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Exploring The Link Between Dietary Fiber, The Gut Microbiota And Estrogen Metabolism Among Women With Breast Cancer

Ayse G. Zengul
University of Alabama at Birmingham

EXPLORING THE LINK BETWEEN DIETARY FIBER, THE GUT MICROBIOTA AND ESTROGEN METABOLISM AMONG WOMEN WITH BREAST CANCER

by

AYSE GUL ZENGUL

WENDY DEMARK-WAHNEFRIED, COMMITTEE CHAIR
ANDREW FRUGE
BRENDA BERTRAND
STEPHEN BARNES

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AYSE G. ZENGUL

NUTRITION SCIENCES

ABSTRACT

Breast cancer continues to be a very prevalent disease and impacts over 260,000 new patients per year in the United States. The gut microbiota composition may affect breast cancer risk by modulating various hormonal metabolites including endogenous estrogens. Dietary factors impact gut microbial ecology and influence the regulation of estrogen metabolism. Current evidence supports the potential role of dietary fiber in breast cancer prevention and its possible modulating influence on serum estrogen levels through the gut microbiota associated with β-glucuronidase activity. However, this mechanism is not clearly understood. In this study, we aimed to explore the associations between dietary fiber, the gut microbiota that are linked with β-glucuronidase activity, and circulating estrogen levels. We hypothesized that higher dietary fiber consumption will be associated with a lower abundance of intestinal microbiota that promotes β-glucuronidase enzyme activity and lower levels of circulating estrogen. This study included 29 newly-diagnosed (stage 0-II), post-menopausal breast cancer patients. For statistical analyses we integrated three data sets: 1) dietary recall data, 2) Illumina MiSeq generated microbiota relative abundance, and 3) HPLC-mass spectrometry-derived estradiol and estrone levels. We performed Spearman's and partial correlations controlling for body mass index and age to assess potential associations. The results suggested the following: (1) total dietary fiber is inversely associated with *Clostridium*

hathewayi (r= -0.419; p=0.024); (2) soluble fiber is inversely associated with *Clostridium* (r=-0.11; p=0.02); and (3) insoluble fiber is positively associated with *Bacteroides uniformis sp.* (r=0.382; p=0.041). Also, serum 17β-estradiol and estrone levels are not correlated with species/genera or dietary fiber, though there is a trend toward an inverse association between soluble fiber and estradiol levels (r= -0.30; p=0.12). These results provide important insights about the underlying mechanisms of the interaction between fiber and estrogen metabolism regarding the breast cancer risk. Further studies are needed to better understand the complex dynamics between these factors.

Keywords: breast cancer, dietary fiber, microbiota, estrogen

DEDICATION

Dedicated to my dear husband Ferhat, and children, Yusuf and Meryem.

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AICR	American Institute for Cancer Research
BMI	Body Mass Index
CNS	Central Nervous System
COMT	Catechol O-Methyltransferase
ER/PR	Estrogen Receptor/ Progesterone Receptor
GSH	Glutathione
QATP1	Organic-anion-transporting polypeptide
SCFA	Short-chain Fatty Acids
UGT	UDP-glucuronosyltransferase
WCRF	World Cancer Research Fund
SHBG	Sex Hormone Binding Globulin

INTRODUCTION

Breast cancer is the most common form of cancer among women residing in industrialized countries. Approximately one in eight American women will develop invasive breast cancer during the course of their lifetime. In the United States, more than 268,000 new cases of breast cancer will be diagnosed, and about 41,760 women will die from this disease in 2019. Even though breast cancer death rates have been decreasing since 1989 as a result of treatment advances and increased awareness, breast cancer continues to be a leading cause of death among women[1]. Therefore, the prevention of breast cancer is of a paramount importance and a primary focus of cancer research.

Healthy lifestyle behaviors are recommended by the World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR) to reduce cancer risk, including breast cancer, and overall mortality. AICR/WCRF recommendations for cancer prevention include being in a healthy weight range and physically active, eating a nutrient-rich diet, limiting consumption of processed foods, red meat, sugar-sweetened drinks/foods, and alcoholic beverages. AICR/WCRF also advises breastfeeding for mothers (Table 1)[2,3]. Among the recommendations for cancer prevention, nutrient-rich diets usually rely on unrefined plant-based foods such as vegetables, fruits, and whole grains[4,5]. Besides being good sources of minerals and vitamins, plant-based foods contain chemical compounds called phytochemicals, beneficial substances in protecting cells from damage that can lead to malignancy, including breast cancer [2,6].

Table 1. AICR/WCRF Recommendations for Cancer Prevention[2,3]

Be a healthy weight	Keep your weight within the healthy range and avoid weight gain in adult life
Be physically active	Be physically active as part of everyday life – walk more and sit less
Eat whole grains, vegetables, fruit & beans	Make whole grains, vegetables, fruit and pulses (legumes) such as beans and lentils a major part of your usual daily diet
Limit 'fast foods'	Limiting processed foods high in fat, starches or sugars helps control calorie intake
Limit red and processed meat	Eat no more than moderate amounts of red meat and little, if any, processed meat
Limit sugar-sweetened drinks	Drink mostly water and unsweetened drinks
Limit alcohol consumption	For cancer prevention, it's best not to drink alcohol
Do not rely on supplements	Aim to meet nutritional needs through diet alone
For mothers: breastfeed your baby, if you can	Breastfeeding is good for both mother and baby
After a cancer diagnosis follow our recommendations, if you can	Check with your health professional what is right for you

A plant-based dietary pattern is promoted by health authorities in breast cancer research because it is strongly associated with a lower breast cancer risk, particularly for ER-PR- tumors[7-10]. Diets rich in plant foods would emphasize whole grains, vegetables, legumes, fruits, seeds, and nuts and dissuade from high meat consumption[11]. Plant foods also tend to be good sources of dietary fiber. Recommendations for fiber intake are

related to gender, age, and energy intake[12]. For adequate intake, 14 g/1000 kcal of dietary fiber consumption is generally recommended [13].

There are two main categories of fiber: soluble and insoluble. Both types have numerous beneficial physiological effects[14]. Fiber may protect against cancer by improving immune function[15], blood glucose control[16], achieving healthy weight[17], and maintaining bowel health[18]. By interacting directly with gut microbes, fiber leads to the production of key metabolites and impacts gut microbial ecology, host physiology, and health. The underlying mechanisms may be understood better by examining the interaction between fiber and the gut microbiota.

Dietary Fiber and Gut Microbiota

Dietary fiber is defined as the portion of plant foods including all carbohydrate polymers with three or more monomeric units that are resistant to digestion by human digestive enzymes[19,20]. Dietary fibers include polysaccharides, lignin, oligosaccharides (e.g., inulin), and resistant starches[13,19,21]. Dietary fiber mainly consists of non-starch polysaccharides which supply 20%-45% of the dry matter in the colon[12]. Fiber consumption influences the intestinal microbiome by altering colony size, species composition, and bacterial fermentation[22]. Fermentability, viscosity, and solubility are physicochemical characteristics of fibers that play a role in maintaining a healthy gut microbiota population. Even though some insoluble fibers can be well fermented, soluble fibers are more likely to be fermented and have a greater viscosity compared to insoluble fibers. However, certain soluble fibers such as acacia gum and partially hydrolyzed guar

gum are not viscous[23]. β-glucan and pectins are some examples of the highly fermentable fibers that also have high solubility and viscosity. These fibers are contained in whole grains such as barley (β-glucan), oats, and fruits, e.g., apples (pectin)[24-26]. Soluble fibers that are non-viscous and readily fermented by intestinal microbiota include resistant maltodextrins, inulin, resistant starch, soluble corn fiber, and polydextrose[27-29]. Inulin-type fructans are found in various plant foods such as agave, asparagus, artichokes, bananas, garlic, chicory root, leeks, onions and wheat[25,30].

In the human large intestine, the microbiota generate energy through fermentation of carbohydrates that are not digested in the small intestine. By using carbohydrate hydrolyzing enzymes, colonic bacteria ferment non-digestible carbohydrates including resistant starch, non-starch polysaccharides (i.e., hemicellulose, celluloses, gums, and pectin), non-digestible oligosaccharides as well as sugar alcohols and, as a result, produce short-chain fatty acids (SCFAs) -mainly butyrate, acetate, and propionate, lactate, and gasses such as carbon dioxide, hydrogen, and methane. The amount of carbohydrate determines the range of these fermented products[23]. SCFAs are the major products of the fermentation process and particularly important since they are the preferred fuel of the colonic epithelial cells. SCFAs are mainly found in the proximal colon[31]. They are either used locally by enterocytes or transported across the gut epithelium into the blood compartment and are taken up by organs -such as liver- where they act as signaling molecules or substrates and affect glucose, lipid and cholesterol metabolism in numerous tissues[32,33]. Two major SCFA signaling mechanisms are: 1- activation of G-protein-coupled receptors (GPCRs) and 2- inhibition of histone deacetylases (HDACs)[31]. SCFAs

are produced by certain bacteria including *Bacteroides, Propionibacterium, Bifidobacterium, Eubacterium, Clostridium, Lactobacillus, Prevotella,* and *Roseburia*[34,35].

