Frying Oil and Frying Oil-Derived Polar Compounds Exaggerate Colitis in Mice

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FRYING OIL AND FRYING OIL-DERIVED POLAR COMPOUNDS EXAGGERATE COLITIS IN MICE

A Thesis Presented
by
XIJING CHEN

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Food Science
FRYING OIL AND FRYING OIL-DERIVED POLAR COMPOUNDS EXAGGERATE COLITIS IN MICE

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ABSTRACT
FRYING OIL AND FRYING OIL-DERIVED POLAR COMPOUNDS EXAGGERATE COLITIS IN MICE
MAY 2020
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Frying in vegetable oil is a popular cooking and food processing method worldwide; as a result, the oils used for frying are widely consumed by the general public and it is of practical importance to better understand their health impacts. To date, the effects of frying oil consumption on human health are inconclusive, making it difficult to establish dietary recommendations or guidelines. Here we show that dietary administration of frying oil, which was prepared under the conditions of good commercial practice, exaggerated dextran sodium sulfate (DSS)-induced colitis in mice. In addition, to explore the potential compounds involved in the actions of the frying oil, we isolated polar compounds from the frying oil and found that administration of the polar compounds also exacerbated DSS-induced colitis in mice. Together, our results showed that dietary administration of frying oil exaggerated development of inflammatory bowel disease (IBD) in mice, and this effect could be mediated by the polar compounds in the frying oil.
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1.1 Fried food and frying oil consumption

Deep frying is a common food procedure worldwide. It brings desirable flavor, brown color and crispy texture to fried food [1]. The temperature of frying can reach 150°C to 190°C, which gets rid of harmful microorganisms in the food products [2]. Therefore, frying is used to produce processed food with long shelf life in food industry [3]. The processed food such as instant noodles, pre-cooked and ready-to-eat products have more and more consumers in western countries as well as in the developing countries [4]. In United States, 17.5% of women and 30.0% of men reported fried-food consumption more than 4 times a week. Report also shows that people who frequently consume fried food at home were more likely to eat fried food when dining out [5].

The choice of frying oil depends on cost, stability of oxidation, resistance to foaming and color change and location [6]. Olive oil is used for frying at home in Spain, while corn oil is used away from home, especially in fast food restaurants. Palm oil and partially hydrogenated soybean oil are commonly used for frying in Costa Rica [1, 3]. Fried food absorbs frying oil during the procedure and become energy-dense. For example, potato chips absorb 9-12% of frying oil, battered and bread foods absorb 15%-20% of frying oil, standard crisps absorb 35-40% and low fat crisps absorb 20% of frying oil [6]. The absorbed oil accumulates on the surface of food during frying procedure and permeates into the interior of food during cooling [7]. A research reveals that an average 138g of fried food including 14g of frying oil was consumed daily in Spain population. Fried food
consumption accounts for 7% in daily food consumption, ranging from 0 to 817g for men and from 0 to 657g for women [8].

1.2 Chemical reactions during frying

1.2.1 Hydrolysis

Triacylglycerols get through hydrolytic degradation during the frying procedure. In this reaction, triacylglycerol reacts with water from the food being fried, producing free fatty acids, diacylglycerol, monoacylglycerol and glycerol [4]. Compared with fresh oil samples, researchers found higher levels of free fatty acids in used frying oils [9]. Frying oils contained 0.38% to 4.3% of free fatty acids, while the fresh oil samples contained 0% to 1.25%.

The production of free fatty acids is related to types of oil, type of fried food and frying time [10]. Free fatty acids levels increased with the time of frying. Soybean oil can produce more free fatty acids than corn oil and olive oil during the frying. Although the moisture content in potato is higher than that in chicken, the oil used for frying chicken products more free fatty acids than the oil for potato. Diacylglycerol, another hydrolytic alteration, increased with the frying time. The replenishment of frying oil reduced the amount of diacylglycerol [11].

Free fatty acids level is regarded as an indicator of frying oil quality. USDA requires that frying oil with more than 2% of free fatty acids content should be discarded. In some European countries, this limitation varies from 1% to 2.5% [9]. The determination of free fatty acids can be measured by titration (AOCS Ca 5a-40) [12]. However, the cleavage and oxidation of double bonds to form carbonyl compounds also produce free fatty acids. The titration method can not differentiate between acids formed by oxidation
and acids formed by hydrolysis [13]. In addition, free fatty acids will react with other degradation products. Research found the free fatty acids remained unaltered when the frying cycles increased [11].

A study analyzed the French fries bought from three major UK fast food stores. Researchers found that French fries contained 13.5% to 15% of fat and 3.1% to 9.1% of free fatty acids (as oleic acid) [14]. There are few researches qualified and quantified the free fatty acids in other fried food.

1.2.2 Lipid oxidation

Frying oil reacts with oxygen at frying temperature (150°C - 190°C). Thermal oxidation follows the same mechanism as autoxidation, but at a faster rate [7]. Briefly, lipid oxidation contains three steps- initiation, propagation and termination. Initiation is the formation of first free radicals. Propagation is the reaction of oxygen and alkyl radical to product peroxyl radical. Peroxyl radical has higher energy than alkyl radicals and can abstract hydrogen from other unsaturated fatty acids resulting in the formation of lipid hydroperoxide and another alkyl radical. Lipid hydroperoxides are primary lipid oxidation products, which are unstable and break down to alkoxy radical. Alkoxy radical steals an electron from an adjacent covalent bond resulting in cleavage of hydrocarbon bond into form secondary lipid oxidation products. When two free radicals form nonradical products, lipid oxidation comes to termination step.

