Study of lung cancer prevention by *Euglena gracilis* extracts

by

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B.S., Tribhuvan University, 2010
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AN ABSTRACT OF A DISSERTATION

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DOCTOR OF PHILOSOPHY

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Abstract

Lung cancer is the leading cause of cancer-related deaths. Because traditional treatment measures are associated with several limitations such as severe side effects and complications, natural products could be explored to develop novel preventative and therapeutic strategies against lung cancer. *Euglena gracilis* (*E. gracilis*) is a unicellular micro-alga used as a nutritional and functional dietary supplement because of its several health benefits. *E. gracilis* has long been known to have medicinal properties for multiple diseases including cancer. However, its anti-tumor properties against lung cancer are unknown. This dissertation examined the effect of water extract of *Euglena gracilis* (EWE) on the growth of lung cancer and demonstrated the mechanisms of its antitumor properties.

In the first part of the dissertation, the antitumor and immunomodulatory effects of partially purified water extract from *E. gracilis* (EWE) against lung cancer were examined. Oral pretreatment with EWE three weeks before the Lewis lung carcinoma (LLC) cell inoculation significantly attenuated the growth of LLC tumors in immunocompetent syngeneic mouse lungs. EWE administration significantly reduced the granulocyte population in mouse peripheral blood and also directly attenuated granulocytic MDSCs (gMDSCs) in primary cell culture with mouse bone marrow cells, which was attributed to the gMDSC-specific apoptosis. These findings indicate that partially purified water extract from *E. gracilis* contains significant bioactive materials that, when consumed, attenuate the lung tumor growth by stimulating host antitumor immunity.

Further, we prepared other fractions by boiling the EWE (bEWE) and then, evaluated the antitumor effects of both EWE and bEWE using orthotopic lung cancer syngeneic mouse models with LLC cells. The treatment with bEWE further attenuated the growth of LLC tumors in the

lungs. Since *Euglena* water extracts were administrated daily *via* drinking water, there is a likelihood of alteration of intestinal microbiota and/or their metabolites. Therefore, we explored the fecal microbial communities from the mice treated with EWE, bEWE, and PBS using 16S rRNA gene sequencing. A more diverse intestinal microbiota composition in both extract-treated groups than that in the PBS group was observed. Particularly, a decrease in the ratio of Firmicutes to Bacteroidetes and significant increases in *Akkermansia* and *Muribaculum* were observed in both EWE- and bEWE-treated groups. The fecal microbiota transplantation (FMT) studies established that the attenuation of lung carcinoma growth in mice by *Euglena* water extracts is associated with the alteration of the gut microbiota.

We also showed the attenuation of lung carcinoma growth using chemical-carcinogen-induced lung tumorigenesis in mice that closely mimics human lung cancer development. The fecal metabolites between the PBS and bEWE treated mice were different. The metabolites such as succinate, malate, triethanolamine, acetylserine, aspartate, glutamate, and threonine that can be metabolized to short-chain fatty acids (SCFAs) were increased with bEWE treatment. More importantly, the levels of fecal SCFAs such as acetate, butyrate, and propionate were increased upon *Euglena* water extract treatment. Further, an *in vitro* study showed inhibition of lung cancer growth with SCFAs treatment *via* apoptosis.

Together, these studies elucidate the understanding of the anti-tumor efficacy of the water extracts from *E. gracilis* on the prevention and treatment of lung carcinoma growth. Further, these studies highlight the role of gut microbiota and its metabolites with the oral administration of *E. gracilis* water extracts in the attenuation of lung carcinoma growth using different lung carcinoma model. These findings may facilitate the usage of water extracts from *E. gracilis* for the development of novel prevention and therapeutic strategies against cancers.

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Dedication

I would like to dedicate this thesis to my mother (Devi Upreti) and my father (Ram Upreti). You have always been an immense support and inspiration to me.

Chapter 1 - Prevention of Lung Cancer by *Euglena gracilis* Extracts: Involvement of Gut Microbiota and Metabolites

Cancer

In 2020 alone, cancer caused the deaths of more than 10 million lives worldwide [1]. Cancer is the second common cause of death in the United States with 1,898,160 new cancer cases and 608,570 cancer deaths projected to occur in 2021 alone [2]. Cancer is defined as a group of diseases that is characterized by uncontrollable growth and the spread of abnormal cells [3]. These cancerous cells are unable to recognize their boundaries, invade the adjoining body parts, and spread to distant organs. This invasive process termed metastasis often leads to death. In fact, cancer is a leading cause of worldwide deaths.

Cancer development is a multi-stage process that progresses from a pre-cancerous lesion to a malignant tumor. This process is governed by several elements including the person's genetic factor and the exposure to external risk factors such as physical, chemical, or biological carcinogens [4]. Because cancer comprises a variety of diseases at the tissue level, it poses a major challenge for its specific diagnosis, and consequent development of efficient preventative or therapeutic measures. Lung cancer is the most lethal form of cancer. However, the risk factors and the molecular pathways are not totally clarified yet. Therefore, critical strategies for the prevention and therapy of lung cancer are still controversial.

Lung cancer

Lung cancer is a malignant tumor with the highest mortality rate because it is detected very late in the course of the disease. Since early detection has been shown to significantly improve patient survival rates and quality of life, late detection leads to a significant reduction in

the quality of life of the patient. This makes it very important for the early detection of lung cancer [5]. Lung cancer is histologically classified into two types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC grows at a slower rate in comparison to SCLC accompanied by few or no symptoms until it reaches the advanced stage. More than 85% of the lung cancer cases are categorized as NSCLC of which 40% are adenocarcinoma, and 25 to 30% are of squamous cell carcinoma [6]. Several risk factors are attributed as the causes of lung cancer. The most important of these causes are smoking which includes active cigarette smoking, exposure to secondhand cigarette smoke (passive smoking), pipe, and cigar smoking. Not only active smokers but also secondhand smokers, exposed to cigarette smoke (passive smoking), make up a sizeable proportion of lung cancer patients. In addition, exposure to indoor and outdoor air pollution, radiation, and occupational exposure to agents such as asbestos, nickel, chromium, and arsenic may also cause lung cancer [7]. On top of being the leading cause of mortality among the smoking population, lung cancer is also the leading cause of death among those who have never smoked [8]. At the molecular level, lung cancer is caused by the alteration of several cell signaling cascades that are induced by the risk factors listed above. The most commonly altered and most clinically relevant oncogenes, tumor suppressor genes, and signaling pathways in lung cancer include KRAS, EGFR, BRAF, MEK, MET, HER2, P53, PTEN, PI3K-Akt-mTOR pathways [9].

Treatment strategies for lung cancer

Current strategies for treating lung cancer mostly consist of surgery, chemotherapy, radiation, and immunotherapy depending on the stages of lung cancer [10]. Non-small cell lung cancer (NSCLC) ranges from stage I-stage IV, indicating that cancer has spread less if it is identified as stage I [11]. Surgical resection is recommended to the patients only with stage I to II

NSCLC, whereas patients with more advanced stages are recommended for non-surgical treatment [12]. However, detection of NSCLC in the early stage is very difficult to attain and is diagnosed only after the tumor has metastasized.

Since more than 70% of NSCLC are diagnosed in advanced stage (stage III and IV), radiotherapy, chemotherapy, and surgical resection followed by chemotherapy (depending on the location of the tumor and whether the tumor is resectable) are the most widely used methods to treat lung cancer. These therapies lessen disease-related adverse effects and improve survival [11]. However, the therapies listed are also associated with several disadvantages such as severe toxicity, limited efficacy, and development of drug resistance [13].

Furthermore, immunotherapy is one of the promising treatment strategies in oncology that boost the immune system in patients to stop or slow down the cancer cell growth as well as inhibit metastasis [10]. Among the several immunotherapy approaches, blocking immune checkpoints using checkpoint inhibitors is the most promising approach. The immune checkpoints play a role in maintaining self-tolerance and regulate the immune response in the body. The inhibition of immune checkpoint molecules such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and its ligands (CD80 and CD86), programmed cell death-1 (PD-1) and its ligand PD-L1 activates the therapeutic antitumor immunity [14]. Although immunotherapy has been increasingly used to treat cancer, one of the major challenges for this treatment strategy is to enhance its efficacy and control the immune modulation to an extent where no adverse side effects such as autoimmunity and nonspecific inflammation are observed [15]. Furthermore, cancer immunotherapies cause several toxicities depending on the mode of action [16].

Combined with the poor prognosis and minimal five-year survival rate of existing therapeutic interventions, there is clearly an urgent need for the development of novel and efficacious preventative, diagnostic, and therapeutic strategies against lung cancer. Thus, phytochemicals and natural compounds that have a long history of medicinal use could prove to be of immense use in developing novel anti-lung cancer therapies.

Natural products in cancer prevention and therapeutics

Phytochemicals and antitumor herbs have a long history of use in the treatment of cancer, especially in eastern and ayurvedic medicine, and continue to be a major source of new drugs [17]. Medically important natural products can be obtained from plants, fungi, and marine sources such as marine algae and marine sponges. Some of the well-known examples of the naturally extracted product used in medical practice are acetylsalicylic acid for the anti-inflammatory agent; quinine for the anti-malarial drug; penicillin from the fungus, *Penicillium notatum*; paclitaxel for the breast cancer drug isolated from the bark of *Taxus brevifolia*; Plitidepsin for melanoma, small cell and non-small cell lung cancer-treating drug isolated from the Mediterranean tunicate *Aplidium albicans* [17]. Herbal medicines have been recognized as one of the attractive approaches for lung cancer therapy because they are less toxic compared to chemotherapy and are proven to be useful and effective in preventing side effects of chemotherapy, prolonging patient survival, and improving the quality of life in lung cancer patients [18]. Unsurprisingly, these agents are being increasingly, and extensively studied and different preclinical and clinical studies have shown the antitumor activity of herbal extracts against lung cancers [19–21]. Studies have shown that the response of lung tumor cells to some of the traditional herbal medicinal extracts was similar to their response to conventional chemotherapeutic drugs [22]. Mechanistically, the herbal medicines targeting lung cancer are

associated with induction of apoptosis, inhibition of angiogenesis and metastasis, reversal of multidrug resistance during chemotherapy, and suppression of reactive oxygen species generation [21]. The natural products are also shown to be effective in modulating the signaling pathways and oncogenes/tumor suppressors listed previously that are vital for the development of lung cancer [19]. In this regard, a naturally occurring unicellular alga called *Euglena* and its metabolites could be of great benefit to human health.

Euglena gracilis and its benefits

Euglena is a genus of single-celled, flagellated, eukaryotic, microalga that are mostly found in stagnant freshwater bodies such as ponds and lakes. They can be seen forming a visible green or red scum on the surface of the water. They lack a cell wall and, therefore, their cell is highly flexible such that its shape can vary from a rod-shaped 100 μm long filament to a sphere with 20 μm diameter. One interesting feature of the organism of the genus *Euglena* is that they are both autotrophic like the plants and heterotrophic like the animals [23]. They use their chloroplasts to generate energy from sunlight via photosynthesis. But they are also capable of consuming food such as green algae and amoebas by phagocytosis to act as heterotrophs. The species of *Euglena* are motile owing to a long, whip-like flagellum protruding out of the cell. Another interesting feature of these organisms is the presence of the "eye spot." These organelles are very similar to the rhodopsin pigments found in our eyes and they help *Euglena* in phototaxis, a movement toward sunlight to optimize autotrophy. Because of their great adaptability and considerable biotechnological potential, these organisms are at the forefront of application-driven research and commercialization. Although several species of *Euglena* exist, *Euglena gracilis* (*E. gracilis*) is the most extensively studied organism of this genera.

E. gracilis is a ubiquitous, freshwater, mixotrophic microalgae that displays all the properties of the genus *Euglena*. Because of its ease of cultivation on a large scale and its ability to produce compounds of various applications as described later, *E. gracilis* research has attracted increasing attention. Although evolutionarily most closely related to the protozoan parasites such as *Trypanosoma* and *Leishmania* that cause severe diseases, *E. gracilis* is not known to cause any disease. Contrastingly, however, mounting evidence indicates the benefit of this organism not only in animal and human health as a dietary supplement and nutraceutical but also in the generation of clean energy as biofuels. Below we will highlight the significance of *E. gracilis* research in aspects ranging from basic molecular levels to the exploration of outer space, with a special focus on its potential use as an anti-tumor agent against lung cancer.

Euglena gracilis as a nutritional source

E. gracilis has been long known to be a rich source of vital vitamins such as A, C, E that are important for the human diet [24–26]. Studies have shown that dried powder of *E. gracilis* contains up to 10-fold more β-carotene than dehydrated carrots, which indicates that it could be used as an excellent source of vitamin A [27, 28]. Depending upon the mode of growth in the culture condition, the production of vitamin C from *E. gracilis* can range from 8 mg/L of culture to 86 mg/L [28, 29] that is enough to fulfill the daily requirement for an adult human. The transcriptomic and proteomic studies have corroborated that *E. gracilis* produces an abundant amount of alpha-tocopherol, also known as vitamin E [29–31]. Under cultured conditions, *E. gracilis* can produce up to 44.2 mg/L culture or 1.1 mg/g dried weight of alpha-tocopherol [32], which is far better than that found in vegetable oils marketed as an excellent source of vitamin E [27].

E. gracilis is also a rich source of dietary protein that could not only fulfill the protein needs of a vegetarian diet but also supplement that of a non-vegetarian diet [27]. *E. gracilis* has similar protein content as animal-based protein such as beef, fish, and chicken and it is also par with other microalga-based protein supplements such as *Chlorella spp.* [27]. Because *E. gracilis* lacks a cell wall, it is more readily digestible and, therefore, acts as an excellent source of single-cell protein [33].

In addition to containing over 14 different vitamins, 20 different amino acids, 9 different minerals such as iron, calcium, and magnesium, *E. gracilis* is also packed with 11 different unsaturated fatty acids like Docosahexaenoic acid (DHA) that could prove beneficial for treating health conditions like cardiovascular diseases and cancer [27, 30, 34, 35]. Loaded with different compounds of high nutritional value [36], there is a huge potential for commercial utilization of *E. gracilis* in the food industry for both human and feedstock [37].

Use of *E. gracilis* in biofuel and biodegradation

In addition to the biomolecules with nutritional value, *E. gracilis* is also a rich source of lipids and wax esters that could be utilized as biofuel [27, 37]. These wax esters are specially produced during anaerobic growth and can endure tough environments such as highly acidic conditions [38–40]. These properties make *E. gracilis* an appropriate candidate to be used in wastewater treatment as well as the production of biofuels [35, 41, 42]. In fact, Euglena Co., Ltd. has already started producing carbon-neutral jet fuel to be used in commercial flights [43]. In addition to being an excellent source of clean energy, the study of survival of this microalga in tough environmental conditions is believed to help scientists understand the evolution of earth by mimicking the relics of the metabolism during anaerobic past [30, 38].

Paramylon: a polysaccharide from *Euglena* with multiple benefits

Paramylon is a water-insoluble storage polysaccharide generated by *Euglena* in the presence of a utilizable carbon source [44], which accounts for approximately 85% of the dry weight of the alga under aerobic conditions [30, 45]. Paramylon is a β-1-3-glucan-type polysaccharide unlike starch, the typical carbon storage polymer, which is α-1,4/6-glucan [30, 46]. In addition to supporting the survival of *Euglena* during nutrient-depleted conditions, paramylon has several economic and health benefits to humans and animals. The β-glucan present in paramylon exhibit several health benefits such as immunostimulatory and antioxidant properties [47, 48]. It is reported to have activities against bacteria such as *Escherichia coli* and *Staphylococcus aureus* and also against viruses such as HIV and Human Influenza virus (H1N1) [49–51]. Studies have shown that paramylon from *E. gracilis* protects mice from hepatic injury and inhibits the development of atopic dermatitis-like lesions [48, 52]. Other studies have shown that the application of an adhesive film made from paramylon is reported to speed up wound healing [53] and daily consumption of paramylon improves the quality of the sperm in male rats [54]. Moreover, because of its homogeneous and fine structure, paramylon is increasingly being used in the cosmetics industry [55].

Anti-cancer properties of *Euglena*

As discussed earlier, the dried powder from *Euglena* or their extracts in water or organic solvents has great medicinal value because of their antimicrobial, antiviral, anti-obesity activities. Moreover, *Euglena* and their extracts are proven to exhibit anti-mutagenic, anti-inflammatory, and anti-cancer properties [45, 56]. Studies from several decades ago have reported the potential involvement of the paramylon from *Euglena* in reducing the risk of cancer [57]. Feeding the paramylon (β-glucan) from *Euglena* results in antitumor activity against preneoplastic colonic aberrant crypt foci in mice [36] highlighting its preventive effects against

colon cancer. Similarly, Euglenophycin, an alkaloid toxin found in certain *Euglena* species has antitumor properties against colorectal cancer [58, 59]. The methanolic extracts of Euglenoids such as *Euglena tuba* showed significant inhibition of human lung (A549) and breast cancer (MCF-7) cells *in vitro* through induction of apoptosis, indicating an anticancer role of the extract [60]. Another *in vitro* study on the ethanol fractionated extract of *Euglena viridis* reported its cytotoxic activity against prostate cancer cell lines such as PC3, Du145, and colon cancer cell lines like HCT-116 [61].

Extensive studies from our lab have shown that a partially purified water extract from *Euglena gracilis* (EWE) devoid of mature paramylon granules suppresses the growth of lung cancer cells *in vitro* and in the orthotopic lung carcinoma allograft model in mice [45]. We found that the EWE treatment inhibited cell growth of both murine Lewis lung carcinoma (LLC) and human lung carcinoma cells (A549 and H1299) in a dose- and time-dependent manner in a two-dimensional cell culture system [45]. The spheroid growth of LLC cells was also significantly attenuated by EWE treatment in a three-dimensional spheroid culture model. Furthermore, in a mouse LLC orthotopic allograft model, EWE pretreatment three weeks prior to the LLC cell inoculation, but not post-treatment after LLC cell inoculation, significantly attenuated the growth of LLC tumors in immunocompetent syngeneic mouse lung highlighting the role of the water extract from *Euglena gracilis* in preventing lung carcinoma growth [45]. The anti-lung cancer properties of *E. gracilis* and possible mechanisms are discussed in the following section.

Role of the microbiome in health and disease

Microbiome refers to the collective genomes of the microorganisms, and microbiota is the community of microorganisms themselves [62]. The human body comprises a diverse range of microbiomes such as bacteria, archaea, viruses, and eukaryotic microbes. These microbiomes

play a vital role in the health and disease of the host. Since microbiota can influence the physiological function of the body such as metabolic functions and immune homeostasis [63], there is growing interest in studying the association of the alteration of microbiota with the development or pathogenesis of several diseases. This research area is emerging, but it is very challenging to establish the cause and effect for the specific disease condition. While many pieces of evidence indicate a fundamental role of microbiota in the susceptibility of diseases, it should also be noted that the diseased condition can alter the microbiota. Thus, there remains a multitude of questions to be answered. Below, we will highlight the gut microbiota and its capability to influence lung cancer. Later, we will discuss the role of *Euglena gracilis* in modulating the gut-lung axis aiming to use *Euglena* as an anti-lung cancer agent.

Gut microbiota

The microbiota colonizes on various body parts such as skin, genitourinary, gastrointestinal, and respiratory tracts. The surface of the gastrointestinal tract (GIT) represents the major area for microbial colonization because it is rich in nutrients for the microbes. The number of microorganisms residing in the GIT is estimated to be more than 10^{14}, which is 10 times more than the number of cells present in the human body [64].

Among the different human symbiotic microbial populations, the gut microbiota that consists mostly of bacteria, some fungi, archaea, and viruses, is known to affect host immune homeostasis locally and systemically [65, 66]. The core of the gut microbiome is comprised of four bacterial phyla: Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria. Other major but less represented phyla include Fusobacteria, Verrucomicrobia, and Spirochaetes [67].

Gut-lung axis

Although the gut and lung are separate organs, emerging experimental evidence has highlighted a crucial intersection between the gut microbiota and the lungs, opening a new field of research termed the "gut-lung axis" [67]. Any alteration in microbial composition and function in either the respiratory tract or GIT has been associated with alterations in immune responses and disease development in the lungs [68]. The gut microbiota is involved in various vital functions, many of which are beneficial for the host health including protection against pathogenic microbiota, production of vitamins, absorption of ions, etc. [69]. More importantly, the gut microbiota is essential for the development, training, and function of the immune system both locally and at distant sites such as the lungs [67, 70]. Any disturbance in the compositions or the functions of the gut microbiota that may be caused by modulation of diet, environment, disease, or therapies result in changes in the immune responses and/or the microbe-derived metabolites that could lead to lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), and lung cancer [71, 72].

Gut microbiota dysbiosis in lung cancer

The increasing number of studies that are characterizing the role of the dysbiosis of gut microbiota in lung cancer have shown significant differences in the gut bacterial composition of lung cancer patients in comparison to healthy controls [71–73]. A 16s rRNA gene sequencing of fecal samples from patients with lung cancer showed increased levels of the phylum: Bacteroidetes, Fusobacteria, Cyanobacteria, Spirochaetes, and Lentisphaerae compared to the healthy volunteers. However, the lung cancer patients had considerably lower levels of Firmicutes and Verrucomicrobia than the healthy control population [73]. At the genera levels, reduced numbers of *Enterobacter*, *Dialister*, *Fecalibacterium*, *Kluyvera*, *Escherichia*–

Shigella, and higher numbers of *Fusobacterium*, *Bacteroides*, and *Veillonella* were found in lung cancer patients compared to the control [73]. Another study has linked reduced numbers of *Actinobacteria*, *Bifidobacterium*, and increased numbers of *Enterococcus* to lung cancer [74]. The levels of *Akkermansia munciphilia*, a member of the Verrucomicrobiota family, are often reduced in patients with lung cancer [75].

Alteration of immune response

The gut microbiota has evolved as a major contributing factor for the pulmonary immune response of the host organism by regulating both innate and adaptive immune systems. Disturbance in the normal gut microbiota exposes the intestinal mucosal barrier to damaging agents and harmful chemicals that may trigger local and systemic inflammation leading to lung cancer [71]. The damage to the gut mucosa by chemicals such as bile acids and invaders such as short filamentous bacteria induces immune cells that, most likely through chemokine-induced homing, migrate from the gut to the lungs to exert their functions [71]. Bile acid exposure in the gut results in increased levels of inflammatory markers such as IL-1β, IL-6, and IL-8 in the lung [76]. Meanwhile, elevated levels of another metabolite/lipokine called 12,13-diHOME decreases the anti-inflammatory responses in the lungs by reducing the number of pulmonary T regulatory cells [77]. Similarly, short filamentous bacteria in the gut distantly stimulate lung pathology by preferentially recruiting the bacteria-induced gut T helper 17 cells (Th17 cells) to the lung owing mostly to the robust expression of the chemoattractant CCL20 in the lungs [78]. Furthermore, postnatal growth restriction induces intestinal dysbiosis and activates intestinal TLR4 increasing the levels of IL-1β in peripheral circulation that stimulates the NF-κB pathway leading to systemic inflammation of the lungs [79]. More importantly, antibiotic-induced dysbiosis in gut microbiota leads to faster progression of melanoma and Lewis lung cancer in mice [80]. This

tumor progression is mainly mediated by the local and systemic suppression of TNFα levels resulting in reduced expression of tumor endothelial intercellular adhesion molecule-1 (ICAM-1) and a subsequent decrease in the number of activated and effector $CD8^+$ T cells in the tumor [80].

Alteration of gut microbiota-derived metabolites

In addition to regulating the host immune functions, gut microbiota could also participate in modulating the metabolism of the host that could affect local and distant organs [81]. There is a dysbiosis in the intestinal flora of patients with lung cancer compared to healthy subjects and these changes in intestinal bacterial composition and metabolism are closely associated with lung cancer [71]. The microbes mostly utilize the accessible carbohydrates in the gut such as fermentable fiber to produce short-chain fatty acids (SCFAs) that can shape lung immunity. The dietary fermentable fibers are known to alter the ratio of Firmicutes to Bacteroidetes that are suggested to be important in the production of SCFA [65]. Acetate, propionate, and butyrate are the major SCFAs that are synthesized in the intestinal lumen of the caecum and large intestine by bacterial fermentation [75, 82]. Although produced in the gut, SCFAs can act on distal sites such as the lungs because of their permeable nature into the bloodstream [83]. The SCFAs generated in the gut then exert pleiotropic functions on the lungs by influencing the immune cells [70].

SCFA-induced T-cell receptor signaling is thought to be one of the primary pathways in the communication between gut and lung [65]. Studies have shown that butyrate, acetate, and propionate protect against airway inflammation by inhibiting the histone deacetylases (HDACs) and by activating the fatty acid receptors GPR43 [65]. Propionate induces the Th2 effector cells in the lungs [84]. Acetate is shown to enhance the number and function of T-regulatory cells via HDAC inhibition leading to the suppression of allergic airways disease [85]. A recent study

showed an association between dysbiosis in the butyrate-producing bacteria in the gut and non-small cell lung cancer (Gui et al., 2020). Furthermore, microbiota-derived butyrate inhibits the growth of lung cancer by inducing various cell cycle-related proteins *via* p53-dependent as well as p53-independent mechanisms [86]. Similarly, butyrate also suppresses the growth of the A549 lung cancer cells by upregulating the microRNA such as miR-3935 [87]. Propionate induces lung cancer cell apoptosis and cell cycle arrest, highlighting the beneficial role of SCFAs against lung tumorigenesis [88].

Gut microbiota as therapeutics

As discussed earlier, gut microbiota plays a vital role throughout the course of lung cancer genesis and progression by modulating the immune system and microbiota-derived metabolites. This modulation is caused by many factors, but presumably, the most important things are in daily food intake. Therefore, it is conceivable that functional foods like prebiotics and probiotics that contain beneficial bacteria will have favorable influences in controlling and preventing cancer.