SCFAs are vital for human health; they have been shown to influence chemotaxis (cell movement in response to a chemical stimulus) and phagocytosis (a process by which phagocytic cells ingest large particles into phagosomes) and alter cell proliferation (an increase of the number of cells). They play a significant role in the immune function by having anti-inflammatory and antimicrobial effects[31]. They also affect the systemic regulation of macronutrient metabolism, secretion of hormones and energy homeostasis[19,36]. These metabolites appear to reduce the precursors of some diseases such as metabolic syndrome, bowel disease and colon cancer in which SCFAs regulate expression of cell cycle-regulating proteins and stimulate apoptosis [19,33,37,38]. According to Thirunavukkarasan et al., SCFAs also play a role in breast cancer; the activation of cognate receptors by SCFAs inhibits metastasis by driving breast cancer cells toward a non-invasive phenotype[39].

SCFAs, as the end products of dietary fiber fermentation, also improve mucosal barrier integrity by increasing mucus secretion. The mucus layer creates a barrier between potentially harmful luminal bacteria and the epithelial cells in the colon and prevents them from causing damage to tissue[40,41]. SCFAs, such as butyrate, are absorbed by the colon and distal ileum and act as key sources of energy for epithelial cells. Butyrate has been shown to stimulate mucosal restitution, promote differentiation,

prevent inflammation and tumor growth and inhibit the development of colorectal cancer [42,43]. To maintain mucosal barrier integrity and to prevent microbial invasion of tissues, having a diverse and large population of beneficial gut microbiota is critical[12]. Fiber- as an important energy source of fermentation and SCFA production- plays a significant role in that mechanism.

Moreover, fiber components that stimulate fermentation lead to bacterial proliferation and consequently increase the fecal mass. For every 100 g of fermented carbohydrate, approximately 30 g of bacteria are produced[23]. Fiber intake is necessary for normal laxation. Because of its physical presence and ability to hold water, fiber - especially incompletely fermented insoluble non-starch polysaccharides such as cellulose- increases stool weight. Larger, heavier and softer stools lessen intracolonic pressure, reduce toxins, shorten transit time and increase the ease of defecation which may help to alleviate constipation[12,23]. These features of fiber are also linked to decreased risk of colorectal cancer [43].

The gut microbiota produces various hormone-like metabolites that are released in the bloodstream to be used at distal sites of the body[44]. For example, as mentioned above, SCFAs act as signaling molecules and influence the production of various hormones. After SCFAs are secreted into the gut lumen, they are transported across the epithelial barrier via the bloodstream and are taken up by effector organs (e.g., liver and brain). More specifically, SCFAs are carried by monocarboxylate transporters, which can be abundantly found at the blood-brain barrier where they may enter the central nervous

system (CNS)[34,45]. In the CNS, they can act as neurotransmitters such as γ-aminobutyric acid (GABA) -an inhibitory transmitter which is produced by several lactobacilli-[46], and monoamines, such as dopamine and noradrenaline[47]. Inulin, a soluble fermentable fiber, increases the production of SCFAs and modulates enteroendocrine serotonin secretion and influences the production of glucagon-like peptide-1 (GLP-1), peptide YY, leptin and ghrelin hormones[48,49]. Moreover, the composition of the microbiota modulates plasma concentrations and the nature of tryptophan catabolites which are precursors to serotonin and essential amino acids[34,50]. In brief, the alteration in microbiota composition through diet is capable of regulating and modifying various hormonal metabolites including endogenous estrogens. A certain set of bacteria involved with estrogen metabolism is called the estrobolome[51].

Estrobolome: The Interplay between Gut Microbiome and Estrogen

The estrobolome is defined by Plottel et al. as "the aggregate of enteric bacterial genes whose products are capable of metabolizing estrogens"[51]. As a steroid hormone, estrogen originates from C27 cholesterol which is required for the steroidogenesis. Cholesterol is then converted to pregnenolone (C21), androgen (C19) and estrogen (C18)[52]. During the ovarian steroidogenesis that is regulated by the steroidogenic acute regulatory protein (StAR), cholesterol is moved into the mitochondrion where it is converted to pregnenolone, a precursor for steroid hormones[53]. This reaction is catalyzed by the mitochondrial side-chain cleavage enzyme complex. Pregnenolone is converted to progesterone (androstenedione) through 17α-hydroxypregnenolone by 17α-hydroxylase and then is transformed into other estrogens or androgens[52,54]. Estrogens are aromatic molecules and consist of a "benzene ring, a phenolic hydroxyl group at C3, and a

hydroxyl group (17β-estradiol) or a ketone group (estrone) at C17"[52]. The main endogenous estrogens are 17β-estradiol (E2), estrone (E1) and estriol (E3)[55]. Estriol is abundant in urine and usually predominant in pregnant women. Estradiol, on the other hand, is mainly secreted by ovarian granulosa cells and regulated via follicle-stimulating hormone (FSH) and it is the predominant estrogen form in nonpregnant women prior to menopause. Estrone is predominant only after menopause and is reversibly converted to estradiol through 17β-hydroxysteroid dehydrogenase enzyme activity. Testosterone is also converted in estradiol in the peripheral tissues via aromatase enzyme (CYP19) [52,56] which is encoded by the CYP19A1 gene. As a rate-limiting enzyme, aromatase takes part in catalyzing the conversion of androgens into estrogens[57]. Blocking the activity of the aromatase enzyme has an instrumental role in the treatment of estrogen-dependent diseases including endometrial cancer, endometriosis, and breast cancer[52].

Estrogen metabolism begins with oxidation/hydroxylation which is mediated by the NADPH-dependent cytochrome P450 enzymes (CYP1A1, CYP1B1, and CYP1A2)[52,57]. CYP enzymes are mostly expressed in the liver; consequently, estrogen metabolism mainly occurs in this organ. For example, CYP1A2 and CYP3A4 catalyze the metabolism of estradiol in the liver. However, some reactions also occur at the other tissues; estradiol is catalyzed by CYP1A1 in extrahepatic tissues, and by CYP1B1 in mammary, uterus, and ovary tissues. Therefore, changes in the level of CYP isoforms would alter estrogen activity and affect the liver and target tissues[57,58]. In the second phase of estrogen metabolism, the hydroxylated estrogens are inactivated through conjugation reactions, including methylation, sulfation, conjugation with GSH or

glucuronidation to be excreted via the liver or kidneys. Conjugation, as a detoxification reaction, can convert hormones into water-soluble compounds to be excreted in urine or feces[52,59].

Methylation of Estrogens

Estrogen methylation is catalyzed by catechol O-methyltransferase (COMT). COMT belongs to the methyltransferase enzyme family and takes part in transferring methyl groups (from *S*-adenosyl methionine) to hydroxyl groups of catechol substrates, such as catechol estrogens. Catechol estrogens are usually rapidly methylated (2- and 4-*O*-methylethers) via COMT and excreted[57,60]. Methylation has an important role in the prevention of cytotoxic and genotoxic damage due to products of the oxidative reactions of catechols[60]. COMT catalyzes the methylation activity of catechol estrogens to methoxy estrogens; consequently, it reduces the catechol estrogens which can be transformed into estrogen quinones[61].

Sulfation of Estrogens

Estrogen sulfation is catalyzed via cytosolic sulfotransferase (SULT) enzymes which convert SO3 from 3′-phosphoadenosine-5′-phosphosulfate -a cofactor- to phenolic acceptor groups. Sulfate conjugation of 17β-estradiol and estrone occurs at the steroidal A ring (3-phenolic group)[62]. Sulfation activity in estrogen metabolism may take place in various tissues, such as liver, kidney, small intestine, placenta, adrenal gland, breast, and uterus[57,60,63].

Conjugation with Glutathione (GSH)

As mentioned earlier, oxidation/hydroxylation is mediated by the NADPH-dependent cytochrome P450 enzymes. Cytochrome P450 enzyme promotes the conversion of the oxidative metabolism of 17β-estradiol (E_2) to catechol estrogens (2-OHE_2 and 4-OHE_2) and quinones (E_2-2,3-Q and E_2-3,4-Q), a highly reactive estrogen. These quinones can be conjugated with GSH either non-enzymatically or catalyzed via enzyme glutathione S-transferase P1 (GSTP1). Catechol estrogen quinones are then converted to mercapturic acid metabolites that can be readily excreted from the cell. This reaction is believed to contribute to the detoxification and prevention of the damage to DNA by catechol estrogen quinones[60,61,64].

