A Doctoral Dissertation

Therapeutic impact of *Schizophyllum commune* derived β-(1,3/1,6)-glucan on cutaneous wounds and metabolic dysbiosis

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치마버섯 유래 베타(1,3/1,6)글루칸의 창상 및 대사성 장균총 불균형에 미치는 치료 효능 연구

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Therapeutic impact of *Schizophyllum commune* derived β-(1,3/1,6)-glucan on cutaneous wounds and metabolic dysbiosis

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LIST OF ABBREVIATIONS

AMOVA - Analysis of molecular variance

CR3 – Complement receptor 3

DB – Degree of branching

DF – Dietary fiber

DP - Degree of polymerization

FAK – Focal adhesion kinase

FAS – Fatty acid synthase

GA - Glutaraldehyde

GI – Glycaemic index

GIP – Gastric inhibitory peptide

GLP – Glucagon like peptide

Gly – Glycitin (4'- hydroxy-6-methoxyisoflavone-7-D-glucoside)

HFD – High fat diet

IFN – Interferon

IL – Interleukin

K10 – Cytokeratin 10

K14 – Cytokeratin 14

LDL-C – Low-density lipoprotein cholesterol

LFD – Low fiber diet

MAMPs – Microbe-associated molecular patterns

MetS – Metabolic syndrome

MW – Molecular weight

NADPH – Reduced nicotinamide-adenine dinucleotide phosphate

NASH – Non-alcoholic steatohepatitis

NF-κB – Nuclear factor kappa light chain enhancer of activated B cells

NK – Natural killer

NMDS - Non-metric multidimensional scaling

NOX – NADPH oxidase

OTU – Operational Taxonomic Unit

PAMPs – Pathogen-associated molecular pattern

PAS – Periodic acid-Schiff

PLGA – Poly (lactic-co-glycolic acid)

PRR – Pattern recognition receptor

PVA – Poly (vinyl alcohol)

PYY – Peptide YY

ROS – Reactive oxygen species

SCFA – Short chain fatty acid

Src – Proto-oncogene tyrosine protein kinase

TG - Triglycerides

TGF-β – Transforming growth factor-beta

TLR – Toll-like receptor

TMF – 4',6,7-trimethoxyisoflavone

TNF-α – Tumour necrosis factor-alpha

WVTR – Water vapor transmission rate

Karthika Muthuramalingam. Therapeutic impact of *Schizophyllum commune* derived β-(1,3/1,6)-glucan on cutaneous wounds and metabolic dysbiosis.

ABSTRACT

β-glucan, the naturally occurring biological response modifier is of much clinical interest in terms of treating medical conditions such as cancer, diabetes, cardiovascular disorders, hyper-cholesterol, obesity etc. With diversities in source of origin (such as bacteria, algae, yeast, protozoan, fungi, oats, barley, rye, wheat etc.,), processing techniques and its influence on physiochemical properties (such as molecular weight, solubility, concentration, viscosity, gel formation, branching, glycosidic linkage etc.), the physiological mechanisms and clinical outcomes of β-glucan differs. Thus, exploring the characteristic modalities of β-glucan obtained from wide spectrum is of high-priority. On contemplating these statements, herein my doctoral study, I have aimed to study the therapeutic impact of fermented *Schizophyllum commune* derived soluble exopolysaccharide β-1,3/1,6 glucan towards wound healing and metabolic disorders.

Traditional wound dressings such as pads, gauze, and cotton wool do not provide an appropriate bed for wound healing, and also periodic dressings is a tedious process as there occurs a chance of disruption of the newly synthesized epidermal layer, while healing takes place. Further, scar development is highly possible when the skin under the process of healing is put under severe tension and in dry environment. To overcome such limitations and to provide a better environment for proper wound healing, I hereby developed immunomodulating β-1,3/1,6 glucan in the form of hydrogel and analyze its impact towards the management of cutaneous wound healing through in-vitro and in-vivo studies. β-glucan based hydrogel dressing exhibited good water-holding capability, optimum WVTR, stability as well as high cytocompatibility. This wet wound dressing significantly accelerated the wound healing rate, minimized cutaneous scarring,

acted as carrier for flavonoids and enhanced the development of skin appendages towards better skin regeneration.

Chronic overconsumption of diets high in calories and low in fiber content serves as a risk factor in the development of metabolic syndrome and its associated metabolic complications such as obesity, gut dysbiosis, insulin resistance, hypertension, hyper-cholesterol, and cardiovascular diseases etc. Dietary interventions with prebiotics, the key modulators of the gut microbiota, is said to have paramount impact on host-associated metabolic disorders. Herein, I analysed the impact of soluble, non-digestible and highly fermentable β-1,3/1,6 glucan towards diet induced metabolic dysbiosis and gut disorders. Consumption of low fiber diet (LFD) and high fat diet (HFD) with β-glucan increased the abundance of butyrate-producing (major 'fuel source' of colonocytes) bacteria such as *Lactobacillus*, *Anaerostipes*, *Coprobacillus*, and *Roseburia* in gut. Significant increase in the length of small intestine and enhanced intestinal goblet cell density along with mucosal layer thickness was observed in LFD+ β-glucan fed mice group. Inclusion of β-glucan mitigated obesity in HFD fed mice and decreased reportedly obesity associated bacteria such as *Parabacteroides* and *Lactococcus* in gut microbiota profile. Further, it ameliorated HFD-induced hepatic stress, colonic motility and intestinal atrophy.

Taken together, observations from in-vitro/in-vivo animal studies showed that β-1,3/1,6 glucan from *Schizophyllum commune* acts as potential candidate in the clinical management of skin wounds, gut microbiota modulations and metabolic distress. With further studies, towards optimizing parameters such as dosage, duration, therapeutic/prophylactic modes, analysing the roles of tissue specific microbiota modulation etc., outreach of this polysaccharide to several human clinical applications is highly guaranteed.

ABSTRACT - HANGUL

치마버섯 유래 베타(1,3/1,6)글루칸의 창상 및 대사성 장균총 불균형에 미치는 치료 효능 연구

베타글루칸은 임상적으로 많은 관심을 받고 있는데, 이는 베타글루칸이 천연 생체반응조절물질로서 암, 당뇨병, 심혈관계 질환, 과다콜레스테롤, 비만 등과 같은 질병을 치료하는 데 있어 관련이 있기 때문이다. 베타글루칸은 세균, 조류, 효모, 원생동물, 진균, 귀리, 보리, 호밀, 밀 등의 다양한 생물에 존재한다. 그리고 베타글루칸은 가공하는 방법에 따라 분자량, 용해성, 농도, 점성, 겔 형성, 분지, 글리코시드 결합 형성 등의 생화학적 특성이 달라지므로 그 생리적 메커니즘과 임상 결과 또한 다르게 나타난다. 그러므로 최우선적으로는, 다양한 곳에서 얻어낸 각각의 베타글루칸의 특징적인 양상을 탐구할 필요가 있다. 이러한 서술을 고려하며 본 박사학위 연구에서는, 치마버섯 균사체 배양액에서 추출한 용해성 세포외다당류인 베타-1,3/1,6 글루칸이 피부 창상과 대사성 장균총 불균형에 대해 갖는 치료 효능을 조사하였다.

패드, 거즈, 면모 등의 기존 창상 피복재는 창상 치료를 위한 적절한 지지대가 되지 못하고, 또한 주기적으로 사용하는 피복재는 그 과정이 너무 오래 걸려, 창상 치료 중 새롭게 형성되는 상피층을 분열시킬 가능성이 있다. 나아가, 치유되고 있는 피부가 심한 긴장상태

그리고 건조한 환경에 놓인다면 흉터가 생길 가능성이 매우 높다. 이러한 한계를 극복하고 적절한 창상 치료에 대한 더 나은 환경을 제공하기 위해, 면역조절성 베타-1,3/1,6 글루칸 함유 수화젤이 피부 창상 치료에 대해 갖는 효험을 세포 수준 그리고 동물 수준 연구 모두에서 분석하였다. 베타글루칸 함유 수화젤 피복재는 높은 세포적합성 뿐만 아니라 훌륭한 보수성, 최적의 투습도와 안정성 또한 보여주었다. 이 수화 창상 피복재는 치료 속도를 상당히 높였고 피부 흉터 형성은 최소화하였으며, 플라보노이드의 운반체 역할을 하고 피부 부속기를 발달시켜 결론적으로 피부재생을 향상시켰다.

현대인의 칼로리가 높고 섬유질 함량이 낮은 식사는 만성적인 대사증후군 그리고 이와 관련된 비만, 장균총 불균형, 인슐린 내성, 고혈압, 과다콜레스테롤, 심혈관계 질환 등과 같은 대사합병증을 유발한다. 이러한 질환들을 극복하는 데 있어, 프리바이오틱은 장내 균 분포를 변화시키는 주요 요인으로서 큰 영향을 미친다. 본 연구의 후반부는, 변비 유도 식이를 섭취한 후 발생한 대사성 장균총의 불균형과 장 질환들에 대해 치마버섯 유래 베타-1,3/1,6 글루칸이 프리바이오틱으로 작용하여 갖는 효능들을 분석하였다. 베타글루칸을 섬유질 함량이 낮고 지방 함량이 높은 식단과 함께 섭취하는 것은, 장세포의 주 연료원인 낙산염을 생성하는 세균들, 예를 들면 *Lactobacillus*, *Anaerostipes*, *Coprobacillus* 그리고 *Roseburia* 의 수를 증가시킨다. 베타글루칸을 섬유질 함량이 낮은 식단과 함께 섭취한 쥐 그룹에서는 소장의 길이가 상당히 증가했고, 장내 술잔세포의 밀도와

점막층의 두께 또한 증가했다. 베타글루칸을 지방 함량이 높은 식단과 함께 섭취한 쥐 그룹에서는 비만이 완화됐고, 비만과 관련되었다고 알려진 세균인 *Parabacteroides* 와 *Lactococcus* 의 수가 장내 균총에서 감소되었다. 나아가, 지방 함량이 높은 식단에서 유발되는 간 관련 질환들, 결장의 운동성 그리고 장 위축도 개선되었다.

종합하면 체외/체내 동물 연구들에서 얻은 관찰 결과들은, 치마버섯 유래 베타-1,3/1,6 글루칸이 피부 창상을 치료하고 장내 미생물상을 조절하여 대사성 질환을 극복시킬 가능성이 매우 크다는 것을 보여준다. 추가 연구에서 투여량, 지속시간, 치료적/예방적 방식의 기준을 최적화하고 조직 특이적인 미생물상 조절을 분석하면, 몇 가지 인체 임상 적용에 있어 이 다당류가 널리 활용될 것임을 매우 확신하는 바이다.

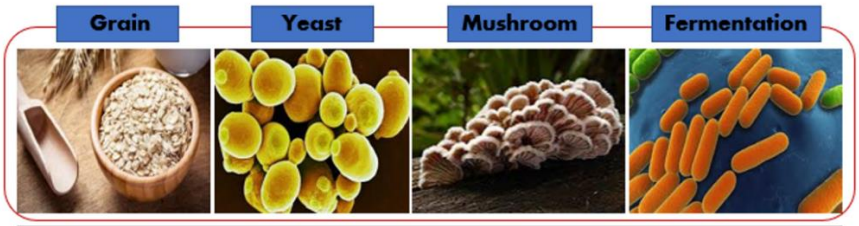
CHAPTER 1

Introduction

"In a medical crisis, if you were limited to only one disease-fighting substance to protect your health and immunity, without exception, beta glucan should be the one you choose" – Professor Somsak Varakamin.

1.1 Description and background analysis of β-glucan

β-glucan, the class of polysaccharides with D-glucose monomeric residues linked together by glycosidic bonds, are naturally occurring bioactive carbohydrates with comprehensive clinical applications. As biological response modifiers, they are of much research interest in terms of treating medical conditions such as cancer, diabetes, cardiovascular disorders, hyper-cholesterol, obesity, immunity etc [1,2]. Many sources for this functional polysaccharide have been reported including bacteria, algae, yeast, protozoan, fungi, cereals such as oats, barley, rye, wheat etc. (**Figure 1. 1**).



Component	Grain	Yeast	Mushroom	Bacteria
Structure	(1→3)(1→4) β-1,4 glucan Linear	(1→3)(1→6) β-1,6 branch Long branched	(1→3)(1→6) β-1,6 branch Short branched	(1→3) linear β-1,3 glucan
Isolated from	Oat, Barley, Rye	S.cerevisiae	Phellinus linteus, Coriolus versicolor, Ganoderma lucidum, Agaricus blazei murill	Agrobacterium sp.
Isolated Part	Surface of grain	Cell wall of yeast	Mycelium fruit body	Exopolysaccharide

Figure 1.1: Description of four kinds of β-glucan.

These array of sources and its genotypes along with spectrum of processing techniques involved in the extraction, purification and functionalization of β-glucan is said to influence their

physiochemical properties such as molecular weight, solubility, concentration, viscosity, gel formation, branching (**Figure 1. 2**), glycosidic linkage etc., which in turn has an impact on their physiological effects (**Figure 1. 3**) [3].

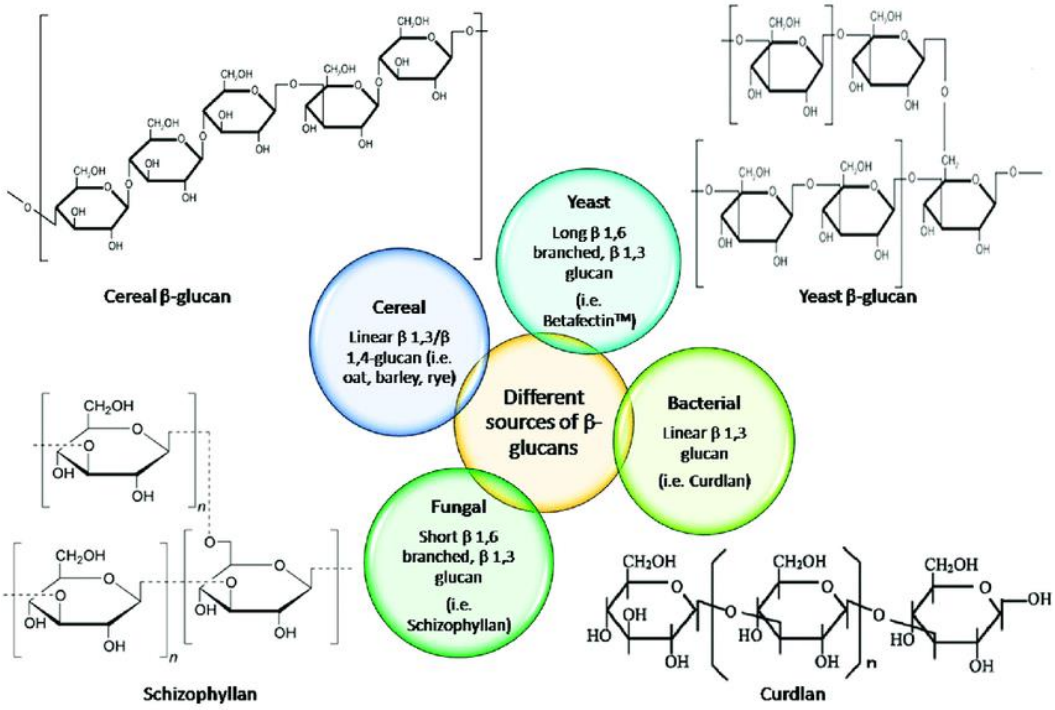


Figure 1.2: Structure and branching degree of β-glucan from different sources. [4]

1.2 Impact of processing and sources on the physiochemical characteristics of β-glucan

With insights into molecular conformations, Woodward et al., in 1983 explained that irregularly spaced glycosidic linkages in the β-glucan structure confers enhanced solubility by overcoming aggregation of the glucan chains [5]. In a study conducted by Lee et al., [6], it was found out that the fraction of soluble-to-total β-glucan was higher in oat genotypes than barley. Gaosong and Vasathan [7] reported that the solubility and content of β-glucan depends on its source cultivar and food-processing technique involved. Hromadkova et al., [8] analysed the

impact of different drying methods such as solvent exchange, spray drying and lyophilization on the physiochemical and immunomodulating effect of particulate (1→3)-β-D-glucan and found out that spray dried β-glucan was efficient as immunomodulator and having limited inter-particle hydrogen bonding leading to low viscosity. Kumagai et al., [9] showed that pre-heating curdlan results in the production of biologically active β-glucan with degree of polymerization (DP) index between 13 and 130. Lan-Pidhainy et al., [10] showed that storage of β-glucan augmented muffin breads using freeze thawing method and the number of freeze-thaw cycles affects the solubility and extractability of β-glucan and thus its effect on controlling post-prandial glycaemic index (GI) with inverse linear relationship between extractability and after-meal peak blood glucose level. Makelainen et al., [11] observed that the amount of extractable β-glucan from food matrix plays a physiological impact on the glycaemic/insulin index rather than the quantity of β-glucan per meal serving. Upon screening 39 species of wild and cultivable mushroom, Sari et al., [12] found that most of the wild types and domesticated species such as A. bisporus, L. edodes and C. cibarius showed greater quantity of β-glucan in them. Nishantha et al., [13] showed that the fraction of β-glucan was comparatively higher in wild (thirty-two breeds) than the domesticated (twenty-eight breeds) barley genotypes. Upon composition analysis of 111 genotypes of barley, 79 genotypes of oats and 14 genotypes of wheat, a maximum of 53.5 g/kg and 57 g/kg of β-glucan were quantified respectively in both of the former cereals, with less than 7g/kg in the later, implying that source of origin plays an important role in the choice of starter material in order to obtain better concentrated end product [14]. Thus, the physiochemical, structural and conformational properties of β-glucan widely vary depending on its source and genotypes of origin, extraction and purification processes, purity of β-glucan, functional modifications etc. Kaur et al., [15] meticulously described the physical and chemical processing techniques employed in the extraction of β-glucan and their influence on structural-functional attributes of β-glucan in the making of bioactive and health beneficial food products.

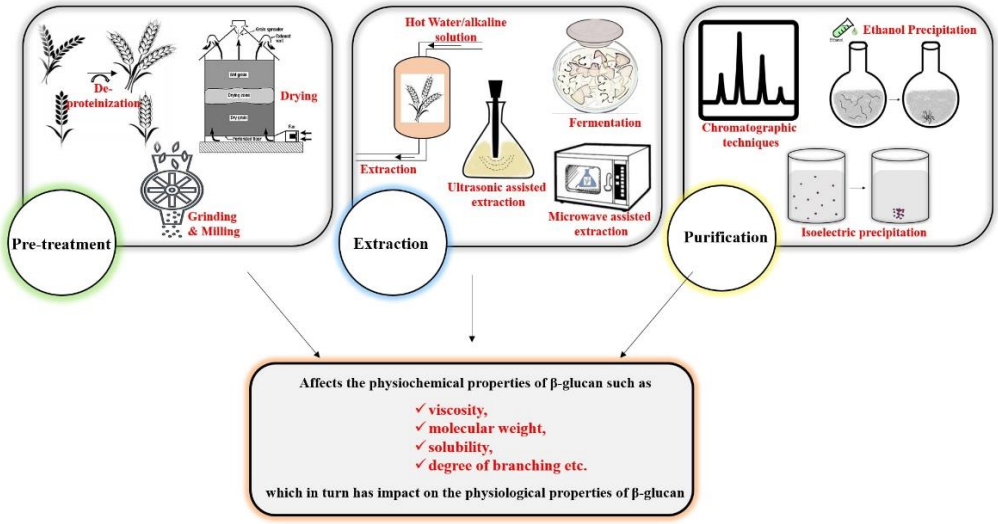


Figure 1.3: Processing techniques impacting the physiochemical properties of β-glucan.

1.3 Impact of physiochemical characteristics on physiological effects of β-glucan

1.3.1 Molecular weight, solubility and viscosity

Anttila et al., [16], Marasca et al., [17] and Henrion et al., [18] intricately described the importance of viscosity and processing parameters involved in the production of β-glucan from cereal based products and their physiological effects. β-glucan with greater molecular weight and high viscosity hinders the rate of food processing in the digestive system resulting in reduced GI, thereby aiding hyper-cholesteraemic and hypo-glycaemic activity [19,20]. A possible mechanism behind the alleviation of cholesterol and post-prandial blood sugar spike is attributed by the ability of β-glucan to form viscous network that hinders the reabsorption of bile acids, resulting in higher bile excretion and cholesterol-to-bile conversion. Upon analyzing the impact of fermentation on barley β-glucan, Xiang et al., [21] observed a decrease in molecular weight and change in microstructure from rod-shape to sheet-like form with increased porosity in the fermented group compared to non-treated β-glucan. Further, the former group was found to be effective at cholesterol adsorption thereby enhancing physiologic lipid and glucose metabolism. Vaclav

Vetvicka and Jana Vetvickova [22] studied the physiological effect of five different β-1,3 D-glucan (yeast-derived (#300, NOW), grain-derived (Glucagel), yeast-fermentate (Epicor) and mushroom-derived (Krestin)) and found that the insoluble Glucan#300 with greater than 95% purity showed enhanced and prolonged potential against inhibiting adipogenic differentiation and skin irritation as well as faster wound area coverage.

1.3.2 Glycosidic linkages and degree of branching

While yeast/fungal derived mixed linkage containing (β-1,3 or β-1,6 or β 1,3/1,6) glucan is said to exerts its action through immunomodulatory property, β-1,4 linkage containing cellulose glucan molecules are not immunomodulators [23]. Branching structures arising out of glycosidic linkages has great deal in the physiological characteristics of glucan polysaccharides. Sato el al., [24] manifested the impact of glycosidic linkages and branching structures in high purity β-glucan molecules on glycosphingolipid mediated neutrophil chemotaxis. While β-(1,3) containing- (curdlan) and β-(1,3) vertebrae with occasional/monoglucosyl β-(1,6) branched residues containing- (Laminarin, Lentinan, grifolan, sonifilan) glucan molecules had no influence, C.albicans derived soluble glucan - β-(1,3) vertebrae branched with longer subunits of β-(1,6) glycosyl residues - had significant contribution towards induction of chemotactic migration of neutrophils via interaction with terminal galactose residue containing glycosphingolipids. In young et al., [25] compared the degree of branching (DB) - the ratio of number of branched glucopyranosyl residues to total number of glycosyl residues in the linear backbone, in β-glucan molecules extracted from different mushrooms and its efficacy towards antitumor properties. Upon analyzing the debranched β-glucan from enzymatic hydrolysis of Chamsong-I mushroom, enhanced secretion of nitric oxide from RAW 264.7 and cellular growth inhibition of cancerous cells such as MCF-7 and Sarcoma 180 was observed as DB gets reduced to 0.32 from 0.67 (branched β-glucan), below which the effect was reversed. In addition, shiitake mushroom with

29% DB containing β-glucan exhibited better nitric-oxide mediated anti-cancerous effect than Chamsong-I derived and cauliflower mushroom derived β-glucan (16% DB). β-glucan with DB between 0.20 and 0.33 are said to be highly bioactive (Savelkoul et al., [26]). Magee et al., [27] investigated whether branching tends to affect the structural characteristics of glucan molecules and thus its biological activity, wherein he demonstrated that upon reducing DB from 0.30 to less than 0.20 in yeast glucan, the triple helical molecular confirmation got transformed into aggregated structures. This encourages the host immune system to recognize these aggregated molecules as microbe-associated molecular patterns (MAMPs), thereby activating several immune defense mechanisms.

1.3.3 Pattern recognition receptors

Humans lack enzymes responsible for digesting glycosidic linkages as there is no synthesis of β-glucan in-vivo; thus making the host immune cells to consider the glucan molecules as foreign body substances via pattern recognition receptors (PRR) such as C-type lectin receptor (dectin-1), toll-like receptor (TLR), complement receptor 3 (CR3), scavenger receptors etc. Binding of β-glucan to PRR elicits activation of specific immune system – innate or adaptive – followed by opsonic/non-opsonic phagocytosis process along with release of several cytokines such as tumor necrosis factor-alpha (TNF-α), interferon (IFN) and interleukin (IL) molecules etc, thereby acting as potent immunomodulator [28]. Synergistic activation of PRRs such as dectin-1 and TLR2 is required for eliciting macrophage mediated pro-inflammatory cytokine production towards mycobacterial infection (Yadav [29]). Upon recognizing β-glucan, one of the major MAMPs, PRR existing on different immune cells of myeloid (monocyte/macrophage cells) and lymphoid (natural killer (NK) cells) origin, dendritic cells etc. activates cascades of immune signals resulting in enhanced production of pro-inflammatory cytokines, antimicrobial resistance, anti-oxidative property, phagocytosis/autophagy, antitumor responses etc [30]. Suzuki et al., [31]

showed that the activation of host complement system depends on the conformation and branching order of glucan molecules. Glucans with triple and single helix conformation triggered alternative and classical complement cascade, respectively. Javmen et al., [32] studied the impact of Saccharomyces cerevisiae derived particulate/soluble β-glucan towards the production of soluble cytokine - IFN-γ using BALB/c mice models. They observed that soluble β-glucan effectively and significantly enhanced the production of IFN- γ than the particulate molecules, upon oral administration. Sahasrabudhe et al., [33] suggested that the ability of particulate β -glucans in mounting stronger immune response than the soluble β -glucan comes from the synergistic activation of Dectin-1 and TLRs, influenced by the physiochemical structure of insoluble glucan molecules. Goodridge et al., [34], Corno et al., [35] and Kim et al., [36] summarized different types of β-glucan and its associated PRR through which it exerts its immunopharmacological activities. Qi et al., [37] reported the difference in the immune system cascade activated by soluble and particulate β-glucan isolated from Saccharomyces cerevisiae. Stier et al., [23] in their review article explained elaborately on how the solubility of β-glucan triggers different PRR and downstream immune systems. While particulate β-glucans primes both innate and adaptive immune cascades through either dectin-1 dependent/independent mechanism, soluble β-glucan binds to CR3 thereby exerting its action via antibody-mediated complement pathway. In addition, glucan with mixed (β-(1,3)/(1,4)) linkages was not discerned by dectin-1 receptors while β-(1,3) core structures gets recognized. Thus, wide range of factors influence the physiological effects of β-glucans in improving human health.

1.4 Physiological effects of β-glucans

1.4.1 Wound healing

In the first line of defense against invading pathogens, skin, the largest integumentary organ of the vertebrate system has prominent roles such as protection from infectious intruders,

thermal- and moisture- regulation, sensation of external milieu through numerous nerve endings that differentiates heat, cold, pressure, injury etc [38]. Upon cross-sectioning of the human skin, the anatomy consists of three layers: epidermis (melanocyte containing outermost physical barrier), dermis (includes sebaceous- and sweat- glands, hair follicles, lymph vessels) and hypodermis (fatty cushion aids in the attachment of dermis to underlying muscles and bones in addition to its role in providing thermal insulation to the body). Breach in the integrity of skin leads to wounds, wherein complications such as infections, inflammations, sores etc. results [39]. In addition, many intrinsic and extrinsic factors such as diabetes, anaemia, debris, chemical and mechanical stress, medications etc., hinders the normal wound healing process [40,41]. Overaggressive wound healing arising out of convolutions such as high intense injury and pathogenic contaminations implicates in scar formation [42]. Thus, wound assessment and management plays paramount role in the complex cascade of wound healing process: haemostasis, inflammation, granulation/wound contraction and remodelling (**Figure 1. 4**) [43,44].

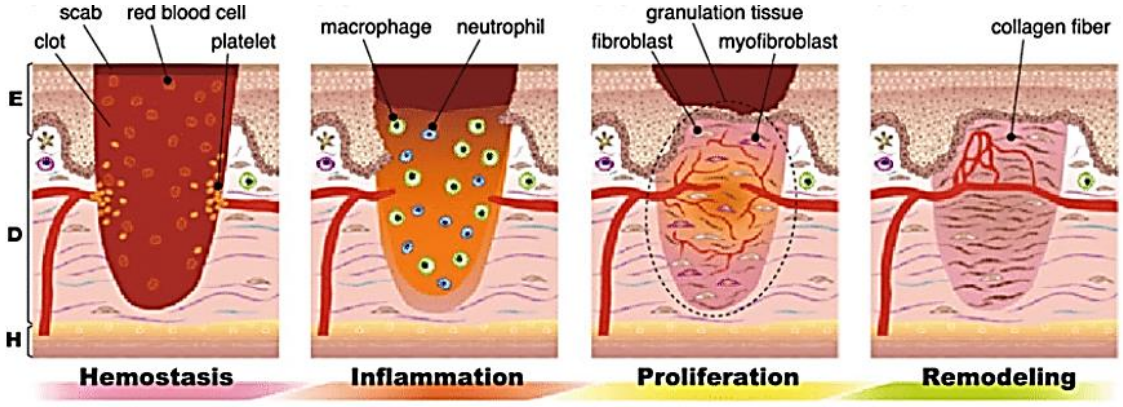


Figure 1.4: Phases of wound healing. [45]

1.4.1.1 Immune and non-immune cells of wound healing mechanism

Different types of cells are involved in the wound healing process (**Figure 1. 5**): platelets (in the haemostasis phase), macrophages and neutrophils (in the inflammatory phase),

keratinocytes and fibroblasts (in the maturation phase). Majtan et al., [46] elaborately reviewed the multi—functional modulation of β-glucan in the area of dermatology, especially wound care. Upon contact with wound surface, β-glucan gets recognised by cell surface receptor molecules such as dectin-1, CR3, TLRs etc. present on immune cells such as neutrophils, macrophages, NK cells, dendritic cells etc. which upon activation stimulates the production of several cytokines, growth factors, chemokines etc. The secreted messenger molecules thus influence the non-immune cells such as keratinocytes, fibroblasts and endothelial cells to proliferate, differentiate and migrate over the wound area, resulting in proper skin remodelling that consists of re-epithelialization, deposition of extra-cellular matrix and collagen, development of skin appendages, wound contraction and closure. Further, the secreted mediators have anti-inflammatory, anti-oxidant, anti-pathogenic, anti-infectious, angiogenic properties.