Experimental studies have shown probiotics to increase the efficacy of anti-lung cancer medicines [89]. *Akkermansia munciphilia* consumption is positively correlated with the antitumor effect of cisplatin in LLC-tumor-bearing mice [90] and it enhances the role of immune checkpoint inhibitor responses in advanced lung cancer patients treated with anti-PD-1 antibodies [91, 92]. *Enterococcus hirae, Barnesiella intestinihominis, and Lactobacillus acidophilus* also increase the anti-cancer effect of both cyclophosphamide and cisplatin in mice with lung cancer [93, 94]. Other probiotics work by suppressing the metastasis of cancers to the lung via immune modulation. For example, *Enterococcus faecium κ50* and *Saccharomyces cerevisiae 14κ* inhibit metastasis in mice with Lewis lung carcinoma and markedly elevate the

efficacy of antitumor vaccine *in vivo* [95]. Studies have also shown the treatment with a probiotic (*Lactobacillus casei* CRL 431) containing milk to reduce the metastasis of breast cancer cells to the lung [96, 97]. *Bifidobacterium infantis* also suppresses the tumor growth and increased the survival of C57BL/6 mice with lung cancer [98] highlighting its potential use as a probiotic.

In addition to the direct consumption of microbiota by prebiotics, probiotics, and fecal microbiota transfer, functional foods rich in microbial metabolites such as SCFAs could also be an attractive alternative to treating lung cancer because of their role in suppressing lung cancer progression as highlighted earlier.

Another exciting and more feasible approach would be the consumption of microalgae such as *E. gracilis* that not only enhance the beneficial bacterial population in the gut but also enhance the levels of microbe-derived metabolites such as the SCFAs. The prospects of using *Euglena* as a lung cancer preventative and therapeutic measure are discussed below.

Euglena gracilis as an anti-lung cancer agent by modulating the gut-lung axis

There is an increasing interest in the usage of algal biomass as well the bioactive compounds derived from algae as functional ingredients to enhance the nutritional value of food and to provide additional health benefits. Mounting experimental evidence has revealed the alteration of intestinal microbiota and/or their metabolites upon administration of microalgae [99–101].

Numerous gut bacteria have been characterized as having protective roles against cancers [69]. More importantly, gut microbiota influences carcinogenesis and immunotherapy in lung cancer [71]. Therefore, it is foreseeable that microalgae-based “functional foods” will gain increasing popularity for preventing and treating certain human diseases such as lung cancers by modulating the gut microbiome and/or metabolites of the host.

As discussed in detail in chapters 2, 3, and 4 of this dissertation, using different models of the lung cancer mouse model, we show that the administration of EWE through drinking water suppresses the growth of lung cancer *in vitro* and *in vivo*. In chapter 2, we provide evidence that EWE pretreatment inhibits lung carcinoma growth mainly by stimulating host antitumor immunity through attenuation of granulocytes. In chapter 3, we establish that the consumption of *Euglena* extracts significantly alters the intestinal microbiota of the host, contributing to the decreased tumor burden. Finally, in chapter 4, we demonstrate oral administration of water extract from *Euglena gracilis* attenuates lung tumorigenesis induced by tobacco-specific carcinogens through modulation of gut metabolites. Our study shows that the bioactive components in *Euglena gracilis* launch attack against cancer from three different frontiers of immunity, gut microbiota, and metabolites. This trifecta of attack makes *E. gracilis* an excellent candidate for furthering the studies on safety and utility as anti-lung cancer therapeutic. Already approved by the US Food and Drug Administration (FDA) as Generally Regarded as Safe (GRAS) for human consumption [102], the outlook of using *E. gracilis* to prevent and treat lung cancer seems promising.

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Chapter 2 - Water Extract from *Euglena gracilis* Prevents Lung Carcinoma Growth in Mice by Attenuation of the Myeloid-Derived Cell Population

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Abstract

The partially purified water extract from *Euglena gracilis* (EWE) was evaluated for its antitumor and immunomodulatory effects in cell cultures and in a mouse orthotopic lung carcinoma allograft model. In two-dimensional cell culture, the EWE treatment inhibited cell growth of both murine Lewis lung carcinoma (LLC) and human lung carcinoma cells (A549 and H1299) in a dose- and time-dependent manner. In contrast, the growth of mouse bone marrow cells (BMCs), but not mouse splenocytes (SPLs), was stimulated by the treatment with EWE. In three-dimensional spheroid culture, spheroid growth of LLC cells was significantly attenuated by EWE treatment. In a mouse LLC orthotopic allograft model, pretreatment with EWE (150–200 mg/kg/day, *via* drinking water) three weeks prior to the LLC cell inoculation, but not post-treatment after LLC cell inoculation, significantly attenuated the growth of LLC tumors in immunocompetent syngeneic mouse lung. This tumor growth attenuation coincided with a significant decrease in the population of myeloid-derived cells, primarily neutrophils. Flow cytometric analysis revealed that the EWE treatment significantly attenuated growth of granulocytic myeloid-derived suppressor cells (gMDSC) in BMCs and that this decrease was due to induction of gMDSC-specific apoptosis and differentiation of monocytic MDSCs (mMDSC) to macrophages. The present study provides evidence that EWE pretreatment inhibits lung carcinoma growth mainly by stimulating host antitumor immunity through attenuation of growth of gMDSCs and decreasing the number of peripheral granulocytes. This study suggests that the partially purified extract derived from *Euglena gracilis* contains significant bioactive materials that prevent lung carcinoma growth.

Keywords: water extract from *Euglena gracilis*; cancer cell growth inhibition; inhibition of pro-tumorigenic immunity; lung cancer; inhibition of MDSC, apoptosis

Introduction

Lung cancer is the leading cause of cancer-related morbidity and mortality in developed countries and remains one of the deadliest cancers even in non-smoking populations [1, 2]. Furthermore, many other cancers tend to metastasize to the lung and form metastatic lung cancer lesions [3-5]. Therefore, novel cancer prevention and therapeutic strategies for both primary and metastatic lung cancers are urgently needed, and successful lung cancer prevention interventions would provide enormous economic and practical benefits to both patients and governments. However, lung cancer prevention is one of the major unmet needs in cancer research. Current strategies for lung cancer prevention have had limited success because only non-specific cancer prevention strategies are available, including avoidance of chemical carcinogens such as tobacco smoke and radon and adherence to a healthy diet and exercise regimen [6].

Anticancer immunity is the central player in spontaneous inhibition of cancer growth [6, 7]. The current success of immunotherapy against multiple immunogenic cancers, including non-small cell lung cancers [8-12] supports this notion. Accordingly, the development of safer, less toxic and more effective enhancers of anticancer immunity would be beneficial for cancer prevention. Although many natural products have been claimed as anticancer agents, including mushrooms [13, 14], multiple types of algae [15, 16] and spirulina [17, 18], their efficacies and molecular mechanisms have not been rigorously established. Importantly, there has been no emphasis on lung cancer prevention despite its current ranking as the deadliest cancer both in the US and worldwide.

Euglena gracilis is a unicellular photosynthesizing green alga, found in both fresh and saltwater, and has features of both animals and plants. This alga is motile by its flagellum and contains a variety of nutrients including amino acids, carbohydrates, vitamins, and minerals;

therefore, it is taken as a nutritional and functional dietary supplement [19]. In addition, it has been shown that whole dried powder and/or water or polar organic solvent extracts of *Euglena species* have medicinal properties, such as antimicrobial [20], anti-mutagenic [21], anti-viral [22], and antitumor [23-25] activities. *Euglena gracilis* is known to store a large amount of β-1,3-glucan as a water insoluble storage granule of paramylon which was shown to be usable as an immunostimulant or an immunopotentiator [26, 27]. These studies suggest that *Euglena* extract and paramylon may be usable as an antitumor agent and that its tumor growth inhibition is attributable to the stimulation of lymphocytes and/or related cytokines [26-29]. However, the molecular mechanism by which *Euglena* extract or paramylon stimulates immune responses is yet to be fully understood. In addition, most studies describing biological functions of *Euglena* or its extract have used *Euglena* extract containing paramylon. It is of interest to study the biological activities of *Euglena* extract without mature paramylon, which is insoluble in aqueous solution [19, 30, 31], in association with cancer prevention and therapy. Here we report for the first time that pretreatment with a water extract derived from *Euglena gracilis*, devoid of both mature paramylon and intact chloroplasts, inhibits the growth of murine lung carcinoma cells in cell culture and in a mouse allograft model *via* a decrease in populations of myeloid-derived immune suppressor cells and peripheral granulocytes.

Materials and Methods

Animals

Wild-type female C57BL/6 mice were obtained from Charles River Laboratories International, Inc. All mice were housed in a clean facility and held for one week to acclimatize. All mice were kept under controlled conditions of temperature (20–26 °C), with 30–70 % relative humidity and light (12:12 h light-dark cycles). The mouse condition was observed every

day. The mouse body weights were monitored every other day. All experiments were carried out under approvals of the Kansas State University Institutional Animal Care and Use Committee (Protocol # 3857) and Institutional Biosafety Committee (Protocol # 1050). All animal experiments were done under strict adherence to IACUC protocols set by Kansas State University (Manhattan, KS).

Materials

The murine Lewis lung carcinoma (LLC) cell line (CRL-1642), human lung carcinoma cell lines H1299 (CCL-5803) and A549 (CCL-185), and human lymphoblast cell line Jurkat (TIB-152) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's Modified Eagle's Medium (DMEM) and RPMI 1640 was obtained from Mediatech, Inc. Ham's F-12 Nutrient Mixture was purchased from Fisher Scientific (Pittsburgh, PA). The fetal bovine serum (FBS) was from EQUITECH-BIO Inc. (Kerrville, TX). Penicillin-streptomycin stock was purchased from Lonza Rockland, Inc. (Allendale, NJ). Fluorescent conjugated antibodies targeting CD45 (30-F11), CD4 (H129.19), CD8b (YTS156.7.7), CD19 (6D5), CD11c (N418), F4/80 (BM8), LY6G (1A8), CD11b (M1/70), Annexin V and isotype IgG, and 7-aminoactinomycin D (7-AAD) were purchased from BioLegend (San Diego, CA).

Preparation of EWE devoid of water insoluble mature paramylons

Dried powder of *Euglena gracilis* was obtained from euglena Co., Ltd. (Tokyo, Japan). It is composed of 29.4% carbohydrates, 42.3% protein, and 19.0% lipid. Approximately 70–80% of the carbohydrate content is paramylon [22]. Water extract (EWE) of the *Euglena gracilis* dry powder was prepared by incubating this powder in autoclaved Milli-Q water (20 mg dry powder/ml) at 37 °C for 30 min with periodic sonication for 30 s (Fig. 1A: whole *Euglena* suspension). The whole mixture was centrifuged at 2000*g* for 20 min. Water insoluble mature

paramylon granules separated, forming a white layer on top of the precipitate (Fig. 1B: paramylon-rich fraction). The supernatant was lyophilized, and the resultant dry material was dissolved in sterile PBS (40 mg dry material/ml) and centrifuged at 12800*g* for 10 min. The half-transparent supernatant was filtered through a 0.22 µm sterile disk filter (Midwest Scientific, Valley Park, MO) (Fig. 1C: filtered EWE). All mature paramylon granules (granule size > 0.2–0.3 µm diameter) were removed by these two steps of centrifugation and membrane filtration. The dry weight was measured after drying a 200 µl aliquot at 55 °C for 48 hrs. This partially purified EWE was stored at −20 °C and was subjected for the current study. To confirm a lack of contamination from bacterial LPS in EWE, the Limulus amebocyte lysate (LAL) assay was conducted using a commercially available kit (Pierce Chromogenic Endotoxin Quantitation Kit, Thermo Fisher Scientific, Waltham, MA, USA). The assay results clarified that EWE contains negligible amount of bacterial LPS (−0.003±0.006 ng in 100 µg/ml EWE).

Electron microscopy

For scanning electron microscopy (SEM), samples of the whole *Euglena* suspension, paramylon-rich fraction and filtered EWE in PBS were fixed with Trump's fixative (1% glutaraldehyde and 4% formaldehyde in a 0.1 M phosphate buffer at pH 7.4) overnight at 4 °C, post-fixed with 1% osmium tetroxide in a 0.2 M phosphate buffer for 1 hrs, and dehydrated with a series of graded ethanol solutions. Ethanol in the dehydrated sample solution was replaced with hexamethyldisilazane by centrifugation and the samples were sputtered with palladium using Denton Vacuum Desk II sputter coater (Denton Vacuum, Moorestown, NJ, USA). Sputter-coated samples were analyzed using Hitachi S-3500N Scanning Electron Microscope (Hitachi Science Systems Ltd., Tokyo, Japan) at an accelerating voltage of 10 kV.

Cell culture

The LLC murine lung carcinoma cell line was cultured with DMEM. The H1299 human lung carcinoma cell line, and Jurkat human lymphoblast cell line were cultured with RPMI1640. The A549 human lung carcinoma cell line was cultured with Ham's F-12. The medium was supplemented with 10% v/v FBS and 1% v/v Penicillin-streptomycin. These cells were cultured at 37 °C in a humidified air atmosphere containing 5% CO_2.

Growth inhibition of lung carcinoma cells by EWE in two-dimensional (2D) cell culture

The murine (LLC; 500 cells/well) and human (H1299, 1500 cells/well, and A549, 3000 cells/well) lung carcinoma cells were seeded into a 96-well plate with 100 µl growth medium. After 24 hrs, the cells were treated with EWE. The dose- (1, 10, 100 and 1000 µg/ml EWE) and time- (48 and 72 hrs) dependent effects of EWE on cell viability were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [32]. PBS was used as the negative control.

EWE-dependent growth stimulation of T lymphoblasts, splenocytes and bone marrow cells *in vitro*

The effect of EWE on the cell proliferation of bone marrow cells (BMCs) and splenocytes (SPLs) was evaluated by MTT assay. C57BL/6 mice were sacrificed by exposing saturated CO_2 followed by cervical dislocation. BMCs and SPLs were cultured as described previously [33]. BMCs (2×10^5 cells/well), and SPLs (5×10^5 cells/well) were cultured in a 96-well plate. Cell proliferation was evaluated dose-dependently (1, 10 and 100 µg/ml EWE) and time-dependently (24, 48, 72, and 96 hrs at 1 and 100 µg/ml EWE). As a positive control, 100 ng/ml LPS was used. PBS was used as the negative control.

Analysis of the effect of combination treatment with EWE and T lymphoblasts on the spheroid growth of LLC cells in three-dimensional (3D) culture

In order to evaluate the combined effect of EWE and T cells, a 3D spheroid assay was performed as described previously [33]. EWE (50 μg/ml) was treated twice at Day 1 and Day 4. Images of the spheroids were taken by inverted microscope IX51 (Olympus America Inc., Center Valley, PA) equipped with cellSens Dimension software (Olympus) at Day 8 after seeding the cells. The spheroid growth was evaluated by spheroid volume at Day 8.

Treatment of mice with EWE *via* drinking water

The cancer prevention effect of EWE was evaluated using an orthotopic LLC cell allograft growth in C57BL/6 mice (n=6). Three treatment regimens were designed: (1) PBS control, (2) 3-week pretreatment of 150 or 200 mg/kg/mouse/day EWE prior to LLC cell injection and (3) 300 mg/kg/mouse/day post-treatment after LLC cell injection (Fig. 4A). The oral administration of EWE (150 and 200 mg/kg/day) *via* autoclaved drinking tap water was carried out at 3 weeks prior to the tail vein inoculation of LLC cells (2×10^6 cells/mouse) and continued throughout the study period of 5 weeks. PBS was used as the control with the same schedule. The EWE doses (0.75, 1 and 1.5 mg/ml) were adjusted based on the average water consumption (4 ml/mouse/day). Daily intake of drinking water containing EWE, which was filtered through a 0.22 μm sterile membrane, was monitored by measuring the weight of water pouches every other day. Two weeks after LLC cell injection, all mice were sacrificed by cervical dislocation after exposure to saturated CO_2 and blood was collected by cardiac puncture for flow cytometry analysis as described below. The lung, spleen, both kidneys, liver, and intestine were collected to examine their weights and fixed in 10% formalin for histological

analysis. The tumor weights in the lungs were calculated by subtracting average weight of an age-matched normal mouse lung (142.3±11.9, *n*=6) from that of the lung in each group.

TUNEL Assay

The TUNEL assay was carried out using the APO-BRDU-IHC (TUNEL) Apoptosis Kit (Novus Biologicals, Centennial, CO) as previously described [33]. The average number of TUNEL positive cells in 10 random fields (*n*=6) were calculated.

Flow cytometry analysis of leukocytes in the peripheral blood of EWE pretreated LLC tumor-bearing mice

Leukocytes in peripheral blood of EWE pretreated LLC tumor-bearing mice were collected at the end of the mouse study. After removing red blood cells using a red blood cell lysing buffer, the leukocytes were immunostained using anti-CD4 (helper T cells), anti-CD8b (cytotoxic T cells), anti-CD19 (B cells), anti-CD11c marker (dendritic cells), anti-F4/80 (macrophages) and anti-LY6G (granulocytes) antibodies, and their population distributions were evaluated by flow cytometry. Mouse IgG was used for the isotype control. The changes of cell populations were analyzed by flow cytometry (BD LSRFortessa X-20; BD Biosciences, San Jose, CA, USA) and analyzed by BD FACSDiva software (BD Bioscience).

Flow cytometric analysis of myeloid cell population and their apoptotic status in the BMCs treated with EWE *in vitro*

BMCs were collected from hind legs of C57BL/6 mice. Bone marrow cells (5×10^6 cells) were seeded into a 6-well plate and incubated with 100 μg/ml EWE. The distribution of myeloid cells was evaluated by flow cytometry using anti-CD11c marker (dendritic cells), anti-F4/80 (macrophages), anti-LY6G (granulocytes), anti-CD11b (myeloid cells), and anti-LY6C (monocytes) antibodies. Monocytic (mMDSC) and granulocytic (gMDSC) myeloid-derived

suppressor cell (MDSC) were identified as populations that show phenotypes of $CD11b^+$ $LY6G^+$ and $CD11b^+$ $LY6C^+$, respectively. Apoptotic status in MDSCs was evaluated using anti-Annexin V antibodies and 7-AAD. Live cells, early apoptotic cells, late apoptotic cells and dead cells were identified as populations which showed phenotypes of Annexin V^- 7-AAD^-, Annexin V^+ 7-AAD^-, Annexin V^+ 7-AAD^+, and Annexin V^- 7-AAD^+, respectively.

Statistical analysis

All values are expressed as the mean ± standard division of mean. For all *in vitro* and *in vivo* experiments, statistical significance was assessed by an unpaired *t*-test or ANOVA followed by Tukey's test. All experiments were conducted with multiple sample determinations. Statistical significance was set at $P<0.05$.

Results

Preparation of EWE devoid of water insoluble mature paramylon granules

To evaluate the effect of an extract from *Euglena gracilis* (EWE) devoid of mature paramylon on the growth of lung cancer, water extraction with two-step centrifugation was conducted as described in the Materials and Methods section. The components from three different fractionations (i.e. the whole *Euglena* suspension, paramylon-rich fraction and filtered EWE) were morphologically examined using SEM. As shown in Fig. 1A, the whole euglena suspension contained whole cell components including a large number of paramylon granules (arrows in Fig. 1A). After the first centrifugation of the whole *Euglena* suspension, a vast majority of water insoluble paramylon granules were segregated as a white layer on top of the whole precipitate. The SEM image of this paramylon-rich fraction clearly showed that the characteristic granules were removed from the EWE. The size of the granules (1–6 μm) and a circular or oval disk shape were consistent with paramylon granules reported previously (Fig.

1B) [19, 34]. The SEM images prepared from filtered EWE suggest that most, if not all of the granules were removed from the EWE (Fig. 1C). These results suggest that EWE prepared in the present study was essentially free of paramylon granules.

EWE treatment attenuated the growth of murine and human lung carcinoma cells in 2D cell culture

To clarify the effect of the water extract from the *Euglena gracilis*, EWE, on the growth of lung cancer cells, an MTT assay was conducted. First, the specificity of EWE's effect on several lung cancer cells was evaluated. Murine lung carcinoma cells (LLC) and A549 and H1299 human lung carcinoma cells were treated with EWE (1–1000 μg/ml) for 48 and 72 hrs in 2D cell culture. The EWE treatment dose- and time-dependently attenuated growth of all three lung carcinoma cells ($P<0.05$, Fig. 2A). Among these three cell lines, growth of both human cell lines (H1299 and A549 cells) was sensitively inhibited at the lower dose (1 μg/ml), but LLC cells required a higher dose to exhibit a significant growth inhibition. The dose-dependent growth inhibition curves clearly indicated that the growth of LLC and H1299 cells was effectively inhibited by the EWE treatment, whereas A549 cell growth was poorly inhibited even at the high dose of EWE (1000 μg/ml). These results suggest that EWE treatment dose- and time-dependently attenuates the growth of both murine and human lung cancer cells in a specific way and is not merely nonspecifically cytotoxic.

EWE treatment stimulated the growth of bone marrow cells but not splenocytes

It has been reported that *Chlorella vulgaris* extract is capable of stimulating the growth of immune cells [35-39]. In the present study, therefore, the growth stimulation effect of EWE on immune cells was evaluated using bone marrow cells (BMCs) and splenocytes (SPLs). EWE treatment (1–100 μg/ml) significantly increased the growth of BMCs in a dose- and time-

dependent manner, but not SPLs (Fig. 2B). This EWE-dependent growth stimulation effect for BMCs was detected at a lower concentration at 1 μg/ml (Fig. 2B). A positive control, LPS exhibited a growth stimulation effect on both BMCs and SPLs. These results suggest that immature leukocytes such as lymphoblasts and myeloblasts are more sensitive to EWE-dependent growth stimulation than peripheral leukocytes.

EWE treatment attenuated the spheroid growth of murine lung carcinoma cells

As shown in Figs. 3A and 3B, EWE treatment (50 μg/ml) significantly attenuated the spheroid growth of LLC cells as compared with the PBS-treated group at days 8 and 10 ($P<0.05$). However, there is no additional growth inhibition was observed at day 10 as compared to the spheroid size at day 8 (data not shown). Although spheroid growth was also attenuated in the presence of T lymphoblasts (Jurkat cells), a significant difference was not confirmed compared with group receiving EWE alone (Fig. 3A). On the other hand, Jurkat cells co-cultured with an LLC spheroid showed significant morphological changes, such as cell enlargement (average of 3.3-fold increase in cell size), granule generation in the cytoplasm, and filopodia morphology, in the presence of EWE (right bottom image in Fig. 3C). These results suggest that EWE may assist only limited T lymphoblast maturation in the tumor microenvironment.

Pretreatment with EWE *via* drinking water attenuated the growth of orthotopic LLC murine lung carcinoma in mice

To evaluate possible antitumor effects of EWE *in vivo*, EWE (150–300 mg/kg/day) was administered by drinking water either three weeks prior to or after LLC cell inoculation *via* tail vein injection (2×10^6 cells/mouse, $n=6$/group, Fig. 4A). Water consumption was monitored every other day and found to be relatively consistent regardless of EWE addition. First, the antitumor effect of pretreatment (150 mg/kg/day) or post-treatment (300 mg/kg/day) with EWE *via*

drinking water was evaluated (Fig. 4A). As shown in Fig. 4B, tumor growth tended to be attenuated for the group pretreated with 150 mg/kg/day EWE (86.5±65.4 mg, n.s.) as compared with the PBS control group (187.3±123.5 mg). However, this attenuation was not observed in the group post-treated with 300 mg/kg/day EWE (190.4±122.0 mg). Further evaluation of the antitumor effect of EWE pretreatment was conducted by administering 200 mg/kg/day EWE to mice using the same pretreatment protocol. As shown in Fig. 4D, the PBS control group developed several macroscopic tumor nodules in the entire lung, whereas the EWE treated group developed only a few small tumor nodules. Average tumor weight (mg) in the EWE treated group (11.7±22.5, $P<0.05$) was significantly smaller than that of PBS treatment group (114.8±43.1) (Fig. 4E). EWE treatment *via* drinking water did not alter growth of the mouse body weight (Fig. 4C) nor the average weight of spleen, kidneys, liver, and intestine (Fig. S1). In the histological analysis using H&E sections, no metastatic tumor or pathological abnormality was detected in these organs. Immunohistochemical analysis of apoptotic cells in tumor nodules clearly suggested that the EWE treatment significantly increased apoptotic cells in EWE-treated LLC tumors compared to PBS-treated tumors (Fig. 4F). These results clearly indicate that EWE treatment is effective in inhibition of lung carcinoma growth in mice. In addition, comparison of various leukocyte populations in peripheral blood collected from tumor-bearing mice pretreated with 200 mg/kg/day EWE indicated a significant decrease of the $LY6G^+$ granulocyte population in EWE treated mice (Fig. 5A). Taken together, these results suggest that EWE *via* oral or intraperitoneal administration possesses a negligible direct antitumor function, whereas pretreatment with EWE oral administration attenuated lung tumor growth through an enhancement of host antitumor immunity by attenuating granulocyte/myeloid-derived cell population.

EWE treatment decreased myeloid-derived suppressor cell (MDSC) population by inducing apoptosis and differentiation in BMCs *in vitro*

MDSCs have been identified as obstacles to anticancer immunity owing to their immunosuppressive effects [40]. Since pretreatment with EWE significantly decreased the $LY6G^+$ granulocyte population in the peripheral blood of tumor-bearing mice, the potential effect of EWE on the granulopoiesis in BMCs was analyzed using flow cytometry. As shown in Fig. 5B, treatment with 100 μg/ml EWE significantly decreased the $LY6G^+$ population in BMCs at 48 hrs after treatment. Further analysis of the data revealed that the $CD11b^+$ $LY6G^+$ granulocytic MDSC (gMDSC) population was decreased with statistical significance by EWE treatment (Fig. 5B). On the other hand, EWE treatment increased the $CD11b^+$ $LY6C^+$ monocytic MDSC (mMDSC) population in BMCs. In addition, the $F4/80^+$ macrophage population, but not the $CD11c^+$ dendritic cell population, was increased by EWE treatment. Furthermore, an analysis of apoptosis determined by Annexin V and 7-AAD clarified that the EWE-dependent decrease of granulocytic MDSC population is mainly due to an induction of early apoptosis (Fig. 6). LPS treatment, used as a positive control for modulation of BMCs, significantly increased the number of $CD11c^+$ dendritic cells, $F4/80^+$ macrophages and $CD11b^+$ $LY6C^+$ mMDSCs, whereas $LY6G^+$ granulocyte and $CD11b^+$ $LY6G^+$ gMDSC populations were markedly decreased by the LPS treatment (Fig. 5B). These results suggest that EWE treatment can directly attenuate the gMDSC population *via* an induction of apoptosis and alter the mMDSC population by inducing differentiation to macrophage. Comparative analysis with the LPS study suggests that the EWE effect on MDSCs is functionally similar to that by the LPS. However, since only a negligible amount of LPS is detected in the EWE (−0.003±0.006 ng in 100 μg/ml EWE), it is suggested that the EWE effect on the MDSC regulation is primarily due to distinct EWE component(s).