Studies revealed that frying oils were highly oxidized evidencing by a high level of peroxide value, p-anisidine value, TBARS value and total polar compounds (TPC) [9, 15]. Peroxide value is the measurement of primary oxidation products (AOCS Cd 8b-90) which indicates the rancidity of oil at the level of 10 meq/kg oil. According to Sebastian and
colleagues, the discard frying oils collected from different restaurants in Canada had peroxide value ranging from 4.7 meq/kg to 247.5 meq/kg [9]. It was found that frying oil at 140°C contained less hydroperoxide than oil at 105°C [16]. Since the lipid hydroperoxides are unstable and decompose at frying temperature, peroxide value is not commonly used as an indicator of frying oil quality. Unsaturated aldehydes, which are secondary oxidation products, can be estimated by p-anisidine value (AOCS Cd 18–90). Malonaldehyde (MDA), as a highly studied secondary lipid oxidation product, can be shown as TBARS value (AOCS Cd 19-90). The upper limit of p-anisidine value in fresh frying oil is 6.0. The acceptable limit for the rancidity development is 1-2 mg MDA/kg of TBARS value. In Sebastian’s study, the p-anisidine value of fresh oil ranged from 0.1 to 2.8, while the range for discard oil was 14.2 to 55.8 [9]. Karimi and colleagues studied the frying oil from Kenya, the discard frying oil had p-anisidine value ranging from 14.6 to 52.7, and TBARS value ranging from 0.44 mg/kg to 0.89 mg/kg [15].

The oxidation products that contain oxygenated functional groups have higher polarity than triacylglycerols. High-performance size exclusion chromatography (HPSEC) can be used to separate and quantify the polar compounds [17]. HPSEC analysis 5 main polar compounds based on their molecular weight: polymerized triglycerides, dimeric triglycerides, oxidized triglycerides, mono- and diglycerides and free fatty acids (Figure 1). As a measurement of hydrolysis and lipid oxidation products, the amount of TPC is widely used as the most accurate indicator of frying oil deterioration [18]. The maximum level of TPC in frying oil has been set at 25% to 27% by mass [19]. A study shows that most of the discard frying oils had the TPC amount ranging from 6.5% to 16% in Canada [9]. However, in a study in Kenya, all the discard frying oil samples contained more than 40% TPC, which
were unsuitable for frying [15]. The accumulation of TPC increased by frying temperature and frying time [17]. It also influenced by the level of saturation [18].

**Figure 1. Analysis procedure of total polar compounds** (modified from references [17, 20])

The rate of oxidation increases as the concentration of oxygen and free radicals increase [7]. Lipid oxidation products volatiles and non-volatiles. 50% of volatiles are aldehydes. GC analysis can identify some of them, such as t-2-octenal, tt-2,4-decadienal, t-2-hexenal, hexanal, t-2-heptenal, heptanal, and nonanal [21]. The non-volatiles include short-chain glycerol-bound aldehydes, acids, ketones and alcohols [22].

**1.2.3 Polymerization**

Polymerization, which occurs in lipid during heating and/or oxidation, forms cyclic monomers, dimers and polymers [19]. Cyclic monomers are produced by the intramolecular cyclization of C18 polyunsaturated fatty acids. Dimers and polymers are originated from -C-C-, -C-O-C-, and -C-O-O-C- bonds through radical reactions. Nonvolatile polar compounds and triacylglycerol dimers and polymers are the main decomposition products from frying oil [7]. HPSEC is used to analyze the composition of TPC, which includes the analysis of polymerized triglycerides and dimeric triglycerides.
Frying temperature, oil types and frying times will influence the formation of dimers and polymers [18]. Dimeric and polymerized triglycerides were more likely to accumulate in the oil that had higher temperature, and longer frying times. Cottonseed oil had a fast rate of dimeric and polymerized triglycerides formation at 204°C, while corn oil was the fast rate at 190°C. Beef tallow was the slowest one in general. The amount of dimeric and polymerized triglycerides should not exceed 10% by mass in Belgium, and 16% in the Netherlands [19]. In the discard frying oils with 24%, 25% and 27% of TPC, the content of dimeric and polymerized triglycerides were 10.4%, 11.1% and 11.6% respectively [23]. The amount of these two compounds were significantly affected by oil types at TPC of 24% and 25%. At the TPC of 27%, the effect of foods that used to be fried in the oil was more significant.

1.2.4 Lipid and protein reactions

Protein-carbohydrates reactions are considered as the main reactions that produce the golden color of fried food [20]. Also, lipid oxidation produce can lead to browning in food in the presence of protein. Carbonyl compounds derived from unsaturated lipids condense with free amino groups to generate imino Schiff bases. Brown macromolecules are produced by polymerization of imino Schiff bases [20]. An additional mechanism proposed that brown pigments are produced by the reaction of epoxyalkenals and unsaturated fatty acids with the reactive groups of proteins. This polypyrrolic polymer explains the formation of the color and fluorescence [24]. In addition, Maillard reaction would take place during frying and product main volatile nitrogen- and sulfur-containing heterocyclic compounds(such as pyrazines, pyridines and pyrroles) and acrylamide [4].
Acrylamide can be analyzed by gas chromatography or liquid chromatography coupled with mass spectrometry. According to FDA estimates, the daily intake is 0.4 µg/Kg b.w./day. In the United States, 35% of the average daily acrylamide intake is from French fries and other potato products [25]. The FDA data shows that French fries and chips contain 20-2762 ppb of acrylamide. There is no regulation that limits the level of acrylamide in frying oil or fried food.

1.2.5 Chemical reactions change the nutritional level of frying oil and fried food

There are several chemical reactions that take place during frying in the presence of food, water and oxygen. Figure 2 conclude those reactions in frying oil.

On one hand, frying products some desirable flavor in fried food. For instance, pyrazines contribute to nutty flavor and 2,4-decadienal together with 2-hepetenal contribute to fried food flavor. On the other hand, degradation causes deteriorative changes in flavor, color, and nutritional value of frying oil. The optical density of frying oil was increased as the frying time increased [22]. Polymers increase oil viscosity, reduce heat transfer, and produce foam and brown color during the frying procedure. In addition, polymers increase the oil absorption in fried food [7]. The decreased ratio of linoleic acid and palmitic acid (C18:2/C16:0) during frying indicated the deterioration of PUFA [22].

The trans-fat also be detected from fried food [26]. The trans-polyunsaturated fatty acids are the major trans fatty acids [27]. Only Denmark regulates the maximum amount of trans fatty acids in unused frying oil as 15% [28].