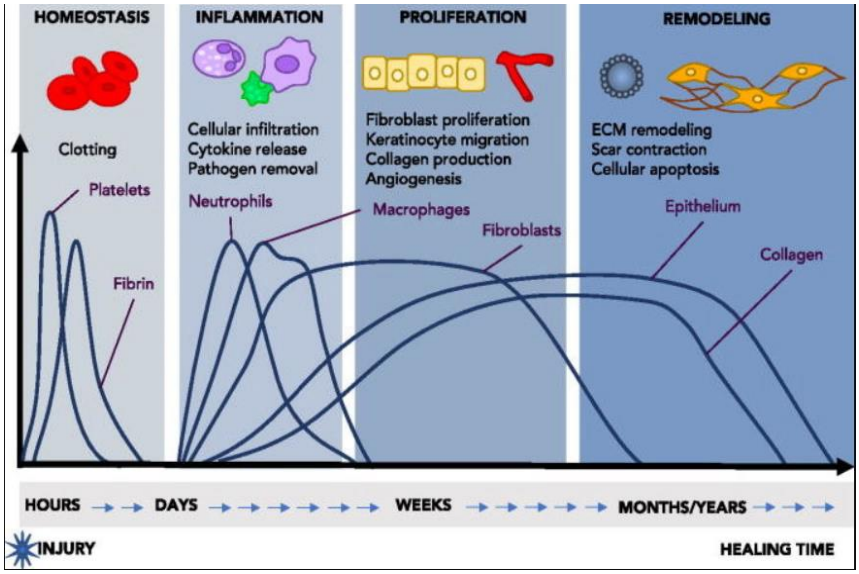


Figure 1.5: Immune and non-immune cells in different phases of wound healing. [47]

1.4.1.2 Experimental outcomes based on in-vitro/in-vivo wound healing studies

A two-year follow up study (Delatte et al., [48]) in a paediatric burn centre showed that usage of β-glucan/collagen wound dressing matrix onto adult partial wounds minimized pain,

improved wound healing and exhibits reduction in water loss and scab formation in addition to the frequency of dressing changes. Locally applied aminated β-(1,3)-D-glucan, a water-soluble derivative of Curdlan stimulated the impaired functions of macrophage cells such as production of growth factors and cytokines towards delayed healing response in diabetic wounds (Berdal et al.,)[49]. Oral administration (1,000 mg/kg body weight) of Sparassis crispa, a medicinal mushroom containing 40% of its content to be β-glucan improved the impeded wound healing in streptozotocin-induced diabetic male Sprague-Dawley rats by enhancing the migration of macrophage and fibroblast cells in the wound area (Kwon et al.,)[50]. A double-blinded, placebo-controlled phase II clinical trial by Zykova et al., [51] provided substantial evidence to show that the topical administration of soluble yeast derived β-(1/3),(1/6) glucan was found to be effective and safe against the treatment of diabetic ulcer wounds in sixty-human patients. Hunt, S.D. [52] did clinical investigation within a mediocre sized cohort of patients with chronic/slow-to-heal wounds and observed that the bioactive soluble β-glucan (Woulgan) hastens wounds healing with better wound care provision. The close resemblance of nanofibers to extra cellular matrix and its ease in facilitating wound healing through better gas exchange mechanism made Grip et al., [53] to develop electrospun soluble β-(1/3),(1/6) glucan loaded nanofiber towards the treatment of chronic diabetic wounds, in-vivo wherein dose-dependent positive impact on re-epithelialization occurred.

Kim et al., [54] developed an artificial skin substitute comprising β-glucan incorporated electrospunned poly (lactic-co-glycolic acid) (PLGA) membrane. They observed that the β-glucan/PLGA membrane enhanced the proliferation of keratinocytes and human dermal fibroblast cells compared to that of PLGA membrane thereby aiding better re-epithelialization and remodelling of the wounded area, together with better vascularization of 34±8.6 capillaries (vs 22.7±8.6 capillaries in PLGA membrane treated wound site). Nair et al., [55] prepared a hydrogel

system made up of Bradyrhizobium japonicum derived cyclic β-(1/3),(1/6) glucan to act as a carrier for encapsulating ciprofloxacin, a hydrophobic antibiotic. The porous nature of the cyclic glucan helps in the attachment and proliferation of fibroblast cells, in vitro. Further, TNF- α induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) dependent collagen biosynthesis by fibroblastic cells ameliorated in-vivo wound contraction using this drug-loaded hydrogel membrane. Zacharski et al., [56] hypothesized that the ability of Lichenan, an Iceland moss derived β-(1/3),(1/4) glucan polysaccharide in inducing terminal differentiation of human keratinocytes possibly involves transforming growth factor-beta (TGF-β) signalling and can be of much interest in the field of wound care management and in the inception of culture conditions in achieving differentiated cellular models. Fuste et al., [57] observed that barley based β-(1/3),(1/4) glucan favored migration over proliferation of fibroblast cells in-vitro and promoted wound closure in-vivo.

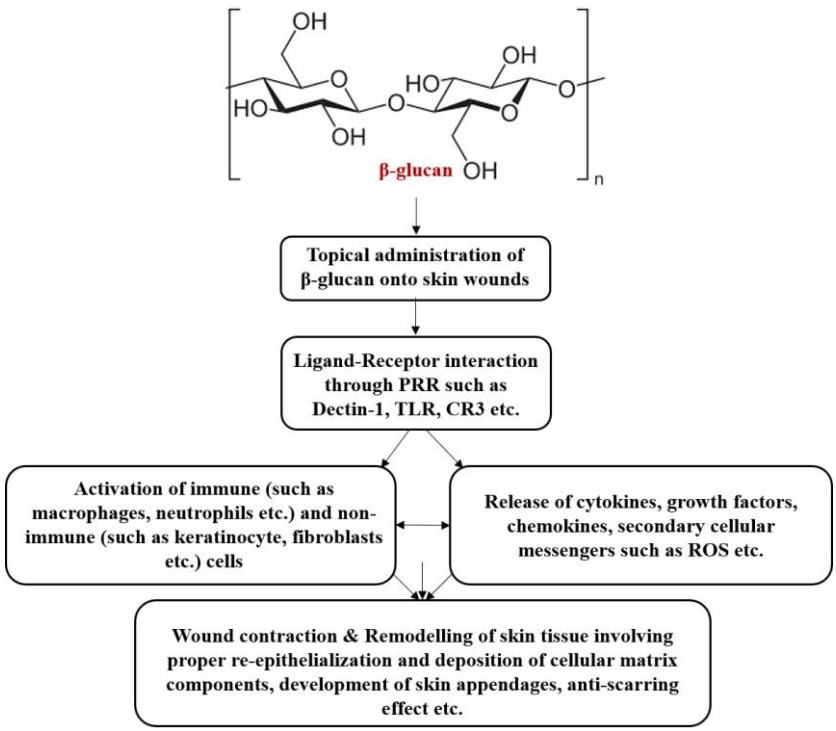


Figure 1.6: General mode of action of β-glucan towards wound healing mechanism.

Thus, β-glucan from different origins has prominent roles in the mechanobiology of wound healing process through immune mediators and non-immune cells of dermal origin as given in (**Figure 1. 6**).

1.4.2 Metabolic dysbiosis

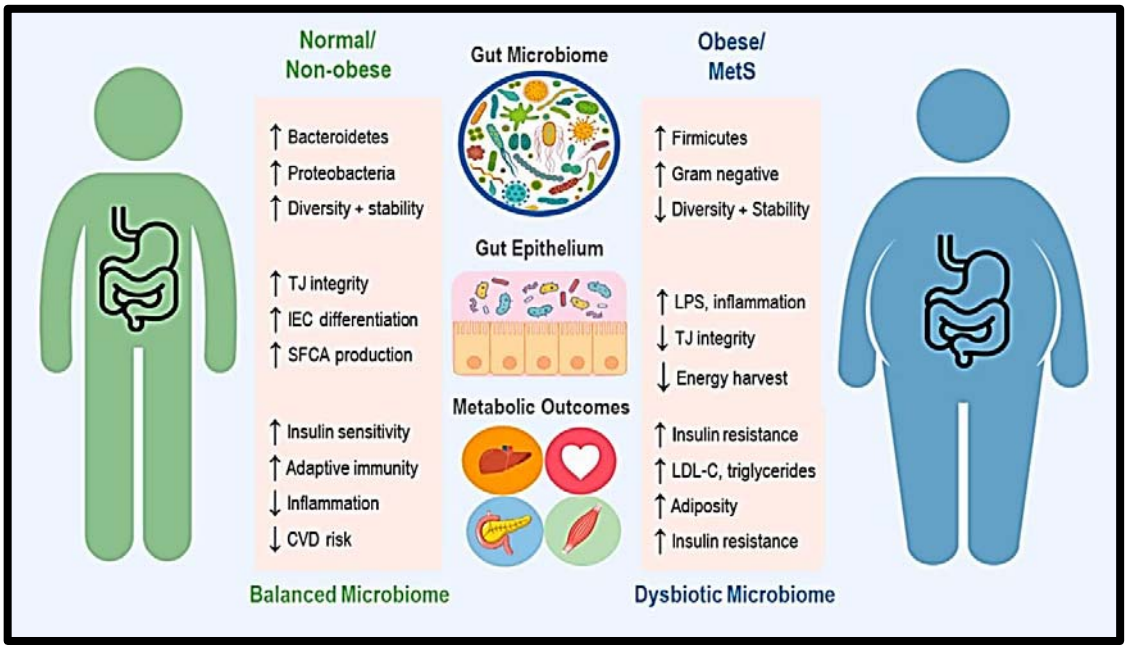


Figure 1.7: Gut microbiome and its complex interactions with host metabolic state. [58]

Metabolic syndrome (MetS) or insulin resistance syndrome or syndrome X is a cluster of metabolic derangements such as upper body/abdominal obesity, atherogenic dyslipidaemia, type II diabetes mellitus, cardiovascular diseases, hypertension etc. affecting one-third of the world's population [59–61]. Variety of factors such as genetic predisposition, over-nutrition, environmental risk factors, oxidative stress, sedentary lifestyles etc. are said to involve in the pathogenesis of MetS [61]. Diet induced MetS is one such alarming factor in the progression of MetS. Consumption of high-carbohydrate, high-fat diet (HFD) and low-fiber diet (LFD) is said to enhance the incidence of MetS wherein increase in body weight, waist circumference, energy

intake, abdominal fat deposition, insulin resistance, high blood pressure, inflammation, fibrosis, atherosclerosis etc. is observed [62–66]. Further, the consumption of westernized diet composing high-fat and low-fiber content is said to bring imbalance in gut microbiome, trigger low-grade inflammatory response and respiratory diseases [67]. In addition, intake of diet lacking fiber has stronger correlation towards intestinal barrier dysfunction by modulating intestinal tight junctions, disproportion in barrier forming/barrier disrupting cytokines, intestinal epithelial cells' association with oxidative imbalance, mucus thinning, colonic inflammation etc [68,69].

1.4.2.1 Dietary fibers

Dietary intervention using dietary fibers (DF) are emerging promising strategy in the management of metabolic syndrome[70]. Dietary fibers are non-digestible carbohydrate polymers with DP ≥ 10 and are resistant to hydrolysis by endogenous digestive enzymes present in the gastrointestinal tract of small intestine and colon. Sekgala et al., [71] coerces the importance of DFs on MetS in a study carried out on young rural populations of South Africa. DF can be classified based on their physiochemical properties into soluble/insoluble, fermentable/non-fermentable, viscous/non-viscous, gel forming, water holding etc., each having its own characteristic advantages according to the underlying clinical conditions [72–74]. Lattimer and Haub [75] and Vetvicka et al., [76] thoroughly explained the mode of action of soluble and insoluble DF in their review article. Soluble DF forms viscous gels when coming in contact with water and thus hinders the absorption of macronutrients, fat and glucose molecules. Further, the increase in viscosity of the processed food delays gastric emptying. These set of events attributed by the soluble DF has potential impact in lowering intestinal cholesterol absorption and post-prandial rise in glucose level. However, insoluble fibers speeds up the rate at which food passes the gastrointestinal tract by exerting fecal bulking effect, thereby curbing digestion and nutrient absorption. The non-digestible DF entering into the large intestine gets fermented by colonic

bacteria, resulting in the release of short chain fatty acids (SCFA)[77]. Outcome of fermentation is said to modulate gut microbial diversity, host immunity and physiology of gastrointestinal tract (**Figure 1. 7** and **Figure 1. 8**). Solubility of dietary fiber affects the degree of fermentation to certain extent (Tiwari et al., [78] and Singh et al., [79]).

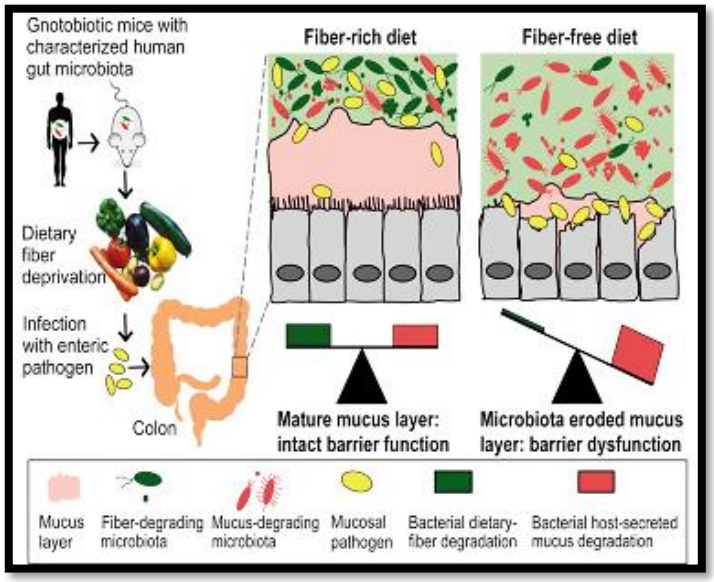


Figure 1.8: Role of dietary fiber towards healthy intestinal mucus barrier. [80]

1.4.2.2 Prebiotics

Some of the dietary fibers are considered as prebiotics – the selectively fermentable ingredient with a potential to modulate gut microbiota and/or gut physiology thereby conferring beneficial effect on human health. Definitions of prebiotics and dietary fibers, their sources, mechanism of action, health benefits are in detail reviewed by Joanne Slavin [81], Holscher [82] and Carlson et al. [83].While candidates such as inulin, fructo-oligosaccharides and galacto-oligosaccharides have sturdy validations to be considered as prebiotics, there exists another group of candidates such as mixed linked glucans, polyphenols etc. those with varying levels of persuading evidence to be accepted as prebiotics (Mitmesser and Combs)[84]. Thus, research

focussed on the search of prebiotics with robust scientific evidence on their effort to improve human health is gaining much interest.

1.4.2.3 Modes of mechanism of β-glucan in alleviating metabolic dysbiosis

In this context, owing to its viscosity, gel-forming property and fermentability, dietary intervention using β-glucan is being studied for its impact and/or mode of action in alleviating the clinical manifestations of metabolic syndrome. The physiological behaviour of β-glucan primarily depends on the physiochemical and structural properties such as molecular weight, existence, dosage etc. which in turn are highly subjectable to variations found in their source of origin, processing conditions, storage and food matrix etc. (Khoury et al., [85]).

Oats derived β-glucans

Kerckhoffs et al. [86] investigated the effect of oats-derived β-glucan on serum cholesterol content in mildly hypercholesterolemic subjects, when administered in the form of processed breads/cookies and with orange juice, wherein the content of β-glucan per serving was approximated to 5g. They observed a significant reduction in low-density lipoprotein cholesterol level in the β-glucan/orange juice matrix group, thereby concluding that the food processing and/or food matrix is influencing the physiological outcome of β-glucan. Duss and Nyberg [87] indicated that the enhanced viscosity of oat β-glucan contributes to its health promoting effect, as the increase in viscosity hinders the digestion rate and fast uptake of nutrients into the bloodstream thereby lowering the glycaemic response and instant release of insulin together with increase/decrease in the rate of bile excretion/lipid absorption respectively. Queenan et al., [88] studied the impact of concentrated β-glucan from oats on hypercholesterolemia patients who are more prone to cardiovascular diseases. An intake of 6.0 g/day of concentrated oat β-glucan results in pronounced reduction of low-density lipoprotein cholesterol (LDL-C). Dong et al., [89] provided substantial evidence on the hypoglycaemic effect of oats derived β-glucan in

streptozotocin-induced diabetic mice, which involves inhibitory effect on intestinal disaccharides thereby potentially regulating glucose metabolism. The increase in the production of butyrate - a SCFA with the ability to hinder intestinal fat absorption and lipid metabolism (Jakobsdottir et al., [90]) – observed using model intestinal fermentation explains the mechanism behind the cholesterol lowering effect of this fermentable oat β-glucan. Zheng et al., [91] observed an increase in hepatic glucokinase activity in the mice group treated with oat β-glucan. The enzyme acts as a biological marker for insulin sensitivity and plays vital role in glucose homeostasis. Ji-Lin [92] found that oat-based soluble and insoluble β-glucan had similar effect in nurturing the growth of Lactobacillus – a probiotic bacterium that could yield a kind of cholesterol reductase. Also, they found that insoluble β-glucan improves weight management while soluble counterpart aids in the control of serum cholesterol levels. All these physiological effects were achieved via increase in the excretion of bile compounds, SCFA production in the large intestine and increase in the number of fat cells resulting in enhanced metabolism of fat.

Barley derived β-glucans

Shimizu et al., [93] conducted a 12-weeks placebo-controlled, double-blinded study to analyse the impact of barley derived β-glucan (intake of 7.0 g/day) on hypercholesterolemic Japanese subjects and found that its consumption significantly reduced the LDL-C level, visceral fat and waist circumference. Kim et al., [94] suggested a possible mechanism behind the reduction of post-prandial insulin response by barley cereal based β-glucan wherein gut hormones such as gastric inhibitory polypeptide (GIP) and glucagon-like peptide 1 (GLP-1), a peptide hormone associated with insulin release is expected to be involved. Miyamoto et al., [95] experimentally suggested that supplementation of barley β-glucan impacts energy metabolism via release of gut hormones GLP-1 and plasma peptide YY (PYY), thus having post-prandial metabolic benefits on insulin-sensitivity and satiety through modulation of gut microbiome, especially SCFA butyrate.

Miscellaneous sources based β-glucan

Kanagasabapathy et al., [96] illustrated the mode of action of P.sajor-caju derived β-glucan in preventing high-fat diet induced obesity and oxidative stress, in-vivo using C57BL/6J mice models. Dietary intervention using this mushroom derived β-glucan induced lipolysis in adipocyte tissue and inhibited its differentiation, resulting in decreased fat mass. Further, upon analysing the anti-oxidant defence mechanism in liver and kidney- the primary organs involved in oxidative metabolism- β-glucan treated test groups exhibited enhanced expression of antioxidant enzymes than the HFD supplemented control mice groups. Thus, P.sajor-caju derived β-glucan helps in the management of HFD induced weight gain, obesity induced oxidative stress and hyperlipidaemia. As intake of high doses of β-glucan ends up with clinical controversies, Xu et al., [97] investigated the synergistic effect of low dose β-glucan and mulberry leaf extract on HFD induced metabolic defects. Apart from the promising outcome against insulin sensitivity, β-glucan/mulberry leaf extract showed beneficial impact towards NASH progression by reducing GSH (reduced glutathione)/GSSG (oxidized glutathione) ratio and reactive oxygen species (ROS) level in the hepatic organ of the HFD-fed mice.

Wang et al., [98] concluded in their 5-week human clinical study that molecular weight of β-glucan plays vital role in the modulation of gut microbiota profile favouring the management of cardiovascular risk factors. Herein they observed a decrease/increase in the abundance of Firmicuites/Bacteroidetes respectively in the diet group supplemented with 3 g/day of high molecular weight (greater than 1.3 x 10^6 Da) barley β-glucan. Qi Sun et al., [99] demonstrated that dietary intake of salecan – a hydro-soluble β-glucan resulted in the decrease of gene profiles associated with de nova lipogenesis and triglyceride (TG) synthesis. Shibakami et al., [100] studied the structure-functional relationship of cationic straight chain polysaccharide made from euglenoid β-1,3-glucan as bile-salt sequestering agent towards HFD associated metabolic distress

in C57BL/6J mice model, wherein prominent increase in the secretion of GLP-1 was observed. With epithelial segments containing follicle-associated cells and villus region, collected from inflammatory bowel disease patients, Mall et al., [101] analysed the mechanism of uptake and sequel of yeast derived β-1,3/1,6 glucan towards the alleviation of gut barrier dysfunction, wherein they observed an enhanced uptake of β-glucan through follicle associated epithelium followed by lipid raft generation, reduction in mast cell-induced intestinal hyperpermeability etc. thereby positively regulating intestinal related health disorders. Penney et al., [102] found that yeast derived β-glucan triggers the activation of CD22 receptor in porcine intestinal model thereby lessening the inflammation.

Thus, with different modes of action, β-glucan has notable characteristics as prebiotics in the management of metabolic syndrome as given in (**Figure 1. 9**).

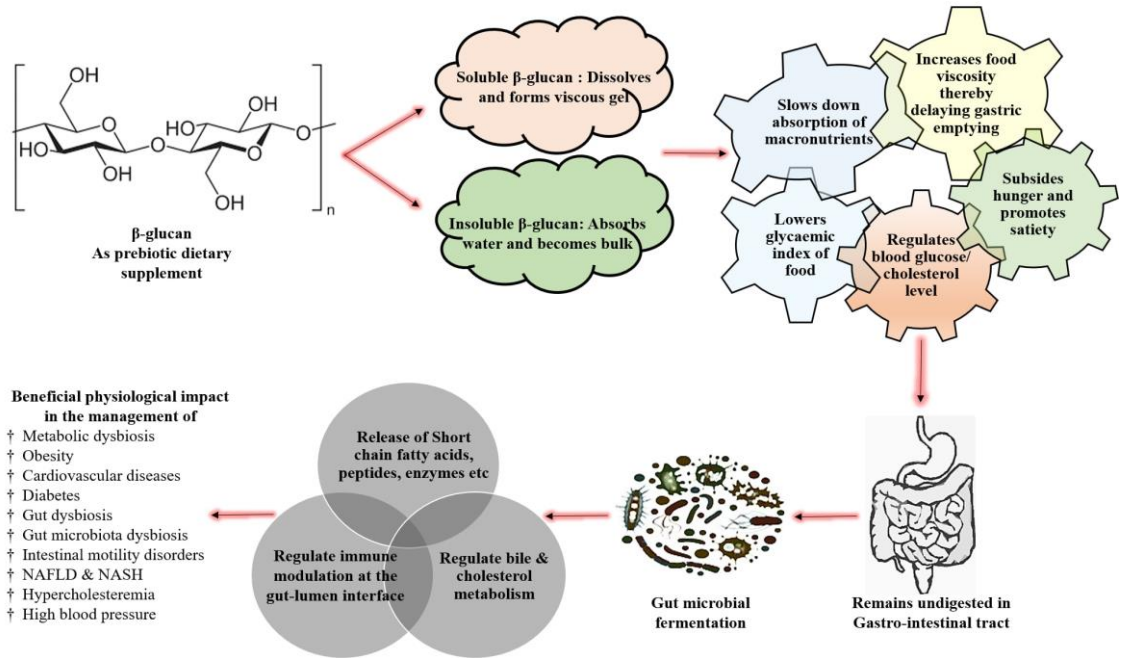


Figure 1.9: General mode of action of β-glucan towards metabolic dysbiosis.

1.5 Objectives and scope of this thesis

Several factors were found to influence the physiochemical and physiological effects of β-glucan on human health. Thus, exploring the bio-functional potential of β-glucan from diverse origin plays pivotal role in the beneficialization of human health. With these considerations, herein my doctoral research, I have aimed to study the therapeutic impact of fermented *Schizophyllum commune* derived exopolysaccharide β-1,3/1,6 glucan (molecular weight greater than 1.7 x 10^6 Da; high purity; degree of branching to be 0.33; water-soluble) on cutaneous wounds and metabolic distress. Research outcome from this thesis may provide a potential competing candidate towards the clinical management of wounds and metabolic dysbiosis.

Characteristics for an ideal wound dressing includes: moisture holding capacity (as it lowers oxygen tension on the wound site aiding better healing process), ease in removal during dressing, thermal-insulating, impermeable to pathogenic agents, assists in better skin remodelling and cellular interactions etc [103,104]. Wet dressings are preferred over dry wound dressings, as the later results in scab formation and also slows down the rate of re-epithelialization over the wounded area. In this frame of reference, hydrocolloids, alginates, gels, hydrogels etc. comes into the wound care management. Stability attributed by highly crosslinked polymeric networks and the ability to absorb enormous amount of water in them provides ease of handling the hydrogel dressings, making them advantageous over gels and colloids [105]. The moist, hydrophilic and immunomodulating characteristics of β-glucan makes them an appropriate choice to be considered as wet wound dressing material. Thus, *S.commune* derived β-glucan will be prepared in the form of hydrogel and will be analysed for its impact on cutaneous wounds.

Chronic overconsumption of diets- rich in fats (and thus calories) and poor in fiber -serves as a risk factor in the development of metabolic syndrome and its associated metabolic complications such as obesity, hypertension, hyper-cholesterol, and cardiovascular diseases. With

appropriate dietary intervention, we can able to reverse the impaired health conditions. The high viscous, hydro-soluble, fermentable and immune modulating characteristics of *S.commune* derived β-glucan intrigued us to look into its mechanism of action towards high fat and low fiber diet induced metabolic distress.

1.6 Structure of this thesis

This thesis is systematized in five chapters as follows:

Chapter -1 presents description about β-glucan, background analysis on the impact of source and processing methods on its physiochemical characteristics, physiological effects and biological mode of action with regards to wound healing and metabolic dysbiosis.

Chapter -2 describes the process involved in the development and characterization of *S.commune* derived β-1,3/1,6 glucan based hydrogel for analysing its efficiency to act as wet wound dressing material.

Chapter -3 describes the biocompatibility and therapeutic impact of *S.commune* derived β-1,3/1,6 glucan based wet dressing through in-vitro and in-vivo wound models.

Chapter -4 describes the therapeutic impact of *S.commune* derived β-1,3/1,6 glucan towards low-fiber diet induced gut barrier dysfunction and healthy gut microbiota disruption.

Chapter -5 describes the therapeutic impact of *S.commune* derived β-1,3/1,6 glucan towards high-fat diet induced metabolic dysbiosis related risk factors such as obesity, intestinal motility disorder, hepatic stress, impaired gut microbiota etc.

CHAPTER 2

Development and characterization of *S.commune* derived β-1,3/1,6-glucan based wet dressing

2.1 Abstract

Treatment towards wound healing, a complex and dynamic process, has been given a great deal of efforts in the last few decades. Focus has been imposed on developing wound dressings that meet the requirements for proper wound healing. In this study, hydrogel made from blends of poly (vinyl alcohol) and β-glucan were synthesized by modified solvent casting method for wound dressing application. Optimization of hydrogel composition and analysis of wound dressing parameters such as stability and fluid uptake capacity (in the presence of water, saline and different pH solutions) has been studied. The result indicated that the PVA/β-glucan hydrogel hold its structural integrity even at alkaline pH (pH~9) and upholds fluids four times of its original weight. Thus, the developed hydrogel is expected to be a promising candidate as wound dressing.

Keywords: poly (vinyl alcohol); β-glucan; hydrogel; wound dressing; solvent-casting

2.2 Introduction

Being the first line of defense, skin forms the protective barrier of the human body with the dense surface and corneous layer. Healing process of the skin wounds is a complex process, aiming to restore several functional damages experienced by the wounded skin [106,107]. From a long time, use of cotton, gauze etc. is used in the treatment of wound healing [108]. However, the resulting dry wound bed leads to the formation of scab thereby extending the healing time through retarding the rate of epithelialization [109]. It was when Winter [110]demonstrated that moist wound bed accelerates the wound healing mechanism, research has been focused on exploring biomaterials that provides moisture to the wound bed along with playing the role of physiological barrier and shield which protects the wound as well as aids in the removal of exudates oozing from the wound site.

Hydrogel are three dimensional polymeric networks that can absorb and retain huge amount of water inside it, when kept in aqueous medium [111]. They are made from homo-/co-polymeric grids synthesized from synthetic and/or natural polymers. Due to its tendency to absorb water, they form soft consistency when swell and thereby closely mimics the functional tissue and cellular environment [112]. Hydrogel made from natural biopolymers such as collagen, cellulose, xanthan, alginate, hyaluronan, carrageenan etc. and partial synthetic/synthetic polymers such as poly (vinyl alcohol), poly (ethylene glycol), poly vinyl pyrrolidone, polyurethane etc. are widely investigated for their application in drug delivery, wound dressings, tissue engineering etc. [113,114] Significant properties such as water holding ability, hydrophilicity, biocompatibility, biodegradability, non-cytotoxicity and non-irritability make the hydrogel one of the promising candidates to be used as wound dressings [115].

PVA is a water soluble- and non-carcinogenic polymer extensively studied in the medical industries in the form of film, fiber, powder, thin coatings, capsule coverings etc [116–118].

Hydrogels made from PVA has been given particular interest in the field of wound dressings due to its transparency, impermeable barrier to bacteria, permeability to small molecules etc. In spite of these substantial properties, PVA based hydrogel suffer from insufficient elasticity and stiffer membrane with imperfect hydrophilicity [119]. Blending PVA with other polymers enhances the setbacks of PVA hydrogel thereby improving the wound healing process. β-glucan, a complex hetero-polysaccharide, is a potent activator of macrophage cells along with inducing the proliferation of dermal cells [120].

In this study, hydrogel made from blends of PVA and β-glucan is synthesized through modified solvent casting method and optimization of the composition of the hydrogel is analysed for desirable characteristics. Using the blends of PVA and β-glucan, it is expected that the resulting hydrogel can act as a promising wound dressing; wherein the fungal polysaccharide accelerates wound healing and mechanical stability is provided by poly vinyl alcohol.

2.3 Materials and Methods

2.3.1 Materials

Poly vinyl alcohol (99+% hydrolyzed) and Glutaraldehyde (GA) (25% in H_2O, Grade 1) was purchased from Sigma-Aldrich. β-glucan (β-1,6-branched-β-1,3-glucan, average MW, 3.5×10^6 Da) was provided by Quegen Biotech Co., Ltd. Glycerol (>99.0% purity, MW 92.09) was purchased from Biosesang Inc, Gyeonggi-do, Korea. All the chemicals were used without further purification.

2.3.2 Optimization of hydrogel

The hydrogel was synthesized through modified solvent casting method. Hydrogel formulation with different composition of the initial precursors were made and allowed to air dry for 48~72h after pouring onto petri dish.

Table 2.1 Optimization parameters in the synthesis of hydrogel and weight of the hydrogel (per 5mL) kept at air drying for 48~72h.

