Pilot study of a mechanistic study focused on the immune function in SARS-CoV-2 of a barley-based diet in mice

TREBALL DE FINAL DE GRAU DE VETERINÀRIA

Student: Ariadna Pey La Haba

Tutor: Carme Piñol Felis (Dpt. De Medicina)

Doble Grau en Veterinària i Ciència i Producció Animal – ETSEA, Universitat de Lleida

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ABSTRACT

ß-glucans are polysaccharides of D-glucose monomers that come from multiple living beings and are known to have multiple beneficial properties such as being immunostimulant, having antitumoral activity and being antioxidant. This *Treball Final de Grau* aims to carry out a pilot study with the two control groups regarding a R+D project titled "*Innovative Barley-Based Functional Foods"* by University of Lleida. The pilot study is done to provide useful information concerning improvements for the large-scale investigation and to try out some of the procedures suggested in the R+D project. For this purpose, 30 Balb/cB&J mice from Charles River Laboratories© (Barcelona, Spain) were randomly distributed into groups of five in a way that we ended up having six groups, three of each sex. Four groups were fed a standard diet supplemented with rice (RC), whereas two groups were fed a maintenance standard chow diet (SC). Every two days for six weeks, animals were weighed and exposed to a health evaluation, as well as their feed intake was monitored. After this period, mice were euthanized and blood, thymus, spleen, intestines, liver, and brain samples were collected for further analysis. Results showed there were no differences between diets regarding the thymus and splenic indexes, as well as the number of Peyer's patches. The flow cytometry analysis and the histological analysis done on the spleen and the Peyer's patches, respectively, did not show any conclusive results. Nevertheless, it has been useful as a pilot study to detect deficits in the design, establishment of animals and obtaining and processing samples.

Key words: 1) Barley; 2) ß-glucans; 3) Pilot study; 4) Mouse model; 5) Immune modulation.

RESUM

Els ß-glucans són polisacàrids de monòmers de D-glucosa que provenen de múltiples éssers vius i se sap que tenen múltiples propietats beneficioses com ser immunoestimulants, tenir activitat antitumoral i ser antioxidants. Aquest *Treball Final de Grau* pretén dur a terme un estudi pilot amb els dos grups control sobre un projecte d'I+D titulat "*Aliments funcionals innovadors a base d'ordi"* de la Universitat de Lleida. L'estudi pilot es fa per proporcionar informació útil sobre millores per a la investigació a gran escala i per provar alguns dels procediments suggerits en el projecte d'I+D. Amb aquesta finalitat, 30 ratolins Balb/cB&J de Charles River Laboratories© (Barcelona, Espanya) es van distribuir aleatòriament en grups de cinc de manera que vam acabar tenint sis grups, tres de cada sexe. Quatre grups van ser alimentats amb una dieta estàndard complementada amb arròs (RC), mentre que dos grups van ser alimentats amb una dieta estàndard de manteniment (SC). Cada dos dies durant sis setmanes, es van pesar els animals i es van exposar a una avaluació de la salut, així com es va controlar la ingesta d'aliments. Després d'aquest període, els ratolins van ser sacrificats i es van recollir mostres de sang, tim, melsa, intestins, fetge i cervell per a una anàlisi posterior. Els resultats van mostrar que no hi havia diferències entre les dietes pel que fa als índexs de tim i esplènic, així com al nombre de plaques de Peyer. L'anàlisi de citometria de flow i l'anàlisi histològica realitzades a la melsa i les plaques de Peyer, respectivament, no van mostrar cap resultat concloent. No obstant això, ha estat útil com a estudi pilot per detectar dèficits en el disseny, estabulació dels animals i obtenció i processament de mostres.

Paraules clau: 1) Ordi; 2) ß-glucans; 3) Estudi pilot; 4) Model de ratolí; 5) Modulació immunològica.