Discussion

While green algae and their extracts are a potential food source and can serve as dietary supplements with high nutritional value, an increasing number of publications indicate that extracts from green algae are also potential sources for therapeutics against various diseases including cancers [41]. *Euglena* is a genus of single cell flagellate eukaryotes that contains at least 800 species. The *Euglena gracilis* species has been widely studied as a model organism in the laboratory. So far, however, neither *Euglena* nor its extracts have seen extensive use in medicinal fields. Furthermore, although *Euglena* has only recently started to be consumed by humans through *Euglena*-based food or beverage supplements, recent discoveries indicate that the extract from *Euglena gracilis* possesses medicinal properties, such as anti-mutagenic [21], anti-viral [22], and antitumor [23-25] activities. *Euglena gracilis* is known to store a large amount of β-1,3-glucan as a paramylon which is an insoluble storage form of β1,3-glucan and was shown to be usable as an immunostimulant or an immunopotentiator [26, 27, 42]. Dectin-1 is shown to be a receptor for β1,3-glucan [42-45] as well as paramylon [46], and appears to be associated with innate immune responses. Therefore, most studies describing biological functions of *Euglena* or its extract have used *Euglena* extract containing paramylon. It is of interest to study the biological activities of *Euglena* extract without mature paramylon in association with cancer prevention and therapy. Accordingly, we have prepared a water extract devoid of water insoluble mature paramylons from *Euglena gracilis* whole power termed EWE (Fig. 1C) and evaluated its antitumor and immune modulatory abilities in *in vitro* and *in vivo*.

The effect of EWE on the growth of murine and human lung cancer cell lines was evaluated in both 2D and 3D cell cultures. EWE significantly and dose-dependently attenuated the growth of LLC murine and H1299 human lung carcinoma cells in 2D cell culture (Fig. 2A).

However, EWE-dependent growth attenuation of another human lung adenocarcinoma cell line, A549, was moderate even at 1000 μg/ml (Fig. 2A). Although both H1299 and A549 cell lines are derived from non-small cell lung carcinoma, H1299 cells were established from metastatic lesions in the lymph node and more malignant, notably possessing the P53 deletion mutation [47, 48], whereas A549 cells are from adenocarcinomic alveolar basal epithelial cells and maintain normal epithelial cell characteristics [49]. LLC cells are also established by transplantation of Lewis lung carcinoma, loosely adherent or floating in culture, and exhibit more aggressive cell growth. Although only three lung cancer cell lines were tested, EWE exhibited stronger growth inhibition in the cell lines possessing malignant characteristics (LLC and H1299) than the cell line with normal epithelial characteristics (A549). A significant growth inhibition effect was also detected in 3D spheroid culture with or without the T lymphocyte coculture (Fig. 3). It is known that 3D culture more closely mimics natural tumor growth than 2D culture [50]. For this reason, 3D culture methods such as clonogenic assay [51-53] and spheroid assay [50] have been applied to the evaluation and screening of novel therapeutics for cancer treatments. The results of the present study, therefore, may suggest that the cytotoxic antitumor effect of EWE alone is strong enough to inhibit 3D spheroid growth of LLC cells within a short time.

On the other hand, EWE stimulated growth of the BMCs for short incubation times, but not SPLs (Fig. 2). At longer times, EWE-dependent growth stimulation is not observed, presumably due to the natural gradual death of BMCs. Although both BMCs and SPLs are primary cultured cells, gradual death of BMCs with time is a typical observation [33]. Distinct responses of BMCs and SPLs appear to be due to their cell types; BMCs contain various cell types including a variety of myeloid cells, whereas the majority of SPLs are composed of

differentiated lymphocytes and their life span is much longer than those of BMCs [54]. These results may also suggest that treatment with EWE influences myeloid cells but not lymphocytes.

The ability of EWE to inhibit the growth of lung carcinoma cells in both 2D and 3D cell culture studies and its growth stimulation of primary cultured BMCs with negligible cytotoxicity (Fig. 2B) compelled an *in vivo* test of the efficacy of EWE. In the present mouse study, the effect of three doses of EWE (150 and 200 mg/kg/day dissolved in PBS for pretreatment and 300 mg/kg/day for post-treatment) against the orthotopic LLC tumor growth was evaluated by administering EWE *via* drinking water. The EWE administrations were started either three weeks prior to (pretreatment) or on the same day as LLC cell inoculation (post-treatment). As shown in Figs. 4B, 4E and 4F, the pretreatment with EWE, but not the post-treatment, dose-dependently and significantly attenuated the growth of murine lung carcinoma cells by inducing apoptosis in tumor cells as compared to the PBS treated mice. The noteworthy observation is that strong tumor growth attenuation was observed only in pretreatment groups of mice. Taken together with flow cytometry analysis of peripheral blood collected from tumor-bearing mice (Fig. 5A), these results suggest that EWE effectively inhibited the growth of lung carcinoma cells directly or indirectly through suppression of myeloid-derived cells, thereby significantly attenuating lung tumor growth in mice. We postulate that one of the major antitumor mechanisms by which EWE inhibited tumor growth in mice is through the attenuation of myeloid cell population. This hypothesized mechanism is supported by a significant decrease of the $LY6G^+$ granulocyte population in EWE treated mouse blood (Fig. 5A) and a significant attenuation of the growth of granulocytic MDSCs by EWE in BMCs *in vitro* (Fig. 5B). Although the EWE-dependent attenuation of the gMDSCs *in vitro* with BMCs is not as much as the granulocyte decrease in the mouse study, this small attenuation is presumably due to the short

duration of the *in vitro* study. In support of this hypothesis, neutrophils, the primary population of $LY6G^+$ granulocytes, have been shown to be pro-tumorigenic [55-57]. Furthermore, decrease of the MDSC population has been shown to increase effector T cell function, thereby attenuating tumor growth [58, 59]. To establish that gMDSCs are the major players in EWE-induced lung cancer prevention further studies by blunting the MDSC by blocking antibody treatment to show a direct cause-and-effect relationship are warranted.

These results suggest that the direct inhibition of cancer cell growth by EWE, observed in the cell culture study (Fig.2A), is very weak or negligible *in vivo* when EWE is administered orally or IP after the tumor cell inoculation. Although it is possible that the negligible direct therapeutic effect of EWE *in vivo* may be due to the poor bioavailability of the active substance in EWE or weak direct inhibition of cancer cell growth *in vivo*, clarification of the therapeutic effect of EWE *in vivo* requires an additional study with multiple EWE doses and evaluation of the pharmacokinetics. In addition, the present study also suggests that EWE might contain multiple bioactive substances; one substance may directly inhibit the growth of lung cancer cells, but a different substance may stimulate growth of BMCs *in vitro* (Fig. 2). Another substance may cause population reductions of both granulocytes and gMDSC *in vivo* (Fig. 5). To the author's best knowledge, the present study is the first to demonstrate that Euglena-derived materials devoid of mature paramylon show antitumor and immunomodulatory activity against lung cancer. Future work to identify EWE's active components will help determine the multiple effects on cancer and immune cells.

Conclusion

The *Euglena gracilis* extract described here, EWE, can dose-dependently inhibit both murine and human lung carcinoma cells, while significantly stimulating the growth of primary

cultured mouse BMCs. However, the EWE treatment significantly attenuated the granulocytic MDSC population in primary cultured mouse BMCs. In a 3D spheroid culture, treatment with EWE significantly attenuated the spheroid growth of murine carcinoma cells. In a murine orthotopic lung carcinoma model with syngeneic mice, EWE pretreatment significantly attenuated tumor growth along with a decrease in granulocyte number in the peripheral blood. Although further studies are required to confirm the *in vivo* safety of EWE by orthodox pharmacokinetics, pharmacodynamics, and multispecies toxicity studies, this data shows that EWE could be an orally administrated agent that can diminish two types of pro-tumorigenic leukocyte population, thereby preventing lung cancer growth.

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Figures- Chapter 2

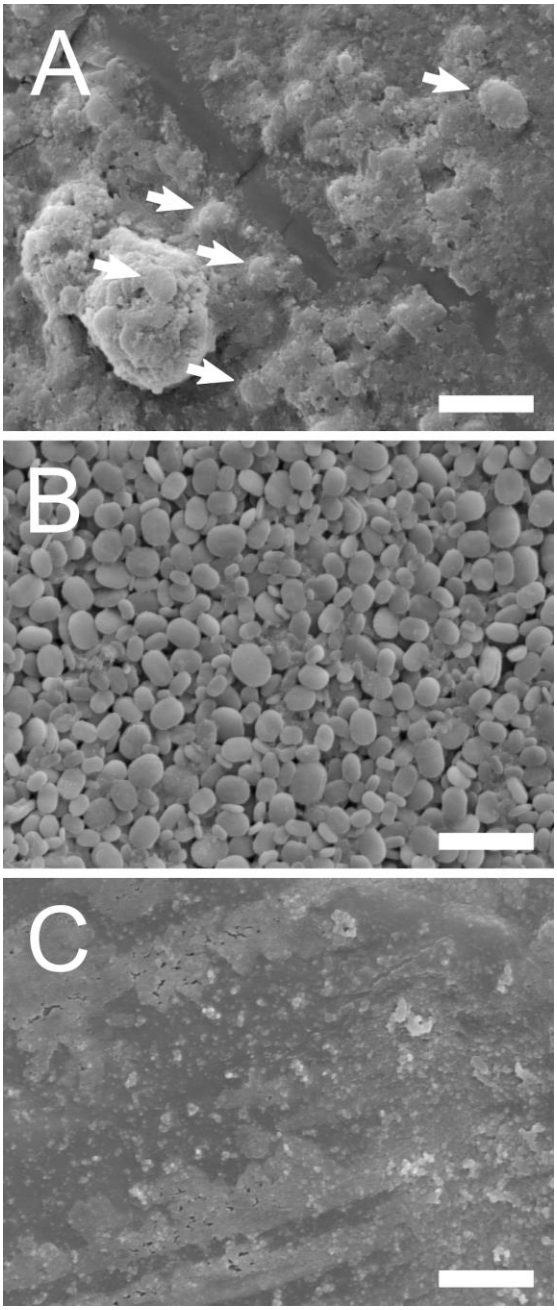

Figure 2.1. Scanning electron micrographs of whole *Euglena* suspension (A), paramylon-rich fraction isolated by centrifugation (B), and the EWE prepared as described in the method (C).

Arrows in panel A indicate paramylons which are buried in other components originating from *Euglena*. Panel C clearly indicates that EWE does not contain any paramylon. The scale bar, 10 μm.

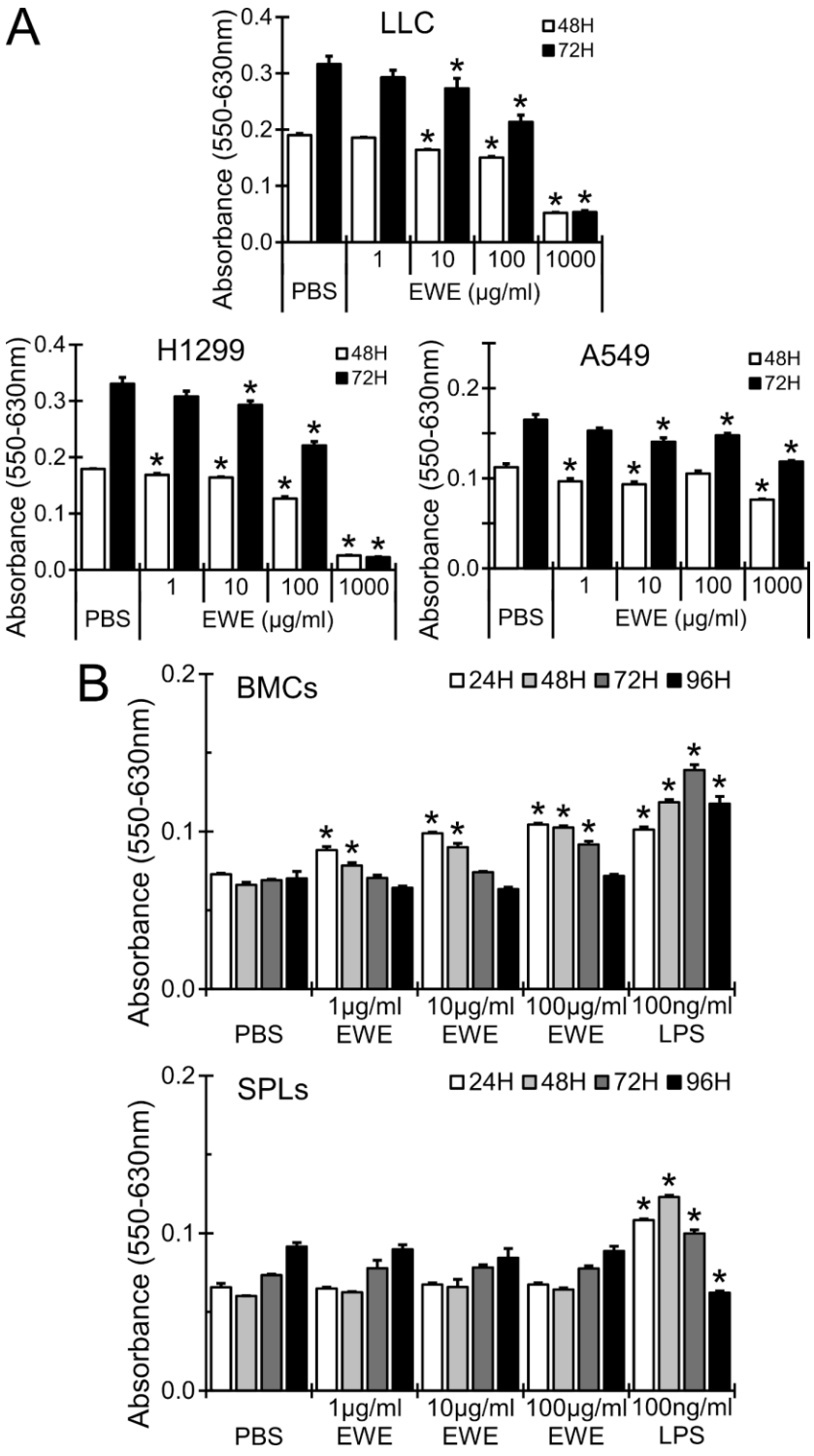

Figure 2.2. EWE treatment dose-dependently attenuated the growth of LLC murine lung carcinoma cells, and H1299 and A549 human lung carcinoma cells, while it dose-dependently stimulated the growth of mouse bone marrow cells (BMCs) but not splenocytes (SPLs) in 2D cell culture.

Results are presented as mean ± SD (n=3). *, P<0.05 compared to naïve untreated cells.

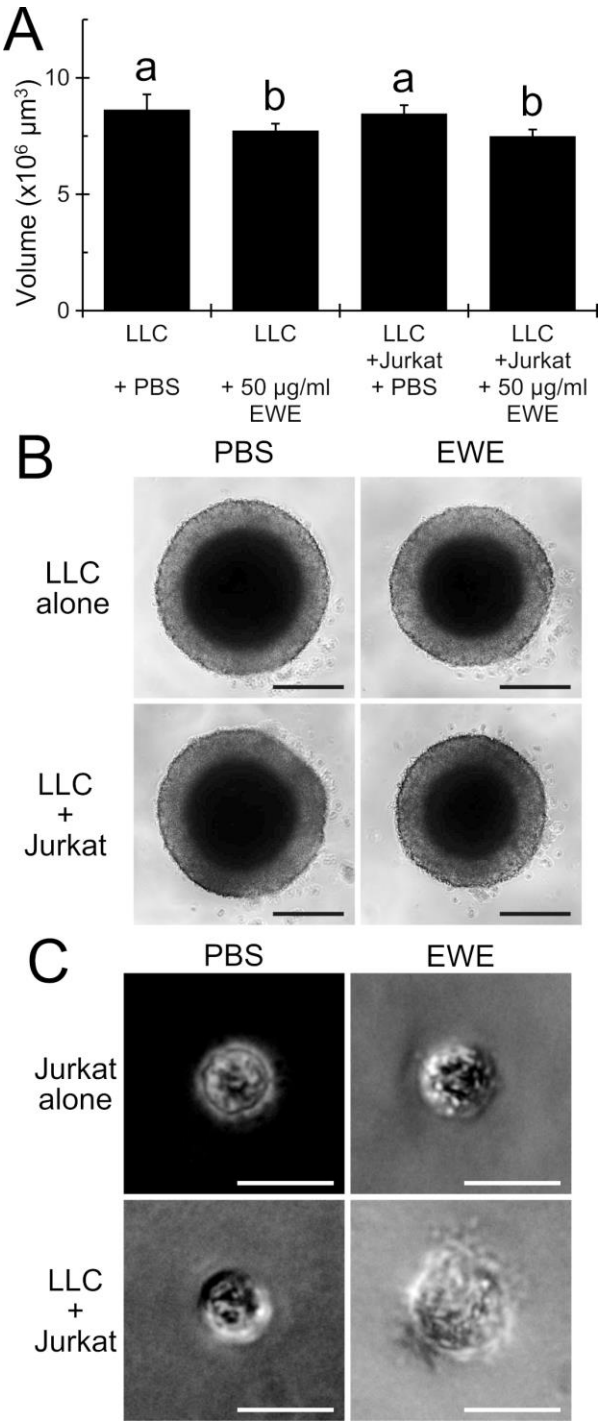

Figure 2.3. EWE treatment attenuated the growth of LLC spheroids.

(A), The volume of the spheroid was measured at Day 8. Results are presented as mean ± SD (*n*=5). a–b, *P*<0.05 between different characters. (B) Typical pictures of a spheroid in each group. The scale bar in each picture represents 100 µm. (C) Typical morphology of Jurkat c cells in agar matrix of each treatment group. The scale bar in each picture represents 20 µm.

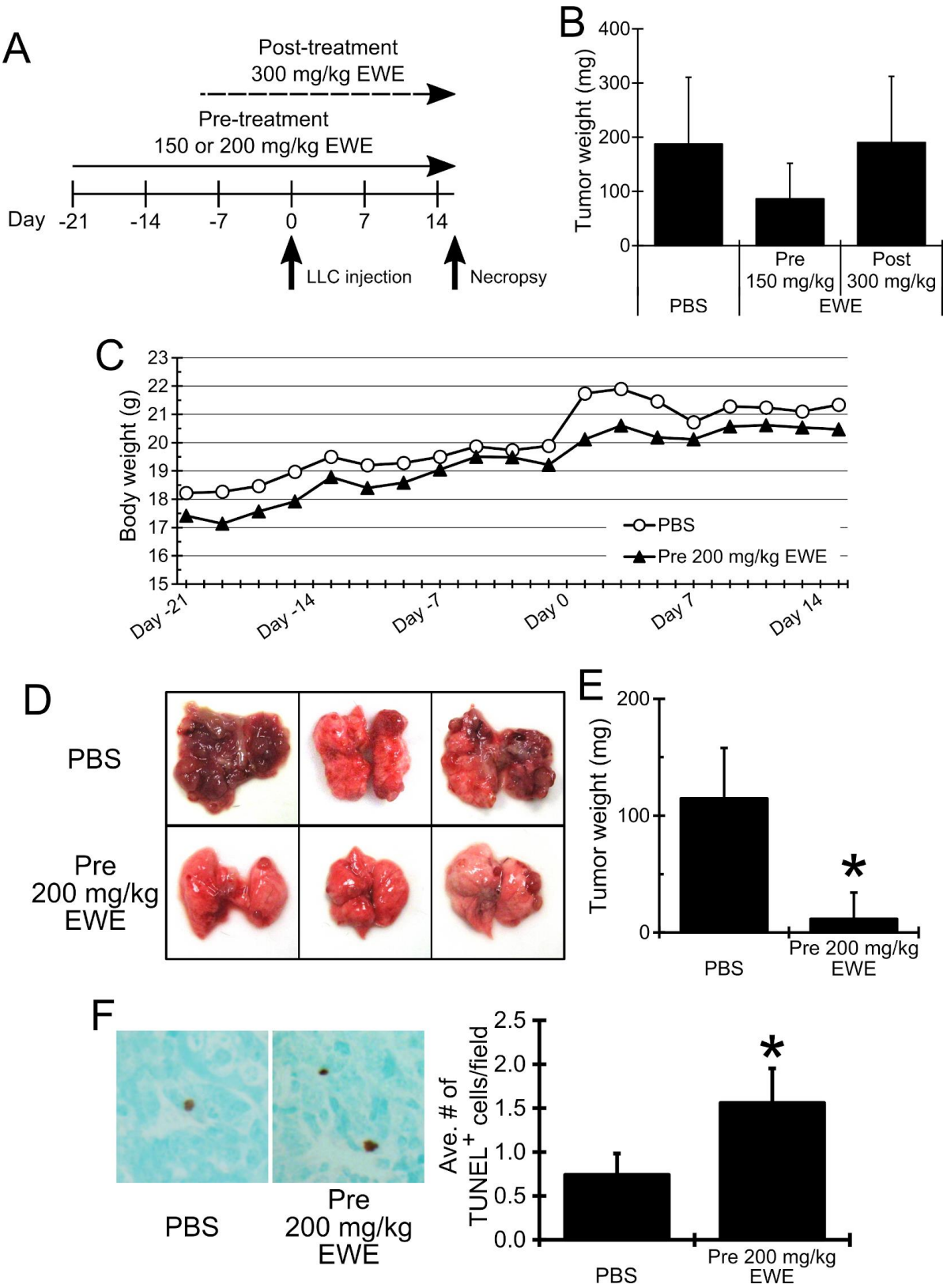

Figure 2.4. EWE treatment significantly attenuated the growth of LLC tumors in mouse lung.

(A), Schematic illustration of the study design. (B), Pretreatment, but not post-treatment, of EWE attenuated LLC tumor growth in the lung. (C), Body weight change in 200 mg/kg/day EWE pretreatment group. PBS served as control. (D), Macroscopic views of the lungs from LLC tumor-bearing mice in PBS or EWE pre-treatment group. The scale bar in each picture represents 5 mm. (E), Average tumor weight in each treatment group. (F), The TUNEL positive cells were significantly increased in the EWE-treated tumors. Results are presented as mean ± SD (n=6). The scale bar in each picture represents 20 μm. *, P<0.05 as compared to the level of the PBS-treated group.

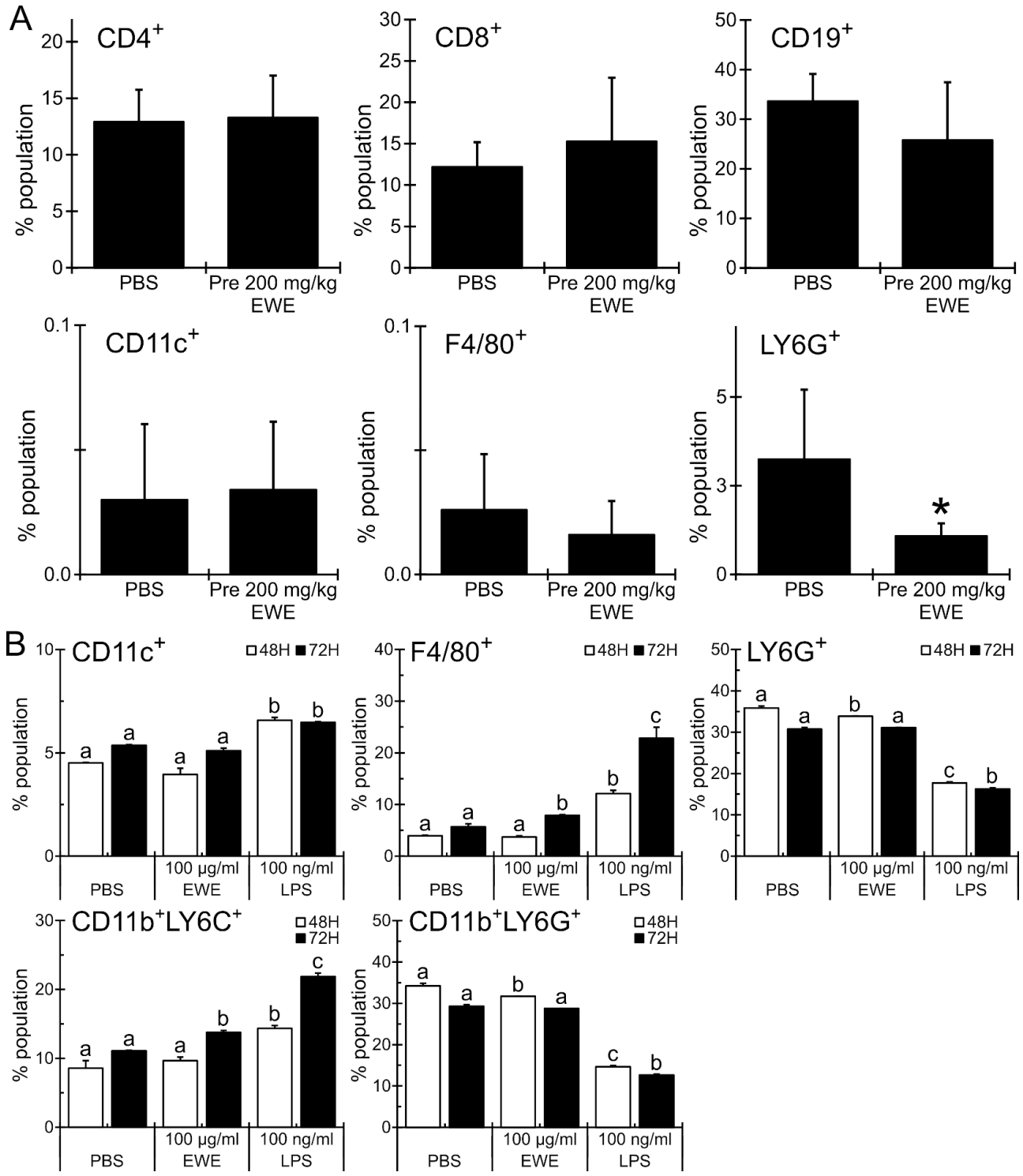

Figure 2.5. EWE treatment decreased granulocyte in peripheral blood of mice bearing LLC cell tumors and granulocytic MDSC in BMCs.

