Study Protocol

Effect of Probiotic Supplementation on the Immune System in Patients With Ulcerative Colitis in Amman, Jordan

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The Objectives of the Study are:

1- To determine the effect of *Lactobacillus* and *Bifidobacterium* probiotics supplementations on immune system response among patients diagnosed with pre-remission mild to moderate UC.

2- To determine the effect of *Lactobacillus and Bifidobacterium* probiotics supplementations on inflammatory response among patients diagnosed with pre-remission mild to moderate UC.

3- To determine the effect of *Lactobacillus and Bifidobacterium* probiotics supplementations on quality of life for pre-remission mild to moderate UC.

The Research Problem:

Ulcerative colitis (UC) is a chronic Inflammatory bowel disease (IBD) that most likely results from the interaction between various environmental and genetic factors (Van Assche *et al.,* 2010). UC is currently defined by continuous mucosal inflammation in the rectum and extended variables in the colon, without the existence of granulomas on mucosal biopsies (Dignass *et al.,*

2012a). UC is a lifetime disease that is characterized by periods of remission and periods of relapse. The relapsing periods often presented with a combination of rectal bleeding, diarrhea, abdominal pain, malaise and weight loss, which are responsible for the decreased quality of life and for most of the disease burden (Dignass *et al.,* 2012b; Levesque *et al.,* 2015). Moreover, newly diagnosed UC patients have a 5-year risk of colectomy of 10–35% (Van Assche *et al.,* 2013), and ultimately, the extensive and persistent inflammation can increase the long-term risk of colorectal cancer (Van Assche *et al.,* 2013). Worldwide, UC is reported to have an overall prevalence range from 7.6 to 245 cases per 10 0,0 0 0 persons/year and an incidence of 1.2–20.3 cases per 10 0,0 0 0 persons/year (Ng et al., 2017; Feuerstein et al., 2019; Gajendran et al., 2019). For specific countries, the UC prevalence in the United States was 286 cases per 100,000 populations (Kaplan and Ng, 2016), in San Marino was 311 per 100,000 populations (Piscaglia et al., 2019), and in Norway 505 of 100,000 populations (Ng et al., 2017). In Jordan, there have been no studies conducted on the prevalence of UC. However, the incidence of UC is growing in Jordan, with changes in the cultural behavior influenced by Westernization and now converted to a country with varied ethnicities (Omran and Barakat, 2016).

The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) nutritional guidelines have defined probiotics as “live microorganisms when administered in adequate quantities confer a health profit to the host cell” (Araya et al., 2002; FAO/WHO,2013; Nolfo *et al.*, 2013). Probiotics have been used in IBD and especially UC (Sartor, 2004). The effect of using probiotics on UC has been studied in a variety of experimental conditions both in vitro and in vivo (Abraham and Quigley 2017; Guandalini and Sansotta, 2019). The rational explanation for using probiotics in UC is based on the hypothesis that intestinal bacteria might be associated with the pathogenesis of IBD in general, and UC in specific (Sartor, 2004). Balance disturbance between the host’s genetic background, enteric microflora, and bowel mucosal immunity might be involved in tissue inflammation in UC (Naidoo *et al.,* 2011).

The use of probiotics to enhance immune responses, attenuate inflammatory responses, improve the intestinal microbial environment and as conventional therapy for patients with IBD in general, and for UC in specific were researched (Mardini and Grigorian, 2014; Shen *et al*., 2018; Dargahi *et al*., 2019). However, results are still inconclusive and insufficient to recommend for or against the use of probiotics either alone or in combination with standard therapy for UC (Mardini and Grigorian, 2014; Shen *et al*., 2018; Dargahi *et al*., 2019). Additionally, in Jordan, no studies have been conducted on the uses of probiotics for UC patients. Therefore, this study aims to evaluate the effect of probiotics supplementations on the immune system and inflammatory responses among mild to moderate UC patients pre-remission in Amman, Jordan.

The importance of the Study:

The importance of the present study lies on establishing information that either validates or invalidates the effectiveness of probiotics supplementation intervention in reducing inflammatory markers and enhancing the immune system response for patients with pre-remission mild to moderate UC. Moreover, the results could provide additional evidence to the beneficial effect of using probiotics as adjuvant therapy for UC patients to elevate their immune response and reduce the inflammatory marker during reaching the remission status.