RESUMEN

Los ß-glucanos son polisacáridos de monómeros de D-glucosa que provienen de múltiples seres vivos y se sabe que tienen múltiples propiedades beneficiosas como ser inmunoestimulantes, tener actividad antitumoral y ser antioxidantes. Este *Treball Final de Grau* tiene como objetivo realizar un estudio piloto con los dos grupos control sobre un proyecto de I+D titulado "*Alimentos Funcionales Innovadores a Base de Cebada*" de la Universidad de Lleida. El estudio piloto se realiza para proporcionar información útil sobre mejoras para la investigación a gran escala y para probar algunos de los procedimientos sugeridos en el proyecto de I+D. Para ello, 30 ratones Balb/cB&J de Charles River Laboratories© (Barcelona, España) se distribuyeron aleatoriamente en grupos de cinco de forma que al final obtuvimos seis grupos, tres de cada sexo. Cuatro grupos fueron alimentados con una dieta estándar suplementada con arroz (RC), mientras que dos grupos G2M y G2F se alimentaron con una dieta estándar de mantenimiento (SC). Cada dos días durante seis semanas, los animales fueron pesados y expuestos a una evaluación de salud, así como también se monitoreó su consumo de alimento. Después de este período, los ratones fueron sacrificados y se recolectaron muestras de sangre, timo, bazo, intestinos, hígado y cerebro para su posterior análisis. Los resultados mostraron que no hubo diferencias entre las dietas con respecto a los índices de timo y bazo, así como el número de placas de Peyer. El análisis de citometría de flujo y el análisis histológico realizado en el bazo y las placas de Peyer, respectivamente, no mostraron ningún resultado concluyente. No obstante, a esto, ha sido útil como estudio piloto para detectar déficits en el diseño, estabulación de los animales, y obtención y procesamiento de las muestras.

Palabras clave: 1) Cebada; 2) ß-glucanos; 3) Estudio piloto; 4) modelo de ratón; 5) Modulación inmunológica.

1. CONTEXT

A R+D project titled "*Innovative Barley-Based Functional Foods"* has been proposed with the following general objectives: (1) To produce innovative barley varieties rich in bioactive compounds (fiber and polyphenols) adapted to Spanish growth conditions; (2) To develop health promoting barley-based ingredients and food products, and (3) To evaluate the potential health effects of barley-based food in animal and human models. Part of this project takes place in the animal facilities at the University of Lleida which allows for work on the mechanistic study focused on the immune function when given a barley-based diet to mice. This part of the project intends to study the role that ß-glucans from barley could play in the activation of cytokines and other inflammation markers with immunomodulatory effects such as Lipopolysaccharide-binding protein (LBP), high-sensitive C-reactive protein and the proliferation of CD4+ and CD8+ Lymphocytes T in the spleen.

For this part of the experiment, 60 Balb/cB&J mice (30 males and 30 females) from Charles River Laboratories© need to be kept in the animal facilities at the University of Lleida. The mice are randomly distributed into five different groups in such a way that we end up with 12 animals in each group: 6 males and 6 females in each. Every group is set a different diet during the entire six-week duration that the experiment lasts. Those are the following: standard chow diet (SD) will be fed to Group nº1, control diet supplemented with whole-grain barley (WBG) will be fed to Group nº2, control diet supplemented with pearled grain (PG) will be fed to Group nº3, control diet supplemented with barley bran (BB) will be fed to Group nº4, and control diet supplemented with white rice (RC) will be fed to Group nº5. Both Group nº2 and Group nº3 will receive 50 mg/kg/day of ß-glucan, and Group nº4 the same amount but of phenol.

Nonetheless, before performing the large-scale investigation, a pilot study was proposed for various reasons. Firstly, to detect any possible issues that could affect the project in a negative way. Because of this method, we could make the necessary adjustments to mitigate or avoid any hypothetical problem detected before starting the large-scale experiment. In addition, it is important to examine the feasibility of this task of the project to avoid wasting unnecessary resources and time. Finally, a pilot study helps the practice of some important techniques that will take place during the research study. The results obtained in the pilot study will be used to guide the methodology of the R+D project.

In conclusion, the aim of this *Treball Final de Grau* is to design a pilot study for a mechanistic study focused on the immune system function when a specific diet is given to mice.

