context of cancer, the microenvironment of the tumour becomes more and more unfavourable for proper tumour growth due to aberrant vascularization and the accumulation of damage from chemotherapy and radiotherapy. This leads cells to undergo changes to survive including EMT (Hapke and Haake, 2020; Huber et al., 2004; Larue and Bellacosa, 2005). Hypoxia, a condition of low oxygen levels, can also stimulate the cells to metastasize by upregulating known E-cadherin inhibitors, such as Zinc finger E-box-binding homeobox 1 (ZEB1) and Twist-related protein 1 (Twist), through overexpression of Hypoxia-inducible factor 1-alpha (HIF1α) (Eger et al.,

2005; Erin et al., 2020). Additionally, EMT and stem cells share core pathways, including Notch, WNT/β-Catenin, Hedgehog, and NFκB (Ouyang et al., 2010). It is even possible to change differentiated cells to a stem-like state using specific genes such as OCT4, SOX2, KLF4, and MYC (Takahashi and Yamanaka, 2006), but unfortunately, silencing these genes does not prevent the cells from developing into cancers (Villodre et al., 2019).

Despite significant advances in cancer treatment, metastasis remains responsible for approximately 66% of deaths in patients with solid tumours (Dillekås et al., 2019). This phenomenon can be easily explained by the CSC theory, which suggests that the process of EMT allows cells to become mobile and more resistant to apoptosis, thereby facilitating the metastatic spread of cancer (Suarez-Carmona et al., 2017). Tumours are heterogeneous and may consist of multiple tumour-initiating cells, with a subset of CSCs being particularly active in specific environments and giving rise to the majority of the primary tumour. However, CSCs also have the potential to migrate and find more suitable environments for proliferation, leading to the formation of metastases (Brabletz et al., 2005). Unfortunately, when tumours metastasize to distant sites, life expectancy decreases significantly and despite significant advances, treatment options for patients with metastatic disease remain limited.

1.5 4T1 *IN-VIVO* BREAST CANCER MODEL

Developing therapies based on our understanding of the underlying mechanisms turning cells into cancer cells and the driving factors in cancer progression is severely limited by our lack of a good model. The use of cancer cell lines grown in vitro allows us to test different compounds and find proteins and pathways that are necessary for the proliferation and survival of those cells but translating those results into real improvement in patients has

not always been successful. Cancer cell lines are severely compromised. For one, many were selected for their hardiness to grow under laboratory conditions, that is their ability to grow without the proper structure found in normal and cancerous tissues like stroma cells, vascularisation and interaction with the immune system (Valkenburg et al., 2018). They also accumulate a large number of mutations through each passage and suffer from selective pressure for the fastest proliferating cell accumulating a genotypic and phenotypic drift compared to the original tumour (Briske-Anderson et al., 1997). This pales in comparison to the idea that the cell lines used could be in fact an entirely different type from a completely different tissue (Capes-Davis et al., 2010).

Animal models offer a more natural environment for studying tumors. While the ethical implications of using animals can be partially alleviated with proper surveillance, planning, and training, the technical limitations have proven more challenging to overcome. Furthermore, the implementation of known and characterized cancer cells in animal models faces similar selection biases as those encountered in in vitro culture.

The 4T1 cell line is a well-established model for studying breast cancer metastasis and is known to form metastases in multiple organs, such as the lung, liver, and brain, mimicking stage IV human breast cancer (Pulaski and Ostrand-Rosenberg, 2001). Derived from a BALB/C mouse tumour, the 4T1 cell line can be easily implemented in other members of the inbred strain of mice (Dexter et al., 1978). 4T1 cells are naturally resistant to the cytostatic antimetabolite 6-thioguanine, which is a useful property that can be used to select for cells that metastasize to distant sites since cells not originating from the primary tumour will not proliferate.

The cell line 4T1 is a murine cell line that provides a suitable background for conducting experiments in an immunocompetent mice model. Models using human-derived cell lines often rely on severe combined immune-deficient (SCID) mice, which have impaired T and B cell lymphocyte development, or NOD SCID mice, which are, in addition, deficient in natural killer (NK) cell. However, these models are deemed less compatible with our research goals of studying immune-dependent mechanisms. By avoiding the use of immunocompromised mice, we can investigate the complex interaction between diets and the immune system, a relationship that is now appreciated as playing an important role in mammary carcinoma development. The 4T1 cell line's ability to metastasize to organs distant from the injection site, combined with its inherent resistance to 6-thioguanine, makes it an excellent model for studying the complex interaction between diet, the immune system, and cancer metastasis (Tao et al., 2008).

1.6 THE EPIGENETIC CONTROL OF CANCER STEM CELLS

Epigenetic mechanisms are believed to play a role in cancer initiation and progression, with deregulation observed at multiple levels, including DNA methylation, histone modifications, and indirectly, microRNA expression (Balassiano et al., 2011; Herceg and Hernandez-Vargas, 2011; Lima et al., 2010). These mechanisms are, by definition, independent of the DNA sequence and heritable from one cell generation to the next. However, unlike genetic changes, epigenetic alterations are reversible and thus represent a promising avenue for targeted intervention. There is growing evidence suggesting that epigenetic profiles are disrupted early during breast carcinogenesis, with various lifestyle and dietary factors potentially contributing to this process (Guttilla et al., 2012; Marotta et al., 2011). Notably, our own research has identified epigenetic-specific changes in CSCs, with methylome analyses

indicating differential methylation of the Jak-STAT signalling pathway in putative CSCs grown as mammospheres compared to the general population compared to the general population of cells present in the parental cell line (Hernandez-Vargas et al., 2011).

1.6.1 MicroRNAs

MicroRNAs are small nucleotide sequences that influence the translation of genes and have emerged as critical regulators of CSCs in drug resistance and cancer metastasis (Schwarzenbacher et al., 2013). These endogenous non-coding RNAs act by controlling gene expression at the post-transcriptional level and have emerged as important regulators of oncogenesis (Hatfield and Ruohola-Baker, 2008). They work by binding to a complementary sequence in the target mRNAs, by either blocking the ribosome from translating the mRNA or cleaving the RNA with the help of the microRNA ribonucleoprotein complex. MicroRNAs are often dysregulated in malignancies (Shi et al., 2008) and some can also function as tumour suppressors or oncogenes (Hatfield and Ruohola-Baker, 2008). MicroRNA networks have been found to establish a permanent feedback loop, involving NF-κB, let-7 microRNA, IL-6, and STAT3, which are responsible for inducing and maintaining the CSC state (Iliopoulos et al., 2010a). Recent research has linked several microRNAs with tumour progression and metastasis, but specific families of microRNAs have been found to be particularly associated with breast carcinogenesis. Among aberrantly expressed microRNAs, let-7 family, miR-125, miR-145, and miR-200 family were found to be significantly downregulated whereas miR-21 and miR-155 were upregulated in breast cancer (Singh and Mo, 2013). Our research has shown that the miR-30 family is involved in eliciting epigenetic-specific changes that prompt cellular transformation, carcinogenesis, and maintenance of stemness (Ouzounova et al., 2013).

Studies have reported that pomegranate juice has an anti-metastatic effect on prostate cancer cells due to the upregulation of anti-invasive microRNAs such as miR-355, miR-205, miR-200, and miR-126, while pro-invasive microRNAs such as miR-21 and miR-373 were downregulated (Banerjee et al., 2012; Wang et al., 2011). The consumption of blueberries has also been shown to have a chemopreventive effect on breast cancer by modulating miR-18a and miR-34c (Jeyabalan et al., 2013). Pterostilbene, a chemical compound that shares structural similarities with resveratrol and is commonly found in blueberries and grapes, has been reported to have the ability to reduce the number of CSCs by increasing the amount of miR-448 in the cells (Mak et al., 2013).

miR-145 is a microRNA associated with the PI3K/AKT pathway that is commonly under-expressed in highly metastatic breast cancer (Iorio et al., 2005; Radojicic et al., 2011). Studies have shown that miR-145 is regulated by Akt in a p53-dependent manner, and suppressing PI3K activity can increase p53 levels and induce miR-145 expression (Sachdeva et al., 2012). In particular, p53 appears to upregulate the expression of tumour suppressor microRNAs such as let-7, miR-34, miR-145, miR-26, miR-30, and miR-146a (Ghose et al., 2011; Stahlhut and Slack, 2015; Suh et al., 2011).

miR-145 has been shown to impact important cancer stem cells and breast cancer proteins, such as c-Myc, OCT4, SOX2, and N-RAS (Cui et al., 2014; Guzel Tanoglu and Ozturk, 2021). Interestingly, OCT4 is of particular interest due to its role in cancer stem cells (Kim and Nam, 2011; Kumar et al., 2012). miR-145 has been shown to inhibit breast cancer metastasis by reducing Cadherin-2 (CDH2) expression (Zeng et al., 2021). Furthermore, miR-145 may be involved in a feedback loop with NF-κB that enables metastasis and angiogenesis

in breast cancer (Zhu et al., 2021). miR-145 and miR-195 also influence angiogenesis, an important factor in tumor progression (Wang et al., 2013, p. 195; Yin et al., 2013).

N-RAS is commonly mutated in other cancers, but not frequently in breast cancer. However, it is highly expressed in a subtype of aggressive TNBC that currently lacks targeted therapies and has a poor prognosis (Zheng et al., 2015). Moreover, miR-145 can potentially reduce angiogenesis by directly targeting N-RAS and inhibiting HIF1 and VEGF in breast and colorectal cancer (Yin et al., 2013; Zou et al., 2012). These findings suggest that miR-145 has potential as a therapeutic target in breast cancer treatment.

miR-210 is consistently induced under hypoxia and commonly over-expressed in solid tumours (Ivan and Huang, 2014). Expression of miR-210 is correlated with a poor prognosis (J. Wang et al., 2014) and is involved in the proliferation of breast cancer stem cells and their escape from the primary tumour (Tang et al., 2018). Several miRNAs, including miR-210, have been shown to either positively or negatively regulate NF-κB activation through their interaction with Toll-like receptors (TLRs), particularly TLR-4. TLRs are known to play a role in cancer development by promoting inflammation and activating anti-apoptotic pathways. TLR-4, for instance, contributes to tumour cell proliferation and helps cancerous cells evade treatments (Oblak and Jerala, 2011; A.-C. Wang et al., 2014). Moreover, intracellular TLR-7/8 can recognize specific miRNAs which can initiate anti-inflammatory effects associated with significant health benefits (Fabbri et al., 2012).

1.7 FOXO

The proteins forming the forkhead family are transcriptional regulators that share a DNA-binding domain aptly called the 'forkhead box'. Originally discovered in adult flies, mutations in the original *fkh* gene led to aberrant head appearance ((Kaestner et al., 2000; Weigel et al., 1989). The discovery of the winged helix domain forming the forkhead box led to the renaming of a mixed group of transcription factors that also contained the same helix-turn-helix pattern as FOX (Clark et al., 1993; Kaestner et al., 2000). To distinguish between members of each subclass, each factor was separated into subclasses using phylogenetic analysis and was renamed FOX followed by a letter, and a number is used to further differentiate between members of each subclass (Kaestner et al., 2000). For instance, Forkhead box protein O1 (FOXO1) was originally identified in alveolar rhabdomyosarcoma and was named forkhead in rhabdomyosarcoma (FKHR) before being renamed (Galili et al., 1993). The Mammalian FOXO subclass comprises four different members, namely FOXO1, FOXO3, FOXO4, and FOXO6. Two factors are not present, FOXO2 is not included because it is nearly identical to FOXO3 and not present in mice (Biggs et al., 2001), and FOXO5 is an ortholog of FOXO3 only found in fishes (Rudd et al., 2003).

FOXO1 is a transcription factor generally known for its role in adipogenesis and the inhibition of the production of glucose in reaction to insulin. FOXO1 is expressed in almost all cells but is more abundant in adipose tissues (Nakae et al., 2003) and ovaries (Shi and LaPolt, 2003). The subcellular localization of FOXO proteins plays a critical role in regulating their activity. Research has shown that phosphorylation of FOXO1 results in its exclusion from the nucleus (Zhang et al., 2002). The FOXO subclass members are regulated by the insulin/PI3K/Akt signalling pathway (Figure 3) (Martinez et al., 2006). Protein phosphatase 2

(PP2A) has been shown to be a FOXO1 phosphatase, which dephosphorylates FOXO1 either in the cytoplasm or in the nucleus (Yan et al. 2008). In addition to insulin receptors (Kops and Burgering, 1999; Nakae et al., 1999) a large number of receptors also affect FOXO1 including insulin-like growth factor I (IGF-I) (Brunet et al., 1999), interleukin 3 (Dijkers et al., 2000), erythropoietin (Kashii et al., 2000), epidermal growth factor (Jackson et al., 2000), nerve growth factor (Zheng et al., 2002) and B cell receptor (Hinman et al., 2007).

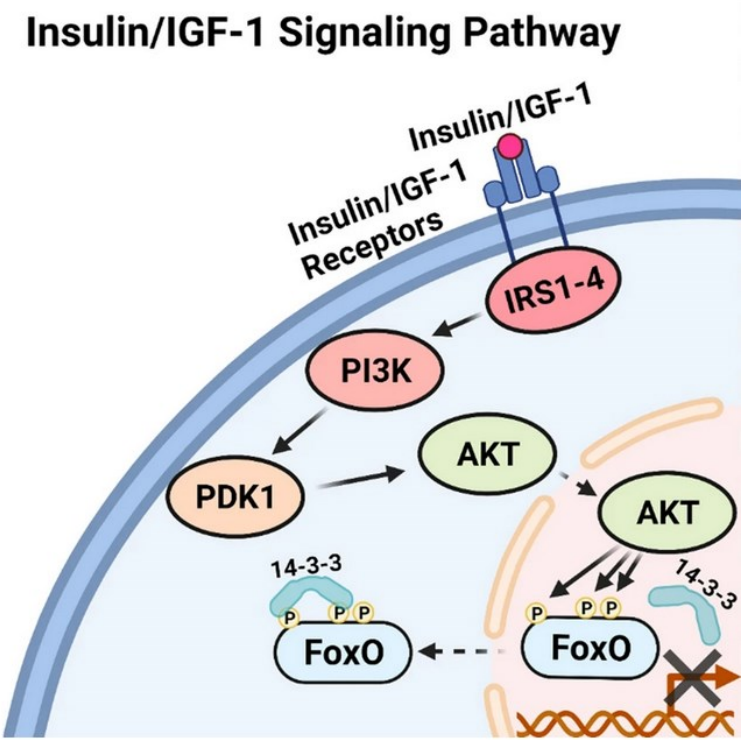

Figure 3: The insulin/PI3K/Akt signalling pathway (Greer and Brunet, 2005)

Specifically, when growth factors or insulin bind to their tyrosine kinase receptors, it activates PI3K, which leads to the activation of Akt and serum- and glucocorticoid-inducible kinase (SGK) family of protein kinases, among others (Cantley, 2002; Ren et al., 2021). The nuclear export of FOXO proteins is facilitated by the conserved C-terminal region, which serves as a nuclear export sequence (NES), and is triggered by the phosphorylation of FOXO

by Akt and SGK (Biggs et al., 1999; Brunet et al., 2002). In the absence of growth factors and when Akt and SGK are inactive, FOXO factors remain localized within the nucleus. However, when cells are exposed to growth factors, the PI3K–Akt/SGK pathway is activated, leading to the phosphorylation of FOXO and its subsequent export to the cytoplasm. This export is facilitated by the interaction of phosphorylated FOXO factors with 14-3-3 proteins, which act as chaperones to escort FOXO out of the nucleus (Brunet et al., 2002, 1999). In summary, the insulin/PI3K/Akt signalling pathway is crucial in regulating FOXO transcription factors by controlling their subcellular localization in response to different growth factors.

FOXO1 is also important in regulating the cell cycle, stress resistance, and tumour suppression, all important factors in cancer stem cells (Lu and Huang, 2011). Like other members of the FOXO family, FOXO1 targets a specific genetic sequence, TTGTTTAC. FOXO proteins contain a Forkhead domain, a 110-amino acid region in the central part of the molecule that enables binding to DNA through recognition of a core motif, typically GTAAA(C/T)A, that is often found in the promoters of genes regulated by FOXO proteins. (Furuyama et al., 2000; Weigelt et al., 2001). A large number of genes are found to contain FOXO-binding sites (DBEs) in their promoters, as evidenced by bioinformatics studies (Xuan and Zhang, 2005). When FOXO factors are present in the nucleus and bound to DNA, they usually act as strong transcriptional activators (Brunet et al., 1999; Kops and Burgering, 1999).

FOXO transcription factors are also subject to regulation by other factors, such as Polo-like kinase 1 (PLK1), a serine/threonine kinase essential for cell cycle progression, which acts on FOXO1 during the late stages of the cell cycle (Gheghiani et al., 2020). PLK1 has been implicated in prostate cancer, and inhibition of PLK1 affects the PI3K/AKT signalling pathway partially through FOXO1 (Zhang et al., 2014).

Furthermore, cytosolic FOXO1 has been found to be required for autophagy in human cancer cells, and this process is independent of its transcriptional activity. FOXO1 is acetylated by SIRT2 and binds to Atg7 to influence autophagy, leading to cell death. This mechanism is associated with tumour suppression in human colon tumours and a xenograft mouse model, linking the anti-neoplastic activity of FOXO1 and the process of autophagy (Zhao et al., 2010).

Although FOXO transcription factors are mainly regulated by changes in subcellular localization, their degradation mediated by the ubiquitin-proteasome pathway is also a critical factor in their regulation (Matsuzaki et al., 2003). Specifically, Akt-mediated phosphorylation of FOXO1 not only exposes its NES and relocates it to the cytoplasm but marks it for degradation by the ubiquitin-proteasome system (Plas and Thompson, 2003). Degradation of FOXO proteins often occurs during cell transformation and may play a critical role in tumourigenesis (Hu et al., 2004; Huang et al., 2005).

FOXO1 and FOXO3 are expressed throughout the body and play overlapping roles in various cellular functions, including tumour suppression. FOXO1 is often repressed in cancer. Cancer often involves constitutive activation of the PI3K/AKT axis (Thorpe et al., 2015) and the loss of its inactivator PTEN by deletion or mutation in cancer which prevents it from repressing AKT signalling (Chalhoub and Baker, 2009). The constitutive activation of AKT continuously marks FOXO1 for transportation and degradation, preventing its slowing effect on the cell cycle. FOXO1 also promotes the expression of miR-145, which controls multiple proteins associated with cancer (Gan et al., 2010). FOXO1 is also a direct target of mir-145 (Jiang et al., 2017) hinting at an autoregulation of the process. While miR-145 can reduce the presence of FOXO1, its ability to repress Akt might be more important, allowing FOXO1 to slow down the cell cycle and prevent proliferation in cancer (Y. Wang et al., 2014).

1.8 N-RAS

Neuroblastoma RAS viral oncogene homolog (N-RAS) is a member of the Ras family of GTPases, which includes H-RAS and K-RAS, all of which are also considered oncogenes due to their role in promoting cancer (Downward, 2003). When activated by various receptor tyrosine kinases, RAS proteins become phosphorylated, leading to the activation of RAF kinases, a group of three protein kinases that participate in the MAPK cascade (Simanshu et al., 2017). N-RAS can activate a wide range of pathways including novel RAS effector 1A (NORE1A), Af6, phospholipase C (PLC), RAS and Rab interactor 1 (RIN1), T cell lymphoma invasion and metastasis-inducing protein (TIAM), and growth factor receptor 14 (Grb14) (Stephen et al., 2014). However, the MAPK cascade and the PI3K pathway are the most studied pathways that ultimately modulate cellular processes such as proliferation, cell death, and motility (Fernández-Medarde et al., 2021; Gupta et al., 2007).

When activated by guanine nucleotide exchange factors (GEFs), Ras proteins undergo a conformational change that enables them to release GDP leaving them free to bind to GTP. Ras can then bind to and activate downstream effector proteins. Ras can be turned off by GTPase-activating proteins (GAPs), which stimulate the intrinsic GTPase activity of Ras and catalyze the hydrolysis of GTP to GDP (Cherfils and Zeghouf, 2013).

Ras proteins can be mutated in ways that render them constitutively active by resisting GTP hydrolysis mediated by GAPs (Grewal et al., 2011). Mutations in *Ras* genes are found in a variety of human tumours, including pancreatic, lung, and colorectal cancers. In fact, *Ras* mutations are among the most commonly observed genetic alterations in cancer, making them an important target for cancer research and drug development (Simanshu et al., 2017). However, *N-ras* is rarely mutated in breast cancer but is activated through other mechanisms

(Cerami et al., 2012; J. Gao et al., 2013). Furthermore, elevated expression of N-RAS has been linked to a greater probability of being diagnosed with triple-negative breast cancer and a poorer prognosis (Banys-Paluchowski et al., 2020).

1.9 BLUEBERRY

Vaccinium angustifolium Aiton, commonly known as the wild lowbush blueberry, is a species native to the eastern coast of Canada and the northeastern United States, with a range extending from Manitoba to Newfoundland (see Figure 4). As a member of the Ericaceae family, blueberries are well-adapted to acidic soils of low quality and low nitrogen and phosphorus availability. This is due in part to the symbiotic relationship between several species of fungi and the root system of the blueberry (Cairney and Meharg, 2003). This interaction, called mycorrhiza, facilitates the absorption of nutrients released by enzymes from the fungi that work only in acidic conditions (Leake and Read, 1989). However, proper establishment of the mycorrhiza makes it challenging for commercial plantations to spread lowbush cultivars since propagation from seed or cutting is slow and yields are typically lower than those of highbush varieties (Yarborough, 2012). Consequently, much of the production of lowbush blueberries comes from managed "wild" patches, rather than from planted cultivated fields. Commercial growers often engage in practices such as controlled burning, pruning, and fertilization to maintain the health and productivity of these patches. Commercial lowbush blueberry production is concentrated primarily in Maine, USA, as well as in Quebec, Nova Scotia, New Brunswick, and Prince Edward Island in Canada (*Statistical Overview of the Canadian Blueberry Industry, 2010*, 2012).

Figure 4: Native Distribution of Vaccinium angustifolium Aiton (U.S. Department of Agriculture, Natural Resources Conservation Service., 2018)

Blueberries are well-known for their high concentration of phenolic compounds, including anthocyanins, which contribute to their high antioxidant capacity (Gibson et al., 2013). Anthocyanins, a type of flavonoid pigment, are responsible for the blue pigmentation of blueberries and are predominantly located in the skin. Members of the genus *Vaccinium* (Ericaceae), such as blueberry and cranberry, are excellent sources of flavonoids, such as anthocyanins, flavonols and proanthocyanidins (Bomser et al., 1996). In addition, wild blueberries are a good source of Vitamin C and dietary fibre, with very little fat, sodium, or cholesterol and only 80 calories per cup of berries (Nadulski et al., 2019; U.S. Department of Agriculture, Agricultural Research Service., 2019). However, variations in the levels of anthocyanins, total phenolics, and antioxidant capacity have been observed between different fields of wild blueberries and even between different years of blueberry production within the same field (Howell et al., 2001; Kim et al., 2013; Lohachoompol et al., 2004). Furthermore, it is worth noting that wild blueberries contain a wider variety and higher levels of anthocyanins

compared to cultivated highbush varieties, likely due to the more stressful growth conditions in the wild and the fact that they have not been selectively bred over generations for sweetness and ability to withstand shipping conditions (Liu, 2013). Recent research has shown that fermenting blueberries with commercially available probiotics leads to only a 2-3 fold increase in antioxidant capacity compared to normal blueberries (Zhong et al., 2021).

Blueberries are well-known for their numerous health benefits. One of their main benefits is due to their antioxidant activities (Kay and Holub, 2002; Mazza et al., 2002). These compounds also protect against DNA damage, which is known to be a contributing factor in various diseases such as cancer (Del Bó et al., 2013; van Breda et al., 2018; Wilms et al., 2007). Moreover, blueberries not only protect against cancer but also increase the effectiveness of certain treatments like radiotherapies and chemotherapies (Davidson et al., 2019; Lin et al., 2019).

Blueberry polyphenols and fibre have been shown to mitigate the risk of diabetes by improving insulin sensitivity and regulating glucose levels (Basu et al., 2021; Delpino et al., 2022; Muraki et al., 2013). Additionally, blueberries have been found to prevent the oxidation of cholesterol, lower blood pressure, and overall protect against heart diseases (Basu et al., 2010; Curtis et al., 2019; Herrera-Balandrano et al., 2021). Polyphenols present in blueberries help inhibit miR-21, miR-146a, and miR-125, miRNAs related to atherosclerotic plaques (Su et al., 2017).

Blueberries have also shown promise in promoting brain function and protecting against neuronal damage and age-related cognitive decline (Boespflug et al., 2018; Krikorian et al., 2022; Tran and Tran, 2021). Like the closely related cranberries, blueberry juice has been found to possess anti-adhesin activity, blocking the binding of bacteria to the urinary tract

wall and preventing urinary tract infections (Cerezo et al., 2020; Ofek et al., 1991). Recent research suggests that blueberry juice may also have a beneficial effect on preventing gastric ulcers by inhibiting the adhesion of Helicobacter pylori to the gastric mucosa and protecting the gastric epithelium from injuries (Shu et al., 2022; Silvan et al., 2022).

1.10 BLUEBERRY POLYPHENOLS

Blueberries are highly regarded for their health benefits, which are largely attributed to their high phytonutrient content, particularly polyphenolic compounds. Polyphenols are a class of natural compounds that contain benzene rings with hydroxyl (OH) groups (Pandey and Rizvi, 2009) and play an important role in the defence and protective mechanisms of plants against various stresses, including UV radiation, tissue damage, heat shock, water restriction, and oxidative stress (Beckman, 2000; Edreva and Velikova, 2008). Blueberries are particularly rich in polyphenols, including flavonoids such as anthocyanins, flavonols, and proanthocyanidins, as well as phenolic acids (Rodriguez-Mateos et al., 2012). These compounds range in size and complexity, from small and easily absorbed like flavonoid aglycones (not bound to a sugar molecule) to more complex structures like anthocyanins (Velderrain-Rodríguez et al., 2014). Anthocyanins, which are responsible for the blueberry's vibrant colour, are one of the most abundant polyphenols present in the fruit (Wu et al., 2006). Blueberries also contain significant amounts of flavonols, primarily composed of quercetin derivatives, and proanthocyanidins, formed by the polymerization of catechin and/or epicatechin units (Cho et al., 2005; Rodriguez-Mateos et al., 2012; Vrhovsek et al., 2012). In addition, phenolic acids, mainly chlorogenic acid, caffeic acid, gallic acid, protocatechuic acid, ferulic acid and salicylic acid, are all present in lowbush blueberries (Kang et al., 2015; Rodriguez-Mateos et al., 2012).