(A), Leukocyte population in peripheral blood collected from LLC tumor-bearing EWE pretreated mice. $CD4^+$ and $CD8^+$ T cells, $CD19^+$ B cells, $CD11c^+$ dendritic cell, $F4/80^+$

macrophage and $LY6G^+$ granulocyte populations were analyzed by flow cytometry. Results are presented as mean ± SD (*n*=6). *, *P*<0.05 compared with PBS control group. (B). Myeloid cell population in EWE-treated BMCs in cell culture. $CD11c^+$, $F4/80^+$, $LY6G^+$, $CD11b^+$ $LY6C^+$ (monocytic MDSC, mMDSC) and $CD11b^+$ $LY6G^+$ (granulocytic MDSC, gMDSC) population were analyzed by flow cytometry after 48 and 72 hrs after EWE treatment (100 μg/ml). EWE treatment significantly decreased number of gMDSC in BMC culture. PBS and 100 ng/ml LPS were treated as negative and positive control, respectively. Results are presented as mean ± SD (*n*=3). a–c, *P*<0.05 between different characters in each time point.

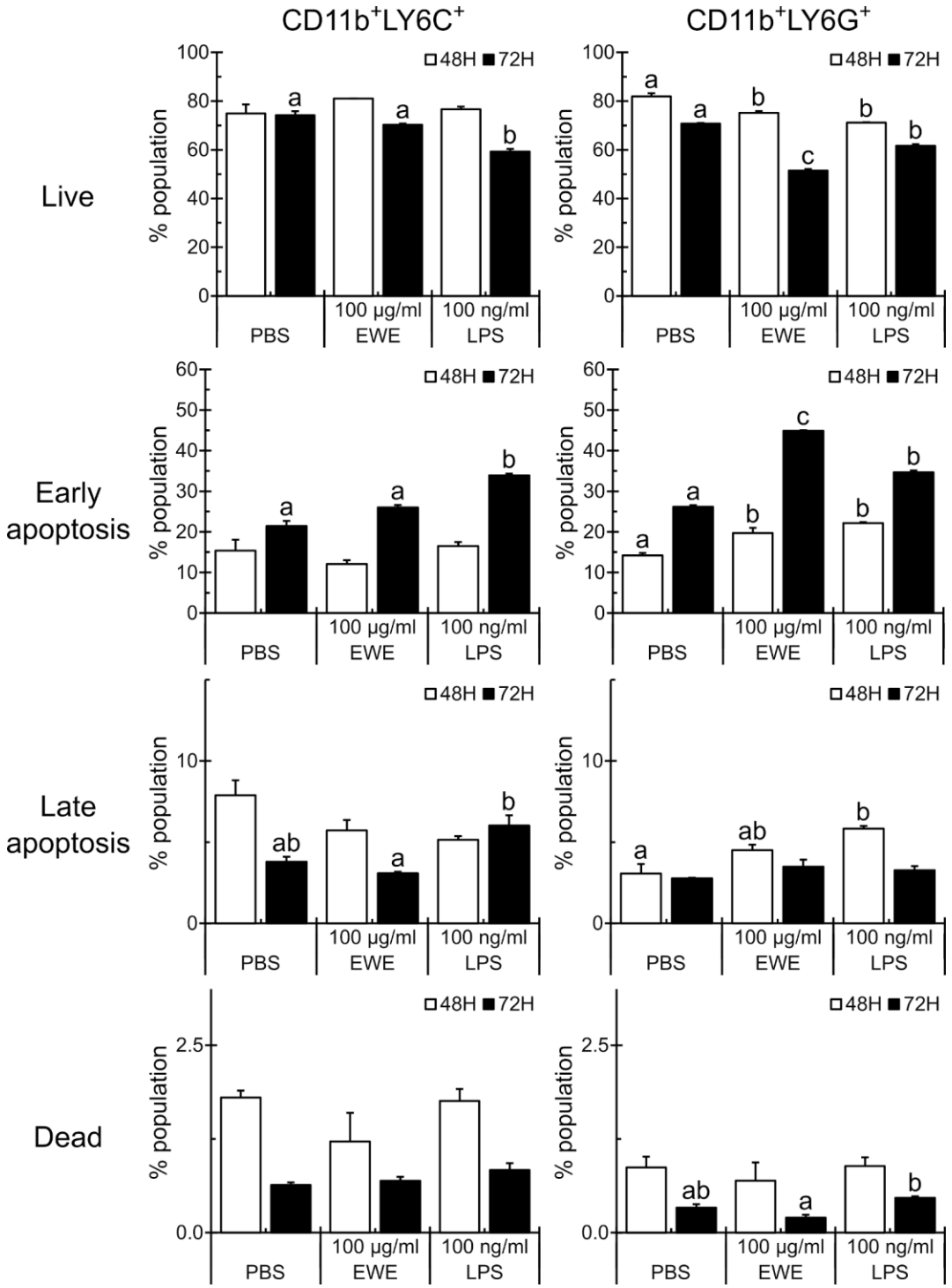

Figure 2.6. EWE treatment significantly increased apoptosis in gMDSC in BMCs.

The MDSC population summarized in Fig. 5B was further analyzed for their apoptotic status by flow cytometry. EWE treatment (100 µg/ml) significantly induced apoptosis in $CD11b^+$ $LY6G^+$ gMDSC population. Results are presented as mean ± SD (n=3). a–c, P<0.05 between different characters.

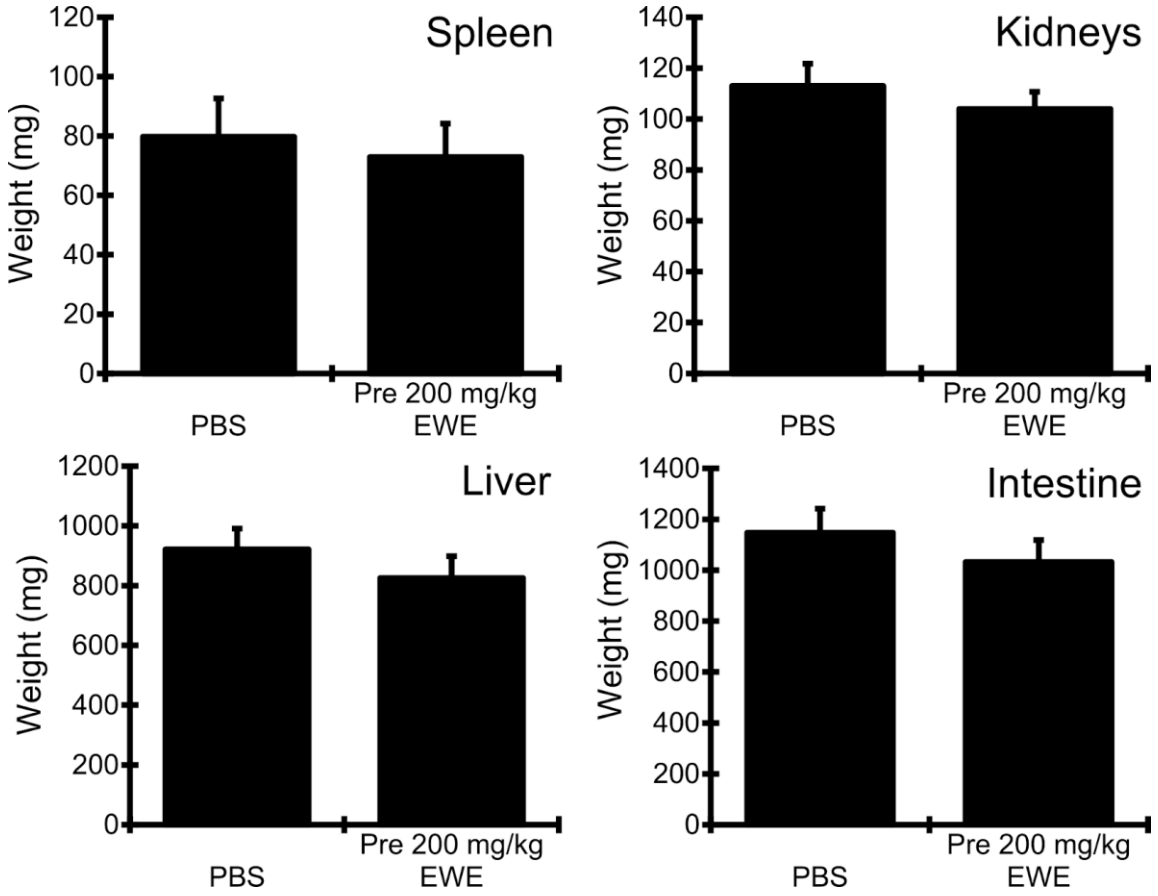

Figure S 2.1. Organ weight from 200 mg/kg/day EWE pretreated LLC tumor-bearing mice.

Spleens, kidneys, livers and intestines were collected and measured the weight. Results are presented as mean ± SD (n = 6).

Chapter 3 - Oral Administration of Water Extract from *Euglena gracilis* Alters the Intestinal Microbiota and Prevents Lung Carcinoma Growth in Mice

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Abstract

The antitumor effects of a partially purified water extract from *Euglena gracilis* (EWE) and EWE treated by boiling (bEWE) were evaluated using orthotopic lung cancer syngeneic mouse models with Lewis lung carcinoma (LLC) cells. Daily oral administration of either EWE or bEWE started three weeks prior to the inoculation of LLC cells significantly attenuated tumor growth as compared to the phosphate buffered saline (PBS) control, and the attenuation was further enhanced by bEWE. The intestinal microbiota compositions in both extract-treated groups were more diverse than that in the PBS group. Particularly, a decrease in the ratio of Firmicutes to Bacteroidetes and significant increases in *Akkermansia* and *Muribaculum* were observed in two types of EWE-treated groups. Fecal microbiota transplantation (FMT) using bEWE-treated mouse feces attenuated tumor growth to an extent equivalent to bEWE treatment, while tumor growth attenuation by bEWE was abolished by treatment with an antibiotic cocktail. These studies strongly suggest that daily oral administration of partially purified water extracts from *Euglena gracilis* attenuates lung carcinoma growth via the alteration of the intestinal microbiota.

Keywords: natural product; water extract from *Euglena gracilis*; lung cancer prevention; gut microbiota alteration; fecal microbiota transplantation

Introduction

Lung cancer is a commonly diagnosed cancer type in both sexes and the leading cause of cancer-related deaths, comprising one in five cancer-related deaths worldwide [1]. Furthermore, lung cancer remains a leading cause of cancer-related death even among individuals who have never smoked [2,3]. Although many new strategies for cancer prevention and treatment are being implemented, lung cancer is predicted to cause the greatest number of deaths in 2021, in comparison to other cancer types [4]. In addition, many cancers tend to metastasize in the lungs [5], resulting in life-threatening conditions. Therefore, novel prevention and therapeutic strategies for lung cancer are urgently needed.

Euglena gracilis (*E. gracilis*) is a single-celled, flagellated, fast-proliferating microalga that is rich in nutrients [6]. This alga can be found in both fresh and saltwater and possesses characteristics of both plants and animals [7]. *E. gracilis* is used as a nutritional dietary supplement because it is rich in carbohydrates, amino acids, minerals, chlorophyll, and vitamins [8]. *E. gracilis* also serves as an excellent source of valuable substances such as paramylon (a water-insoluble granule or linear form of β-1,3-glucan), tocopherol, and carotenoids with pharmaceutical potentials [9]. *E. gracilis* with paramylon is known to have an immunomodulatory effect in both humans and animals, playing a role as an immunostimulant or an immunopotentiator [10,11]. Studies have revealed that *E. gracilis* has potential as a therapeutic due to its antimicrobial [12], antioxidant [13], anticancer [14], antiviral [15–17], and antihyperglycemic properties [18]. Furthermore, our previous study showed that the partially purified extract derived from *E. gracilis* prevents lung carcinoma growth in mice by stimulating host antitumor immunity through the attenuation of myeloid-derived suppressor cell (MDSC) populations [19].

The gut is colonized by complex bacterial populations, many of which are beneficial for the health to the host, including protection against pathogenic microbiota, production of vitamins, absorption of ions, etc. [20]. More importantly, the gut microbiota is essential for the development, training, and function of the immune system both locally and at distant sites [21,22]. Moreover, gut microbiota-derived metabolites, such as short-chain fatty acids (SCFA), can influence host immune responses [23]. Emerging evidence demonstrates vital crosstalk between the gut microbiota and the lung through a shared mucosal immune system termed the "gut–lung axis" [21], highlighting the impact of gut microbes in lung diseases such as asthma, chronic obstructive pulmonary disease, and lung cancer [24]. In fact, there is a significant difference in the gut bacterial composition of lung cancer patients in comparison to healthy controls [25]. These studies highlight the potential of manipulating the gut microbiota and/or metabolites as a therapeutic approach for lung diseases.

Our previous finding that the oral administration of partially purified extracts of *E. gracilis* prevents the growth of lung cancer in mice indicates a possible interaction along the gut–lung axis [19]. Another study reported that the components of *E. gracilis* stimulate the growth of gut microbes such as *Faecalibacterium* to improve digestive health by producing butyrate, an SCFA [26]. Here we examine the mechanism behind the prevention of lung carcinoma in mice upon daily administration of *E. gracilis* water extract. We find that the oral administration of *Euglena* water extract alters the gut microbiota and plays a role in the attenuation of lung carcinoma growth. Our findings indicate that water extract from *E. gracilis* could be used as a prebiotic for the prevention of lung cancer.

Materials and Methods

Animals

Wild-type female C57BL/6 mice were purchased from Charles River Laboratories International, Inc. and were housed in a clean facility under controlled conditions of temperature (20–26 °C), with 30–70% relative humidity and light (12:12 h light-dark cycles). All mice were held for a week to acclimatize before the treatment. All mice were housed humanely according to university, state, and federal guidelines (AAALAC) in the AAALAC-accredited animal resource facilities of the Kansas State University College of Veterinary Medicine. The condition of the mice was observed daily, and body weights were measured every three days. All experiments were carried out under approvals of the Kansas State University Institutional Animal Care and Use Committee (Protocol # 4346) and Institutional Biosafety Committee (Registration # 1317).

Preparation of Extracts from E. gracilis

Dried powder of *E. gracilis* was obtained from Euglena Co., Ltd. (Tokyo, Japan). Water extracts of *E. gracilis* were prepared by suspending this dried powder in sterile phosphate buffered saline (PBS) and incubating it at 37 °C for 30 min with periodic sonication for 30 s. The whole suspension was centrifuged at a low-speed of 34× *g* for 1 min. While the precipitate was separated and used to prepare paramylon water extract (PWE), the supernatant was used to prepare the *Euglena* water extract (EWE) and boiled *Euglena* water extract (bEWE). The precipitate was suspended in PBS, followed by centrifugation at 1240× *g* for 10 min. To obtain the substance denoted PWE, the precipitate derived from the first low speed centrifugation was boiled for 10 min, followed by a high-speed centrifugation at 11,405× *g* for 10 min. The resulting supernatant was used as the PWE. To obtain EWE and bEWE, the supernatant was centrifuged at 11,405× *g* for 10 min and then filtered by a 0.22 μm sterile disk filter (Midwest

Scientific, Valley Park, MO, USA). The difference between EWE and bEWE is that a boiling process was carried out before the filtration step during the preparation of bEWE. The dry weight was calculated after drying a 300 μL aliquot at 55 °C for 48 h from which the dry weight of PBS was subtracted (0.125 mg dry powder/mL). All the partially purified water extracts from *E. gracilis* were stored at −20 °C until further use.

Cell Culture

The mouse Lewis lung carcinoma (LLC) cell line (CRL-1642) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Mediatech, Inc. (Manassas, VA, USA). Fetal bovine serum (FBS) was obtained from Biowest (Riverside, MO, USA). The penicillin-streptomycin stock was obtained from Lonza Rockland, Inc. (Allendale, NJ, USA). The cells were cultured at 37 °C in a humidified air atmosphere containing 5% CO_2. The cell line was authenticated by short tandem repeat (STR) DNA profiling. Both the cells were maintained in low passages (<15) for this study.

Treatment of Mice with *Euglena* Extracts via Drinking Water

The effect of oral administration of EWE and bEWE on lung cancer growth was evaluated using an orthotopic LLC cell syngeneic model in C57BL/6 mice (n = 4–5). Four treatment regimens were used: (1) PBS control, (2) 150–250 mg/kg/mouse/day EWE, (3) 150–250 mg/kg/mouse/day bEWE, (4) 15–25 mg/kg/mouse/day PWE (Figure 1A). The extracts were orally administered daily by drinking water three weeks before LLC cell inoculation via tail vein injection (2×10^6 cells/mouse). Consumption of drinking water containing extracts was monitored by measuring the weight of the water pouches every three days. Based on the average water consumption (Figure S2), doses of each extract were adjusted to maintain the desired

dosage. Each mouse was monitored daily for signs of illness. Three weeks after LLC cell injection, all mice were sacrificed by cervical dislocation following exposure to saturated CO_2. The lung, spleen, both of the kidneys, liver, and intestine were collected to examine their weights and fixed in 10% formalin for histological analysis. The tumor weights in the lungs were calculated by subtracting the average weight of an age-matched normal mouse lung (142.3 ± 11.9, $n = 5$).

Fecal Sample Collection and 16S rRNA Amplicon Sequencing

Feces were collected from mice receiving each of the *Euglena* water extracts (EWE, bEWE, or PBS), at three time-points; Day 0 (before the treatment), Day 21 (before LLC cell injection), and Day 42 (before necropsy) and kept at −80 °C for later use. DNA was isolated from fecal pellets (200 mg) from the treated groups using the DNeasy PowerSoil Kit (Qiagen, Germantown, MD, USA). Genomic DNA samples were quantified using the Nanodrop system (Thermo Scientific, Waltham, MA, USA) and kept at −80 °C for further use.

The DNA samples were then processed for 16S rRNA sequencing by the Next Generation Sequencing Service of the Veterinary Diagnostic Lab (Kansas State University). The V3/V4 hypervariable region of the 16S rRNA gene was sequenced as specified by the manufacturer (Illumina, San Diego, CA, USA). Libraries were sequenced on an Illumina Miseq. Raw reads were trimmed for quality using FastQC v0.11.9 and analyzed using Mothur v1.44.1 [27–29]. The unique 16S reads, the output of Mothur (operational taxonomic units (OTUs)), were aligned to reference sequences from the SILVA rRNA database [30]. Near-identical sequences were merged using VSEARCH v2.15.1 [31].

Fecal Microbiota Transplantation

Fecal microbiota transplantation (FMT) was performed to determine the effect of the gut microbiota on the prevention of lung cancer in orthotopic LLC cell allografted mice. Four treatment regimens were performed: (1) PBS control, (2) 150–250 mg/kg of bEWE for 5 weeks prior to and 3 weeks after LLC cell injection, (3) FMT for 2 weeks prior to and 3 weeks after LLC cell injection, (4) 150–250 mg/kg of bEWE treatment throughout the experiment along with antibiotics (ABX) for 2 weeks prior to and 3 weeks after LLC cell injection.

After 3 weeks of bEWE administration via drinking water, feces were collected under sterile conditions and stored at −80 °C until further use for FMT. About 5–6 fecal pellets (about 100 mg) were thawed on ice and re-suspended in 1 mL Milli-Q water. The solution was vigorously mixed for 30 s by vortex and then centrifuged at 800× *g* for 3 min. About 500–600 μL of supernatant was collected and administered by oral gavage (100 μL/mouse/day) immediately after the collection [32]. Depletion of the gut microbiota was performed using a cocktail of ABX consisting of the following: ampicillin (1 g/L) (USBiological Life Sciences, Salem, MA, USA), kanamycin (1 g/L) (Thermo Scientific, Waltham, MA, USA), metronidazole (1 g/L) (Cayman Chemical Company, Ann Arbor, MI, USA), and vancomycin (0.5 g/L) (Cayman Chemical Company, Ann Arbor, MI, USA) as previously described [33,34]. ABX treatment was conducted out via oral gavage (100 μL/mouse/day).

Statistical Analysis

The diversity of microbial populations within samples (alpha diversity) was assessed using the Chao1 estimator and observed indices. Comparison of microbial communities between samples (beta diversity) was evaluated using Principal coordinate analysis plots (PCoA), based on Bray-Curtis distances which demonstrated the dissimilarity of microbiota between samples

and treatments. This was compared using the nonparametric analysis of similarities (ANOSIM) test. Microbiota statistical analysis was carried out using MicrobiomeAnalyst software [35]. All values are expressed as the mean ± standard deviation of the mean. For all the experiments, statistical significance was assessed by an unpaired *t*-test and was conducted with multiple sample determinations. A *p*-value of <0.05 was considered statistically significant.

Results

Pretreatment with EWE and bEWE via Drinking Water Attenuated the Growth of Orthotopic Lung Carcinoma in Mice

The oral administration of water extracts from *E. gracilis* (EWE, bEWE, and PWE) via drinking water in mice was performed to evaluate their possible antitumor effects (Figure 1A). Body weights and water consumption were monitored every three days throughout the study period, and these were relatively consistent (Figures S1 and S2). The average body weights at the end of the study period were 20.6 ± 0.6, 20.6 ± 0.7, 20.6 ± 0.6, and 20.5 ± 0.7 g with PBS, EWE, bEWE, and PWE treatment, respectively. Water consumption was 3.8 ± 0.3, 4.2 ± 0.2, 4.4 ± 0.3, 4 ± 0.3 mL/mouse/day with PBS, EWE, bEWE, and PWE treatment, respectively. As shown in Figure 1B, tumor growth was attenuated in the group pretreated with an average of 202 ± 12 mg/kg/day EWE (tumor weight = 107.0 ± 103.0 mg, $p > 0.05$) as compared with the PBS control group (tumor weight = 203.2 ± 91.6 mg). Treatment with an average of 213.1 ± 13.3 mg/kg/day bEWE further attenuated the growth of LLC tumors in the lungs of the mice (tumor weight = 19.9 ± 31.3 mg, $p < 0.05$). However, this attenuation was not observed in the group treated with an average of 19.7 ± 2 mg/kg/day PWE (tumor weight = 152.8 ± 199 mg, $p > 0.05$). Although the dosage of the PWE is 1/10 of those of EWE or bEWE, this is reflective of the yield of PWE from the same amount of *Euglena gracilis*. As shown in Figure 1C, the EWE- and bEWE-treated

mice developed fewer and smaller tumor nodules as compared to the PBS control group. Either EWE or bEWE treatment via drinking water did not alter mouse growth as measured by body weight (Figure S1). These results indicate that *Euglena* water extracts (EWE and bEWE) are effective in inhibiting the lung carcinoma growth in mice. A noteworthy finding in this study is that the bEWE is significantly more effective than the EWE in inhibiting lung tumor growth. This suggests that the heat treatment of EWE for 10 min in boiling water may be an important step in recovering the anticancer activity of *Euglena gracilis*.

Euglena Water Extracts Alter the Gut Microbiota Composition

Euglena water extracts (EWE and bEWE) reduce lung carcinoma growth in mice (Figure 1), which concurs with our recent study [19]. Since *Euglena* water extracts were administered daily via drinking water, there is a possibility of alteration of intestinal microbiota and/or their metabolisms. We, therefore, analyzed the gut microbiome using 16S rRNA sequencing with the Illumina MiSeq platform in feces from the following groups: PBS (control), mice treated with EWE, and mice treated with bEWE, at three different time points (Day 0: before any treatment, Day 21: before LLC cell injection, Day 42: after LLC cell injection).

At a 97% similarity level, we obtained 25620 OTUs and 11 phyla, 53 families, 104 genera after statistical analysis of 16S rRNA sequences. Statistical analyses including alpha and beta diversity were quantified using observed index and Chao1 index and a principal coordinates analysis (PCoA), respectively. Chao1 estimates the total richness of the microbial community in a sample [36,37]. Our results showed no significant difference in alpha diversity between EWE- or bEWE-treated LLC tumor-bearing mice as compared to the PBS-treated group at both Day 21 and Day 42 (Figure 2A).

Beta diversity with principal coordinate analysis (PCoA) showed that bEWE- and EWE-treated mice had significantly different gut microbiome compositions (Figure 2B. ANOSIM, R = 0.51, $p < 0.01$) as compared with PBS treated mice at Day 42 (ANOSIM, R = 0.18, $p < 0.01$); however, some overlap between EWE and bEWE was observed, likely because both of these extracts were prepared from dried *Euglena* powder. ANOSIM based on Bray-Curtis distance indicated notable differences in microbial communities recovered from the fecal samples of LLC tumor-bearing mice treated with PBS, EWE, and bEWE.

Euglena Water Extracts Caused Alteration in the Gut Microbial Communities

A taxon-dependent analysis was conducted to explore the gut flora phylum and genus that potentially mediate or are closely associated with the attenuation of lung carcinoma growth upon *Euglena* water extracts administration. At the phylum level, the analysis showed the Bacteroidota and Firmicutes were the most abundant phyla identified. We observed that Bacteroidota abundance was significantly higher but Firmicutes abundance was significantly lower in the groups treated with *Euglena* extracts at Day 42 (after LLC cell inoculation) than in the PBS-treated group (Figure 3B,G). Furthermore, bEWE treatment at Day 42 was associated with a significantly higher abundance of Actinobacteriota and Proteobacteria when compared to PBS treatment (Figure 3A,H). We also observed a significantly higher abundance of Verrucomicrobiota, but a significantly lower abundance of Deferribacterota, in groups treated with *Euglena* water extracts at Day 42, when compared to the PBS treated group (Figure 3I,E). Currently, *Akkermansia muciniphila* is the only identified representative species of the phylum Verrucomicrobiota, [38] and *Akkermansia* abundance was also significantly higher with EWE or bEWE treatment (Figure 4A). Further, significantly higher abundance of *Muribaculum* (phylum

Bacteroidota) and significant decrease of *Mucispirillum* (phylum Deferribacterota) was observed at Day 42 with EWE or bEWE treatment, rather than with PBS treatment (Figure 4B,C).