2. INTRODUCTION

ß-glucans are polysaccharides of D-glucose monomers that come from multiple living beings such as bacteria, yeast, mushrooms, algae, and some plants. The main plants from which ß-glucans can be isolated from, are barley and oats (Jayachandran et al., 2018; Lei et al., 2015). Barley is becoming one interesting ingredient to produce healthy food, due to its nutritional value and its bioactive compounds. Many studies claim that mainly because of the presence of ß-glucans in barley, positive effects in the glycemic index, cholesterol and heart diseases can be achieved. More recent investigations state that an enhancement of the innate and adaptive immune system, thanks to the interaction between ß-glucans and activated macrophages, has been observed (Chan et al., 2009). These macrophages trigger numerous reactions that occur in the modulation of the immune and inflammatory responses. Other cells like neutrophils and dendritic cells that play a key role in the innate immune system also have several receptors capable of identifying ß-glucans (Goodridge et al., 2009).

2.1. TYPES OF ß-GLUCANS

ß-glucans have different sizes and characteristics depending on the organism it is extracted from and the method used for its extraction and purification (Nakashima et al., 2018). ß-glucans found in plants, such as barley, include linear ß-1,3 and ß-1,4 but not ß-1,6 glycosidic bonds like ß-glucans extracted from fungi or some types of yeast have. Other ß-glucans-containing organisms have a lower quantity of ß-1,4 glycosidic bonds.

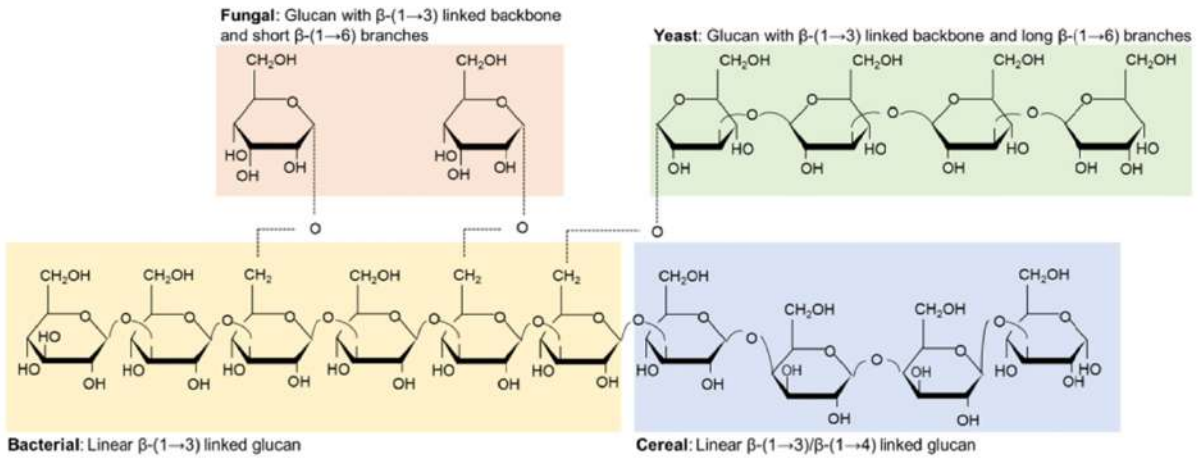

Figure 1. Representation of the chemical structure of different types of ß-glucans extracted from a fungi, yeast, bacteria, and cereal. [Source: de Graaf et al., 2018].

Because of the different molecular structures in each type of ß-glucan (*Figure 1),* other characteristics are attributed, such as solubility, the number of side chains size and the composition of the glucan backbone. These traits will directly translate into diverse physiological functions in the human body. However, there are some physiological activities that are common for all types of ß-glucans (Nakashima et al., 2018). Firstly, because of ß-glucans' viscosity, they

act as dietary fiber that helps eliminate unbeneficial contents from the gut. Secondly, ß-glucans are considered antioxidants because of their ability to scavenge reactive oxygen species (ROS). Lastly, pro- and anti-inflammatory properties have been attributed to all kinds of ß-glucans (Goodridge et al., 2009).