Glucuronidation

After sulfates, glucuronides are the most abundant estrogen conjugates in circulation. Glucuronidation of estrone (E1) and 17β-estradiol (E2) and their derivatives 2/4-OHCE and 2/4-methoxy-catecholestrogen (MeOCE) in humans is catalyzed by microsomal UDP-glucuronosyltransferase (UGT) enzymes including 1A1, 1A3, 1A4, 1A8, 1A9, and 1A10[60]. These enzymes are expressed in the liver but also in estrogen target tissues such as biliary epithelium, ovary, kidney, prostate, gut, and breast. UGTs are type-I transmembrane glycoproteins that are mainly present in the endoplasmic reticulum. E1 and E2, as steroid hormones, are hydrophobic. UGT enzymes with a glucuronic acid group which is derived from uridine diphosphate-glucuronic acid (UDPGA) conjugate these hormones and turns them into more polar, water-soluble and less toxic substrates for various membrane transporters; thus, they can be more easily

eliminated from the body via the bile and urine[65]. The biliary and renal anion transport systems (e.g., Oatp1) have a high affinity for glucuronide estrogen conjugates; they enable secretion into bile and urine by recognizing the glucuronide[60,66]. The transfer of glucuronic acid moiety to the functional group can occur at either the 3 or 17β hydroxyl group which is the predominant site for 17β-estradiol. The change in the structure of the compounds also alters parent molecules' biological activity and prevents them from binding the receptors. Consequently, the parent estrogens are inactivated to be excreted out of the body[60,66,67]. A study in which radioactively labeled C14-estradiol and C14-estrone and estriol have been injected into 22 women revealed that around 65% of estradiol, 48% of estrone, and 23% of estriol are recovered in the bile[68]. Around 10% to 15% of intravenously injected radioactively labeled estradiol, estrone, and estriol are expressed in conjugated form in stool[69,70]. This indicates that a substantial proportion of endogenous estrogens are reabsorbed in the circulation[55]. One of the reasons for this reabsorption is that gut microbiota can influence the estrobolome. Some products of various bacterial genes are capable of metabolizing endogenous estrogens through deconjugation and conjugation reactions. The β-glucuronidase enzyme, for example, is produced by specific bacterial species and involved in deconjugation of inactivated estrogens[51,71].

Intestinal β-glucuronidase Activity in Estrogen Metabolism

ß-glucuronidase activity in the gut can lead hepatically conjugated estrogens to be reabsorbed and appear again in the circulation. In other words, ß-glucuronidase is capable of hydrolyzing estrogen molecules that had undergone hepatic glucuronidation and

biliary excretion into the gastrointestinal (GI) tract. As a lysosomal enzyme, β-Glucuronidase is a "332-kD glycosyl hydrolase that hydrolyzes β-glucuronic acid from the non-reducing termini of GAGs in the lysosome"[72,73]. Like most of the lysosomal proteins, each monomer of β-glucuronidases is synthesized on a membrane-bound ribosome and then targeted and translocated into the endoplasmic reticulum where they undergo post-translational modifications. The polypeptide chain of β-glucuronidase enzyme is co-translationally glycosylated and transported to microsomal lumen[73-75]. According to Gloux et al., 19 positive metagenomic clones for β-glucuronidase were identified. Among these clones, only H11G11-BG exhibited a strong β-D-glucuronidase activity. Compared to known β-D-glucuronidases , H11G11-BG has "distant amino acid sequence homologies and an additional C terminus domain"[76]. β-glucuronidase has an essential role in modifying the extracellular matrix components regarding physiological and inflammatory indices[73]. Deficiency of β-glucuronidase can cause mucopolysaccharide storage disease (MPSVII or Sly syndrome) which leads to cellular damage and organ dysfunction due to partial degradation of chondroitin sulfate, heparan sulfate, and dermatan sulfate, and accumulation in lysosomes of related tissues. This condition may cause progressive disability, mental retardation, dysmorphism, organ dysfunction, behavioral deficits, and reduced life span[73,77,78].

Moreover, β-glucuronidase enzymes, acting on β-glucuronides of steroid hormones, promotes recirculation of vitamin D, bilirubin, thyroid, and estrogen hormones. As a glycosyl hydrolases, β-glucuronidase is capable of cleaving off the glucuronic acid as glycone (sugar) moiety due to change in its local pH[73,79]. Considering

that toxic substances are bound to glucuronic acid to increase the solubility to be excreted, breaking this bond through hydrolyzation of conjugated estrogens can release the parent aglycone forms within the enterohepatic cycle and prevents the removal of circulating hormones from the body[76,80]. This enzyme also hydrolyzes bilirubin glucuronide into glucuronic acid and free bilirubin. As a result of deconjugation, β-glucuronidase enzyme activity can release various potential hormones, toxins, and various drugs in the circulation[55,73].

The β-glucuronidase gene is expressed in most of the body fluids and tissues. According to Kwa et al., in humans, there are 15 bacterial genera that colonize the colonic tract, which encode ß-glucuronidase enzyme[55,81]. Several studies also have demonstrated that ß-glucuronidase activity is expressed in various bacteria, including *Bacteroides* spp., *Bifidobacterium* spp., in strains belonging to *R. hominis* and *R. intestinalis*, Lachnospiraceae (cluster XIVa), *Clostridium leptum* group (cluster IV) and in the bacterial phyla *Firmicutes* and *Bacteroidetes*[55,76,82,83]. The evidence indicates that ß-glucuronidase enzyme activity is controlled by diet and bacterial population density. In healthy human subjects who consume foods high in protein or fat, increased fecal ß-glucuronidase activity has been reported[84,85]; on the other hand, a fiber-rich diet is associated with the decreased activity[55,82]. Exploring the capacity of fiber-rich plant food diets in estrobolome metabolism is crucial to understand the effect of β-glucuronidase activity on estrogen metabolism and modulation of risk factors for breast cancer.

The Influence of Dietary Fiber on Estrobolome via Inhibition of ß-glucuronidase Activity

Studies suggest that plant-based diets lead to higher fecal weight due to the decreased ß-glucuronidase activity of fecal bacteria and increased fecal excretion of endogenous estrogens[86,87]. Considering the high consumption of dietary fiber among vegetarians, examining the influence of soluble and insoluble fiber on ß-glucuronidase activity is important to better understand estrogen metabolism. It is known that D-glucaric acids reduce the circulating estrogen levels, possibly due to glucuronidation and increased excretion. *In vivo*, D-glucaro-1,4-lactone, formed from D-glucaric acid, can lead to an increase in detoxification of carcinogens. It can inhibit chemically-induced carcinogenesis in rats, partly by inhibiting β-glucuronidase activity and reducing estrogen levels in circulation[87,88]. Another study that was also conducted on rats demonstrated that the dietary fiber isolated from black gram or coconut has a potential role in reducing the activity of the intestinal and fecal beta-glucuronidase enzyme[89].

Besides animal studies, human trials have shown that people who consume higher levels of dietary fiber daily had decreased β-glucuronidase activity and higher fecal excretion of estrogens[87]. Specifically, several botanical vegetables and fruit groups including Rosaceae, Cucurbitaceae, and Leguminosae were inversely correlated with serum β-glucuronidase activity[87]. Also, diets containing oat and wheat bran have been associated with a significant reduction in the activities of β–glucuronidase and serum estrone and estradiol[90 91]. Moreover, Adlercreutz found positive correlations between grain fiber and total fiber, and fecal estrone and estradiol excretion in postmenopausal women. Fat consumption, on the other hand, was negatively associated with fecal

excretion of estrogens. Consequently, he suggested that the dietary fat/fiber ratio can influence the enterohepatic circulation of steroids[92]. Further, the results in the study of Lampe et al. indicate that β-glucuronidase activity is inversely associated with the consumption of fruit, plant protein, and dietary fiber such as whole grains and/or nuts[87]. The preponderance of evidence suggests that fiber-rich plant-based diets can be good sources of β-glucuronidase inhibitors, such as D-glucaric acid. In short, high fiber intake reduces reabsorption of estrogens in the colon by decreasing β-glucuronidase activity in the feces. In addition, by binding to estrogens in the intestine, fiber increases their fecal excretion. Further, fiber's influence on estrogen metabolism subsequently impacts other menstrual hormones such as progesterone synthesis and episodic gonadotropin secretion which regulates hormonal fluctuations during the menstrual cycle[93]. Considering that elevated levels of circulating estrogens may lead to diseases such as colon and breast cancer, the relationship between fiber and estrobolome is increasingly gaining in importance.