Transplantation of Fecal Microbiota from EWE-Treated Mice Resulted in Attenuation of Lung Cancer Growth

To further understand the role of microbiota in the prevention of lung carcinoma growth in mice by daily oral treatment with *Euglena* extract treated by boiling (bEWE) (Group #1 in Figure 5A), experiments with fecal microbiota transplantation (FMT) (Group #2 in Figure 5A) and antibiotics treatment (ABX) (Group #3 in Figure 5A) were performed as shown in Figure 5A. After 3 weeks of bEWE administration via drinking water, feces were collected and used for FMT for 5 weeks (Group #2 in Figure 5A). From this experiment, we observed that FMT-treatment significantly attenuated the growth of tumors in the mouse lung (349.3 ± 232.0 mg, $p < 0.05$) as compared with the PBS control group (591.2 ± 194.1 mg). The FMT-induced reduction of the lung tumor growth was similar to that of bEWE treated mice, which also experienced a significant decrease in the tumor burden (289.9 ± 98.1 mg, $p < 0.05$) in comparison to the burden of the PBS groups. On the contrary, the ABX treatment reversed the bEWE treatment-induced tumor growth attenuation and the tumor burden (452.7 ± 302.9 mg) was similar to that of the control group. Although a little variation in body weight was observed between FMT and ABX groups (administered through oral gavage) and PBS and bEWE groups (administered via drinking water, Figure S3), these variations were not statistically significant. These results strongly suggest that the intestinal microbiota plays a role in the attenuation of lung carcinoma growth in mice by oral administration of bEWE.

Discussion

Compounds present in organisms of the genus *Euglena* have considerable potential as pharmaceuticals and nutraceuticals. Since *Euglena gracilis* is easy to cultivate and has a faster growth rate than other Euglenophyceae species, it is widely used in basic research as a model organism [9]. Although several studies have been conducted to determine the therapeutic properties of substances obtained from *Euglena* against various diseases, including cancers [14,15,39,40], their effect on the gut microbiota is still not well understood. In the present study, we investigated the effect of oral daily administration of *E. gracilis* water extracts devoid of paramylons on the gut microbiota of mice and its association with the attenuation of lung cancer growth. Pretreatment with two types of *Euglena* water extracts (EWE and bEWE) via drinking water attenuated the growth of orthotopic lung carcinoma in syngeneic mice (Figure 1B). Among two types of extracts, bEWE (EWE with boiling)-induced tumor growth attenuation was much stronger than that by EWE. This result indicates that heat treatment and boiling for 10 min altered either the quality or quantity of antitumor substances in the EWE. Although it is difficult to pinpoint the specific mechanism by which the heat treatment increased the antitumor effect of EWE, due to the unknown chemical nature of this antitumor substance it could be attributed to following possible mechanisms. Firstly, it is conceivable that boiling may have resulted in increasing the solubility and diffusion rate of the bioactive molecules, which could lead to their increased bioavailability. Secondly, boiling and the subsequent centrifugation may have removed a potential inhibitor against antitumor substances in the EWE. Indeed, large denatured molecules have been removed by this process (data not shown). Moreover, boiling has long been a traditional method of extraction of chemical compounds from natural products. Nonetheless, this

experiment warrants a follow-up study to identify the antitumor substance(s) present in *Euglena* extracts.

The mouse study revealed that FMT using stools from bEWE-fed mice attenuated lung carcinoma growth at the same level as in mice who were orally administered bEWE (Figure 5B). We observed that the administration of *Euglena* water extracts (bEWE and EWE) increased the ratio of Bacteroidetes to Firmicutes along with a significant increase in the genera *Akkermansia* and *Muribaculum* in lung tumor-bearing mice (Figures 3B,G and 4). These results suggest that oral administration of partially purified water extracts from *E. gracilis* alters the intestinal microbiota and induces a significant suppression of lung carcinoma growth, presumably due to changes in the bacteria itself and/or their metabolites.

The association between gut microbiota and lung cancer has received increased appreciation after recent studies indicated the involvement of the gut–lung axis in the development of cancers by altering the immune system [41]. In the present study, we found no significant difference in the alpha diversity of gut microbiota in mice treated with *Euglena* water extracts in comparison to the PBS-treated mice. However, the beta diversity analysis showed a significant difference in the microbial composition (Figure 2). It has been reported that the gut microbial composition in healthy humans is significantly different from that in patients with cancers, including lung cancer [25,41,42]. Therefore, an alteration of the gut microbiota composition appears to be significantly influential on carcinogenesis in the lung.

In the current study, we observed significant alterations in the gut microbiota at Day 42 (end of the study period) after LLC cell injection in mice treated with *Euglena* water extracts (Figure 3). The alterations were characterized as a significant increase in the relative abundance of microbes of the phyla Bacteroidota, Verrucomicrobiota, Actinobacteriota, and Proteobacteria,

while there was a significant reduction for the phyla Firmicutes and Deferribacterota (Figure 3). Since bacteria of the phyla Bacteroidota and Firmicutes are most abundant in the gut of humans and mice, any alteration in these bacteria potentially disrupts immunological homeostasis, thereby leading to various disease conditions. In support of this notion, an increase has been reported in the ratio of Firmicutes to Bacteroidota in patients with irritable bowel syndrome (IBS), autism, hypertension, chronic fatigue syndrome, and cancer patients, in comparison to healthy humans [43–46]. Similarly, another study found a higher ratio of Firmicutes to Bacteroidota in lung cancer patients than in healthy controls [47]. The current study found that administration of *Euglena* water extracts significantly decreased lung tumor growth and the ratio of Firmicutes to Bacteroidota. Therefore, it is suggested that daily oral administration of *Euglena* water extracts induces an attenuation of lung carcinoma growth, at least in part by the alteration of the gut microbial ratios of Firmicutes and Bacteroidota.

The current study also found that the abundance of organisms of phylum Deferribacteres and genus *Mucispirillum* was significantly lower in the LLC tumor-bearing mice treated with the *Euglena* water extracts at Day 42 (Figures 3E and 4C). Deferribacteres are intestinal flora that play a role in iron metabolism and are associated with bowel iron balance [48]. Increased iron metabolism is associated with malignant transformation and cancer progression, as cancer cells are highly dependent on iron compared to normal cells [49,50]. Therefore, higher iron metabolism increases the risk of cancers and promotes tumor growth [48]. Although the underlying mechanisms are still not clear, imbalances in pulmonary iron levels are associated with the development of lung cancers [51]. Results in the current study suggest that *Euglena* water extract-induced growth attenuation of lung cancer is possibly associated also with the disruption of iron metabolism.

In our study, the abundance of genera *Akkermansia* and *Muribaculum* was found to be significantly higher in mice treated with *Euglena* water extracts than those treated with PBS at Day 42 (Figure 4). Although the study did not include the species-level identification within *Akkermansia* and *Muribaculum*, the significant increase in abundance for these genera maybe linked to the attenuation of lung carcinoma growth. Currently, *Akkermansia muciniphila* is the only representative species of the phylum Verrucomicrobiota, which typically reside in the large intestine of humans and other animals [38]. *A. muciniphila* has been reported as a beneficial microorganism due to its displayed role in increasing anti-inflammatory functions, improving host metabolism, and enhancing cancer treatment [52,53]. A human study revealed that *A. muciniphila* is less abundant in patients with lung cancer and supplementation of *A. muciniphila* improves the efficacy of immune checkpoint inhibitor (PD-1 blockade) therapy in lung, skin, and renal cancer treatments [54]. In addition, the present study also found that the abundance of *Muribaculum*, a genus belonging to the phylum Bacteroidota, was increased in the LLC tumor-bearing mice treated with *Euglena* water extracts (Figure 4B). Because an increase in *Muribaculum* is associated with better therapeutic efficacy of natural compounds in LLC tumor-bearing mice [55], it is suggested that *Akkermansia* and *Muribaculum* are the gut bacteria that could play a significant role in the *Euglena* water extracts-induced attenuation of lung carcinoma growth in mice.

Conclusions

In summary, the present study revealed that oral administration of *Euglena* water extracts (EWE and bEWE) prevents lung cancer growth in an orthotopic syngeneic mouse model. Daily oral administration of *Euglena* water extracts caused significant alteration in the gut microbiota, including an increase in the ratio of Bacteroidota to Firmicutes, an increase in

Verrucomicrobiota, a decrease of Deferribacteres, and an increase of the genera *Akkermansia* and *Muribaculum*. The FMT and ABX studies confirmed that the attenuation of lung carcinoma growth in mice by *Euglena* water extracts is associated with the alteration of the gut microbiota. The alteration of the gut microbiome may induce microbial metabolite-dependent suppression in cancer growth inhibition. Future studies are needed to identify the bacterial metabolites involved in lung cancer growth attenuation and the mechanisms by which they attenuate the growth of lung cancer. The current study strongly suggests that oral daily administration of *Euglena* water extracts alters the gut microbiota, thereby attenuating lung carcinoma growth. This study indicates that the water extract from *E. gracilis* is a promising prebiotic for the prevention of lung cancer. This study also provides insights for developing a new rationale for lung cancer prevention strategy which would be of great value for human health.

Author Contributions

Conceptualization: M.T. Resources: A.N., K.S. Methodology: D.U., S.I., N.R., M.T. Investigation: D.U., S.I., N.R., M.T. Validation and Formal Analysis: D.U., S.I. Data Curation: D.U., S.I., M.T. Writing—Original Draft Preparation, Writing—Review & Editing and Visualization: D.U., S.I., J.C., M.T. Supervision, Project Administration and Funding Acquisition: J.C., M.T. All authors have read and agreed to the published version of the manuscript.

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Figures- Chapter 3

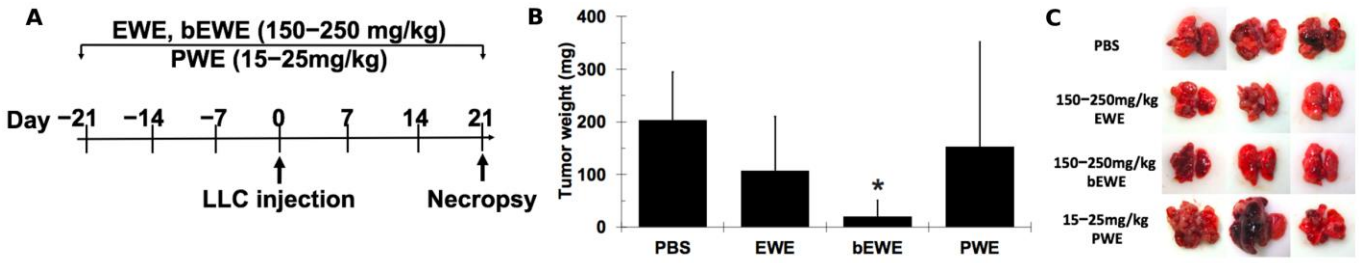

Figure 3.1. Pre-treatment with *Euglena* water extract (EWE) and boiled *Euglena* water extract (bEWE) attenuated growth of Lewis lung carcinoma (LLC) tumors in murine lungs.

(**A**) Schematic illustration of the study design. (**B**) Average tumor weight in each treatment group; phosphate buffered saline (PBS) served as a control. (**C**) Macroscopic views of the lungs from LLC tumor-bearing mice in PBS or extract pretreatment groups. The scale bar in each picture represents 5 mm. Results are presented as mean ± SD (n = 5). *: $p < 0.05$ as compared to the PBS group. PWE: paramylon water extract.

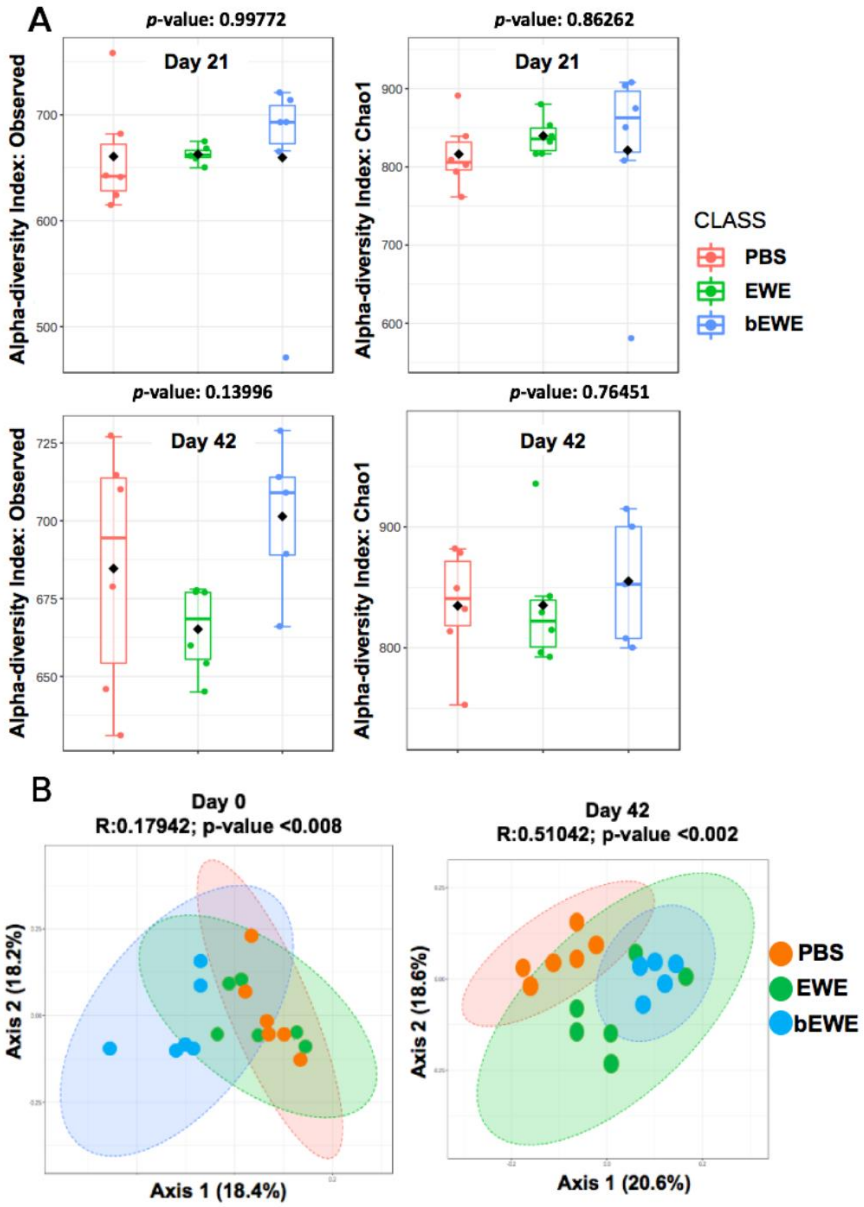

Figure 3.2. Alpha and beta diversity of gut microbiota in *Euglena* water extracts and PBS treated mouse feces before (Day 21) and after LLC cell inoculation (Day 42).

(**A**) The difference in alpha diversity; measured as observed index and Chao1 index for Day 21 (upper panels in **panel A**) and Day 42 (lower panels in **panel A**). Box-and-whisker plots show high, low, and median values, with lower and upper edges of each box denoting first and third quartiles, respectively. (**B**) Principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity representing the difference in gut microbial communities. Each PCoA plot is accompanied by an analysis of similarity (ANOSIM) using an appropriate distance matrix. The R-values closer to zero indicates no difference between groups.

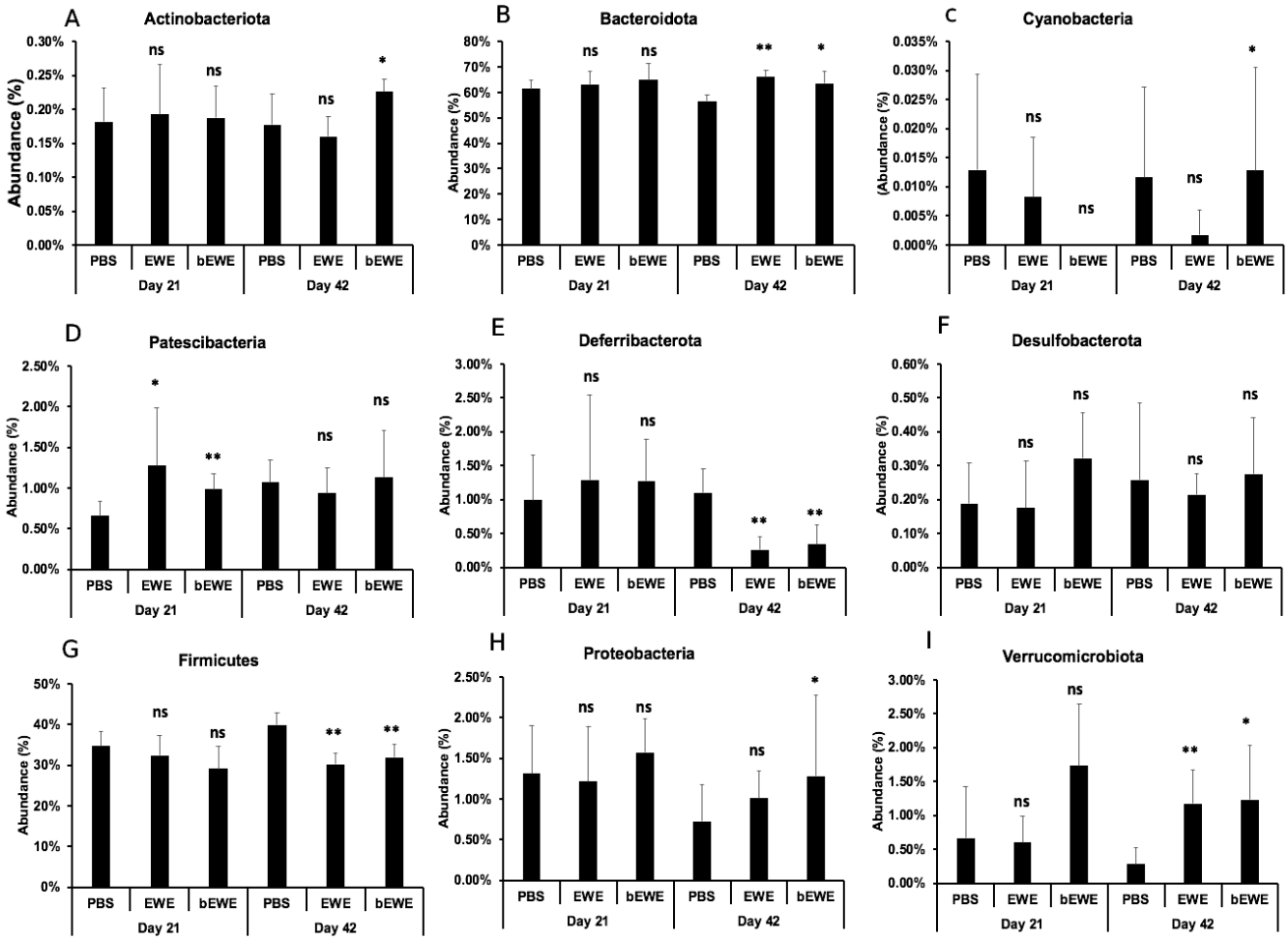

Figure 3.3. Relative abundance of bacterial taxa at the phylum level in mice treated with *Euglena* water extracts at Day 21 (before LLC cell inoculation) and Day 42 (after LLC cell inoculation).

Percentage of taxa abundance of (A) Actinobacteriota, (B) Bacteroidota, (C) Cyanobacteria, (D) Patescibacteria, (E) Deferribacterota, (F) Desulfobacterota, (G) Firmicutes, (H) Proteobacteria, and (I) Verrucomicrobiota is presented. The difference in taxa abundance for each phylum between PBS and EWE or bEWE was compared at Day 21 and Day 42 using a *t*-test. Results are presented as mean ± SD (n = 5–6). *: $p < 0.05$; **: $p < 0.01$; ns: $p > 0.05$.

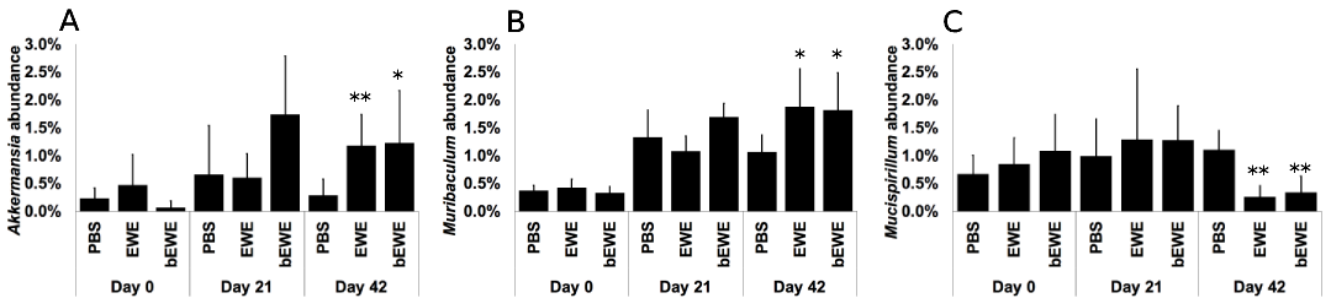

Figure 3.4. Relative abundance of bacterial taxa at the genus level in mice treated with *Euglena* water extracts at Day 21 (before LLC cell inoculation) and Day 42 (after LLC cell inoculation).

Percentage of taxa abundance of (**A**) *Akkermansia*, (**B**) *Muribaculum*, and (**C**) *Mucispirillum* between PBS and EWE or bEWE were compared at Day 21 and Day 42 using t-test. Results are presented as mean ± SD (n = 5–6). *: $p < 0.05$; **: $p < 0.01$.

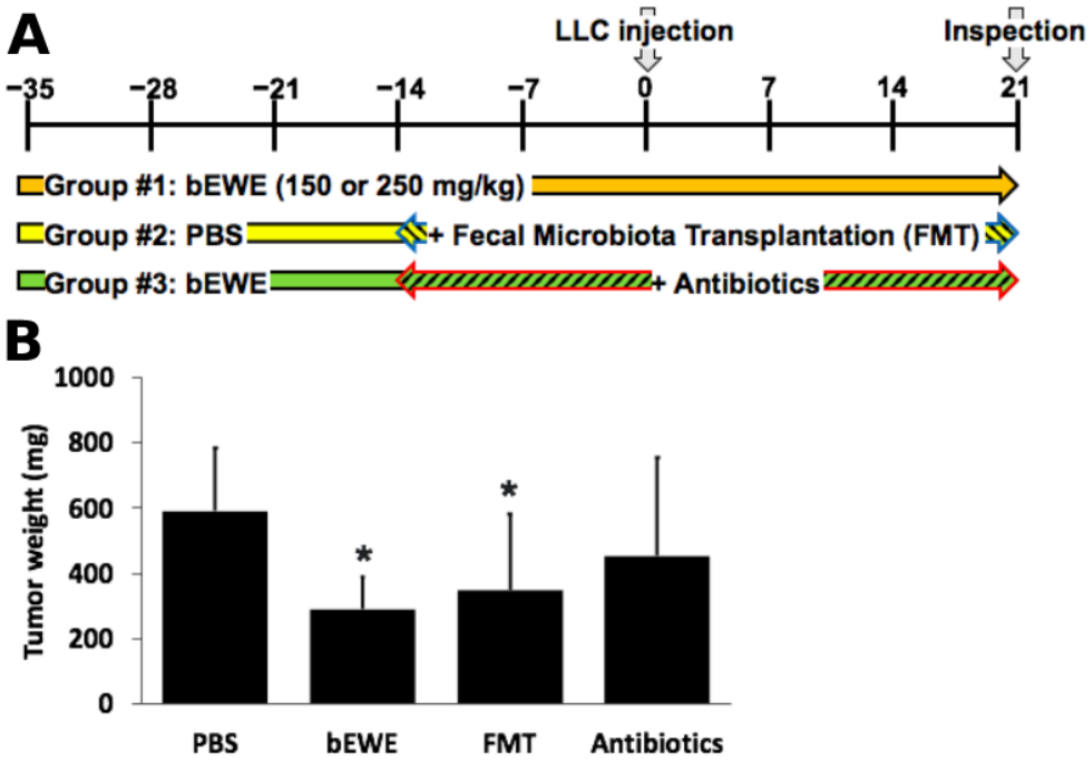

Figure 3.5. Fecal microbiota transplantation (FMT) from bEWE treated mice significantly attenuated the growth of tumors in the murine lung.

(**A**) Schematic illustration of the study design. (**B**) Average tumor weight in each treatment group. PBS served as control. Results are presented as mean ± SD (n = 5). *: $p < 0.05$ as compared to PBS-treated group.

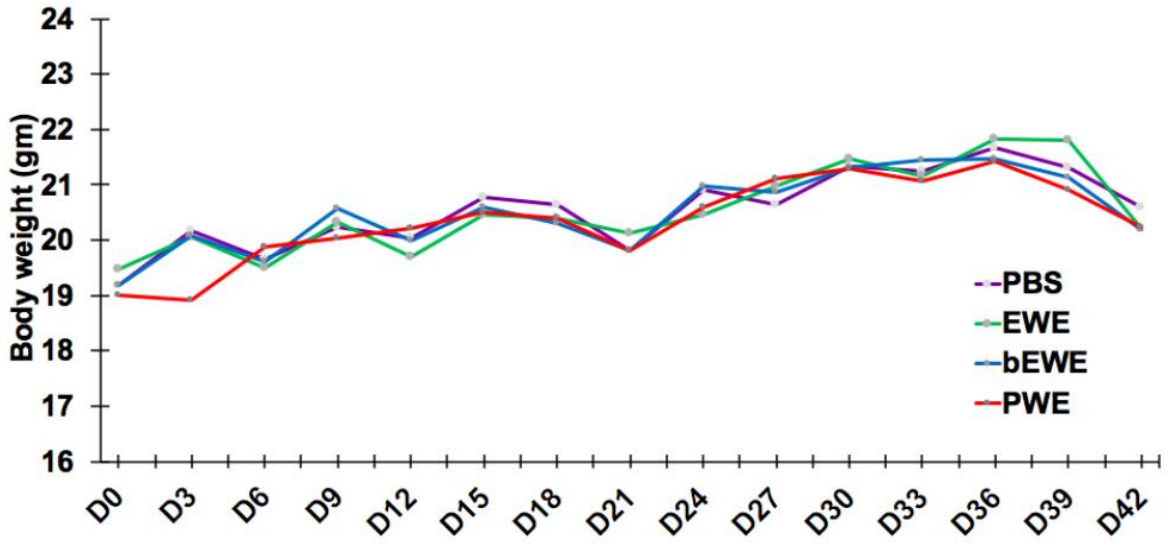

Figure S 3.1. Treatment of mice with water extracts from *E. gracilis* didn't alter the body weight.