2.2. ß-GLUCANS' RECEPTORS

Barley's ß-glucans are soluble, which means that can pass through the epithelial lumen using passive or active transport mediated by the Peyer's patches' M cells (*Figure 2)*. When ß-glucans are absorbed by the intestinal gut, they can bind with their receptors which are expressed on macrophages and dendritic cells. The main receptors on immunological cells for ß-glucans are Dectin-1 and Complement Receptor 3 (CR3) (Mumby et al., 2001). On the one hand, Dectin-1 has a strong affinity to bind with the ß-1,3 glycosidic bond although it has been confirmed that can also bind with less strength to a ß-1,4 glycosidic bond. On the other hand, CR3 responds to the union of small soluble ß-glucans and to the insoluble ß-glucan molecules but, with a minor response (Chan et al., 2009).

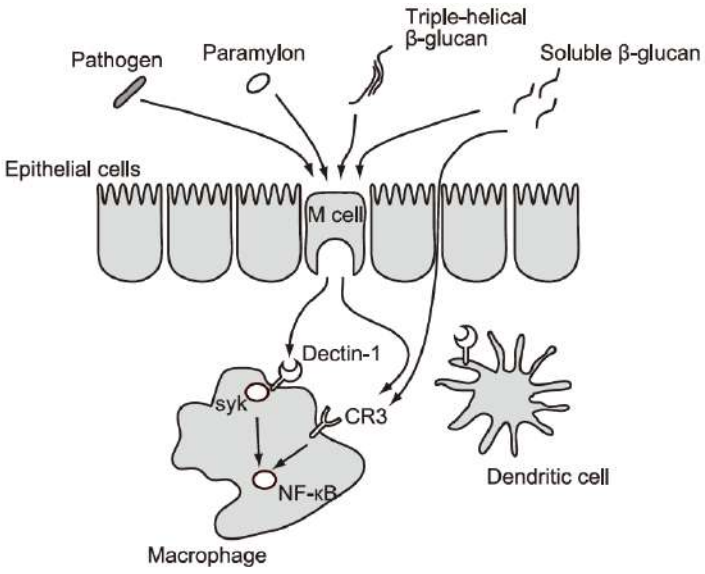

Figure 2. *Representation of the mechanisms of absorption of ß-glucans by the intestinal gut epithelial lumen and how then act on receptors expressed on macrophages and dendritic cells. [Source: Nakashima et al., 2018].*

When the orally administered ß-glucans are absorbed by the intestinal gut epithelia, the ß-1,3 glycosidic bond binds to the Dectin-1 receptor expressed by macrophages, and less frequently by dendritic cells and neutrophils. Macrophages, with the ß-glucan fragment, will travel throughout the systemic blood to get to the spleen, lymph nodes and bone marrow. Once there, the whole fragment of ß-glucan will be cut into smaller and more soluble pieces and released to be taken to the complement receptor 3 (CR3) from other granulocyte cells, such as neutrophils which will be later activated and can trigger an immunological cascade.

2.2.1. DECTIN-1 RECEPTOR

Dectin-1 receptor is a type-II transmembrane receptor from the C-type lectin receptors family (CLRs), which can extracellularly recognize ß-1,3 glycosidic bonds from multiple glycans (for instance, ß-glucans) on pathogens thanks to its carbohydrate-recognition or C-type lectin-like domain (Goodridge et al., 2009; Willment et al., 2005). Dectin-1 receptor can bind to some ligands on T cells (Ariizumi et al., 2000), co-stimulating the proliferation of CD4+ and CD8+ T lymphocytes and IFN-γ (Grünebach et al., 2002).