1.10.1 Anthocyanins

Anthocyanins, a type of flavonoid molecule, are water-soluble pigments that can display a range of colours, including red, purple, blue, and black depending on pH levels. They are responsible for the blue hues in various fruits and plants, such as blueberries, raspberries, black rice, and black soybeans, as well as the autumnal colours of leaves. (Archetti et al., 2009; Mazza, 1993). These pigments have important roles in plant reproduction, attracting pollinators, and protecting plants from environmental stressors such as UV light, drought, and cold (Edreva and Velikova, 2008; Lev-Yadun and Gould, 2009). Blueberries contain a complex anthocyanin profile, containing 5 of the 6 common anthocyanidins found in food: malvidin, cyanidin, delphinidin, petunidin, and peonidin (Figure 5) (Cho et al., 2005; Prior et al., 2001).

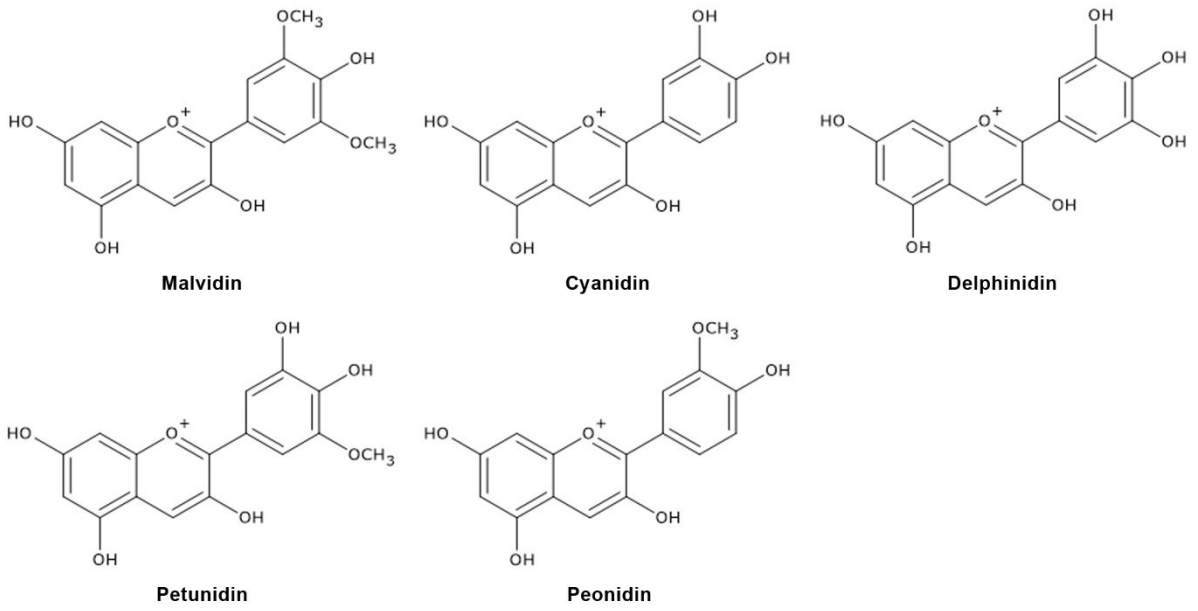

Figure 5: Anthocyanins present in blueberries.

Studies have shown that anthocyanins can have a beneficial effect on obesity-related diseases, such as diabetes (Cao et al., 2019), as well as inflammation (Lee et al., 2017). They may also reduce inflammation and the risk of heart disease by improving endothelial function and reducing blood pressure (Vendrame and Klimis-Zacas, 2019). Moreover, anthocyanins may have a protective effect against neurological diseases by promoting blood flow to the brain and maintaining synaptic function (Gratton et al., 2020; Vauzour et al., 2021).

Studies have also suggested that anthocyanins have anti-cancer properties, with potentially protective effects against colorectal and breast cancers (Bobe et al., 2006; Mazzoni et al., 2019; Paramanantham et al., 2020; X. Wang et al., 2019). These effects may be mediated through the regulation of signal cascades, such as the Ras-MAPK and PI3K/Akt pathways (Lin et al., 2017).

1.10.2 Protocatechuic acid

Protocatechuic acid (PCA), also known as 3,4-dihydroxybenzoic acid, is a naturally occurring phenolic acid found in a wide variety of plants. It is structurally similar to other well-known antioxidant compounds such as gallic acid, caffeic acid, vanillic acid, and syringic acid (Figure 6) (Kang et al., 2015). PCA has been shown to have antioxidant (Li et al., 2011) and anti-inflammatory properties (Lende et al., 2011), as well as the ability to interact with various enzymes, which may contribute to its pharmacological effects.

Figure 6: Example of phenolic acid present in blueberries.

Interestingly, PCA has been found to exhibit dual-directional roles in regulating many pharmacological activities, acting as both an antioxidant and oxidant as well as stimulating

both cell apoptosis and proliferation depending on the dose used (Nakamura et al., 2000; Yin et al., 2009). This highlights the importance of careful consideration of PCA dosage in its use as a potential therapeutic agent.

Studies have shown that PCA has a wide range of pharmacological activities, including anticancer (Lin et al., 2011; Semaming et al., 2015), neuroprotective (Z. Li et al., 2021; Zhang et al., 2015), antibacterial (Fifere et al., 2022; Wu et al., 2022), antiviral (Ou et al., 2014; Wang et al., 2022) and anti-osteoporotic (Rivera-Piza et al., 2017; Zhang et al., 2020). However, more research is needed to fully understand the pharmacokinetics and toxicity of PCA to ensure its safe and effective use.

1.10.3 Gallic Acid

Gallic acid is a widely distributed phenolic acid in the plant kingdom that has numerous applications in the food and pharmaceutical industries (Figure 6) (Kahkeshani et al., 2019). It has antioxidant properties, making it useful as a food additive to protect oils and fats (Choubey et al., 2015).

Studies have shown that gallic acid has anticancer effects and may prevent the development of cancer (Raina et al., 2008; Verma et al., 2013) possibly by inhibiting the activation of NF-κB and AKT, both of which are involved in the regulation of cell survival and proliferation. (Ho et al., 2013). Additionally, it induces cell cycle arrest by reducing the expression of proteins involved in cell cycle progression and increasing the levels of CDK inhibitor p27KIP (Huang et al., 2012).

Furthermore, at certain doses, gallic acid can reduce the levels of overexpressed miR-421 in certain types of cancer, such as gastric and non-small cell lung cancer (Li et al., 2020;

Paolini et al., 2015; Xu et al., 2022). It can also increase PTEN expression by downregulating miR-21 and reduce the expression of Ras and RhoA by upregulating miR-143 and miR-145 (Chung et al., 2020).

1.10.4 Catechin

Catechin is a type of flavonoid that is present in a variety of plants and fruits, including apples, blueberries, gooseberries, grape seeds, kiwi, strawberries, and acai, as well as products derived from them, such as green tea, red wine, beer, cacao liquor, chocolate, cocoa, and vinegar (Gadkari and Balaraman, 2015; Gálvez et al., 1994). It belongs to the Flavan-3-ol subgroup, with (–)-epicatechin and (+)-catechin being the most abundant isomers found in nature. Related molecules, such as epigallocatechin, gallocatechin (which possesses an additional phenolic hydroxyl group), and catechin gallates (gallic acid esters of catechins), also exist. (Figure 7). Blueberries naturally contain several catechins, with the most abundant being (+)-catechin. Notable quantities of (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate, and (-)-epigallocatechin gallate are also present (Bhagwat et al., 2016). Catechins are known for their antioxidant and anti-inflammatory properties, which have been extensively studied for their potential health benefits and cancer prevention (Almatroodi et al., 2020). However, recent research has also highlighted additional mechanisms by which catechins may help prevent cancer, including inhibition of angiogenesis (Negrão et al., 2013; Yee et al., 2017), prevention of the degradation of the extracellular matrix (Kim et al., 2004; Yang et al., 2022), and inducing apoptosis (Gianfredi et al., 2017) making it an interesting candidate for further research as a chemopreventive agent.

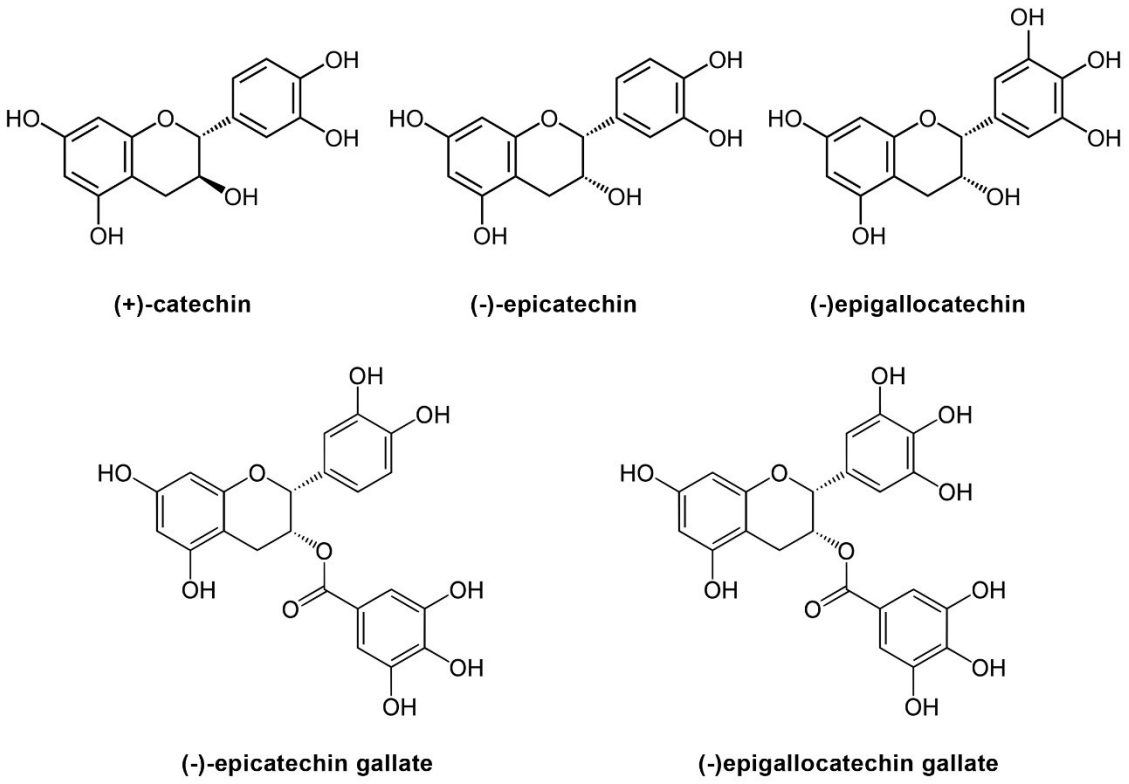

Figure 7: Example of catechins present in blueberries.

1.11 BIOTRANSFORMED BLUEBERRY JUICE BY *ROUXIELLA BADENSIS* SUBSP. *ACADIENSIS*

Blueberries are commonly recognized as a "superfood" due to their high content of antioxidant polyphenols and anthocyanins, which have been shown to have antiproliferative and apoptotic effects on cancer cells (Bornsek et al., 2012; Bunea et al., 2013; Srivastava et al., 2007). Blueberries are subject to intense research due to their value-added characteristics and antioxidant capacity. Despite their well-known health benefits, many formulations of blueberries with high antioxidant activity have failed to show an effect in vivo due to the low bioavailability of complex polyphenol chains and poor absorption in the intestinal tract

(Manach et al., 2005). Our lab has discovered a natural fermentation process that increases the antioxidant activity, polyphenol content, and bioefficacy of many berry preparations.

Through a series of screening experiments involving fermentations with bacteria isolated from the microflora of lowbush blueberries, our lab identified two promising isolates of bacteria from the microflora of lowbush blueberries. One of these isolates, a Gram-negative, catalase-positive, facultatively anaerobic coccobacillus, was found to have the ability to ferment blueberry juice and increase the level of naturally occurring polyphenols four-fold (Martin and Matar, 2005).

The bacterium was first identified by analyzing a partial sequence (1500 nucleotides) of the 16S rRNA gene which showed a genetic difference of 1.82% compared to the closest match at the time, *Serratia proteamaculans quinovora* (Martin and Matar, 2005). Although the difference in genetic makeup was too high to definitively identify it as *Serratia proteamaculans quinovora*, its physical properties and biochemical profile indicated that it could potentially be a new Serratia strain and was provisionally named *Serratia vaccinii* for its association with blueberries (Martin and Matar, 2005).

Further investigation using whole-genome shotgun sequencing revealed that the bacterium was closely related to a newly described species of Serratia (Le Flèche-Matéos et al., 2017; Salvetti et al., 2023). This isolate was named *Rouxiella badensis* subsp. *acadiensis* after the region in which it was discovered (Salvetti et al., 2023; Yahfoufi et al., 2021), and the resulting fermented blueberry juice was named Polyphenol Enriched Blueberry Preparation (PEBP) (Vuong et al., 2016).

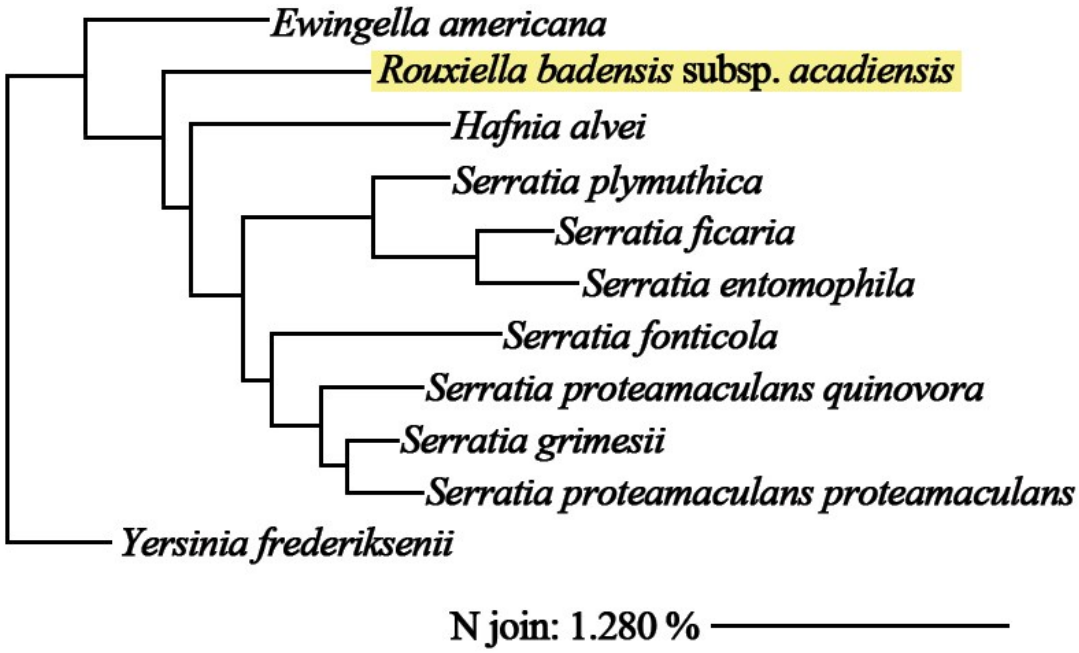

Figure 8: Phylogenetic analysis based on the partial 16S rDNA fragment determined by MIDI Laboratories in 2005 (Martin and Matar, 2005).

PEBP has demonstrated multiple beneficial effects on various biological systems. For instance, it has been shown to reduce weight gain in diabetic and obesity model KKAy mice and exhibits antidiabetic effects by mimicking metformin's anti-inflammatory effects. The mechanism of action is thought to involve anti-inflammatory effects, increasing 5' adenosine monophosphate-activated protein kinase (AMPK) activity, adiponectin levels, and possibly IL-6 (Vuong et al., 2009, 2007) but this effect may be dose-dependent (Sánchez-Villavicencio et al., 2017). In addition, PEBP and some of its compounds have shown antidiabetic effects in vitro (Nachar et al., 2017). Furthermore, PEBP has been shown to prevent oxidative stress on neurons and decrease nitric oxide production by macrophages (Vuong et al., 2010, 2006).

Additionally, our studies indicate that PEBP has the ability to increase adiponectin secretion (Vuong et al., 2009). This effect is likely due to its ability to counteract reactive oxygen species (Furukawa et al., 2004) and inhibit pro-inflammatory cytokines (Bruun et al.,

2003). Inflammation is a known contributor to obesity, diabetes, and cancer (Lashinger et al., 2014).

Moreover, PEBP has demonstrated significant anticancer effects, including the ability to decrease mammosphere development in various cell lines, delay tumour growth, and reduce metastasis to the lungs in animal models. Its mechanism of action involves regulating the PTEN/PI3K/AKT pathway, which is a critical node in cancer stem cell (CSC) signalling and homeostasis (Vuong et al., 2016). It has also been shown to suppress CSCs in skin cancer by upregulating miR-200b and reducing the expression of ZEB1, a transcription factor that promotes epithelial-mesenchymal transition (Alsadi et al., 2021).

Genetic analysis of *R. badensis* subsp. *acadiensis* revealed no potentially pathogenic genes, suggesting it is safe for human consumption. In vitro and in vivo experiments have shown that this bacterium has immunomodulatory effects on immune cells and reduces inflammation in the gut. Additionally, it has been found to improve markers for intestinal barrier integrity, which could have implications for gut health (Yahfoufi et al., 2021).

2 HYPOTHESIS

Naturally occurring dietary compounds are gaining increasing attention for their efficacy in cancer chemoprevention (Matar et al., 2001; Vinderola et al., 2007b). Furthermore, our preliminary results have demonstrated that blueberry can repress CSCs (cancer stem cells) of breast cancer cells, supporting a diet-mediated targeting of CSCs. Additionally, we have reported that consuming probiotics can modulate the immune system and maintain it in a state of surveillance against breast carcinoma in mice (de Moreno de Leblanc et al., 2007; de Moreno de LeBlanc et al., 2005b; Duru et al., 2012), as well as inflammatory diseases (de Moreno de LeBlanc et al., 2007; Leblanc et al., 2004; Martin and Matar, 2005; Vinderola et al., 2007a; Vuong et al., 2010, 2009).

Our research has revealed that a bacterium *Rouxiella badensis* subsp. *acadiensis* (formerly known as *Serratia vaccinii)* isolated from the biota of blueberry can increase the level of polyphenols present in blueberry juice. This novel product named Polyphenol-Enriched Blueberry Preparation (PEBP) has been shown to have anti-inflammatory, anti-diabetic, and anticarcinogenic properties.

At the molecular level, breast carcinogenesis is regulated by a subset of aggressive cells known as CSCs. Evidence suggests that cellular transformation, carcinogenesis, and stemness maintenance are driven by epigenetic-specific changes involving microRNAs. Polyphenol compounds can influence signalling pathways important in maintaining CSC phenotype like AKT/mTOR, STAT3 and ERK1/2, the hypothesis of this study is that PEBP and a mixture of polyphenols found in blueberries can control the development of CSCs by modulating inflammatory pathways and miRNAs. Therefore, the main objective of this thesis was to gain

a better understanding of the underlying mechanisms through which PEBP offers protective effects against breast carcinoma in mice.

3 AIMS

The primary objective of this thesis is to gain a deeper understanding of the mechanisms underlying the protective effects of the polyphenol-enriched preparation in mitigating breast cancer progression. The specific objectives are as follows:

1. Investigate the potential of the polyphenol-enriched blueberry preparation to prevent the development of breast cancer stem cells in both cell models and in vivo. Additionally, explore the involvement of the STAT3 and MAPK signaling pathways.
2. Assess the impact of the polyphenol-enriched blueberry preparation on breast cancer by regulating the expression signatures of miRNA involved with cell proliferation, survival, and CSC self-renewal pathways in in vitro experiments.
3. Characterize PEBP and investigate the effect of a subset of its components on miRNA expression. Furthermore, validate the role of those components in regulating the functional behavior of breast cancer stem cells through experiments using a 4T1 animal model.

4 ROLE OF A POLYPHENOL-ENRICHED PREPARATION ON CHEMOPREVENTION OF MAMMARY CARCINOMA THROUGH CANCER STEM CELLS AND INFLAMMATORY PATHWAYS MODULATION.

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Key words

Polyphenols, breast cancer stem cells, tumor, metastasis, STAT3, MAPKs.

ABSTRACT

Background: Naturally occurring polyphenolic compounds from fruits, particularly from blueberries, have been reported to be significantly involved in cancer chemoprevention and chemotherapy. Biotransformation of blueberry juice by *Serratia vaccinii* increases its polyphenolic content and endows it with anti-inflammatory properties.

Methods: This study evaluated the effect of a polyphenol-enriched blueberry preparation (PEBP) and its non-fermented counterpart (NBJ), on mammary cancer stem cell (CSC) development in in vitro, in vivo and ex vivo settings. Effects of PEBP on cell proliferation, mobility, invasion, and mammosphere formation were measured in vitro in three cell lines: murine 4T1 and human MCF7 and MDA-MB-231. Ex vivo mammosphere formation, tumor growth and metastasis observations were carried out in a BALB/c mouse model.

Results: Our research revealed that PEBP influence cellular signaling cascades of breast CSCs, regulating the activity of transcription factors and, consequently, inhibiting tumor growth *in vivo* by decreasing metastasis and controlling PI3K/AKT, MAPK/ERK, and STAT3 pathways, central nodes in CSC inflammatory signaling. PEBP significantly inhibited cell proliferation of 4T1, MCF-7 and MDA-MB-231. In all cell lines, PEBP reduced mammosphere formation, cell mobility and cell migration. In vivo, PEBP significantly reduced tumor development, inhibited the formation of ex vivo mammospheres, and significantly reduced lung metastasis.

Conclusions: This study showed that polyphenol enrichment of a blueberry preparation by fermentation increases its chemopreventive potential by protecting mice against tumor development, inhibiting the formation of cancer stem cells and reducing lung metastasis.

Thus, PEBP may represent a novel complementary alternative medicine therapy and a source for novel therapeutic agents against breast cancer.

INTRODUCTION

Life-style changes significantly contribute to cancer prevention and are considered an important paradigm in translational medicine (Slattery et al., 2014). For example, a dietary intervention showed that a few months of following a Mediterranean diet are sufficient to favorably modify the metabolic/endocrine characteristics of breast cancer survivors (Villarini et al., 2012). In fact, breast cancer patients are among the highest users of integrative medicine in conjunction with conventional oncology care (Greenlee et al., 2014). Currently, cancer preventive phytochemicals are receiving increasing attention regarding their impact on Cancer Stem Cell (CSC) self-renewal pathways (Rossi et al., 2014). In line with these reports, our preliminary results have shown that repression of breast CSCs by fermented blueberry preparation, named Polyphenols-Enriched Blueberry Preparation (referred hereafter as PEBP), supports diet-mediated targeting of CSCs. The chemopreventive effects of blueberry polyphenolics on breast cancer are well-known (Kanaya et al., 2014; Montales et al., 2012). For example, phenolic extracts from European blueberry were shown to inhibit proliferation and induce apoptosis in breast cancer cells (Nguyen et al., 2010). Therefore, increasing the phenolic content of blueberry might enhance its anticancer properties and reduce its metastatic potential. Indeed, biotransformation of blueberry juice with a novel strain of bacteria isolated from the blueberry flora increases its phenolic content and antioxidant activity (Martin and Matar, 2005).

CSCs, a highly tumorigenic cell subtype, are emerging as key drivers of cancer (Graziano et al., 2008; O'Connor et al., 2014). CSCs in breast cancer have been identified as $CD44^{+}/CD24^{low}$ phenotype and are able to grow as spheres named, in this case, mammospheres (Camerlingo et al., 2014; Podberezin et al., 2013). Interleukin 6 (IL-6) and its major effector, the signal transducer and activator of transcription 3 (STAT3), are part of an important inflammation-associated pathway in malignancies, and are highly involved in CSC development and progression (Chang et al., 2013). STAT3 has been recently recognized as a key therapeutic target to reduce tumor growth (Lamy et al., 2012) and metastasis (Zhao et al., 2012) in different types of cancer. The persistent self-renewal observed in CSCs was reported to be epigenetically controlled in the IL-6/STAT3/phosphatidylinositol 3-kinase (PI3K) signaling pathway (Hernandez-Vargas et al., 2011). STAT3 with PTEN is part of the positive feedback loop that underlies the epigenetic switch that links inflammation to cancer. Thus, prevention or inhibition of deregulation in the PI3K/STAT3/PTEN signaling pathway could be beneficial for the treatment and better outcome of breast cancer. Several signal transduction pathways, such as the extracellular-signal-regulated kinase/mitogen-activated protein kinase (Erk/MAP) pathway and PI3K pathway have been implicated in mammary carcinogenesis (Vivanco and Sawyers, 2002).

Moreover, members of the mitogen-activated protein kinase (MAPK) pathways have been well studied for their role in controlling cellular responses to the environment and in regulating gene expression, cellular growth and apoptosis in cancer (Chen et al., 2012; Yip et al., 2011). The extracellular signal-regulated kinases (ERKs)-1/2 were linked to cell proliferation and survival, whereas the stress-activated MAPKs, p38 and c-Jun N-terminal kinase (JNK), were connected to apoptosis (Wagner and Nebreda, 2009). Controlling MAPK

pathways was shown to impact CSC-promoting IL-6 and modify CSC-like behavior (Balko et al., 2013).

Different studies have shown that the fermentation of PEBP greatly increased its antioxidant potential (Martin and Matar, 2005; Vuong et al., 2006) and endowed it with novel anti-inflammatory (Vuong et al., 2010), antidiabetic (Vuong et al., 2009, 2007) and other biological activities (Vuong et al., 2010). Importantly, the anti-inflammatory effects of PEBP seemed to be connected to IL-6 related pathways, as demonstrated by decreasing hyperglycemia, activating AMPK pathways and mimicking Metformin metabolic effects (Vuong et al., 2009). Additionally, our studies have revealed that PEBP increases adiponectin secretion (Vuong et al., 2009), probably by counteracting reactive oxygen species (Furukawa et al., 2004) and inhibiting the pro-inflammatory cytokines (Bruun et al., 2003); two mechanisms that contribute to the inflammatory response. Indeed, inflammation is linked to obesity, diabetes and cancer (Lashinger et al., 2014). The goal of this study was to investigate the anticarcinogenic effects of PEBP on breast cancer stem cell development in cell models and *in vivo*, as well as to study the involvement of STAT3 and MAPKs signaling pathways in its chemopreventive activities.