Test Group / Precursors	1	2	3	4	5	6	7	8
Beta Glucan (w/v)	-	0.004	-	0.004	-	0.004	0.004	-
PVA (w/v)	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
Glycerol (v/v)	0.04	0.04	0.04	0.04	-	-	0.04	0.04
1% GA (v/v)	0.04	0.04	-	-	-	-	0.1	0.1
Weight (g) of hydrogel (per 5mL) after 48~72h of atmospheric drying	1.1438	1.3034	0.7735	1.2493	0.2172	0.7211	1.2632	0.4975

Briefly, the initial precursor molecules, poly (vinyl alcohol) and β-glucan were mixed, according to the composition given in **Table 2.1**, under magnetic stirring for 10 min at 60°C, such that the final concentration of PVA and β-glucan in the final solution is 2% (w/v) and 0.2% (w/v) respectively. Glycerol and Glutaraldehyde were added to the solution sets as per the volume indicated in the above-mentioned table and stirring was continued for another 30 min in-order to achieve homogenous solution. The solution was then poured onto petri dish and was allowed to air dry for a period of 2~3 days, later which it can be peeled off evenly as a hydrogel film. The color of the film was noted down after peeling the film from the petri dish. The weight of the hydrogel per 5mL of the initial solution were noted down after 48h of air drying. With the use of tissue paper, the film was blotted and the water absorbed in the tissue paper was classified as high, medium and low upon comparing with each other.

2.3.3 Stability of the hydrogel

The hydrogels were cut into 1cm x 1cm and were immersed in water for 24 hours at 37 ºC. At the end of 24 hours, the samples were taken out from the medium and were blotted using tissue paper. The ability of the hydrogel to remain in its stable form was noted down.

2.3.4 Fluid uptake capacity

$$Swelling\ ratio\ (\%) = (W_t/W_i\ ) * 100$$

→ (Equation 2.1)

where, W_i and W_t are the weights of the hydrogel sample before immersion and at particular intervals of time after immersion respectively.

The stable hydrogel with PVA/B glucan formulation was taken for fluid uptake study. Briefly, 1cm x 1cm was cut from the hydrogel film and immersed in solution (distilled water and 0.9% NaCl) at 37 ºC. At particular intervals of time, the film was taken out from the immersion

medium and the surface water was gently blotted using tissue paper. The weight of the sample before and after immersion in immersion medium was noted. The swelling ratio was calculated using the following formulae:

2.3.5 Effect of pH on fluid uptake capacity

The effect of pH on swelling ratio of the hydrogel samples were observed at five different pH conditions (pH 4, pH 5.8, pH 7, pH 7.4 and pH 9). Briefly, the hydrogel samples were weighed and immersed in different pH solution at 37 ºC. At specific time intervals, these samples were taken out from the immersion medium and after removing the surface water, the weights were again noted down. The effect of pH on swelling ratio was calculated using the formulae given in equation 2.1.

2.4 Results and Discussion

The prepared hydrogel solutions (from **Table 2.1**) poured onto the petri-dish forms hydrogel when left to atmospheric air drying for 48~72h. The test groups to which glutaraldehyde solution was added, showed switch in color (**Figure 2.1**) from transparent solution to translucent yellow shades, marking the crosslinking process (between functional groups of poly-(vinyl alcohol) and β-glucan molecules) taking place in the hydrogel formation step. The remaining test groups in which glutaraldehyde is absent, remained transparent even after the formation of hydrogel. Also, addition of β-glucan and Glycerol to the hydrogel precursor solution, increases the weight of the hydrogel (measured per 5mL of the prepared solution after 48~72h of air drying during the hydrogel formation process).

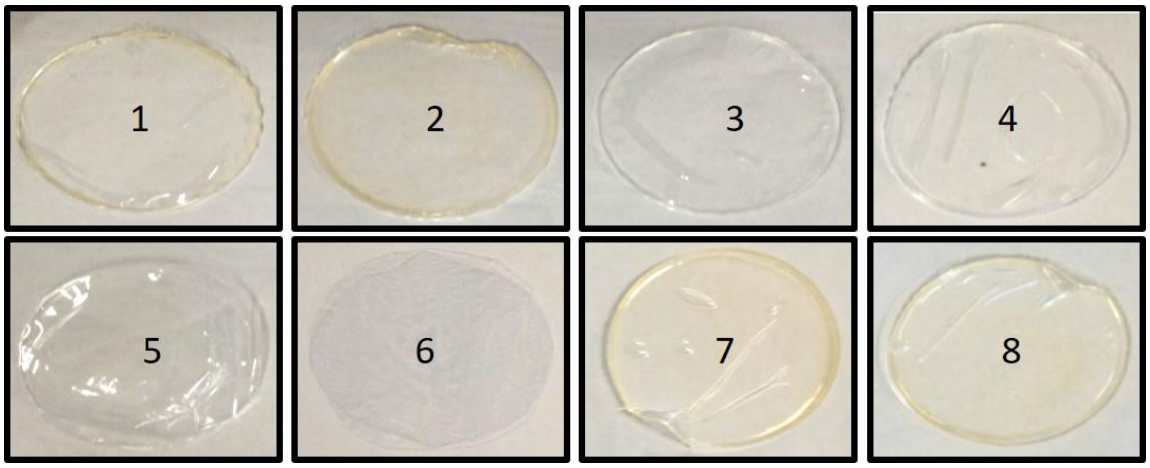


Figure 2.1: Visual observation of the different hydrogel groups (prepared as mentioned in Table 2.1).

In addition, all the test groups containing β-glucan showed high surface moisture while the rest of the test groups exhibited no moisture content (showed in **Table 2.2**). In order to test the stability of the formed hydrogel, 1cm x 1cm sample from each test group were immersed in water solution for 24h and were looked for their ability to hold the structure, even after uptake of the

fluid. It was found out that the test groups 2 and 7 exhibited good stability (**Figure 2.2**) apart from the hydrogel made of PVA alone (either with or without glutaraldehyde and glycerol).

Table 2.2 Physical characteristics of the as-prepared hydrogel

Set	Colour	Surface water
1	Yellow transparent	Lowest
2	Yellow transparent	High
3	Colorless transparent	Medium
4	Colorless transparent	High
5	Colorless transparent	No
6	Colorless transparent	High
7	Yellow transparent	High
8	Yellow transparent	Lowest
Inference	Addition of GA leads to transparent yellow colour	Adding β-Glucan & Glycerol gives moist nature to the gel

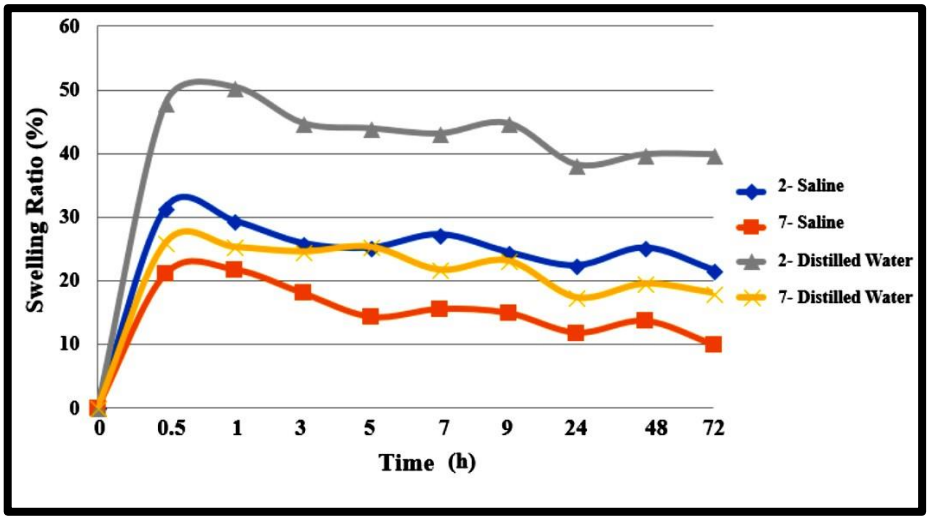


Figure 2.2: Fluid uptake capacity of the hydrogel (Test group 2 and 7 given in Table 2.1).

The starter materials used for hydrogel synthesis were acidic (β-glucan [pH 3.5 to 4.0] and PVA [pH 5.6 to 5.8]), making the synthesized hydrogel also acidic. Thus, the milieu provided by this acidic hydrogel is expected to favor the activation and survival of neutrophils and migration of epidermal cells to the wound site, which in turn helps in intensifying the pace of wound healing[121]. Apart from the soluble β-glucan, glycerol, a trihydroxy alcohol, was added to synthesize the hydrogel. Glycerol helps to improve skin hydration and epidermal barrier repair during the complicated multistep wound healing process [122]. In addition, the stiffness imparted by the PVA to the hydrogel was lessened with the addition of glycerol, in addition to the effect of β-glucan.

It was reported earlier that higher the glucan content, the less wound healing in the inflammatory phase; whereas higher the PVA content, the stiffer and moisture-less the hydrogel was, making it unsuitable for wound dressing application. The blending ratio of the polymers used in this study (polyvinyl alcohol and β-glucan) were carefully selected so that the effects of the hydrogel on wound healing, as well as its mechanical stability in body fluids, was greatly enhanced. Thus, the hydrogel (test groups 2 and 7) with appropriate polymer blends and composition with suitable moisture and flexible nature were selected for further analysis of the fluid uptake capacity, a desirable parameter in wound dressing application.

Swelling ratio acts as an index for analyzing the fluid uptake capacity of the hydrogel. Solutions such as de-ionized water, saline solution and solutions with different pH were used for studying the effect of solutions on water uptake ability of the hydrogel. As the former two solutions are used to clean wounds and the saline solution resembles body fluid, these solutions were selected for the swelling study. In addition, normal human skin is slightly acidic to prevent microbial invasions. However, some medical conditions may alter the skin; therefore, the swelling behavior of the hydrogel was studied (**Figure 2.3**) using solutions with solutions with different

pH values (4.0, 5.8, 7, 7.4, and 9.0). Thus, solutions with different pH conditions were involved in analyzing the fluid uptake capacity of the hydrogels. Briefly, the hydrogel was cut and immersed into different solutions. At the required time intervals, the immersed hydrogel pieces were removed from each solution and, with blotting paper, the solution adsorbed onto the hydrogel surface was carefully removed. The weight of the individual pieces was determined before and after immersion, and the swelling ratio was calculated.

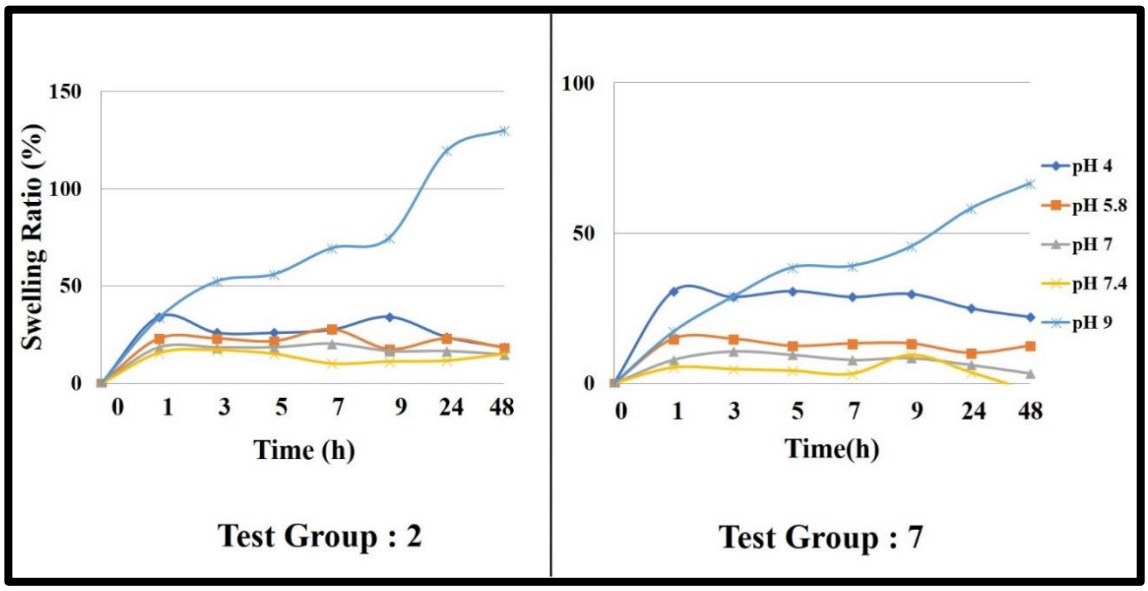


Figure 2.3: Effect of pH on fluid uptake capacity of the hydrogel (Test group 2 and 7 given in Table 2.1).

It was found out that the test group '2' surpasses the water uptake capacity upon comparing the test group '7', when immersed in all the three types of solution. Also, the water uptake capacity of the hydrogel is higher for both the groups upon immersing in distilled water when comparing the saline solution. Infected wound beds and exudate fluid have pHs ranging from 7.5 to 8.9. Hence, it is important for the hydrogel to hold its structural integrity together with removing the exudate fluids from the alkaline wound bed. When testing the effect of pH on

swelling ratio of the hydrogel, the hydrogel immersed in a solution with an alkaline pH showed drastic swelling of more than four times its original weight followed by water uptake capacity at pH 4.0 and 7.4. At pH 5.8 and a neutral state, the swelling capacity of the hydrogel was reduced. In all the pH conditions, the hydrogel remained stable. In addition, the as-prepared membrane (Test group: 7) was subjected to drying by keeping it in hot air oven for a period of 7 days, followed by analyzing the swelling capacity of the dried membrane. It was found out that the dried membrane still holds its capacity to swell as comparable to the swelling ratio exhibited by non-heat-treated membrane (**Figure 2.4**).

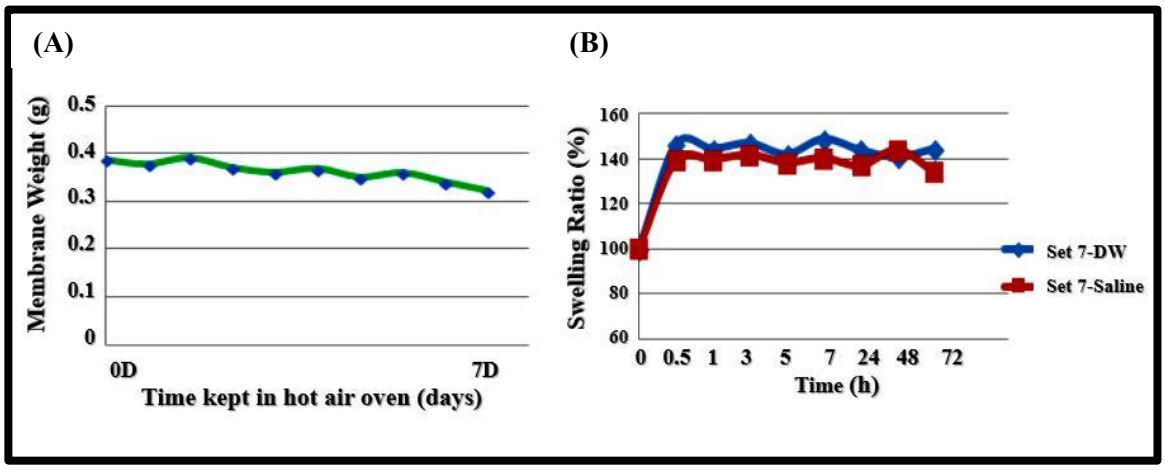


Figure 2.4: Assessment of swelling ratio after drying the hydrogel (test group: 7) at 60 ºC. **(A):** Measurement of weight loss of 1cm x 1cm hydrogel membrane when kept in hot air oven (60 ºC) for a period of 1 week. **(B):** Fluid uptake capacity of the dried hydrogel membrane for a period of 3 days.

2.5 Conclusions

Herein, it is demonstrated that the PVA/β-glucan hydrogel developed in this study can be a promising candidate for wound healing application owing to its composition (immune stimulating β-glucan polymer), stability (provided by PVA) and fluid uptake capacity.

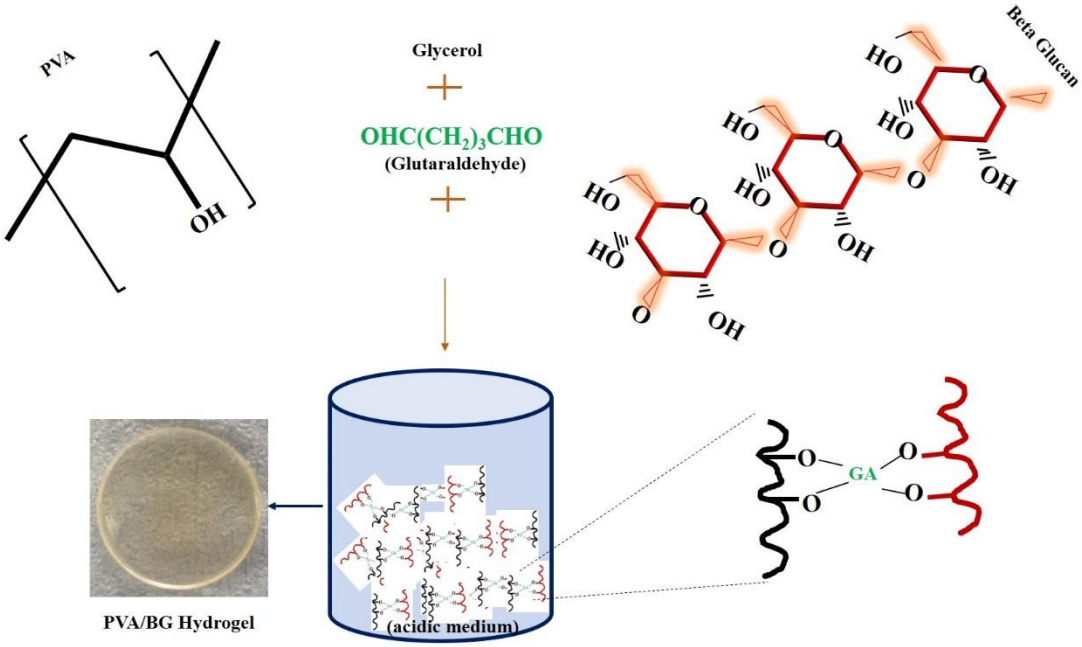


Figure 2.5: Schematic representation of PVA/β-glucan hydrogel formation and possible mechanism thereof.

CHAPTER 3

Therapeutic impact of *S.commune* derived β-1,3/1,6-glucan based wet dressing for cutaneous wound healing

3.1 Abstract

Recognized as pathogen-associated molecular patterns (PAMPs), β-glucans, a naturally occurring heterogeneous group of polysaccharides, were investigated for their ability to accelerate wound healing in the form of high water-retaining hydrogel dressing. For this, **i**mmunomodulating β-glucan (responsible for fighting infections at the wound site, and enhancing the migration and proliferation of keratinocytes and fibroblasts) in the form of a three-dimensional hydrogel membrane that retains a high-water content (responsible for cooling and soothing effect around the wound site, thereby reducing pain) was prepared. Full-thickness wounds on the dorsal side of mice created using a 5- mm biopsy punch was treated with β-glucan-based hydrogel for 2 weeks. Standardized photographs of the wound site were taken at regular time intervals to calculate the percentage of wound closure. Tissues isolated from the wound area were subjected to histological examination and immunoblot analysis. β-Glucan-based hydrogel significantly accelerated the duration of wound healing and enhanced the development of skin appendages in the regenerated skin tissue. Increased expression of transforming growth factor- β3 in the skin tissue isolated from the healed wound site indicated that skin regeneration rather than skin repair occurred, thereby minimizing cutaneous scarring. The expression level of cytokeratin 10 and cytokeratin 14 in the isolated skin tissue revealed that the wounds treated with hydrogel showed proper differentiation and proliferation of keratinocytes in the epidermal layer. β-Glucan-based hydrogels are promising as wet wound dressings in the health care industry.

Keywords: anti-scarring; wet dressing; β -glucan; immunomodulating; wound healing; hydrogel

3.2 Introduction

The skin is the largest organ of the integumentary system, and acts as a protective barrier for the human body by providing electrolytic balance, heat regulation, and evaporation and microbial control, among other benefits [106]. According to the World Health Organization, skin injuries are expected to be the leading cause of death worldwide by 2020, overwhelming other infectious diseases [123]. Globally, around 6 million people had sought medical treatment annually for burns according to the World Burn Foundation until 2014 [124]. Burns, blisters, punctures, abrasions, contusions, and superficial injuries to the skin can result in trauma or septic shock, causing the skin to lose its ability to protect the human body. In addition, when a wound becomes ‘‘chronic’’ as a result of inflammation, hypertrophic scarring is more likely to occur. Treatment strategies for acute and chronic wounds arising from accidents, surgical injuries, burns, and ulcers include autologous skin transplant, sutures, staples, adhesive sealant, physiologic dressings, and administration of antibiotics. The treatment strategy plays a significant role in the outcome of the healing process, and lessening pain and discomfort experienced by the injured patients. Because of its low availability and scar formation potential, autologous skin transplant is less widely applied compared with dressing materials[116].

After Yannas’ group first developed dressings based on synthetic hydrogels modified with polymeric materials, researchers worldwide examined polymeric materials as biomedical devices[116]. Hydrogels, which are three-dimensional polymeric materials, can retain high water content, prolonging exposure of the wound to a moist environment. This favors the migration and proliferation of fibroblasts during the proliferative phase of wound healing, leading to re-epithelialization at the wound site[111,112]. A higher water content in a hydrogel improves its ability to cool, sooth, and reduce pain in the wound area [125]. In addition, the humid conditions provided by the hydrogel aid in spontaneous detachment of dead tissue from the healthy tissue

below. Although the moist environment provided by a hydrogel promotes faster wound healing, bacterial propagation can occur at the wound site. β-Glucan has antibacterial and antiviral properties, and may be useful for overcoming this limitation [126].

Recognized as pathogen-associated molecular patterns (PAMPs), β-glucans are a heterogeneous group of polysaccharides that are potent innate immune inducers [120,127]. Through their ability to attract macrophages, neutrophils, and other immune cells, these water-soluble PAMPs fight infections at the wound site, and enhance the migration and proliferation of keratinocytes and fibroblasts, which are critical events in the complicated wound healing process. In addition, β-glucan shows anti-inflammatory and antioxidant effects, which are essential for injury healing.

In this study, a hydrogel prepared from blends of immune-modulating β-glucan and poly (vinyl alcohol) (PVA) was developed using the solvent casting method, and its effect on wound healing was evaluated in detail. In addition, the ability of the developed PVA/ β-glucan hydrogel to act as a carrier for flavonoids was examined.

3.3 Materials and Methods

3.3.1 Wound dressings

The hydrogel was prepared by a solvent casting method, wherein cross-linked blends of polymer blends were poured onto a Petri dish, followed by atmospheric air drying. In brief, at a 1:1 volume ratio, 4% (w/v) PVA (MW 89,000–98,000, 99+% hydrolyzed) solution was mixed with 0.4% β-glucan solution ([β-1,6-branched, β-1,3-glucan, average MW, $3.5x10^6$ Da] originated from *Schizophyllum* spp.) while stirring for 10min at 60°C. Glycerol and 1% glutaraldehyde were mixed into the solution at 1:25 and 1:10 volume ratios, respectively, and further stirred for 30min to obtain a homogeneous solution. In the place of β-glucan, an equal volume of distilled water was added to the 4% PVA solution to obtain PVA hydrogel. Flavonoid incorporated hydrogels were prepared by adding a 1:1 molar concentration (100μM) of Glycitin (4'- hydroxy-6-methoxyisoflavone-7-D-glucoside) and 4',6,7-trimethoxyisoflavone (TMF) to the PVA/β-glucan hydrogel solution before casting.

3.3.2 Wound-dressing properties

One of the crucial aspects of a wound treatment strategy is providing an appropriate bed for the healing process while clearing exudates from the wound surface, thereby increasing the rate of wound healing [128]. These properties of an ideal dressing were analyzed by determining the water vapor transmission rate (WVTR) and swelling index of the topical hydrogel. An ideal wound dressing prevents excessive dehydration at the wound site. The potential to transmit bodily fluid or wound exudates is reflected in the WVTR. Each hydrogel was cut into a circular shape and mounted on the mouth of a cylindrical bottle (diameter of 18mm) containing 30mL of deionized water. After weighing (W_i), this apparatus was incubated at 37°C for 24 h. After 24 h, the mass of the bottle was noted (W_f).

The WVTR of the hydrogel was determined as follows:

$$WVTR\ (g/m^2.day) = \frac{W_i - W_f}{Area\ of\ the\ membrane\ under\ study}$$

→ (Equation 3.1)

The ability of the hydrogel to absorb wound exudates was analyzed by determining the swelling index of each hydrogel exposed to various solutions. To measure the swelling ratio of each hydrogel, the hydrogels were cut into 1 cm x 1 cm pieces, and then immersed in distilled water and 0.9% saline. In addition, the effect of pH on the water holding capacity of the PVA/β-glucan hydrogel was studied using buffers with different pH values (pH 4.0, 5.8, 7.0, 7.4, and 9.0). At specific time points, the immersed hydrogel pieces were removed carefully from the solutions and weighed after removing the adsorbed surface water using blotting paper. The following formula was used to calculate the swelling ratio:

$$Swelling\ ratio\ (\%) = (W_t/W_i\ ) * 100$$

→ (Equation 3.2)

where W_t and W_i correspond to the weights of the sample at specific time points during immersion and weight of the sample before immersion, respectively.

3.3.3 In vitro cytocompatibility studies

The cytocompatibility of the hydrogels was investigated by observing the cellular morphology and viability of the dermal and epidermal layers. The experiments were performed using extracts of the hydrogel in Dulbecco's Modified Eagle's Medium (DMEM) as reported previously with slight modifications [129]. First, 0.12 g of hydrogel was weighed and sterilized under ultraviolet light for a period of 30 min, followed by immersion in 12mL of DMEM for 24 h at 37°C. After 24 h, the medium was collected and sterilized using a 0.20-μm filter membrane. The filtered hydrogel extract was used for in vitro studies. Cells cultured in growth medium were used as controls. Primary human dermal fibroblasts were seeded at a density of 1 x 10^4 cells per

well in a six-well plate. After allowing the cells to grow for 24 h at 37°C in 5%CO_2 incubator, the growth medium was exchanged for the hydrogel extract collected in the growth medium. After 48 h, cell morphologies were observed under an optical microscope (Olympus IX70 microscope, Tokyo, Japan, equipped with a digital camera). Human keratinocytes (HaCaTs) were cultured at an initial density of 5 x 10^3 cells per well for 24 h. After removing the growth medium, 200 μL of the prepared hydrogel extracts were added. Cellular viability was then measured after 24 h using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide] assay with six replicates.

3.3.4 In vitro wound healing ability

HaCaT cells were seeded into six-well cell culture plates at a cell density of 5 x 10^4 cells per well and grown in DMEM containing 10% fetal bovine serum for 24 h. After 24 h, a sterile pipette was used to make a scratch on the monolayer at the center of the well. The well was washed carefully with 1 · phosphate-buffered saline, followed by incubation with PVA and PVA/β-glucan hydrogel extract. At 0, 24, and 48 h, the scratched area was photographed under an optical light microscope at 100x magnification. The migration rate for wound closure was calculated using ImageJ software (NIH, Bethesda, MD) based on the change in the margins of the scratch area.

3.3.5 Cutaneous wound model—in vivo approach for wound healing

Six-week-old Institute of Cancer Research mice were used for the experiment. All procedures were approved by the Animal Care and Use Committee, Jeju National University, Jeju, Republic of Korea. The mice were adapted to the laboratory animal room for 1 week at 23°C ± 2°C with constant humidity (55% – 15%) on a 12-h light/12-h dark cycle. At the end of the adaptation period, the mice were randomly placed into five groups (n= 6 for each group) as follows: negative control group (no treatment), test group (PVA hydrogel, PVA/β-glucan hydrogel, flavonoid-incorporated PVA/β-glucan hydrogel), and MeditouchH (positive control group) (Ildong Bioscience Co. Ltd., Gyeonggi-do, Republic of Korea). Elastic tape was used to hold the

hydrogel to the wound. In brief, the dorsal side of each mouse was shaved, and a wound was created using a 5-mm punching tool. On days 0, 4, 7, 10, and 14, the wound area was photographed using a digital camera (ruler was used to have the same field of view of the wound area) to determine the percentage of wound closure. The images were processed using ImageJ software to calculate the percentage of wound closure at each time point by analyzing the wound area left exposed.

3.3.6 Histology

To analyze skin regeneration, tissues collected on days 17 and 21 (three mice from each group) of the treatment period were subjected to histopathological staining. Tissues were isolated from the wound area fixed in 10% (v/v) formalin solution for a minimum of 2 days, followed by embedding in paraffin wax. The embedded tissue samples were sectioned at 4-µm thickness and stained for granulation tissue by hematoxylin and eosin (H&E) staining. The stained samples were visualized under an Olympus BX51 microscope at different magnifications. From the obtained image, epidermal thickness was measured with ImageJ software by considering the thickness at ten different locations along the re-epithelialized skin tissue on days 17 and 21. Localization of cytokeratin proteins in the skin tissue isolated on day 17 was carried out by immunohistochemical analysis using the primary antibodies: cytokeratin 10 (sc-53252) and cytokeratin 14 (sc-17104).

3.3.7 Protein expression analysis

Proteins were extracted from the skin tissue isolated on day 17 using T-per buffer (Thermo Scientific, Waltham, MA) as reported previously[130]. Equivolume samples containing 20 µg of protein per lane were used to examine protein expression by western blotting. Samples were tested for expression of the following proteins (Catalog No. and concentration of the primary antibody used is given in parentheses): cytokeratin 10 (K10) (sc-53252; 1:500), cytokeratin 14 (K14) (sc-17104; 1:4000), transforming growth factor-b3 (TGF-β3) (sc- 166861; 1:1000), and β-actin (sc-

47778; 1:1000) as the loading control. All primary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). The blots were developed using a chemiluminescence ECL kit (LPS solution, Daejeon, Korea). The bands were then quantified using ImageJ software (https://imagej.nih.gov/ij/).

3.3.8 Statistical analysis

All results were expressed as the mean – standard error. The wound area left exposed for the indicated time and thickness of the epidermal layer at days 17 and 21 based on H&E staining and protein expression analysis were quantified using ImageJ software. Graphs were plotted using Microsoft Excel. Significant differences between test groups were determined using independent t-test (between two groups) and one-way analysis of variance (for more than two groups) with a cutoff significance of $p < 0.05$.

3.4 Results and Discussion

Traditional wound dressings such as pads, gauze, and cotton wool do not provide an appropriate bed for wound healing, and periodic dressing changes are tedious, and may disrupt the newly synthesized epidermal layer during healing. Further, scar development is likely when the skin under the healed area is subjected to severe tension or a dry environment. To overcome these limitations and provide a better environment for complete wound healing, researchers have focused on developing wound dressings that can stimulate cells participating in the dynamic wound healing process and have anti-scarring effects.