Literature Review:

Ulcerative Colitis (UC):

In the 21st century, Inflammatory bowel disease (IBD) is a global disease that includes ulcerative colitis (UC), Crohn's disease (CD), and indeterminate colitis (IC) (Bamias *et al*., 2012; Ng *et al.,* 2017). In contrast of Crohn's disease, which are healthy parts of the intestine mixed in between inflamed areas that can occur in all the layers of the bowel walls (Dignass *et al*., 2012a; Gajendran *et al*., 2019). Ulcerative colitis (UC) is a continuous inflammation of the colon, lifelong, chronic

IBD that only affects the inner most lining of the colon and most likely results from the interaction between genetics and various environmental factors (Dignass *et al*., 2012a; Gajendran *et al*., 2019). UC is defined as a chronic inflammatory condition resulting in a continuous mucosal inflammation starting with mucosal inflammation of the rectum and extending proximally to the colon without the existence of granulomas on mucosal biopsies, characterized by a period of remission and period of relapse (Dignass *et al*., 2012a; Gajendran *et al*., 2019).

UC Prevalence:

Ulcerative colitis (UC) is the most common IBD worldwide (Gajendran *et al*., 2019). It can develop at any age but has an incidence peak occurs in the 2nd or 3rd decades of life (between 10 and 29 years of age), followed by a second peak between 50 and 80 years of age (Feuerstein *et al*., 2019). UC is more common in developed countries including North America and Western Europe, and recently there has been increased incidence in Asia (Feuerstein *et al*., 2019; Gajendran *et al*., 2019). Additionally, the incidence of UC increased in newly industrialized countries whose societies have become more westernized (Ng *et al*., 2017). The overall incidence of UC is reported to be 1.2–20.3 cases per 10 0,0 0 0 persons/year, while the prevalence is reported to be 7.6–245 cases per 10 0,0 0 0 persons/year (Ng *et al*., 2017; Feuerstein *et al*., 2019; Gajendran *et al*., 2019). A systematic review of population-based studies conducted on the incidence and prevalence of inflammatory bowel disease worldwide in the 21st century as well as other studies on the prevalence of UC, reported that the prevalence of UC was 286 cases per 100,000 populations in the United States (Kaplan and Ng, 2016), 311 of 100,000 in San Marino (Piscaglia *et al*., 2019), 505 of 100,000 in Norway, and a low of 6.67 per 100,000 in Malaysia (Ng *et al*., 2017). Also, they found that the prevalence of IBD (UC) exceeded 0·3% in North America, Oceania, and many countries in Europe (Ng *et al*., 2017).

In Jordan, there is very scare information about the UC disease and the information about its prevalence and incidence are is not known (Omran and Barakat, 2016). However, the UC is not rare and occur among all age groups, with a peak incidence in the second and third decades (Ghazzawi and Al-Marayat, 2007; Omran and Barakat, 2016).

UC Risk Factors and Clinical Manifestations:

The key risk factors associated with the development of UC are genetics, environmental factors, autoimmunity, and gut microbiota (Dignass *et al*., 2012a; Gajendran *et al*., 2019). The genetic predisposition plays a role in 8–14% of UC cases where patients have family history of UC (Gajendran *et al*., 2019). The environmental risk factors associated with the development and severity of UC are ethnic and racial differences, which have reported to be more related to environmental influences, food habits and lifestyle rather than the true genetic differences (Gajendran *et al*., 2019). Other environmental risk factors include smoking, age, and diet ("Western" style diet) differences (Dignass *et al*., 2012a; Gajendran *et al*., 2019). Intestinal microbiota populations composition alteration associated with dysregulation of the immune response to bacterial antigens (Lepage *et al*., 2011; Gajendran *et al*., 2019).