Vitamin C gets less deterioration when frying, comparing with baking or boiling [28]. Researchers found vitamin E is lost in frying oil because of oxidation and absorption
by food [29]. The level of vitamin E was increased in chicken nuggets and breaded shrimp after frying in palm Olein [30].

![Chemical reactions in frying oil](modified from reference [31])

**Figure 2. Chemical reactions in frying oil** (modified from reference [31])

### 1.3 Health concern of fried food consumption

Numerous studies deal with health concerns about frequent fried food consumption. In a study, rats were fed with 10% unheated oil or frying oil that under a good commercial condition in their diet. It took 10 years to fed three generations and the results showed no difference between two groups [32]. Furthermore, another study fed rats with 20% of fresh sunflower oil, heated oil, non-polar fraction and polar fraction for 18 months. They found that the polar group gained less body weight than other three groups but had higher weight of livers and kidneys. Also, their glutamic pyruvic transaminase and glutamic oxaloacetic
transaminase were higher in serum [32]. Some other animal studies found that repeatedly heated vegetable oils increased the risk of cardiovascular disease [2].

In human studies, researchers revealed around 25-36% of adults in North American consume food from restaurants every day. In the United States, the consumption of fried chicken and fried fish/shellfish increased their risk of all cause cardiovascular mortality in postmenopausal women [33]. Due to the differences in the genetic predisposition, the association between adiposity and fried food consumption varies from person to person, and the fried food consumption might modify the genetic influences on adiposity [34]. However, some studies found that consumption of fried food did not associate with developing chronic diseases in Mediterranean countries. For instance, in Spain, the consumption of fried food did not increase the risk of coronary artery disease [3]. Similarly, the null relation between fried food consumption and colorectal cancer was reported in Italy [35]. The overall healthy dietary pattern that rich in fruits, vegetables, whole grain, low red meat can be an explanation. In addition, the choice of olive oil as frying oil reduces the lipid oxidation intensity during frying, because olive oil has lower degree of unsaturation.

1.4 Biological activities of frying oil derivatives

Several chemical reactions result in products including volatile compounds, hydrolysis products, oxidized triacylglycerol monomers, cyclic compounds, trans configuration compounds and acrylamide [4]. It is not practical or essential to identify and quantify all the degradation products in frying oil [19]. Some degradation products are discussed below in terms of in vitro biological activities and in vivo biological activities.
1.4.1 Trans, Trans-2,4-Decadienal (tt-DDE)

Due to the oxidation of polyunsaturated fatty acid (PUFA), cooking oil generates a high level of aldehydic products [36]. The main constituent of that cooking oil fumes is Trans, Trans-2,4-Decadienal (tt-DDE), which is more than 100-fold higher than that of polycyclic aromatic hydrocarbon [37]. tt-DDE has been reported to induce cell proliferation in gastrointestinal epithelial [38]. The expression and release of pro-inflammatory cytokines such as IL-1β and tumor necrosis factor-α were increased [38] and p27 was reduced in human bronchial epithelial cell BEAS-2B due to the tt-DDE exposure [39]. At the concentration from 50 to 200 µM, tt- DDE induce the DNA damage in human lung carcinoma A-549 cell was related to reactive oxygen species formation [40].

In order to study the in-vivo activities of tt-DDE, CD-1 mice were instilled with 8 or 24mg/kg of tt-DDE for 8 weeks. Both bronchioloalveolar junctions hyperplasia and enhanced pSTAT3 accumulation were observed, which suggested that tt-DDE potentially increase the risk of lung adenocarcinoma development [37]. In the study conducted by Hira et al., the oral administration of tt-DDE at the dose of 100mg/kg was used in rats and was not found to be very toxic. tt-DDE ingested together with other macronutrients could delay gastric emptying, which contributes to efficient digestion and absorption of nutrients and attenuation of postprandial hyperglycemia and/or hyperlipidemia[41].

1.4.2 4-Hydroxy-trans-2-nonenal (4HNE) and 4-Hydroxy-trans-2-hexenal (4HHE)

4 HNE originates from ω-6 fatty acids and 4 HHE is oxidized from ω-3 fatty acids [42]. They are oxygenated α,β-unsaturated aldehydes and generate both in biological system and in foods [43]. The absorption of toxic aldehydes from the diet has scarcely been studied, while the pathological significance of endogenous lipid peroxidation has
been highly discussed [42, 43]. It is because those aldehydes generated endogenously has a higher risk of causing diseases. An in vitro digestion model studied the bioaccessibility of 4HNE from thermodegraded fluid deep-frying fat. 4HNE persisted after digestion and was likely absorbed by the gastrointestinal tract, which makes it possible to reach the systemic circulation [43]. A study found that 4-HHE and 4-HNE caused human umbilical vein endothelial cells death at concentrations of 25-50 µM. At the concentration of 5 µM, 4-HHE increased the expression of antioxidant enzyme HO-1 through the activation of Nrf-2 [44].

Although many studies deal with endogenously generated aldehydes, 4HNE was orally administrated by some experimental animals to study the potential toxicity. Mice treated with 5mg/kg/day 4-HNE enhanced activation of TLR4 signaling and increased Inflammatory bowel disease (IBD) [45]. In another in vivo study, Drosophila melanogaster was used, due to its genetic similarity to human. 4-HHE penetrated the intestine of the larvae and affected midgut cells and hemocytes [46]. Similarly, the potential genotoxic risk of 4-HHE is studied by the Drosophila. Exposure to 4-HHE induced mutation and somatic recombination in the wing imaginal disc cells of larvae [47].

1.4.3 Acrolein

Acrolein is an α, β-unsaturated aldehyde originated from amino acids, fats or carbohydrates under heat. It can be formed via Maillard reaction when preparing the carbohydrate-containing foods [48]. The study also found that acrolein is formed from PUFAs, especially linolenic acid, when vegetable oil is heated to 180°C [49]. It is also generated endogenously. A review by Abraham et al. discussed the toxicology and risk assessment of acrolein [48]. In cell culture studies, ≥ 10 µM acrolein reduces the viability
of cell lines such as bronchial endothelial and epithelial cells. In human lung cells, acrolein causes cell death via apoptotic processes.