In this study, we developed a bioactive and biocompatible hydrogel that promotes cutaneous wound healing while fighting infections at the wound site, promoting cellular migration to increase the rate of wound closure, and providing a moist bed for highly complex wound healing dynamics.

The water vapor diffusion ability of the hydrogel must be optimal; high or low values are associated with excessive dehydration or leakage of exudate from the wound site, respectively. The WVTR of healthy skin is 204 – 12 g/m^2/day, whereas in traumatized skin, these values are 279 – 26 and 5138 – 202 g/m^2/day for burned skin and granulating wounds, respectively. For the PVA/β-glucan hydrogel, the WVTR was in the suggested range (2000–2500 g/m^2/day) of ideal wound dressings, thereby providing requisite moisture without risking wound bed dehydration. The PVA and PVA/β-glucan hydrogels fabricated in this study (**Figure 3.1 (A)**) showed WVTRs of 2930.80 – 101.78 and 2427.83 – 36.58 g/m^2/day, respectively (**Figure 3.1 (B)**). The ability of the hydrogel to absorb wound exudates was analyzed by determining the swelling index of each hydrogel in various solutions. The swelling ratio of PVA/β-glucan hydrogel reached a maximum in distilled water than that observed in saline solution when immersed in the respective medium (**Figure 3.1 (C, D)**) with p-value <0.1. In addition, the swelling ratio was higher for PVA/β-glucan

hydrogel than for the PVA hydrogel. As infected wound beds and exudate fluid have pH values ranging from 7.5 to 8.9, the higher swelling ratio of the PVA/β-glucan hydrogel under alkaline conditions makes it a better candidate for removing oozes from the wound bed while maintaining a moist environment [131]. Analysis of the impact of pH on the fluid uptake capacity (given in **Figure 3.1 (E)**) indicated that the PVA/β-glucan hydrogel immersed in alkaline medium (pH of 9) showed significant swelling of more than fourfold its original weight.

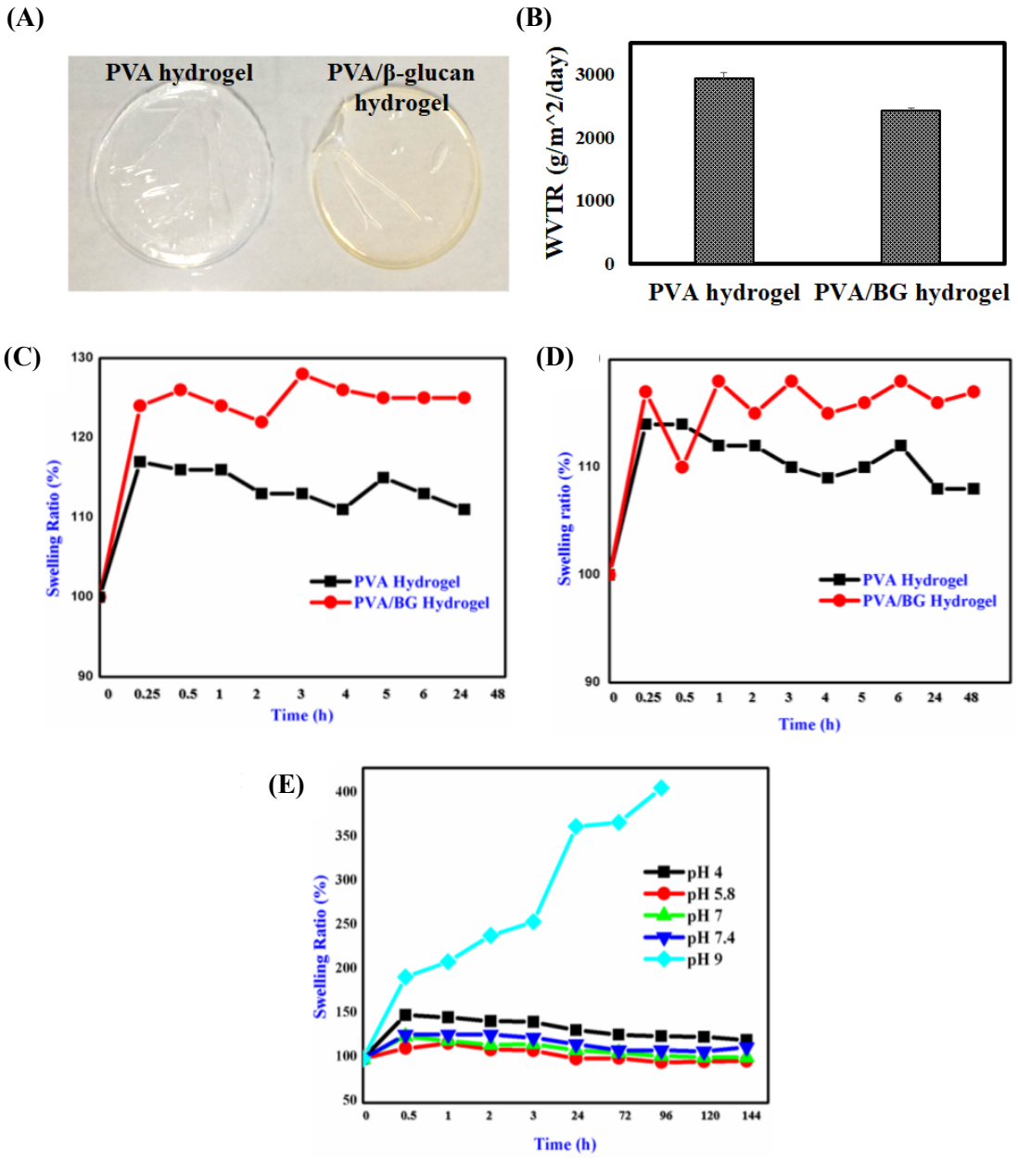


Figure 3.1: In-vitro characteristics of the developed hydrogel. **(A):** Photograph of the developed PVA and PVA/β-glucan hydrogel. (**B):** Moisture permeation capacities of the hydrogels (n = 3). The results are shown as the mean ± standard error. The fluid absorption capacity of PVA and PVA/β-glucan hydrogels in **(C):** distilled water and (**D):** 0.9% saline solution (p value < 0.1). (**E):** swelling ratio of the PVA/β-glucan hydrogel immersed in PBS solution at different pH. The values were significant at $p < 0.05$.

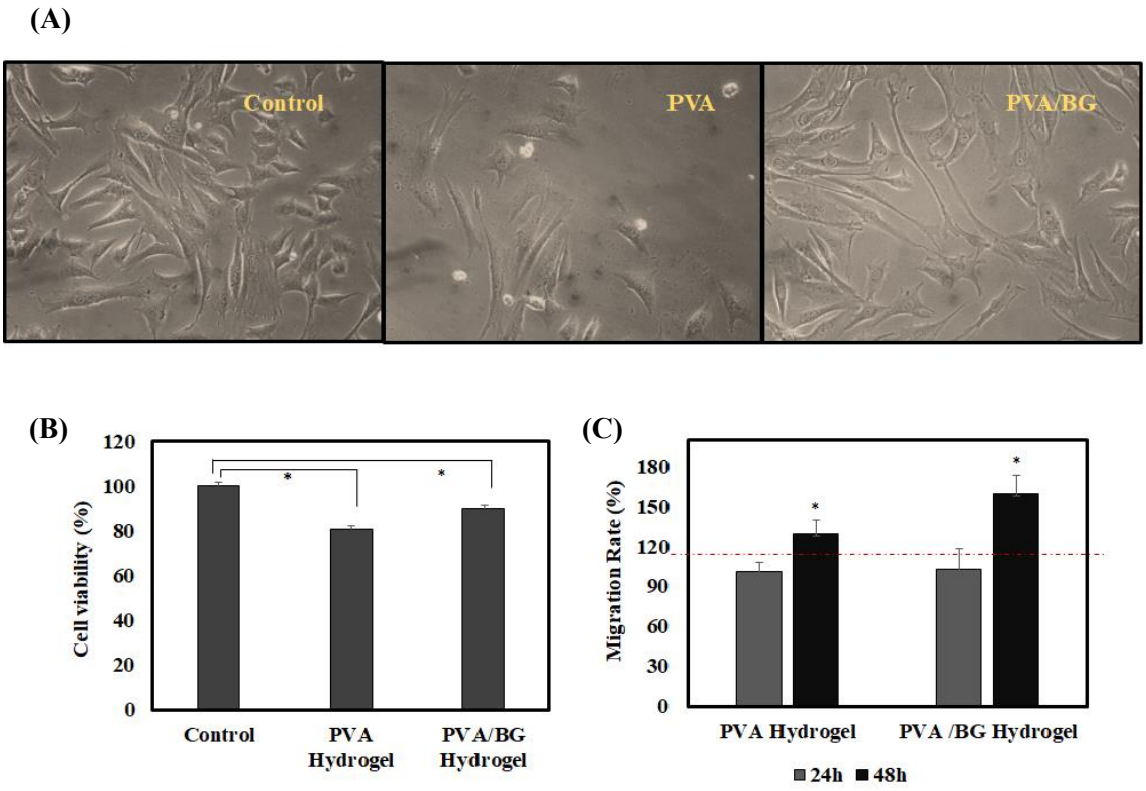


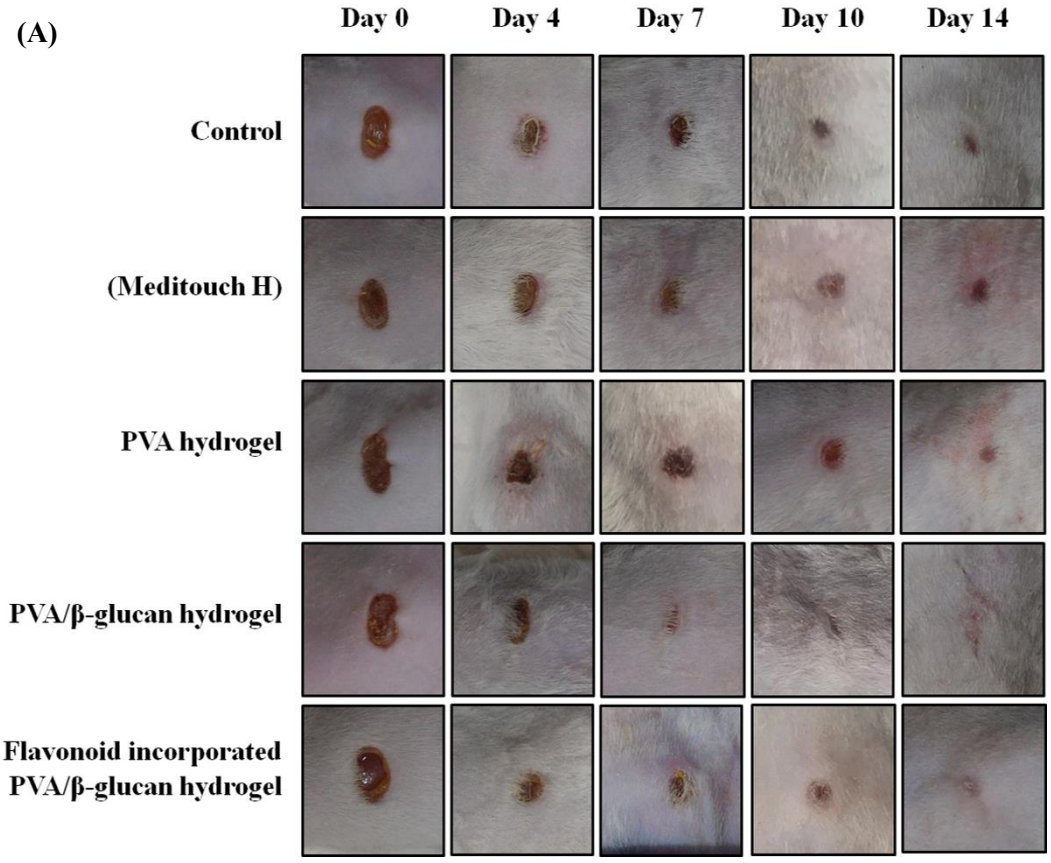
Figure 3.2: Cellular compatibility of the hydrogels. (**A):** Morphology of human dermal fibroblast cells cultured in DMEM (control) and hydrogel extract in DMEM (magnification = 200×) are shown. (**B):** Cell viability of HaCaT keratinocytes grown in hydrogel extract for 24 h as determined by MTT assay (n = 6). (**C):** *In vitro* wound closure attributed to the effects of hydrogel extract on keratinocyte cells (n = 2). The red dashed line indicates the migration rate under the

influence of hydrogel extract compared to that of the control. * represents $p < 0.05$ relative to that of control and was considered significant.

The effect of hydrogel extract on cellular attachment and morphology of the human dermal fibroblasts was observed using microscopic images. The morphology of cells grown under the influence of PVA/β-glucan hydrogel extract showed no detectable changes (**Figure 3.2 (A)**), with similar or slightly enhanced growth rates compared with cells grown in DMEM; in contrast, cells grown under the influence of PVA hydrogel showed few cells with slightly malformed degenerative structures. According to ISO 10993-5:2009, any material that shows cellular viability >70% of that of the control is considered nontoxic with good cytocompatibility. Quantitative cell viability results from the MTT assay (**Figure 3.2 (B)**) showed that in the PVA and PVA/β-glucan hydrogel groups, cell viability was >80% of that of tissue-culture-grade polystyrene dishes-grown keratinocytes (statistically significant), suggesting that these hydrogels are excellent materials for biomedical applications. Further, the in vitro scratch wound healing assay showed that HaCaT cells cultured in the PVA/β-glucan hydrogel extract had a faster migration rate than cells grown in PVA hydrogel extract medium (**Figure 3.2 (C)**).

Full-thickness wounds created on the dorsal sides of mice were treated with PVA hydrogel, PVA/β-glucan hydrogel, flavonoid-incorporated (1:1 molar concentration of glycitin and TMF) PVA/ β-glucan hydrogel, MeditouchH (positive control), and not treated. As shown in **Figure 3.3 (A, B)**, wound closure was faster in the PVA/β-glucan hydrogel treated mice group, with >75% of the wound areas closed on day 10, followed by the positive control, PVA hydrogel, and nontreated control groups. Further, the flavonoid-incorporated PVA/β-glucan hydrogel showed significantly (p-value <0.01) faster wound closure (20%) compared with the nontreated control at day 4 postinjury. While scabs appeared starting on day 4 of the treatment period in the PVA/ β-glucan hydrogel-treated mice, the scabs disappeared with the onset of re-epithelialization on day

10. In contrast, the remaining groups showed scabs beyond day 10. Healing was indicated as red pinkish skin on the wound bed.



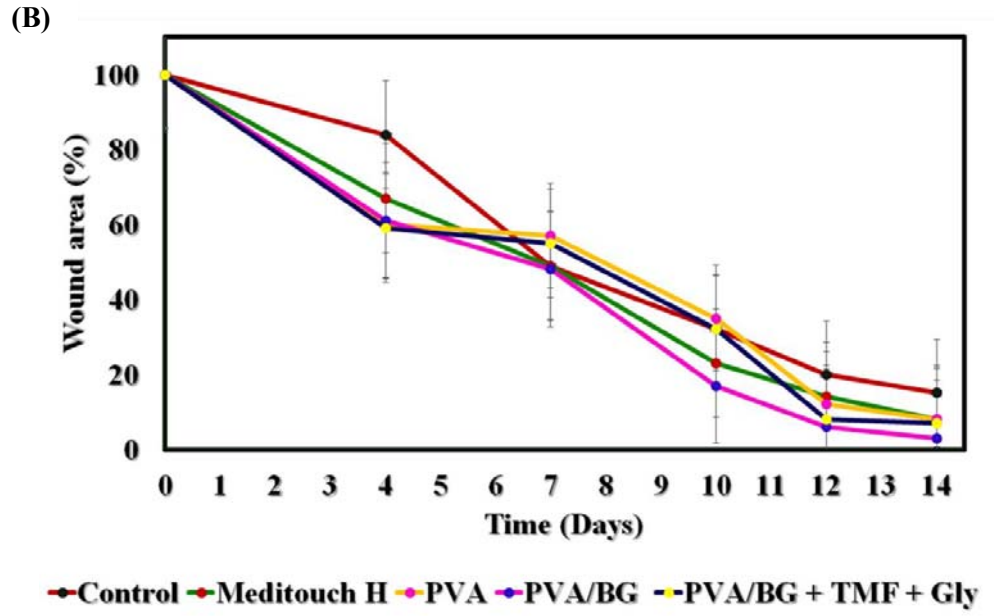
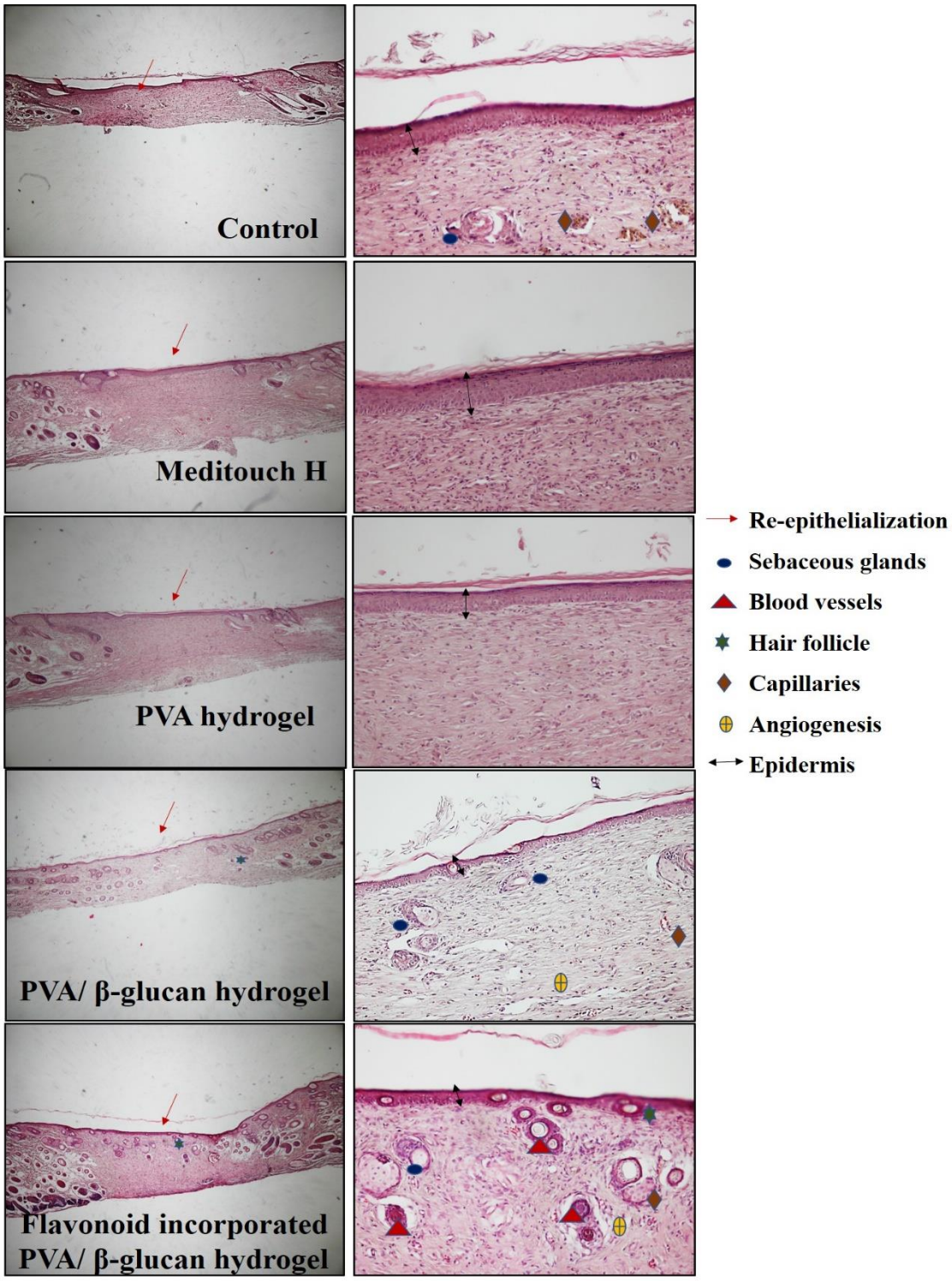


Figure 3.3: Effects of the hydrogels on *in vivo* wound healing evaluated using a mouse wound model. (**A):** Standardized photographs of wound healing with different dressings at various times are shown. (**B):** Open wound areas on the indicated days after injury (n = 6) are represented graphically. The result is presented as the mean ± standard error.

As a potent macrophage stimulator, β-glucan plays a vital role in the dynamic wound healing process by releasing wound growth factors through macrophage stimulation [132]. Histological analysis (**Figure 3.4 (A)**) showed that mice treated with PVA/β-glucan and flavonoid incorporated PVA/β-glucan hydrogel onto the cutaneous wound were in the remodeling phase of the healing process with well-developed skin appendages, glandular substances, capillary vessel formation, better granulation, hierarchical arrangement of dermal layers, and re-epithelialization similar to that of normal skin. Flavonoid incorporation enhanced wound healing during the initial phase of the process and promoted proper skin regeneration around the wound site. On days 17–21 of the remodeling phase postinjury (**Figure 3.4 (B)**), the thickness of the epidermal layer in the test groups approached that of the normal skin, with the PVA/β-glucan hydrogel treated group showing the fastest rate, and the results were significant.

(A)



(B)

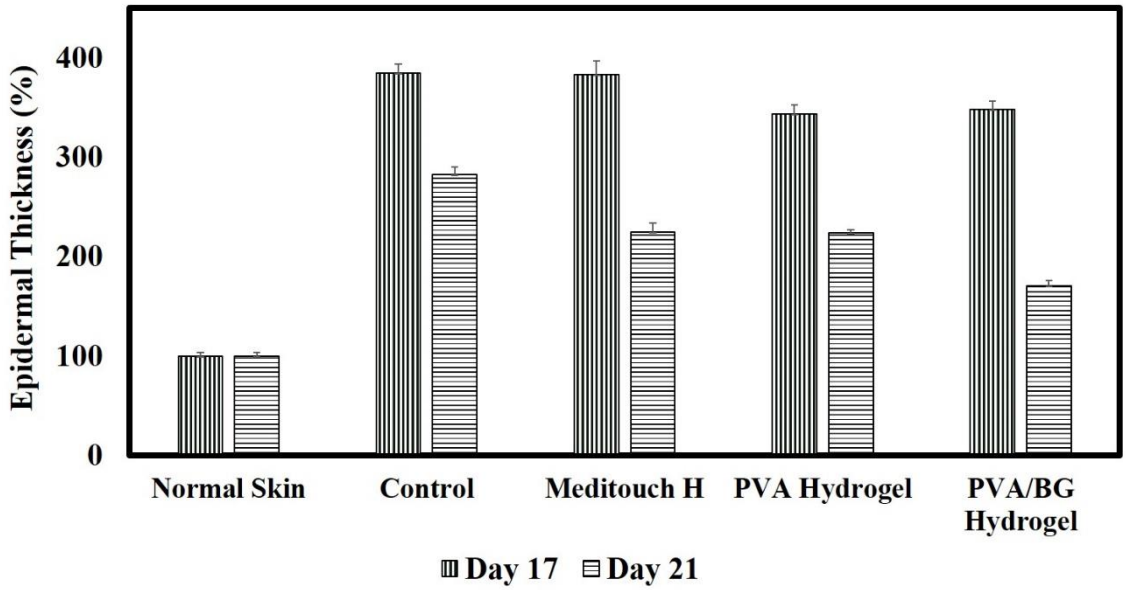
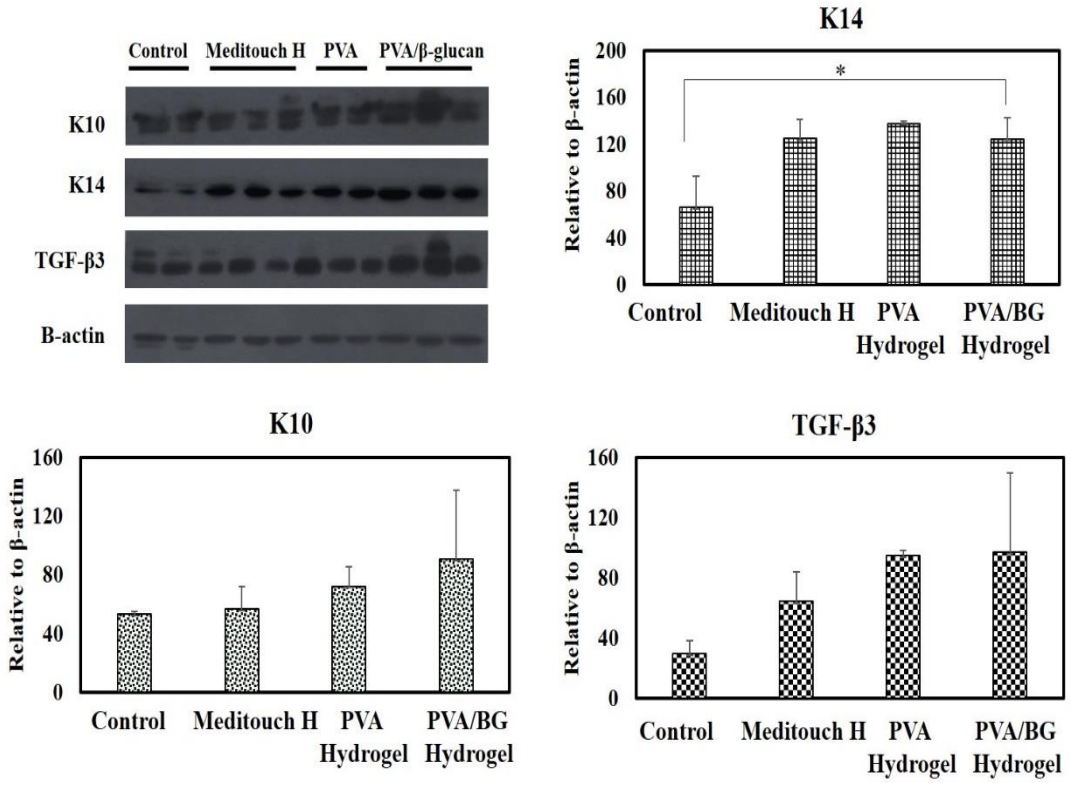


Figure 3.4: Histological analysis of healed wound tissue, in-vivo. **(A):** Pathological examination using H&E staining of regenerated skin tissue at the wound site treated with different dressings (magnification: 40x and 200x). (**B):** Epidermal thickness of the skin tissue isolated from the healed wound area on days 17 and 21. The results were significant at $p < 0.05$. Skin tissues were isolated from the wound site on days 17 and 21, and the effects of the hydrogels on re-epithelialization with proper granulation were examined. The epithelial thickness was calculated using H&E-stained tissue samples (magnification = 100×) using ImageJ software.

Protein expression analysis of the isolated skin tissue on or around the wound site on day 17 postinjury revealed enhanced translational levels of K10, K14, and TGF-β3 proteins in mouse groups treated with PVA/β-glucan hydrogel (**Figure 3.5 (A)**). Localization of keratin proteins in the isolated skin tissue was observed through immunohistochemical analysis (**Figure 3.5 (B, C)**), and it was found out that K10 and K14 were circumscribed to the supra-basal and basal layer of the skin tissue, respectively.