MATERIALS AND METHODS

Preparation of blueberry juices

Mature lowbush blueberries (*Vaccinium angustifolium* Ait.) were purchased from Cherryfield Foods Inc. (Cherryfield, ME) as fresh and untreated fruits. Blueberry juice was extracted by blending the fruit (100 g) in a Braun Type 4259 food processor. The fruit mixture

was then centrifuged at 500 x g for 10 min to remove insoluble particles. The resulting juice was sterilized using 0.22 μm Express Millipore filters (Millipore, Etobicoke, ON).

Serratia vaccinii bacteria were cultured as previously described (Martin and Matar, 2005). Blueberry and PEBP preparation have been partially characterized elsewhere (Martin and Matar, 2005; Matchett et al., 2006).

Cell culture

Murine 4T1, a 6-Thioguanine resistant cell line, human MCF-7 and human MDA-MB-231 cell lines were obtained from American Type Cell Collection (ATCC; Chicago, IL). ATCC authenticated the human cell lines by using short tandem repeat profiling and the mice cell line was confirmed to be from mice by cytochrome C oxidase 1 gene assay. MCF-7 cells were cultured in MEM, 4T1 and MDA-MB-231 in RPMI-1640, media containing FBS (10%, v/v) (ATCC), penicillin (100 μU/ml), streptomycin (100 μg/ml) (Sigma-Aldrich, Oakville, ON) at 37ºC in a humidified atmosphere with 5% CO_2.

Cell viability

Cell viability was assessed by water soluble tetrazolium salts (WST-1) and Lactate Dehydrogenase (LDH) assays (Roche, Laval, QC). After a 24 h treatment, supernatants were collected for LDH assay following the manufacturer's instructions. The absorbance was measured with the μ-Quant plate reader (Bio-Tek, Winooski, VT).

Cell motility

Cells were plated in a six-well plate at density of 1 x10^6 cells/0.2 ml/well and allowed to form a confluent monolayer for 24 h. The monolayer was then scratched with a pipette tip, washed with RPMI-1640 to remove floating cells, and photographed (time 0). The cells were

treated with NBJ or PEBP for 24 hours. The cells were then photographed again at three randomly selected sites per well. Cell motility was expressed as a percent of the surface area covered by migrating cells compared with time 0.

Cell invasion

The cell invasion assay was performed on a polyethylene terephthalate (PET) membrane (8 μm pore size) in a Tissue Culture (TC) insert (BD biosciences, Mississauga, ON) according to the manufacturer's instructions. In short, cells were incubated in the superior chamber for 24 hours. The insert was then transferred to a new plate containing HBSS supplemented with 4 μg/ml of Calcein AM for 1 hour. The intensity of the fluorescence is measured and is expressed as a ratio of the control well without treatment (Partridge and Flaherty, 2009).

Mammospheres formation

Adherent cells were detached by trypsin and single cells were counted using the Countess automated cell counter (Invitrogen, Burlington, ON). For tumor tissue, approximately 0.05 g of each tumor was minced and dissociated in RPMI-1640 media containing 300 U/ml collagenase (Sigma), and 100 U/ml hyaluronidase (Sigma) at 37 °C for 2 hours. The cells were sieved sequentially through a 100 μm and a 40 μm cell strainer (BD Biosciences) to obtain a single cell suspension, and counted in a hemocytometer.

Single cells were plated in ultralow attachment 96-well plates (Costar) at 10^3 cells/0.2 ml/well, in the presence/absence of PEBP and NBJ, in DMEM-F12 (Invitrogen), supplemented with 10 ng/ml EGF, 20 ng/ml bFGF, 5 μg/ml insulin, 1 mM sodium pyruvate, 0.5 μg/ml hydrocortisone, and penicillin/streptomycin (0.05 mg/ml) (Sigma) (Hernandez-

Vargas et al., 2011). Cells grown in these conditions as non-adherent spherical clusters of cells or mammospheres were counted after 4-7 days.

IL-6 determination

BD OptEIA Mouse IL-6 ELISA sets (BD Biosciences) were used to measure extracellular IL-6 production by mammospheres following the manufacturer's instructions.

Western Blot analysis

Cells in mammospheres formation conditions were collected and lysed after 1, 2, 6 and 24 h treatment with/without PEBP and NBJ. Cell lysates were run on a 10 % acrylamide gel, transferred to a PVDF membrane, and probed with either anti-phosphorylated STAT3 (1:1000), PI3K (1:1000), Akt (1:1000), PTEN (1:1000), p38 MAPK (1:1000), ERK1/2 (1:1000), SAPK/JNK (1:1000), β-Actin (1:1000) (Cell Signaling Tech. Inc., Danvers, MA, USA). Bands were visualized via chemiluminescence using horseradish peroxidase-conjugated secondary antibodies. Bands were quantified using β-actin as loading control by Bio-Rad Quantity One software (Bio-Rad, Mississauga, ON).

Animals

Six- to eight-week-old BALB/c female mice weighing 18–20 g (Charles River, Montreal, QC) were randomly distributed into seven experimental groups: control, NBJ 12.5%, NBJ 25%, NBJ 50%, PEBP 12.5%, PEBP 25% and PEBP 50%. Each experimental group consisted of 8 mice housed in a controlled atmosphere (temperature 22 ± 2 °C; humidity 55 ± 2%) with a 12 h light/dark cycle. Mice were maintained and treated in accordance with the guidelines of the Canadian Council on Animal Care. The protocol (ME-289) was approved by the Animal Care Committee of University of Ottawa.

While mice in the control group received normal water, mice in NBJ- and PEBP-groups received either NBJ or PEBP, incorporated in their drinking water at three concentrations: 12.5%, 25% and 50% (v/v) respectively. After two weeks of treatment, all mice received a subcutaneous injection of 4T1 cells (1400 cells/0.1 ml/mice) into the abdominal mammary gland fat pad. Three weeks after the inoculation, tumors and lungs were collected and weighed (Pulaski and Ostrand-Rosenberg, 2001). Mice consumed an average of 2.9 ml of juice each day and both blueberry juices were well tolerated and did not affect mice body weight.

Lung metastasis

Lungs were minced and dissociated in RPMI-1640 media containing 300 U/ml collagenase (Sigma), at 37 °C for 15 min. After filtration through a 40 μm cell strainer (BD Biosciences), cells were collected and suspended in RPMI-1640 containing 10% FBS (ATCC), penicillin/streptomycin (0.05 mg/ml) and 60 μM 6-Thioguanine (Sigma). The cells were plated in 10-cm culture dishes (Corning) at 37 °C in a humidified atmosphere with 5% CO_2. After 14 days, the lung cells were fixed by methanol and stained with 0.03% methylene blue solution. All blue colonies were counted, one colony representing one clonogenic metastatic cell (Pulaski and Ostrand-Rosenberg, 2001).

Statistical analysis

Statistical analysis of the data by ANOVA and Bonferroni's post hoc tests were performed using GraphPad Prism software version 5.04 (San Diego, CA, USA). Statistical significance was set at $p \leq 0.05$. Data are reported as mean ± SEM.

RESULTS

At a concentration of 200 μM Gallic Acid Equivalent (GAE), PEBP significantly inhibited the proliferation of 4T1, MDA-MB-231 and MCF-7 cancer cells by 34%, 24% and 33% respectively (Figure 9), whereas the same concentration of NBJ only showed an inhibition of 32% in 4T1 cell proliferation (Figure 9, panel A). No significant effects of NBJ were observed in MDA-MB-231 and MCF7 (Figure 9, panel B-C). Both PEBP and NBJ did not show any toxicity on the three cell lines at tested concentrations, as determined by an LDH assay (data not shown).

Both NBJ and PEBP at 150μM GAE significantly reduced the invasive ability of 4T1 and MDA-MB-231 (Figure 10, panel D and E). However, only PEBP exhibited an inhibitive effect on the motility of all three breast cancer cell lines (Figure 10, panel A-C). NBJ did not show any significant effect on cell motility as compared to the control.

PEBP significantly decreased the formation of mammospheres in all three cell lines (

Figure **11**), and nearly total inhibition was observed at 150 μM GAE of PEBP. A treatment with the same concentration of NBJ only exhibited an inhibition of 75% in MDA-MB-231 (

Figure **11**, panel B), whereas it significantly increased the formation of mammospheres in 4T1 and MCF-7 by 60% and 96%, respectively (

Figure **11**, panel A and C).

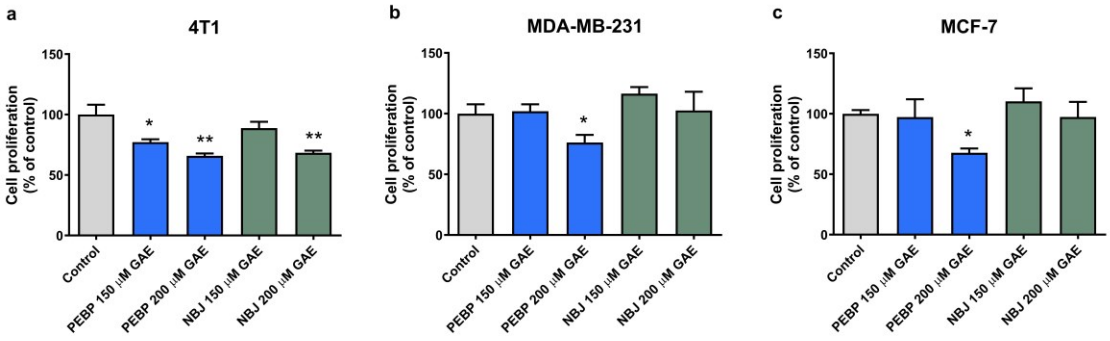

Figure 9: PEBP suppressed the growth of mammary carcinoma cell lines. Proliferation of 4T1 (**a**), MDAMB-231 (**b**), and MCF-7 (**c**) cells after treatment with either 150 or 200 μM GAE (gallic acid equivalent) of either polyphenol-enriched blueberry preparation (PEBP) or normal blueberry juice (NBJ) for 24 h. All values are means of 3 separate experiments ±SEM. *Denotes statistical significance at $p \leq 0.05$ vs. control. **Denotes $p \leq 0.01$ vs. control

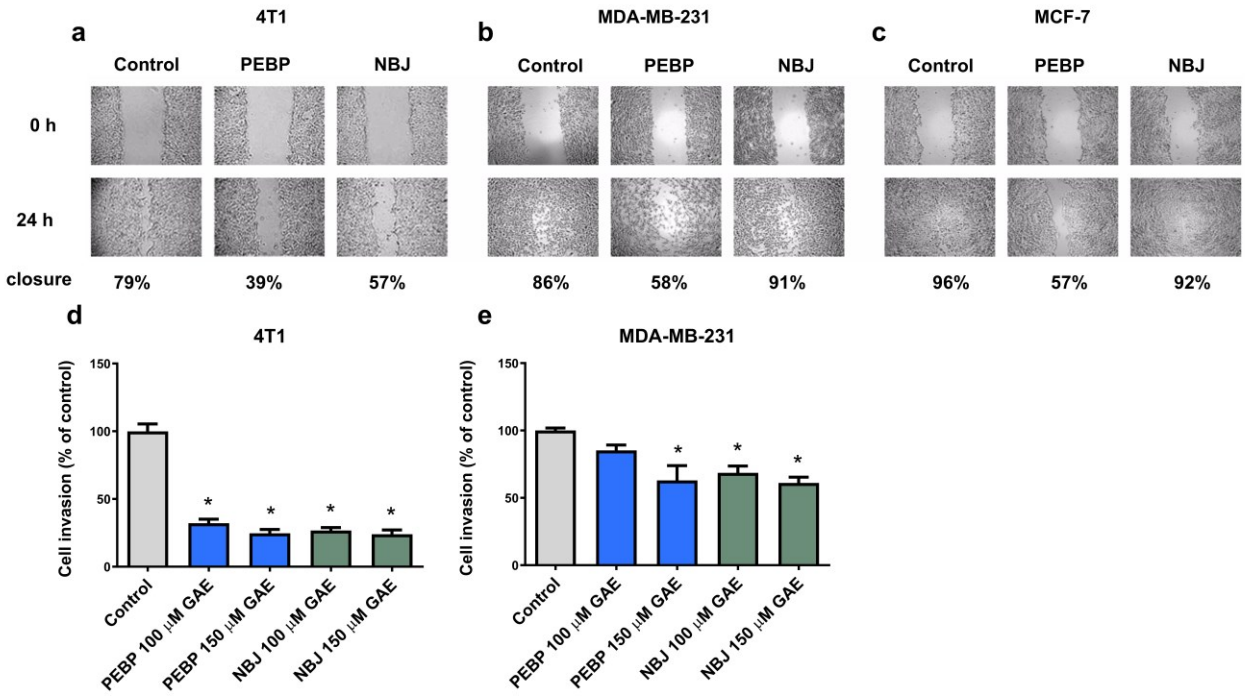

Figure 10: PEBP decreased motility and invasiveness in gel invasion experiment. Cell mobility of 4T1 (**a**), MDAMB-231 (**b**), and MCF-7 (**c**) cells after treatment with 100 µM GAE (gallic acid equivalent) of either polyphenol-enriched blueberry preparation (PEBP) or normal blueberry juice (NBJ) for 24h and cell invasion of 4T1 (**d**) and MDAMB-231 (**e**) cells after treatment with either 100 or 150 µM GAE of PEBP or NBJ for 24 h. Contrast was enhanced to better show cell motility. All values are means of 3 separate experiments ±SEM. *Denotes statistical significance at $p \leq 0.05$ vs. control

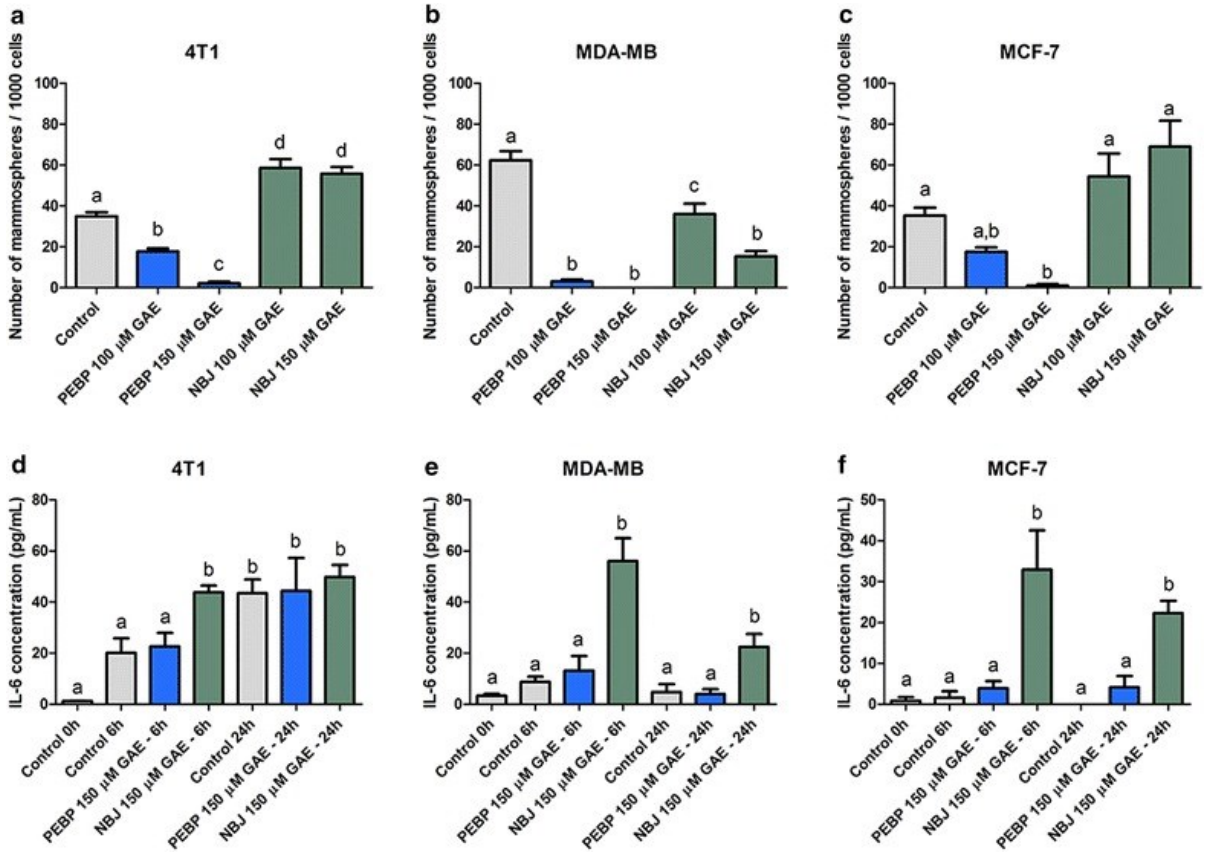

Figure 11: PEBP and NBJ decreased the formation of mammospheres in cell culture. Mammospheres formation of 4T1 (**a**), MDAMB-231 (**b**), and MCF-7 (**c**) cells after treatment with either 100 or 150 µM GAE (gallic acid equivalent) of either polyphenol-enriched blueberry preparation (PEBP) or normal blueberry juice (NBJ) for 4–7 days and IL-6 production by 4T1 (**d**), MDAMB-231 (**e**), and MCF-7 (**f**) cells after treatment with 150 µM GAE of PEBP or NBJ for 6 and 24 h. All values are means of 4 separate experiments ±SEM. *Bars* that have no letter in common are significantly different from each other ($p \leq 0.05$)

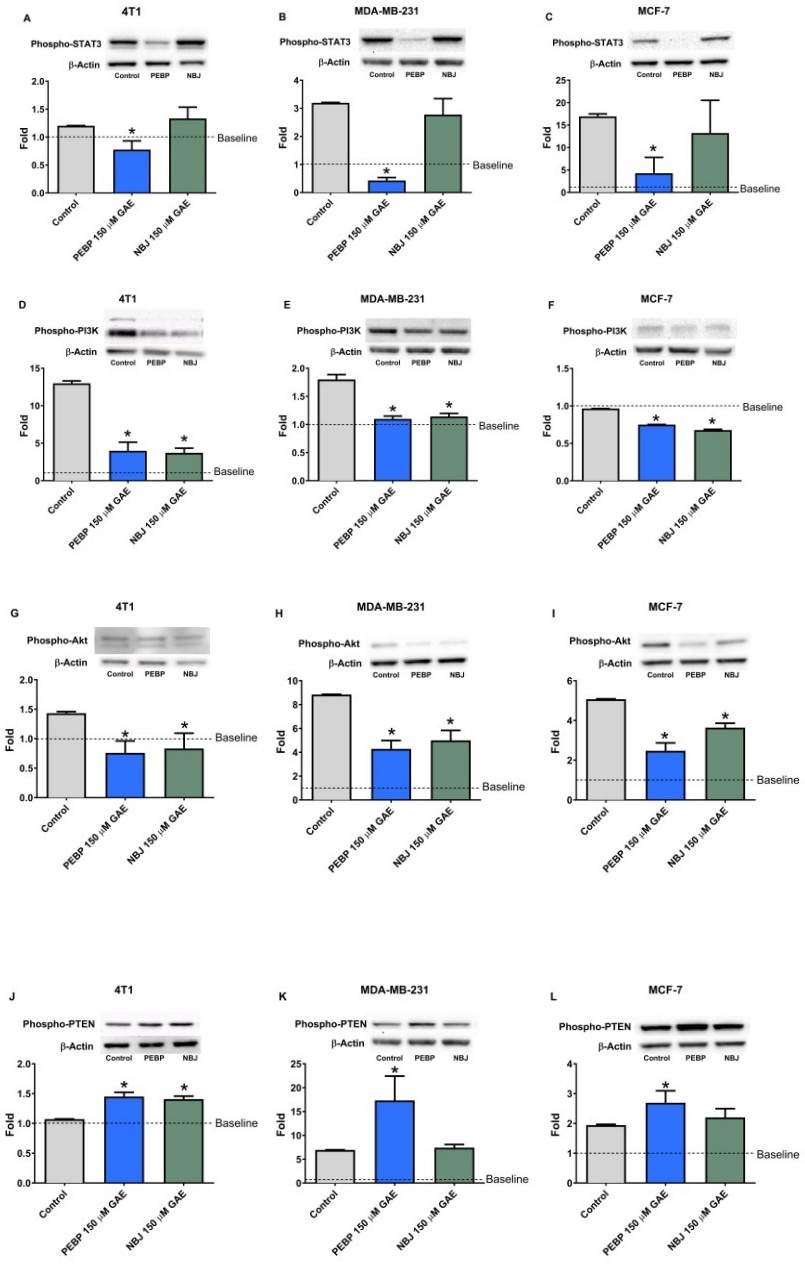

Figure 12: PEBP inhibited STAT3/PI3K/Akt signaling pathway. Phosphorylation of STAT3, PI3K, Akt, and PTEN in 4T1 (a, d, g, j), MDAMB-231 (b, e, h, k), and MCF-7 (c, f, i, l) mammospheres after treatment with 150 μM GAE (gallic acid equivalent) of either polyphenol-enriched blueberry preparation (PEBP) or normal blueberry juice (NBJ) for 6 h. All values are means of 3 separate experiments ±SEM. Baseline represent the level of phosphorylation present in cells not exposed to the mammospheres forming medium. *Denotes statistical significance at $p \leq 0.05$ vs. control at 6 h

A 6 h-treatment with NBJ in mammosphere formation conditions significantly elevated the secretion of IL-6 in all three cell lines (Figure 11, panels D-F), while PEBP did not induce any modification as compared to the control cells.

Moreover, PEBP significantly inhibited the phosphorylation of STAT3 and PI3K/Akt in all three cell lines. This inhibition started after a 6 h-treatment (Figure 12 panel A-I) and lasted up to 24 h (data not shown), whereas NBJ only decreased the phosphorylation of PI3K/Akt. Both PEBP and NBJ significantly enhanced the activity of PTEN in 4T1 (Figure 12, panel J), but only PEBP increased PTEN phosphorylation in MDA-MB-231 and MCF-7 (Figure 12, panel K-L).

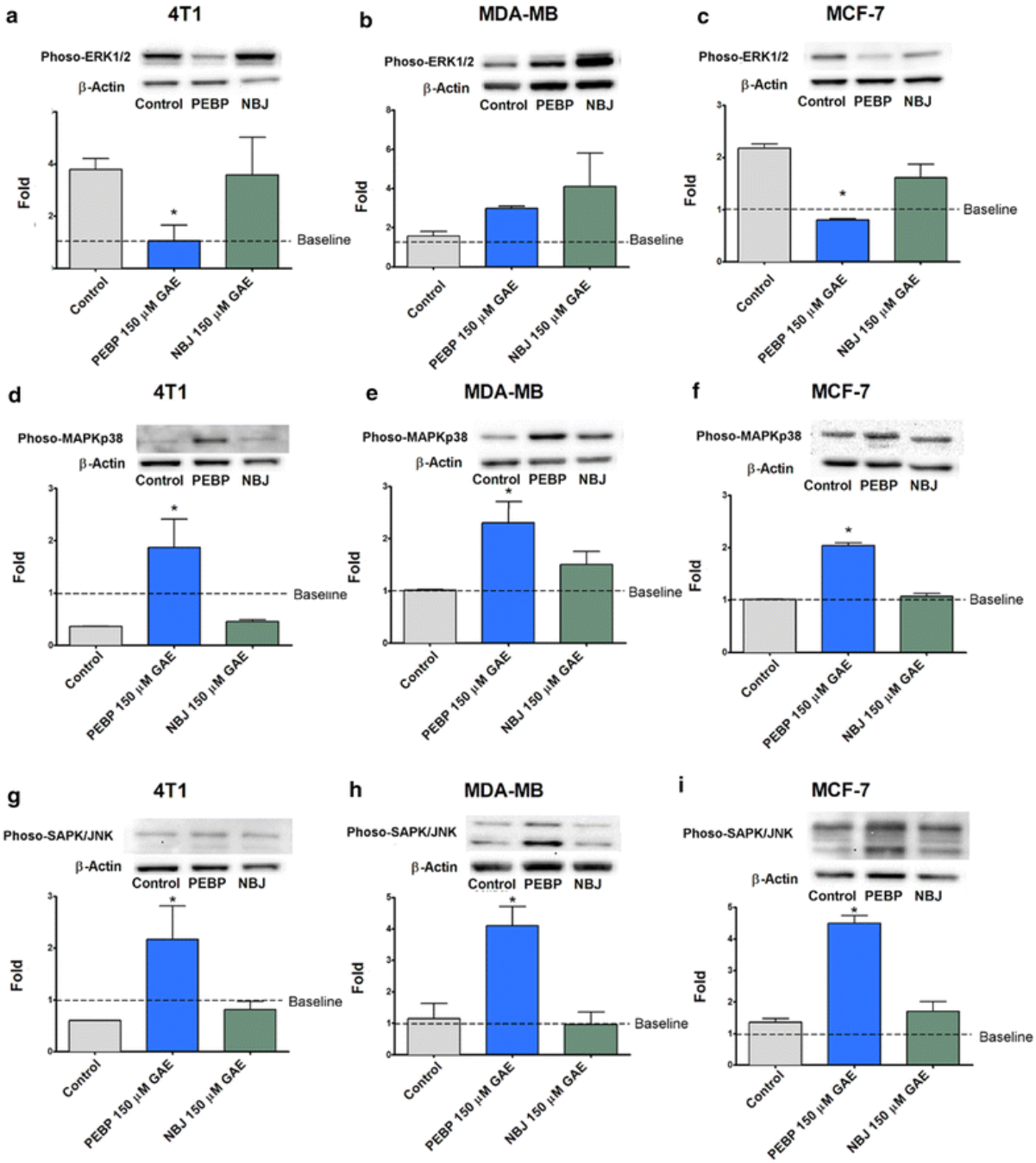

Figure 13 : PEBP inhibited ERK1/2 but enhanced MAPKp38, and JNK signaling. Phosphorylation of ERK1/2, MAPK p38, and JNK in 4T1 (**a**, **d**, **g**), MDAMB-231 (**b**, **e**, **h**), and MCF-7 (**c**, **f**, **i**) mammospheres after treatment with 150 µM GAE (gallic acid equivalent) of either polyphenol-enriched blueberry preparation (PEBP) or normal blueberry juice (NBJ) for 2 h. All values are means of 3 separate experiments ±SEM. Baseline represent the level of phosphorylation present in cells not exposed to the mammospheres forming medium. * Denotes statistical significance at $p \leq 0.05$ vs. control at 2 h