Abraham et al. also conclude the in vivo study about acrolein in their review [48]. There is no evidence concluded that oral intake of acrolein in a certain amount has the toxic effect in bladder, kidney or brain. Oral exposure leads to gastrointestinal symptoms, gastric ulcers and/or gastric bleeding. Frequent vomiting and reduction of calcium, albumin and total protein levels in the serum were observed in Beagle dogs with the exposure of 0.1, 0.5 and 1.5mg acrolein/kg bw/day for one year. In a study of chronic acrolein toxicity, reduction of creatinine phosphokinase in serum and increasing mortality rates were observed in rats with gavage doses of 0.05, 0.5 and 2.5mg acrolein/kg bw/day for 2 years.

1.4.4 Malondialdehyde (MDA)

Primary lipid peroxidation products have low digestibility while the secondary lipid peroxidation products are highly digestible. MDA is oxidized from both ω-6 fatty acids and ω-3 fatty acids and commonly used as a biomarker for lipid peroxidation [50]. MDA also can be generated in vivo by enzymatic processes [51]. MDA has the ability to create DNA-protein crosslink resulting in its genotoxicity [51]. The reaction between MDA, proteins and DNA may cause impaired cellular function and integrity [52].

In vivo study, the serum MDA concentrations of rats increased significantly when fed with oxidation oil in diet. This suggests that the ingestion of oxidation oil leads to in vivo lipid peroxidation [53]. It is not clear that the MDA in plasma is related to diet or endogenous formation. MDA is able to react with proteins and DNA in the human body as well [51]. Del Rio et al. concluded a positive association between MDA and human disease.
The higher level of plasma MDA is related to lung cancer, breast cancer, cervical cancer, gastric cancer, etc.
CHAPTER 2
EFFECTS OF FRYING OIL ON DSS-INDUCED COLITIS IN MICE

2.1 Introduction

Frying in vegetable oil (e.g. canola, soybean, and corn oils) is a popular cooking and food processing method worldwide. As a result, the frying oils or fried food are widely consumed by the general public. The Nurses’ Health Study (NHS) showed that ~14% of women and ~23% of men consume 4-6 times per week of fried food [5]. In the Spanish cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC) study, it was estimated that the average daily consumption of fried food is as high as 123 grams, with 14 grams of frying oils [8]. During the frying process, the vegetable oils undergo an array of chemical reactions, resulting in formation of various oil degradation-derived products [7, 32]. Notably, vegetable oils are rich in polyunsaturated fatty acids such as linoleic acid (LA, 18:2), which are susceptible to oxidation; and previous studies have shown that the oils used for frying contain many LA oxidation-derived compounds, such as 2,4-decadienal, 4-hydroxynonenal (4-HNE), and malondialdehyde [4, 49]. In addition, the frying oils could also have other types of oil-degradation compounds, including free fatty acids derived from breakdown of triglycerides, dimeric and polymerized triglycerides from polymerization reaction, and acrylamide from Maillard reaction [4, 49]. Since frying oils are widely consumed by the general public, it is of practical importance to better understand their impacts on human health.

To date, the effects of frying oil consumption on human health are inconclusive, making it difficult to establish dietary recommendations or guidelines [32, 42]. Early animal experiments showed that treatment with frying oils could cause adverse effects in
test animals, however, many of these studies used extremely heated oils which have limited relevance with human consumption [32]. Recent studies generally support that the oral toxicity of frying oils, which are prepared under the conditions of good commercial practice, is low [32, 42]. Nolen et al. performed a 2-year feeding study in rats and found that dietary administration of frying oils, which were prepared under practical restaurant-type frying conditions, induced no pathological disorders [54]. The results from this study are supported by many other animal experiments, though there are inconsistent results (see review papers in Ref [32, 42]). Some human studies have shown that frequent consumption of fried food or frying oil is associated with increased risks of human disorders [3, 5, 55]. It remains unclear, however, whether frying oil consumption is causally involved in the elevated risks of these diseases. With a lack of definitive toxicology data, currently there are no governmentally or industrially established limits to regulate the use of frying oils in food preparation. A better understanding of the health impacts of frying oils could lead to a significant impact on public health and regulatory policy.

Most previous studies were performed to investigate the effects of frying oils on disease initiation in healthy animals, the effects of frying oil on disease development are not well understood [32, 42]. Notably, a recent study showed that treatment with frying oil accelerated tumor metastasis in a late-stage breast cancer model in mice [56], suggesting potential promoting effects of frying oil on tumorigenesis. After oral consumption of frying oils or fried food, they have direct interactions with gastrointestinal tract, especially the colon tissues; therefore, it is of practical importance to study the effects of frying oils on gut health. To date, the effects of frying oil consumption on colonic inflammation are
unknown. Here in this study, we treated mice with a frying oil sample, and studied its effects on the development of dextran sulfate sodium (DSS)-induced colonic in mice.

2.2 Materials and methods

2.2.1 Collection and characterization of frying oil

A sample of frying canola oil, which was used for one week to fry Falafel (a deep-fried ball made from ground chickpeas, fava beans, or both) in a standard commercial fryer, was collected from UMass-Amherst Dining Commons. The fryolator could hold 50 pounds of oil. The oil was fried about 6-8 times per day, and the total number of frying hours was approximately 8 hours per day. The oil was fried with a temperature of 325F (163°C). We performed the following assays to characterize the frying oil: (1) the oxidative status of the oil was determined using a lipid peroxide assay, as described [57], (2) the fatty acid profiles of the oils were analyzed by GC-MS [58], and (3) the levels of free fatty acids in the oil were determined using titration [59].