Estrogen and Breast Cancer

High circulating estrogen levels are associated with an enhanced incidence of a variety of sex-hormone driven cancers including cervical[94], prostate[95], endometrial[96], ovarian[97], and breast cancer[98]. Microbiome constituents are likely to be altered in many of these cancers[51]. This alteration could play a role in regulating the estrobolome and promoting carcinogenesis by influencing various hallmarks of malignancy such as cell proliferation and apoptosis[99]. Deconjugation of estrogens through β-glucuronidase activity enabling unbound estrogens to bind to estrogen receptors in which cell

proliferation is promoted due to the increased number of G0/G1 cells during the cell cycle[100]. Hormone receptor-positive (HR+) tumors are the most common subtype of breast cancer (approximately 2/3 of breast tumors), which are significantly mediated by estrogen[52,101] that is recognized as a causal factor in the initiation and promotion of neoplastic growth[55,102-104]. Both estrogen receptor positive (ER+) and progesterone receptor positive (PR+) tumors are common and may be influenced by a number of factors. The literature suggests that breast cancer patients have an altered gut microbiota composition and higher levels of circulating estrogens[52,105-107]. Among the endogenous estrogens, estradiol is the most potent and biologically active hormone and is a major determinant of breast cancer risk[108,109]. Even though it plays a key role in breast cancer progression, the specific mechanisms of estradiol in breast cancer development are not well-documented. However, experimental evidence proposes that estradiol, by binding estrogen receptor alpha (ERα) and promoting cell proliferation, leads to mutations that occur due to errors during DNA replication[104,110]. The initiation of mutations in cell growth ultimately results in cancer.

Yadger and Davidson explain the mechanisms of estrogen carcinogenesis through two different and complementary pathways[111]. In the first pathway, estradiol can lead to altered gene expression and increased cell proliferation by acting through estrogen-receptor-mediated genomic (transcriptional), non-genomic (second messenger) and mitochondrial (transcriptional) signaling. In the second way, estrone and estradiol are converted to quinone metabolites through the catechol pathway in which cytochrome P-450 enzyme takes part in the reactions. Consequently, the estrogen 3,4-quinone can bind

to DNA and form unstable adducts with guanine and adenine compounds and lead to depurination and mutation[111-115]. Also, catechol estrogen metabolites undergo redox cycling and produce oxygen free radicals which can lead to oxidative DNA damage. Estrogen–quinone adducts and oxidative DNA damage can exert genotoxic effects and contribute to the progression of the carcinogenic process in breast cancer[110,111,113,116]. In some studies, women who have a high risk for breast cancer (or with breast cancer) have higher levels of depurinating estrogen–quinone DNA adducts in their urine than healthy women[110,117,118].

AIMS AND HYPOTHESES

Accumulating evidence suggests that dysbiosis in the gut microbiota and estrogen metabolism could potentially favor oncogenesis and tumor progression; dietary fiber metabolites, on the other hand, are capable of altering microbiome composition and influencing estrobolome metabolism which is linked to breast cancer incidence. However, the role of fiber in affecting estrogen activity is still obscure. Epidemiological studies have evaluated the intake of total fiber in relation to overall breast cancer risk in numerous studies; however, the results are not consistent. Investigating the influence of dietary fiber on fecal microbiota and estrogen metabolism would provide valuable data and opportunities to explore different aspects of the complex interaction between diet and estrobolome. By utilizing the microbiome, estrogen and dietary data -that includes soluble, insoluble, and total fiber-, this study will enable us to explore our hypotheses relating to dietary fiber intake, fecal bacteria composition and serum estrogen levels in breast cancer patients.

The overall hypothesis of this study is that there is a significant association between dietary fiber intake and estrogen metabolism, and this association is mediated through the gut microbiota responsible for β-glucuronidase enzyme activity.

More specifically, in our null hypothesis (H0), we state that there is no statistically significant relationship between dietary fiber, gut microbiota and circulating estradiol. In

our first alternative hypothesis (H1), however, we postulate that the higher levels of dietary fiber will be associated with a lower abundance of intestinal microbiota that promotes β-glucuronidase enzyme activity, i.e., an inverse association. Moreover, in our second alternative hypothesis (H2), we propose that the lower abundance of intestinal microbiota will be significantly associated with lower levels of circulating estrogen.

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AYSE G. ZENGUL, WENDY DEMARK-WAHNEFRIED, STEPHEN BARNES, CASEY D. MORROW, BRENDA BERTRAND, TAYLOR F. BERRYHILL, AND ANDREW D. FRUGÉ

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ABSTRACT

Breast cancer is a hormonally-driven cancer, and various dietary factors are associated with estrogen metabolism, including dietary fiber. Several studies report associations between dietary fiber and breast cancer; however, research on whether fiber influences circulating estrogens through the gut microbiota is rare. The objective of this cross-sectional study among 29 newly-diagnosed (stage 0-II), post-menopausal breast cancer patients is to examine associations between dietary fiber and the gut microbiota that are linked with β-glucuronidase activity, and purportedly increase circulating estrogens. Spearman's and partial correlations controlling for body mass index and age were performed using dietary recall data, Illumina MiSeq generated microbiota relative abundance, and HPLC-mass spectrometry-derived estradiol and estrone levels.

Major findings are: (1) total dietary fiber is inversely associated with *Clostridium hathewayi* (*r= -0.419; p=0.024*); (2) soluble fiber is inversely associated with *Clostridium* (r=-0.11; p=0.02); (3) insoluble fiber is positively associated with *Bacteroides uniformis sp*. (*r=0.382; p=0.041*); and (4) serum estradiol and estrone levels are not correlated with species/genera or dietary fiber, though there is a trend toward an inverse association between soluble fiber and estradiol levels (*r= -0.30; p=0.12*). More studies are needed to understand the complex interaction between dietary fiber, intestinal microbiota, and hormonal levels in older females.

Key words: Breast cancer, Dietary fiber, Microbiota, Estrogen

INTRODUCTION

Breast cancer affects many people worldwide. It is estimated that in 2018, approximately 266,120 US women will be diagnosed with invasive and 63,960 with non-invasive breast cancer [1]. Roughly 40,920 American women will die of this disease, making it the second leading cause of cancer-related death among U.S. females [1]. While breast cancer claims the lives of many women, many more are survivors. Currently, it is estimated that more than 3.1 million women are alive in the U.S. with a history of breast cancer. These women are either cancer-free or continue to live with active disease [2]. Considering the threat breast cancer poses to so many women, the concern for the prevention of this disease has become forefront in today's society.

Both weight status and dietary factors appear to be associated with breast cancer risk among post-menopausal women. Numerous studies have shown that post-menopausal obese women have a 20% to 40% increased risk of developing breast cancer compared to women of normal weight [3,4]. Each 5-unit increase in BMI is associated with a 12% increase in the risk of breast cancer in postmenopausal women [3-5]. It appears that a high-fat diet, and increased circulating levels of total cholesterol and triglycerides play a role in increasing breast cancer risk [6,7]. Furthermore, high protein intake, particularly increased red meat consumption, is associated with a 13% increase in the risk of breast cancer [6,8]. On the other hand, consumption of a plant-based dietary pattern is associated

with a decreased risk of breast cancer and provides protection against this prevalent disease [9].

In plant-based diets, fiber intake is prominent and associated with significantly lower breast cancer risk [10,11]. A high-fiber diet provides many health benefits. It may enhance weight loss and lower high cholesterol levels, as well as decrease insulin sensitivity [12]. Considering that increased estrogen levels are associated with breast cancer development [13], the relationship between fiber and estrogen metabolism may play an important role in breast cancer prevention. It is postulated that fiber reduces circulating estrogen levels by altering the gut microbiota and decreasing deconjugation and reabsorption of estrogen. By accelerating intestinal transit and binding to estrogen in the intestine, fiber decreases serum estrogen concentrations and prevents free hormones, such as 17β-estradiol, to be reabsorbed (See Figure 1) [10,14].