Body weight was 20.6 ± 0.6, 20.6 ±0.7, 20.6 ± 0.6, and 20.5 ± 0.7 g in average with PBS, EWE, bEWE, and PWE treatments, respectively.

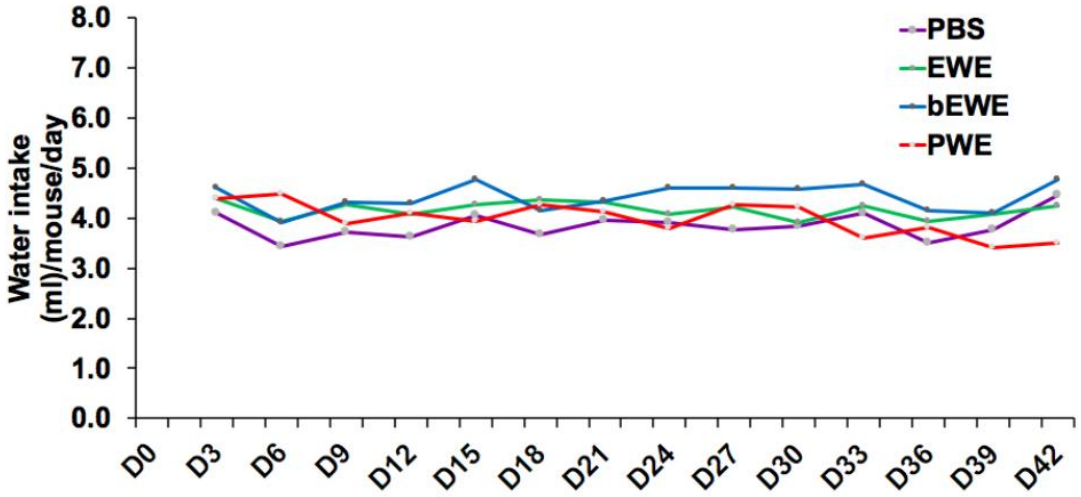

Figure S 3.2. Consumption of water with different treatments was relatively consistent throughout the study.

Water consumption were 3.8 ± 0.3, 4.2 ± 0.2, 4.4 ± 0.3, 4 ± 0.3 mL/mouse/day with PBS, EWE, bEWE, and PWE treatments, respectively.

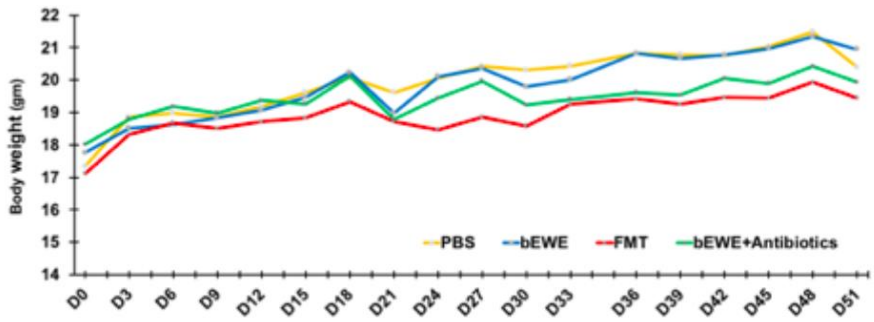

Figure S 3.3. The body weight of mice treated with PBS, bEWE, FMT, or antibiotics was comparable.

The average body weights at the end of the study period were 19.9 ± 1.0, 19.8 ± 1.0, 18.9 ± 0.6, and 19.4 ± 0.6 g with PBS, bEWE, FMT, and bEWE + Antibiotics treatments, respectively.

Chapter 4 - Oral Administration of Water Extract from *Euglena gracilis* Attenuates Lung Tumorigenesis Induced by a Tobacco-Specific Carcinogen Through the Modulation of Gut Microbial Metabolites

Abstract

Euglena water extract's anti-cancer properties and effects on the gut metabolic landscape were investigated in a tobacco smoke carcinogen: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung carcinoma mouse model. Oral administration of boiled *Euglena* water extract (bEWE) 2 weeks prior (pre-bEWE) or 10 weeks post (post-bEWE) NNK injection greatly attenuated NNK-induced tumorigenesis in the mice's lungs. A partial least-squares discriminant analysis demonstrated a clear difference in fecal metabolites between the phosphate-buffered saline (PBS) and bEWE treated groups. High-throughput metabolic profiling identified 27 metabolites that were significantly altered in the feces collected from pre- and post-bEWE treated mice as compared to that from control PBS treated mice. Specifically, succinate, malate, triethanolamine, acetylserine, glyceric acid, aspartic acid, glutamic acid, and threonine, which can be metabolized to short-chain fatty acids (SCFAs), were increased with the bEWE treatment compared to the PBS treatment. Furthermore, using the targeted analysis, a significant increase in SCFAs, such as acetic acid, butyric acid, and propionic acid, was observed in the feces collected from either pre- or post-bEWE treated mice. Moreover, treatment with SCFAs significantly suppressed the proliferation of both human and murine lung cancer cell lines in cell culture *via* induction of apoptosis. The present study suggests that oral administration of *E.*

gracilis water extract suppresses the growth of tobacco smoke carcinogen-induced lung cancer by altering the gut metabolites to increase SCFA production.

Keywords: natural product, water extract from *Euglena gracilis*, lung cancer prevention and therapy, gut metabolites, short-chain fatty acids

Introduction

Lung cancer is one of the most commonly diagnosed cancers in both men and women and the leading cause of cancer-related deaths worldwide [1]. An estimated 130,180 Americans are expected to die from lung cancer in 2022, accounting for over 21 percent of all cancer-related deaths [2]. Tobacco smoke is the leading cause of lung cancer and associated deaths due to long-term exposure to carcinogens. Compared with non-smokers, smokers have as much as a 14-fold increased risk of developing lung cancer [3, 4]. However, lung cancer also remains a leading cause of cancer-related death among non-smokers, occurring in 10–15% of people who have never smoked [5]. Although many preventive and therapeutic strategies are being implemented to limit the casualties, lung cancer patients still have a lower rate of survival, less than five years, than any other cancer [6]. Therefore, the development of novel and efficacious preventative, diagnostic, and therapeutic strategies against lung cancer is urgently required.

While the traditional therapeutic strategies for lung cancer involve side effects and complications, natural products are investigated as sources of effective and safe therapeutic remedies. *Euglena gracilis* (*E. gracilis*), a unicellular micro-alga, is found in both fresh and saltwater and has features of both animals and plants. This alga is used as a nutritional and functional dietary supplement and has also been known to have a wide range of medicinal properties [7]. It is, therefore, expected that E. gracilis could function as one of the natural sources used to improve the efficacy of therapeutic strategies against diseases such as lung cancer and aid in its prevention.

There is an increasing interest in the modulation of the gut microbiota using prebiotics and probiotics to develop preventative and therapeutic strategies against cancer. The gut microbiota plays a role in the modulation of the host immune system by microbiota-derived

metabolites. Short-chain fatty acids (SCFAs) are one of the typical metabolites produced by microbiota. It has been reported that SCFAs can regulate host physiological functions and influence the development of immune and inflammatory responses, both locally and at distant sites such as the lungs leading to the lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), and lung cancer [8, 9][8–11]. A recent study reported that the intake of dry powder of E. gracilis alters microbiota composition in the human gut and increases the production of butyrate, a SCFA, resulting in improved digestive health and defecation [7]. Therefore, the use of natural products that target the alteration of gut microbiota and metabolites could open a new avenue for cancer prevention and/or therapy.

We have previously shown that the oral administration of a partially purified water extract from *Euglena gracilis* (EWE) suppresses lung carcinoma growth by attenuating the myeloid-derived cell population in orthotopic mouse lung tumor models [12]. Our recent study demonstrated that oral administration of either EWE or boiled EWE (bEWE) alters intestinal microbiota compositions, thereby attenuating the growth of lung tumors in mice [13]. These two findings suggest that EWE-induced attenuation of lung tumor growth appears to be mediated through an enhancement of antitumor immunity caused by an alteration of gut microbiota and their metabolites. However, the detailed mechanisms by which *Euglena* water extract-induced alteration of gut microbiota inhibits lung tumor growth are still unknown. Additionally, whether EWE is also effective in inhibiting tobacco smoke carcinogen-induced lung cancer, which more closely mimics human clinical lung cancer [14–17], is not known.

In the present study, we report the tumor growth preventive effects of EWE on lung tumorigenesis in tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced mouse lung cancer. Since this NNK-induced lung cancer mouse model mimics

the human lung adenocarcinoma in morphology and mutation characteristics [14–17], the outcome of this research would be an applicable translational study. In this study, the high-throughput metabolic profiling revealed that oral administration of bEWE significantly increased intestinal microbial metabolites: short-chain fatty acids (SCFAs) and their precursors. In addition, treatment of the SCFAs significantly suppressed proliferation of both human and murine lung cancer cell lines in cell culture via induction of apoptosis, suggesting the anti-cancer effects of bEWE are attributed to changes in the intestinal metabolites.

Materials and methods

Animals

All the procedures for the handling of animals were approved by the Kansas State University Institutional Animal Care and Use Committee (Protocol # 4346) and Institutional Biosafety Committee (Protocol # 1433). Wild-type male and female A/J mice were purchased from Charles River Laboratories International, Inc. and were housed in a clean facility with controlled humidity and temperature on 12-hour light-dark cycles. The temperature of the room was set at 20–26 °C and the relative humidity was 30–70 %. Before treatment, all the mice were acclimatized in the facility for a week. The mouse condition was observed every day and their body weights were monitored every three days.

Reagents

4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Sort chain fatty acids: sodium acetate, sodium butyrate, and sodium propionate were from Sigma-Aldrich (St. Louis, MO, USA). Fluorescent conjugated antibodies targeting Annexin V and 7-aminoactinomycin D (7-AAD) were purchased from BioLegend (San Diego, CA, USA).

Cell culture

The mouse Lewis lung carcinoma (LLC) cell line (CRL-1642), human H1299 lung carcinoma cell line (CCL-5803) and normal human bronchial epithelial cell line (BEAS-2B) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM) and RPMI 1640 were obtained from Mediatech, Inc. (Manassas, VA, USA). Fetal bovine serum (FBS) was from Biowest (Riverside, MO, USA). The penicillin-streptomycin stock was obtained from Lonza Rockland, Inc. (Allendale, NJ, USA). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. The cell lines were authenticated by short tandem repeat (STR) DNA profiling. Both the cells were maintained in low passages (<15) for this study.

Preparation of *Euglena* water extract

The boiled *Euglena* water extract (bEWE) was prepared using a protocol described previously [13]. Briefly, dried powder of *Euglena gracilis* (5 g) obtained from Euglena Co., Ltd. (Tokyo, Japan) was suspended in sterile PBS (40 ml) and incubated at 37 °C for 30 min with periodic sonication for 30 s. The suspension was centrifuged at 11,405 × *g* for 20 min and the supernatant was filtered through one layer of water-soaked filter paper to remove floating materials. The filtrate was boiled for 10 min. After the centrifugation at 11,405 × *g* for 10 min, the supernatant was filtered again through a 0.22 μm sterile disk filter (Midwest Scientific, Valley Park, MO, USA). The dry weight was calculated after drying a 400 μL aliquot at 55 °C for 48 h from which the dry weight of PBS was subtracted (0.125 mg dry powder/mL). The bEWE was stored at −20 °C until further use.

Effect of orally administrated bEWE on the growth of NNK-induced lung carcinoma

To evaluate the effect of orally administrated bEWE on the growth of NNK-induced lung carcinoma, three treatment regimens were performed: (1) PBS control, (2) 150–250 mg/kg of bEWE pre-treatment for 2 weeks prior to NNK injection until the end of the experiment (designated as Pre-bEWE), (3) 150–250 mg/kg of bEWE post-treatment after NNK injection (designated as Post-bEWE) (Fig. 1A). PBS or bEWE were administered orally *via* drinking water. The weight of water pouches was measured every three days to monitor the daily intake of the drinking water containing bEWE. Based on the average water consumption (4 mL/mouse/day), the doses of bEWE were adjusted.

For the induction of lung tumors, the A/J mice (n=10; male=5 and female=5 per group) were intraperitoneally injected with 100 mg/kg of NNK as described previously [18]. The mice were visually inspected for any signs and symptoms on a routine basis after the NNK injection. After 19 weeks of NNK injection, the mice were euthanized by exposure to saturated CO_2 followed by cervical dislocation, and the lungs were collected. The whole lung was stained by injecting India Black ink solution through the trachea and the tumor nodules in the lungs were counted under the stereo microscope. All of the measurements throughout this study were carried out by a single observer in a blinded fashion.

Metabolic profiling

To analyze the profile of primary metabolites in the fecal samples collected from PBS control or bEWE-treated NNK tumor-bearing mice, fecal samples were collected at the end of the study. These samples were analyzed by automatic liner exchange/cold injection gas chromatography-time of flight mass spectrometry (ALEX-CIS GC-TOF-MS) at the West Coast

Metabolomics Center (University of California, Davis, Davis, CA, USA) using their proprietary methods. Briefly, 10 mg of the fecal pellet was homogenized, and the metabolites were extracted using the cold extraction buffer (isopropanol, acetonitrile, and water in 3:3:2 ratio at −20 °C). The samples were then dried and derivatized to increase volatility and loaded through a capillary column. Proprietary analytical procedures were carried out to ensure high-quality data after minimizing the system artifacts, misassignments, and background noise among the samples. The raw reading from the acquired spectra was normalized in terms of peak heights. Values for each sample were normalized by the total ion of the chromatogram (mTIC) of the samples to avoid using potential non-biological artifacts as described in the filtering algorithm implemented in the Fiehn Lab BinBase database [19–21] and matched against the Fiehn Lab Mass Spectral Library of authentic metabolite spectra. Because the mTIC was used to normalize the spectra according to the peak height of the metabolites, the value of the metabolite levels are only relative, semi-quantifications, and are expressed as arbitrary units (au) in this study.

Following the normalization and filtering using proprietary algorithms, the obtained values were log-transformed and only the metabolites with known annotations were included in the analysis for this study. Multivariate analysis was used to clarify the clustering pattern based on the covariance of the data. The difference in overall metabolite profile between the groups was visualized by a partial least squares-discriminant analysis (PLS-DA) score plot. The ANOVA contrasts and PLS-DA were conducted using the MetaboAnalyst online tool [22]. Based on the variable importance in projection (VIP) score of each metabolite, which was generated from the PLS-DA model, the varied/feature metabolites that were different between the groups were further identified. The VIP score equal to or greater than 1.5 was considered as a different variable/feature between the groups and the difference was visualized by heatmap based

on the abundance ratio of metabolites. Further, univariate statistical analysis was conducted to analyze the features that vary significantly between the treatment groups. A Student's t-test was performed to identify the metabolites that were significantly different in between the treatments and the data was plotted in GraphPad Prism (version 9.3.1).

Absolute quantification of short-chain fatty acids

The absolute quantification of SCFAs was determined by a targeted metabolite analysis at the West Coast Metabolomics Center (UC Davis). Briefly, the metabolites were extracted from the feces (10 mg) of three groups of mice using a proprietary method with a mixture of 5:1:1 ratio of water, hydrochloric acid, and methyl *tert*-butyl ether. Extracted samples were analyzed using an Agilent 5977A GC-quadrupole mass spectrometer (Agilent, Clara, CA, USA). The data was acquired by selected ion monitoring (SIM) mode and the raw data was processed using the Agilent Mass Hunter Quantitative Analysis software (B.07.00). The absolute value of SCFA in the fecal sample was quantified using authentic SCFA standards.

Growth inhibition of lung carcinoma cells and normal bronchial epithelial cells by SCFA in cell culture

The LLC murine (2×10^3 cells/well) and H1299 human (2×10^4 cells/well) lung carcinoma cells and BEAS-2B normal human bronchial epithelial cell (2×10^4 cells/well) were seeded into a 24-well plate with a 1 ml growth medium. After 48 h, the cells were treated with SCFA: sodium acetate, sodium butyrate, and sodium propionate with different concentrations of 1, 5, and 10 mM. PBS was used as the negative control. Trypan blue staining was performed according to the manufacturer's instructions and the number of viable cells (trypan blue negative cells) were counted using a hemocytometer under an inverted microscope.

Flow cytometric analysis of the apoptotic status of lung cancer cells treated with SCFAs *in vitro*

Anti-Annexin V antibodies/7-Aminoactinomycin D (7AAD) was used to quantify the percentage of apoptosis in Lewis lung cancer cells (LLC) treated with SCFAs (sodium acetate, sodium butyrate, and sodium propionate. The LLC cells were seeded into a 12-well plate ($0.5*10^5$ cell/ml) and incubated with different SCFAs with three different concentrations at 0.1 mM, 1 mM, 5 mM for 24 h and 48 h. The cells were collected and washed with PBS and co-stained with Annexin V and 7AAD in binding buffer. The sample was then analyzed by flow cytometry (BD LSRFortessa X-20; BD Biosciences, San Jose, CA, USA) and analyzed by BD FACSDiva software (BD Bioscience). Early apoptotic cells and late apoptotic cells were identified as populations that showed phenotypes of Annexin V^+ 7-AAD^- and Annexin V^+ 7-AAD^+, respectively.

Statistical analysis

The data were presented as mean ± standard deviation (SD) of the mean. The experiment was conducted with multiple sample determinations with several sampless. For the global profiling of metabolites in mouse feces, 10 biological repeats were used for each condition. For the targeted quantification of SCFAs, 8 biological repeats were used per treatment condition. The data were analyzed and visualized using MetaboAnalyst software [22], GraphPad Prism (v9.3.1), and Microsoft Excel (v16.16.27). Any statistical significance was assessed by a two-tailed unpaired t-test. The following convention for symbols to indicate statistical significance was set. ns: $p>0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p<0.0001$.

Results

Oral administration of bEWE attenuated the growth of NNK-induced lung tumorigenesis

In our previous study, oral administration of *Euglena* water extract (EWE and bEWE) significantly attenuated the growth of orthotopic Lewis lung carcinoma (LLC) allografts in mice [12, 13]. In this study, the antitumor effects of bEWE were further investigated using chemical carcinogen (NNK) -induced lung tumorigenesis in mice. No differences were observed in mouse body weight (Fig. S1) or water consumption (Fig. S2) among the three mouse groups described in Fig. 1A; PBS control group, pretreatment group with bEWE for 19 weeks (bEWE treatment was started 2 weeks prior to the NNK injection and continued throughout the study), and the post-treatment group with bEWE for 9 weeks (bEWE treatment was started 10 weeks after the NNK injection and continued till the end of the study). As shown in Fig. 1B, both pre- (3.32 ± 1.6, $p<0.01$) and post-bEWE (3.0 ± 1.7, $p<0.01$) treatment significantly reduced the numbers of NNK-induced lung tumor nodules in the lung as compared with that of the control PBS (5.67 ± 1.8). It has been reported that a single dose of NNK (100 mg/kg, i.p.) induces tumor formation 6-14 weeks after the treatment [23–25]. Because bEWE treatments were started 2 weeks before (Pre-bEWE) or 10 weeks after (Post-bEWE) NNK injection, these results clearly indicate that the daily administration of bEWE prevents tobacco smoke carcinogen-induced lung tumorigenesis, even after microtumor formation.

Multivariate analysis of fecal metabolites

In our previous study, oral administration of both EWE and bEWE *via* drinking water attenuated lung carcinoma growth in orthotopic allograft models by altering their intestinal microbiota compositions [13]. In the present study, therefore, further analyses of the metabolic

profiling of fecal samples were conducted in bEWE-treated NNK-induced tumor-bearing mice. A multivariate statistical analysis was conducted using a supervised PLS-DA model, to see the difference between metabolite profiles of the three mouse groups that received NNK. Two-dimensional PLS-DA scores plot visualized a clear separation of the overall fecal metabolite profile between the bEWE (both pre- and post-) and PBS treatment groups (Fig. 2A), indicating that marked metabolomic changes occurred due to bEWE administration. VIP scores based on the PLSD-DA model exhibit the overall coefficient scores for the metabolites. A higher VIP value represents a stronger contribution to the discrimination among the treatment groups. Based on the VIP scores, a total of 25 metabolites: salicylic acid, urea, triethanolamine, dihydro-3-coumaric acid, 3-(4-hydroxyphenyl)propionic acid, aspartic acid, inosine, lactose, allantoic acid, adenine, 3-hydroxyphenylacetic acid, hippuric acid, N-acetylserine, guanine, malic acid, 2,8-dihydroxyquinoline, tocopherol-alpha, galactinol, succinic acid, pinitol, maltose, pseudouridine, citric acid, 6-hydroxynicotinic acid, and alanine-alanine appeared to be the most important fecal metabolites for distinguishing the differences between the PBS, pre- and post- bEWE groups (Fig. 2B).

Oral administration of *Euglena* water extract altered the fecal metabolite levels in NNK-induced lung tumor-bearing mice

Further comparison of metabolite levels among the individual groups was performed and 27 metabolites were found to be significantly altered in either pre- or post-bEWE treated mice when compared with the PBS control group (Figs. 3 and S3). Of those, 17 metabolites were significantly higher (Fig. 3), and 10 metabolites were significantly lower (Fig. S3) in either pre- or post-bEWE treated mice. Triethanolamine, salicylic acid, desaminotyrosine, N-acetyl serine, glycolic acid, aspartic acid, succinate, galactinol, and malate were significantly higher in both

pre-and post-bEWE treated mice. Similarly, glyceric acid, glutamic acid, deoxycholic acid, adenine, and 4-aminobutyric acid were significantly higher in only post-bEWE treated mice. Guanine, lactose, and threonine were significantly higher in only pre-bEWE treated mice (Fig.3). Overall, the metabolic profiling of pre- and post-bEWE-treated fecal samples was observed to be similar to one another.

Euglena water extract-induced metabolite changes strongly contributes to SCFA metabolism

As shown in Fig. 3 and S3, oral administration of bEWE altered microbiota metabolites. It was reported that SCFAs from gut microbiota attenuate lung carcinogenesis [26]. In the present study, therefore, the change in SCFAs and the metabolic pathways involved in the gut microbial production of SCFAs were further investigated. As depicted in Fig. 4, the microbial accessible carbohydrates in the mammalian gut are broken down to phosphoenolpyruvate *via* glycolysis or the pentose phosphate pathway. Phosphoenolpyruvate is then converted into either pyruvate or malate *via* microbial enzymes. Malate and succinate are the key metabolites for the biosynthesis of SCFAs such as propionic acid, and butyric acid [27]. Furthermore, amino acids such as aspartic acid, glutamic acid, and threonine can be metabolized to acetate, butyrate, and propionate [28, 29]. Other metabolites such as glyceric acid, acetylserine, and triethanolamine can also contribute to the synthesis of SCFAs like butyrate and acetate [30].

As shown in Fig. 3, metabolic profiling revealed that 5 metabolites were significantly increased in both of the pre- and post-bEWE treatment groups (triethanolamine, malate, succinate, aspartic acid, and acetylserine, which are highlighted in blue in Fig. 4). Further, glyceric acid and glutamic acid were higher in both of the pre- (n.s.) and post-bEWE ($p<0.05$) treatment groups, compared to the PBS control group. Threonine was also higher in both of the

pre- (p<0.05) and post-bEWE (n.s.) treatments (highlighted in blue in Fig. 4). The increase of the SCFA precursor metabolites suggests the possibility of alteration of SCFA metabolism occurring in the bEWE treated mice.

Euglena water extract treatment increases the production of SCFAs by gut microbiota in NNK-induced lung tumor-bearing mice

The results from the metabolic profiling (Figs. 3 and 4) suggest the possibility of the involvement of SCFA metabolism in the antitumor effects of bEWE. To test this hypothesis, a separate targeted quantitative analysis of SCFA levels in the feces from either bEWE or PBS treated NNK tumor-bearing mice was conducted. SCFAs are the major metabolites produced in the gut by anaerobic bacterial fermentation of dietary fibers [27]. As shown in Fig. 5, acetic acid was significantly higher in both pre- and post-bEWE treated groups compared to the PBS control group (Fig. 5A). On the other hand, propionic acid and butyric acid were significantly higher in only post-bEWE treated mice (Figs. 5B and 5C). However, there was no significant difference in valeric acid, formic acid, or isovaleric acid levels among the three treatment groups (Figs. 5D, 5E, and 5F). These results suggest that there were associations between fecal primary SCFA metabolism and the antitumor effects of *Euglena* water extract in NNK-induced tumorigenesis in the mouse lung.

SCFA treatment inhibits the proliferation of murine and human lung carcinoma cells by inducing apoptosis *in vitro*

A quantitative analysis of SCFA levels in the feces clearly indicated that oral administration of bEWE increased the production of SCFAs in the gut of NNK-induced tumor-bearing mice. To clarify the involvement of SCFAs in the antitumor effects of bEWE, the effect of the SCFAs on the proliferation of lung cancer cells was investigated by a trypan blue staining

assay in cell culture. Murine (LLC) and human (H1299) lung carcinoma cells were treated with sodium acetate, sodium butyrate, and sodium propionate at 1 mM, 5 mM, 10 mM, and 30 mM concentrations for 48 h. The SCFAs treatment dose-dependently attenuated the growth of both types of lung carcinoma cells (Fig. 6A and B). Butyrate treatment inhibited the proliferation of both lung cancer cell lines more efficiently in comparison to acetate and propionate. On the other hand, SCFAs treatment did not significantly attenuate the normal human bronchial epithelial cell growth except butyrate treatment at 10mM (Fig. 6C). These results suggest that SCFA dependent cytotoxicity is specific to cancer cells more than normal bronchial epithelial cells. Furthermore, these results strongly support our hypothesis that the antitumor effects of bEWE are induced by the stimulation of SCFA production in the gut of mice.