(A)



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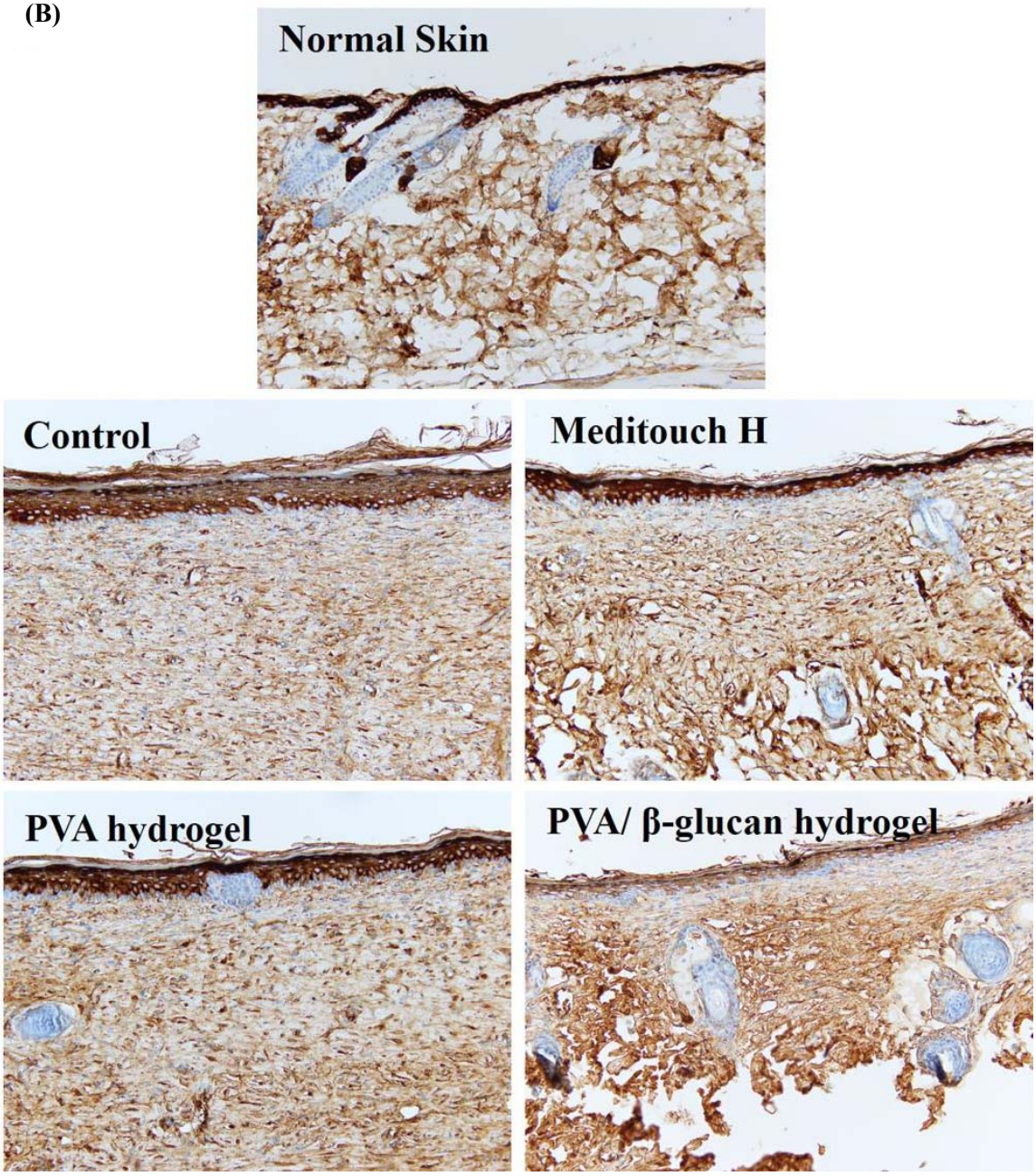

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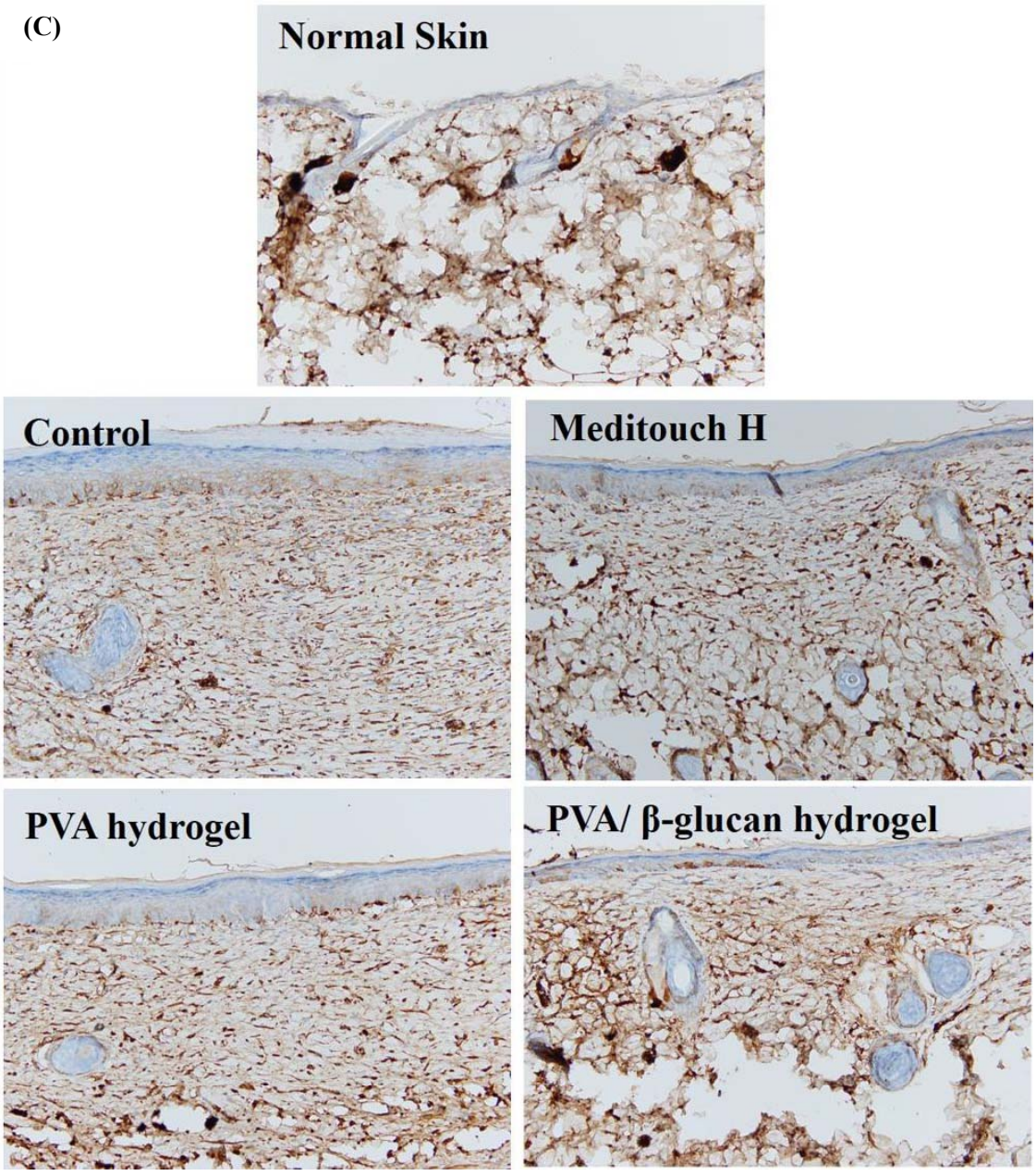


Figure 3.5: Protein expression analysis from the skin wound tissue, post-injury. **(A):** Protein immunoblot assay of the skin tissue isolated from the wound site post-injury on day 17 to determine the expression of K10 (terminal keratinocyte differentiation marker), K14 (basal keratinocyte proliferation marker), and TGF-β3 (anti-scar marker). The blots were processed with ImageJ software, and the corresponding values were plotted on a graph using Excel. * represents $p < 0.05$ relative to that of control and was considered significant. Localization of keratin proteins through immunohistochemical analysis of the skin tissue isolated on day 17 (**B):** Cytokeratin 10 and **(C):** Cytokeratin 14.

The translational expression levels of K10, K14, and TGF-β3 proteins in the skin tissue isolated on or around the wound site were found to be highest in the PVA/β-glucan hydrogel-treated group. The increase in expression of protein K10, a keratinocyte differentiation marker, in the PVA/β-glucan hydrogel-treated group agrees with the results of the epidermal thickness study. During the remodeling phase, keratinocytes covering the wound bed began to get flattened, matured, and differentiated, forming a thin, multilayered stratified epithelial layer[133]. The rapid approach of the epidermal thickness in the PVA/β-glucan hydrogel-treated group to that of the normal skin indicates that the β-glucan-incorporated hydrogel has a good wound healing capacity with proper epidermal reorganization. Expression of the proliferation marker K14, found in the stratum basal of the epidermal layer, was similar in all groups except for in the untreated control group. Proper coverage of the wound bed with basal keratinocytes was marked by keratinocyte proliferation factors such as keratin 5 and 14, which promote reorganization of the underlying epidermal and dermal layers [134]. The lower K14 expression in the control group suggests that re-epithelialization was not well developed, posing the risk of scar development. This agrees with the reduced expression of TGF-β3, an anti-scarring mammalian isoform of TGF-β, by coordinating the remodeling phase of the skin wound healing process. When TGF-β3 is highly

expressed, skin regeneration is greatly facilitated rather than skin repair, thereby minimizing cutaneous scarring[135–137]. Based on the proper differentiation and proliferation of keratinocytes in the epidermal layer (as indicated by increased expression of K10 and K14), along with minimal or no scar formation (as indicated by increased expression of TGF-β3) and proper development of skin appendages (as indicated by H&E staining), the β-glucan-incorporated hydrogel showed good potential for clinical applications in wound healing.

Isoflavones, TMF, and Glycitin showed synergistic effects on wound healing at a 1:1 ratio. A cutaneous wound model treated with these flavonoid-incorporated PVA/β-glucan hydrogels showed enhanced wound closure (a difference of 20% wound closure compared with the control on day 4 postinjury) during the initial stage. Thus, flavonoid incorporation plays an important role in wound treatment support by increasing the healing rate.

3.5 Conclusions

In this study, a hydrogel prepared from immunomodulating β-glucan was synthesized as a wet-dressing material. The developed hydrogel exhibited good water-holding capability and stability as well as high cytocompatibility of >80%. An in vitro wound healing study showed that the epidermal cells migrated 1.5-fold faster under the PVA/β-glucan hydrogel extract than with the control. The hydrogel significantly promoted skin wound healing, not only in vitro but also in vivo, with better remodeling of newly formed skin tissue. In addition, the hydrogel can act as a carrier for flavonoids to enhance the development of skin appendages in the regenerated skin and increase the rate of wound closure during the initial phase of wound healing. Protein immunoblot assays against anti scarring (TGF-β3), proliferation (K14), and differentiation (K10) markers along with histopathological study suggested that the hydrogel is a promising candidate as a wound care management strategy.

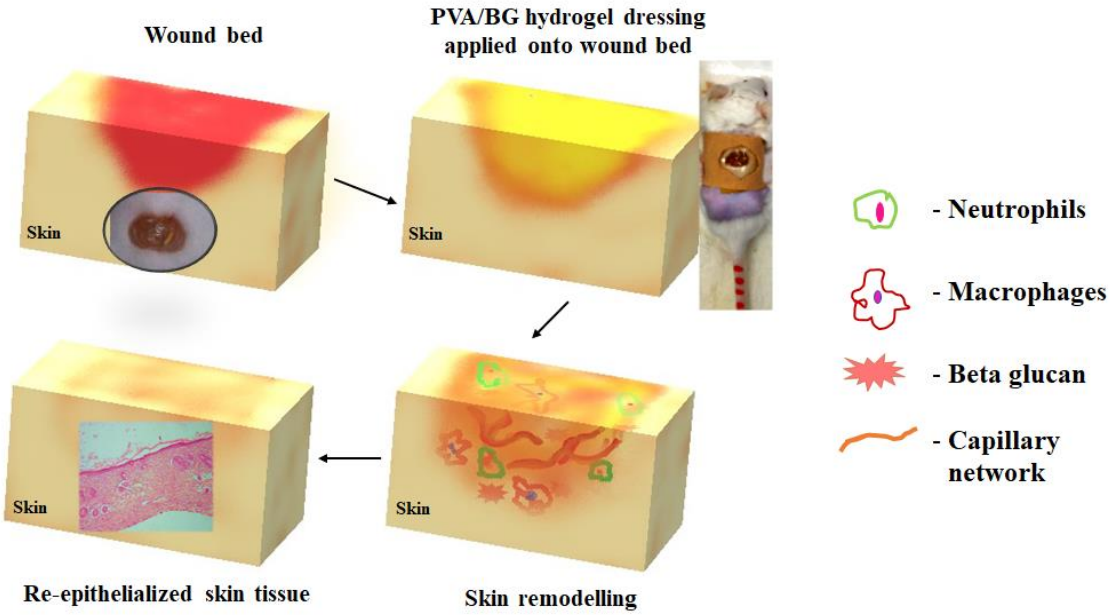


Figure 3.6: Graphical abstract representing the effect of PVA/β-glucan hydrogel towards cutaneous wound healing.

CHAPTER 4

Therapeutic impact of *S.commune* derived β-1,3/1,6-glucan on low-fiber diet induced metabolic distress

4.1 Abstract

Dietary pattern has paramount importance in shaping the gut microbiota and its associated host health. Herein this study, long term (12 weeks) impact of mushroom derived dietary fiber, β-glucan, is investigated for its effect on low fiber diet consumption. Inclusion of dietary fiber into the low fiber diet (LFD) increased the abundance of genera Lactobacillus and Anaerostipes, the microbes responsible for butyrate (major 'fuel source' of colonocytes) production. Mice fed LFD with β-glucan showed significant increase in the length of small intestine compared to that of the LFD group without β-glucan. Further, dietary fiber consumption enhanced goblet cell density along with mucosal layer thickness. These results indicate promising effects of β-glucan towards maintenance of healthy gut and gut microbiota.

Keywords: β-glucan; Dietary fiber; Gut Dysbiosis; Gut microbiota; Low-fiber diet

4.2 Introduction

Diet is one of the major factors that modulate gut microbiome. With the rapid changing lifestyle toward westernization, such as high consumption of calories, refined carbohydrates, sugar, salt, fat, animal proteins, energy dense and highly processed foods etc., the gut microbiota can dramatically get altered, leading to gut dysbiosis. Further, diet components lacking microbiota-accessible carbohydrates ensue irremediable loss of gut microbial population, thereby contributing dysfunctions, chronic inflammatory disorders, metabolic syndromes etc [138]. The susceptibility to these diseases, at-least in part, can be prevented by adding dietary fibers to the diet, thereby establishing a healthy host-microbe relationship and host immunity.

Dietary fiber, as defined by the Institute of Medicine (US), consists of non-digestible carbohydrate and lignin that are intrinsic and intact in plants[139]. On an average, the recommended intake of fiber content for a young man and woman is around 38 g/day and 25 g/day respectively[140]. Diets that lack the required amount of fibers will make the people more prone towards obesity, heart disorders, diabetes, bowel cancers, constipation etc. Foods such as whole-grain cereals, fruits, vegetables, legumes etc. are said to be rich in fiber content[141]. Consumption of dietary fibers such as cellulose, arabinoxylan, inulin, soluble corn fiber, resistant starch, guar gum, gum-arabic etc., is found to be inversely linked with the risk of microbiome-associated non-communicable disorders such as obesity, cardiovascular diseases, allergies, cancer, inflammatory diseases etc [142,143].

β-Glucan, an indigestible- and hydro soluble- dietary fiber has shown to exhibit much physiological benefits on cholesterol reduction, weight management, cardiovascular risks, diabetes mellitus, metabolic syndrome etc [85,144]. Being a major soluble fiber found in the cell walls of oats and barley endosperm, the β-glucan is commercially extracted from Saccharomyces cerevisiae and also from other sources such as Phellinus linteus or Sparassis crispa [145,146].

While oat and barley based β-glucan presents metabolic effects such as postprandial glycemic response, fungi derived β-glucan exerts immunological modulation; thereby making the sources of β-glucan as one of the prime factors contributing to its clinical outcome [95,147]. Further, the fermentation of this indigestible fiber in the lower gastrointestinal tract is said to alter the gut microbial composition, whose metabolites is found to have prime importance on host health [98,148].

Herein this study, we aimed to investigate the long-term impact of mushroom derived β-glucan, a soluble dietary fiber consumption on the gut dysbiosis induced by low fiber consumption in animal model.

4.3 Materials and Methods

4.3.1 Animal care

C57BL/6J male mice (5 weeks old) were used in this study. With a laboratory acclimation period of 1 week, the mice were 6 weeks old when they were subjected for experimentation. 12 h/12 h of light/dark cycle with ambient atmosphere was provided to the mice. All the animals were taken care of according to the rules framed by Animal Care and Use Committee (ACUC No: 2018-0018).

4.3.2 Experimental design

Four experimental groups with n=11 mice per group was classified as: normal diet group (ND), low fiber diet group-AIN 76A (LFD), LFD with 3 g/kg of β-glucan (LFD + BG1) and LFD with 5 g/kg of β-glucan (LFD+BG2). β-Glucan (β-1,6/1,3 glucan isolated from *Schizophyllum* spp. with average molecular weight of 1.78~1.79×106 Da) was provided by Quegen Biotech Co. Ltd., Republic of Korea. During the experimental study period, mice were provided with ab libitum of water and corresponding diets. At two time points in the scale of 12 weeks experimental period (6th week and 12th week), feces from individual mice in all the groups were collected and subjected for microbiota analysis. On the day prior to the sacrifice of mice, they were left starving through the night. The mice were orally administered 100μL of activated charcoal solution prepared in 1X PBS, 20 min prior to sacrificing it. The distance travelled by the charcoal in the intestine was observed and the intestinal transit rate was calculated accordingly:

$$Intestinal\ transit\ rate\ (\%) = \left(\frac{Distance\ tavelled\ by\ the\ activated\ charcoal\ (cm)}{Length\ of\ the\ small\ intestine\ (cm)}\right) * 100$$

→ (Equation 4.1)

Once the mice were sacrificed, the internal organs were isolated and weighed. Small intestine samples collected from the mice were fixed using 4% formaldehyde solution for further histopathological analysis.

4.3.3 Physiological measurements

At the start of every week, measurement on body weight, weekly consumption of food and water were measured. In addition, 25 pieces of feces from each cage was collected and weighed. The fecal moisture content was calculated by weighing the difference in feces weight before and after drying at 60°C for 2 days.

4.3.4 Histopathological analysis

The formaldehyde fixed small intestine samples were sectioned and subjected to Alcian blue and PAS staining, in-order to observe the effect of different diets on intestinal architecture and mucin abundance. The stained sectioned were imaged using Olympus BX51 microscope. The thickness of mucus layer is quantified using ImageJ.

4.3.5 Fecal microbiota analysis

Feces from individual mice were collected on 6th week and 12th week of the diet-based experimental study. 16S rRNA targeted PCR amplification was done to the QIAamp, PowerFecal DNA kit extracted total DNA samples and the sequenced data (sequencing was performed by Macrogen, Seoul, Republic of Korea) was processed using MOTHUR [149], a bioinformatics software package. Briefly, raw sequence reads assembled using make.contigs command were aligned to SILVA database using align.seqs, followed by trimming the rare sequences using split.abund and pre.cluster. Further, chimeric sequences were removed using chimera.vsearch command and non-bacterial sequences such as mitochondria and chloroplasts were removed before clustering the resulted clean reads using opti.clust at the distance 0.03. Nonmetric multidimensional scaling (NMDS), using Bray-curtis dissimilarity distance, computed with nmds

MOTHUR subroutine was employed to identify the bacterial Operational Taxonomic Units (OTUs).

4.3.6 Statistical analysis

Values are given as mean ± standard error and the statistical significance was calculated using Student t-test. Significance in the microbial population difference was examined using Analysis of molecular variance (AMOVA).

4.4 Results and Discussion

Compared to that of the normal diet, mice fed with low fiber diet showed a relative 10% increase in body weight (**Figure 4.1 (A, B)**). This result gets reflected with the increase in the Firmicutes/Bacteroidetes ratio, a "obese type microbiota from the fecal analysis (shown in **Figure 4.7 (A)**). In general, high fiber intake is associated with weight management by increasing gastric distension and satiety, whereas poor-fiber diet enhances hunger thereby increasing total energy intake resulting in overweight and obesity. Although not significant ($p > 0.05$), inclusion of β-glucan to LFD exhibits promising result towards weight reduction. Mice fed with LFD + BG2 showed relatively higher water consumption than the rest of the groups (**Figure 4.1(C)**) while there was no significant difference in the food consumption (**Figure 4.1(D)**) among the low fiber diet groups (LFD, LFD + BG1, LFD + BG2) at the 6th and 12th week of the experimental period.

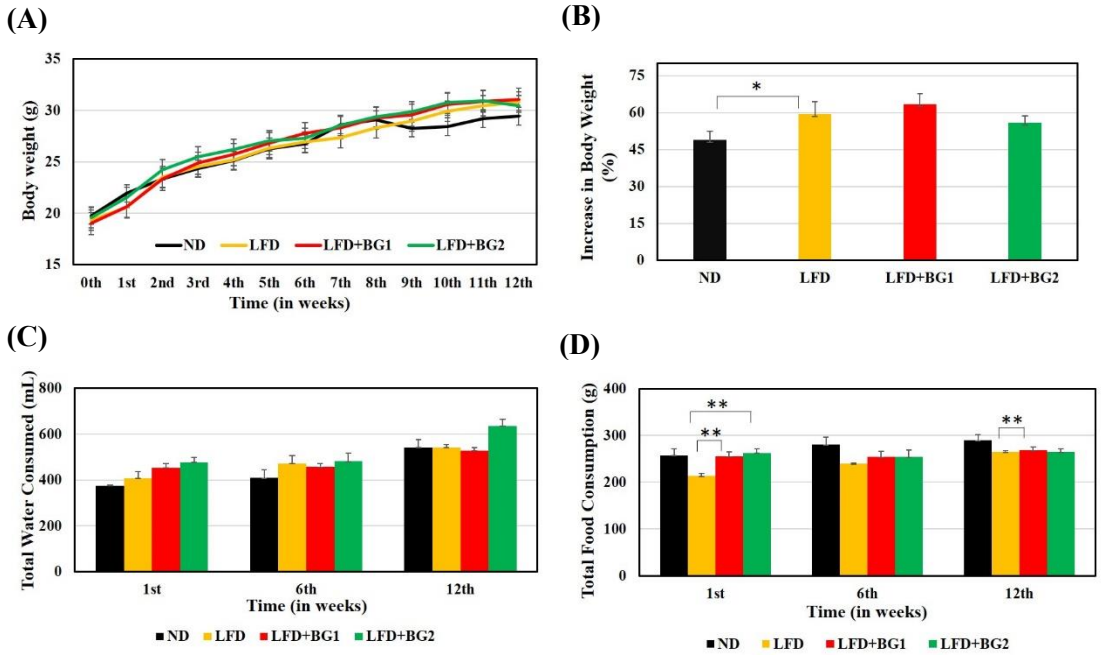


Figure 4.1: Effect of low fiber diet with/without β-glucan on physiological parameters of the mice. **(A):** Body weight. **(B):** percentage increase in body weight. **(C):** water consumption per week. **(D):** food consumption per week.

LFD and LFD + BG2 showed almost similar transit percentage (**Figure 4.2 (A)**), however, it was reduced with LFD + BG1. Fecal weight is one of the key indicators of intestinal health[150]. There was a significant fecal weight reduction in the β-glucan included LFD groups compared to that of the LFD control group in a dose dependent manner (**Figure 4.2 (B)**). Thus, mushroom derived β-glucan doesn't have much impact on bowel movement associated intestinal health. In addition, the mice fed with LFD showed lowest moisture content in the fecal samples (**Figure 4.2 (C)**); whereas the dietary fiber included LFD groups showed relatively higher moisture content. This leads to conclude that the reduction in fecal weight doesn't come from its moisture content and further investigation on other factors such bacterial biomass, protein or nitrogenous or undigested dietary matters is required. Mice fed with higher concentration of β-glucan incorporated LFD showed increase in small intestine length (**Figure 4.2 (D)**). This may aid in higher nutrient absorption by the increased cross-sectional area of intestinal mucosal barrier. However, no significant changes were observed in the liver/body weight of the low fiber diet groups (**Figure 4.2 (E)**).

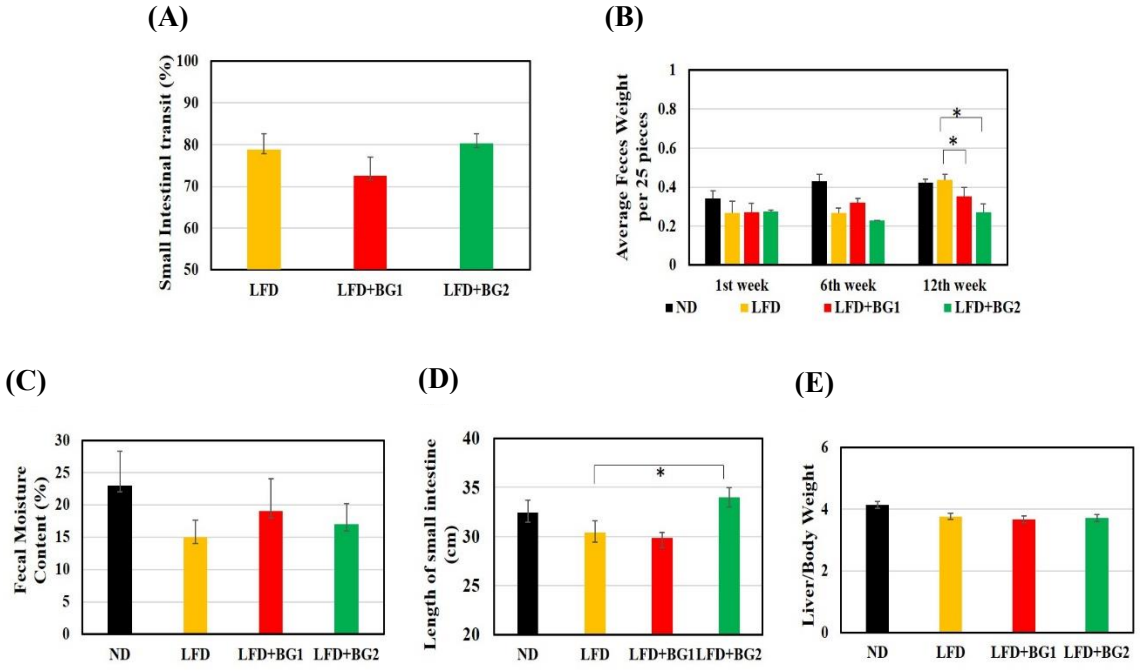
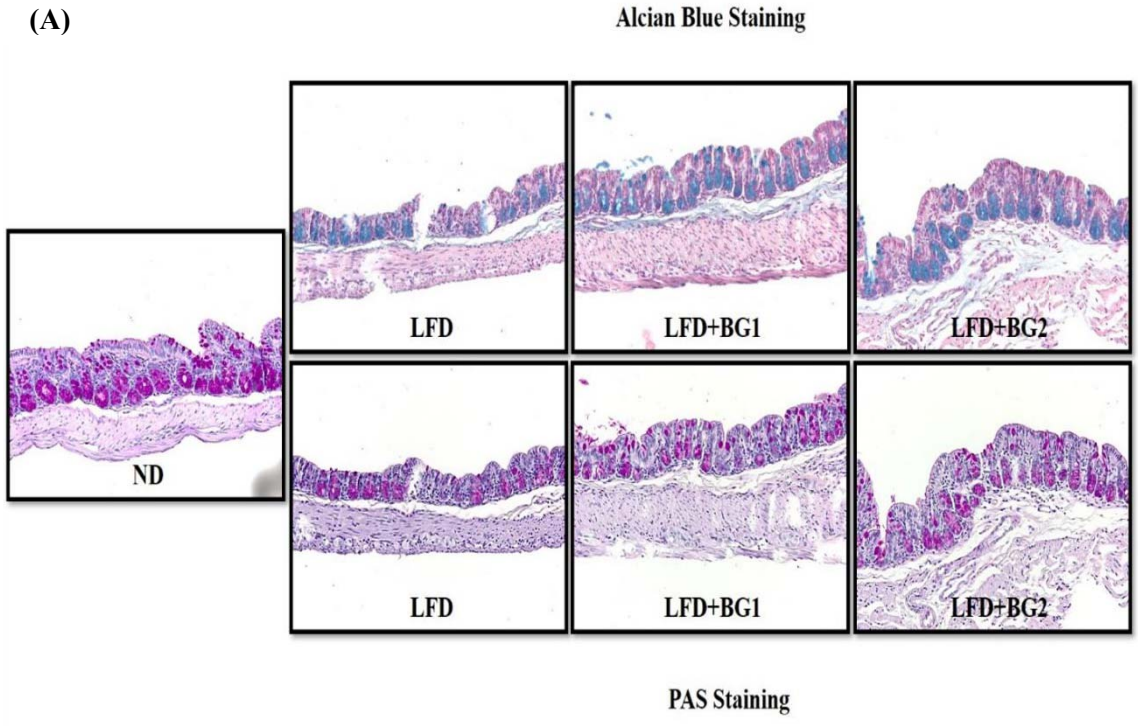


Figure 4.2: Effect of low fiber diet with/without β-glucan on **(A):** intestinal transit rate. **(B):** feces weight. **(C):** fecal moisture content. **(D):** length of small intestine and **(E):** Liver/body weight.

Fiber-deprived diets urge the gut microbiota to rely on the 'highly glycosylated' intestinal mucus layer, whose primary function is to protect the underlying gastrointestinal tract (comprising of epithelial cells) from bacterial invasion [80,151]. From the histopathological staining of the small intestine sample (**Figure 4.3 (A)**), it was observed that β-glucan incorporated LFD groups showed enhanced goblet cell count with significantly higher mucosal layer thickness (dose-dependent increase) (**Figure 4.3 (B)**) and mucosa supporting submucosal layer than the LFD control group, thereby presaging that consumption of diet lacking in fiber content may result in bacterial invasion in the mucus layer, leading to 'the leaky' gut wall; whereas inclusion of dietary fiber will ameliorate the condition. Though not significant, we observed a dose dependent decrease in serum triglyceride level together with enhanced high-density lipoprotein level in the β-glucan incorporated LFD groups than the LFD group (**Figure 4.4**).



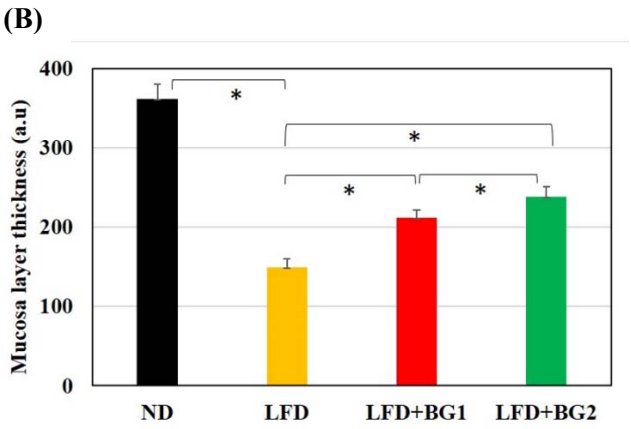


Figure 4.3: Effect of low fiber diet with/without β-glucan on histological analysis of intestinal sample. **(A):** Alcian blue and PAS staining. **(B):** mucosa layer thickness.

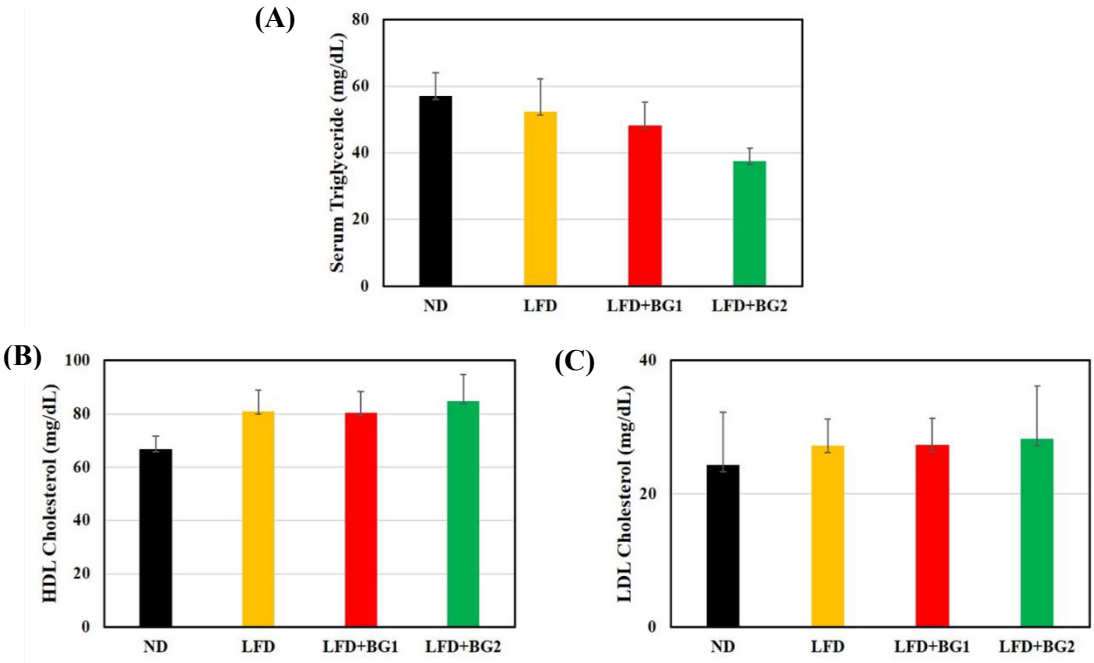


Figure 4.4: Effect of low fiber diet with/without β-glucan on blood profiles. **(A):** serum triglyceride. **(B):** HDL cholesterol. **(C):** LDL cholesterol.