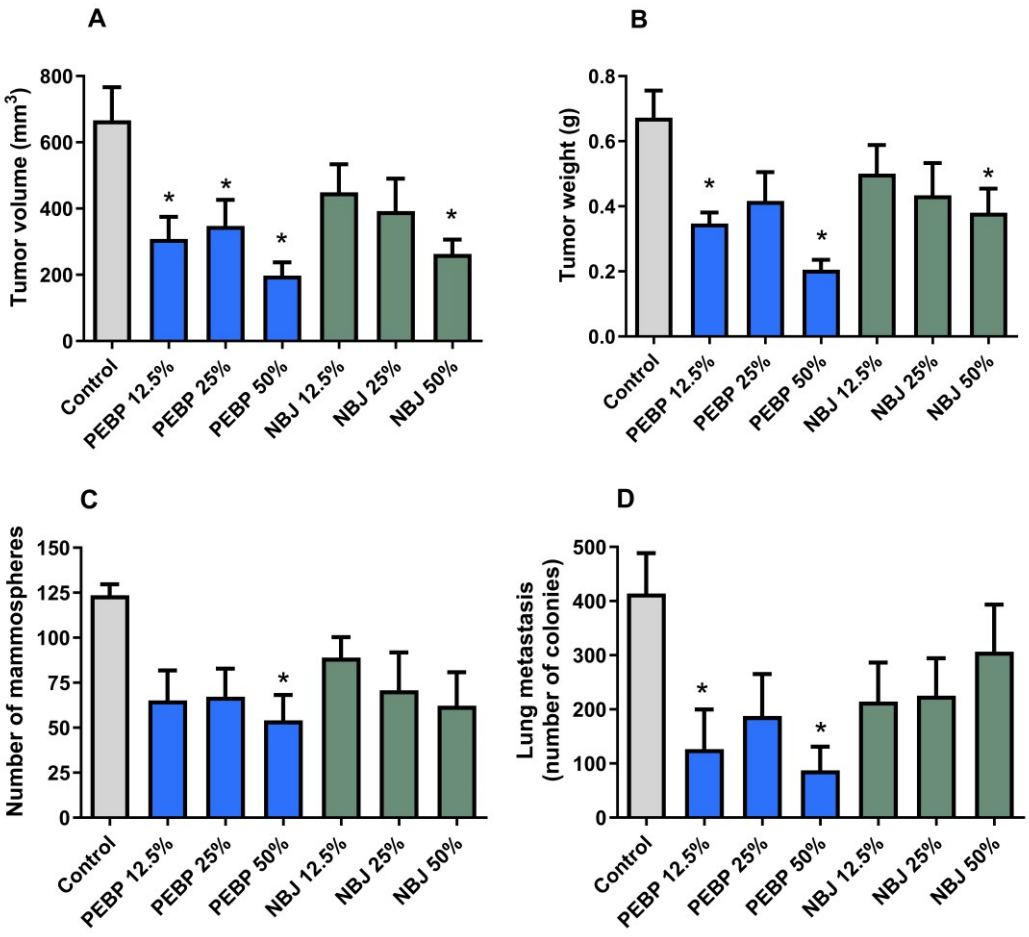

Figure 14: Antitumoral effects of PEBP in BALB/c mice model with 4T1 cell challenge. Tumor volume (**a**), tumor weight (**b**), mammospheres formation from primary tumor cells (**c**) and metastasis present in lungs (**d**) of mice that received a 2-week pre-treatment and a 3-week post-inoculation treatment with either polyphenol-enriched blueberry preparation (PEBP) or normal blueberry juice (NBJ) incorporated in drinking water at concentrations of 12.5 % (NBJ 12.5 % and PEBP 12.5 %), 25 % (NBJ 25 % and PEBP 25 %), and 50 % (NBJ 50 % and PEBP 50 %). All values are means of 2 separate experiments ±SEM (n = 16). *Denotes statistical significance at $p \leq 0.05$ vs. control

Starting from one hour after the addition of PEBP, a significant inhibition of ERK1/2 phosphorylation was observed in 4T1 and MCF-7 (Figure 14, panels A and C). PEBP also increased MAPK p38 and JNK/SAPK phosphorylation in all three cell lines. Their inhibited- or activated-state attained the maximal level after 2 h of treatment and remained stable up to

24 h (Figure 13, panels D-I). NBJ did not show any significant modification of the three MAPKs family members.

As illustrated in Figure 14, when administered chronically over a 5-week period, NBJ reduced tumor volume and weight in a dose-dependent manner. However, significant effects were only observed in the NBJ50 group, whereas all three doses of PEBP-treated mice displayed significant delays of tumor growth (Figure 14, panels A-B). Moreover, the mammosphere formation from tumoral primary cells was significantly reduced exclusively in tumors of PEBP50-treated animals (Figure 14, panel C). Similarly, the treatment with PEBP significantly reduced the metastasis in lungs of PEBP-treated mice, while all of the other groups did not show a significant difference as compared to control animals (Figure 14, panel D).

DISCUSSION

Chemoprevention is an important part of integrative and translational medicine in oncology. Naturally occurring compounds, such as polyphenols in fruits, are increasingly recognized for their effects in controlling aberrant signaling pathways and inflammatory signals in CSCs. Our group has discovered that the fermented, probiotic-like product PEBP greatly accentuates its antioxidant potential and endows it with novel anti-inflammatory (Vuong et al., 2006), antidiabetic (Vuong et al., 2009, 2007) and neuroprotective (Vuong et al., 2010) biological properties. The common mechanisms underlining the multiple beneficial effects of PEPB are probably related to its capability to modulate the activity of global regulators that are associated with cellular transformation and inflammation. In addition,

biotransformations involving fermentation and catabolic breakdown have been suggested to enhance bioavailability (Yang et al., 2008).

In fact, PEBP was found to inhibit adipogenesis and increase glucose uptake in muscle cells and adipocytes (Vuong et al., 2007) through the activation of the AMP-activated kinase, mimicking Metformin activities (Davis et al., 2006; Guigas et al., 2006). Particularly, the anti-inflammatory effect of PEBP is pointing to the blockade of the STAT3 pathways (essential in CSCs, and inflammation) and the activation of AMPK, which in turn inhibits MAPK downstream (essential in diabetes and cancer).

In addition, PEBP mimics Metformin anti-inflammatory/antitumoral activities by inactivation of PI3K/AKT pathways. Metformin is now proposed as a major adjunct therapy in cancer with a powerful inhibitory effect on CSCs (Hirsch et al., 2009; Jung et al., 2011). This observation led us to further investigate the effect of PEBP on CSCs.

The antiproliferative effect of PEBP was observed in all three breast cancer cell lines at 200 μM, whereas NBJ, at the same concentration, only had an effect in 4T1. NBJ did not show any antiproliferative effect in MDA-MB-231 as previously reported (Adams et al., 2010; Faria et al., 2012). This might be due to the low tested-doses in our study. Moreover, PEBP significantly inhibited the motility of all three cancer cell lines, which prompted further investigation for its antimetastatic activity *in vivo.* As expected, PEBP significantly reduced metastasis potential to the lung when tested in a murine breast cancer model.

There is now substantial evidence that many cancers, including breast cancer, are driven by a cellular subpopulation, identified as cancer stem cells, which mediate tumor metastasis and resistance to conventional therapies. Therefore, controlling CSC growth in

breast cancer is a possible avenue to prevent tumor development and metastasis. Thus, the investigation of PEBP-induced molecular mechanisms that mediate CSC growth was important to clarify its anticancer and anti-metastatic activities. Indeed, our data indicated that PEBP significantly inhibited mammosphere formation *in vitro*. Moreover, its inhibitory effect was further confirmed by the reduction of *ex vivo* mammosphere development from PEBP-treated animals.

Polyphenols naturally have multi-target actions/mechanisms, which explain their wide spectrum of biological activities (Fraga et al., 2010). Their anti-inflammatory property is the key factor in the interface between inflammation and neoplasia (Subbaramaiah et al., 2013). At the cross road of cancer and inflammation, the STAT3 and MAPK pathways have been reported as crucial for CSC growth and their acquired EMT characteristics during metastasis (Chang et al., 2013; Iliopoulos et al., 2011). Depending on the cell type, the IL-6/STAT3-dependent pathways, such as the JAK/STAT (Chang et al., 2013), PI3K/AKT/NF-κB (Iliopoulos et al., 2009a), or p38 MAPK (Koul et al., 2013), can enhance tumor growth and refractoriness to chemotherapy (Chang et al., 2013). Therefore, our studies were conducted to examine the involvement of these pathways in PEBP's antitumor activities. We demonstrated that IL-6 production, as well as STAT3 and PI3K phosphorylation, were decreased in CSC culture after PEBP treatment, when compared to the non-fermented control. Although, polyphenols from blueberry have demonstrated inhibitory activities on cancer cells via the control of inflammatory cytokines such as IL-6 (Kanaya et al., 2014), dramatic and biphasic increase of IL-6 occurs early in CSC cellular transformation (Hirsch et al., 2013), independently of STAT3 decrease. STAT3 signaling, an important inflammation-associated pathway in malignancies, has been recognized as a key therapeutic target to reduce tumor

growth and metastasis (Zhao et al., 2012). Several signal transduction pathways such as STAT3, PI3K/AKT/NF-κB cascade, p38/MAPK/ERK, or the AMPK pathways play an important role in inflammation-mediated response at all stages of cancer development and refractoriness to chemotherapy (Mauer et al., 2015). Moreover, downstream effectors of the PI3K pathway include Akt, which is overexpressed in many cancer types and is associated with increased tumorigenicity (Bellacosa et al., 1995; Cheng et al., 1992). Our preliminary results showed that PEBP delayed the formation of CSCs in different types of cell culture and *in vivo*, through modulation of IL-6/STAT3, the PTEN/PI3K/AKT axis, and ERK/p38 in MAPK signaling pathways, which are all central nodes in CSC signaling and homeostasis (Korkaya et al., 2011) (Fig. 3,5). We have demonstrated that STAT3, AKT, and PI3K are decreased, PTEN (a tumor suppressor gene upregulated by p53) is increased in a non-cell type dependent manner, and ERK1/2 was significantly inhibited in 4T1 and MCF7 (Fig. 5)**.** In MAPK pathways, ERK1/2 is the most relevant to breast cancer. Increased expression of ERK1/2 was recently reported as driving endocrine resistance and breast cancer progression in an obesity-associated experimental model (Bowers et al., 2013). In fact, both PEBP and NBJ inhibited the phosphorylation of PI3K. These findings are consistent with previous reports, which attributed the inhibition of PI3K activity to the anticancer effects of blueberry (Adams et al., 2010; Montales et al., 2012). In our study, PEBP and NBJ also enhanced the activity of PTEN, an upstream inhibitor protein of PI3K, possibly via the inhibition of miRNA-21 expression (Liu et al., 2013). These alterations, unfound with NBJ, could be exerted by the novel compounds that were produced during biotransformation and acted in concert on different types of receptors.

Treatment with PEBP rapidly increased p38-MAPK- and JNK- phosphorylation, which significantly reached its highest level at 2 h, and remained elevated for up to 24 h. PEBP reduced ERK1/2 phosphorylation in the same kinetic and cell-type independent manner. Modifications in MAPK family enzymes might contribute to the abolition of stem cell growth afforded by PEBP. Indeed, prolonged activations of JNK and MAPKp38 and/or inhibition of ERK1/2 induced apoptosis in most cancer cell lines (Chen and Sun, 2012; Leisner et al., 2013; Na et al., 2012; L.-H. Yang et al., 2012). The mechanisms by which PEBP modified MAPKs' activities are unknown. In addition, PEBP-induced alterations of upstream MAPK members might inhibit the downstream STAT3/PI3K/Akt signaling, indicating an extensive crosstalk and interplay between the MAPK cascade and STAT3 pathways.

We further confirmed the *in vivo* anticancer and antimetastatic potential of PEBP using the 4T1-induced breast cancer model in BALB/c mice. The 4T1 tumor is highly tumorigenic and invasive and, unlike most tumor models, can spontaneously metastasize from the primary tumor in the mammary gland to multiple distant sites (Lelekakis et al., 1999, p. 199; Pulaski and Ostrand-Rosenberg, 1998).

Chronic administration of PEBP via incorporation in drinking water significantly reduced tumor volume and breast cancer stem cell development derived from the tumor. This diminution supports the low count of metastasis in lungs of PEBP-treated animals. Especially, PEBP anticancer and antimetastatic effects were observed at a therapeutic dose as low as 12.5%, which, according to dose translation from animal to human using body surface area, corresponds to 1.2 cups of juice per day for humans (Reagan-Shaw et al., 2008). In contrast, NBJ at the same dose did not show any significant effect. NBJ could show a decrease in tumor size and weight only at the dose of 50%, which represents a substantial consumption of

blueberry juice for humans. These results are consistent with findings from previous studies, which reported that feeding mice with blueberry extracts or whole fruit powder has an impact on inflammation and could delay tumor growth (Adams et al., 2010; Montales et al., 2012; Mykkänen et al., 2014). However, NBJ failed to achieve the reduction of breast cancer stem cells and metastasis observed with PEBP. Nonetheless, the process of preparing PEBP, which greatly increases the content in total phenolic compounds, could clarify its effectiveness at a low therapeutic dose as compared to NBJ. Furthermore, the novel antimetastatic potential of PEBP could be explained by the change of phenolic composition from NBJ to PEBP during the biotransformation process. Indeed, the biotransformation of blueberry juice not only increases its phenolic content, but also produces novel compounds (Martin and Matar, 2005). One interesting possibility is that these novel compounds may possess more potent anticancer and antimetastatic properties that could have contributed to the observed reduction in tumor size and metastasis, as opposed to components of NBJ. In addition, the biotransformation process has probably broken down long polyphenol chains, which are poorly absorbed into gastro-intestinal tracts, increasing their bioavailability, and rendering PEBP highly functional (Perez-Jimenez et al., 2011)**.**

CONCLUSION

The results of the present study demonstrate that polyphenol-enriched blueberry preparation potently reduced the tumor growth and metastasis in mice. We have demonstrated that repression of breast CSCs by fermented blueberry supports a diet-mediated targeting of CSCs. We have provided evidence that PEBP selectively inhibits the inflammatory signature in CSCs through signaling pathways linked to the maintenance stemness and metastasis. The mechanisms of action involve, at least in part, alterations in the MAPKs cascade and inhibition

of the STAT3 signaling pathway, involved in inflammatory pathways. The results convincingly demonstrated that PEBP, indeed, holds great promise as a chemopreventive agent and may represent a novel complementary therapy against breast cancer and metastasis. Conclusively, the prospective modulation of CSCs by nutrition will probably mark a major advance in preventing breast cancer and further optimizing the management of this significant disease. It is an important approach in translational medicine for specific integrative therapies that can be recommended as evidence-based supportive care for cancer patients.

Authors' contributions

TV prepared the PEBP, carried out the cell culture experiment and animal experiment and drafted the manuscript. JFM participated in the animal experiment, and carried out the multiplex assay and the lung metastasis and drafted part of the manuscript. SR carried out the western blot. MO, HHV and ZH contributed to the design of the mammosphere experiment and revised the manuscript. CM drafted part of the manuscript and contributed to the conception and design of this research.

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Competing interests

Dr. Matar and Dr. Vuong have applied for a patent on the bacteria *Serratia vaccinii*. The other authors do not have any conflict of interest to report.

5 POLYPHENOL-ENRICHED BLUEBERRY PREPARATION CONTROLS BREAST CANCER STEM CELLS BY TARGETING FOXO1 AND MIR-145

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ABSTRACT

Scientific evidence supports the early deregulation of epigenetic profiles during breast carcinogenesis. Research shows that cellular transformation, carcinogenesis, and stemness maintenance are regulated by epigenetic-specific changes that involve microRNAs (miRNAs). Dietary bioactive compounds such as blueberry polyphenols may modulate susceptibility to breast cancer by the modulation of CSC survival and self-renewal pathways through an epigenetic mechanism, including the regulation of miRNA expression. Therefore, the current study aimed to assay the effect of polyphenol enriched blueberry preparation (PEBP) or non-

fermented blueberry juice (NBJ) on the modulation of miRNA signature and the target proteins associated with different clinical-pathological characteristics of breast cancer such as stemness, invasion, and chemoresistance using breast cancer cell lines. To this end, 4T1 and MB-MDM-231 cell lines were exposed to NBJ or PEBP for 24 h. miRNA profiling was performed in breast cancer cell cultures, and RT-qPCR was undertaken to assay the expression of target miRNA. The expression of target proteins was examined by Western blotting. Profiling of miRNA revealed that several miRNAs associated with different clinical-pathological characteristics were differentially expressed in cells treated with PEBP. The validation study showed significant downregulation of oncogenic miR-210 expression in both 4T1 and MDA-MB-231 cells exposed to PEBP. In addition, expression of tumor suppressor miR-145 was significantly increased in both cell lines treated with PEBP. Western blot analysis showed a significant increase in the relative expression of FOXO1 in 4T1 and MDA-MB-231 cells exposed to PEBP and in MDA-MB-231 cells exposed to NBJ. Furthermore, a significant decrease was observed in the relative expression of N-RAS in 4T1 and MDA-MB-231 cells exposed to PEBP and in MDA-MB-231 cells exposed to NBJ. Our data indicate a potential chemoprevention role of PEBP through the modulation of miRNA expression, particularly miR-210 and miR-145, and protection against breast cancer development and progression. Thus, PEBP may represent a source for novel chemopreventive agents against breast cancer.

INTRODUCTION

Breast cancer is the world's most commonly diagnosed cancer, making up 11.7% of total cases in 2020. Reproductive and hormonal factors, as well as lifestyle risk factors such as alcohol intake, obesity, and physical inactivity, contribute to breast cancer pathogenesis

(Sung et al., 2021). Histological and molecular subtypes are used to classify breast cancer. Molecular subtype classification is based on the presence or lack of estrogen receptors, progesterone receptors, and human epidermal growth factor receptor-2 (HER2) (Cava et al., 2015). Triple-Negative is an aggressive subtype of breast cancer and because of the absence of estrogen, progesterone, and HER2 receptor expression, no targeted therapy has been developed for this subtype (Khaled and Bidet, 2019).

In recent years, different targeted therapies, such as targeting epigenetic changes associated with cancer, have been developed to increase the efficacy of cancer treatment (Sung et al., 2021). Epigenetic mechanisms appear to play a key role in cancer establishment and progression, and their deregulation has been reported at multiple levels, including DNA methylation, histone modifications, and, indirectly, microRNAs (miRNAs) expression (Balassiano et al., 2011; Herceg and Hernandez-Vargas, 2011; Lima et al., 2010). In contrast to genetic mechanisms, epigenetic changes are reversible and are therefore capable of being targeted for intervention and cancer therapy, and can therefore be used as a biomarker for early detection of cancer (Cava et al., 2015; Guttilla et al., 2012; Marotta et al., 2011).

Mounting evidence supports the idea that healthy eating habits can control and reduce many epithelial cancers, including breast cancer (Jeyabalan et al., 2013; Link et al., 2013). In this sense, natural products such as probiotics, polyphenols, and fermented plant foods are known for their anti-inflammatory effects (Shahbazi et al., 2021, 2020, 2018) and may control neoplasia (Verma and Shukla, 2013). Blueberries are a well-known source of antioxidants and polyphenols (Bornsek et al., 2012). Polyphenols found in blueberries are shown to reduce inflammation, oxidative stress (Shahbazi et al., 2021), and metastasis (Adams et al., 2010), while promoting apoptosis in cancer cells (Mei et al., 2018).

During the fermentation process, bioefficacy, bioavailability, and content of blueberry polyphenols increase (Shahbazi et al., 2021). We have previously shown that fermentation of blueberry juice with *Rouxiella badensis* subsp. *acadiensis* (previously identified as Serratia vaccinia), a probiotic isolated in our lab from the natural microflora of lowbush blueberry, significantly raised the quantity of polyphenols present naturally in the juice (Martin and Matar, 2005). This novel product, polyphenol enriched blueberry preparation (PEBP), was shown to reduce weight gain in the diabetic and obesity model KKA(y) mouse and exerted an antidiabetic effect by mimicking metformin anti-inflammatory effects. The effect was thought to be modulated by increasing AMPK activity and adiponectin level (Vuong et al., 2009). PEBP was also shown to prevent oxidative stress from hydrogen peroxide on neurons and to reduce nitric oxide production by macrophages (Vuong et al., 2010, 2006). Accordingly, we demonstrated that PEBP decreased the formation of cancer stem cells (CSCs) by controlling phosphatase and tensin homolog/phosphatidylinositol-3 kinase/protein kinase B (PTEN/PI3K/AKT), interleukin 6/ signal transducer, and activator of transcription 3 (IL-6/STAT3), and mitogen-activated protein kinase (MAPK) pathways, which are central nodes in CSC signaling and homeostasis (Vuong et al., 2016).

Various mechanisms have been attributed to the protective effects of naturally occurring compounds such as PEBP against breast cancer. Modulation of the CSC self-renewal pathways is one important mechanism (Vuong et al., 2016). CSCs are a subpopulation of cells within tumors that are highly tumorigenic and can form spheres, termed mammospheres, with a CD44+/CD24−/low phenotype, under defined culture conditions. These cells can self-renew, differentiate into various types of cells composing the tumor (Duru et al., 2012), and are

believed to be a major cause of relapse in many cancers, having been found in a large number of cancers (Dave et al., 2012).

Epigenetic-specific changes in CSCs have previously been reported (Hernandez-Vargas et al., 2011). We have shown that cellular transformation, carcinogenesis, and stemness maintenance are regulated by epigenetic-specific changes that involve miRNAs (Ouzounova et al., 2013). miRNAs are small nucleotide sequences that influence gene translation and have emerged as critical regulators of CSCs in drug resistance and cancer metastasis (Schwarzenbacher et al., 2013). They work by binding to a complementary sequence in the target mRNAs, either by blocking the ribosome from translating the mRNA or by cleaving the RNA with the help of the miRNA ribonucleoprotein complex. Their expression changes in many malignancies (Shi et al., 2008), and some of them can function as tumor suppressors or oncogenes (Hatfield and Ruohola-Baker, 2008). miRNA networks have been reported to create a permanent feedback loop involving nuclear factor-κB (NF-κB), let-7 miRNAs, IL-6, and STAT3, which induce and maintain the CSC state (Iliopoulos et al., 2010a).

Given the significant role of epigenetic regulation in breast cancer formation and, as we have previously shown in the repression of breast CSCs by PEBP (Vuong et al., 2016), the current study aimed to investigate the possible mechanism of action of PEBP against the development of breast cancer through the regulation of the miRNAs expression signature involved in cell proliferation, survival, and CSC self-renewal pathways in vitro using breast cancer cell lines.

RESULTS

Effect of PEBP on miRNAs Expression in 4T1 and MDA-MB-231 Cell Cultures

For the microarrays experiment, we first performed an MTT assay using different doses of PEBP. The dose–response curve showed a significant effect of PEBP ranging from 40 to 200 µM gallic acid equivalent (GAE) (data not shown). Then, 60 µM GAE was used to assay the miRNAs profile. We examined the expression levels of many miRNAs by microarrays in 4T1 cells exposed to 60 µM GAE of PEBP for 24 h. Our results revealed that several miRNAs associated with different clinical-pathological characteristics of breast cancer, such as stemness, invasion, and chemoresistance, were differentially expressed (Table 1). In particular, miR-210, the most consistently and robustly induced miRNA under hypoxia, which is generally over-expressed in solid tumors (Ivan and Huang, 2014), was found to be highly downregulated. Furthermore, miR-145, a PI3K/AKT-cancer-associated miRNA (Ye et al., 2019), was over-expressed following exposure of the cells to the PEBP. miR-145 is associated with IL-6/STAT3 pathways and is under-expressed in breast cancer with high metastatic capability (Zou et al., 2012).

Overexpressed		**Underexpressed**	
microRNA	Fold change	microRNA	Fold change
miR-145	3.04	miR-7	0.37
miR-34b	2.13	miR-450	0.40
miR-26a	1.97	miR-23b	0.44
miR-216b	1.95	miR-214	0.46
miR-101	1.86	miR-210	0.51

let-7g	1.77	miR-301	0.52
miR-150	1.73	miR-297	0.54
miR-365	1.70		
miR-195	1.65		
miR-182	1.44		

Table 1 Expression of selected miRNAs in 4T1 cells exposed to 60 µM gallic acid equivalent (GAE) of PEBP for 24 h compared to non-treated cells.

We selected miR-210 and miR-145 as two of the predominant miRNAs regulated by PEBP treatment which are involved in the PI3K/AKT and STAT3 signaling pathways, for our functional analysis. 4T1 and MDA-MB-231 cells were treated with 60 µM GAE of either PEBP or NBJ for 24 h. The validation study by qRT-PCR revealed and confirmed the downregulation of oncogenic miR-210 and upregulation of tumor suppressor miR-145 (Figure 15 and Figure 16). miR-210 was significantly down-regulated in both 4T1 and MDA-MB-231 cells exposed to 60µM GAE of PEBP ($p < 0.001$ and $p < 0.01$, respectively). This effect was not seen in 4T1 cells when exposed to NBJ ($p > 0.05$) but was reduced by 50% in MDA-MB-231 cell culture ($p < 0.05$) (Figure 15A and Figure 16A). Furthermore, miR-145 expression was significantly increased in both cell lines treated with PEBP ($p < 0.05$), while treating cells with NBJ did not significantly change miR-145 expression in cells (Figure 15B and Figure 16B).