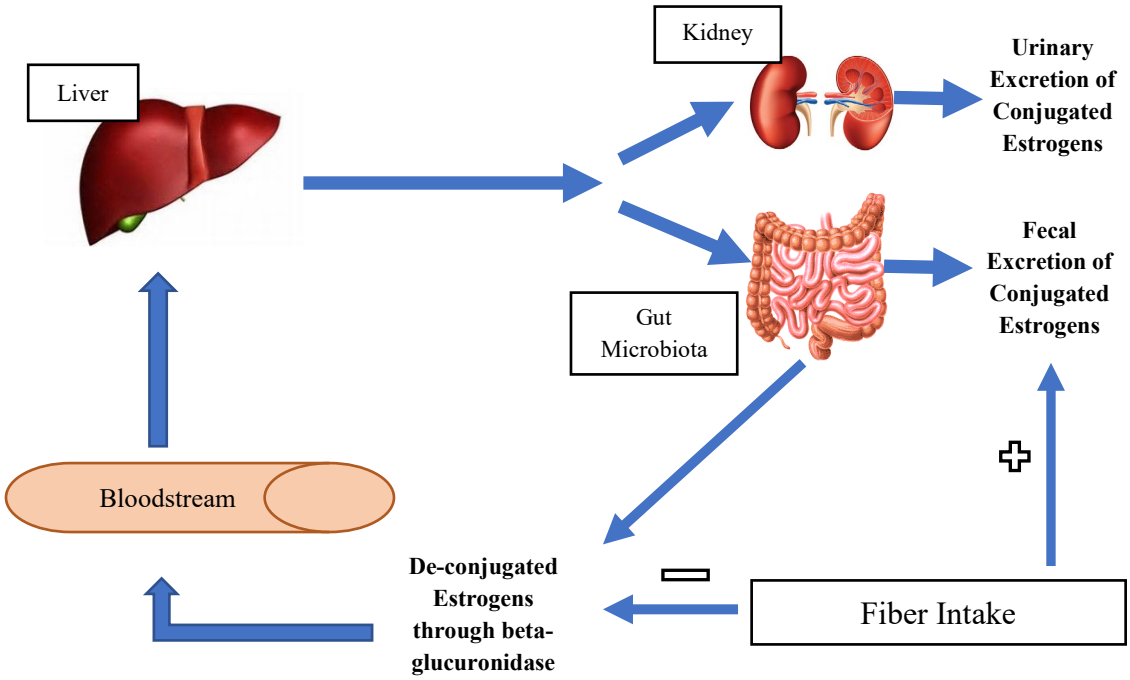
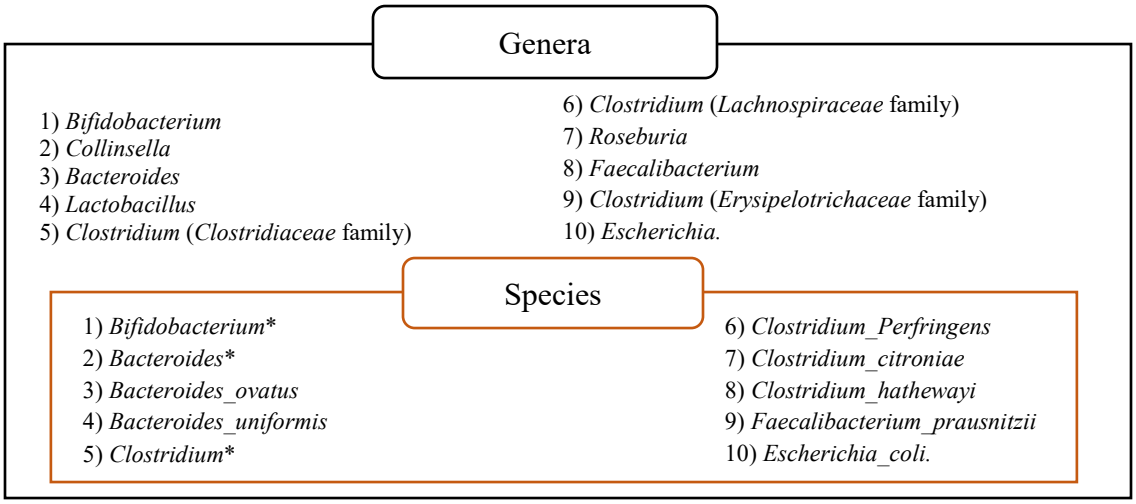


Figure 1: Conceptual Framework Exhibiting the Relationship between Fiber Intake, Gut Microbiota and Estrogen Metabolism (Adopted from Kwa, *et al.* 2016) (15)

Further, dietary fiber may alter the gut microbiota and influence estradiol metabolism through specific enzyme activities, such as β-glucuronidase [14]. Normally, estrogens circulate throughout the body until they reach the liver where they are inactivated through conjugation. Inactivated estrogens are then transported to the intestine for excretion into the stool. However, specific bacterial genera encode β-glucuronidase, which re-activates conjugated estrogens in the gut (see Figure 2). Deconjugated estrogens are reabsorbed and influence estrogen metabolism which is associated with hormone-dependent cancers, such as breast cancer [15,16]. To better understand the association between fiber intake and breast cancer, this study investigates associations between dietary fiber and the gut microbiota that promote β-glucuronidase

activity and explores associations with estrogen levels in the blood. We hypothesize that higher levels of dietary fiber will be associated with lower abundance of intestinal microbiota that promote β-glucuronidase activity while lower abundance of intestinal microbiota will be significantly associated with lower levels of circulating 17β-estradiol and estrone.



*Represents unnamed but previously identified bacterial species/OTUs within the genera

Figure 2: ß-glucuronidase encoding bacteria (Adapted from Kwa, *et al.* 2016) (15)

MATERIALS AND METHODS

Experimental Plan

Study Design

This cross-sectional study includes baseline data from a randomized controlled trial of weight loss conducted among 29 post-menopausal women who were newly-diagnosed with stage 0–II breast cancer and treatment naïve. The detailed methods of this trial have been published previously and are summarized below [17].

Recruitment/consent

Study subjects were recruited from the University of Alabama at Birmingham (UAB) Kirklin Interdisciplinary Breast Health Clinic (Birmingham, Alabama, USA). The trial was registered with the National Clinical Trials database (NCT02224807) and approved by the UAB Institutional Review Board (IRB-130325009).

Eligibility criteria for the study included being overweight or obese (BMI of 25–60 kg/m^2) with histopathologically-confirmed stage 0–II breast cancer and scheduled for surgery as primary treatment. Patients were screened to ensure there were no pre-existing medical conditions that would prevent adherence to unsupervised exercise. Also, physician clearance for any conditions, such as resting blood pressures >99 diastolic or >159 systolic, or cardiac anomaly was obtained. Participants had no current medical conditions that would affect weight status, such as Cushing's syndrome or untreated

hypothyroidism, nor additional active malignancy. Those who met these criteria were informed about the study, provided written informed consent, and were enrolled.

Baseline Assessment

Study staff collected and recorded data for demographics, height/weight, medical history, and medication use (including recent use of antibiotics) [17]. A multiple-pass method was used to collect two 24-hour dietary recalls that represented normal eating habits for one weekday and one weekend day. The data were entered and analyzed using the Nutrition Data System for Research (NDSR 2014, Minneapolis, Minnesota, USA). Phlebotomy was performed after a 12-hour fast and serum was obtained. Stool samples were collected using a sterile wipe after a bowel movement prior to the baseline assessment. All samples were stored at −80°C.

LC-MS analysis of 17β-estradiol and estrone

Assays for 17β-estradiol and estrone in serum were performed in the UAB Targeted Metabolomics and Proteomics Laboratory. Estrogen analyses were determined by isotope dilution HPLC-electrospray ionization-multiple reaction ion mass spectrometry adapted from the method of Tai and Welch [18]. 17β-Estradiol and estrone standards were prepared in 0.05% BSA. Sera (500 μl) were diluted 1:1 with MilliQ H_2O. Samples and standards were spiked with 0.5 ng/ml $^{13}C_6$-estradiol (CIL, Tewksbury, MA) internal standard. Diluted samples (1 ml) and standards were loaded onto individual 30 mg Polymeric Strata-X Solid Phase Extraction cartridge columns (Phenomenex, Torrance, CA). The cartridges were washed with MilliQ H_2O (1 ml) and 40% methanol

(1 ml) followed by elution of 17β-estradiol with 1 ml of methanol. Sample eluents were dried under a gentle stream of N_2. Sodium bicarbonate (50 µl, 100 mM, pH 10.5) and dansyl-chloride (50 µl, 1 mg/ml) in acetone were added to samples which were incubated at 60°C for 10 min. Samples were dried once more under a gentle stream of N_2 followed by reconstitution in 100 µl of 40% methanol/0.1% formic acid (FA). Chromatography was carried out using an Ace Excel C_{18}-Aromatic 1.7 µm 50 x 3.0 mm IS column at 50°C using a 20AD Prominence HPLC (Shimadzu, Kyoto, Japan) in tandem with 6500 Qtrap mass spectrometer (SCIEX, Framingham, MA). LC-MS operation and data collection were under the control of Analyst 1.6.2 software (SCIEX). The mobile phases were composed of (A) 0.1% FA and (B) acetonitrile 0.1% FA; the flow rate was 300 µl/min. Gradient starting conditions were 50% B which was held for 1 min, a linear increase of B to 100% B at 4 min, held at 100% B until 4.75 min, and returned to 50% B at 5 min to equilibrate to starting conditions until 7 min. LC flow was diverted to waste for the first 1.8 min to prevent salt contaminating the MS front end. The MS was operated in positive electrospray ionization mode with the following parameters: curtain gas 30, collision gas medium, temperature 500, ion spray voltage 5000, collision energy 25, GS1 60 and GS2 60. Mass transitions for multiple-reaction-monitoring mode were *m/z* 506/171 for dansyl-17β-estradiol, 504/171 for dansyl-estrone and *m/z* 512/171 for $^{13}C_6$-dansyl-17β-estradiol. The standard curve ranged from 5 – 5000 pg/ml over 7 points. All data were processed, and concentration factors were corrected using Multiquant 3.0.1 (SCIEX).