The typical symptoms of UC are bloody diarrhea, abdominal pain, fecal urgency, tenesmus, and mucus in the stool (Feuerstein *et al*., 2019; Gajendran *et al*., 2019). The diagnosis of UC is made based on a combination of clinical features, Lab results, and endoscopic findings that show a continuous and diffuse colonic inflammation which starts in the rectum, as well as histological findings where biopsies of the colon documenting a chronic inflammation that confirm the diagnosis of UC (Feuerstein *et al*., 2019; Gajendran *et al*., 2019).

UC Managements:

The management of UC is multi-disciplinary and changing process, aiming mainly to induce clinical remission and then the maintenance of remission, which defined as normalization of bowel

movements and cessation of bleeding (Dignass *et al*., 2012b; Vanga and Long, 2018). The suitable treatment approach for each patient is personalized based on the activity, distribution (proctitis, left-sided, extensive1), pattern of disease (relapse frequency, course of disease, response to previous medications, side-effect profile of medication and extra-intestinal manifestations) and the age at onset and disease duration (Dignass *et al*., 2012b). Most patients are treated with pharmacological therapy to first induce remission and then to maintain a corticosteroid-free remission. There are multiple classes of drugs used to treat the disease (Vanga, and Long, 2018). However, the patients in which medical therapy fails or have developed dysplasia secondary to their long-standing colitis require surgical treatment (Ray and Sagar, 2017; Feuerstein *et al*., 2019).

In addition to the progression in the treatment of inflammatory bowel diseases (IBD) specially UC, adjuvant and alternative options are continually seeking and needed to enhance and improve the pharmacological treatment (Guandalini and Sansotta, 2019). These options include dietary modifications, food supplements, and, more recently, probiotics (Guandalini and Sansotta, 2019). Mucosal homeostasis or chronic inflammation are occurring as a result of the imbalance between beneficial and harmful intestinal microorganisms which known to be associated with IBD occurrences (Guandalini and Sansotta, 2019). The UC and IBD patient's microbiota differ from healthy individuals, as it is characterized by a reduced diversity, reduced abundance of *Firmicutes and Bacteroidetes*, and increased the abundance of *Enterobacteriaceae* (Guandalini and Sansotta, 2019).

Probiotics

As the demands for healthy and functional foods that promote health and prevent or cure illness increased during the last decades, probiotics have received attention in the field of self-care and complementary medicine (Shokryazdan *et al*., 2017a). The word "probiotic" comes from the

Greek words “pro” and “biotic,” meaning “for the life” (Shokryazdan *et al*., 2017a). In 2001, the Food and Agriculture Organization/World Health Organization (FAO/WHO) defined probiotics as “live microorganisms which, when administered in adequate amounts confer a health benefits on the host.” (FAO/WHO, 2001). This concept was updated in 2013 to include the three main key aspects of probiotics: microbial, viable, and beneficial to health, to became “live microorganism that, when administered in adequate amounts, confer a health benefit on the host” (Hill *et al*., 2014). Microorganisms that are considered as probiotics should have several characteristics including resistance to gastrointestinal environment (low pH and bile salt), antimicrobial activity, multidrug resistance, and antioxidant activity (Biradar *et al.,* 2008). The chief and most widely used probiotics bacterial species belong to the genera *Lactobacillus* and *Bifidobacterium*, which belong to lactic acid bacteria group (Ritchie and Romanuk, 2012; Sharif *et al*., 2018).

Probiotics Health Benefits

The human gastrointestinal tract (GIT) is a complex microbial ecosystem inhabited by more than 400 bacterial species, which can be influenced by different factors, and one of the most important factors is the diet of the host (Holzapfel and Schillinger, 2002; Graf *et al.,* 2015). Recent scientific research showed that the deficiency or imbalance of intestinal microbiotate leads to some of the infections and disorders (Shokryazdan *et al.,* 2017a; Sánchez *et al.,* 2017). Probiotics have been considered as one of control strategies for several gastrointestinal disorders such as GIT infections, constipation, irritable bowel syndrome, inflammatory bowel disease (Crohn’s disease and ulcerative colitis), antibiotic-induced diarrhea, food allergies, and certain cancers such as colorectal cancer (Shokryazdan *et al.,* 2017a; Sánchez *et al.,* 2017).