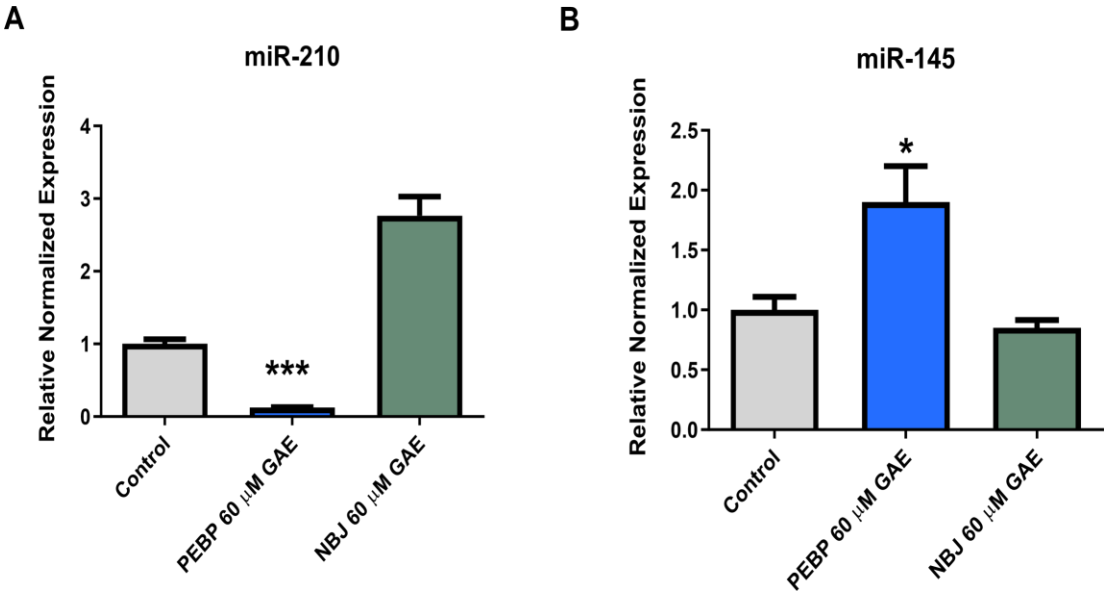

Figure 15: (A) Relative expression of miR-210 and (B) miR-145 by 4T1 cells after 24 h treatment with 60 μM gallic acid equivalent (GAE) of either polyphenol-enriched blueberry preparation (PEBP) or non-fermented blueberry juice (NBJ). The control consisted of using the same vehicle media used for the treatment groups. One-way ANOVA and Bonferroni's post-hoc tests were used to compare groups. All values are mean ± SEM of 3 separate experiments. * $p < 0.05$ and *** $p < 0.001$ vs. control.

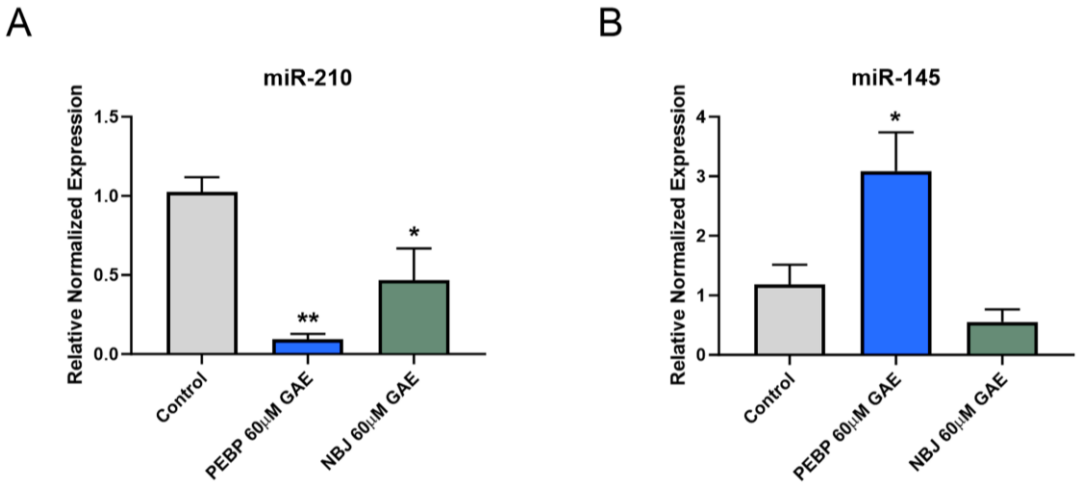

Figure 16: (A) Relative expression of miR-210 and (B) miR-145 by MDA-MB-231 cells after 24 h treatment with 60 μM gallic acid equivalent (GAE) of either polyphenol-enriched blueberry preparation (PEBP) or non-fermented blueberry juice (NBJ). The control consisted of using the same vehicle media used for the treatment groups. One-way ANOVA and Bonferroni's post-hoc tests were used to compare groups. All values are mean ± SEM of 3 separate experiments. * $p < 0.05$ and ** $p < 0.01$ vs. control.

Effect of PEBP on FOXO1 Expression in 4T1 and MDA-MB-231 Cell Cultures

For assaying the expression of target proteins, we first treated cell lines with different doses of NBJ and PEBP, ranging from 40 μM to 200 μM GAE to optimize the doses for Western blot analysis (data not shown). Subsequently, 100 μM and/or 150 μM GAE were used in related experiments. To determine the level of forkhead box protein O1 (FOXO1), 4T1 and MDA-MB-231 cells were exposed to 100 μM and 150 μM GAE of NBJ or PEPB for 24 h. FOXO1 is a transcription factor of PI3K/AKT known to influence the expression of miR-145 (Gan et al., 2010). PEBP has been shown to inhibit PI3K/AKT activation in three different breast cancer cell lines, potentially affecting miR-145 through FOXO1(Vuong et al., 2016). FOXO1 was over-expressed in both cell lines exposed to 100 μM GAE ($p < 0.001$ for 4T1 and $p < 0.01$ for MDA-MB-231) or 150 μM GAE of PEBP ($p < 0.001$) (Figure 17A, B). While treatment with NBJ had no significant effect on FOXO1 expression in 4T1 cells (Figure 17B), exposure to 150 μM GAE of NBJ significantly increased expression of FOXO1 in MDA-MB-231 cells ($p < 0.05$) (Figure 17B).

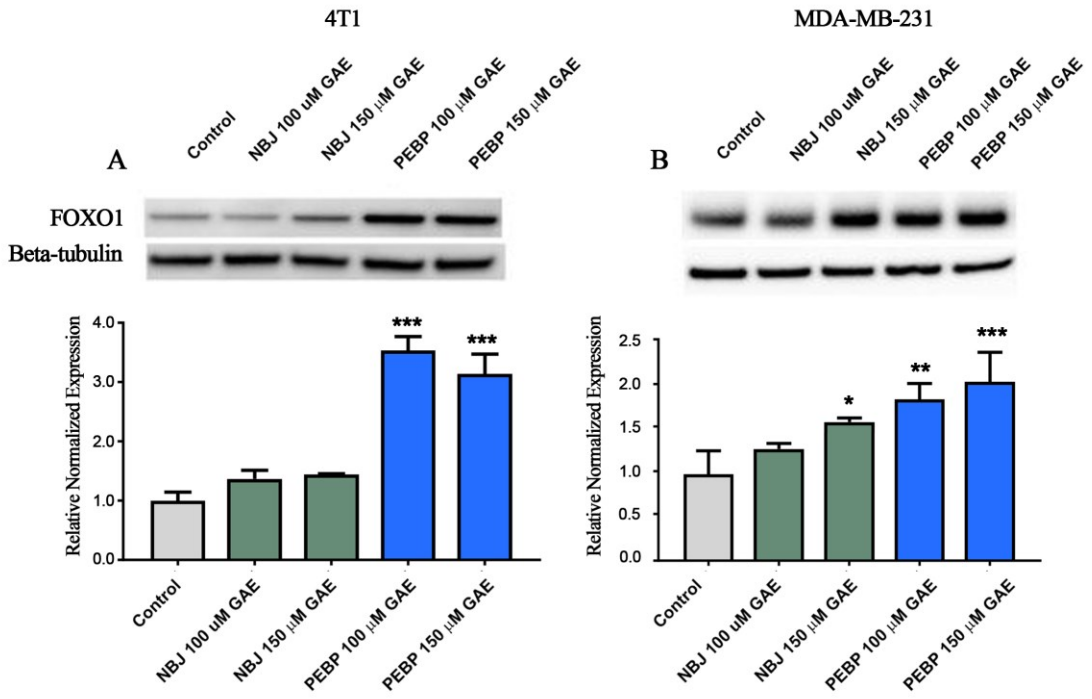

Figure 17: Relative expression of FOXO1 in (A) 4T1 and (B) MDA-MB-231 cells exposed to 100 μM or 150 μM gallic acid equivalent (GAE) of either polyphenol-enriched blueberry preparation (PEBP) or non-fermented blueberry juice (NBJ) for 24 h. The control consisted of using the same vehicle media used for treatment groups. Western blot images are from one representative experiment. One-way ANOVA and Bonferroni's post-hoc tests were used to compare groups. All values are mean ± SEM of 3 separate experiments. * $p< 0.05$, ** $p < 0.01$ and *** $p \leq 0.001$ vs. control.

Effect of PEBP on N-RAS Expression in 4T1 and MDA-MB-231 Cell Cultures

N-RAS has been found to be overexpress in some subtypes of breast cancer leading to the formation and progression of breast cancer (Zheng et al., 2015). To examine the effect of PEBP on N-RAS expression, 4T1 and MDA-MB-231 cells were treated with 100 μM GAE of NBJ or PEBP for 24 h. Expression of N-RAS was significantly reduced in 4T1 and MDA-MB-231 cells ($p < 0.05$) following 24 h treatment with PEBP ($p < 0.05$) (Figure 18A,B). In addition, a significant decrease was observed in the N-RAS expression in MDA-MB-231 cells treated with NBJ ($p < 0.01$), while N-RAS expression significantly raised in 4T1 cells in the presence of NBJ ($p < 0.01$) (Figure 18A,B).

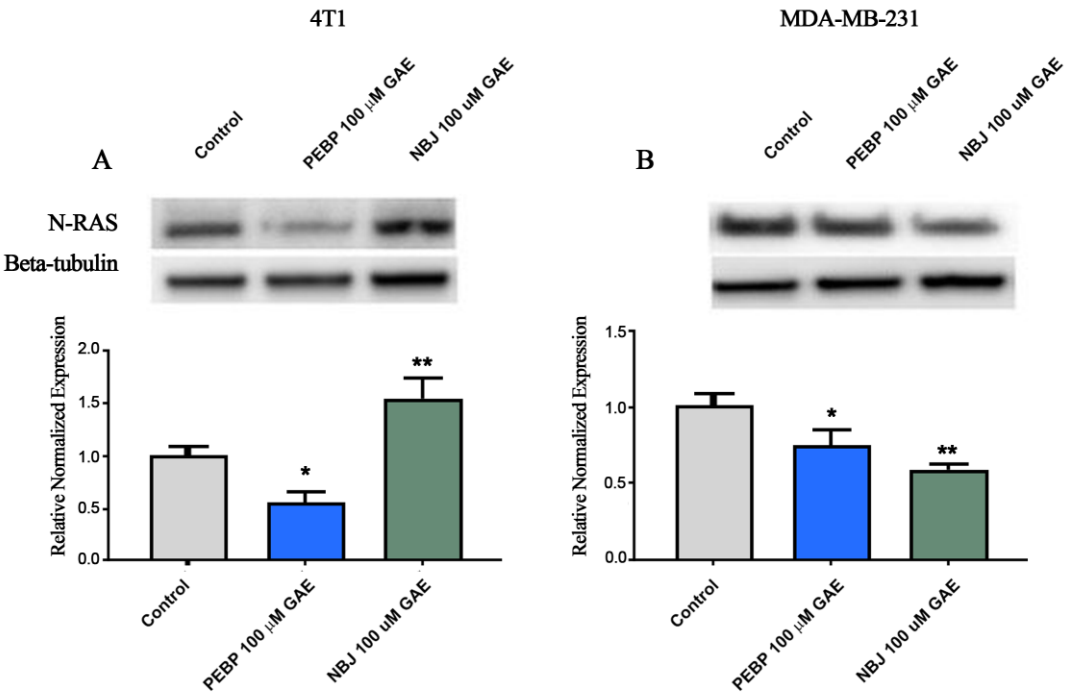

Figure 18: Relative expression of N-RAS in (A) 4T1 and (B) MDA-MB-231 cells exposed to 100 μM gallic acid equivalent (GAE) of either polyphenol-enriched blueberry preparation (PEBP) or non-fermented blueberry juice (NBJ) for 24 h. The control consisted of using the same vehicle media used for treatment groups. Western blot images are from one representative experiment. One-way ANOVA and Bonferroni's post-hoc tests were used to compare groups. All values are mean ± SEM of 3 separate experiments. * $p < 0.05$ and ** $p < 0.01$ vs. control.

Effect of miR-145 on N-RAS Expression in 4T1 and MDA-MB-231 Cell Cultures

miR-145 exhibits significant inhibitory activity against breast cancer malignancy and tumor growth through negatively regulating N-RAS signaling (Zou et al., 2012). To examine the role of miR-145 in N-RAS expression, 4T1 and MDA-MB-231 cells were transfected with either a miR-145 mimic or a miR-145 inhibitor. Expression of N-RAS decreased in the presence of the miR-145 mimic and increased in the presence of the miR-145 inhibitor in both 4T1 and MDA-MD-231 cell lines, although the results were not significant compared with control ($p > 0.05$). There was a significant difference in N-RAS expression between groups

transfected with the miR-145 inhibitor and the miR-145 mimic in both 4T1 and MDA-MB-231 cell cultures ($p < 0.001$ and $p < 0.05$, respectively) (Figure 19A,B).

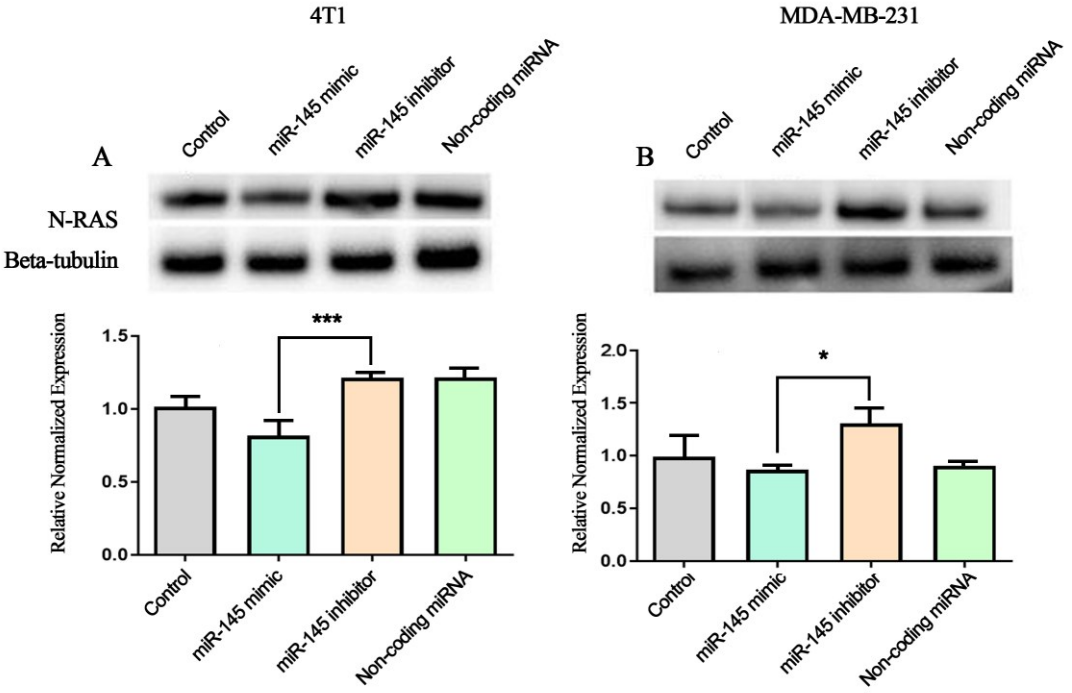

Figure 19: Relative expression of N-RAS in (A) 4T1 and (B) MDA-MB-231 cells transfected with a miR-145 mimic or inhibitor. The control consisted of using the same vehicle media used for treatment groups. Western blot images are from one representative experiment. One-way ANOVA and Bonferroni's post-hoc tests were used to compare groups. All values are mean ± SEM of 3 separate experiments. * $p < 0.05$ and *** $p < 0.001$ miR-145 mimic vs. miR-145 inhibitor.

DISCUSSION

Although breast cancer is considered a complex disease with a multifactorial etiology, emerging data about the importance of diet in the prevention of breast cancer are currently the subject of intense research (Teng et al., 2021). Adhering to a healthy eating style may be associated with a significant reduction in the risk of breast cancer (Buja et al., 2020). Biological and epidemiological evidence supports an inverse association of polyphenols intake and breast cancer, with more emphasis on subclasses of individual compounds of phenolic acids and the

risk of postmenopausal breast cancer (Bars-Cortina et al., 2022; Romanos-Nanclares et al., 2020).

Evidence indicates an early deregulation of epigenetic profiles during breast carcinogenesis (Giovannelli et al., 2012; Guttilla et al., 2012; Marotta et al., 2011). In this sense, various dietary regimes are thought to modulate susceptibility to breast cancer by altering normal epigenetic states and reversing abnormal gene activation or silencing (Hardy and Tollefsbol, 2011). Our pioneering research in integrative oncology has shown that probiotic and prebiotic intake has tremendous immunoprotective and chemopreventive effects against breast cancer (de Moreno de LeBlanc et al., 2006, 2005a, 2005b; Rachid et al., 2006; Vinderola et al., 2007a, 2006a, 2006b, 2005a, 2005b). The underpinning mechanisms are thought to involve miRNAs and epigenetic-specific changes controlling breast cancer stem cells (Hernandez-Vargas et al., 2011) and metastasis in vivo (Graham et al., 2017; Ouzounova et al., 2013; Vuong et al., 2016).

A panel of 38 miRNAs has been found to be differentially expressed between molecular subtypes of breast cancer (Blenkiron et al., 2007). Among aberrantly expressed miRNAs, miR-125 and miR-145 were significantly down-regulated, whereas miR-21 and miR-155 were up-regulated (Iliopoulos et al., 2010b, 2009b). Recent studies have shown that natural agents, including resveratrol, could alter miRNA expression profiles (Bao et al., 2012b), leading to the enhancement of the efficacy of conventional cancer therapeutics. In addition, it has been shown that the anti-metastatic effect of pomegranate juice on prostate cancer cells is partly due to the expression and up-regulation of anti-invasive miRNAs, such as miR-355, miR-205, and miR-200, whereas pro-invasive miRNAs such as miR-21 and miR-373 were down-regulated by the juice (Banerjee et al., 2012; Wang et al., 2011). Furthermore,

the anticancer effect of other natural compounds such as melatonin and tocotrienols by regulation of miRNAs expression, including miR-145 and miR-210, and miR-429 and their target genes has been reported in breast cancer cells. These genes are associated with apoptosis, cellular senescence, and cell proliferation (Aggarwal et al., 2019; Chuffa et al., 2020).

Cytokine-mediated cross-talk, led by IL-6, has been reported to play a role in tumor-elicited inflammation and the development of CSCs (Balassiano et al., 2011; Gueron et al., 2012). More precisely, the IL-6 pathway is subject to epigenetic modifications involving STAT3 signal transduction (D'Anello et al., 2010). The interface by which IL-6 controls stemness strongly involves miRNAs. An inverse relationship has been reported between let-7 miRNA and IL-6 expression in breast cancer tissues, suggesting the importance of inflammatory activation of miRNAs-related to IL-6 pathways and regulatory circuits in stemness (Iliopoulos et al., 2010a, 2009b, 2009a).

In the current study, we performed miRNA profiling in CSC cultures of mammary carcinoma cell lines exposed to PEBP. We revealed that several miRNAs associated with different clinical-pathological characteristics of breast cancer, such as stemness, invasion and chemoresistance, were differentially expressed (Table 1). Some clusters of these regulated miRNAs, such as hypoxamirs (regulating hypoxia) and metastamirs (regulating metastasis), are strongly involved in sustaining the inflammatory microenvironment that resolves in neoplasia (Gee et al., 2014; Hurst et al., 2009). Importantly, we demonstrated that the most prominent hypoxamir, miR-210, was remarkably downregulated following treatment of breast cancer cell lines with PEBP. miR-210 is involved in hypoxia-induced aggressiveness and resistance of CSCs (Tang et al., 2018). Importantly, the differential expression of this overlapping set of miRNAs reinforces the hypothesis that PEBP is controlling CSC

development by the deactivation of STAT transcription factors which control pro-inflammatory cytokine production and aberrant oncogenic signaling pathways. Our data also indicated overexpression of let-7g, miR-195, and miR-145 tumor suppressors that inhibit invasion and metastasis (Nadeem et al., 2017; Qian et al., 2011, 2011). We therefore postulate that PEBP induces epigenetic-specific changes by modulating miRNA regulatory networks (tumor-suppressive or oncogenic miRNAs) and inhibiting CSC-dependent survival/stemness pathways. We also identified miRNAs associated with IL-6/STAT3 pathways such as miR-365 and miR-145 in CSC cultures. Thus, as one of the predominant group of miRNAs regulated by PEBP treatment, miR-210 and miR-145, and related signaling, were selected for further functional analysis in this study. Validation studies by qRT-PCR revealed that miR-210 was substantially decreased in 4T1 and MDA-MB-231 cell cultures compared to the negative control, while miR-145 was significantly increased in MDA-MB-231 cell cultures compared to control.

miR-210 plays an important role in mammary tumorigenicity and is controlled by STAT3 transcriptional activity (Iliopoulos et al., 2010a). miR-210 is over-expressed in various human tumors and cancer cell lines in hypoxic conditions, a vital feature of the tumor microenvironment (Kulshreshtha et al., 2007; Volinia et al., 2012). Hypoxia promotes genomic instability in tumor cells. miR-210 may likewise control the DNA repair capacity of tumor cells during hypoxia (Crosby et al., 2009) by specifically decreasing pro-apoptotic signals (Rothé et al., 2011). In addition, hypoxia has been found to induce expression of vascular endothelial growth factor (VEGF), IL-6, and CSC signature genes such as Nanog and Oct4 with increased cell migration/invasion, concomitant with the upregulation of miR-210 expression in human pancreatic cancer cells (Bao et al., 2012a). Upregulation of miR-210 is

associated with poor prognoses for breast cancer patients and plays a role in the cancer's invasion and transition (Hong et al., 2012). Furthermore, downregulation of miR-210 has been reported to significantly suppress cell viability, increase apoptosis rate, and enhance radiosensitivity in hypoxic human hepatoma and lung cancers (Grosso et al., 2013; W. Yang et al., 2012).

Interestingly, miR-210 expression is correlated with metastasis of breast and melanoma tumors (Zhang et al., 2009) under the control of STAT3 in mammary carcinoma (Iliopoulos et al., 2009a). This observation perfectly aligns with the results of our previous study since we reported that the probiotic-like product, fermented blueberry juice, decreased the formation of CSCs in different types of mammary carcinomas cell lines as well (Vuong et al., 2016). In a recent review, miR-210 was shown to be a part of five immune-related miRNAs that can subvert the physiological immune response toward oncogenesis (Tili et al., 2013). Many identified targets of miR-210 such as suppressor anaphase-promoting complex, cyclin-dependent kinase 10, SERTA Domain Containing 2 are involved in cell cycle regulation and correlate with aggressiveness of breast cancer (Fasanaro et al., 2009).

miR-145, a PI3K/AKT-cancer-associated miRNA, was also over-expressed in our study. miR-145 is under-expressed in breast cancer with high metastatic capability (Zou et al., 2012). Since we have previously shown that the PEBP-inhibited PI3K/AKT pathway was also accompanied by a decrease in STAT3 activation (Vuong et al., 2016), it could be argued that regulation of miR-145 will potentially lead to tumor control and regression. miR-145 is also regulated by Akt in a p53-dependent manner. Suppression of PI3K activity substantially increases p53 levels and, at the same time, induces miR-145 (Sachdeva et al., 2012). In

particular, p53 is involved in the upregulation of the expression of tumor suppressor miRNAs such as let-7, miR-34, miR-145, miR-26, miR-30, and miR-146a (Boominathan, 2010).

FOXO1, a transcription factor generally known for its role in adipogenesis and the inhibition of glucose production in response to insulin, is a target of phosphorylation by AKT (Martinez et al., 2006). Since the PI3k/AKT axis is often constitutively activated in cancer (Thorpe et al., 2015) and its deactivator PTEN is often mutated or deleted in cancer, preventing it from repressing AKT signaling (Chalhoub and Baker, 2009; Ren et al., 2012), FOXO1 is often repressed in cancer. FOXO1 is also essential in cell cycle regulation, stress resistance, and tumor suppression, all crucial in cancer stem cells (Lu and Huang, 2011). In this study, FOXO1 was shown to increase in 4T1 and MDA-MB-231 cell cultures after treatment with PEBP compared to the control group in a dose-dependent manner. Lack of FOXO3A expression in breast cancer patients is associated with an increased recurrence rate (Smit et al., 2015). Inactivation of FOXO3A by the PI3K/AKT pathway favors cell survival, proliferation, and expansion of the CSC population and increases self-renewal and tumorigenic capacity, such as enhanced mammosphere formation, inhibition of differentiation, and increase in CD133 expression (Smit et al., 2015). FOXO1 also promotes the expression of miR-145 (Gan et al., 2010) to control multiple proteins associated with cancer.

N-RAS is a vital effector for tumor growth (Malaney and Daly, 2001). A correlation has been seen between high N-RAS levels and the most aggressive of breast cancer subtypes, the triple-negative phenotype (Banys-Paluchowski et al., 2020). In this study, we found that N-RAS was significantly down-regulated by the treatment with PEBP in 4T1 and MDA-MB 231 cell lines. The 4T1 cell represents a murine cell line mimicking the advanced stage of breast cancer, and MDA-MB-231 represents the triple-negative cell line. miR-145 has been

reported to block the activation of AKT and ERK1/2 pathways, directly targeting N-RAS (Yin et al., 2013; Zou et al., 2012). Figure 20 illustrates the possible mechanism of action of miR-145 and miR-210 in cancer cells.