2.2.2 Preparation of un-oxidized vegetable oil

Because many commercial canola oil options were already oxidized with varied degrees of lipid oxidation, we purchased a freshly opened commercial canola oil from a local market in Hadley, MA and purified it using a silicic acid-activated charcoal chromatography, as described [59]. This is a well-established technique to remove oxidized compounds from the oils. Briefly, three layers, including 400 g silicic acid (Clarkson Chromatography Products, South Williamsport, PA), 100 g activated charcoal (Sigma-Aldrich, St. Louis, MO), and another 400 g silicic acid, were sequentially packed into a column (3-inch internal diameter x 18-inch height, 2000 ml reservoir capacity) using hexane as the mobile phase. One liter of the canola oil dissolved in the same volume of
hexane was loaded onto the column and eluted with hexane. The oxidized compounds are more polar and were thus absorbed onto the column, and the un-oxidized triglycerides were eluted out by hexane and evaporated to dryness using a rotary vacuum evaporator. The obtained un-oxidized oil was fortified with 500 ppm tert-butyldihydroquinone (TBHQ, Sigma-Aldrich) as an antioxidant to prevent oxidation, then stored at -80°C for the animal experiment. The fatty acid profiles, levels of free fatty acids, and the concentrations of lipid peroxides of the prepared fresh oil were analyzed, as described above.

2.2.3 Animal experiment

All animal experiments were conducted in accordance with the protocols approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Amherst.

The mice were maintained in a specific-pathogen-free (SPF) facility of the University of Massachusetts. At the end of the experiment, the mice were sacrificed by CO2 overdose and the tissues were collected in a clean operation room located in the SPF facility.

C57BL/6 male mice (Charles River, Shrewsbury, MA) were randomly assigned to two groups, and treated with: (1) a diet containing 10 wt/wt % fresh oil which was fortified with 500 ppm TBHQ, and (2) a diet containing 4% frying oil (fortified with 500 ppm TBHQ) and 6% fresh oil (fortified with 500 ppm TBHQ), throughout the whole experiment. The composition of the experimental diet is shown in Table 1. The diets were prepared and changed every other day, in order to prevent oxidation during the animal feeding. After 3 weeks, the mice were stimulated with 2 wt/vol % DSS (MP Biomedicals, Solon, OH) in
drinking water for 1 week to induce colitis, then the mice were sacrificed for biochemical analyses.

**Table 1. Composition of experimental diets to study the effects of frying oil on colitis**

(All ingredients except the fresh/frying canola oils were purchased from Dyets Inc. Bethlehem, PA)

<table>
<thead>
<tr>
<th>Ingredient (g/kg)</th>
<th>Control diet</th>
<th>Frying oil diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Dyetrose</td>
<td>132</td>
<td>132</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>367.5</td>
<td>367.5</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Mineral Mix #210025</td>
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<td>35</td>
</tr>
<tr>
<td>Vitamin Mix #310025</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Fresh canola oil (fortified with 500 ppm TBHQ)</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>Frying canola oil (fortified with 500 ppm TBHQ)</td>
<td>0</td>
<td>40</td>
</tr>
</tbody>
</table>

**2.2.4 Flow cytometry quantification of immune cell infiltration in tissues**

The distal colon tissues were dissected, cleaned and digested using Hank's-balanced salt solution (Lonza, Basel, Switzerland) supplemented with 1 mM dithiothreitol (DTT) and 5 mM EDTA overnight at 4°C, then filtered through 70 μm cell strainer (BD Biosciences, San Jose, CA) to afford single cell suspensions. The cells were stained with FITC-conjugated anti-mouse CD45 antibody, PerCP/Cy5.5-conjugated anti-mouse F4/80 antibody, PE/Cy7-conjugated anti-mouse Ly-6G/Ly-6C (GR-1) antibody, isotype control antibody, and Zombie Violet™ dye according to the manufacturer’s instructions (Bio Legend, San Diego, CA). The stained cells were analyzed using BD LSRFortessa™ cell
analyzer (BD Biosciences) and data were analyzed using FlowJo software (FlowJo LLC, Ashland, OR).

2.2.5 qRT-PCR analysis

Tissues were ground after freezing in liquid nitrogen, and total rRNA was isolated using TRIzol reagent (Ambion, Austin, TX). The concentration of RNA was measured by NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The RNA was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to manufacturer’s instructions. qRT-PCR was carried out with a DNA Engine Opticon system (Bio-Rad Laboratories, Hercules, CA) with Maxima SYBR-green Master Mix (Thermo Fisher Scientific). The sequences of mouse-specific primer (Thermo Fisher Scientific) are listed in Table 2. The results were normalized to glyceraldehyde-3-phosphate dehydrogenase (Gapdh) using the $2^{-\Delta\Delta C_T}$ method.

Table 2. Sequences of primers in qRT-PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh</td>
<td>AGGTCCGTTGTGAACGGATTTG</td>
<td>TGTAAGACCATGTAGTTGAGGTCA</td>
</tr>
<tr>
<td>Tnf-α</td>
<td>CCCTCACACTCAGATCATCTCTCT</td>
<td>GCTACGACGTGGGCTACAG</td>
</tr>
<tr>
<td>Il-6</td>
<td>TAGTCCTTCTACCCCAATTTCC</td>
<td>TTGGTCCCTAGCCACTCCTTC</td>
</tr>
<tr>
<td>Il-1β</td>
<td>GCAACTGTTCCTGAACTCAACT</td>
<td>ATCTTTTGGGGTGTCGTAACACT</td>
</tr>
<tr>
<td>Mcp-1</td>
<td>TTTAAAACCTGGATCGGAACCAA</td>
<td>GCATTAGCTTCAGATTTACGGGT</td>
</tr>
<tr>
<td>Ifn-γ</td>
<td>ATGAACGCTACACACTGCATC</td>
<td>CCACCTTTTGGCCAGTTTCTC</td>
</tr>
</tbody>
</table>

2.2.6 ELISA analysis of cytokines in plasma

Blood samples were collected via cardiac puncture and the plasma samples were obtained by centrifugation of the blood samples at 1,500 g for 10 min at 4°C. The
concentration of cytokines in plasma were determined using a CBA Mouse Inflammation Kit (BD Biosciences) according to the manufacturer’s instructions.