The health promoting benefits of probiotics include modulation of the immune system reducing colitis and inflammation, antioxidant activity, toxin-binding and detoxification activity, maintenance of mucosal integrity, decreasing incidence and duration of diarrhea, and regulation of

gut motility to control constipation or irritable bowel syndrome (Tannock, 1999; Orrhage and Nord, 2000; Zoghi *et al.,* 2014). Additionally, probiotics may reduce allergy symptoms, improve nutrient absorption, alleviate symptoms of lactose intolerance, and produce beneficial compounds, such as vitamins, short-chain fatty acids (SCFAS), and conjugated linoleic acid (Shokryazdan *et al.,*2014; Isolauri *et al.,* 2015; Onubi *et al.,*2015; Liu *et al.,*2017; Shokryazdan *et al.,* 2017b). Regarding the safety, there is no evidence that consumption of *lactobacilli* probiotics causes any risk of infection greater than that associated with commensal strains and it is impossible to propose a risk of death for *lactobacilli* consumption (Borriello *et al.,* 2003).

Probiotics and Immune Response

The protection of gastrointestinal health is an essential medical issue because 70% of the human immune system is placed in the intestine (Sharif *et al*., 2018). Probiotics help to enhance intestinal barrier function and maintain the integrity of the gut and other organs by secreting antioxidative and anticarcinogenic compounds that help in maintaining and supporting the immune system (Sharif *et al*., 2018; Dargahi *et al.,* 2019). Probiotics stimulate the immune system function by producing anti-inflammatory cytokines; stimulating dendritic cells (DCs) to produce inflammatory cytokines interleukin-12 (IL-12) and regulatory IL-10; down-regulating IL-12 levels through surface layer proteins that interact; and stimulating the innate cells (Kanmani *et al.,* 2013; Zhong *et al.,* 2014; Witkowski *et al.,* 2018). Furthermore, probiotics enhance the total numbers of T cells, natural killer (NK) cells, histocompatibility complex (MHC) class II+ cells, and CD4-CD8+ T cells and increase the production of cytokines, such as interferon (IFN)-γ, interleukin1-β (IL-1β) and tumor necrosis factor alpha (TNF-α) (Kanmani *et al.,* 2013; Zhong *et al.,* 2014; Dargahi *et al.,* 2019).

Studies of animal and human subjects indicated that several isolates of *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus rhamnosus* GG, and/or *Lactobacillus acidophilus* can mediate anticancer effects through different mechanisms, including maturation of dendritic cells, release of

probiotic-derived ferrichrome, or activation of natural killer cells (Hu *et al*., 2015; Cai *et al*., 2016; Konishi *et al*., 2016; Dargahi *et al.,* 2019). Additionally, Lactobacillus mixture shifted the gut microbial community toward *Prevotella* and *Oscillibacter*, which are known to produce anti-inflammatory metabolites that decrease Th17 polarization and enhance the differentiation of anti-inflammatory Treg/Tr1 cells in the gut (Li *et al*., 2016; Dargahi *et al.,* 2019).

Probiotics Role in Ulcerative Colitis

The uses of probiotics for UC have been explored under a variety of experimental conditions both in vitro and in vivo (Abraham and Quigley 2017; Guandalini and Sansotta, 2019). Studies revealed that using of probiotics as an adjunct to medical therapy might be useful in the treatment of UC (Shen *et al*., 2018; Guandalini and Sansotta, 2019). This beneficial effect of Probiotics may be explained through at least two mechanisms (Witkowski *et al*., 2018). First one, their ability to enhance the mucosal barrier function, which shown to promote the secretion of IgA and mucins (Kabeerdoss *et al*., 2010) and tight junction function (Karczewski *et al*., 2010; Kotzampassi and Giamarellos-Bourboulis, 2012). Secondly, through their interaction with the local immune system to enhance regulatory T cell responses, decreased the pro-inflammatory cytokines such as TNF-α and IL-1β and increased anti-inflammatory factor IL-10 (de Moreno *et al*., 2011; Zhao *et al*., 2013), consequently improving the symptoms of UC, and this mediated through PI3K/Akt and NF-B signaling pathway (Shen *et al*., 2018; Witkowski *et al*., 2018). *Bifidobacterium* probiotic is one of beneficial microbiota that promotes antitumor immunity and prevents recurrence of UC (Ventura *et al*., 2014). Anther probiotic that studied and used for treating UC *are E. coli Nissle 1917*, which widely used in Europe (Scaldaferri *et al*., 2016), *Saccharomyces boulardii* (Thomas *et al*. 2011; Limketkai *et al*., 2018) and lactic acid-producing bacteria (*L. plantarum, L. delbrueckii subsp. bulgaricus, L. casei, L. acidophilus, Bifidobacterium breve, B. longum, B. infantis, L. paracasei subsp. paracasei, Lactobacillus casei strain Shirota and Streptococcus salivarius subsp. Thermophilus*) (Limketkai *et al*., 2018; Guandalini and Sansotta, 2019).