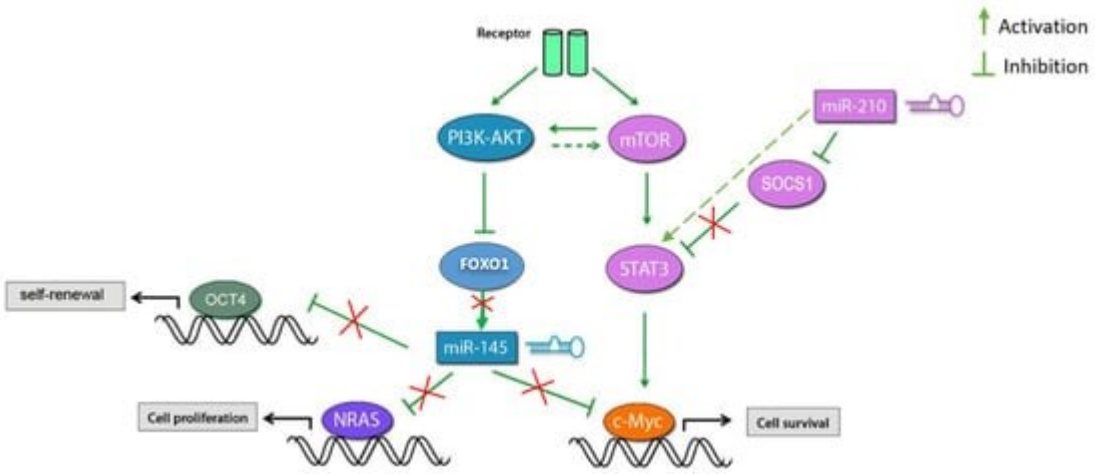

Figure 20: The potential mechanism of action of miR-145 and miR-210 in cancer cells. PI3k/AKT signaling is often constitutively activated in cancer. Inactivation of FOXO1 and subsequent inhibition of miR-145 expression by the PI3K/AKT pathway favors cell survival, proliferation, and expansion of the CSC population and increases self-renewal and tumorigenic capacity. Activation of STAT3 induces cancer cell survival by inducing the expression of c-Myc. miR-210 inhibits SOCS1 and activates the STAT3 pathway favoring cell survival. The red X marks the mechanism that would no longer be active by the treatment with PEBP.

In conclusion, our data validate the potential chemoprevention role of enriched polyphenol blueberry mixture through modulation of miRNAs, in particular miR-210 and miR-145. Our data indicate that these miRNAs may be involved in FOXO1 and NRAS modulation, breast cancer development, and progression. Therefore, polyphenol-enriched blueberry preparation may represent a novel complementary alternative medicine therapy.

MATERIALS AND METHODS

Preparation of Blueberry Juices

Mature lowbush blueberries (Vaccinium angustifolium Ait.) were purchased from Cherryfield Foods Inc. (Cherryfield, Maine, USA) as fresh and untreated fruits. Blueberry juice was extracted by blending the fruit (100g) in a Braun Type 4259 food processor. The fruit mixture was then centrifuged at 500 × g for 10 min to remove fruit skin and insoluble particles. The resulting juice was sterilized using 0.22 μm Express Millipore filters (Millipore, Etobicoke, Ontario, Canada). *Rouxiella badensis* subsp *acadiensis* (Canan SV-53), (*Rouxiella badensis* subsp. *acadiensis* has been filed in a U.S. Provisional Application No. 62/916,921 entitled "Probiotics Composition and Methods" for its potential probiotic effects) was cultured as previously described (Martin and Matar, 2005). The juice was inoculated with a saturated culture of the bacterium corresponding to 2% of the total juice volume. After a four day fermentation period, the transformed juice was sterilized by 0.22 um filtration. The total phenolic content was then measured by the Folin–Ciocalteau method using gallic acid as standard and hence expressed as μM gallic acid equivalent (GAE). The total phenolic content was increased from 5.9 mM GAE to 30.7 mM GAE, confirming successful transformation. Blueberry and biotransformed blueberry juice have been partially characterized elsewhere (Martin and Matar, 2005; Matchett et al., 2006).

Cell Culture

Murine 4T1, and human MDA-MB-231 cell lines were obtained from American Type Cell Collection (ATCC; Chicago, IL, USA). The cells were grown in RPMI-1640, media containing FBS (10%, v/v) (Sigma–Aldrich, Oakville, ON, Canada), penicillin/streptomycin (0.05 mg/mL) at 37oC in a humidified atmosphere with 5% CO_2.

miRNA Profiling

The miRNA profiling experiment was completed in vitro in 4T1 breast cancer cells using the Affymetrix GeneChip miRNA array 2.0 and validated using real-time quantitative reverse transcription PCR.

Real-time Quantitative Reverse Transcription PCR

4T1 and MDA-MB-231 cell lines were treated with 60 μM GAE of either PEBP or non-fermented blueberry juice (NBJ) for 24 h. Then cells were collected, and RNA was extracted using miRNeasy kit (Qiagen, Toronto, ON, Canada). Samples underwent a reverse transcription reaction to produce cDNA using individual probes. The cDNA was synthesized by Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Invitrogen, Burlington, ON, Canada). The expressions of miR-145 and miR-210 were measured by RT-qPCR using Taqman primers (Applied Biosystems, Burlington, ON) and a FastStart Taq Polymerase (Roche, Mississauga, ON, Canada) in a CFX96 machine (Bio-Rad, Mississauga, ON, Canada). Gene expression was normalized to gene reference U6 small non-coding RNA (Applied Biosystems, Burlington, ON).

miRNAs Transfection

4T1 and MDA-MB-231 cells were cultured in a medium without any antibiotics until they achieved approximately 30% confluence. They were then transfected with a miR-145 mirVana™ mimic or inhibitor (Ambion, Burlington, ON, Canada) using Lipofectamine (Invitrogen, Burlington, ON, Canada). The media was changed after 17 h, and the cells were grown without Lipofectamine until they reached 80% confluence.

Western Blot Analysis

After treatment with PEBP or NBJ, cells were collected and lysed. Cell lysates were run on a 4–12% acrylamide gel, transferred to a PVDF membrane, and probed with anti-FOXO1, anti-N-RAS, and anti-β-tubulin (Cell Signaling Tech. Inc., Danvers, MA, USA). Bands were visualized via chemiluminescence using horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Bands were quantified using β-tubulin as loading control using Bio-Rad Quantity One software.

Statistical Analysis

GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA) was used to perform statistical analysis. One-way analysis of variance (ANOVA) and Bonferroni's post-hoc tests were used to compare groups. $p \leq 0.05$ was considered statistically significant. Data are reported as mean ± SEM.

Author Contributions

J.-F.M. performed all experiments and contributed to the writing and data analysis; R.S. contributed to the writing, editing and submitting the manuscript. N.A. contributed to the data analysis; C.M. designed and supervised the work and contributed to the writing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

The data presented in this study are available on request from the corresponding author.

Acknowledgments

Special thanks to the University of Ottawa library.

Conflicts of Interest

The authors declare no conflict of interest.

Sample Availability

Samples of the compounds PEBP and NBJ are available from the authors.

6 ROLE OF A MIXTURE OF POLYPHENOL COMPOUNDS RELEASED AFTER BLUEBERRY FERMENTATION IN CHEMOPREVENTION OF MAMMARY CARCINOMA: *IN VIVO* INVOLVEMENT OF MIR-145

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Abstract: Epigenetic mechanisms such as microRNA (miRNA) deregulation seem to exert a central role in breast cancer initiation and progression. Therefore, targeting epigenetics deregulation may be an effective strategy for preventing and halting carcinogenesis. Studies have revealed the significant role of naturally occurring polyphenolic compounds derived from fermented blueberry fruits in cancer chemoprevention by modulation of cancer stem cell development through epigenetic mechanisms and regulation of cellular signaling pathways. In this study, we first investigated the phytochemical changes during the blueberry fermentation process. Fermentation favored the release of oligomers and bioactive compounds such as protocatechuic acid (PCA), gallic acid, and catechol. Next, we investigated the

chemopreventive potentials of a polyphenolic mixture containing PCA, gallic acid, and catechin found in fermented blueberry juice in a breast cancer model by measuring miRNA expression and signaling pathways involved in breast cancer stemness and invasion. To this end, 4T1 and MDA-MB-231 cell lines were treated with different doses of the polyphenolic mixture for 24 h. Additionally, female Balb/c mice were fed with this mixture for five weeks; two weeks before and three weeks after receiving 4T1 cells. Mammosphere formation was assayed in both cell lines and single-cell suspension obtained from the tumor. Lung metastases were counted by isolating 6-thioguanine-resistant cells present in the lungs. In addition, we conducted RT-qPCR and Western blot analysis to validate the expression of targeted miRNAs and proteins, respectively. We found a significant reduction in mammospheres formation in both cell lines treated with the mixture and in tumoral primary cells isolated from mice treated with the polyphenolic compound. The number of colony-forming units of 4T1 cells in the lungs was significantly lower in the treatment group compared to the control group. miR-145 expression significantly increased in tumor samples of mice treated with a polyphenolic mixture compared to the control group. Furthermore, a significant increase in FOXO1 levels was noted in both cell lines treated with the mixture. Overall, our results show that phenolic compounds found in fermented blueberry delay the formation of tumor-initiating cells in vitro and in vivo and reduce the spread of metastatic cells. The protective mechanisms seem to be related, at least partly, to the epigenetic modulation of mir-145 and its signaling pathways.

INTRODUCTION

Transformation of medicinal plant products by microbial fermentation to produce new nutraceuticals is a common practice in Asia and Europe (Vuong et al., 2006). In recent years, fermented plant products have become popular globally due to their unique sensory properties

and health benefits (Shahbazi et al., 2021). Fermented foods are a rich source of probiotics, prebiotics, and polyphenols with known health-promoting properties that potentially work by modulating gut microbiota and the immune system (Robichaud et al., 2021; Shahbazi et al., 2021, 2020). Microbial fermentation of plant products generates bioactive compounds by metabolizing fermentable macronutrients and improves the nutritional value, polyphenol levels, and antioxidant capacity (Li et al., 2022; Shahbazi et al., 2021). Due to their high concentration of bioactive compounds, fermented products play a significant protective role against chronic inflammatory diseases such as type 2 diabetes, cancers, and cardiovascular disease (Shahbazi et al., 2021; Vuong et al., 2016).

Blueberries are a well-known source of phenolic compounds (Bornsek et al., 2012). We previously showed that fermenting native North American blueberries (*Vaccinium corymbosum* (highbush blueberry) or *V. angustifolia* Aiton (lowbush blueberry)), using a novel bacterium, *Rouxiella badensis* subsp *acadiensis* (known as Canen SV-53) isolated from the blueberry skin microflora, significantly increases the amount of polyphenols present in the blueberry juice, raises its antioxidant potential (Martin and Matar, 2005; Vuong et al., 2006), improves its anti-inflammatory properties, and health-promoting activities (Vuong et al., 2010, 2009, 2007). This fermented blueberry juice, known as polyphenol-enriched blueberry preparation (PEBP), decreases the formation of cancer stem cells (CSCs) and notably suppressed the metastasis of breast cancer cells to the lungs in a mouse model of breast cancer (Vuong et al., 2016).

Furthermore, we have shown that PEBP has potential chemopreventive properties through the epigenetic modulation of CSCs' self-renewal pathways (Alsadi et al., 2021; Mallet et al., 2021; Vuong et al., 2016). CSCs are a small subset of neoplastic cells which may

contribute to tumor growth, maintenance, and recurrence (Alsadi et al., 2021). Moreover, we have obtained evidence that carcinogenesis in breast cancer was regulated by epigenetic-specific changes that involved microRNAs (miRNAs) (Hernandez-Vargas et al., 2011). Epigenetic changes mediated by miRNAs contribute to CSCs characteristics, including the self-renewal ability, mammospheres formation, and chemoresistance by modification-specific signaling involved in their survival and proliferation (Khan et al., 2019). Our previous research found an upregulation of tumor suppressor miR-145 expression and a significant downregulation of oncogenic miR-210 expression in 4T1 and MDA-MB-231 breast cancer cell lines treated with PEBP (Mallet et al., 2021). We also found an increase in the Forkhead box O1 (FOXO1) level and a decrease in the N-ras level in cells exposed to PEBP (Mallet et al., 2021). However, no detailed studies of the phytochemical changes that occurred after fermentation have been published thus far.

In this research, our primary objective was to identify quantitative phytochemical changes during the fermentation process. As a first step, we employed untargeted metabolomics using ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-MS-QTOF) analysis to identify compounds present in fermented and unfermented juice. Discriminant analysis was used to identify significant phytochemical markers present in the fermented juice compared to non-fermented juice. Next, targeted analysis allowed us to measure the amounts of changes caused by the fermentation. Using our library of compounds and a modified validated methods we have used before for blueberry products, we applied targeted analysis to better characterize the full range of changes in the fermented product. Our results showed that fermentation favored the release of small oligomeric and bioactive compounds like protocatechuic acid (PCA), gallic acid, and catechol.

Our secondary objective was to investigate the possible mechanism of action of a polyphenol mixture (PCA mix) containing PCA, gallic acid, and catechin that was released after blueberry juice fermentation against the development of breast cancer. To this end, we measured the effect of the mixture on the expression of targeted miRNAs and proteins involved in breast cancer cell proliferation, CSCs' self-renewal, and tumor formation using a 4T1cell-induced breast cancer model. In this paper, we report that some polyphenol compounds found in fermented blueberry juice decreased the formation of cancer stem cells, delayed the development of mammary carcinoma tumors, and inhibited the metastasis of the highly metastasizing 4T1 cells to the lungs in animals receiving the polyphenolic compounds.

RESULTS

2.1. UPLC-QTOF Analysis of Fermented and Non-Fermented Blueberry Juice

The total phenolic content was increased from 5.9 mM Gallic Acid Equivalent (GAE) to 30.7 mM GAE in fermented juice, confirming successful biotransformation. Untargeted metabolomics using UPLC MS QTOF was used to identify compounds present in fermented and non-fermented juice. Gradient separation was developed using reversed-phase UPLC and sub-two micron particle size stationary phase. By applying this high-resolution separation, the compounds were well separated within 18 minutes. Negative electrospray ionization was the best approach for phenolic acids and flavonoids while positive ionization was preferable for anthocyanins using quadrupole time of flight mass spectrometry. Although the profiles of fermented and unfermented juice are similar (Figure 21A, B), qualitative changes in specific compounds were visible in the chromatograms. Once the analysis was completed using optimal conditions, the identification of individual metabolites was carried out.

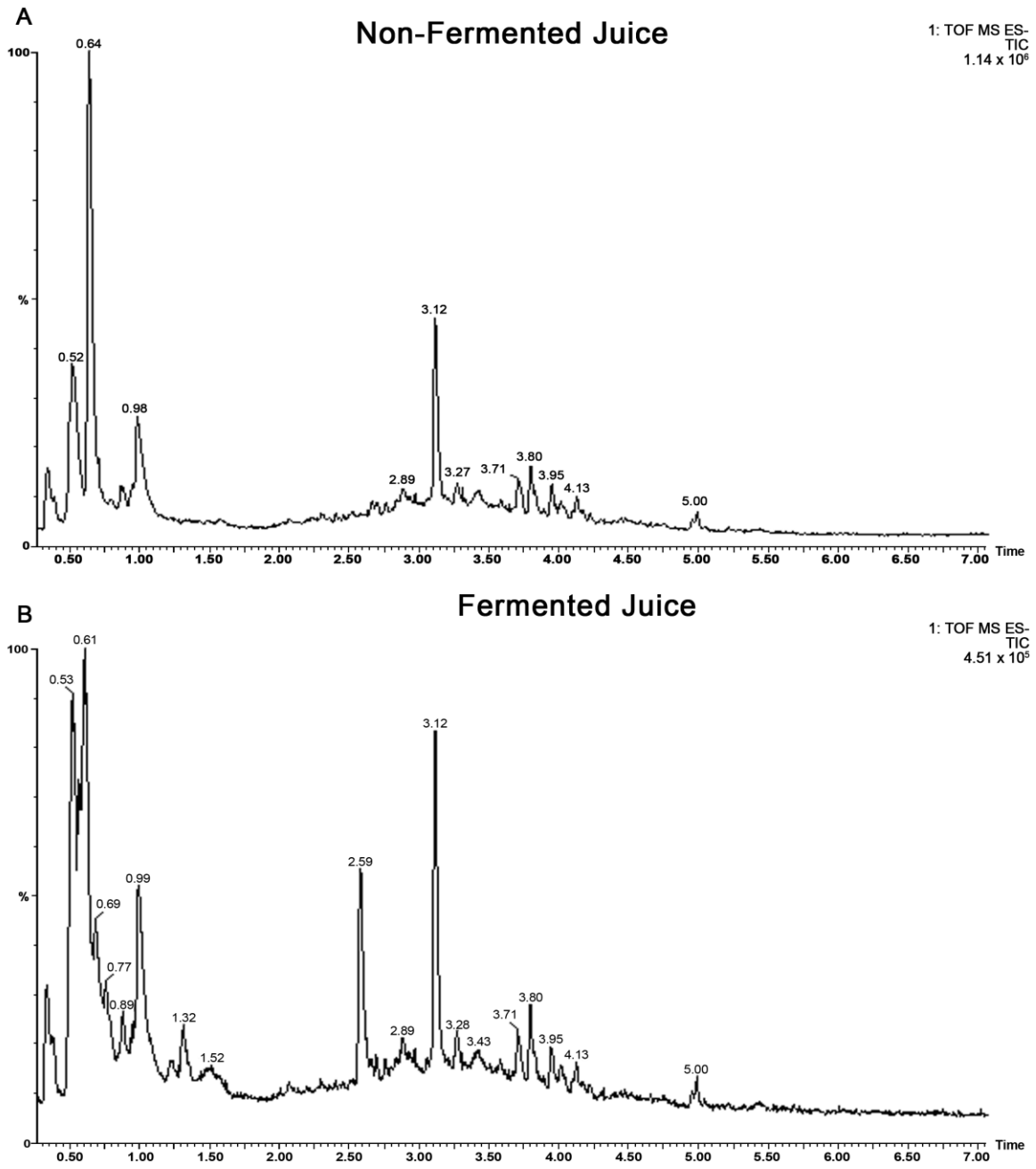

Figure 21 : Total ion chromatograms (TOF ESI negative) of **(a)** non-fermented and **(b)** fermented blueberry juice by SV-53. The numbers above the peaks represent retention times in minutes.

2.2. Identification, Quantification, and Discriminant Analysis of Metabolites

Figure 22: A illustrates metabolites detected in fermented blueberry juice. The use of discriminant analysis with metabolomics data is a means of identifying potentially bioactive compounds in fermented plant extracts and is complementary to bioassay-guided isolation (Choi et al., 2021; Zhang et al., 2018). Therefore, a search was conducted for at least 114 small molecules (mostly secondary metabolites), known in Vaccinium species, by UPLC-QTOF electrospray ionization (positive and negative modes). Of these compounds, confirmed identification was made for 22 compounds detectable in the study materials within 5 ppm mass accuracy and based on retention matching with authentic standards. These confirmed identified compounds mainly include phenolic acids, flavonoids, epicatechin, Myricetin-3-*O*-galactoside, Myricetin-3-*O*-glucoside, Quercetin-3-*O*-galactoside, Quercetin-3-*O*-glucoside, Quercetin-3-*O*-rhamnoside, Quercetin, anthocyanins, and procyanidins (Figure 22A). See Table 2 for more details about the compounds' features. In addition to these compounds with confirmed identification, tentative identification of several compounds was made using spectral matches with online databases. These compounds included carbohydrate metabolites galactonic acid, glucuronic acid, 4-*O*-β-δ-glucopyranosyl-δ-glucose, a bacterial secondary metabolite, pramicidin, and gallotanin.

Furthermore, the metabolomes of fermented and non-fermented juice were subjected to discriminant analysis to identify key markers that differentiate the two samples. The most significant markers in the fermented juice were catechol, gallic acid, and gluconic acid. Catechol (RT 2.59) and gallic acid (RT 1.32) were visible in the large peaks in fermented juice in Figure 21B but absent in the non-fermented juice in Figure 21A.

We also observed a decrease in the level of rutin and the rise of its aglycone counterpart, quercetin. This suggest that rutin is a possible substrate for SV-53. (Figure 22B, C).

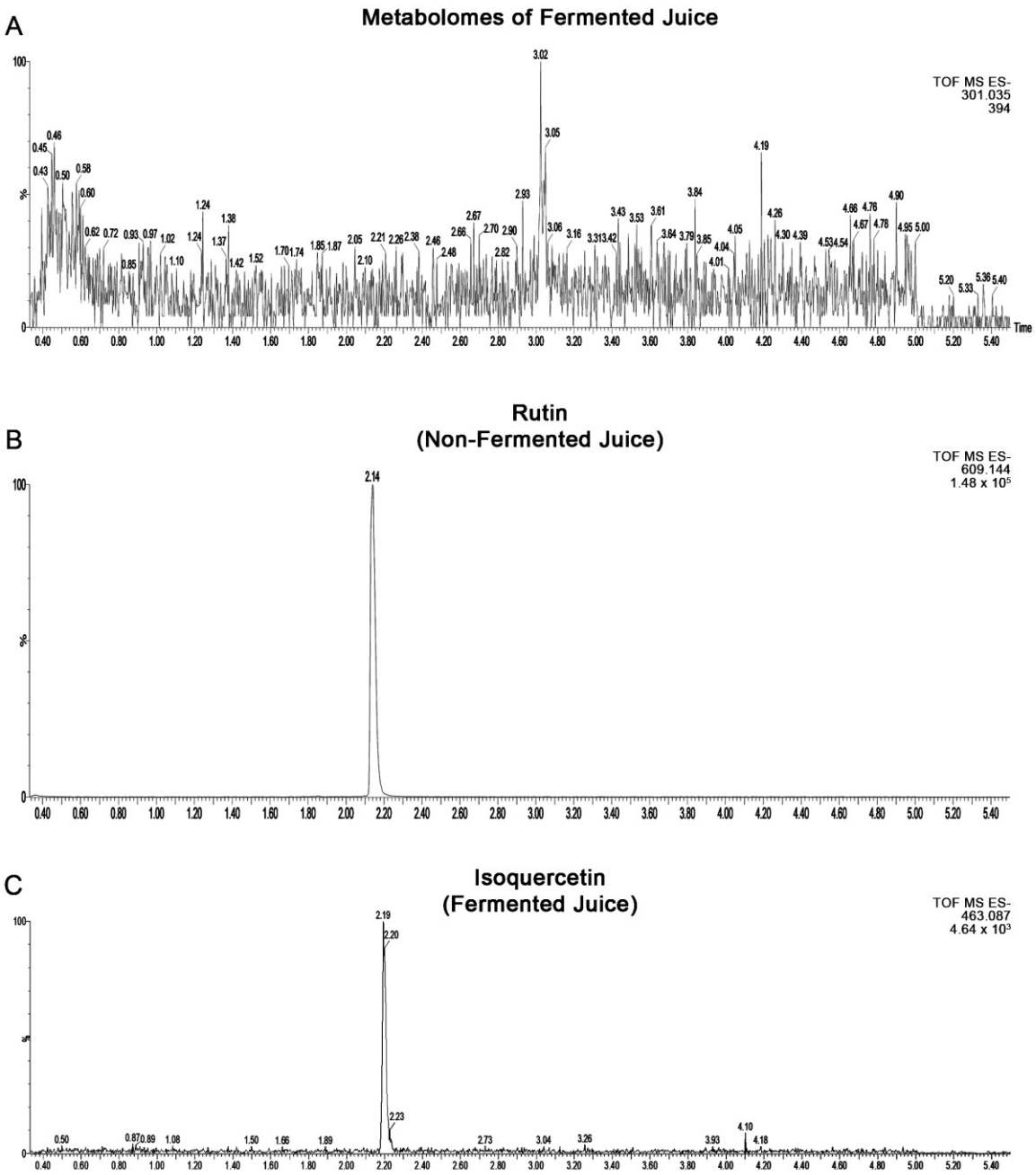

Figure 22 (a) Metabolomes detected in fermented blueberry juice by SV-53. Extracted ion spectrum of (b) rutin in non-fermented and (c) isoquercetin in fermented blueberry juice. The numbers above the peaks represent retention times in minutes.

Table 2 Characteristics of the identified metabolites in the fermented blueberry juice

Metabolite	Elemental Compound	Accurate mass	Rt (min)	Ions detected
Gallic acid	C7H6O5	170.0215	1.30	171.0293 (1+)/171.0295; 169.0137 (1-)/169.0131
Delphinidin-3-*O*-galactoside	C21H21O12+	465.1033	2.30	465.1033 (1+)/465.1025; 463.08752 (1-)/463.0873
Protocatechuic acid	C7H6O4	154.0266	2.31	155.0344 (1+)/155.0350; 153.0188 (1-)/153.0185
Idaein	C21H21O11+	449.1084	2.50	449.1084 (+)/449.1081;447.0930 (2-)/447.0928
Catechol	C6H6O2	110.0368	2.58	111.0446 (1+)/112.9555, 130.9658; 109.0290 (1-)/109.0289
Cyanidin-3-*O*-glucoside	C21H21O11+	449.1084	2.58	449.1084(+)/449.1070; 447.0912 (2-)/447.0911
Salidroside	C14H20O7	300.1209	2.66	301.1287 (+)/323.1111 [M+Na]+1; 299.1131 (1-)/299.1138, 398.0308
Pyrocatechol-*O*-β-D-glucopyranoside	C12H16O7	272.2600	2.69	273.0974 (1+)/295.0791 [M+Na]+, 326.0083; 271.0818 (1-)/271.0818
Primulin	C23H25O12+	493.1346	2.83	493.1346(+)/493.1347, 331.0813
Oxycoccicyanin	C22H23O11+	463.1240	2.84	464.1319 (+)/463.1225; 462.1162 (2-)/461.1078
(+)-Catechin	C15H14O6	290.0790	2.88	291.0869 (1+)/291.0868,311.0532* [M+Na]+1; 289.0712 (1-)/289.0717
p-hydroxybenzoic acid	C7H6O3	138.0317	2.88	139.0395 (1+)/139.0395;137.0239 (1-)/137.0237
Oenin	C23H25O12+	493.1346	2.89	493.1346 (+)/493.1351; 491.1183(2-)
Procyanidin B2	C30H26O12	578.1424	3.08	579.1503 (+)/579.1509;577.1346 (2-)/577.1359
Chlorogenic acid	C16H18O9	354.0951	3.12	355.1029(1+)/355.1008, 378.0843 [M+Na]+1; 353.0873 (1-)/353.0856,
(-)-Epicatechin	C15H14O6	290.0790	3.17	291.0869(1+)/291.0866; 289.0712 (1-)/298.0710
Myricetin-3-*O*-galactoside	C21H20O13	480.3800	3.47	481.0982(1+)/481.0973,503.0801*; 479.0826 (1-)/479.0826
Myricetin-3-*O*-glucoside	C21H20O13	480.3800	3.51	481.0982 (1+)/481.0980,;479.0826 (1-)/479.0826
Quercetin-3-D-galactoside	C21H20O12	464.0955	3.80	465.1033(1+)/465.1035,303.0509*,487.0849; 463.0877 (1-)/463.0871
Quercetin-3-glucoside	C21H20O12	464.0955	3.83	465.1033 (1+)/465.1012, 303.0496*;463.0877 (1-)/463.0877
Quercetin-3-*O*-rhamnoside	C21H20O11	448.1006	4.13	449.1084 (1+)/449.1086, 471.0895, 303.0502*;447.0927 (1)/447.0923
Quercetin	C15H10O7	302.0427	4.91	303.0505 (1+)/303.0498; 301.0348 (1-)/301.0354

2.3. Effect of the Polyphenolic Mixture on Mammospheres Formation in 4T1 and MDA-MB-231 Cell Cultures

First, we treated 4T1 and MDA-MB-231 cell lines with different concentrations of the polyphenolic mixture, ranging from 0.5 mM to 3 mM GAE to optimize the best doses for treating cells to conduct subsequent experiments. Cell viability was assessed by water-soluble tetrazolium salts (WST-1) and Lactate Dehydrogenase (LDH) assays (Roche, Laval, QC) (data not shown). Then, we selected 1- and 2-mM GAE concentrations of the polyphenolic mixture to perform our experiment. Treatment of 4T1 cells with 1- and 2-mM GAE of the polyphenolic mixture for 24 h significantly decreased the formation of mammospheres in this cell line (Figure 23A). However, only higher concentrations (2 mM GAE) significantly inhibited mammospheres formation in MDA-MB-231 cells (Figure 23B).