Fecal microbe analysis

Stool samples were collected by participants after defecation using a sterile wipe, placed in a plastic bag, and kept in their home freezer until baseline assessment, at which time the samples were collected and stored at −80°C until analyzed.

Fecal DNA extraction was carried-out using Zymo Fecal DNA Miniprep kit. Microbiome analysis targeting the V4 region of the 16S rRNA gene was performed using an Illumina MiSeq [19]. The post DNA sequence analysis used Quantitative Insight into Microbial Ecology (QIIME) suite, V.1.7 with modifications as described in Kumar et al. [19] and Fruge et al. [20,21].

Variables and Statistical Analyses

This study was a secondary analysis that utilized data on 29 post-menopausal women. The outcome variables of interest were dietary intake of fiber, serum 17β-estradiol, BMI, and the bacterial genera and species that colonize the human intestinal tract that encode for ß-glucuronidase based on the findings of Kwa et al. [15].

Normality tests for all variables of interest were assessed. Due to non-normal distribution, the Spearman rank test was utilized to reveal any potential associations between gut microbiota, 17β-estradiol, estrone, and dietary fiber (total, soluble and insoluble). In addition to the individual correlations between gut microbiota at the genera and species levels, the microbiota linked at phylum, family and class levels were combined, and the correlations between these, 17β-estradiol, estrone and the dietary fiber types were explored. Further analyses, including partial correlation tests between the

dietary fiber types and specific microbiota, were performed by controlling for BMI and age. All analyses were performed using IBM SPSS (version 24.0).

RESULTS

A total of 32 women enrolled in the study and provided fasting blood and fecal samples. We excluded premenopausal (n=2) and perimenopausal (n=1) women because fluctuating hormone levels affect circulating estrogen concentrations. Demographic characteristics of participants are provided in Table 1. The study participants had a mean age of 62.4 years and 82.1% were obese. Most were Non-Hispanic White or African-American, the remainder were of mixed race. Most were recently diagnosed with invasive cancers that were both estrogen- and progesterone-positive.

Table 1. Characteristics of post-menopausal treatment-naïve women with stage 0-II breast cancer (n=29)

Variable	**Mean (SD)**	**Range**
Age (years)	62.3 (8.5)	51-85
BMI (kg/m^2)	34.6 (5.8)	25.9-47.8
Weight (kg)	90.1 (16.6)	58.4-124.7
	N	**%**
Clinical Stage		
In situ	7	24.1
Invasive	22	75.9
Biopsy Grade		
Low	2	6.9
Low-Intermediate	1	3.5
Intermediate	8	27.6
Intermediate-High	9	31
High	9	31
Hormone receptor status		
Estrogen Receptor Positive	26	89.7
Progesterone Receptor Positive	20	69
Comorbidities		
0	1	3.4
1-2	10	34.5
3+	18	62
Race		
African-American	12	41.4
Non-Hispanic White	15	51.7
More than one race	2	6.9
Cardiovascular disease	4	13.8
Diabetes mellitus	9	31
Smoker	2	6.9

Table 2 presents data on dietary fiber, microbiota, estradiol concentrations, and the relative abundance of investigated species and genera. Total dietary fiber intake was roughly 14 g/day of which insoluble fiber comprised the major proportion. The relative abundance values of each phylum for each subject are presented in figure 3. The most common phyla were *Firmicutes* and *Bacteroidetes.* At the genus and species levels, a higher abundance of *Faecalibacterium* and a lower abundance of *Bifidobacterium, Clostridium-perfringens,* and *Clostridium hathewayi* were detected in the samples (Table 2).

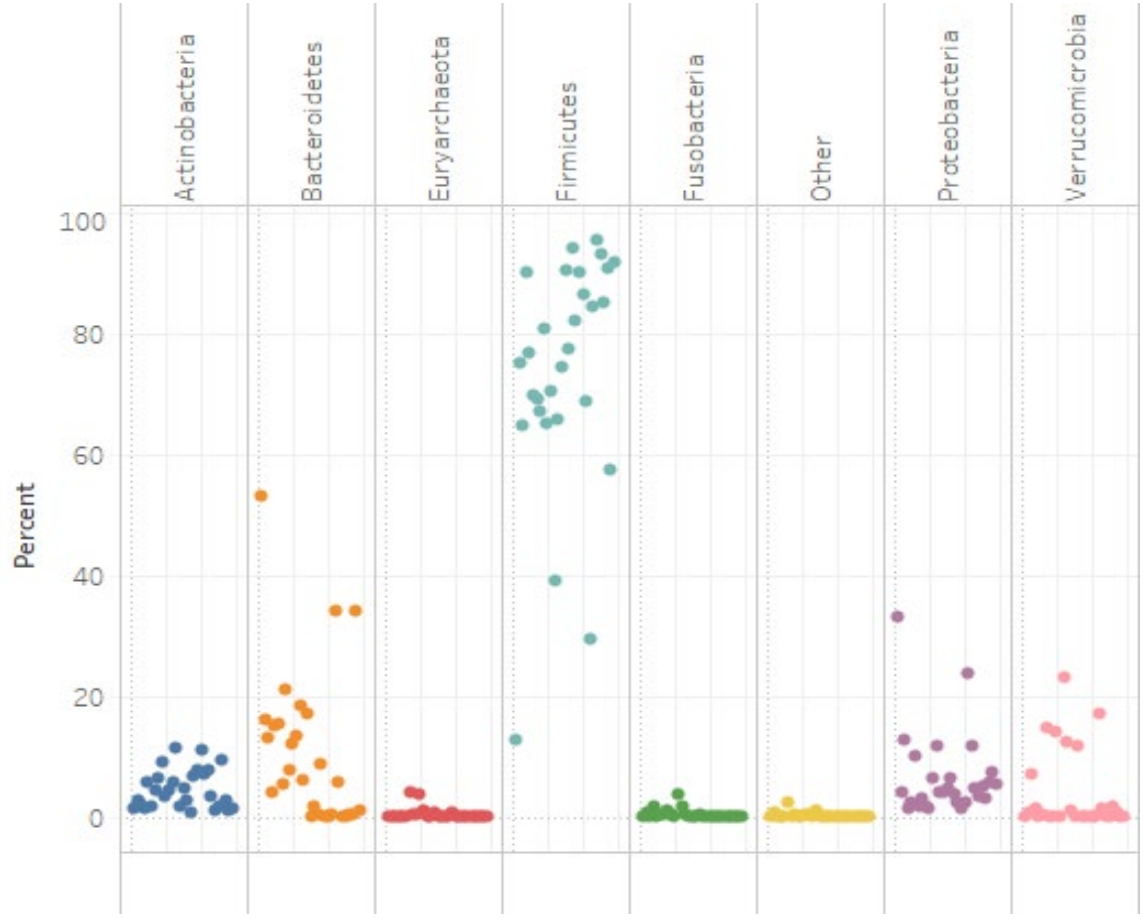


Figure 3: Phylum Level Relative Abundance of fecal bacteria from 29 treatment-naïve post-menopausal women with stage 0-II breast cancer.