For microbiota analysis, 19,590 reads were randomly sampled from each fecal sample to normalize the number of reads. More than 99% Good's coverage was observed in each sample;

thus, this random sampling hardly affected the number of observed species. Results in **Figure 4.5** showed that the use of low-fiber diet (LFD) significantly decreased species richness and evenness. In this study, 12th week samples showed lower richness and evenness compared to those at the 6th week. Since dietary fibers are the source of energy for bacteria in the large intestine, lack of this fiber in diet could cause reduction of certain species that feed on dietary fibers. Previously, it has been reported that dietary fibers are fermented by the intestinal bacteria to produce short chain fatty acids such as butyrate and propionate, resulting in improving the intestinal immune system and host's health [138].

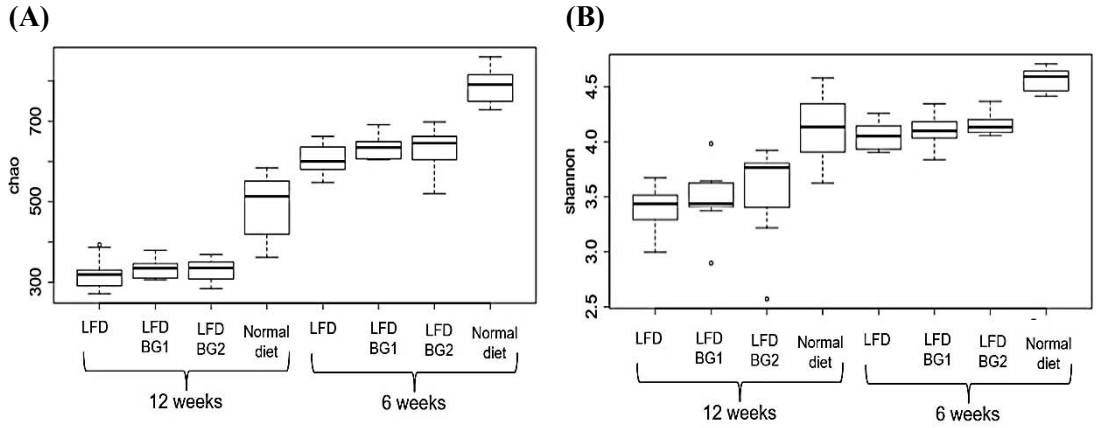


Figure 4.5: Ecological indices of mice gut microbiota observed in this study. **(A):** species richness. **(B):** species evenness.

Results in **Figure 4.6** show that the dysbiosis caused by LFD are more obvious at the 12th week than at the 6th week as NMDS plots were more scattered. On the other hand, LFD + BG1 samples are less scattered and plotted relatively closer to the normal diet at the 12th week, suggesting that LFD + BG1 may have slightly ameliorated gut dysbiosis. While distribution of LFD + BG2 samples were similar to that of LFD samples at the 12th week, LFD + BG1 showed significantly different microbiota distribution when compared with that of LFD samples ($p < 0.001$). These shifts observed in LFD + BG1 group were not observed at the phylum level

(**Figure 4.7 (A)**). At the family level, the lower abundance of the family S24-7 was observed in LFD, suggesting that lack of fiber cause a decrease in the abundance of S24-7 (**Figure 4.7 (B)**). While distribution at the family level for normal diet showed a clear separation between the 6th and 12th week, LFD groups did not, suggesting that unbalanced intestinal microbiota among LFD groups.

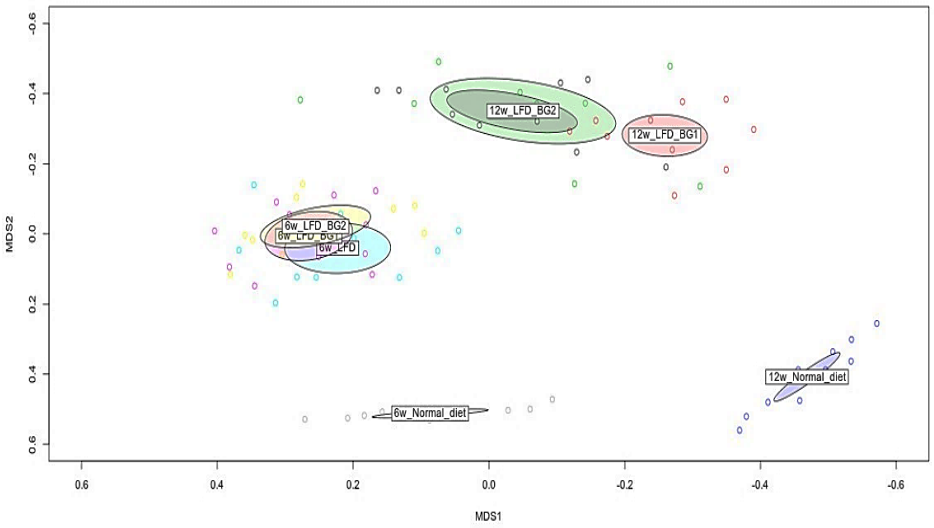
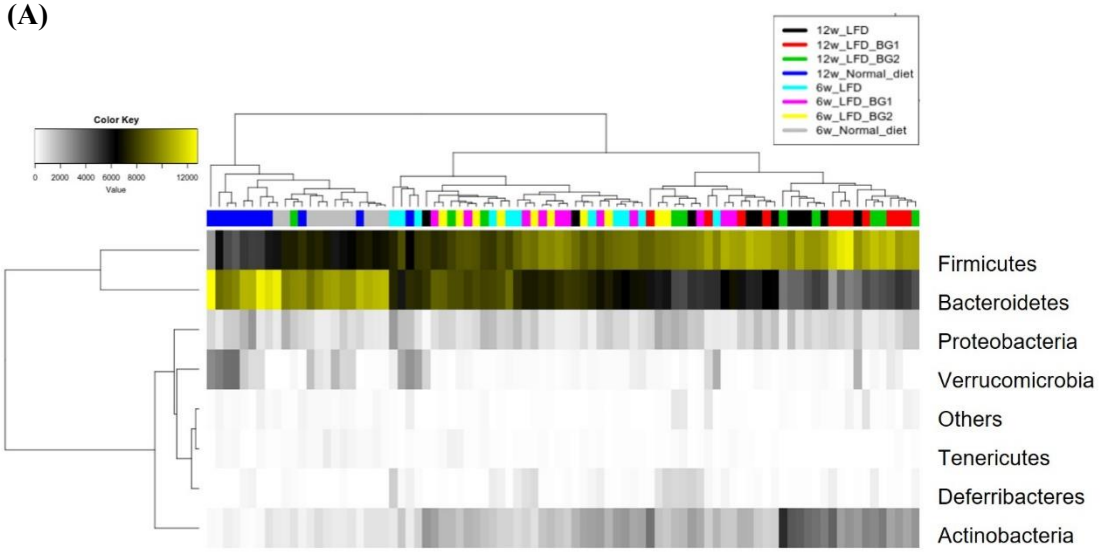


Figure 4.6: Analysis of microbial community comparison by non-metric multidimensional scaling (NMDS).

(A)



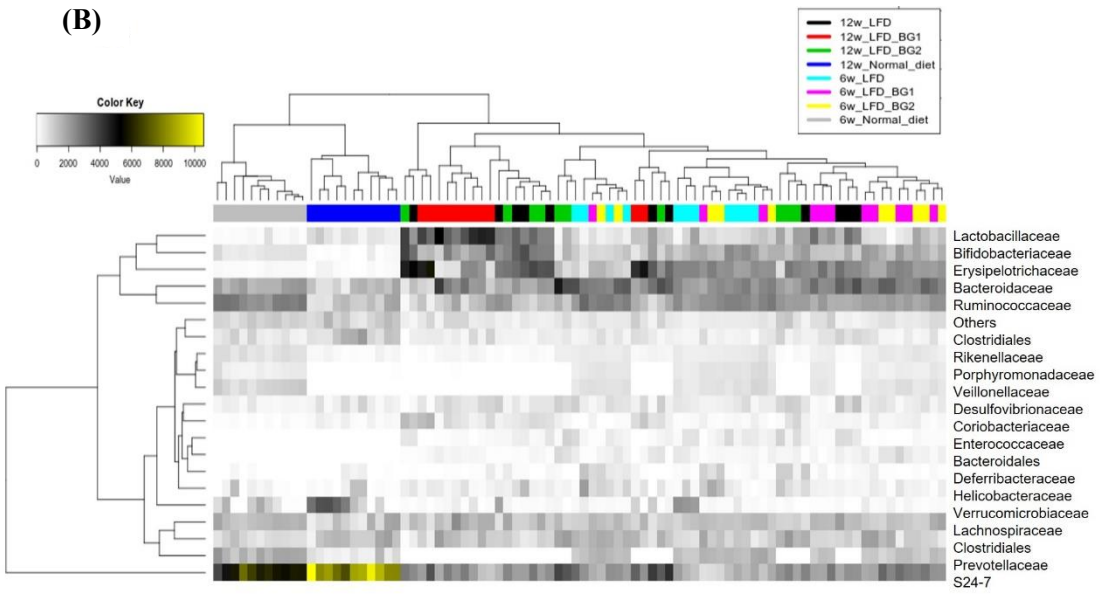


Figure 4.7: Taxonomy composition of gut microbiota at the **(A):** phylum level and **(B):** family level.

At the 12th week, LFD + BG1 samples were clustered due to the increased abundance of genera, Lactobacillus, and Anaerostipes (**Figure 4.8**). The genus Lactobacillus is a well-known probiotic bacterium, while some species of the genus Anaerostipes are known to produce butyrate by utilizing lactate [152,153], suggesting that the increase of these two genera may cross-feed to produce butyrate in the gut. The roles of butyrate produced in the gut include being the major energy source for colonocytes, maintaining the colonial mucosal health, and regulating the intestinal tight junctions along with imparting immunomodulatory and anti-inflammatory properties on the gut barrier. Therefore, our results suggest that long-term use of β-glucan may shift microbiota toward preferred environment for butyrate production in the gut. It should be noted that the higher dose of β-glucan did not increase Lactobacillus, thus beneficial effects of β-glucan may be dose-independent.

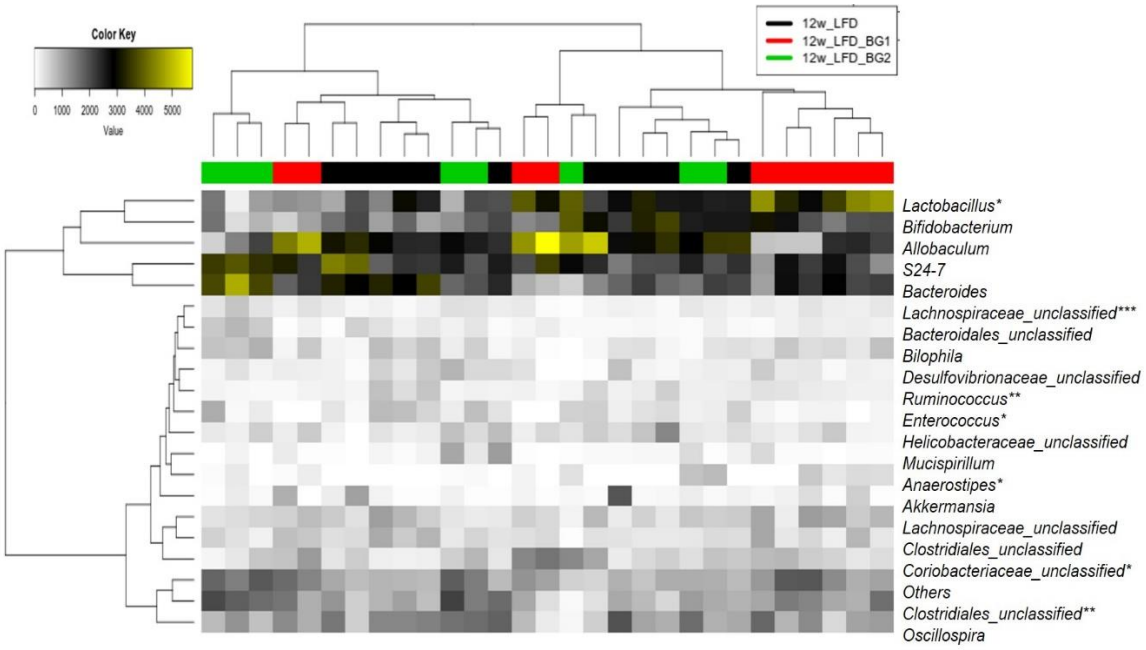


Figure 4.8: Comparison of the taxonomic composition at the genus level. *, **, and *** indicate significant difference between LFD and LFD+BG1, LFD and LFD+BG2, and LFD and both LFD+BG1 and LFD+BG2, respectively.

Figure 4.9 show differentially abundant OTUs between LFD samples and LFD + BG samples. There are 3 OTUs (0040, 0036, and 0050) increased and 4 OTUs (0024, 0017, 0056, and 0087) decreased in both BG groups, indicating that β-glucan dose independently increased Anaerostipes, unknown genera in F16 and Clostridiales and decreased Oscillospira, Ruminococcus gnavus, and unknown genera in S24-7 and Lachnospiracea. The abundance of Oscillospira, Ruminococcus, and Lachnospiracea is highly correlated with the pathogenesis of Type 1 Diabetes by decreasing FoxP3+ regulatory T cells[154]. Herein our study, the LFD + BG groups has the tendency to decrease their abundance thereby exhibiting fiber restricted diet associated anti-diabetic effect. It is interesting that the abundance of Lactobacillus was increased in LFD + BG1 but decreased in LFD + BG2. As there are more OTUs that were significantly increased in LFD + BG2 group, some of these OTUs may have competed against Lactobacillus

over substrates, or the decrease of some OTUs in LFD + BG1 may have helped Lactobacillus to increase. Further study is needed to investigate who competes against whom over substrates in the gut. This is important to maintain the healthy gut microbiota and it should be noted that the dose determined based on mice experiment should not be applied to human case as previously reported[155].

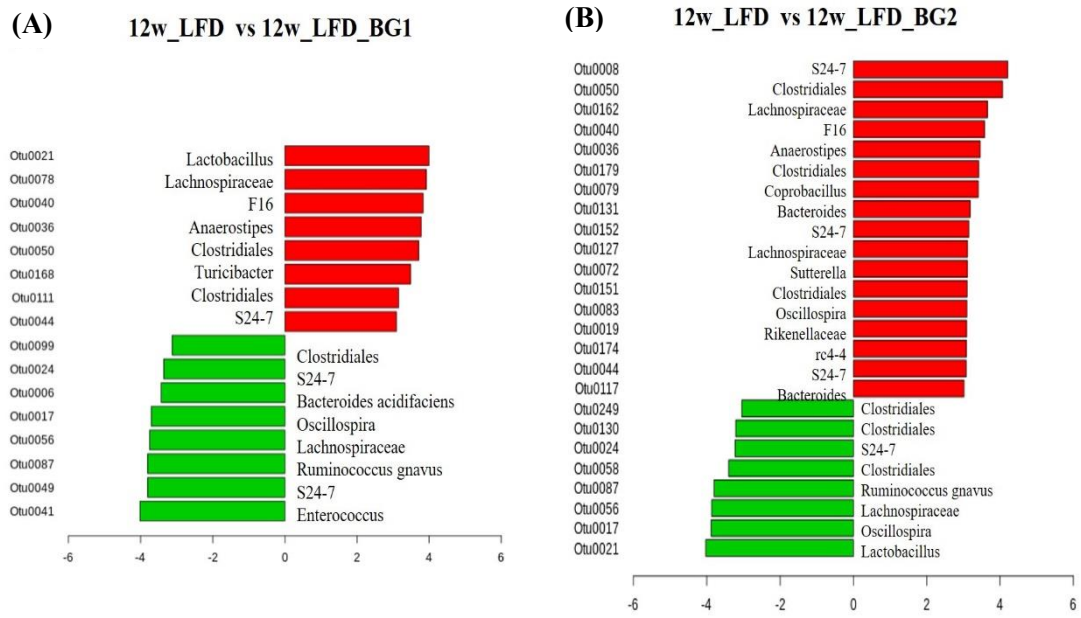


Figure 4.9: Differential abundance analysis among the 12th-week samples. **(A):** LFD and LFD+BG1. **(B):** LFD and LFD+ BG2. (p <0.05, LDA >3).

4.5 Conclusions

To summarize, the use of β-glucan improves LFD induced gut dysbiosis in terms of goblet cell production and enhanced mucosa layer protective barrier. LFD consumption significantly changed gut microbiota compared to that of a normal diet. While the changes were seen in increased Firmicutes/Bacteroidetes ratio, it was rather random at the family level, suggesting that the use of LFD disrupt the healthy gut microbiota in various ways. The lower dose of β-glucan increased Lactobacillus and Anaerostipes, which may have contributed to stabilizing the gut microbiota, while a higher dose of β-glucan decreased Lactobacillus thus failed to stabilize the gut microbiota. Further studies are needed to find the optimum dose of β-glucan toward human application, but here, we report the promising effects of β-glucan in maintaining healthy gut microbiota.

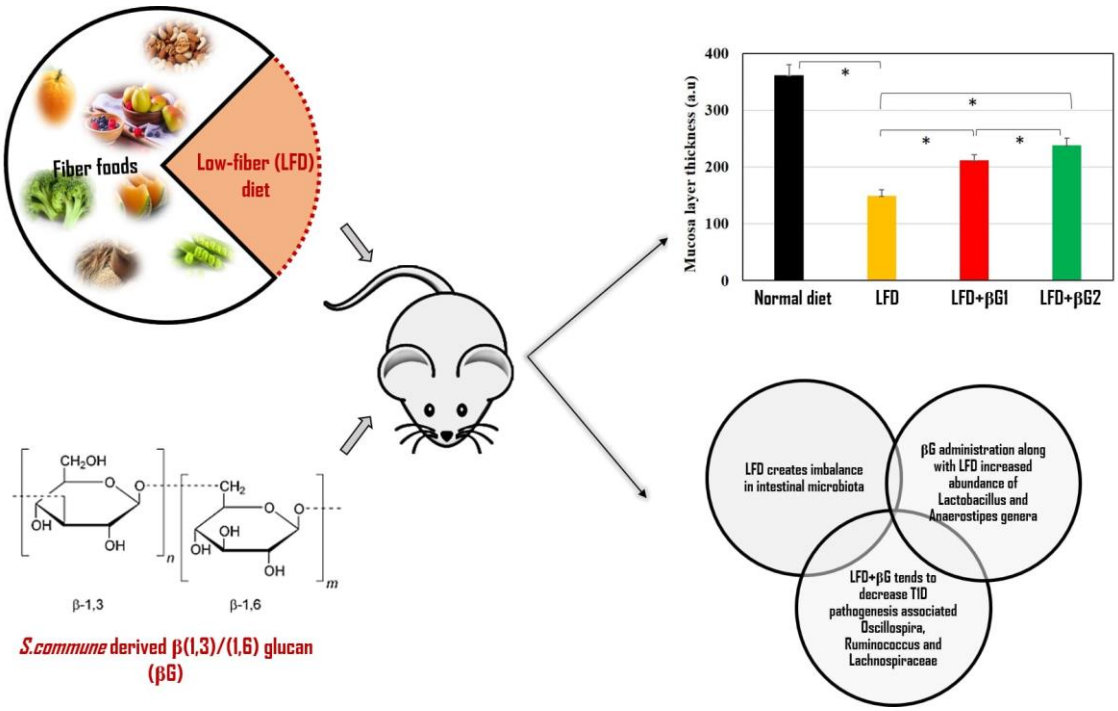


Figure 4.10: Graphical abstract representing the impact of *S.commune* derived β-(1,3/1,6)-glucan towards LFD induced gut- and gut microbiota- dysbiosis.

CHAPTER 5

Therapeutic impact of *S.commune* derived β-1,3/1,6-glucan on high-fiber diet induced metabolic distress

5.1 Abstract

Western diet, rich in carbohydrates and fat, is said to be a major factor underlying metabolic syndrome. Interventions with prebiotics, the key modulators of the gut microbiota, have paramount impact on host-associated metabolic disorders. Herein, we investigated the effect of fungus-derived (1,3)/(1,6)-β-glucan, a highly soluble dietary fiber, on high-fat diet (HFD)-induced metabolic distress. Male C57BL/6 J mice were fed with different diet groups (n = 11): control diet, HFD, 3 g/kg or 5 g/kg of β-glucan incorporated HFD. At the end of experimental study period (12th week), body weight, feces weight and fecal moisture content were observed. Further, colonic motility was measured using activated charcoal meal study. Proteins extracted from liver and intestine tissues were subjected to western blot technique. Paraffin-embedded intestinal tissues were sectioned for histochemical [Periodic acid-Schiff (PAS) and Alcian blue (AB) staining] analysis. Fecal microbiota analysis was performed using MOTHUR bioinformatic software. β-glucan consumption exhibited anti-obesity property in mice groups fed with HFD. In addition, β-glucan ameliorated HFD-induced hepatic stress, colonic motility and intestinal atrophy (reduction in colon length, goblet cells, and mucosal layer thickness). Further, β-glucan incorporation shifted bacterial community by increasing butyrate-producing bacteria such as *Anaerostipes*, *Coprobacillus*, and *Roseburia* and decreasing reportedly obesity-associated bacteria such as *Parabacteroides* and *Lactococcus.* Altogether, the outcomes of this present pre-clinical animal study show β-glucan to be a promising therapeutic candidate in the treatment of HFD-induced metabolic distress. Further comprehensive research has to be conducted to brace its clinical relevance, reproducibility and efficacy for aiding human health.

Keywords: High-fat diet; Gut microbiota; β-glucan; Prebiotics; Obesity; Metabolic syndrome

5.2 Introduction

Chronic overconsumption of diets high in fats and calories serves as a risk factor in the development of metabolic syndrome and its associated metabolic complications such as obesity, hypertension, hyper-cholesterol, and cardiovascular diseases[156,157]. Overweight/obesity, a progressive disorder, is getting much attention as a global pandemic owing to its detrimental effect on health and mortality[158]. A high body mass index (BMI) is considered the fourth leading cause of mortality, with no signs of decreasing. The Organization for Economic Co-operation and Development (OECD) projected a rapid increase in the obesity rate in the upcoming years in countries such as Korea and Switzerland, according to a report released in 2017[159]. The report also indicated that by the year 2030, a significantly high obesity rate of around 47%, 39%, and 35% will be observed in the USA, Mexico, and England, respectively. According to the World Health Organization (WHO), the level of obesity and overweight in children and adults of age 5–19 years has quadrupled over the last 40 years to 18% in 2016 (https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight). The WHO indicated that the imbalanced state between energy consumption and energy burning would further worsen obesity.

Decades-long research on microbiome revealed that gut microbial diversity and richness have influential roles in the onset of metabolic diseases associated with obesity, type II diabetes, atherosclerosis, and other related noncommunicable diseases[160,161]. Amidst several studies, Turnbaugh et al. [162] provided substantial evidence that showed correlation between microbiota and obesity, by transferring the microbial community from genetically obese mice to germ-free mice. It was determined that the obese mouse microbiome has a greater ability to harvest energy from diet materials than the lean mouse microbiome, which happens to be transmittable between animals. Therefore, it was marked that modulating the gut microbiota feasibly revamps microbiome perturbations and may aid in mitigation of its associated metabolic distress.

Dietary elements shape the gut microbiota and its corresponding microbiome[163,164]. As the driving fuel of fermentation, dietary carbohydrates such as fibers, polyphenols, and glycan compounds act as substrate for gut microbes along with endogenous mucous that has highly glycosylated mucin as its building blocks[165,166]. Upon fermentation, prebiotics (a group of non-digestible carbohydrates) releases energy that selectively stimulates the growth and/or activity of some health-beneficial microbes, thereby changing the microbiota compositions in the colon. Short-chain fatty acids (SCFAs), one of the several beneficial end products of colonic fermentation of such carbohydrates, exhibit numerous favorable roles in the host system such as maintenance of intestinal integrity and its associated organic processes; in the energy metabolism of heart, brain, muscles, and colonocytes, and providing protection against metabolic disease states such as obesity by influencing the production and regulation of leptin hormone[167]. Among the SCFAs produced, butyrate, propionate, and acetate are considered to be dominant with their roles as substrates in the metabolism of glucose, cholesterol and lipid[168]. Any dysbiosis in such gut microbiomes along with imbalanced microbiota instigates metabolic complications by modulating the immune system, altering resumptions in host gut epithelial cells, tempering digestive capacity, etc. Hence, harmonizing the gut microbiota with dietary interventions using prebiotics is one way to beneficially impact the high-fat-induced deregulated metabolic conditions[169].

The C57BL/6J mice tend to overconsume high-fat diet and, thus, exhibit drastic susceptibility toward becoming obese, starting from the early days of the diet[170,171]. In the due course of feeding them with high-fat diet, they develop features commonly linked with metabolic syndrome in human[172,173]. Herein, the long-term impact of fungus derived β-glucan, a soluble- and non-digestible fiber, on alleviating high-fat diet (HFD)-induced metabolic distress was analyzed using the C57BL/6 J animal model. Further, the effect of this dietary fiber on gut motility-associated clinical disorders was also studied in detail.

5.3 Materials and Methods

5.3.1 Animals and animal care

Five-week-old male C57BL/6 J mice were used as the animal model in this study. Animal handling and experimentation were ethically approved and performed as per the guidelines framed by the Animal Care and Use Committee (ACUC No.: 2018-0018) at Jeju National University. Mice were nursed initially for a period of 1 week in our laboratory animal room with 12/12 h of a light/dark cycle to acclimate to the experimental environment with perennial humidity of 55 ± 15% at temperature of 23 ± 2 °C. After 7 days of nursing, the mice, with average initial body weight of 21 g, were placed into 4 groups [control diet, HFD, and HFD with β-glucan at a concentration of 3 g/kg (HFD + BG1) or 5 g/kg (HFD + BG2)] with n = 11 per group. HFD + BG1 and HFD + BG2 were made by adding β-glucan as a direct additive to the high-fat diet ingredients, which is given in **Table 5.1**. Quegen Biotech Co. Ltd., (Republic of Korea) provided us with the purified and pharmacologically active (1,3)/(1,6)-β-glucan isolated from *Schizophyllum* spp. The mice were housed with ad libitum access to water and food. Mice bedding in the animal cage was changed every week so that fresh fecal samples could be collected for the calculation of fecal moisture content.

5.3.2 Physiological measurements

On the first day of every week, the body weights of the mice in all the groups were measured using a weighing balance and recorded. Further, the amount of water and food consumed the previous week was also calculated based on the amount filled and the amount remaining. 25 pieces of feces (excreted within 24 h after placing the mice in clean cage) were randomly collected per cage and their weights were measured using weighing balance with four decimal readability. Photographs of the feces were taken to observe the size/morphological variations as well as the

differences in color of the fecal samples. The experiment was carried out over a time period of 12 weeks. The percentage increase in body weight was calculated according to the formula:

$$Percentage\ increase\ in\ body\ weight\ (\%) = \left(\frac{Body\ weight\ in\ the\ n^{th}\ week - Initial\ body\ weight}{Initial\ body\ weight}\right) * 100$$

→ (Equation 5.1)

Table 5.1 Composition and energy value of different diet groups used in this study

Ingredient	AIN 93M Purified Control diet (Control diet)		AIN 93M Modified High Fat Diet (45% Kcal) (HFD)		AIN 93M Modified High Fat Diet (45% Kcal) with 3g/Kg of β-glucan (HFD+BG1)		AIN 93M Modified High Fat Diet (45% Kcal) with 5g/Kg of β-glucan (HFD+BG2)	
	gm	Kcal	gm	Kcal	gm	Kcal	gm	Kcal
Casein, lactic	140	560	140	560	140	560	140	560
L-Cystine	1.8	7.2	1.8	7.2	1.8	7.2	1.8	7.2
Corn Starch	465.692	1863	263	1052	263	1052	263	1052
Sucrose	100	400	100	400	100	400	100	400
Maltodextrin	155	620	155	620	155	620	155	620
Cellulose	50	0	50	0	50	0	50	0
Soybean Oil	40	360	43	387	43	387	43	387
Lard	-	-	200	1800	200	1800	200	1800
β-glucan	-	-	-	-	3	0	5	0
t-Butylhydroquinone	.008	0	.008	0	.008	0	.008	0
AIN-93M Mineral Mix	35	0	35	0	35	0	35	0
AIN-93 Vitamin Mix	10	40	10	40	10	40	10	40
Choline Bitartrate	2.5	0	2.5	0	2.5	0	2.5	0
Total	1000	3850.2	1000.308	4866.2	1003.308	4866.2	1005.308	4866.2

5.3.3 Fecal moisture content analysis

To calculate the moisture content of the feces, equal amounts (grams) of feces were removed from each cage and kept in a hot air oven (60 °C) for 48 h. At the end of 48 h, the weight of the feces was recorded. Fecal moisture content was calculated indirectly from the loss in fecal weight (which indicates the moisture content held by the feces) as follows:

$$Fecal\ moisture\ content\ (\%) = \left(\frac{Initial\ weight - weight\ at\ n^{th}\ hour}{Initial\ weight}\right) * 100$$

→ (Equation 5.2)

5.3.4 Feed efficiency calculation

Feed efficiency was calculated as follows:

$$Feed\ efficiency\ (\%) = (Increase\ in\ body\ weight\ (g)\ up\ to\ n^{th}\ week) / (Total\ food\ consumed(g)\ up\ to\ n^{th}\ week)$$

→ (Equation 5.3)

5.3.5 Gastrointestinal transit rate

To calculate the intestinal transit rate, activated charcoal (100 µl) was administered 20 min prior to mice killing (the mice were starved overnight). The length of the small intestine and the distance travelled by the charcoal in the small intestine were measured using a ruler, and the transit rate was calculated according to the following formula:

$$Intestinal\ transit\ rate\ (\%) = \left(\frac{Distance\ tavelled\ by\ the\ activated\ charcoal\ (cm)}{Length\ of\ the\ small\ intestine\ (cm)}\right) * 100$$

→ (Equation 5.4)

Further, organs such as the liver and lungs were separated from the killed mice and weighed individually. Moreover, the length of the small intestine was measured from the

duodenum up to the terminal ileum, whereas the colon length was measured from the cecum to the rectum using a ruler.