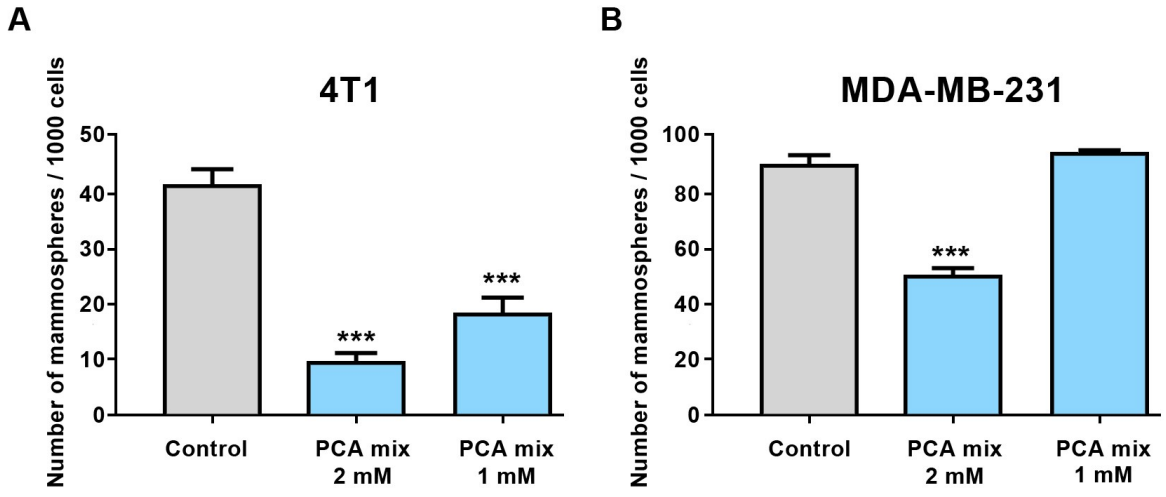

Figure 23 The number of mammospheres formation from (**a**) 4T1 and (**b**) MDA-MB-231 cell lines in a low attachment environment exposed to 1 or 2 mM gallic acid equivalent of the protocatechuic acid-based mixture (PCA Mix) for 4-7 days. One-way ANOVA followed by Dunnett's post hoc test was used to compare groups. All values are means of 3 separate experiments ±SEM. ***$p \leq 0.001$ vs. control.

2.4. Effect of the Polyphenolic Mixture on FOXO1 and N-ras Expressions in 4T1 and MDA-MB-231 Cell Lines

4T1 and MDA-MB-231 cells were exposed to 1 mM and 2 mM GAE of the mixture for 24 h in order to examine the level of FOXO1 and N-ras expression in cell cultures. FOXO1 is a major tumor suppressor which controls cell proliferation. Dysregulation of FOXO1 is thought to contribute to the progression of a variety of cancers, including breast carcinoma (Yu et al., 2014). Furthermore, FOXO1 might inhibit N-ras activation by regulating miRNA expression mainly miR-145. N-ras overexpression has been linked with the formation and progression of breast cancer (Mallet et al., 2021). Treatment of 4T1 and MDA-MB-231 cells with the 1- and 2-mM GAE of the polyphenolic mixture significantly elevated the expression of FOXO1 in cells ($p \leq 0.001$) (Figure 24A, B). We also observed a significant increase in N-ras levels in MDA-MB-231 cells treated with 2mM GAE of the polyphenolic mixture ($p \leq 0.01$) while no change was observed in 4T1 cells (Figure 24C, D).

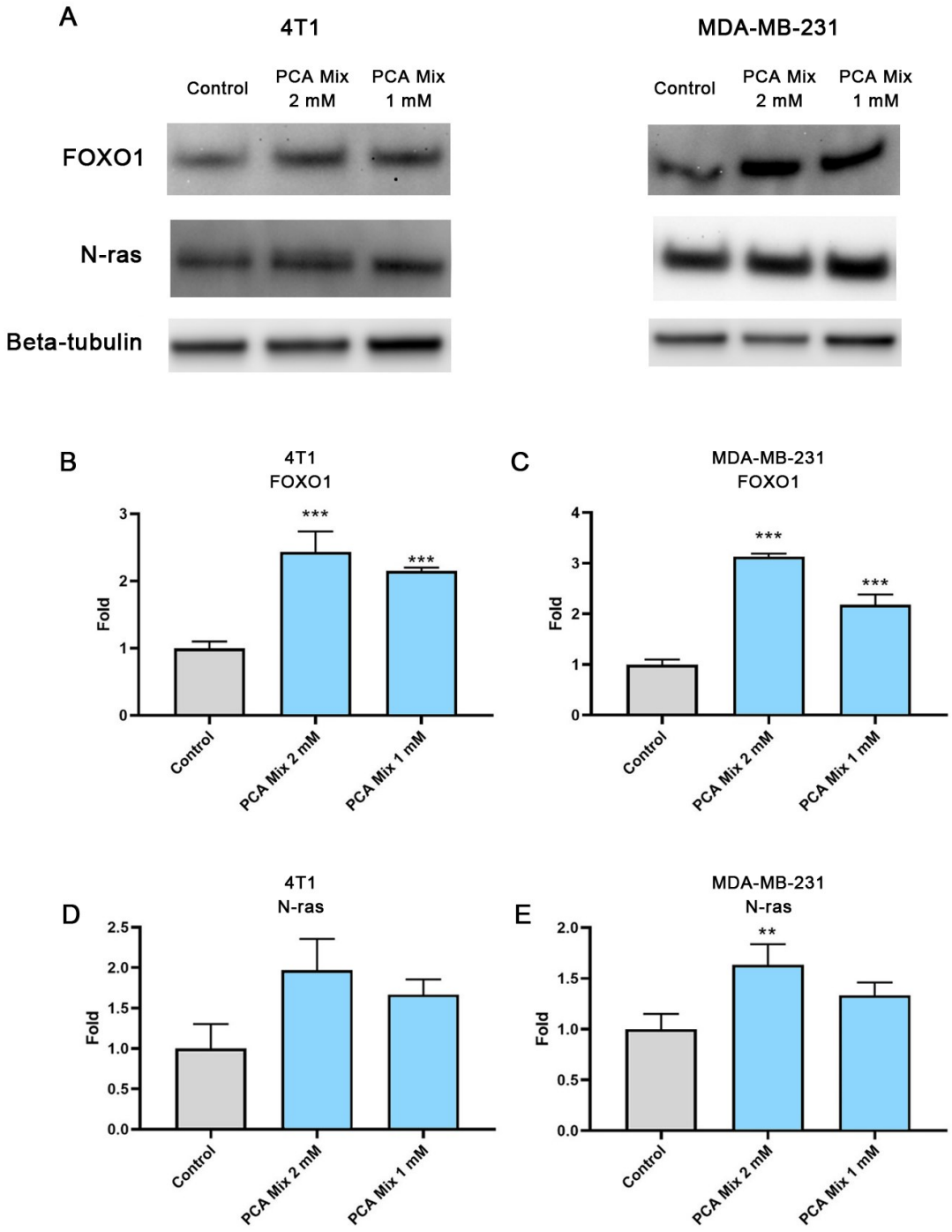

Figure 24 Relative expression of FOXO1 and N-ras in 4T1 and MDA-MB-231 cells exposed to 1 or 2 mM gallic acid equivalent (GAE) of a protocatechuic acid-based mixture (PCA mix) for 24 hours. (a and b) Relative expression of FOXO1 in 4T1 and MDA-MB-231 cells, respectively, and (c and d) relative expression of N-ras in 4T1 and MDA-MB-231 cells, respectively. One-way ANOVA followed by Dunnett's post hoc test was used to compare groups. All values are means of 3 separate experiments ±SEM. ** $p \leq 0.01$ and ***$p \leq 0.001$ vs. control.

2.5. Effect of the Polyphenolic Mixture on miR-145 and miR-210-5p Expressions in Tumor Samples

We previously conducted a microarray experiment to find the differentially expressed miRNAs in the 4T1 cell line exposed to PEBP for 24 h (Mallet et al., 2021). Our microarray analysis, followed by validation using qRT-PCR, revealed and confirmed the over-expression of the tumor suppressor miR-145 and under-expression of the oncogenic miR-210 in 4T1 cells (Mallet et al., 2021). Therefore, in the present study, we assayed the expression of miR-145 and miR-210-5p in 4T1-induced mammary tumors collected from mice treated with our polyphenolic mixture for a five-week period. Our result revealed a significant increase in miR-145 expression in tumor samples of mice treated with polyphenolic mixture compared to the control group ($p<0.05$) (Figure 25A); however, no significant difference was observed in the expression level of miR-210-5p (Figure 25B).

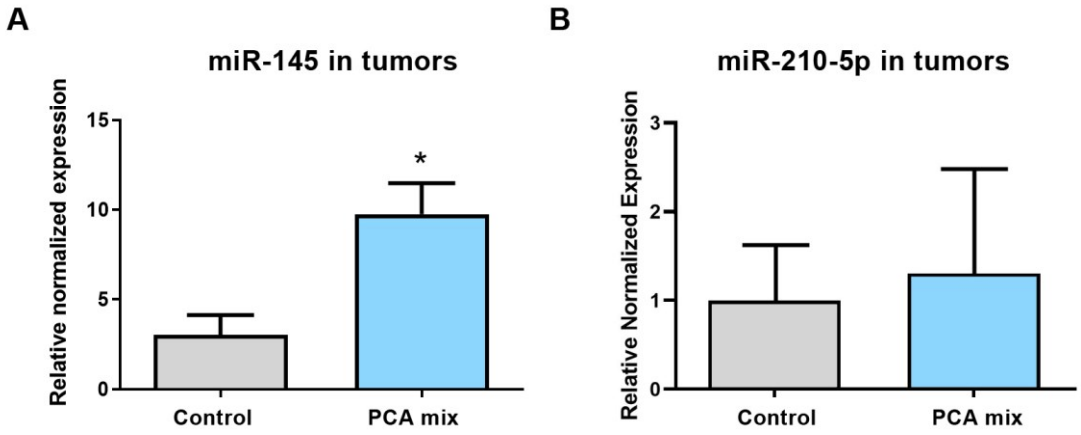

Figure 25 Relative expression of (**a**) miR-145 and (**b**) miR-210-5p in tumors from mice received either drinking water (control group) or a polyphenolic mixture (PCA mix) for five weeks. Independent T-test was performed to compare groups. All values are means of 3 separate experiments ±SEM (for a total of 12 animals in each group). N=12 in each group. * $p<0.05$ vs. control.

2.6. Effect of the Polyphenolic Mixture on Spheroids Formation and Metastasis ex vivo

Spheroids formation from tumoral primary cells was significantly reduced in tumors removed from animals fed with polyphenols ($p<0.05$) (Figure 26A). Similarly, the number of colony-forming units of 4T1 cells present in the lungs of mice was significantly lower in the treatment group compared to the control group indicating the reduction of the metastasis in the lungs of polyphenols-treated mice ($p<0.05$) (Figure 26B).

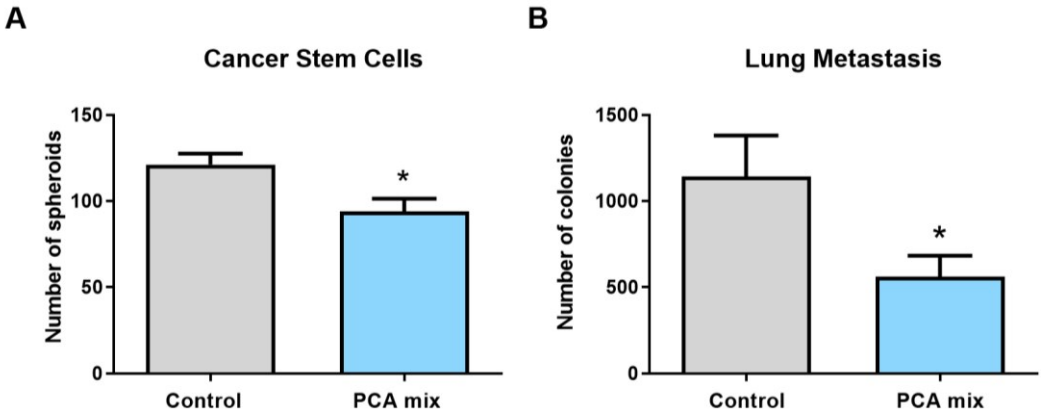

Figure 26 (**a**) The number of spheroids from cells isolated of the 4T1 cells-induced tumors and (**b**) the number of colony-forming units of 4T1 cells present in the lungs of mice received either drinking water (control group) or a polyphenolic mixture (PCA mix) for five weeks. Independent T-test was performed to compare groups. All values are means of 3 separate experiments ±SEM. N=12 in each group. *$p<0.05$ vs. control.

DISCUSSION

Naturally occurring compounds, mainly polyphenols, have gained immense attention because of their ability to target key inflammatory signaling pathways (Mallet et al., 2021; Vuong et al., 2016; Yahfoufi et al., 2018). Numerous studies are currently focused on developing innovative phytochemical-based treatment options for the prevention and treatment of cancer (Mazurakova et al., 2022). We have provided evidence that fermented blueberry juice, referred to as PEBP, exhibits a potential chemopreventive role in cancer (Vuong et al., 2016). The molecular mechanisms underlying the pleiotropic activities of fermented products produced by SV-53 involve the regulation of global cell regulators at

various levels of cell signaling, which are implicated in the inflammatory response and immune homeostasis (Alsadi et al., 2021; Mallet et al., 2021; Vuong et al., 2016).

Herein, we first aimed to study phytochemical changes in blueberry juice following the fermentation process. The transformation by SV-53 leads to an increase in bioactive components resulting in PEBP having four times more antioxidant activity than normal blueberry juice (Vuong et al., 2006). One hypothesis that underlines the higher beneficial effects of PEBP is related to tannin degradation, which converts large polyphenols to smaller oligomers. Small oligomers are known to be better absorbed, greatly affecting their bioavailability and consequently physiological effects (Manach et al., 2005). Small oligomers of polyphenols may then exert their activity as prebiotics or natural ligand for toll-like receptors (TLRs) involved in immune regulation. In fact, there is a growing body of evidence to support the notion that some polyphenolic ingredients act as prebiotics. For example, quercetin has proven to positively influence microbiota (Porras et al., 2017). Quercetin is an important flavonol with known anti-inflammatory activities. Interestingly, quercetin might exert its anti-inflammatory activity via the blockade of the TLR4-mediated signaling pathway (Han et al., 2016; Vuong et al., 2016). In addition, quercetin, has been found to increased anti-inflammatory miR-200b and miR-145 in pancreatic and ovarian cancer stem cells, respectively (Liu et al., 2017; Nwaeburu et al., 2017). Along this line, the presence of isoquercetin in the fermented blueberry juice might indicate that SV-53 is able to hydrolyze the sugar moiety in rutin and thereby enrich it with bioactive phenolic acids such as PCA. This is one of many examples of how the fermentation of blueberries might yield bioactive compounds positively influencing ligands found on non-immune and immune cells and differentially influencing miRNAs profile.

Blueberry polyphenols have been widely studied for their wide range of health benefits (Shahbazi et al., 2021). Although more than 8000 polyphenols have been discovered (Del Rio et al., 2010), research has focused on a specific class of flavonoids known for their beneficial effects, including quercetin, rutin, catechin, and PCA (Serra et al., 2012). The protective effects of flavonoids are not only due to intact flavonoids, as their bioavailability in their native form is low, but also or exclusively due to other bioactive substances formed after microbial degradation by gut microbiota (Manach et al., 2005). PCA, a metabolite of quercetin, has a remarkable antiatherogenic effect. PCA, as the gut microbiota metabolite of cyanidin-3- *O*-β-glucoside (Cy-3-G), exerts its antiatherogenic effect partially through miRNA-10b (Wang et al., 2012). PCA was also shown to have an apoptotic effect on cancer cells (Yin et al., 2009).

In perfect alignment with these observations, we showed that our biofermentation process mimics a healthy colonic fermentation of flavonoids by colon microbiota. In fact, phytochemical studies using UPLC-QTOF analysis revealed a significant change in the fermented product compared to conventional juice. The biofermentation process led to the appearance of novel peaks of oligomeric phenols. We have also shown the release of gallic acid, catechol, chlorogenic acid, and PCA in fermented blueberry juice (Nachar et al., 2017). Additionally, we have demonstrated that our probiotic can transform rutin into its aglycone counterpart quercetin. Furthermore, the biofermentation of quercetin generated a wide range of metabolites, including *p*-hydroxyphenylacetic acid, PCA, 3-(4-hydroxyphenyl) propionic acid, *p*-hydroxybenzoic acid, and *p*-coumaric acid (Lin et al., 2016). Notably, the main metabolite produced through the colonic fermentation of quercetin is PCA (Lin et al., 2016).

Next, we examined the preventative effect of a PCA-based polyphenolic mixture, consisting of protocatechuic acid, gallic acid, and catechin, which are the main polyphenolic

compounds found in fermented blueberry juice produced by the novel probiotic bacterium SV-53. Our main goal was to study the inhibitory effect of this mixture on CSC formation and metastasis through the regulation of specific signaling pathways and miRNA expression both *in vitro* and *in vivo*.

CSCs are the key drivers of cancer and play a role in relapse, resistance to anticancer therapies and tumor recurrence (Podberezin et al., 2013). CSCs derived from breast cancer cells with CD44+/CD24 low/- phenotype have the ability of heterogeneous differentiation, initiating diverse tumors and forming mammospheres (Dontu et al., 2003b; Liu et al., 2021; Manuel Iglesias et al., 2013; Wicha, 2006). The mammosphere formation assay has been used as a useful method for studying stem cell-like characteristics in breast cancer cell cultures (Manuel Iglesias et al., 2013). Polyphenols such as resveratrol and curcumin have been found to exhibit cytotoxic effects on CSCs, eliminate CSC populations from tumors, inhibit the formation of mammospheres, and thus prevent tumor formation (Taylor and Jabbarzadeh, 2017). Accordingly, we have previously demonstrated that PEBP delays the formation of cancerous stem cells in different types of cell cultures and *in vivo*, through modulation of IL-6/STAT3, as well as the extracellular regulated kinase (ERK) and p38 in mitogen-activated protein kinase (MAPK) signaling pathways (Vuong et al., 2016). The STAT3 and MAPK pathways play a crucial role in CSCs growth and metastatic characteristics (Vuong et al., 2016). In accordance with our previous results, we found that our polyphenolic mixture prevented mammosphere formation *in vitro* in 4T1 and MDA-MB-231 cell lines, and *ex vivo* in the cells isolated from mammary tumors.

Epigenetic mechanisms, such as DNA methylation, histone modifications, and miRNAs contribute to the development of CSCs (Liu et al., 2021). miRNAs can play either

inhibitory or stimulatory roles in CSCs development (Khan et al., 2019). For instance, miR-145, miR-200c, miR-494, and miR-34 have been shown to inhibit CSCs, while miR-19, miR-501-5p miR-21 and miR-221/222 promote CSC development (Khan et al., 2019). We have previously reported epigenetic-specific changes in CSCs that involve miRNAs (Alsadi et al., 2021; Mallet et al., 2021). We identified several differentially expressed clusters of miRNAs involved in maintaining the inflammatory microenvironment and are associated with various clinical-pathological characteristics of breast cancer, such as stemness, invasion, and chemoresistance (Mallet et al., 2021). We have also reported that the regulation of breast cancer stemness may be controlled by PEBP, particularly through the upregulation of anti-inflammatory miR-145 and the downregulation of oncomiR-210 expression *in vitro* (Mallet et al., 2021). Additionally, we found that PEBP increases the expression of miR-200b in metastatic B16F10 skin cancer cells, a miRNA that is commonly downregulated in the melanoma cell line (Alsadi et al., 2021). Consistent with our previous findings, we found that the PCA-based mixture significantly upregulated the expression of tumor suppressor miR-145 in tumor samples of mice.

miR-145 is downregulated in various types of tumors, including breast tumors (Zou et al., 2012). It plays an important role in the anti-tumorigenic functions of the FOXO1 transcription factor pathway, which regulates cellular proliferation, differentiation, apoptosis, and metastasis (Zeinali et al., 2019). miR-145 suppresses metastasis in cancer by targeting various signaling pathways and suppressing multiple oncogenes. For instance, N-cadherin is a direct target of miR-145 (P. Gao et al., 2013), and its expression has been shown to be closely linked with invasion and metastasis in breast cancer tumors (Hazan et al., 2000). Moreover, the suppression of N-cadherin by miR-145 has been found to reduce cell invasion in breast

cancer (Zhao et al., 2016). Additionally, the inhibition of ZEB2 by miR-145 allows for the expression of E-cadherin, which is known to inhibit cell migration in breast cancer (Jiang et al., 2016; Younis et al., 2007). We have previously shown that PEBP significantly inhibits the metastasis of 4T1 cells to the lungs in Balb/c mice (Vuong et al., 2016). The highly metastasizing 4T1 cell line typically forms metastasis in multiple organs such as the lungs, liver, and brain (Pulaski and Ostrand-Rosenberg, 2001). Consistent with our previous finding, we demonstrated that a polyphenolic mixture could inhibit the invasion of 4T1 cell to the lungs in a mouse model of breast cancer.

Furthermore, we studied the pathways related to miR-145, including FOXO1 and N-ras, in the breast cancer cell lines. FOXO1 downregulation occurs in various types of cancers (Prasad et al., 2014). For example, Dong et al., (2017) demonstrated that FOXO1 can inhibit cell motility, invasion *in vitro*, lung metastasis *in vivo*, and suppressed epithelial-to-mesenchymal transition (EMT) induced by ZEB2 (Dong et al., 2016). Additionally, Li et al., (2019) reported that FOXO1 reduced tumor stemness and EMT signals in nasopharyngeal carcinoma by inducing miR-200b (Y. Li et al., 2019). PI3K/AKT-mediated suppression of FOXO3A leads to expansion of the CSC population and promotes their self-renewal and mammospheres formation abilities (Smit et al., 2015). Our previous results revealed the effectiveness of PEBP in inhibiting CSC formation and suppressing cellular motility and invasiveness by upregulating miR-200b and downregulating ZEB1 in skin cancer cell lines (Alsadi et al., 2021). Moreover, we demonstrated the role of PEBP in inhibiting breast cancer stemness by upregulating FOXO1 and downstream miR-145 in breast cancer cell lines (Mallet et al., 2021). Similarly, in this study, we observed a significant increase in FOXO1 expression in cancer cell lines exposed to different doses of our polyphenolic mixture.

Ras proteins upregulation might be associated with tumorigenesis, invasion, and metastasis (Malaney and Daly, 2001). Oncogenic N-ras elevation correlates with poor clinical outcomes and poor breast cancer-specific survival. Evidence show overexpression of N-ras in the triple-negative subtype of breast cancer as the most aggressive breast cancer subtype (Banys-Paluchowski et al., 2020). Epigenetic modifications participate in N-ras expression and activity in breast cancer. For example, a study found that miR-145 exhibited antitumor activity by inhibiting tumor angiogenesis, cell invasion, and tumor growth through post-transcriptional modification of N-ras and growth factors (Zou et al., 2012). In our research, we found an increase in N-ras levels in MDA-MB-231 cells exposed to the higher concentration of polyphenolic compounds despite the higher level of miR-145 observed in the tumors. This contradicts our previous finding, where PEBP reduced N-ras levels in the same cell lines (Mallet et al., 2021). PEBP is a highly complex product, and this discrepancy may be due to the presence of components in PEBP that are not present in our mixture. Further research is necessary to understand and optimize the composition of our mixture.

In conclusion, our findings show the chemoprevention potential of a PCA-based polyphenolic mixture works, at least partly, by decreasing the number of tumor-initiating cells and preventing metastasis through the upregulation of miR-145. Our data might suggest this polyphenolic mixture could act as a potent chemo-preventive agent. Finally, nutritional approaches enriched with bioactive polyphenol compounds may be a viable strategy for preventing cancer.

MATERIALS AND METHODS

4.1. Preparation of Blueberry Juices

Fresh and untreated lowbush blueberries (*Vaccinium angustifolium* Ait.) were purchased from Cherryfield Foods Inc. (Cherryfield, Maine, USA). Following blending the fruit (100 g) in a Braun Type 4259 food processor, the mixture was centrifuged at 500 x g for 10 min to remove skin and other insoluble particles and extract fruit juice. The resulting juice was sterilized using 0.22 µm filters (Millipore, Etobicoke, Ontario, Canada). *Rouxiella badensis* subsp *acadiensis* SV-53 formally known as *Serratia vaccinii* bacterium was cultured as previously described (Martin and Matar, 2005). The juice was inoculated with a saturated culture of the bacterium corresponding to 2% of the total juice volume. After four days of fermentation, the transformed juice was sterilized by 0.22 um filtration. The total phenolic content was then measured by the Folin-Ciocalteau method using gallic acid as standard and hence expressed as µM Gallic Acid Equivalent (GAE). Blueberry and biotransformed blueberry juice have been partially characterized elsewhere (Martin and Matar, 2005; Matchett et al., 2006).

4.2. Metabolite Selection

An extensive literature survey was carried out to select the compounds previously reported in *Vaccinium* species using online databases KEGG, NIST, Scifinder, and Chemspider. This resulted in a wide variety of chemical classes, including anthocyanins, flavonoids, phenolic acids, phenolic glycosides, tocopherols, tocotrienols, terpenoids, and procyanidins. The abundance of metabolites was measured in fermented blueberry juice and compared with controls (non-fermented). Previously unreported compounds in *Vaccinium* were identified as the metabolites that were discriminant and changed in response or produced due to fermentation.