Furthermore, an apoptosis analysis carried out using Annexin V and 7-AAD demonstrated that the SCFA dependent inhibition of lung cancer cells is due to the induction of apoptosis (Fig. 7). The results obtained from the LLC cells treated with SCFAs (sodium acetate, sodium propionate, sodium butyrate) at 0.1 mM, 1 mM, 5 mM concentrations for 24 h and 48 h showed an increase in the apoptotic cells. When compared to the PBS control, the total apoptosis rate was higher in cells treated with butyrate in comparison to acetate and propionate for both 24 h and 48 h time points. These results explain that the apoptosis induced by SCFAs contributes to attenuating the growth of lung cancer cells.

Discussion

Because of its wide variety of nutritional components, *Euglena gracilis* is often used as a nutritional dietary supplement. It has been shown that *Euglena* extract and its main components, β-1,3-glucan, called paramylon, contain biological properties including immunomodulatory, anti-bacterial, anti-viral, and therapeutic properties for various diseases, including cancer [7, 31–34].

While there is an increasing interest in the exploration of the therapeutic potentials of E. gracilis and paramylon, the detailed mechanisms of their anti-cancer properties are yet to be clarified. Our previous study showed the anti-cancer effects of orally administrated *Euglena* water extract, devoid of paramylon, on lung cancer growth in an orthotopic LLC cell allograft model by altering immune suppressor cell populations: myeloid-derived suppressor cells [12], as well as altering gut microbiota compositions [13]. However, the effect of *Euglena* water extracts without mature paramylon (water-insoluble aggregates of β-1,3-glucan) on the fecal metabolites in lung tumor-bearing mice remains unknown.

Among the various carcinogens involved in the induction of lung cancer, tobacco-specific nitrosamine: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) has been documented as a potent lung carcinogen in mice [15], which induces the formation of DNA adducts and, therefore, results in multiple mutations in critical cancer control genes [16, 35]. NNK-induced tumorigenesis in mice mimics human lung cancer development and has served as an excellent model for studying the mechanisms and development of preventative and therapeutic measures against lung cancer. It has been reported that the inhalation of the toxic chemicals in smoke induces dysbiosis of the human gut microbiota [36–38]. In addition, there is a strong correlation between NNK-induced lung adenocarcinoma and the disruption of gut microbiota and metabolite profiles in mice, signifying that this avenue should be researched further to develop better interventional targets against lung cancers [39]. Therefore, the anti-cancer properties and underlying mechanisms of *Euglena* water extract against lung tumorigenesis by NNK were investigated.

Microorganisms and their metabolites in the gut play crucial roles in human and animal health [40]. Emerging evidence shows the importance of the interaction between gut microbiota

and metabolites in lung diseases such as asthma, acute infectious diseases, fibrosis, and lung cancer [9]. Thus, the prospect of developing a therapeutic approach for lung cancer via improving the gut microenvironment, including gut microbiota compositions and/or metabolites, is promising. The present study was conducted to clarify two subjects; (1) to evaluate the antitumor effects of orally administrated bEWE in NNK-induced lung tumor models, and (2) to identify the mechanism by which bEWE inhibits lung cancer development based on changes in gut metabolites.

In the present study, the effect of bEWE administration was evaluated with two regimes: treatment started from 2 weeks before (Pre-bEWE) or 10 weeks after (Post-bEWE) NNK injection. Both treatment types were continued until the end of the experiment. Both pre- and post-bEWE treatments significantly reduced NNK-induced tumor development in comparison to the PBS treatment, and the attenuation was similar in both pre- and post-bEWE groups (Fig. 1B). In our previous study with the murine LLC allograft model, tumor growth was attenuated only with pre-treatment (3 weeks prior to the LLC cell inoculation), but not post-treatment (immediately after LLC cell inoculation for 3 weeks) [12]. However, in the present study, bEWE reduced tumor burden when administered 10 weeks after NNK injection; at this point, the tumors had already been developed [24]. The mechanism of tumor induction was different between the LLC allograft model and the NNK-induced lung tumor model. In addition, a longer administration of the extract (8-19 weeks), in comparison to the 3 weeks in the previous study [12], could be the reason for the differences in the tumor attenuating effects of post-treatment. This result suggests that bEWE could potentially be applied to lung cancer treatments associated with tobacco smoke carcinogens if administered in the early stages of tumor development. This

result may increase usage of bEWE, not only for the prevention of lung cancer but also in the treatment of lung cancer, even after clinical diagnosis.

Analysis of metabolomics often helps our understanding of the association of phenotypic characteristics of multifactorial diseases, including cancer [41]. Multivariate statistical analysis showed a clear separation of the fecal metabolites between the bEWE treatment groups and the PBS control group. On the other hand, there was an overlap of fecal metabolites between both pre-bEWE and post-bEWE treatment groups (Fig. 2A). This overlap between pre- and post-bEWE treatment shows the bEWE treatment induces similar alterations in the gut microenvironment, regardless of the timing and the term of treatment. Furthermore, analysis of variable importance in projection (VIP) scores, which is an index showing significant separation of metabolites, showed a marked increase in the phenolics such as 3-(4-hydroxyphenyl) propionic acid, 3-hydroxyphenylaceticacid, dihydro-3-coumaricacid, and salicylic acid in the bEWE treated groups (Fig. 2B). Phenolic compounds, the secondary metabolites in algal metabolites, have nutritional, antimicrobial, anti-inflammatory, and anti-cancer properties [42, 43]. Further, salicylic acid, which had the highest VIP score of 3.5, has been reported as an apoptosis-inducing agent in melanoma [44], breast cancers [45], B-chronic lymphocytic leukemia cells [46], and lung cancer cells [47]. Clarifying the involvement of these phenolic compounds in the anti-cancer effects of bEWE is a future study of interest.

The metabolomic profiling also identified a significant increase in SCFA precursor metabolites in bEWE treated groups compared to PBS treated groups (Figs. 3 and 4). To further understand the role of SCFAs in bEWE-induced anti-tumorigenesis, a quantitative analysis targeting SCFA levels in feces from bEWE-treated NNK-induced tumor-bearing mice was conducted. The results showed significant increases in acetate, propionate, and butyrate, while no

significant change in formic acid, isovaleric acid, and valeric acid in the post- or pre-bEWE treatments compared with the PBS treatment in the NNK-induced tumor-bearing mice (Fig. 5). SCFAs are gut microbial metabolites that play a role in various physiological processes and impact the immune cell functions of the host [48]. Acetate, propionate, and butyrate are the major SCFAs that are synthesized in the lumen of the caecum and large intestine by the bacterial fermentation of dietary fibers [49]. While SCFAs are produced in the intestinal compartment, they can permeate the bloodstream and affect functions at distal sites, such as the lungs [50]. Moreover, SCFAs have pleiotropic functions, which, either directly or indirectly, influence various cells types, tissues, and immune cells [11], and can induce lung cancer cell apoptosis and cell cycle arrest, especially by propionate [51]. Therefore, the present results suggest that the SCFAs acetate, propionate, and butyrate, derived from the bEWE-stimulated microbiota, may play an important role in the attenuation of NNK-induced lung carcinoma growth.

Since our results indicate that the increase of biosynthesis of SCFAs (acetate, propionate, and butyrate) by bEWE-stimulated gut microbiota may correlate with the antitumor activities of bEWE, the effects of SCFAs on the growth of lung carcinoma cell lines was evaluated in cell culture. The treatment with SCFAs dose-dependently attenuated the growth of both murine and human lung carcinoma cells. Furthermore, the flow cytometric analysis of apoptosis in LLC cells treated with SCFAs showed an increase in the total (early and late apoptotic) percentage of apoptotic cells when compared with the PBS treated group. The apoptotic cells were dose-dependently increased by acetate, butyrate, and propionate treatments at 48 h post-treatment. Strong induction of apoptosis was observed by the butyrate treatment, especially, as compared to the other two SCFAs (Fig. 7). These results suggest that induction of apoptosis is a potential mechanism for the inhibition of lung cancer cell growth. These findings align with previous

studies that report propionate and butyrate attenuating lung cancer cells by inducing apoptosis [51–53]. Taken together, these in-vitro studies support our hypothesis that an increase of SCFAs is attributable to the attenuation of NNK-induced lung tumorigenesis by bEWE treatment.

In summary, the present study revealed that *Euglena* water extracts (bEWE) can be orally administered for the prevention and treatment of tobacco smoke carcinogen (NNK)-induced lung tumorigenesis in mice. Daily oral administration of bEWE caused a significant alteration in several fecal metabolites, either with pre- or post-treatment, including an increase in the malate, succinate, triethanolamine, glyceric acid, acetyl serine, aspartic acid, glutamic acid, and threonine, which can be metabolized to SCFAs. Furthermore, the increase in fecal SCFAs (acetate, propionate, and butyrate) with bEWE treatment, and the inhibition of lung cancer cells with SCFAs, confirmed that the attenuation of NNK-induced lung tumorigenesis is associated with the alteration of gut microbiota metabolites. The present study strongly suggests that daily oral administration of bEWE alters gut metabolites, thereby attenuating NNK-induced lung tumorigenesis.

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Figures- Chapter 4

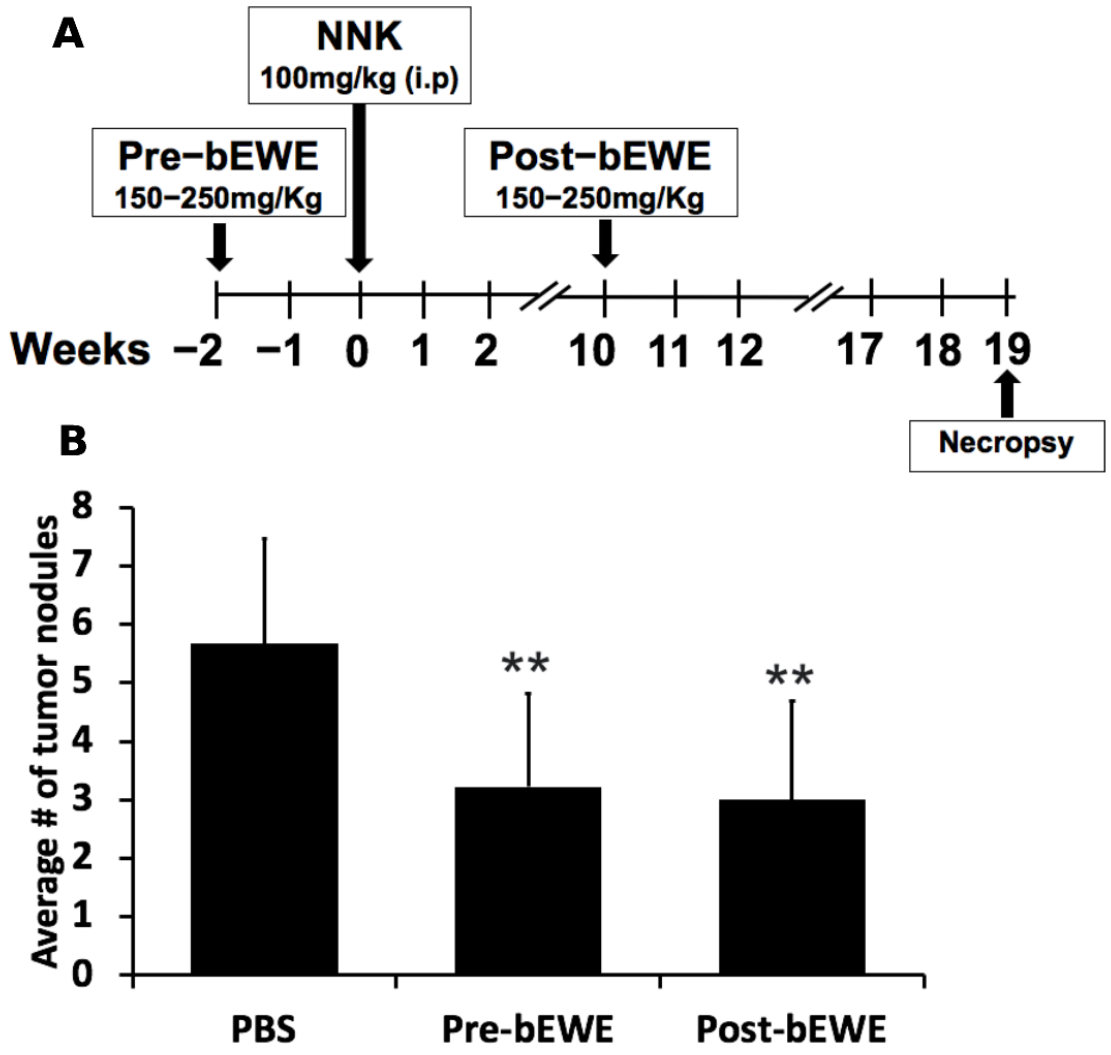


Figure 4.1. Pre- and post-treatment with bEWE significantly attenuated the growth of NNK-induced lung tumorigenesis in mice.

(A) Schematic illustration of the study design. (B) Average lung tumor nodule numbers in each treatment group. PBS served as a control. Results are presented as mean ± SD (n = 10). **: p<0.01 as compared to the PBS group.

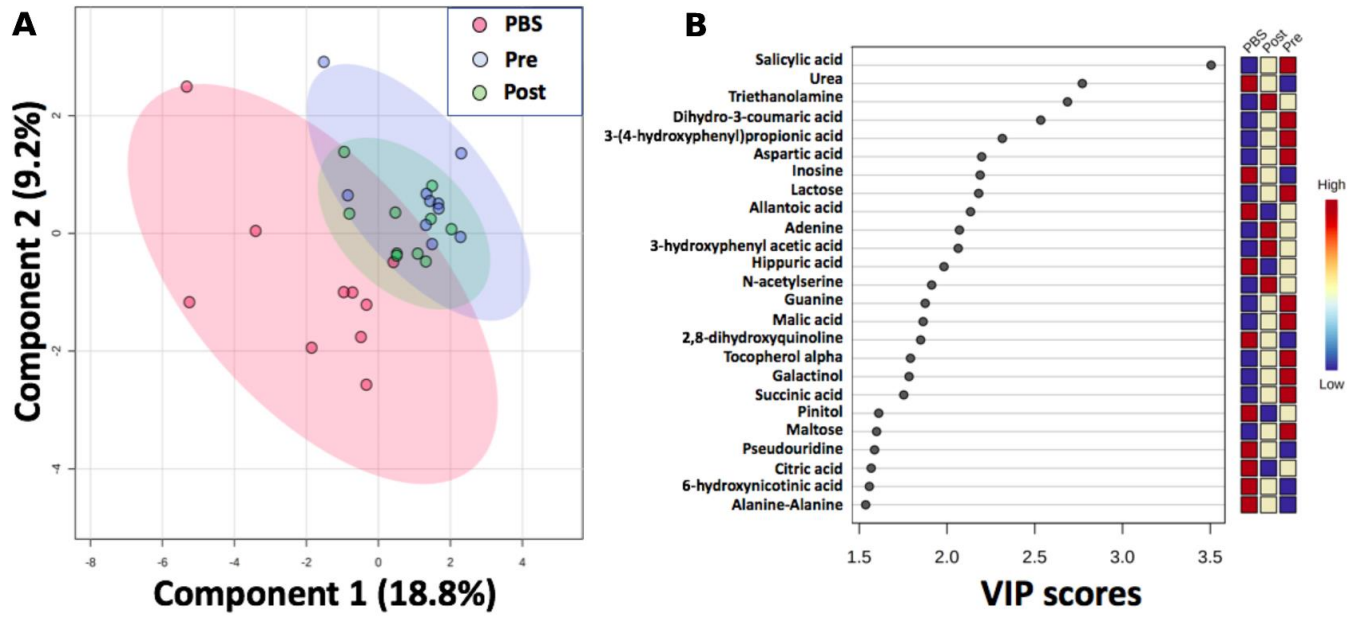


Figure 4.2. Partial least squares discriminant analysis (PLS-DA) score plot and VIP score of fecal metabolites in the pre-bEWE, post-bEWE, and PBS-treated mice (n=10/group).

(A) A 2-D PLS-DA scores plot visualized differences in metabolite profiles in each group. (B) A variable importance in projection (VIP) plot depicted 25 most important fecal metabolites for distinguishing the differences between the treatment groups. The significance of the contributors was tested with a VIP score and a value equal to or greater than 1.5 was used as a threshold of significance. A heat map with an abundance ratio is shown to the right.

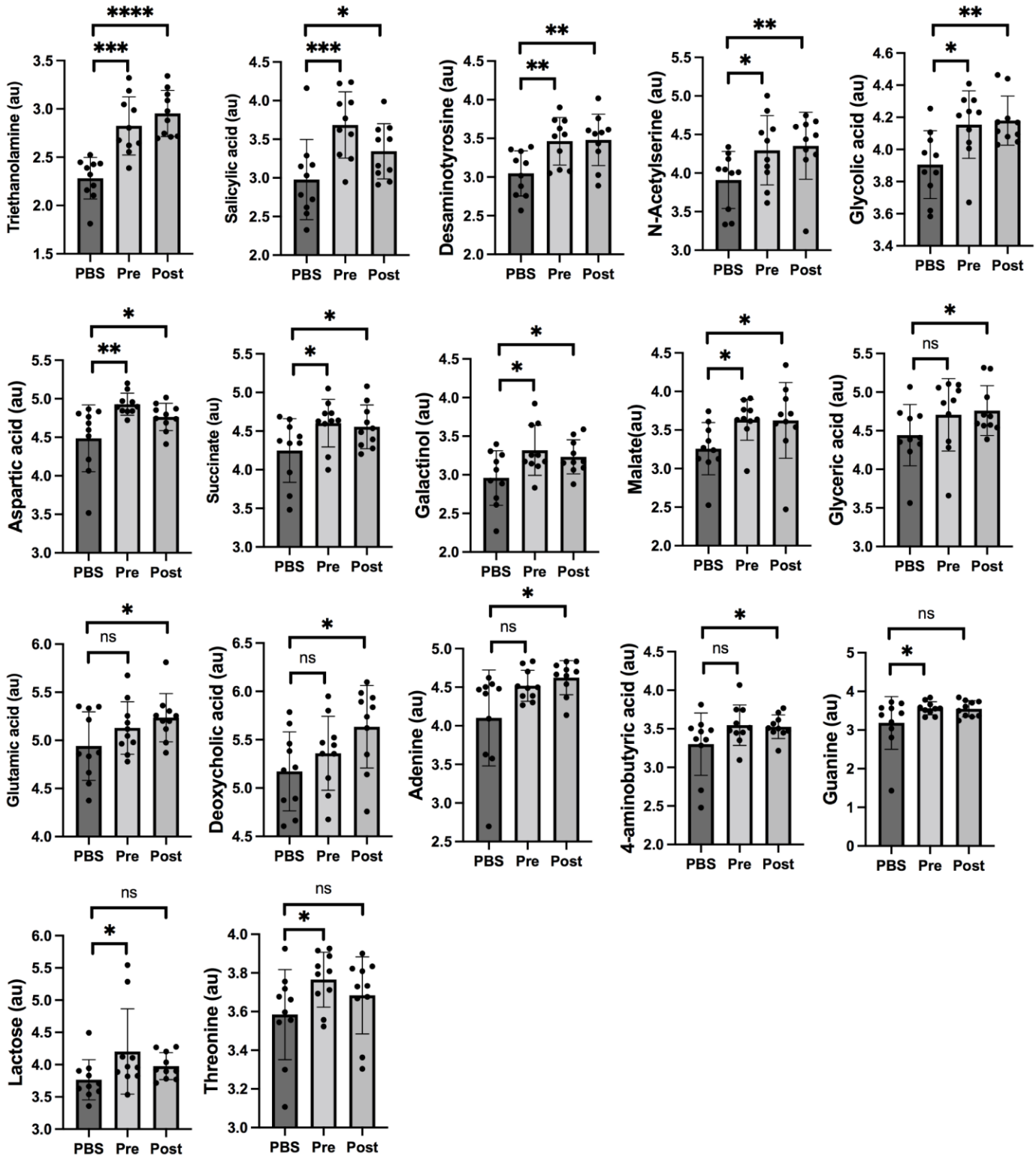


Figure 4.3. Oral administration of bEWE altered the fecal metabolite levels in NNK-induced tumor-bearing mice.

The difference in each metabolite level between the three groups: PBS, pre-bEWE (Pre), and post-bEWE (Post), was compared using a *t*-test. Results are presented as means ± SD (n = 10). ns: $p>0.05$, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$.

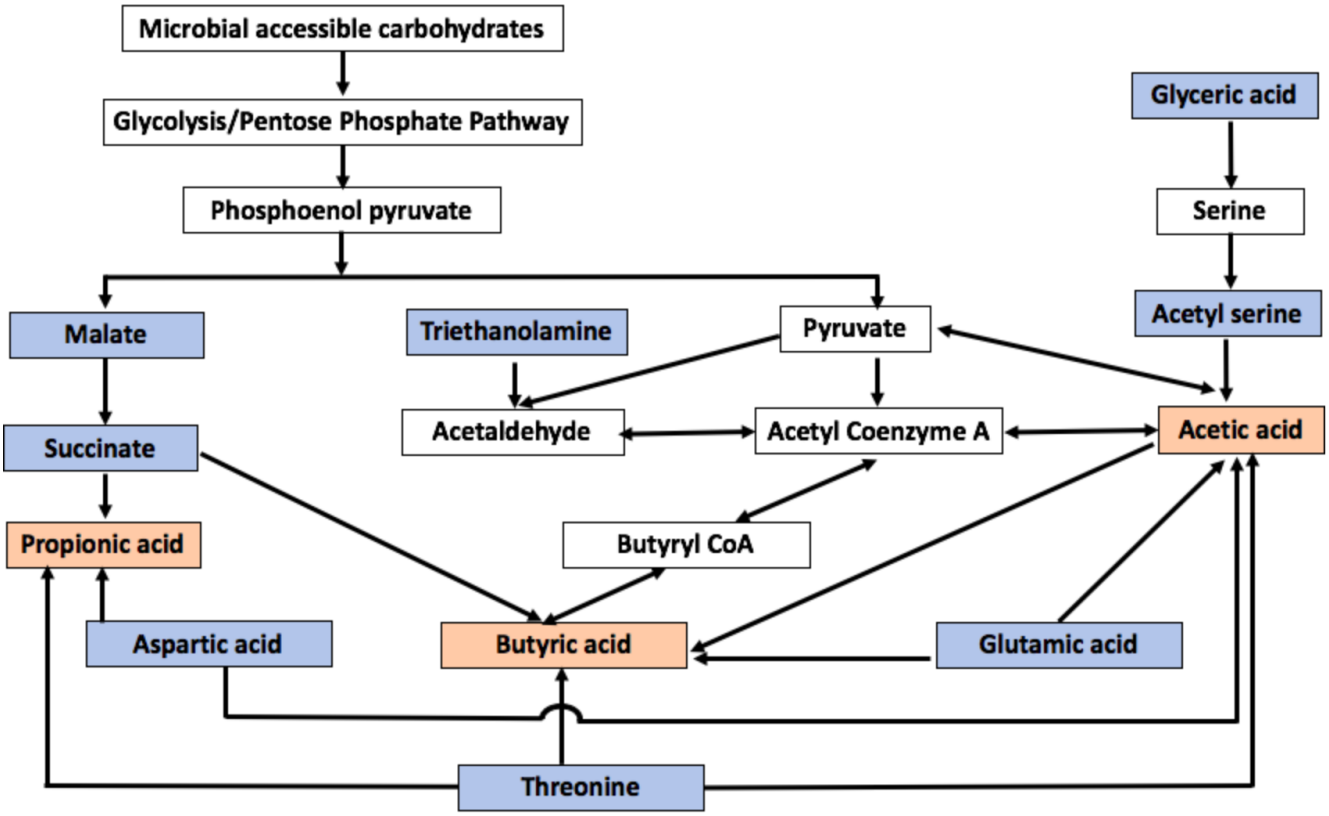


Figure 4.4. An outline of the metabolic pathways involved in the gut microbial production of SCFAs.

The three primary SCFAs, acetic acid (acetate), propionic acid (propionate), and butyric acid (butyrate) (highlighted in orange) are produced by various bacterial fermentation of microbial accessible carbohydrates and metabolism of different amino acids. The metabolites highlighted in blue are significantly increased with both pre- and post-bEWE treatment groups, except glyceric acid (p<0.05 in post-bEWE: n.s. in pre-bEWE), glutamic acid (p<0.05 in post-bEWE: n.s. in pre-bEWE), and threonine (p<0.05 in pre-bEWE: n.s. in post-bEWE).