2.2.7 Histological analysis

The dissected colon tissues were fixed in 10% neutral buffered formalin (Thermo Fisher Scientific) for 48h. After dehydration, the tissues were embedded in paraffin and sliced (5 μm) by Rotary Microtome (Thermal Fisher Scientific). The slices were dewaxed in serial xylene and rehydrated through ethanol solutions, stained with hematoxylin and eosin (Sigma-Aldrich), and examined with a light microscopy (Nikon Instruments, Melville, NY). The histological scores were evaluated by a blinded observer according to the following measures: crypt architecture, degree of inflammatory cell infiltration, muscle thickening, goblet cell depletion, and crypt abscess. The histological damage score is the sum of each individual score.

2.2.8 Data analysis

All data are expressed as means ± SEM. Shapiro-Wilk test was used to verify the normality of data and Levene's mean test was used to assess equal variance of data. Statistical comparison of two groups was performed using Student’s t-test, or Wilcoxon-Mann-Whitney test (when normality test fails). The statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC).

2.3 Results

2.3.1 Collection and characterization of frying oil

To study the effects of frying oil on gut health, we collected a “real-life” frying oil sample, which has been used for 1 week to prepare Falafel, from the Dining Commons of UMass-Amherst. We analyzed the oxidative status of the frying oil: the concentration of
lipid hydroperoxide (a marker of fat oxidation) is $1.03 \pm 0.26$ mEq/kg for the frying oil versus $0.08 \pm 0.01$ mEq/kg for the control fresh oil, and the level of free fatty acid (a marker of triglyceride breakdown) was $0.17 \pm 0.00\%$ for the frying oil versus $0.07 \pm 0.00\%$ for the fresh oil (Table 3). These results demonstrate enhanced fat oxidation and degradation in the frying oil. We have to point out that the concentration of lipid hydroperoxide is not a reliable marker of oxidative status, since the lipid peroxides could decompose [60].

Consistent with the increased oxidative status of the frying oil, GC-MS analysis showed that compared with the control fresh oil, the frying oil had lower levels of polyunsaturated fatty acids: the level of LA (18:2ω-6) in the frying oil is $14.20 \pm 0.11\%$ versus $19.06 \pm 0.09\%$ in the fresh oil, and the level of α-linoleic acid (α-LA, 18:3ω-3) in the frying oil is $1.82 \pm 0.03\%$ versus $6.82 \pm 0.09\%$ in the fresh oil (Table 3).

Table 3. Fatty acid profile and oxidation status of fresh oil and frying oil

<table>
<thead>
<tr>
<th></th>
<th>Fresh oil</th>
<th>Frying oil</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>**Fatty acid profile (%) *</td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
</tr>
<tr>
<td>C16:0</td>
<td>4.37</td>
<td>0.03</td>
<td>4.14</td>
</tr>
<tr>
<td>C18:0</td>
<td>1.95</td>
<td>0.03</td>
<td>2.00</td>
</tr>
<tr>
<td>C18:1 (n-9)</td>
<td>62.48</td>
<td>0.19</td>
<td>72.50</td>
</tr>
<tr>
<td>C18:1 (n-7)</td>
<td>3.49</td>
<td>0.02</td>
<td>3.48</td>
</tr>
<tr>
<td>C18:2 (n-6)</td>
<td>19.06</td>
<td>0.09</td>
<td>14.20</td>
</tr>
<tr>
<td>C18:3 (n-3)</td>
<td>6.82</td>
<td>0.09</td>
<td>1.82</td>
</tr>
<tr>
<td>C20:0</td>
<td>0.60</td>
<td>0.02</td>
<td>0.62</td>
</tr>
<tr>
<td>C20:1</td>
<td>1.24</td>
<td>0.03</td>
<td>1.24</td>
</tr>
<tr>
<td><strong>Oxidation status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid hydroperoxide (mM/kg oil)</td>
<td>0.08</td>
<td>0.01</td>
<td>1.03</td>
</tr>
<tr>
<td>Free fatty acid (%)</td>
<td>0.07</td>
<td>0.00</td>
<td>0.17</td>
</tr>
</tbody>
</table>

* The results are expressed as % of individual fatty acid to total fatty acids, assessed by GC-MS analysis. The data are mean ± SEM. (n = 3 per group).
2.3.2 Effects of frying oil on DSS-induced colitis

We studied the effect of the frying oil on colonic inflammation, using a well-established DSS-induced colitis model in C57BL/6 mice (see scheme of animal experiment in Figure 3). We treated mice with a completely defined isocaloric diet containing 10% (wt/wt) fresh oil, or a combination of 4% frying oil and 6% fresh oil. We determined the level of frying oil in the experimental diet (4 wt/wt %) to mimic human consumption: a previous study estimated that the average intake of fried foods (containing ~10% wt/wt frying oil) is 0-817 g per person per day, leading to a calculated intake of frying oil to be ~0-4% wt/wt of the diet [8].

![Scheme of animal experiment about dietary administration of frying oil](image)

**Figure 3. Scheme of animal experiment about dietary administration of frying oil**

We found that dietary administration of the frying oil enhanced DSS-induced colitis. Mice in frying Oil group lost body weight faster than the mice that consumed fresh oil during DSS treatment (Figure 4). The frying oil group tended to have reduced colon length (Figure 5A) and increased weight both in spleen (Figure 5B) and kidney (Figure 5C). Compared with the DSS mice treated with the fresh oil, the DSS mice treated with the frying oil had exaggerated crypt damage (Figure 6A), enhanced colonic infiltration of CD45+ F4/80+ and CD45+ Gr1+ immune cells (Figure 6B), increased colonic expression
of pro-inflammatory cytokines (Il-1β, Il-6, and Mcp-1) (Figure 6C). Together, these results demonstrate that dietary administration of frying oil exaggerated DSS-induced colitis in mice.

Figure 4. Effect of dietary administration of frying oil on body weight change during DSS treatment. The data are mean ± SEM, n = 7 mice per group.