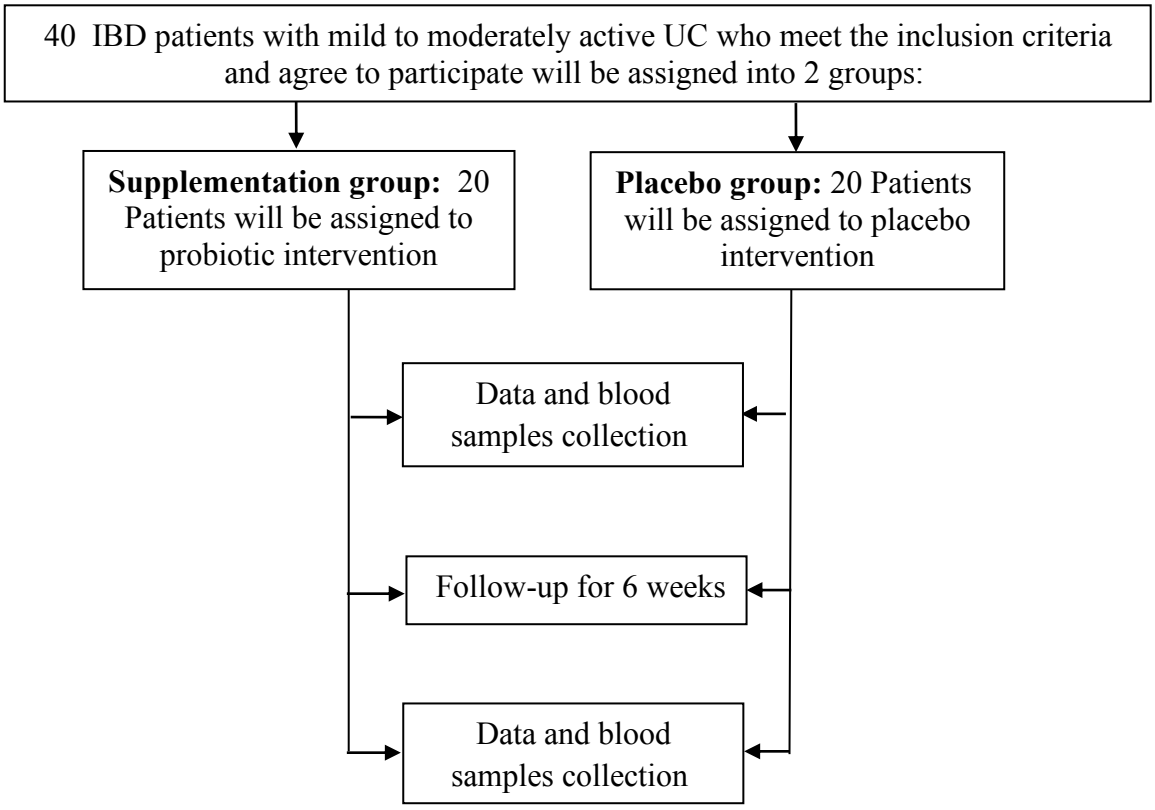
Probiotics also found to have an effect in inducing, maintaining and increasing the remission period in UC and this effect suggested to be also associated with the immunomodulator role of certain microorganisms (Tamaki *et al*. 2016). For example, *in vitro* studies, *Lactobacillus casei* strain Shirota found to have anti-inflammatory effect by inhibition of IL-6 and interferon c (IFN-c) synthesis in lipopolysaccharide (LPS)-stimulated murine chronic IBD cells (Matsumoto *et al*. 2005), and *Lactobacillus delbrueckii* had anti-inflammatory effect by inhibition of NF-kB pathway in large intestinal lamina propria mononuclear cells (Santos Rocha *et al*. 2012). *In vivo*, *Lactobacillus delbruekii* and *Lactobacillus fermentum* supplementation, in UC patients undergoing treatment with sulfasalazine (an anti-inflammatory medicine) showed a reduction in several pro-inflammatory parameters in the colon, such as IL-6 levels, tumor necrosis factor TNF-α and NF-kB p65 expression, and leukocyte recruitment, in comparison with the placebo and medical treatment groups (Hegazy and El-Bedewy 2010). In another study where *Bifidobacterium breve* was administrated as a part of a symbiotic, the results showed a significant decrease in the inflammatory parameters and improvement in the clinical status evaluated by colonoscopy in patients with mild to moderate UC (Ishikawa *et al*. 2011). Moreover, administration of probiotic product Bifid Triple Viable, that contains *Lactobacillus acidophilus, B. bifidum and Streptoccocus*, for 2 months, lead to reduce the expression of IL-1b and increased the expression of IL-10 and immunoglobulin A (IgA) in the colon of UC patients (Li *et al*. 2012).