Table 2. Dietary fiber intake, fecal microbiota, and serum estradiol concentrations (N=29)

	Mean	**SD**	**Range**
Total Dietary Fiber (g/day)	14.22	6.94	5.23 - 39.67
Soluble Dietary Fiber (g/day)	4.54	1.79	2.01 - 8.88
Insoluble Dietary Fiber (g/day)	9.67	5.96	2.32 - 33.81
Serum estradiol (pg/ml)*	17.68	15.12	4.3 – 79.5
Serum estrone (pg/ml)*	198.30	114.43	21.8 - 535.1
Bacteroides;s__	2.63%	4.77%	0.002 - 22.301%
Bacteroides;s__ovatus	0.02%	0.03%	0.000 - 0.157%
Bacteroides;s__uniformis	0.69%	1.11%	0.000 - 4.352%
Clostridium;s__	0.47%	0.51%	0.002 - 1.959%
Clostridium;s__perfringens	0.01%	0.04%	0.000 - 0.198%
Clostridium;s__citroniae	0.92%	3.95%	0.000 - 21.321%
Clostridium;s__hathewayi	0.01%	0.03%	0.000 - 0.106%
Faecalibacterium;s__prausnitzii	9.24%	8.56%	0.011 - 29.509%
Escherichia;s__coli	1.63%	4.46%	0.002 - 21.331%
g__Bifidobacterium	1.26%	2.00%	0.004 - 7.285%
g__Collinsella	1.48%	1.91%	0 .000 - 6.333%
g__Bacteroides	5.79%	8.63%	0.005 - 31.364%
g__Lactobacillus	0.69%	1.20%	0.031 - 6.360%
g__Clostridium (*Clostridiaceae* family)	0.56%	0.56%	0.002 - 1.959%
g__Clostridium (*Lachnospiraceae* family)	0.94%	3.95%	0.002 - 21.353%
g__Roseburia	3.61%	4.03%	0.020 - 16.531%
g__Faecalibacterium	9.32%	8.64%	0.010 - 29.688%
g__Clostridium (*Erysipelotrichaceae* family)	0.17%	0.44%	0.000 - 1.830%
g__Escherichia	1.63%	4.46%	0.002 - 21.331%

*Sample size reduced from 29 to 28 from lack of blood sample for one participant.

Correlations between dietary fiber types, gut microbiota, 17β-estradiol and estrone concentrations are displayed in Table 3. The results indicate that total dietary fiber intake is significantly and inversely associated with *Clostridium hathewayi sp*. (*r= -0.419; p=0.024*). While the strength of association was somewhat weaker, soluble dietary fiber was significantly and inversely associated with *Clostridium (r=-0.11; p=0.02*). Both of these inverse relationships continued to be observed after controlling for age and BMI. Also, insoluble dietary fiber was significantly and positively associated with *Bacteroides uniformis sp*. (*r= 0.382; p=0.041*), again an association that remained after controlling for age and BMI. There were marginally significant correlations between certain gut microbiota and dietary fiber types (Table 3). For example, the association between insoluble dietary fiber and *Clostridium hathewayi sp*. was marginally significant (*r= -0.31 p=0.066*). There also were marginally significant positive associations between *Escherichia coli sp., Escherichia* and total dietary fiber (*r=0.35*; *p=0.059*). Relatedly, there was a trend toward an inverse association between soluble dietary fiber and 17β-estradiol levels (*r= -0.30; p=0.12*), albeit statistically insignificant. Serum 17β-estradiol and estrone levels were not correlated either with species/genera nor with dietary fiber types. Moreover, combined genera and species at the phylum, family and class levels and the summative proportion of the microbes at the genus and species levels did not reveal any significant associations.

Table 3. Associations between dietary fiber intake, gut microbiota, and serum estradiol concentrations among stage 0-II breast cancer patients (n=29)

	Total Dietary Fiber (g/day)	Soluble Dietary Fiber (g/day)	Insoluble Dietary Fiber (g/day)
Serum Estradiol (pg/ml)**	-0.21 (p=0.28)	-0.30 (p=0.12)	-0.08 (p=0.70)
Serum Estrone (pg/ml)**	-0.08 (p=0.70)	-0.17 (p=0.40)	0.07 (p=0.74)
Species			
Bifidobacterium;s__	0.07 (p=0.71)	0.11 (p=0.56)	0.02 (p=0.91)
Bacteroides;s__	0.18 (p=0.35)	0.00 (p=1.00)	0.28 (p=0.14)
Bacteroides;s__ovatus	0.14 (p=0.46)	-0.01 (p=0.96)	0.22 (p=0.26)
Bacteroides;s__uniformis	0.30 (p=0.11)	0.12 (p=0.54)	**.382* (p=0.04)**
Clostridium;s__	0.17 (p=0.39)	0.10 (p=0.62)	0.22 (p=0.26)
Clostridium;s__perfringens	0.08 (p=0.69)	0.06 (p=0.77)	0.04 (p=0.83)
Clostridium;s__citroniae	-0.12 (p=0.53)	-0.24 (p=0.22)	-0.08 (p=0.68)
Clostridium;s__hathewayi	**- .419* (p=0.02)**	-0.31 (p=0.10)	-0.35 (p=0.07)
Faecalibacterium;s_prausnitzii	0.09 (p=0.63)	0.00 (p=0.98)	0.12 (p=0.54)
Escherichia;s__coli	0.35 (p=0.06)	0.14 (p=0.48)	0.30 (p=0.12)
Genera			
g__Bifidobacterium	0.27 (p=0.16)	0.29 (p=0.13)	0.19 (p=0.31)
g__Collinsella	-0.08 (p=0.67)	-0.16 (p=0.41)	-0.07 (p=0.70)
g__Bacteroides	0.25 (p=0.20)	0.06 (p=0.77)	0.34 (p=0.07)
g__Lactobacillus	-0.07 (p=0.71)	-0.12 (p=0.54)	-0.03 (p=0.89)
g__Clostridium (*Clostridiaceae* family)	0.20 (p=0.29)	0.15 (p=0.43)	0.23 (p=0.23)
g__Clostridium (*Lachnospiraceae* family)	-0.21 (p=0.28)	-0.22 (p=0.25)	-0.17 (p=0.36)
g__Roseburia	-0.08 (p=0.66)	-0.17 (p=0.37)	-0.10 (p=0.62)
g__Faecalibacterium	0.09 (p=0.63)	0.00 (p=0.98)	0.12 (p=0.54)
g__Clostridium (*Erysipelotrichaceae* family)	-0.16 (p=0.40)	**-0.11* (p=0.02)**	-0.15 (p=0.44)
g__Escherichia	0.35 (p=0.06)	0.14 (p=0.48)	0.30 (p=0.12)
SUM_Species	0.29 (p=0.12)	0.22 (p=0.26)	0.29 (p=0.13)
SUM_Genera	0.33 (p=0.08)	0.22 (p=0.26)	0.31 (p=0.10)

*significant at the p<.05 level; these associations were stable after controlling for BMI and age
** n=28 for this analysis since serum was unavailable for one participant

DISCUSSION

Our study is one of the few to examine the relationship between the gut microbiota that promote ß-glucuronidase activity, dietary fiber and circulating 17β-estradiol and estrone. Contrary to our hypothesis, the results of this study indicate that dietary fiber intake had no relationship to estrogen levels in the blood. However, we found that higher levels of total and soluble dietary fibers correlate with lower levels of *Clostridium hathewayi sp*. and *Clostridium* (*Erysipelotrichaceae* family), respectively. These bacteria promote ß-glucuronidase activity and the inverse relationship between them and dietary fiber continued to be observed after controlling for age and BMI. Contrary to these findings, we also found a positive and significant relationship between insoluble fiber and *Bacteroides uniformis* which also promotes ß-glucuronidase activity. Given the important role of ß-glucuronidase enzyme in luminal hormone metabolism, this exploratory analysis provides valuable insights for future studies.

To date, no studies have found a link between *Clostridium hathewayi* and dietary fiber. However, our results demonstrated the significant inverse relationship between total dietary fiber intake and *Clostridium hathewayi,* a newly discovered *Clostridium* species [22], which has been implicated in clinical diseases, such as sepsis and infection [23-27]. Thus, the association between total dietary fiber and *Clostridium hathewayi* may have important implications regarding the prevention of both infectious diseases, as well as those that are hormonally driven.

Another inverse relationship observed was between soluble dietary fiber and *Clostridium*. This result is not in accordance with findings reported by Chinda et al. [28] and Bang et al. [29] who reported that pectin, which is a soluble fiber, was associated with higher levels of *Clostridium*. These studies were conducted exclusively in healthy males, unlike our study which included female postmenopausal breast cancer patients, and included far fewer study participants, i.e., n=14 and n=3, respectively. Also, our outcome variables included total soluble dietary fiber, rather than pectin alone. However, the *Clostridium* genus is known to produce SCFAs, mainly butyrate, which are the major products of the fiber fermentation process [29 31]. Therefore, the production of SCFAs by *Clostridium* indicates that higher fiber intake might increase the relative abundance of these bacteria and consequently the activity of the β-glucuronidase. The role of *Clostridium in* the production of the SCFAs -acetate and butyrate- might be important for enhancing intestinal peristalsis and in preventing/treating constipation, diarrhea, and colitis [29]. The interaction between the *Clostridium* genus and fiber regarding SCFA production is a possible confounder in our study and needs to be explored in future studies.