Figure 5. Effect of dietary administration of frying oil on organs. (A)Colon length. (B) Spleen weight. (C) Kidney weight. The data are mean ± SEM, n = 7 mice per group.
Figure 6. Dietary administration of frying oil exaggerates DSS-induced colitis in mice. (A) Left panel: representative images of H&E staining of the colon in c(magnification 600×, scale bar = 50 μm). Right panel: quantification of H&E staining. (B) FACS quantification of immune cell infiltration in the colon. (C) Colonic expression of pro-inflammatory cytokines. The data are mean ± SEM, n = 7 mice per group.
2.4 Discussion

The incidence and prevalence of inflammatory bowel disease (IBD) have risen dramatically in recent decades in the United States and other countries [61]. The rapidity of the development suggests that environmental and dietary factors, which remain to be identified, could be primarily responsible for the elevated risks of IBD. It is of practical importance to identify novel risk factors of IBD, which could lead to a significant impact on public health and regulatory policy. Here our central finding is that dietary administration of a frying oil sample exaggerated DSS-induced IBD in mice. Overall, these results support that the frying oils could be potential dietary risk factors of IBD. In our study, we studied the effects of frying canola oil; it is expected that other types of frying oils (e.g. soybean oil) could induce similar adverse effects since they also have high levels of LA which is a probe to lipid oxidation. Based on our finding, the individuals with or prone to IBD cancer could be susceptible to the adverse effects of frying oils and may need to reduce the dietary intake of frying oils or fried foods, though more studies are needed to validate the impact of frying oils on gut health before any dietary recommendations could be established.

In our experiment, we used a frying oil sample, which has been used for 1 week to prepare Falafel, from UMass-Amherst Dining Commons. The objective was to get an oil that truly represented oxidized oil that is consumed by the general public. Commercial deep fat fryers are designed to handle large volumes of food meaning that they must be able to maintain a constant temperature. This is accomplished by having a large oil reservoir and a heating apparatus with a large surface area for rapid heat transfer. These conditions are difficult to mimic in a laboratory setting even with small household frying appliances.
Because the initial heating of the fryer takes so long, fryer is left on all day in a restaurant setting. Therefore, restaurant fryers undergo periods where they are held at high temperature without frying meaning that they are close to anaerobic followed by periods where foods are cooked resulting in aeration and large amounts of oxygenation. These conditions as well as the large volume of food cooked in a restaurant are also difficult to accurately reproduce in a laboratory setting. Thus, we felt that to study an oil that represents dietary fat from frying, it was critical to use actual food service oil. To date, the accurate assessment of exposure and absorption of frying oil in human populations is under-studied. In our study, we treated mice with a diet containing 4 wt/wt % of the frying oil sample, since a previous study estimated that the average intake of fried foods (containing ~10% frying oil) is 0-817 g per person per day, leading to a calculated intake of frying oil to be ~0-4% wt/wt of the diet [8]. Together, these results support that the pro-colitis effects of frying oils in mouse models, and more studies are needed to characterize their impacts on human health.
CHAPTER 3
EFFECTS OF FRYING OIL-DERIVED POLAR COMPOUNDS ON DSS-INDUCED COLITIS IN MICE

3.1 Introduction

During the frying process, several types of chemical reactions could happen, including lipid peroxidation and hydrolysis. The oxidation products that contain oxygenated functional groups have higher polarity than triacylglycerols. As a measurement of hydrolysis and lipid oxidation products, the amount of total polar compounds (TPC) is widely used as the most accurate indicator of frying oil deterioration [18]. The maximum level of TPC in frying oil has been set at 25% to 27% by mass [19]. The accumulation of TPC increased by frying temperature and frying time [17]. It also influenced by the level of saturation [18].

Polar compounds from lipid oxidation are consumed with food and absorbed by the intestinal tract, eventually present in the blood circulation. This may increase the risk of cardiovascular disease [62]. Other studies also showed that the lipid hydroperoxides could promote tumor in the colon [42]. To date, the effects of frying oil-derived polar compounds consumption on colonic inflammation are unknown. Here in the second study, we treated mice with polar compounds that isolation from frying oil and studied its effects on development of dextran sulfate sodium (DSS)-induced colonic in mice.
3.2 Materials and methods

3.2.1 Isolation of polar compounds from frying oil

The frying oil sample (500 g) dissolved in 500 mL hexane was loaded onto a silica gel column, then eluted with a mixed solvent of ethyl acetate and hexane (Figure 7A). The obtained fractions were pooled based on thin-layer chromatography (TLC) analysis using a mobile phase of hexane: ethyl acetate = 15:1 (vol/vol). The polar compounds were combined, evaporated to dryness using a vacuum rotary evaporator, and used for animal experiment.

3.2.2 Animal experiments and analysis

C57BL/6 male mice were randomly assigned to two groups and treated with a diet with or without 0.1 wt/wt % of frying oil-derived polar compounds (see the composition of the experimental diet in Table 4) throughout the whole experiment. The diets were prepared and changed every other day, in order to prevent further oxidation during animal treatment. After 3 weeks, the mice were stimulated with 2% DSS in drinking water for 1 week to induce colitis. At end of the experiment, the mice were sacrificed for analyses.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Polar compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
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<tr>
<td>Dextrose</td>
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<tr>
<td>Cornstarch</td>
<td>367.5</td>
<td>367.5</td>
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<td>Cellulose</td>
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</tr>
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<td>Mineral Mix #210025</td>
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<td>35</td>
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<tr>
<td>Vitamin Mix #310025</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Corn oil</td>
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<td>99</td>
</tr>
<tr>
<td>Polar compounds</td>
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<td>1</td>
</tr>
</tbody>
</table>
The preparation of un-oxidized vegetable oil followed the steps described above in 2.2.2. After animal scarification, the tissues were collected and analyzed by following the steps in 2.2.4 (Flow cytometry quantification of immune cell infiltration), 2.2.5 (qRT-PCR analysis), 2.2.6 (ELISA analysis of cytokines in plasma), 2.2.7 (Histological analysis), and 2.2.8 (Data analysis).