5.3.6 Protein immunoblotting

Equal amounts (grams) of liver and small intestine samples from all the three high-fat groups (HFD, HFD + BG1, and HFD + BG2) with $n = 4$ were weighed, and tissue lysates were collected by homogenizing the samples using an equi-volume of T-per buffer. The homogenized tissue was centrifuged, and the debris-free tissue lysate was collected by centrifugation for 20 min at 13,000 rpm, 4 °C. Protein separation was carried out using the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analytical method. Equal concentrations (30 µg) of protein were loaded into each well, and western blotting was performed. Transfer buffer was used to transfer the proteins separated on a weight basis onto nitrocellulose blotting membrane. Non-fat skimmed milk (5%) was used for blocking the unoccupied sites in the membrane, thereby reducing background interference. Primary antibodies used in this study were prepared in 1X Tween 20-Tris-buffered saline (TTBS) solution containing 5% skimmed milk powder, while secondary antibodies were prepared in 1X TTBS solution. Primary antibodies were purchased from Sigma-Aldrich [Claudin-1 (sc 166338;1:1000), Cyclin D1 (sc 246; 1:1000), Cyclin E (sc 247; 1:1000), Cytokeratin-14 (K14) (sc 17104; 1:4000), GAPDH (sc 25778; 1:2000), Klotho (sc 22218; 1:1000), Occludin (sc133256; 1:1000), p27 (sc 1641; 1:500), phospho P38/MAPK (sc 7973; 1:1000)], Cell Signalling Technology [E-cadherin (cs 3195; 1:500), phospho AKT (cs 9271; 1:500), p21(WAF1/CIP1) (cs 2947; 1:500), P38/MAPK (cs 9212; 1:1000), phospho AMPK-α (cs 2535; 1:1000)]. Secondary antibodies [rabbit (1:10000), mouse (1:5000), and goat (1:20000)] were bought from Koma Biotech, Seoul, Republic of Korea. Bands detected using chemiluminescent ECL kit were quantified using ImageJ software.

5.3.7 Blood profiling

Upon killing the mice at the end of the experimental study period, blood was drawn using cardiac puncture method followed by centrifuging it at 500 × g for 10 min. The separated serum samples were sent to ChemOn Inc. (Republic of Korea) for analysis of serum biomarkers associated with liver/kidney injury, body glucose level, total bilirubin content, etc. such as aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total bilirubin (TBIL), triglyceride (TG), blood glucose level (GLU), blood urea nitrogen (BUN) and creatinine (CRE). The above-mentioned parameters were measured using clinical biochemical analyzer (AU680, BECKMAN Coulter, Japan.

5.3.8 Histopathological analysis

Small intestine samples were sectioned and taken for histological analysis after periodic acid-Schiff (PAS) and Alcian blue staining. The stained sections were imaged using Olympus BX51 microscope at different magnifications. Thickness of the mucus layer (ten measurements per section per animal; four animals per group; thickness measurement comprising mucosa with submucosa layer identified from the stained sections of the samples) was quantified using ImageJ software.

5.3.9 Fecal microbiota analysis

Fecal microbiota analysis was conducted for feces collected at the 6th and the 12th week. Fecal bacterial DNA was extracted using the QIAamp, PowerFecal DNA kit (QIAGEN, Hilden, Germany) from feces according to the manufacturer's instructions. Total DNA obtained from mice feces was PCR amplified targeting the V4 region of 16S rRNA genes. The MiSeq library was prepared by 2-step PCR, and sequencing was performed by Macrogen (Seoul, Republic of Korea) according to the manufacturer's instruction (Illumina, CA, USA). MiSeq data were processed using MOTHUR[149]. Briefly, erroneous reads were trimmed based on quality scores and aligned

against the SILVA database[174], 2 bp mismatches were also corrected using pre.cluster MOTHUR subroutine, UCHIME[175] was used to remove chimeric sequences, and taxonomic classification was performed using the Greengene database [176] version 13.5.99. Sequences that were classified as eukaryote, cyanobacteria, and mitochondria were removed. Clustering was performed using Opticlust [177] to assign operational taxonomic units (OTUs). Bray–Curtis distance [178] was used to measure the dissimilarity between microbial communities. Non-metric multidimensional scaling (NMDS) was performed using the nmds MOTHUR subroutine. Ecological indices (Good's coverage, Shannon, and Chao) were obtained using the summary.single MOTHUR subroutine.

5.3.10 Quantification of the genus *Bifidobacterium* based on qPCR

Quantification PCR (qPCR) was carried out using TB green premix (Takara, Tokyo, Japan) according to the manufacturer's instructions. qPCR was performed with Bifidobacterium-specific primer set (F:5′- CGC GTC YGG TGT GAAAG -3′ and R:5′-CCC CAC ATC CAG CAT CCA -3′) [179] using a TB Green™ Premix Ex Taq™ (Takara, Japan). Amplification and detection of DNA were performed using Thermal cycler dice real time system (Takara, Japan) with the following conditions: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s.

5.3.11 Statistical analysis

Statistical significance was analyzed using *t* test in most of the experiments. Analysis of molecular variance (AMOVA) was applied to examine the significant difference between two groups of microbial communities based on the NMDS plots. Differentially abundant genera were identified using LEfSe [180], with the significance level set at $P < 0.05$. Significant difference for species richness and evenness was examined based on ANOVA and $p < 0.05$ was considered to be significantly different.

5.4 Results and Discussion

In this study, the long-term impact of fungus-derived dietary β-glucan on the detrimental effects imposed by HFD consumption was studied in detail. High dietary fat intake along with less physical exercise results in the development of metabolic syndrome, characterized by high body fat content, increased lipogenesis, distortion in the balance between pro and anti-inflammatory cytokines, and increased susceptibility to intestinal permeability[181]. Further, an HFD is found to induce gastrointestinal dysmotility as a result of intestinal dysbiosis through lipopolysaccharide-induced triggering of the TLR4 and SAPK/JNK signaling cascade [182,183].

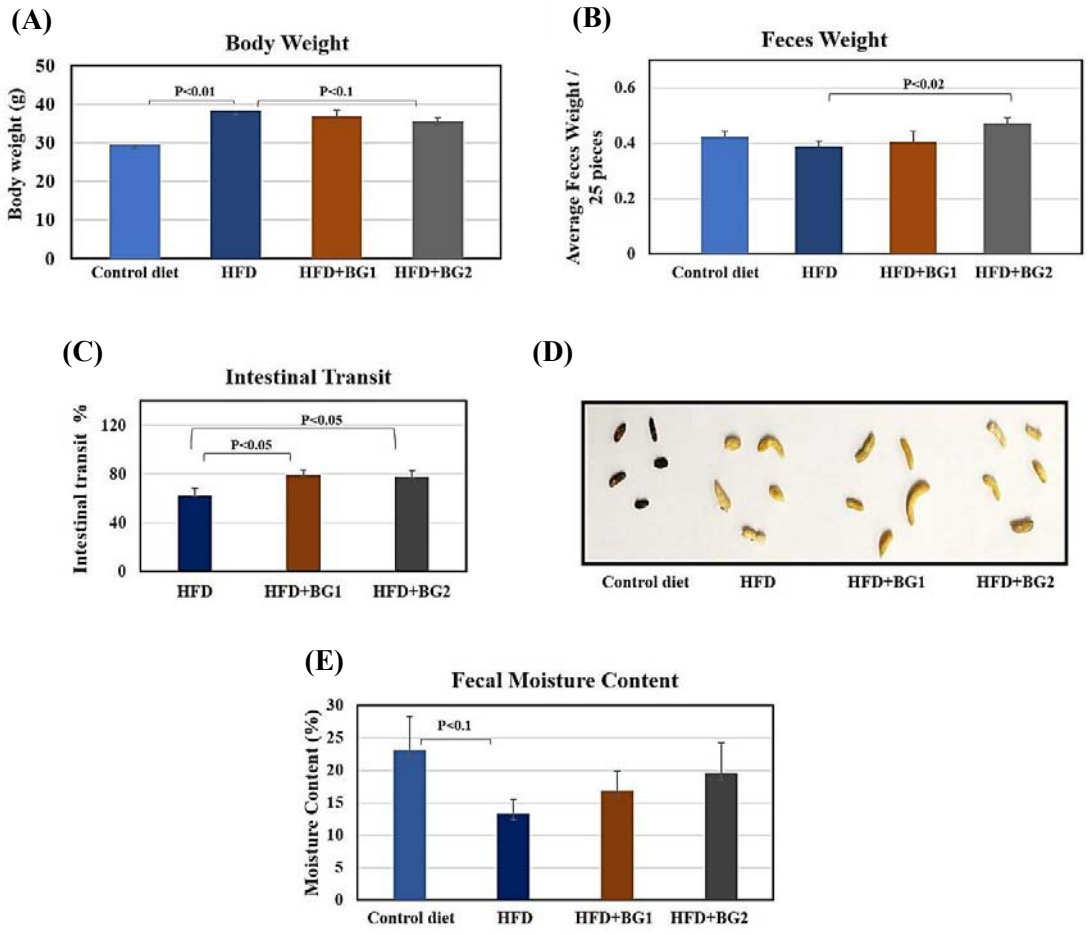


Figure 5.1: Effect of dietary β-glucan on HFD-induced obesity, colonic motility, and fecal outcome. At the end of 12 weeks, several parameters of the experimental mice (n = 11 per group)

was measured and compared between the different diet groups. **(A):** Body weight. **(B):** Feces weight. **(C):** Intestinal transit rate (Colonic motility was calculated by administering activated charcoal to the mice 20 minutes prior to sacrifice. By measuring the distance travelled by the administered charcoal and the total small intestine, the transit rate was calculated). **(D):** Morphology and color of the fecal samples. **(F):** Fecal moisture content (Fecal samples were collected and processed by keeping the collected feces in a hot air oven. By observing the change in weight of the sample, fecal moisture content was calculated).

At the end of the 12-week study period, wherein the mice were fed different diets (control diet or HFD with or without β-glucan at two different concentrations), there was a marked increase in body weight ($p < 0.01$) in the HFD group compared to that of the control diet group. However, inclusion of β-glucan along with the HFD helped decrease weight gain (p value < 0.1) (**Figure 5. 1 (A)**). With the increasing β-glucan concentration in the HFD group, the weight loss increased at the 12th week.

In addition, although not significant, feed efficiency (body weight gain/total food intake) was found to be higher in HFD than the control diet, while those with β-glucan incorporated (HFD + BG1 and HFD + BG2) diet showed lesser value than HFD but not control diet (**Figure 5. 2 (A)**), suggesting that β-glucan may play a prominent role in weight management. To investigate further, we examined the metabolic activities and food consumption level. The results shown in **Figure 5.2 (B)** suggest that HFD groups tended to have lower food consumption than the control diet group. In addition, there was a trend of increasing water consumption in the HFD + BG groups compared to that of the HFD group (**Figure 5. 2 (C)**).

Increased stool weight and thus enhanced intestinal transit rate were observed in the β-glucan incorporated HFD groups than the corresponding control groups (**Figure 5. 1 (B) and Figure 5. 1 (C))**, respectively). Increase in the weight of the stool (bulky stool) facilitates its

passage via colon to the outside of the body, thereby relieving problems such as constipation. Herein, the increased stool weight in the HFD + BG group compared to that of HFD group may be attributed to the ability of β-glucan in absorbing water, thereby adding bulk to the stool and thus tending to aid constipation-related sickness. Mice fed with control diet and HFD had brown and pale feces colors, respectively. However, increasing the concentration of β-glucan in the HFD group resulted in feces with more shades of brown color (**Figure 5. 1 (D)**). Pale stool could possibly be the result of alterations or dysregulations in the bile system, leading to malabsorption of fat in the intestine followed by intensified fat excretion[184]. Further, the stool collected from the mice fed the HFD was found to be relatively bigger in size than that in the control group. Loss in feces weight is directly proportional to the fecal moisture content. As shown in **Figure 5. 1 (E)**, it was found that β-glucan incorporated HFD diet groups had feces with greater moisture content than that in the HFD group.

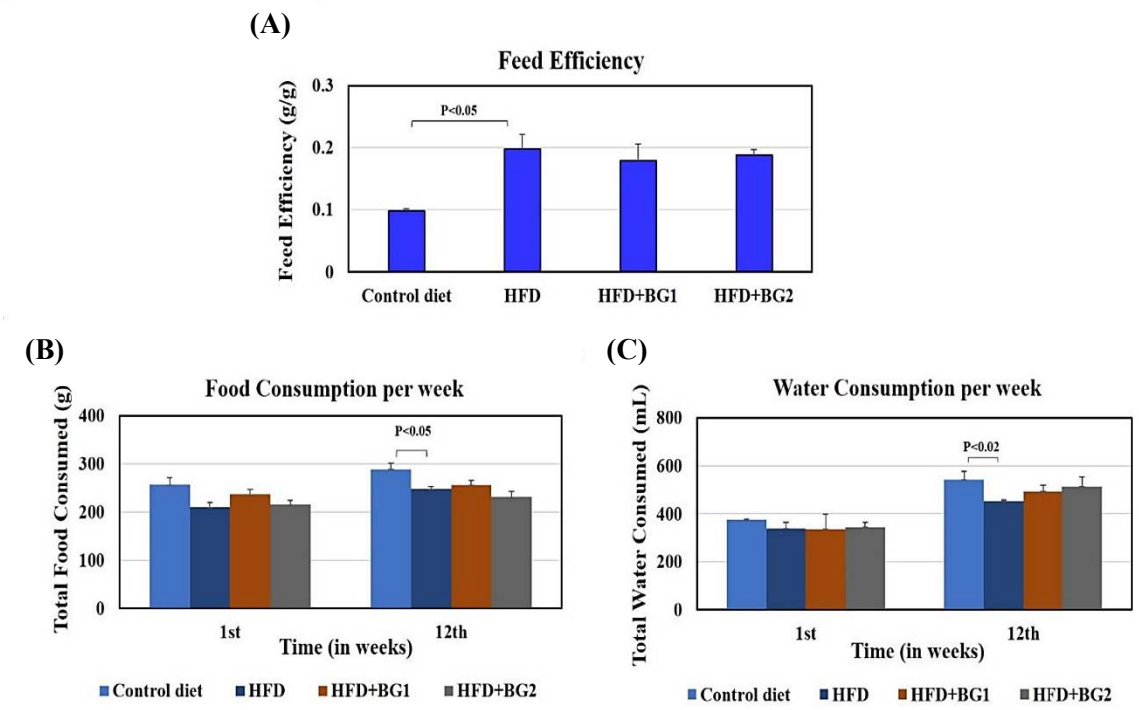


Figure 5.2: Effect of dietary β-glucan on energy intake. **(A):** Feed efficiency. **(B):** Food consumption. **(C):** Water consumption.

Changes in the organ size/mass are often related to histopathological status. Mice in the β-glucan-incorporated HFD groups had lengthier small intestine (up to 2.5 cm; *p* value < 0.05) and colon (up to 0.4 cm; *p* value < 0.05), compared with those in the HFD groups (**Figure 5. 3**).

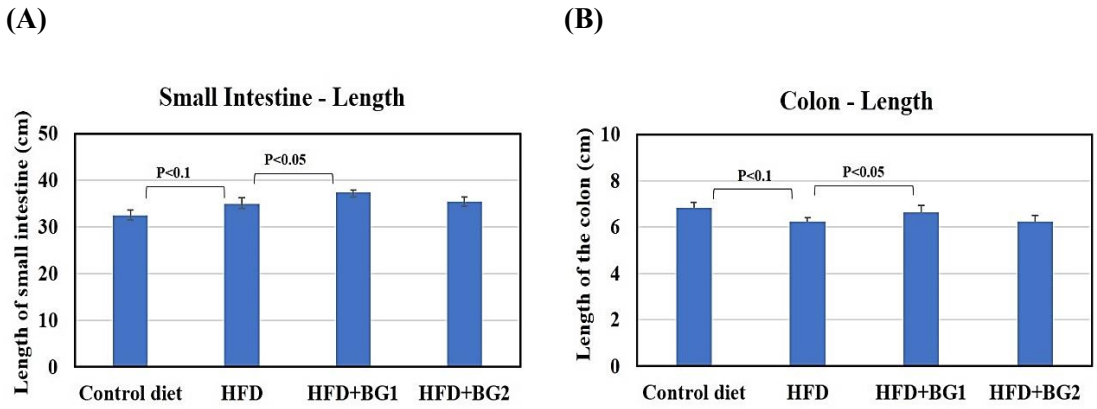


Figure 5.3: Effect of dietary β-glucan on gastrointestinal organs of mice. **(A):** Length of small intestine. **(B):** length of colon. Briefly, mice were sacrificed at the end of the experimental study period followed by organ isolation and weight measurement.

At the end of 12 weeks, mice were sacrificed using the cardiac puncture method, and blood was drawn using a sterilized syringe and needles. The collected blood samples were sent to ChemOn Inc. for the analysis of different biochemical markers as indicated in the **Table 5.2** (AST-aspartate transaminase, ALT-alanine transaminase, ALP- alkaline phosphatase, TBIL- total bilirubin, GLU- blood glucose level, TG-triglyceride, BUN- blood urea nitrogen and CRE-creatinine. (*-compared to that of control diet; $-compared to that of HFD)). Analysis of the blood biochemical markers indicated that the parameters associated with fatty/damaged liver or kidney together with increased risk of heart disease are high in HFD group when compared to control diet

group, as significant difference in ALT, ALP, TBIL, GLU, BUN and TG were observed. However, with inclusion of β-glucan into the HFD, the condition gets reversed to certain extent. ALT (whose level increases in blood when there is liver damage) and TG (elevated TG level increases the risk of heart disease, obesity, metabolic syndrome, etc.) were significantly lower in the HFD + BG groups when compared to the HFD group.

Table 5.2 Blood biochemical markers profiling

Test	Control diet	HFD	HFD+BG1	HFD+BG2
AST (U/L)	231.0 ± 46.3	298.1 ± 55.2	228.8 ± 36.3	236.3 ± 29.0
ALT (U/L)	37.7 ± 2.6	47.5 ± 3.3/*	46.4 ± 6.6	40.8 ± 2.5/$
ALP (U/L)	77.8 ± 2.2	46.2 ± 3.7/*	50.0 ± 2.3/*	35.4 ± 3.6/*/$
TBIL (mg/dL)	0.08 ± 0.04	(0.3) ± 0.04/*	(0.3) ± 0.05/*	(0.2) ± 0.1/*
GLU (mg/dL)	170.4 ± 6.1	139.25 ± 5.9/*	104.543 ± 9.6/*/$	87.9 ± 3.6/*/$
BUN (mg/dL)	39.0 ± 2.0	27.9 ± 1.8/*	24.1 ± 0.7/*/$	25.4 ± 0.8/*
CRE (mg/dL)	0.32 ± 0.004	0.31 ± 0.01	0.32 ± 0.01	0.34 ± 0.012/$
TG (mg/dL)	58.0 ± 1.58	94.0 ± 1.93/*	81.7 ± 5.33/*	77.14 ± 5.8/*/$

Hepatic expression of activated AMPK-α and AKT, the proteins involved in fatty acid oxidation[185] and glucose uptake[186], was found to be enhanced in mice treated with the β-glucan-incorporated HFD compared to that of HFD (**Figure 5. 4**). A significant reduced expression level of activated p38 mitogen-activated protein kinase (p38) to p38 ratio and higher expression of Klotho was found in liver samples of β-glucan-incorporated HFD (HFD + BG1 and HFD + BG2, respectively) groups compared to that of HFD diet group. A reduced expression level of activated p38 mitogen-activated protein kinase (p38), a cellular stress sensor, indicated that

there was a reduction in the stress ambiance in the liver. Further, higher expression of Klotho, an essential protein in bile acid homeostasis, in the β-glucan-incorporated diet group may indicate the synthesis of bile acid from cholesterol, thereby aiding in the abrogation of fatty liver formation.

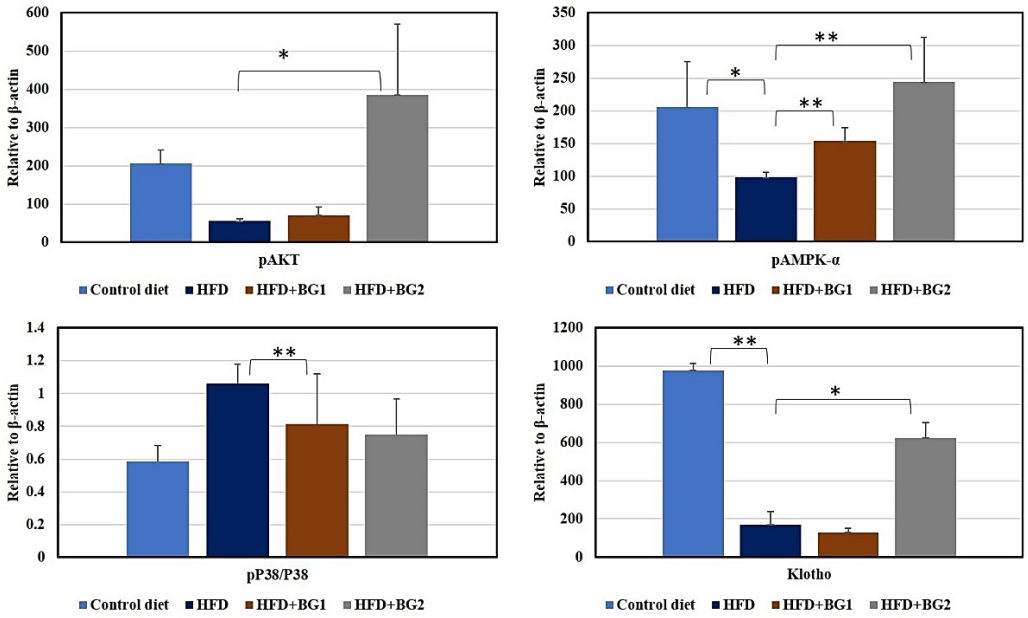


Figure 5.4: Quantitative analysis of hepatic proteins - involved in fatty acid oxidation, glucose uptake, and bile acid homeostasis. Proteins isolated from processed liver samples were subjected to western blot analysis, and quantitatively compared between control diet, HFD, HFD + BG1, and HFD + BG2 groups using ImageJ software.

The lower gastrointestinal tract, with anatomical division of small intestine and colon, is lined with continuously renewing intestinal epithelial cells (IECs), with the major function of sustaining a homeostatic relationship between the host system and gut microbiota[187]. This is regulated through two distinct mechanisms: segregation and mediation[188]. Intestinal mucosa (microvilli's glycocalyx) intact intracellular junctions form the basis for segregation of microbes and prevent them from invading the IECs, thereby preventing inflammation-related conditions in the intestine. Further, the signaling cascade between the microbes and host immune system often

results in the release of antimicrobial peptides, chemokines, and cytokines, which have roles in shaping the bacterial colonization. Consumption of an HFD is said to distort the effective functioning of the IECs in nutrient absorption, mucosal permeability, transport of solutes and ions, and protection against microbial invasion (thinning of mucus layer causes the detrimental effect of inflammatory bowel disease (IBD), as it leads to enhanced invasion of gut microorganisms into the mucus membrane resulting in bowel inflammation [189], thereby establishing an imbalance between the host system and gut microbiota[190,191].

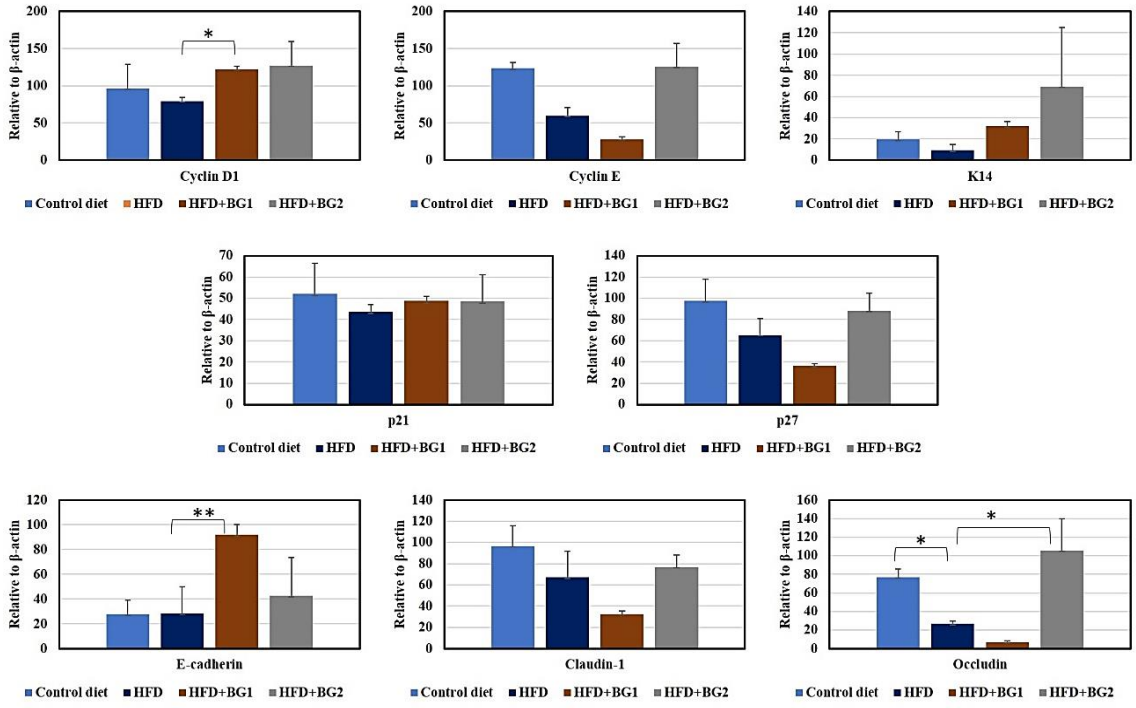
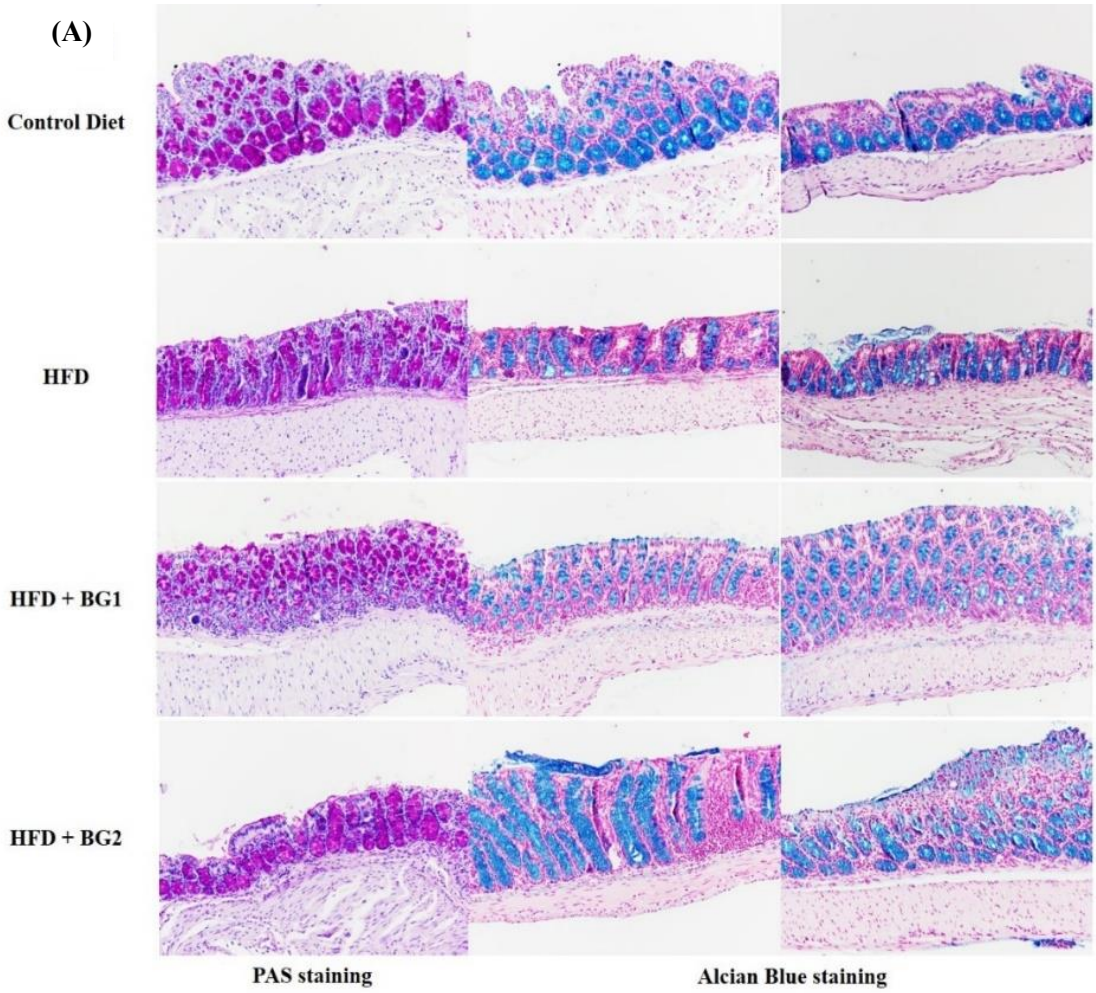


Figure 5.5: Assessment of expression of intestinal proteins involved in colon cell proliferation, differentiation, and tight junction markers in HFD, HFD + BG1, and HFD + BG2 groups.

We observed a trend of increased expression of proliferation indicators (Cyclin E, and K14) and differentiation indicators (p21 and p27) in the intestinal tissue of β-glucan-incorporated HFD groups (**Figure 5.5**) compared to that of the HFD group (although the results were not significant). Both p21 and p27 have been implicated as regulators of intestinal epithelial cell

differentiation. In this study, we observed increased expression of proliferation markers such as Cyclin D1, Cyclin E, and K14 and differentiation indicators such as p21 and p27 in the β-glucan-incorporated HFD groups compared to that of the HFD group. This likely indicates that the intestinal cells were in a state of proliferation and terminal differentiation, thus allowing for a better absorptive capacity of the intestine. Further, enhanced expression level of tight junction proteins (Occludin and Claudin-1) and adhesion junction proteins (E-cadherin) was observed in the HFD + BG groups compared to that of the HFD group, thereby indicating that β-glucan intake preserves epithelial linings of the small intestine to a better degree.