Among the three dietary fiber types, the only significant positive relationship we found was between insoluble fiber and *Bacteroides uniformis*. Higher levels of insoluble fiber were associated with higher levels of *Bacteroides uniformis*. This finding may relate to the influence of *Bacteroides uniformis* on glycolysis pathways. According to a study that was conducted by Benítez-Páez on 75 full-term newborns, "*B. uniformis* strains exhibit an expanded glycolytic capability when compared with other *Bacteroides*

species" [30]. Enhanced glycolysis is related to higher glucose uptake, and it is currently used as an indicator of malignancy since it represents an evident characteristic of many cancers [31,32]. Considering the relationship between *Bacteroides uniformis* and glycolysis, our results, thereby, might shed light on understanding the influence of *Bacteroides uniformis* on glycolysis and its possible physiological effects in breast cancer.

Although we found some significant associations, we did not find support for our hypothesis regarding the relationship between dietary fiber and levels of circulating estrogens. We found only a weak inverse association between soluble fiber and 17β-estradiol. This result is consistent with the findings of Gaskins et al. (2009) who observed a significant and inverse relationship between dietary fiber consumption and 17β-estradiol concentrations among 250 premenopausal healthy women (aged 18–44 y) [13]. In this much larger prospective cohort study, both soluble and insoluble fiber had an inverse relationship with 17β-estradiol concentrations ($\beta = -0.222$, $p = 0.01$; $\beta = -0.057$, $p = 0.02$, *respectively*) [13]. Despite highly fluctuating 17β-estradiol levels among premenopausal women, the significant associations they found suggest that if this relationship was explored among larger samples of postmenopausal women, significant results might be attained.

In 17b-estradiol and estrone analyses, we analyzed total estrogen values at baseline by LC-MS for postmenopausal women. When we compared our results with the previous studies, the circulating estradiol concentrations of postmenopausal women – determined by LC/SRM mass spectrometry– were reported within the range of 6.2 to

51.5 pg/ml (mean 26.3 pg/ml); and serum estrone concentrations were reported to be in the range of 61.3 to 442.1 pg/ml (mean 176.6 pg/mL) [33]. In our analyses, serum estradiol and estrone levels were in a range 4.3 – 79.5 (with a mean of 17.68) and 21.8 to 535.1 (with a mean of 198.30) respectively. The greater range in our analyses might be due to a higher proportion of younger subjects (age 51-52) who had higher values of estradiol and estrone concentrations. This might indicate that those subjects might actually be perimenopausal. Higher estrogen values might have affected the correlation results.

To clearly understand the lack of significant findings related to the interaction between fiber intake, beta-glucuronidase activity, and estrogen concentrations, we also consider other sources of biological variation that might influence the results. For example, sex hormone binding globulin (SHBG) is a major carrier protein for free sex hormones in the human plasma. It is produced by the liver, and it binds to dihydrotestosterone (DHT), testosterone, and estradiol found in both women and men. In women, SHBG plays an important role by transporting these hormones in the blood and regulating the levels of androgens and estrogens. SHBG carries sex hormones as biologically inactive forms; thus, reduces the levels of free estradiol[34]. Dietary fiber consumption increases the concentrations of SHBG in the blood and promotes the excretion of estrogen[35]. However, there is evidence that some factors such as age, high BMI, and fasting insulin might negatively influence the concentrations of SHBG[36]. Considering our study was conducted among postmenopausal women who were overweight or obese, the potential impact of carrier proteins, especially SHBG, can be a

possible confounder of the results since they can reduce plasma SHBG levels which will lead to an increase in the proportions of free estrogens and androgens in the blood.

Another possible confounder is that according to *in vitro* research, β-glucuronidase, as a lysosomal enzyme, requires an acidic medium for its catalytic activity. As the end product of glycolysis, lactate increases the acidity of the cells[37]. The elevated level of lactate is usually considered as an indicator of mitochondrial dysfunction. In this situation, the acidification around the macrophage cells might also induce the β-glucuronidase activity and assist the deconjugation of the glucuronide metabolites[37]. Considering the complexity of estrogen metabolism, such biological variations might have influenced our results.

Furthermore, in animal studies, bacterial glucuronidase activity mainly occurs in the caecum and the large intestine[38, 39]. In the bowel lumen, the inactivated estrogens are deconjugated by bacterial glucuronidase; thus, free estrogens are reabsorbed across the bowel mucosa into the blood circulation[40]. However, the evidence shows that the fecal/luminal and mucosal bacteria compositions are significantly different. Both the outer mucus and the intestinal lumen of the large intestine are capable of forming a unique microbial niche in which same bacterial species present differential proliferation and resource utilization. Therefore, careful consideration in choosing a sampling method matters when analyzing the microbiota composition[41]. In our study, we only analyzed fecal microbiota abundance. Thus, the lack of a more comprehensive sampling method could have influenced the results we achieved.

Finally, our results may have been influenced by potential bias in the collection of dietary data. For this study, we collected two 24-hour dietary recalls, which rely on subjects' self-reported dietary intake. Even though the multiple-pass method was applied to enhance complete and accurate food recall, the subjects' responses to dietary questionnaires may be influenced by social desirability. Previous studies on the accuracy of dietary measures reveal that some participants tend to under-report numbers of foods and portion sizes they consume while reporting more frequent intakes of healthy foods[42,43]. Also, relying on human memory might frequently lead to imprecise reports. According to Krebs-Smith et al.[43] and Briefel et al.[44], underreporting in dietary surveys may affect up to 15% of all 24 hour recalls even if a multiple-pass method is used. Age, gender, obesity, educational level, and personal tendency are some of the factors that may influence the responses on dietary questionnaires[43,44,45]. Compare to men; women have a higher tendency towards social desirability[46]. This study included overweight or obese women subjects and cognitive, perceptional and emotional influence on the 24 hours recalls must also be considered in the evaluation of the results.

Besides the above mentioned potential biological variations, it is important to recognize some other limitations including small sample size and cross-sectional design that resulted in a limited ability to detect associations and reduced the statistical power. Although we adjusted for potential confounders for breast cancer, our results could still have been affected by residual or unmeasured confounding. The range of 17β-estradiol and estrone levels in post-menopausal women, for example, is narrow and levels are low - thus the distribution may not have been sufficient to detect correlations. Finally,

multiple testing and the chance of uncovering associations that are spurious is a limitation of this study. Despite these limitations, this study makes a unique contribution, first because very few studies have investigated microbiota among populations with breast cancer, and second, this study relies on dietary data that includes soluble, insoluble, and total fiber.

CONCLUSION

The role of bacterial ß-glucuronidase activity in breast cancer risk is still obscure. The influence of dietary fiber on this activity has an important bearing on understanding the link between estrogen metabolism and breast cancer. By being one of the few studies investigating the triad of these overlapping relationships among dietary fiber, ß-glucuronidase activity and serum estrogens, our study provides insight and direction for future studies.

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GENERAL CONCLUSION

The aim of this study was to investigate the relationship between dietary fiber, specific gut microbiota that promote ß-glucuronidase enzyme and circulating estrogens in breast cancer patients. Twenty-nine post-menopausal women, ages 51-85 years, newly-diagnosed with stage 0–II breast cancer and treatment naïve, were enrolled in the study. By utilizing an integrated approach that combined dietary, fecal microbiota and estrogen data, this study aimed to explore any potential mechanistic relationship between these factors.

The present study revealed that serum estradiol and estrone levels were not correlated either with species/genera or dietary fiber types. However, there was a trend toward an inverse association between soluble dietary fiber and estradiol levels. The results also indicated significant associations between dietary fiber intake and certain fecal bacteria composition, i.e., total dietary fiber is inversely associated with *Clostridium hathewayi* and soluble fiber is inversely associated with *Clostridium*. Also, insoluble fiber is positively associated with *Bacteroides uniformis sp*. Similar to some studies, our findings suggest that fiber consumption can alter gut microbiota composition; they also reinforce the link between ß-glucuronidase activity and higher levels of circulating estrogen. However, some other studies suggest contradictory results regarding these relationships. Considering the small sample size and cross-sectional design of this study,

further research is needed to see if dietary fiber consumption takes part in estrogen metabolism through altering ß-glucuronidase activity in the gut. Findings from this study contribute to advancing the literature and developing understanding of the link between estrogen metabolism and breast cancer.

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