3.3 Results

3.3.1 Isolation and characterization of polar compounds from frying oil

To determine the extent to which the observed biological effects of the frying oil are mediated by lipid oxidation-derived compounds, we isolated the polar compounds from the frying oil. The rationale of this approach is that during lipid oxidation, the lipids are derivatized with polar moieties (e.g. hydroxyl, keto, or epoxide groups), resulting in formation of lipid oxidation-derived compounds which are generally more polar than the un-oxidized lipids. Using a silica gel-based column chromatography, we isolated the polar compounds from the frying oil (see scheme of experiment in Figure 7A). The isolated polar compounds represent ~2.6 wt/wt % of the frying oil. Compared to the frying oil, the isolated polar compounds have higher concentrations of lipid oxidation-derived compounds: the concentration of lipid hydroperoxides was 10.02 ± 0.08 mEq/kg for the isolated polar compounds versus 1.03 ± 0.26 mEq/kg for the frying oil (Figure 7B).
Figure 7. Isolation and oxidation analysis of frying oil-derived polar compounds. (A) Isolation of polar compounds from frying oil. The frying oil sample was purified using a silica gel flash column, the polar fractions (F5-F11) were combined and used as “polar compounds” in the animal experiment. (B) Lipid hydroperoxide analysis (n = 3 per group). The data are mean ± SEM.

3.3.2 Effects of frying oil-derived polar compounds on DSS-induced colitis

To study the biological actions of the frying oil-derived polar compounds, we treated the mice with a diet with or without 0.1 wt/wt % of the isolated polar compounds, then stimulated the mice with DSS to induce colitis (see scheme of animal experiment in Figure 8). We have determined the level of the polar compounds in the diet (0.1 wt/wt %), since the experimental diet with 4% frying oil (containing ~2.6% polar compounds) as described above contains similar amounts of the polar compounds, allowing us to study the roles of the polar compounds in the actions of the frying oil.
Figure 8. Scheme of animal experiment about dietary administration of frying oil-derived polar compounds.

We found that dietary administration of the polar compounds exaggerated DSS-induced colitis in mice. Mice treated with polar compounds lost body weight slightly faster than the mice that consumed fresh oil during DSS treatment (Figure 9). There was no significant differences between two groups in colon length (Figure 10A), spleen weight (Figure 10B) and kidney weight (Figure 10C). Compared with the control DSS-stimulated mice, the DSS-stimulated mice treated with the polar compounds had exaggerated crypt damage (Figure 11A), increased expression of pro-inflammatory genes (Tnf-α, Il-1β, Il-6, Mcp-1 and Ifn-γ) in the colon (Figure 11C), and increased concentrations of pro-inflammatory cytokines (TNF-α and IFN-γ) in the plasma (Figure 11D). Together, these results demonstrate that polar compounds, which are enriched with lipid oxidation-derived compounds, have similar pro-colitis effects as the frying oil, supporting that these compounds could contribute to the observed biological actions of the frying oil.
Figure 9. Effect of dietary administration of frying oil-derived polar compounds on body weight change during DSS treatment. The data are mean ± SEM, n = 10 mice in ctrl group, and n = 8 mice in polar compounds group.

Figure 10. Effect of dietary administration of frying oil-derived polar compounds on organs. (A) Colon length. (B) Spleen weight. (C) Kidney weight. The data are mean ± SEM, n = 10 mice in ctrl group, and n = 8 mice in polar compounds group.
3.4 Discussion

During the frying process, several different types of chemical reactions, including the breakdown of triglycerides to generate free fatty acids, lipid oxidation, and reactions between lipids with other food components such as food proteins, could happen, resulting in the formation of an array of complicated products [49]. Compared with the first animal experiment, the treatment with polar compounds resulted in less severe symptom. The loss of some semi-volatile compounds that with medium polarity caused by the rotary evaporation can be an explanation [36]. Our results showed that dietary inclusion of “polar compounds” isolated from the frying oil, which had high concentrations of lipid peroxides, exaggerated DSS-induced colitis in mice, supporting that lipid oxidation-derived compounds could contribute to the pro-IBD effects of frying vegetable oils. Consistent with this notion, a recent study from our lab showed that systemic treatment (via intraperitoneal injection) with 4-HNE, a lipid oxidation-derived compound, exaggerated DSS-induced IBD in mice [45]. To date, the biological fates of the lipid oxidation-derived compounds in systemic circulation are controversial [63]. It is feasible that some of these compounds could locally interact with the gut cells or microbiota, resulting in exaggerated colonic inflammation. It should be noted that the body has a battery of antioxidant systems (e.g. glutathione, catalase, and superoxide dismutase), to counteract the potential adverse
effects of lipid oxidation-derived compounds. However, previous studies have shown that the mice and human patients with IBD have significantly elevated tissue levels of reactive oxygen species and enhanced severity of oxidative status, suggesting that the antioxidant protective system could be compromised in the IBD patients [64-66]. These results suggest that these individuals could be highly sensitive to the adverse effects of oxidized lipids, which are commonly present in frying oils.
Our animal experiment studied the effect of frying oil and frying oil-derived polar compounds on C57BL/6 male mice. Our results all together showed that dietary intake of frying vegetable oils, as well as frying oil-derived polar compounds, increased the severity of experimental colitis in mice, supporting that the frying oils could be novel risk factors of IBD and associated diseases. This part of result had been published in 2019 [67]. Furthermore, another study from our lab found that dietary intake of frying oil can aggravate azoxymethane (AOM)/DSS-induced colon tumorigenesis in mice. In addition, dietary administration of frying oil impaired intestinal barrier function, enhanced translocation of lipopolysaccharide (LPS) and bacteria from the gut into the systemic circulation, and increased tissue inflammation. Recent research found the dietary intake of frying oil can accelerate tumor metastasis in late-stage breast cancer. Therefore, more studies are needed to figure out the effect of frying oil consumption on the patients with other diseases, such as diabetes, cardiovascular disease, Alzheimers disease or hepatic disease. Meanwhile, researches about the diet of patients who suffer from different diseases may help to develop a specific diet for them in order to relieve their pain, and these efforts could lead to a significant impact on public health and regulatory policy.