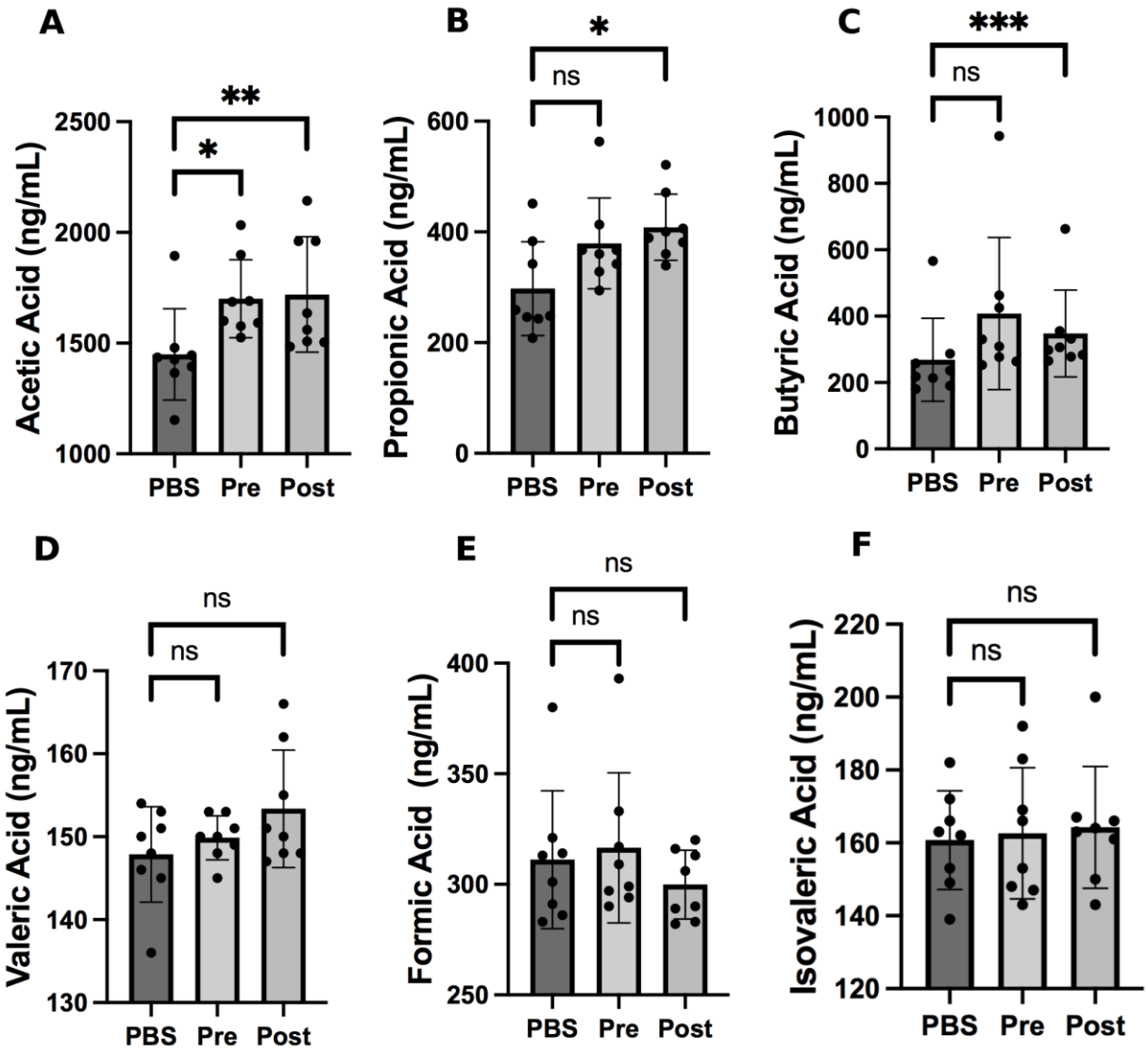
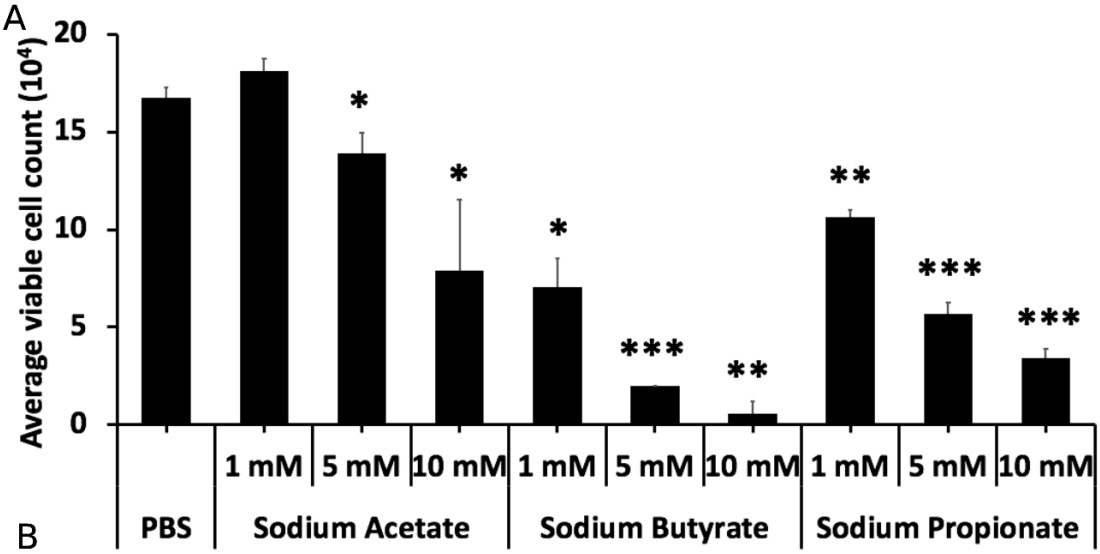


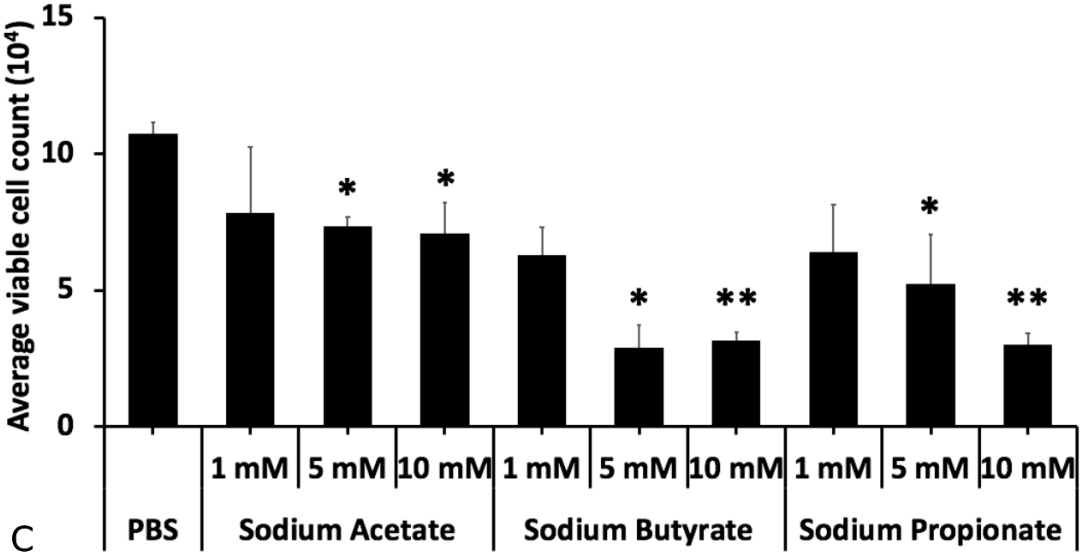
Figure 4.5. bEWE treatment increased short-chain fatty acid (SCFA) metabolism in the gut of NNK-induced tumor-bearing mice.

The amount of SCFAs (ng/mL) in the feces of PBS, Pre-bEWE (Pre), and Post-bEWE (Post) treated mice are shown in the graphs. PBS served as the control. Results are presented as means ± SD (n = 8/group). ns: $p>0.05$, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

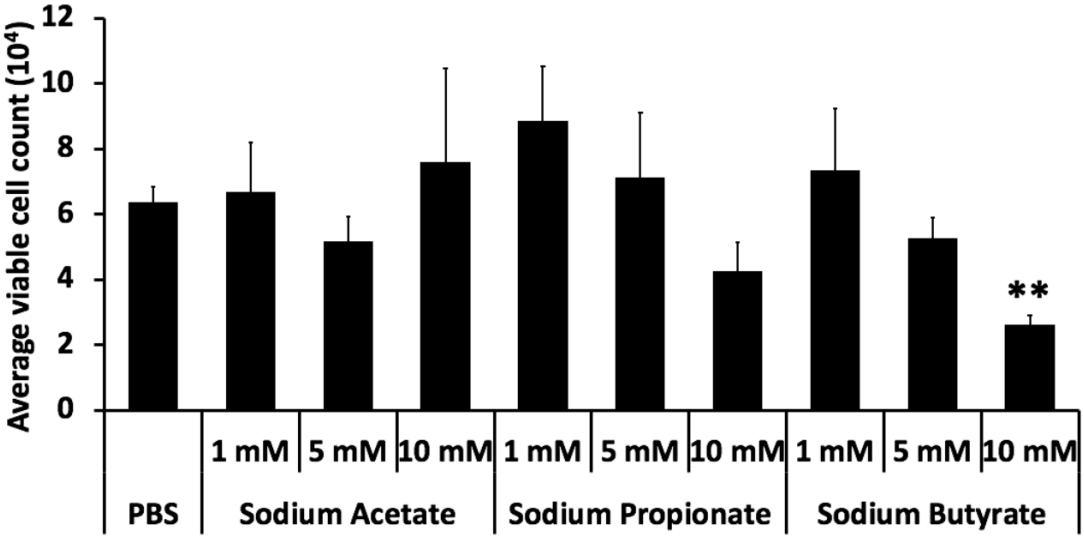
A


Average viable cell count (10^4)
20
15
10
5
0
*
*
*
***
**
**
***
***
1 mM
5 mM
10 mM
1 mM
5 mM
10 mM
1 mM
5 mM
10 mM
PBS
Sodium Acetate
Sodium Butyrate
Sodium Propionate

B


Average viable cell count (10^4)
15
10
5
0
*
*
*
**
*
**
1 mM
5 mM
10 mM
1 mM
5 mM
10 mM
1 mM
5 mM
10 mM
PBS
Sodium Acetate
Sodium Butyrate
Sodium Propionate

C


Average viable cell count (10^4)
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10
8
6
4
2
0
**
1 mM
5 mM
10 mM
1 mM
5 mM
10 mM
1 mM
5 mM
10 mM
PBS
Sodium Acetate
Sodium Propionate
Sodium Butyrate

Figure 4.6. SCFA treatment dose-dependently attenuated the growth of murine and human lung carcinoma cells.

The average viable cell number of (A) LLC murine lung carcinoma cells, (B) H1299 human lung carcinoma cells are presented, and (C) BEAS-2B normal human bronchial epithelial cell. PBS served as the control. Results are presented as means ± SD (n = 3). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

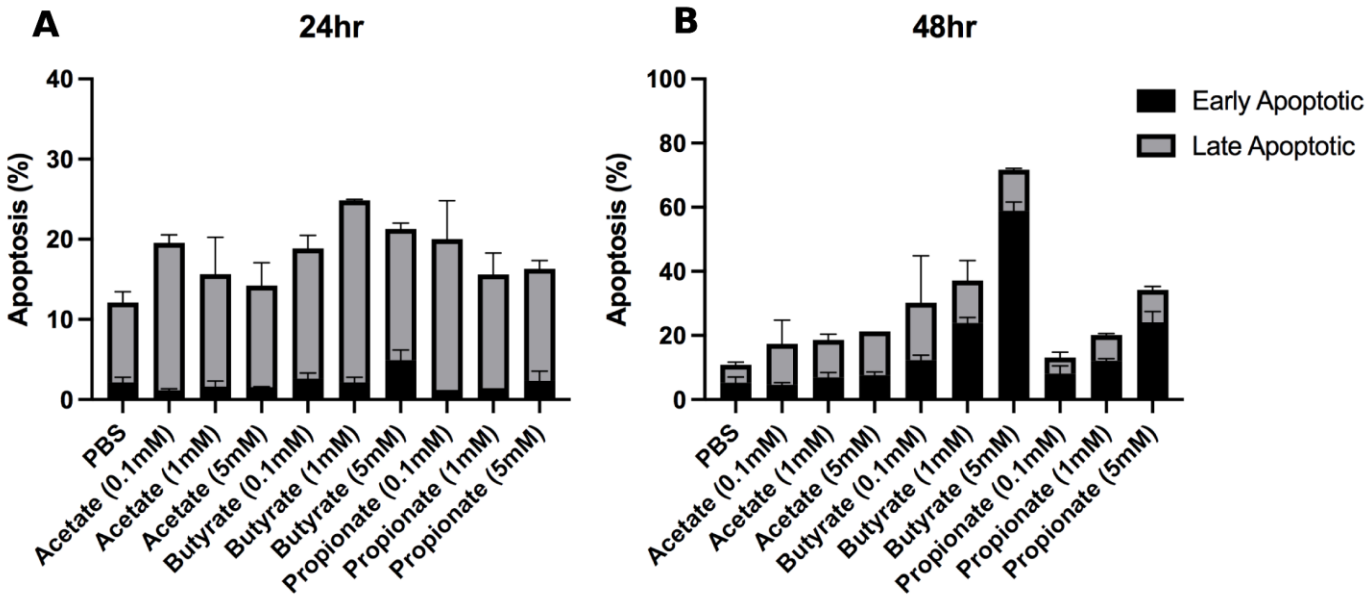

Figure 4.7. SCFA treatment increased apoptosis in LLC murine lung carcinoma cells. LLC cells were treated with SCFAs (sodium acetate, sodium butyrate, sodium propionate) at 0.1–5 mM for 24–48 h and the percentage of apoptotic cells was determined using flow cytometry. PBS served as a control. Results are presented as means ± SD (n = 2).

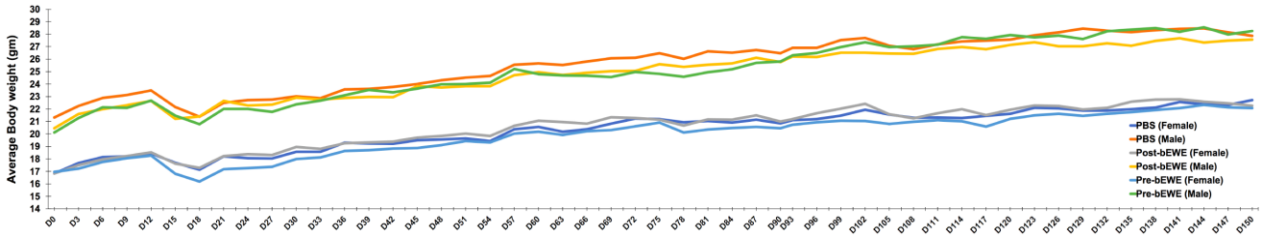

Figure S 4.1. Treatment of mice with water extracts from *E. gracilis* didn't alter the body weight.

The average body weights at the end of the study period were 25.6 ± 2.2, 25.1 ± 2.4, 24.9 ± 2.1 g in male A/J mice of PBS, pre-bEWE, and post-bEWE treatment groups, respectively. Similarly, the average body weights at the end of the study period were 20.3 ± 1.6, 19.9 ± 1.7, 20.5 ± 1.7 g in female A/J mice of PBS, pre-bEWE, and post-bEWE treatment groups, respectively.

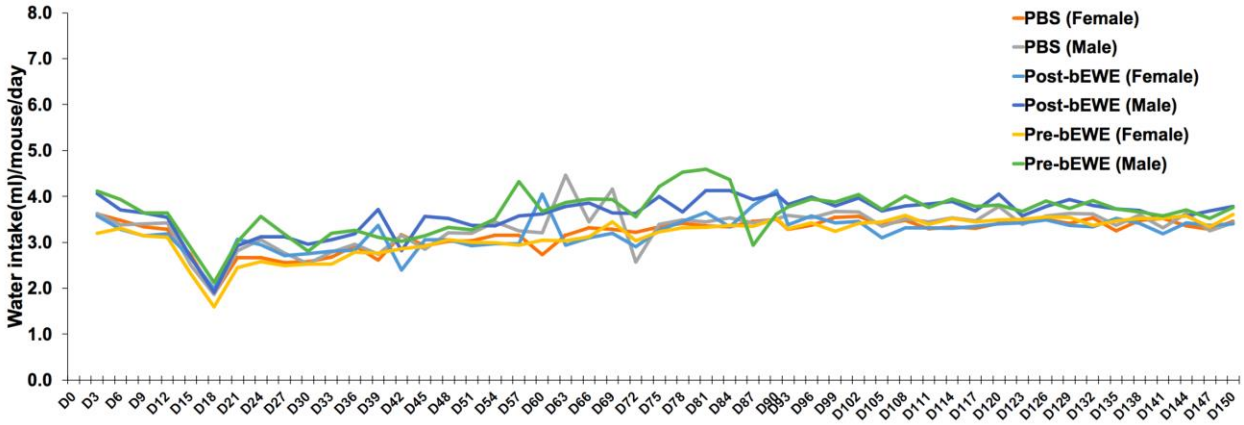

Figure S 4.2. Consumption of water with different treatments was relatively consistent throughout the study.

Water consumption was 3.3 ± 0.4, 3.6 ± 0.5, 3.6 ± 0.4 ml/mouse/day in male A/J mice in PBS, pre-bEWE, and post-bEWE treatment groups, respectively. Similarly, water consumption was 3.2 ± 0.4, 3.2 ± 0.4, 3.2 ± 0.4 ml/mouse/day in female A/J mice in PBS, pre-bEWE, and post-bEWE treatment group, respectively.

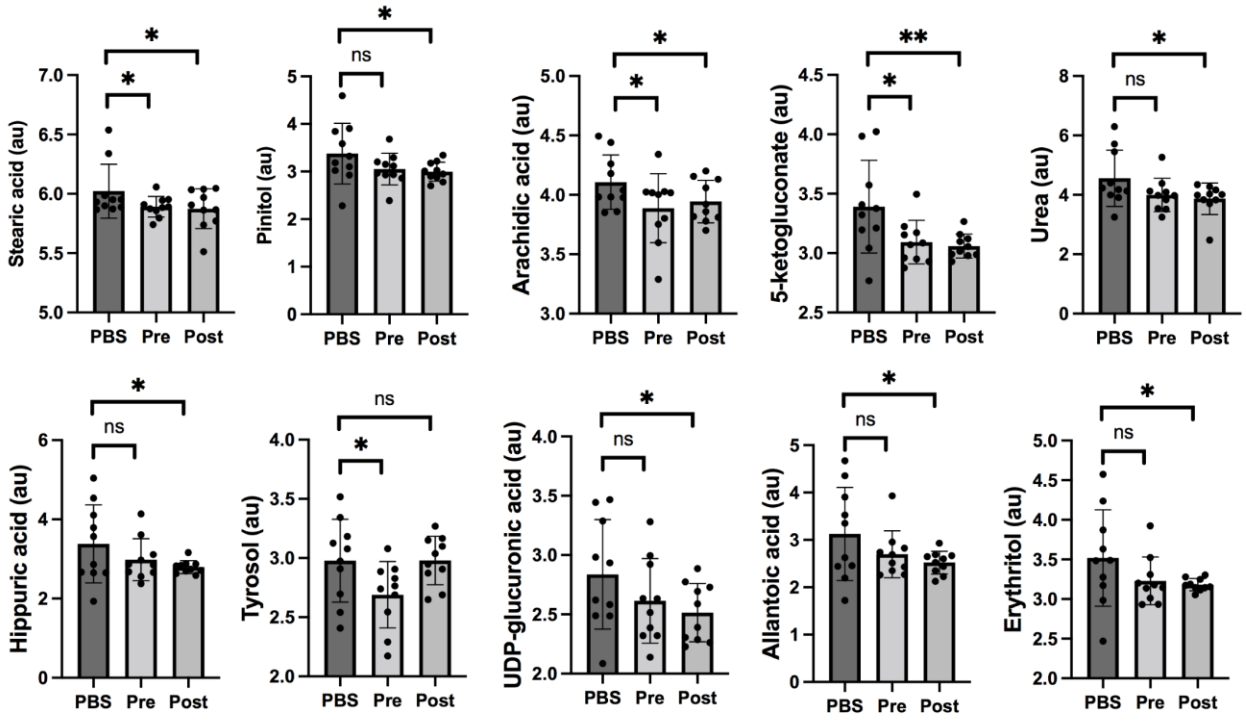

Figure S 4.3. Consumption of bEWE altered the fecal metabolite levels in NNK-induced tumor-bearing mice.

The difference in each metabolite level between the three groups: PBS, pre-bEWE (Pre), and post-bEWE (Post), was compared using a *t*-test. Results are presented as means ± SD (n = 10). ns: $p>0.05$, *: $p<0.05$, **: $p<0.01$.

Chapter 5 - Conclusion

Despite the advancement of chemotherapy and other treatment strategies, lung cancer remains a leading cause of death in cancer patients. Because of the several limitations in the existing therapeutic approaches as discussed in detail in chapter one of this dissertation, novel considerations are needed while designing anti-lung cancer therapies. *Euglena gracilis*, one of the frontrunners in the field of the "functional food" industry, is rich in nutritional and medicinal value. It possesses antimicrobial, antiviral, anti-inflammatory properties. Studies for over 50 years have noted the anti-cancer property of bioactive compounds in *E. gracilis*. In this dissertation, we demonstrated that water extracts from *E. gracilis* act as a potent anti-lung cancer agent and also highlighted some of the major mechanisms it utilizes in functioning as an anti-tumor agent. In this chapter, we aim to highlight how the findings reported in this dissertation could further the usage of natural products as anti-cancer agents and contribute to therapeutic development. We also aim to propose future directions based on the findings reported in this dissertation.

Euglena gracilis has a long history of research for use as a dietary supplement and also for the development of novel drugs against different diseases. In this dissertation, we showed that consumption of *E. gracilis* water extract prevents and protects the mice from lung cancers as shown by our studies using a different model of lung cancer studies. We found that *Euglena* water extract consumption develops favorable conditions in the mice from an immunological, gut microbial, and metabolic viewpoint.

At the immunological frontier, we found that oral administration of EWE significantly reduces the granulocyte population in mouse peripheral blood and also directly attenuates granulocytic MDSCs in primary cell culture with mouse bone marrow cells. Because

granulocytes such as neutrophils are the major mediators of an innate immune response, our findings that EWE consumption attenuates lung tumor growth by generating a favorable condition for antitumor immunity in the host.

From the gut microbial standpoint, we show that EWE consumption through drinking water significantly alters the microbiota composition in the gut of the orthotopic lung cancer syngeneic mouse models with Lewis lung carcinoma (LLC) cells. Particularly, we observed a decrease in the ratio of Firmicutes to Bacteroidetes and significant increases in *Akkermansia* and *Muribaculum* in the EWE-treated groups as compared to PBS treatment control. As discussed in detail in the previous chapters, the bacterial population that was different in our study plays a beneficial role in protection against lung cancer indicating that the EWE-dependent suppression of lung cancer growth is mediated by these alterations in intestinal microbiota and the metabolites that they generate. Furthermore, fecal microbiota transplantation of bEWE-treated mouse feces reduced tumor growth to a level comparable to bEWE treatment itself. This attenuation in tumor growth was lost upon treatment with an antibiotic cocktail. Our findings give a strong indication that daily oral administration of partially purified water extracts from *Euglena gracilis* attenuates lung carcinoma growth *via* the alteration of the intestinal microbiota.

From the metabolic viewpoint, we determined the metabolic landscape of the gut of *E. gracilis* water extract fed mice with tobacco-specific nitrosamine-induced lung tumor using a high-throughput metabolomics approach. Strikingly, we found that *Euglena* water extract administration markedly alters the fecal metabolite levels of the tumor-bearing mice in comparison to the PBS control treatment. The levels of the SCFA and their precursors were significantly increased upon EWE treatment. Furthermore, SCFA treatment remarkably suppressed the growth of lung carcinoma cells. SCFAs have been known to play the anti-tumor

role through their various beneficial functions as discussed in previous chapters. Our study further establishes an important role of SCFAs in the attenuation of NNK-induced lung tumorigenesis with the *Euglena* water extract treatment.

In sum, our study has significantly advanced our understanding of *E. gracilis* and its anti-lung cancer properties. Combined with further optimization and testing in animal models, our findings have the potential to be applied in the development of novel strategies for the prevention and treatment of lung cancer.

Future Perspectives

Although we show that administration of *Euglena* water extract has a preventative and protective effect against lung cancer-induced using different mouse models, further studies are warranted before it can go from the laboratory bench to the bedside of a patient. In the following section, we will discuss how we can follow up on our findings to further our understanding of EWE-induced anti-cancer properties.

While we show that daily oral administration of partially purified water extracts from *Euglena gracilis* causes significant changes in the composition of the intestinal microbiota of treated mice and the FMT from EWE treated mice also reduced lung tumor growth, it would be interesting to identify the potentially beneficial bacterium/bacteria. Because our 16s rRNA profiling revealed a significant increase in the genus *Akkermansia* in the EWE-treated groups as compared to PBS treatment control, the usage of *Akkermansia* could be a good starting point. Because *Akkermansia* is shown to exert anti-cancer properties, it would be interesting to examine the effect of administration of the pure culture of *Akkermansia munciphilia* in suppressing lung cancer in mice.

Another question that needs to be answered is the role of SCFAs in modulating the host cell immune system during lung cancer. There is a consensus among the scientific community that SCFAs exhibit immunosuppressive properties. While this idea aligns with our findings that EWE treatment reduces the granulocyte population in mice and attenuates granulocytic MDSCs in culture, further experiments need to be done to examine the effect of SCFA on myeloid-derived suppressor cell (MDSC) population in BMCs. It would also be interesting to test the effect of SCFA treatment on the activation of LLC-specific $CD8^+$ T-cells. Although we observed a reduction in lung cancer cell growth upon SCFA treatment in cell culture, further *in vivo* studies of the effect of SCFA treatment in lung cancer growth are warranted to establish a more direct correlation.

Another question that remains unanswered is the identity of the exact nature of the chemical from *Euglena* that exerts suppressive action on lung cancer. As discussed earlier, *E. gracilis* contains several beneficial biomolecules, many of which could potentially have anti-cancer properties. One of the major candidates among them is paramylon, a storage granule made of β-1,3-glucan. While some studies have shown paramylon to have anti-cancer properties, in our studies the *Euglena* water extract reduced lung cancer even after removal of the majority (if not all) of the paramylon granules by multistep centrifugation and filtration through a 0.22 μm pore size filter. Moreover, in our parallel mouse studies, boiled water extracts prepared from purified paramylon granules did not show any reduction in lung cancer growth. These findings indicate that the paramylon or other water-soluble oligomers of β-1,3-glucan are most likely not responsible for the anti-cancer property of *E. gracilis* water extracts. Further studies are required to evaluate the *in vivo* safety of *E. gracilis* water extracts by pharmacokinetics, pharmacodynamics, and multispecies toxicity studies. Because *E. gracilis* is approved as

generally safe for consumption by the FDA, the exact identity of the chemical in *Euglena* and pharmacokinetics study, although helpful, is not a must for its administration. Regardless of the chemical identity of the compound, the findings from our several studies have established a beneficial role of water extracts from *E. gracilis* against lung cancer in mice.