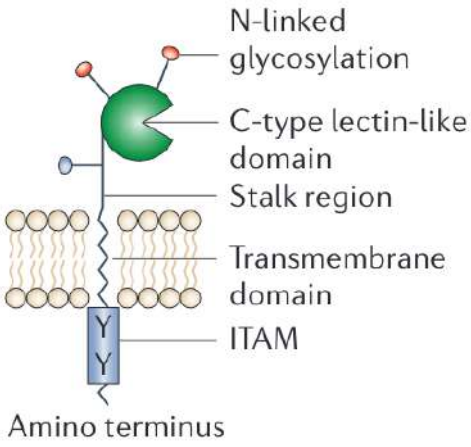

Figure 3. *Schematic representation of Dectin-1 structure. Dectin-1 contains N-linked glycosylation sites in the C-type lectin-like domain in mice (red) and in the stalk region in humans (blue). The C-type lectin-like domain is extracellular and binds the membrane through a stalk region, which might disappear in the isoform of Dectin-1 in humans. Intracellularly there is a cytoplasmatic tail that contains immunoreceptor tyrosine-based activation motif (ITAM), that modulates the signaling from inside of the cell. [Source: (Brown, 2006)].*

Dectin-1 receptor can be found in macrophages, dendritic cells, and neutrophils' membranes, as well as some types of T cells (Taylor et al., 2002). In humans, Dectin-1 receptor is also expressed on B lymphocytes and eosinophils (Brown, 2006). It has two main parts, the extracellular and the intracellular part (*Figure 3)*. Externally, there is a C-type lectin-like domain linked to the stalk region where a N-linked glycosylation site can be found in humans. On the contrary, mice have N-linked glycosylation sites on the C-type lectin-like domain. Internally, there is an immunoreceptor tyrosine-based activation motif, which is involved in cellular signaling and activation (Brown, 2006; Gantner et al., 2003; Goodridge et al., 2009). Additionally, Dectin-1 receptor is responsible for mediating inflammatory responses, phagocytosis, secretion of cytokines and chemokines and modulating the immune cells (Goodridge et al., 2009).

Dectin-1 receptor is expressed in many tissues; however, it is mainly found in tissues related to pathogens' entry such as the lungs and the gut (Brown, 2006; Taylor et al., 2002). This confirms the important role Dectin-1 plays in immune surveillance and immunity activation.

2.2.2. COMPLEMENT RECEPTOR 3 (CR3)

Complement receptor 3 (CR3) is a membrane-bound receptor with multiple binding domains. It is expressed on neutrophils, macrophages, and even Natural Killer cells (NK cells), which the expression of this receptor helps potentiate their cytotoxicity effect (Di Renzo et al., 1991; Willment et al., 2005). ß-glucans bind to the lectin site of the CR3, specifically to the CD11b subunit. This union activates the I-domain, making it possible for microorganisms, whose walls are made of ß-glucans, to also bind to the iC3b unit. The double union results in the initiation of the killing-process mediated by the immune cell (*Figure 4A)* (Zhang et al., 2018).

As explained in *Section 2.3.4.,* CR3 receptor is also involved in the antitumoral property activation that ß-glucans develop. As explained before, ß-glucans bind to the lectin site, which results in the activation of the I-domain. Thanks to that, tumoral cells will be able to bind to the iC3b unit and be phagocytized (*Figure 4B)* (Zhang et al., 2018).