4.3. Sample Preparation

Standards (>95% purity) of blueberry compounds, purchased from Sigma (St Louis, MO, Canada) and Extrasynthese Inc. (Lyon, France), were prepared at three dilutions that bracket the metabolite response in the samples. Blueberry juice was diluted 10-fold by Milli-Q water in a 5 mL glass tube, sonicated for 5 min, incubated at room temperature for 5 min, pipetted into a 96 well plate for analysis, and 5 µL of juices were injected.

4.4. Ultra-Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (UPLC-MS-QTOF) Analysis

Analyses were undertaken on an Acquity UPLC coupled with XevoG2 QTOF system (Waters Inc., Milford, MT, USA). UPLC analyses were performed on a Waters Acquity System. Separations were performed on a BEH C18 1.7 µm, 2.1 ×100 mm column (part #186002352; serial #02113226415705, LANSET# General Purpose 2.1 × 100 BEH) connected with a VanGuard pre-column 2.1 x 5 mm with following characteristics: mobile phase A, water+0.1% formic acid, B-acetonitrile+0.1% formic acid (Fisher Optima LC-MS), flow rate 0.8 ml/min (back pressure at starting conditions=10,000 PSI), column temperature, 65°C, sample temperature 4°C. Mobile phase B composition was 0-1 min 2% isocratic, 1-4 min linear gradient 2-20%, 4-9 min 20-40%, 9-11 min 40-60%, 11-14 min 60-100%, 14-18 min 100% isocratic. A 5 µL PLUNO injection was through 10 uL loop followed by strong wash 200 µL (50% acetonitrile+50% water) and weak wash 600 µL (10% acetonitrile+90% water).

Optimized Q-TOF analysis conditions were as follows: MassLynx software, MSe ESI+; and ESI- modes, lock mass Leucine Enkephalin ^{12}C 556.2615, source temperature 150°C; desolvation temperature 500°C; cone gas (N2) flow 50 L/hr; desolvation gas (N2) flow 1200 L/hr; Mse conditions, mass range 100-1500 Daltons; Low energy F1 conditions (CE, 6V,

F2 CER 10-30V, cone voltage 20V, Scan time 1 sec); Instrument calibration; 50-1000 Da sodium formate.

4.5. Cell Culture

Murine 4T1 and human MDA-MB-231 cell lines were obtained from the American Type Cell Collection (ATCC; Chicago, IL, USA). Cells were grown in RPMI-1640 media containing FBS (10%, v/v) (Sigma-Aldrich, Oakville, ON, Canada), penicillin/streptomycin (0.05 mg/mL) (Fisher Scientific, Toronto, ON, Canada) at 37ºC in a humidified atmosphere with 5% CO_2. 4T1 and MDA-MB-231 were treated with 1- and 2-mM GAE of a polyphenolic mixture (PCA mix) containing PCA, gallic acid, and catechin for 24 h. Then, cells were collected to conduct relevant experiments.

4.6. In-vivo Breast Cancer Model

In this experiment, mice were maintained and treated in accordance with the guidelines of the Canadian Council on Animal Care. The protocol (Hse-3178) was approved by the Animal Care Committee of the University of Ottawa.

A total of 24 female Balb/c mice (Charles River, Montreal, QC) aged 6-8 weeks and weighed 18–20 g, were divided into two experimental groups (12 mice per group), including 1-control; receiving drinking water and 2- receiving a polyphenolic mixture (a protocatechuic acid-based mixture) dissolved in drinking water. The mixture consisted of PCA (70 mg/kg BW), gallic acid (35 mg/kg Bw), and catechin (1.5 mg/kg Bw). After 2 weeks of feeding, animals were subcutaneously injected with 4T1 cells (1400 cells /0.2 ml/mouse) into the abdominal mammary gland fat pad and nutritional intervention continued for three weeks. Then, mice were monitored for 3 weeks for tumor growth and health. At the end of the

experiment, mice were euthanized and the tumors and lungs were collected for further testing. All the tissues were digested using collagenase and the resulting cells were cultured either to form mammospheres or in a 6-thioguanine enriched medium to detect the lung metastasis.

4.7. Mammospheres Formation

4T1 and MDA-MB-231 cell lines were cultured in RPMI-1640 media until they reached 70% confluency. Then, adherent cells were detached using trypsin and single cells were counted using Countess (Invitrogen, Burlington, ON, Canada). The cells were then seeded in ultra-low attachment 96-well plates (Corning, Saint-Laurent, QC, Canada) at 103 cells/0.2 ml/well, in the presence/absence of the PCA mixture (1- or 2-mM GAE), in DMEM-F12 Thermo Fisher Scientific. ON, Canada), supplemented with 10 ng/ml EGF (Millipore Sigma, ON, Canada), 20 ng/ml bFGF (Millipore Sigma, ON, Canada), 5 µg/ml insulin, 1 mM sodium pyruvate (Millipore Sigma, ON, Canada), 0.5 µg/ml hydrocortisone (Millipore Sigma, ON, Canada), and penicillin/streptomycin (0.05 mg/mL). Formed spheroids were counted after 2 to 3 days by light microscopy.

For tumor tissues, approximately 0.05g of each tumor was minced and dissociated in RPMI-1640 media containing 300 U/ml collagenase (Millipore Sigma, ON, Canada), and 100 U/ml hyaluronidase (Millipore Sigma, ON, Canada) at 37ºC for 2h. Cells were sieved sequentially through 100 µm and 40 µm cell strainers (Fisher Scientific, Toronto, ON, Canada) to obtain a single cell suspension. Then, the single cells were plated at the same condition as above. Cells grown in these conditions form non-adherent spherical clusters of cells or mammospheres, which were counted after 4-7 days.

4.8. Lung Metastasis

Lung metastasis was assayed as previously described (Vuong et al., 2016). Briefly, lungs were dissociated in RPMI-1640 media containing 300 U/ml collagenase, at 37 °C for 15 min. After filtration through a 40 μm cell strainer, the cells were gathered and resuspended in RPMI-1640 medium supplemented with 10 % FBS, penicillin/streptomycin (0.05 mg/ml), and 60 μM 6-thioguanine (Millipore Sigma, ON, Canada). The cells were plated in 10-cm sterile culture dishes and incubated at 37 °C and 5 % CO2 for 14 days. Then, after fixation in methanol, cells were stained with 0.03 % methylene blue solution. All blue colonies were counted, one colony representing one clonogenic metastatic cell in the lungs (Pulaski and Ostrand-Rosenberg, 2001).

4-9. MicroRNAs Expression

The expression of miRNAs in breast tumors collected from mice was measured using qRT-PCR. Tumor samples' RNA was extracted using miRNeasy kit (Qiagen, Toronto, ON, Canada). Samples underwent a reverse transcription reaction to produce cDNA using individual probes. The cDNA was synthesized by Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Invitrogen, Burlington, ON, Canada). The expressions of miR-145 (TaqMan® MicroRNA Assays 002278, Applied Biosystems, Burlington, ON, Canada) and miR-210 (TaqMan® MicroRNA Assays 462444_mat, Applied Biosystems, Burlington, ON, Canada) were measured by RT-qPCR using Taqman primers (Applied Biosystems, Burlington, ON, Canada) and a FastStart Taq Polymerase (Roche, Mississauga, ON, Canada) in a CFX96 machine (Bio-Rad, Mississauga, ON, Canada). Gene expression was normalized to U6 small non-coding RNA as reference gene (Applied Biosystems, Burlington, ON, Canada).

4.10. Western Blot Analysis

4T1 and MDA-MB-231 cell lines were treated with different doses of the above-mentioned polyphenolic mixture (protocatechuic acid-based mixture) for 24 h. Cell lysates were extracted and run on a 4–12% acrylamide gel (Life Technologies, Burlington, ON, Canada), transferred to a PVDF membrane, probed with anti-FOXO1 (1:1000), anti-N-ras (1:1000), and anti-β-tubulin primary antibodies (1:1000) (Cell Signaling Tech. Inc., Danvers, MA, USA) and incubated at 4ºC overnight. The next day, blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10000) (Jackson Immuno Research Laboratories, West Grove, PA, USA) at room temperature for 1 hour. Then, bands were visualized by chemiluminescence technique using ECL substrate (Bio-Rad, Mississauga, ON, Canada). Bands were quantified by the Bio-Rad Quantity One software using β-tubulin as the loading control.

4.11. Statistical Analysis

GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA) was used to perform statistical analysis. Independent T-test was conducted to compare the means of two experimental groups and one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test was performed to compare the means of more than two groups. Statistical significance was set at $p \leq 0.05$. Data are reported as mean ± SEM.

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C.M. designed and supervised the work. All authors have read and agreed to the published version of the manuscript.

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Sample Availability: Samples of the polyphenolic mixture used in the current study are available on request from the corresponding author.

7 DISCUSSION

Chemoprevention, which involves the use of drugs, diet, or natural agents to decrease the risk of cancer development, prevent recurrence, and delay tumor progression, can significantly improve a patient's quality of life (Sporn and Suh, 2002). Interventions such as adopting a Mediterranean diet, enriched with polyphenol compounds, with its increased vegetable consumption and reduced meat intake have been shown to reduce the incidence of breast cancer by 20% (Turati et al., 2018). Furthermore, naturally occurring compounds, particularly polyphenols, have garnered considerable attention due to their ability to target key signaling pathways involved in CSCs. Ongoing research studies in laboratories and clinics aim to develop innovative phytochemical-based treatment options for cancer.

The investigation into the role of naturally occurring bioactive compounds in cancer chemoprevention and alleviation of chemotherapy-induced symptoms is an important area of research with the potential to improve cancer patients' lives and prognoses. However, the limited efficacy observed so far suggest a lack of understanding of the underlying mechanisms related to cell signalling pathways.

Polyphenols, a diverse group of naturally occurring compounds that contain hydroxyl groups on aromatic rings, are considered a good candidate with potential anticancer properties (Pandey and Rizvi, 2009). Previous studies have linked natural polyphenols to a range of effects, including antioxidant and anti-inflammatory activities (Lee et al., 2017; Lende et al., 2011; Li et al., 2011). Additionally, these compounds have shown the ability to influence molecular targets and signalling pathways associated with crucial cellular processes such as survival, proliferation, differentiation, migration, angiogenesis, hormone activities, detoxification enzymes, and immune responses (Sharma et al., 2018; Zhou et al., 2016).

7.1 PEBP ON BREAST CANCER

PEPB with its higher antioxidant and polyphenol concentration (Martin and Matar, 2005) has been shown to prevent neurodegeneration (Vuong et al., 2010), NOS damage (Vuong et al., 2006) and to have anti-diabetic and anti-obesity effects (Vuong et al., 2009). Considering the proven therapeutic effects of metformin on cancer, it was hypothesized that that PEPB might have a similar impact on cancer.

PEBP was first investigated to determine its effect on 3 breast cancer cell lines, 4T1, MDA-MB-231 and MFC-7. The ability of PEPB to decrease the proliferation (Figure 9) and reduce the mobility (Figure 10) of all the tested cell lines was reported. The mammospheres assay use cells that can form low-attached or floating spheres when grown in low attachment conditions. CSCS present the phenotype CD44+/CD24low. They are widely used for testing products and drugs for their effect on CSC-like cells (Cioce et al., 2010; Dontu et al., 2003a). The effect of PEBP on the number of mammospheres present in cell culture exposed to 150 μM GAE of PEBP compared to control and non fermented counterpart was significant (

Figure **11**).

In order to better decipher the role of PEBP on cancer stem cells, signaling pathways that are known to be involved in inflammation were studied. Because it is already known that PEBP can activate AMP-activated kinase (Vuong et al., 2007) and that AMPK can inhibit the activation of MAPK by phosphorylating the RAS kinase family (Yuan et al., 2020), the effect of PEBP on the regulation of the MAPK pathway ERK1/2 through AMPK was studied. The phosphorylation of multiple proteins in the STAT3/PI3K/Akt pathway and MAPK pathway were analyzed. The phosphorylation of STAT3, AKT, and PI3K was significantly decreased in all cells exposed to PEBP and in the case of STAT3 it was only observed in PEBP and not

NBJ (Figure 12). The tumour suppressor PTEN was increased in all cells by PEPB while for the control of the non-fermented preparation only had an effect in 4T1 cells (Figure 12). The activation STAT3 and the PI3K/AKT pathways has been linked to maintenance of CSCs (Xia and Xu, 2015) and the ability of PEBP to inhibit the activation of those pathways explain partially the effect of PEBP on the mammosphere numbers. In addition, , the inhibition of ERK1/2 in 4T1 and MCF7 by PEPB (Figure 13) further provides evidence that PEPB affects pathways implicated in maintaining CSC phenotype (Rybak et al., 2015). In MAPK pathways, ERK1/2 is the most relevant to breast cancer. Increased expression of ERK1/2 was recently reported as leading to endocrine resistance and breast cancer progression in an obesity-associated experimental model (Zhong et al., 2023).

All these effects in vitro were verified in vivo on a BALB/c model. Mice fed PEPB and NBJ mixed in their drinking water saw a reduction in tumour burden (Figure 14a and b) and a reduction of metastasis to the lung (Figure 14d) but only PEBP had any effect at the lowest concentration, showing a improved efficacy. The lower presence of cell presenting CSC-like phenotype in tumour from mice drinking PEBP can explain the reduction in tumour size and metastasis.

Altogether, PEBP is able to reduce the number of CSC present in a tumour by regulating pathway important in the maintenance of CSC like JAK/STAT3, PI3K/AKT and ERK1/2, thus reducing the ability of the tumour to proliferate and metastasis.

7.2 PEBP AND MICRORNAS

MicroRNAs (miRNAs) are a subset of small non-coding RNAs that play a crucial role in tumor development, drug resistance, and metastasis, acting either as tumor suppressors or

oncogenes. In breast cancer, the expression of specific miRNAs such as miR-145, miR-146a, and miR-34a has been found to be significantly downregulated, while miR-21 and miR-210 are upregulated (Iliopoulos et al., 2009b). Importantly, the expression of miRNAs can be modulated by dietary polyphenols, thereby potentially enhancing the effectiveness of conventional therapeutics (Bao et al., 2012b).

In our study, we observed significant changes in the expression levels of miR-145 and miR-210 in 4T1 cells following exposure to PEBP. Notably, the tumor suppressor miR-145, which is associated with AKT signaling in cancer, was found to be overexpressed (Table 1 and Figure 15), whereas it is typically underexpressed in highly metastatic breast cancer (Zou et al., 2012). miR-145 is also regulated by Akt in a p53-dependent manner. Suppression of PI3K activity substantially increases p53 levels and at the same time induces miR-145 (Sachdeva et al., 2012). PI3K was shown to be downregulated in our study with PEBP (Figure 12). In particular, p53 has been shown to upregulate the expression of several tumor suppressor miRNAs, including let-7, miR-34a, miR-145, miR-26, and miR-146a, all of which were observed to be regulated in our study (Table 1). Mechanistically, FoxO exerts its suppressive effect on c-Myc by upregulating miR-145, thereby establishing the FoxO/c-Myc/miR-145 axis as a significant barrier to tumor progression (Gan et al., 2010).

Hypoxia-related miR-210, the most consistently and robustly induced miRNA under hypoxia (W. Yang et al., 2012) was found to be highly down-regulated in our study (Table 1 and Figure 16). Hypoxia promotes genomic instability in tumor cells resulting in downstream phosphorylation of ERK1/2 (Kang et al., 2014), increased expression of IL6, and CSC signature genes such as Nanog, and Oct4 (Covello et al., 2006; Petruzzelli et al., 2014). High levels of miR-210 have been linked to invasion, and persistence of CSCs (Devlin et al., 2011).

Up-regulation of miR-210 in most solid tumors is negatively correlated with clinical outcomes (Huang and Zuo, 2014). Moreover, STAT3 was reported to be under the control of miR-210 (Fan et al., 2020). ERK1/2 as well as STAT3 were both downregulated after PEBP (Figure 12 and Figure 13).

Altogether, our findings reinforce the hypothesis that microRNAs could be regulated by natural chemopreventive agents such as PEBP, leading to the inhibition of CSCs, blockade of inflammation-related microRNAs, such as hypoxia-induced microRNAs that are oncogenic to CSCs development or activate oncogenic suppressor microRNAs such IL-6/STAT3 negative regulators and metastasis (Table 1).

miR-210 is induced by hypoxia and is strongly induced in multiple cancers (Radojicic et al., 2011). The induction of mir-210 protects the cancer cells from radiation (Grosso et al., 2013), and high levels of circulating miR-210 are associated with poor prognosis (Hong et al., 2012). miR-145 has been shown to target a large number of cancer-related proteins but the most important for breast cancer are c-Myc, RTKN, OCT4, and N-RAS (Cui et al., 2014). OCT4 is particularly interesting for its importance in cancer stem cells (Kim and Nam, 2011; Kumar et al., 2012).

7.3 POLYPHENOL DEGRADATION BY *ROUXIELLA BADENSIS* SUBSP. *ACADIENSIS*

A possible explanation for the better efficacy of PEPB compared to NBJ in vivo is the degradation of tannins by *R. badensis* subsp. *Acadiensis* to smaller and more easily absorbed polyphenols **(**Figure 21**)**. These smaller oligomers are known to be more easily absorbed, significantly impacting their bioavailability and physiological effects (Manach et al., 2005)**.** Polyphenols, in general have a poor bioavailability. For example, 75 percent of the resveratrol

consumed is absorbed in the intestine but is quickly excreted in urine (Walle et al., 2004). The rest is either transformed in the intestine or by the liver to resveratrol glucuronides and sulphates (Kuhnle et al., 2000). The low bioavailability/high bioactivity paradox take into consideration this element (Di Lorenzo et al., 2021). The presence of certain metabolites suggest interaction with bacteria in the intestine (Walle, 2011). Thus, there is mounting evidence that polyphenols act as a prebiotic for beneficial bacteria (Plamada and Vodnar, 2021).

An UPLC-MS-QTOF analysis of PEBP revealed that fermentation promotes the emergence of novel peaks representing oligomeric phenols that may synergistically target specific pathways (Figure 21). Furthermore, our research has demonstrated the bacterium's capability to release quercetin from its precursor rutin (Figure 22). Quercetin is a significant flavonol with well-known anti-cancer properties. In a study conducted by Wei et al.(2011), quercetin treatment demonstrated a significant reduction in the number of breast cancer stem cells (including the ALDH+ population), inhibited cell migration, and suppressed mammosphere formation. Additionally, quercetin induces mitochondrial apoptotic-dependent growth inhibition by blocking the phosphoinositide 3-kinase (PI3K)-Akt signaling pathway in gastric cancer stem cells (Shen et al., 2016). Interestingly, a recent study suggested that quercetin's anti-cancer activity may be exerted through the blockade of the Toll-like receptor 4 (TLR4)-mediated signaling pathway (Han et al., 2016).

Small oligomers of polyphenols might then exert their activity as prebiotic or a natural ligand for Toll-like receptors (TLRs), involved in immune regulation. In fact, there is a growing body of evidence to support the notion that some polyphenolic ingredients act as prebiotic. For example, Quercetin has proven to positively influence microbiota (Lan et al.,

2021). Quercetin is an important flavonol with known anti-inflammatory activities (Lin et al., 2016). Interestingly, Quercetin might exert its anti-inflammatory activity via blockade of the TLR4-mediated signaling pathway (T. Li et al., 2019). In addition, quercetin, increased anti-inflammatory miR-200b and miR-145 in pancreatic and ovarian cancer stem cells, respectively (Nwaeburu et al., 2017; Zhou et al., 2015). Research has been focusing on a particular class of flavonoids known for their beneficial effects, including quercetin, rutin, catechins and protocatechuic acid (Serra et al., 2012). The protective effects of flavonoids are not only due to intact flavonoids, because intact flavonoids cannot be absorbed in their native form, but also or exclusively due to other bioactive substances formed after microbial degradation by gut microbiota (Manach et al., 2005). PCA, a metabolite of quercetin, has a remarkable antiatherogenic effect. PCA, as the gut microbiota metabolite of Cy-3-G, exerts its antiatherogenic effect partially through miRNA-10b (Wang et al., 2012). Thus, phenolic metabolites of flavonoids play a preventive and therapeutic role in disease.

We have identified a number of those smaller peaks (Table 2) and many of them have known beneficial properties against cancer. We selected 3 compounds for the abundance and known effect on cancer, protocatechuic acid, gallic acid and catechin. This mix was able to significantly decrease the formation of mammospheres in the 4T1 cell line (Figure 23A). However, only higher concentrations (2 mM GAE) significantly inhibited mammospheres formation in MDA-MB-231 cells (Figure 23B). This might be caused by a slightly less effective effect on mir-210 by the PCA mixture (Figure 26) because the proteins affected by mir-145 were still significantly changed by the mixture (Figure 25). In contrast to PCA and gallic acid, which are categorized as benzoic acids, PEPB also contains acids from different families, including cinnamic acids derivative like chlorogenic acid (Table 2). Chlorogenic acid

has been reported to offer health benefits similar to PEPB, including anti-diabetic and anti-carcinogenic properties (Tajik et al., 2017). In addition, chlorogenic acid can modulate the gut microbiota (Z. Wang et al., 2019) and is degraded by the microbiome and produce a lot of metabolites that could have an effect on health (Gonthier et al., 2003).

In an in vivo model, feeding mice with the PCA mixture helped reduce the number of cancer stem cells present in the tumour (Figure 27b) but was slightly less effective than the PEBP (Figure 15d).

The project falls in perfect alignment with increased worldwide interest in "Medical Foods" or nutraceuticals as adjunct therapies to help prevent and improve survivorships for breast cancer patients. Those patients are among the highest users of integrative medicine in conjunction with conventional oncology care (Greenlee et al., 2014). The scientific importance of this project is 2-fold; 1) shedding the light of underlining mechanisms for protective effects of fermented products that contain polyphenols, and 2) promote healthy diets. The findings may help to propose a more optimal diet plan that not only reduces inflammation but also acts on decreasing the predisposition to cancer. In addition, an integrated approach to considering the evidence shows that most diets that are protective against cancer are rich in foods of plant origin, thus consolidating the evidence of the long-term beneficial effects of balanced diet in prevention of cancer.

8 LIMITATIONS

Our research into the influence of PEPB on the cancer stem cell population has provided valuable insights into its potential for breast cancer prevention. However, the techniques employed in this study come with inherent limitations.

One significant limitation of our research lies in the inherent nature of mammosphere assays as an indirect measure of the stem cell population. While these assays are commonly employed for studying cancer stem cells, they select cells capable of self-renewal and growth in a low-attachment environment. This selection may introduce a bias since it might not fully represent cancer stem cells and tumour initiating cells, particularly those reliant on specific microenvironments and interactions with other cells present in vivo. This lack of full representativeness could potentially introduce biases into our findings, as the selected cells may not accurately reflect the overall heterogeneity of the cancer stem cell population. Furthermore, high cell densities can lead to cellular aggregation in mammosphere assays, which can adversely affect the accuracy of the assay. Cellular aggregation hinders the formation of distinct mammospheres and complicates the quantification and characterization of the stem cell population.

Another limitation in our study is the use of Western Blot analysis. While it is valuable for detecting and quantifying specific proteins, it's important to note that Western Blot analysis provides only semi-quantitative data. It offers relative, rather than absolute, quantification of protein levels. Despite adhering to established guidelines for Western Blot analysis (Taylor et al., 2013), this semi-quantitative nature can introduce variability and uncertainty into the measurement of protein expression levels.

Our research utilized a 4T1 mouse model to study cancer progression and cancer stem cells. Mouse models are invaluable in cancer research, but they have limitations. In our case, the mouse model exhibited rapid tumor growth and a high metastatic rate. However, the primary tumor often grows so rapidly that it significantly impacts the animals' well-being before metastases can be observed. While some techniques, such as primary tumor removal after implantation, can be employed, they are invasive and require specialized expertise. Furthermore, despite the model's immunocompetence, mouse immune responses differ from those in humans. These disparities can influence studies in cancer immunology and immunotherapy. Consequently, findings from mouse models may not always directly translate to human applications.

9 FUTURE DIRECTIONS

The fermentation of blueberry by *Rouxiella badensis* subsp. *acadiensis* favourably increase the release of small oligomer of polyphenols However, many other metabolites-nonrelated to the degradation of large polyphenol compounds released during the fermentation may also present an important potential in chemoprevention of breast cancer. For example, the anti-fungal Procyanidin B2 has shown promise in the prevention of cancer and may function by inhibiting the AKT/mTOR signaling pathway (Y. Li et al., 2021). Moreover, one could argue that the inclusion of quercetin in the mixture of polyphenol compounds may lead to better potentiation of polyphenol effects and improvement in the effectiveness. Quercetin itself has been shown to inhibit PI3K in hepatocellular carcinoma (Maurya and Vinayak, 2015) and could potentially reduce CSC through its effect on AKT/mTOR (Pratheeshkumar et al., 2012) and STAT3 (Yu et al., 2017).

While identifying the active compounds in PEBP is important, it does not diminish the importance of further testing PEBP itself. The complexity of PEBP makes it impossible to fully replicate, and its efficacy in the mouse model demonstrated the need to continue testing it in various cancer types. Colon cancer would be an interesting area of research towards which the research could be further targeted. It is important to note, that fermentation converts large polyphenols to smaller oligomers. Small oligomers are known to be better absorbed, greatly affecting bioavailability and consequently physiological effects (Sahakyan et al., 2020). . Along this line, these small oligomers such as quercetin might impact favorably on microbiota and exert their anti-inflammatory activity via blockade of the TLR4-mediated signaling pathway. Toll-like receptors (TLRs) are a key family of microbial sensors involved in inflammation. Polyphenols, such as quercetin, are among the numerous regulatory molecules

that are able to fine-tune the TLR-signaling pathways. Many of these molecules have been reported to be responsible for playing a role in inflammation and in regulating microbiota by preventing dysbiosis-induced TLR4-mediated inflammation. It is important to further study of role of polyphenols in inhibiting cancer cell growth by investigating how they block inflammation-dependent pathways and examine their impact on microbiota. Polyphenols contribute to intestinal homeostasis in the gut-breast axis. Any signaling initiated at the gut level can be propagated systematically through the mucosal associated tissue that includes the mammary glands. Ongoing research in our lab are now investigating this triad of microbiota-miRNAs-immune signaling and cancer stem cells.

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