Materials and Methods:

Study Design

An interventional double-blind randomized controlled trial (RCT) design will be used in this study. A placebo group will be included parallel with the treatment group in this study. Forty IBD patients with mild to moderately active UC aged 18-65 will be recruited conveniently from the gastroenterology section, the IBD clinic at the Jordan University Hospital and Al-Bashir Hospital, Amman, Jordan. Patients who meet the inclusion criteria and agree to participate will be centrally

randomized to probiotic supplementation group or placebo group using computer-generated



random numbers, stratified by age and gender. The duration of intervention will be 6 weeks. **The study scheme:**

The Interventions

The probiotics and placebo treatments will be given at weekly basis in double blind manner for the six weeks. The patients in the probiotics group will receive 3 oral viable capsules of Jamieson Probiotic 10 Billion product, each of which contain ($1*10^{10}$ CFU/g) of *lactobacillus* (*Lactobacillus rhamnosus* HA-111, *Lactobacillus acidophilus* HA-122, *Lactobacillus salivarius* HA-118, *Lactobacillus paracasei* HA-196) and *bifidobacteria* (*Bifidobacterium breve* HA-129, *Bifidobacterium bifidum* HA-132 HA, *Bifidobacterium longum* HA-135) species three times a day. Similarly, the placebo group will receive 3 placebo capsules containing maltodextrin three times a day.

Human Participants:

Inclusion and Exclusion Criteria:

Inclusion criteria will be patients with age between 18 -70 years and diagnosed with UC established by colonoscopy and histology, and suffering from mild to moderate UC as defined by Modified Mayo Disease Activity Index (MMDAI, table 1) (score 3–9), the diagnoses will be done by a physician (Ng *et al.,* 2010; Palumbo *et al*., 2016; Tamaki *et al.* 2016). Patients will be excluded if they had age <18 years, >70 years, pregnancy, planned pregnancy, breastfeeding, evidence of severe disease (MMDAI >10), concurrent enteric infection, use of antibiotics within the past 2weeks, change in dose of oral 5-aminosalicylic acid (5-ASA) within the past 4weeks, and use of rectal 5-ASA or steroids within 7 days before entry into the study, or the use of probiotic preparations during study, received any investigational medicines within 3months, and if they have significant hepatic, renal, endocrine, respiratory, neurological, or cardiovascular diseases were also excluded (Ng *et al.,* 2010; Bengtsson *et al*., 2016; Tamaki *et al*. 2016; Palumbo *et al*., 2016).

Table 1. Modified Mayo Disease Activity Index.