(B)

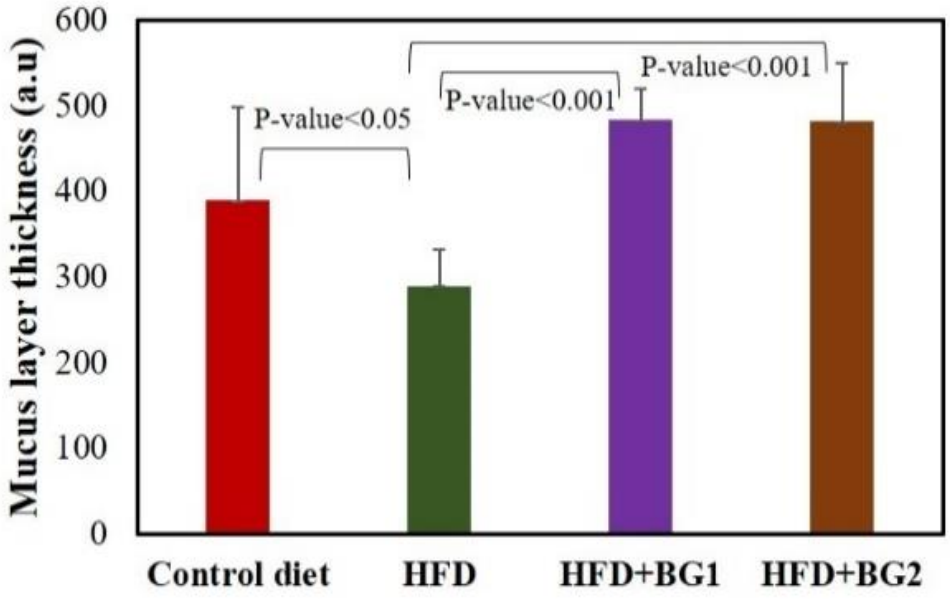


Figure 5.6: Histopathological analysis of the small intestine sample. **(A):** Sectioned intestine samples were processed with Alcian blue and the PAS staining method for analyzing the gut architecture relating to mucin production. **(B):** Graph showing differences in the mucosal layer thickness. For quantification of the membrane thickness, the sectioned intestine samples were imaged and processed using ImageJ software.

From the histological staining (PAS staining followed by Alcian Blue staining) of the isolated small intestine, we could observe a marked reduction in the intensity of goblet cells (which stains bright blue and magenta representing the acid and neutral mucin, respectively, secreted by goblet cells) in the HFD groups compared with that in the control diet group (**Figure 5.6 (A)**), which was reversed to normal by supplementation with β-glucan, suggesting that the mucin production (a primary function of goblet cells) was increased by β-glucan supplementation to the HFD. Further, HFD consumption reduced the mucus thickness, while HFD + BG groups

replenishes the mucous membrane, suggesting that β-glucan may play a key role in regulating gut permeability (**Figure 5.6 (B)**) in addition to its role in providing a lubricating intestinal mucin layer that aids in relieving HFD-induced constipation (supporting the enhanced intestinal transit rate described in **Figure 5.1 (C)**).

After the erroneous reads were removed, we normalized the number of reads per sample by randomly sampling 19,560 reads (the lowest number of reads per sample) from each sample. Results from the rarefaction curve analysis suggested that the sequence depth in this study was sufficient. Moreover, Good's coverage observed for each sample suggested that 19,560 reads per sample was enough to capture more than 99% of the total fecal microbial community. Samples at the 12th week showed lower species richness and evenness compared to those at the 6th week. In addition, the use of an HFD lowered species richness and evenness (**Figure 5.7**). The inclusion of β-glucan did not affect species richness, while some samples in the BG2 group showed variations in species evenness. The results shown in **Figure 5.8** suggest that feeding an HFD shifted the gut microbiota at the 6th week, while the effects of β-glucan only became clear at the 12th week. AMOVA, however, indicated a significant microbial community difference among samples with β-glucan from the 6th week ($P < 0.001$).

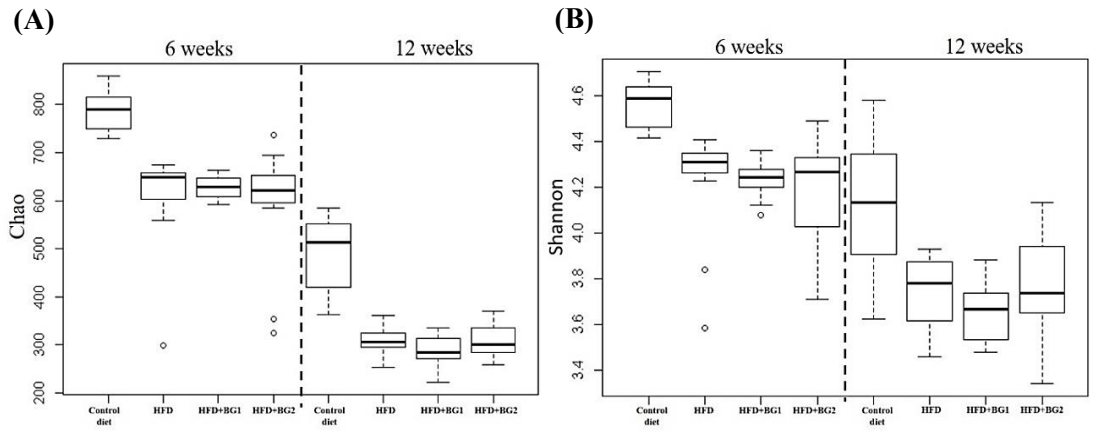


Figure 5.7: Effects of feeding duration and diets on **(A):** species richness and **(B):** evenness in the mouse gut microbiota.

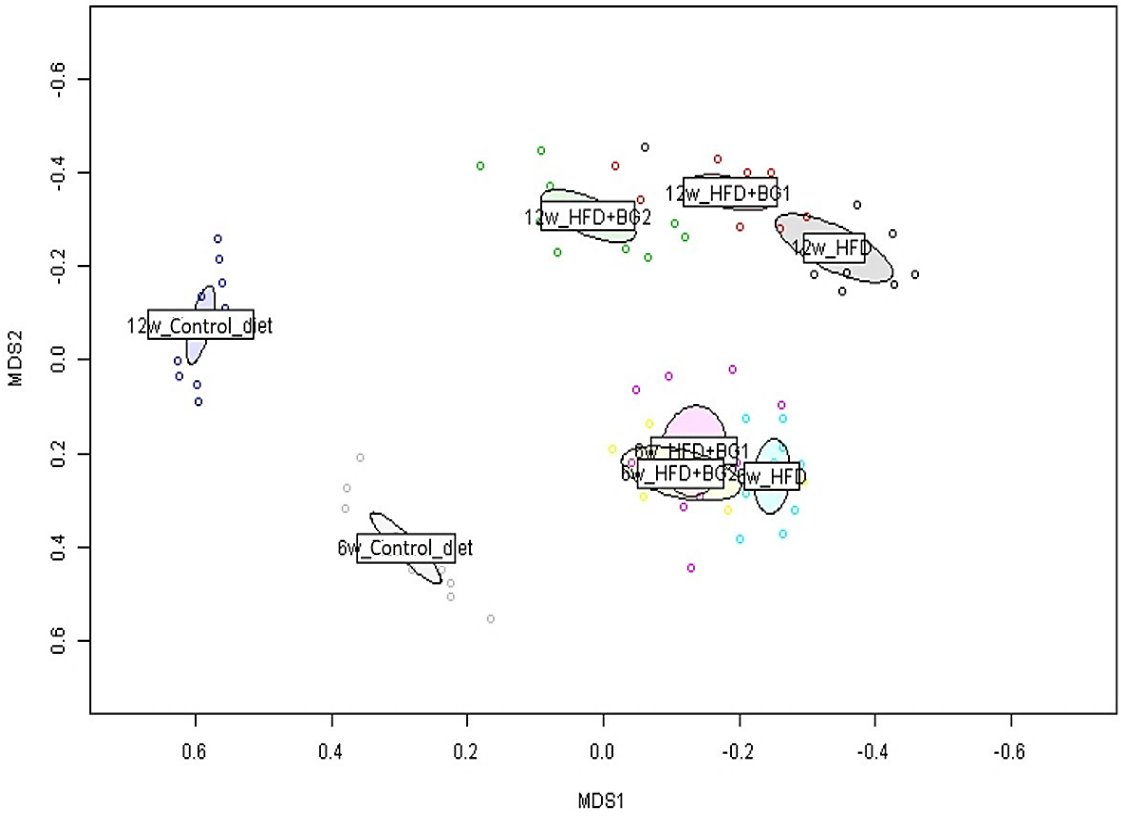


Figure 5.8: Effects of feeding duration and diet on mice gut microbiota analyzed with non-metric multidimensional scaling (NMDS) analysis. Total DNA extracted from mice fecal samples collected at the indicated times was PCR amplified targeting the V4 region of 16S rRNA, and the obtained sequencing data was subjected to NMDS analysis for the observation of bacterial community. Each dot represents the individual bacterial community composition.

Bacterial composition was analyzed using heatmap clustering. At the phylum level, mice fed an HFD showed relatively higher Firmicutes and lower Bacteroidetes (**Figure 5.9**), which are often used to indicate the obese microbiota[192]. There seemed to be no other effects at the phylum level, as samples did not cluster according to the feeding period or the dietary treatment. However, at the family level, there were clear differences shown among each dietary group and feeding period (**Figure 5.10**). The control diet groups had a higher abundance of the family S24-7 and fewer Erysipelotrichaceae compared to the HFD groups. Among the HFD groups, the families of

Prevotellaceae, Porphyromonadaceae, and Veillonellaceae were not observed at the 12th week, while levels of Bacteroidaceae and S24-7 were increased. Inclusion of β-glucan did not seem to affect the bacterial composition at the family level.

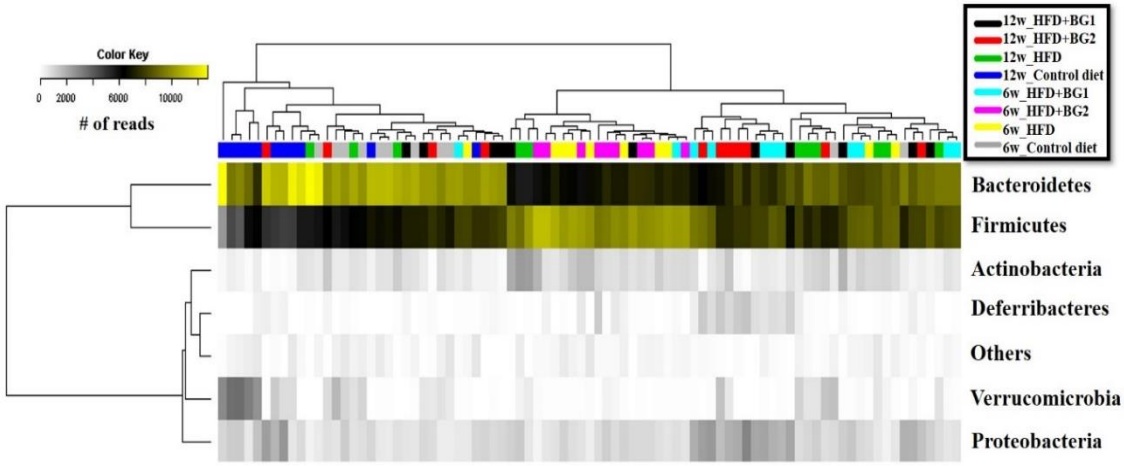


Figure 5.9: Bacterial composition analysis at the phylum level.

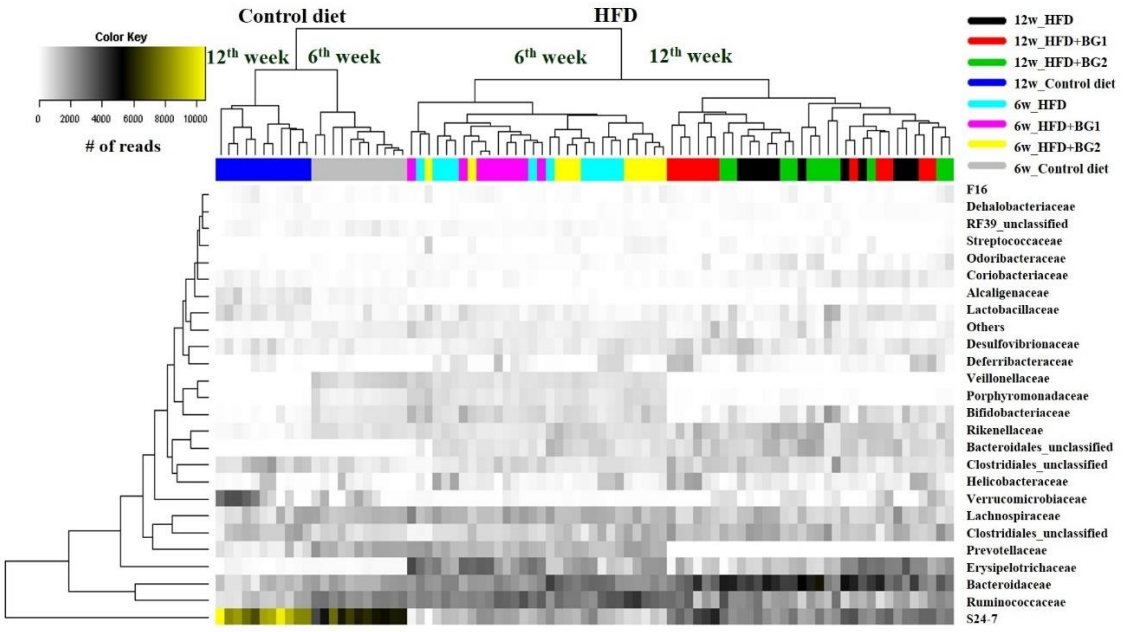


Figure 5.10: Bacterial composition analysis. Total DNA extracted from mice fecal samples collected at the indicated times was PCR amplified targeting the V4 region of 16S rRNA and the

obtained sequencing data was subjected to heatmap clustering for analyzing the bacterial composition at the family level.

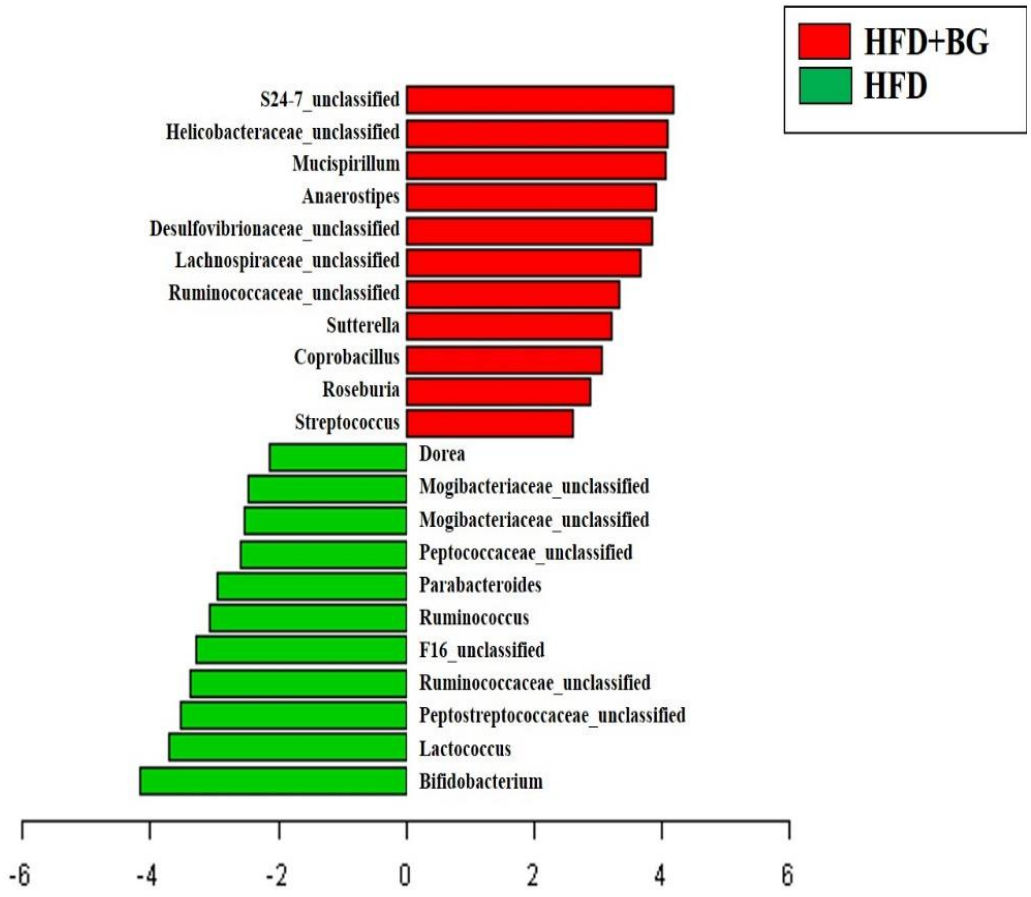


Figure 5.11: Differentially abundant genera at the 12th week in control and β-glucan groups.

To investigate whether β-glucan increases or decreases bacteria of a specific genus, we conducted the differential abundance test at the genus level using LEfSe analysis at the 12th week for the high-fat diet groups. LEfSe detected 12 and 11 genera that were significantly increased and decreased by the addition of β-glucan, respectively (**Figure 5.11)**.

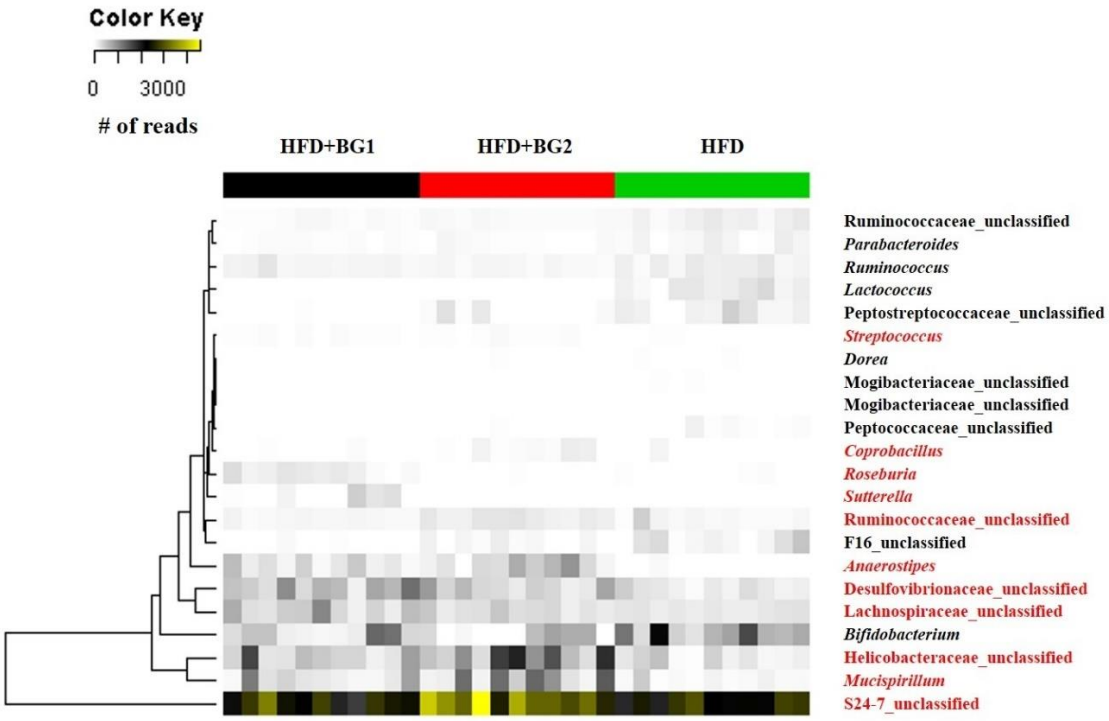


Figure 5.12: β-glucan uptake significantly affected the bacterial genera. Read abundance of differentially abundant genera at the 12th week investigated using LEfSe analysis. Genera shown in red and black indicate a significant increase and decrease, respectively, due to the addition of β-glucan.

The results presented in **Figure 5.12** show that the average relative abundance of the family S24-7_unclassified was approximately 14%, whereas the other genera ranged from less than 0.1% to 3.0%. Although little information is available regarding this species, Ormerod et al. [193] conducted a bioinformatic investigation and suggested that these bacteria may be capable of degrading a variety of glycans including glucan and are also involved in host–microbe interactions that impact gut function and health. Butyrate produced in the colon is known to provide an energy source for epithelial cells, enhance intestinal integrity, and increase mucin production. In this study, we observed that the addition of β-glucan significantly increased the butyrate-producing bacteria such as *Anaerostipes*, *Coprobacillus*, and *Roseburia*. It has been reported that *Coprobacillus* is

capable of fermenting glucose and producing lactate[194], while *Anaerostipes* and *Roseburia* utilize lactate and produce butyrate[152,195], suggesting that these bacteria may cross feed to maintain butyrate in the colon. It has been reported that these butyrate producers often cross feed with *Bifidobacterium* [196], which was found to be decreased by the addition of β-glucan. Although the use of diverse β-glucan increases the abundance of *Bifidobacterium* in vitro [197], samples supplied with β-glucan showed lower abundance of *Bifidobacterium* in this study. Although the *Bifidobacterium* species is a well-known probiotic and is used as an indicator of a healthy gut microbiota, previous studies have reported that the quantification of this bacteria may not be accurate, depending on the choice of 16S rRNA gene variable regions used for identification [198–201]. To complement this, we conducted qPCR to quantify the *Bifidobacterium*. The results shown in **Figure 5.13** indicate that inclusion of β-glucan did not affect the abundance of *Bifidobacterium* in this study. Another significantly increased bacteria, *Mucispirillum*, is a mouse-specific bacterium that feeds on mucin [202]and the increase in this bacterium may suggest that the mucin availability in the colon was increased by β-glucan. *Mucispirillum* is known to cause inflammation; however, we did not observe inflammation, suggesting enhanced intestinal integrity due to the mucin increase. In addition, we observed that β-glucan decreased reportedly obesity-associated bacteria such as *Parabacteroides* [203] and *Lactococcus* [204], suggesting that long-term supplementation with β-glucan may have ant obesity effects.

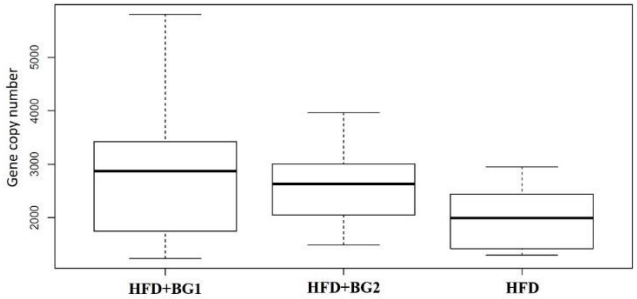


Figure 5.13: Enumeration of Bifidobacterium based on qPCR for HFD group samples at the 12th week.

5.5 Conclusions

In summary, β-glucan, a soluble and highly viscous fiber, was found to exhibit anti-obesity property, in the animal model. Further, mice fed with β-glucan-incorporated HFD were relieved from HFD-induced intestinal motility disorder with increased colonic transit time, owing to its fecal bulk-forming and intestinal lubricative properties. With continuous turnover in the absorptive intestinal cells along with regulation of tight and adherent junctions, the inclusion of β-glucan in the HFD helps in the restoration of an HFD-induced compromised intestinal barrier in the C57BL/6 J mice model. In addition, stress imposed on the liver while consuming HFD was reduced by β-glucan intake. Altogether, the outcomes of this present pre-clinical animal study show β-glucan to be a promising therapeutic candidate in the treatment of HFD-induced metabolic distress. However, comprehensive research has to be conducted to brace its clinical relevance, reproducibility and efficacy for aiding human health.

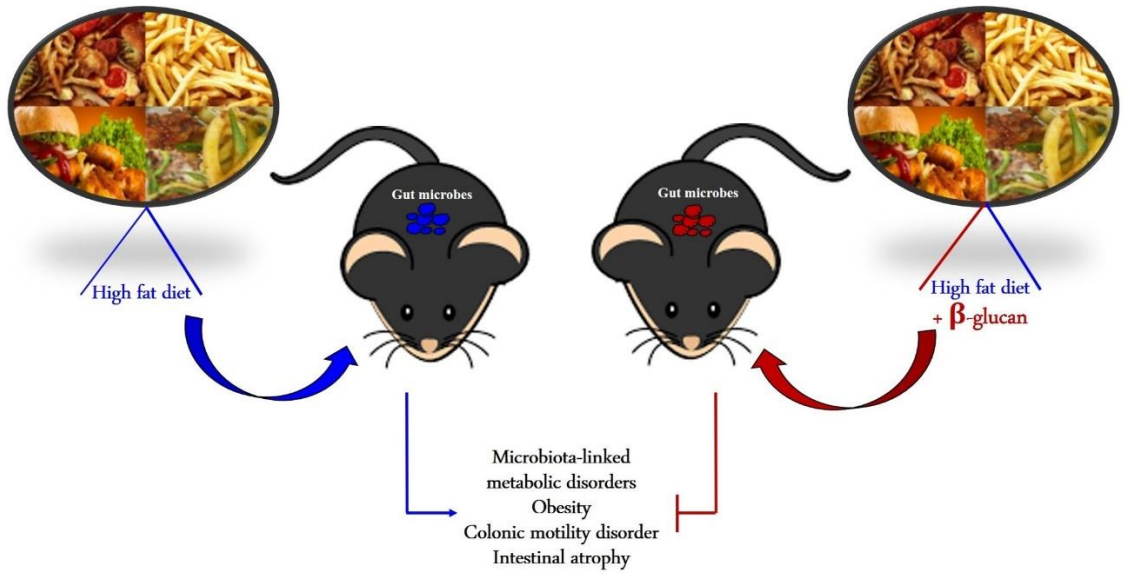


Figure 5.14: Graphical abstract representing the impact of *S.commune* derived β-(1,3/1,6)-glucan towards HFD induced metabolic distress.

SUMMARY OF THE THESIS

Studies conducted on in-vitro and in-vivo animal model showed that *Schizophyllum commune* derived β-1,3/1,6 glucan-based hydrogel enhanced wound healing, re-epithelialization with proper skin appendages and imposed anti-scarring effect. Further, high fat and low-fiber diet induced metabolic distress and impaired gut microbiota in in-vivo animal models were rescued through long-term (12-weeks) dietary intervention containing *S.commune* derived β-1,3/1,6 glucan. LFD induced gut dysbiosis and disruption in healthy gut microbiota were improved with the inclusion of *S.commune* derived β-1,3/1,6 glucan. HFD induced- obesity, intestinal motility disorder, compromised intestinal barrier, hepatic stress were relieved when dietary intervention using *S.commune* derived β-glucan was administered.

Overall, the scientific outcomes from my doctoral dissertation showed that *S.commune* derived β-1,3/1,6 glucan has promising therapeutic impact towards the management of wound care and metabolic dysbiosis. With further studies on this *S.commune* derived immunomodulating polysaccharide - β-1,3/1,6 glucan, there is high possibility for exploring its hidden clinical impact towards medical conditions such as NAFLD, NASH, organ-specific fibrosis etc.

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Karthika Muthuramalingam

APPENDIX A: List of publications

- **Karthika Muthuramalingam**, Sanggyu Park and Moonjae Cho (2018) **Synthesis and optimization of immunomodulating hydrogel for biomedical application**. Journal of Applied Biological Chemistry. 61(4):351-355 (Chapter 2)
- **Muthuramalingam Karthika**, Choi Seung In, Hyun Changlim, Kim Young Mee and Cho Moonjae (2019) **β-Glucan-Based wet dressing for cutaneous wound healing**. Advances in Wound Care. 8(4):125-135 *(Q1 journal)* (Chapter 3)
- **Karthika Muthuramalingam**, Vineet Singh, Changmin Choi, Seung In Choi, Sanggyu Park, Young Mee Kim, Tatsuya Unno and Moonjae Cho (2019) "**Effect of mushroom (*Schizophyllum* spp.) derived β-glucan on low-fiber diet induced gut dysbiosis**" Journal of Applied Biological Chemistry. 62(2):211-217 (Chapter 4)
- **Karthika Muthuramalingam**, Vineet Singh, Changmin Choi, Seung In Choi, Youngmee Kim, Tatsuya Unno and Moonjae Cho (2019) "**Dietary intervention using (1,3)/(1,6)-β-glucan, a fungus-derived soluble prebiotic ameliorates high-fat diet-induced metabolic distress and alters beneficially the gut microbiota in mice model**" European Journal of Nutrition. doi: 10.1007/s00394-019-02110-5 *(Q1 journal)* (Chapter 5)

APPENDIX B: Conference presentations

- Presented **poster** on "Beta glucan based wet dressing for wound care management" at 24th IUBMB Congress & 15th FAOBMB Congress, Seoul, Republic of Korea on June 4th – 8th, 2018.
- Presented **poster** on "Immunomodulating wet dressing for wound healing application" at 2018 International Symposium and Annual Meeting of the KSABC, Republic of Korea on June 18th – 20th, 2018
- Presented **poster** on "Prebiotics in the management of high fat diet induced metabolic syndrome" at KSBMB International Conference, Republic of Korea on June 2 ~ 5th, 2019.
- Abstracted in **poster** on "Prebiotics mitigate high-fat diet induced metabolic disorders" at International Scientific Conference Probiotics, Prebiotics, Gut Microbiota and Health, Prague Congress Centre, Prague, Czech Republic on June 17~20th, 2019.
- **Oral** presentation on "Mushroom derived β-glucan ameliorates high fat diet induced metabolic syndrome in mice" at 2019 International Symposium and Annual Meeting of the KSABC, Republic of Korea on June 20th – 22nd, 2019
- Abstracted in **poster** on "Long term impact of mushroom derived β-glucan on obesity and gut microflora in mice fed with high fat diet" at ACS Fall 2019 National Meeting & Exposition, San Diego, CA, United States on August 25~29th, 2019

DECLARATION

I, **Karthika Muthuramalingam**, as a graduate student of Jeju National University, hereby declare that I have not committed any acts that may damage the credibility of my research. These include, but are not limited to: falsification, thesis written by someone else, distortion of research findings or plagiarism. I affirm that my thesis entitled "**Therapeutic impact of *Schizophyllum commune* derived β-(1,3/1,6)-glucan on cutaneous wounds and metabolic dysbiosis**" submitted to Jeju National University, in the partial fulfillment of the requirements for the award of the degree of **Doctor of Philosophy** under **Faculty of Medicine** is a record of original research work done and published by me during the period **March 2017 to August 2020** under the supervision and guidance of my thesis advisor **Prof. Moonjae Cho**.

Karthika Muthuramalingam