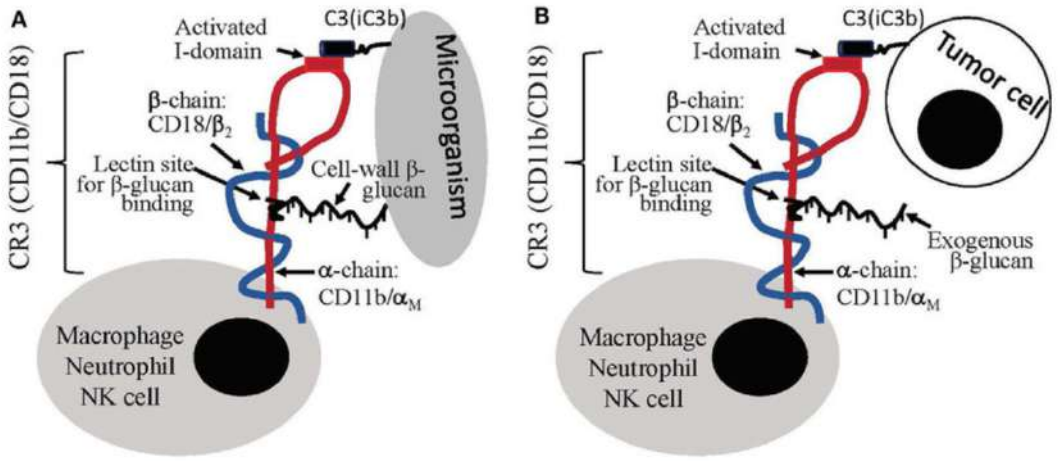

Figure 4. *Schematic representation of the immune cells' activation by ß-glucans. (**A**) Complement receptor 3's (CR3) lectin site binds to microorganism's cell-wall ß-glucans which results in the activation of the I-domain and consequently, a double union is formed with the microorganism and the iC3b unit from CR3. (**B**) CR3's lectin site binds to an exogenous ß-glucan which results in the activation of the I-domain and consequently, tumoral cells can form a union to the iC3b unit of the CR3. [Source: Zhang et al., 2018].*

2.3. IMMUNOMODULATORY AND OTHER BENEFICIAL EFFECTS OF ß-GLUCANS

The immunomodulatory activities and effects of ß-glucans have been widely studied, mainly for its pro- and anti-inflammatory activities due to cytokines secretion, and the functional changes that it causes in immune cells (Jin et al., 2018). However, ß-glucans have plenty of other beneficial effects on the human body when consumed, including the lowering of cholesterol levels, the antitumoral activity and the antioxidant activity (*Figure 5)*.

For many years, it has been believed that only ß-glucans extracted from mushrooms were able to stimulate the immune system. However, more recent studies have showed the possibility to work

with plant ß-glucans and obtain similar results to the ones extracted from fungi (Arcidiacono et al., 2019).

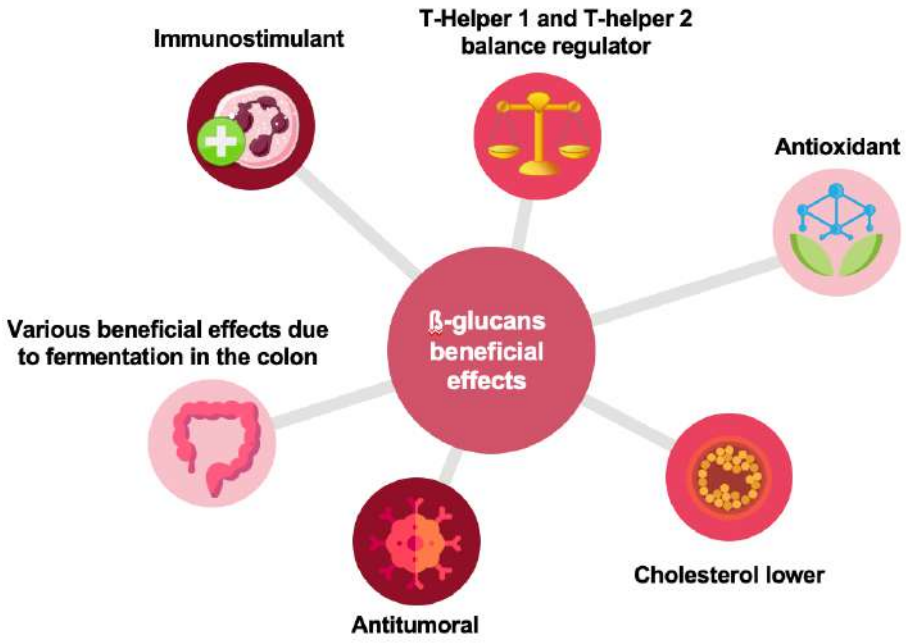

Figure 5. List of beneficial effects of ß-glucans in human health.

2.3.1. THE IMMUNOSTIMULANT PROPERTY OF ß-GLUCANS

It has been proved that less than 0.5% of the amount of ß-glucan administered orally were found in systemic blood. Even with such a low percentage, ß-glucans are significantly able to activate multiple immune responses; both humoral and cellular responses (Chan et al., 2009). The immunostimulant property of ß-glucans is primarily mediated by the innate immune system, specifically the macrophages and less common neutrophils and dendritic cells.