Grade	Bowel frequency	Rectal bleeding	Physician's global assessment	Endoscopy/sigmoidoscopy finding
0	Normal number of stools per day for this patient	No blood seen	Normal	Normal or inactive disease
1	1 or 2 more stools than normal	Streaks of blood with stool less than half the time	Mild disease	Mild disease (erythema, decreased vascular pattern)
2	3 or 4 more stools than normal	Obvious blood with stool most of the time	Moderate disease	Moderate disease (marked erythema, absent vascular pattern, friability, erosions)
3	5 or more stools than normal	Blood alone passed	Severe disease	Severe disease (spontaneous bleeding, ulceration)

Palumbo *et al*., 2016.

Ethical Approval:

This study will seek approval by Research Ethics Committee at the University of Jordan and by the Institution Research Board (IRB) of Jordan University Hospital and Al-Bashir Hospital, Amman, Jordan. The participants will be provided with information consent containing a brief description of the study and written informed consent will be obtained from all participants before their interview and blood drawl (10ml) (Appendix 1, 2). Participants' information will be treated confidentially. However, only the researcher (PhD student) will know participants' names and she is the only one who will give them identification (ID) number.

Personal Questionnaire:

The personal questionnaire will contain questions related to age, gender, education, employment, family income/month, residency area, smoking status, family history of IBD, medications, previous and current health problems. As well as, questions about using probiotics and certain eating habits will be asked (Appendix3). Anthropometric measurements including weight (measured to the nearest 0.1 kilogram) and height (measured to the nearest 1 cm) will be taken from the existing medical record. Body mass index (BMI) will be calculated.

Biochemical Measurements:

The resercher will take the permission form the participants to draw blood sample (10ml) and perform biochemical specialized labs. Venous blood samples will be withdrawn by specialized medical laboratory technicians. Complete blood count (CBC) will be analyzed using standard precedure. Blood serum will be separated by centrifugation (3000 rpm for 10 min) after clotting at room temperature (27 °C) and stored at -18°C until analysis. Immunoglobulin G(IgG), Immunoglobulin M (IgM), Immunoglobulin (IgA), interleukin (IL)-6, IL-1, IL-10, C-Reactive Protein (CRP), and TNF-α will be analyzed using commercially available enzyme-linked immunosorbent assay (ELISA) kits.

Data Collection and Quality of Life Questionnaire

Three parts package was used for collecting data that met the purpose of this study, including a personal questionnaire, physical activity questionnaire, and a short inflammatory bowel disease questionnaire (SIBDQ). A face-to-face interview technique was used as a method for data collection. Data on age, body weight, educational level, previous and current health problems, family history of IBD, and probiotic use were collected. The physical activity level was measured using a 7-day Physical Activity Recall (PAR) which was originally developed by Sallis *et al.*, 1985 (Sallis *et al*., 1958). For assessing the quality of life, a disease-specific, validated, and reliable Arabic version of the SIBDQ obtained from the McMaster short IBD questionnaire at McMaster University was used (Mahalli and Alharthi, 2017). The SIBDQ contains ten questions covering the four HRQOL domains (systemic, social, bowel, and emotional) which were calculated and scored. The response to each question of the ten was graded on a 7-point Likert scale ranging from 1 (worst aspect) to 7 (best aspect). The total SIBDQ scores range from 10 to 70, with higher scores indicating better HRQOL (Mahalli and Alharthi, 2017). The summation of the responses to Q4, Q6, and Q9 was used for the bowel domain, the sum of the responses to Q2 and Q3 was for the social domain, the sum of the responses to Q1 and Q7 was for the systemic domain, and the sum of the responses to Q5, Q8, and Q10 was for the emotional domain. The total score of SIBDQ was composed of the sum of the responses to all ten questions (Guyatt et al., 1989; Mahalli and Alharthi, 2017).

Statistical Analysis:

Statistical analysis will be done using IBM SPSS Statistics 20.0 software (version 20.0, IBM, lnc., Chicago, IL, USA). Mean ± standard deviation (SD) will be used to express the continuous data and percentage to express the categorical data. The categorical variables will be compared using the Chi square and the continuous variables will be compared using the one -way ANOVA. A

paired *t*-test will be used to assess within-group changes in outcomes from baseline to end of intervention. For all data, $P< 0.05$ will be considered statistically significant.

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