When ß-glucans enter the small intestine, they are absorbed by the M cells in Peyer's patches or the intestinal epithelia by passive transportation (*Figure 6)*. Macrophages and other antigen-presenting cells will bind with their Dectin-1 receptor to the ß-glucan molecule by its ß-1,3 glycosidic bond. This union will trigger an immune response, the production of pro-inflammatory factors and phagocytosis (Chan et al., 2009). These cells will also transport the ß-glucan to the bone marrow and other lymphoid tissues and cut the ß-glucan molecule into smaller fragments so they can be detected by the CR3 receptor expressed by neutrophils. This will cause a cellular activation. Pro-inflammatory cytokines and interleukins will start being secreted by neutrophils to enhance the production of different lymphocytes (Goodridge et al., 2009).

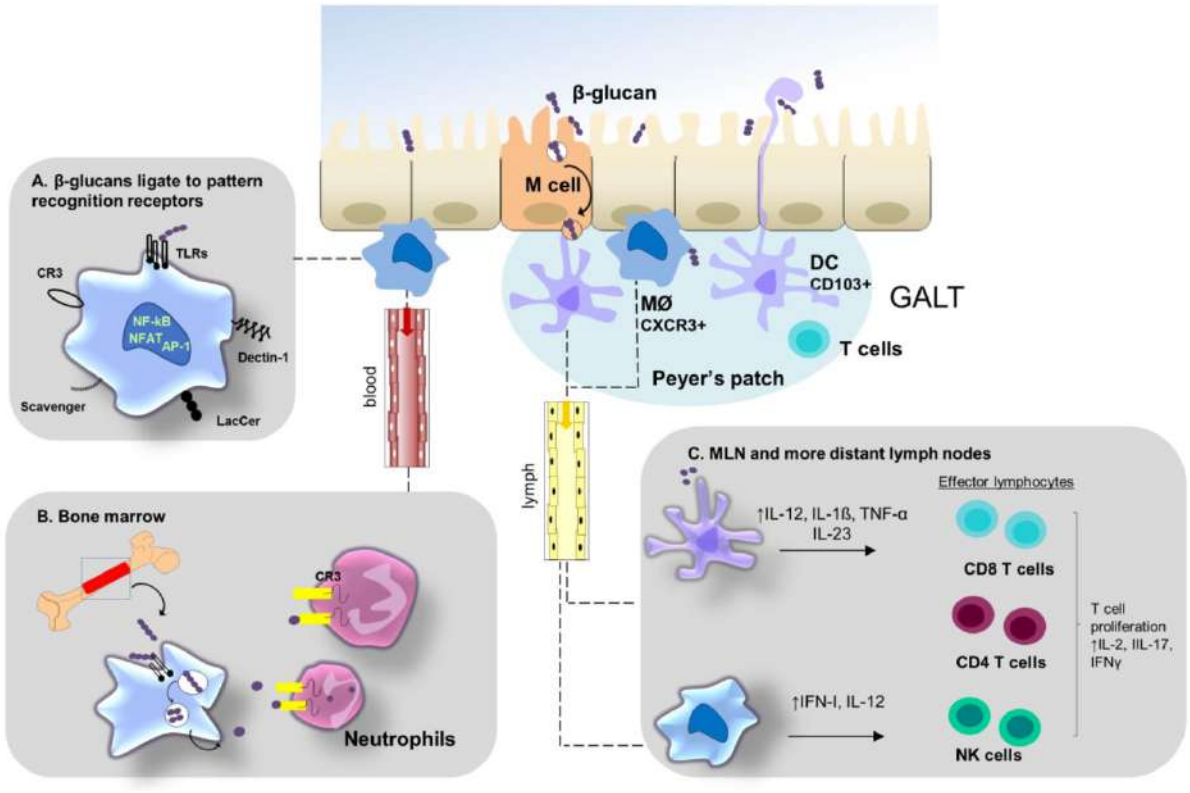

Figure 6. Immune route activation by ß-glucans when absorbed by the intestinal lumen. (A) ß-glucans will be recognized and will bind to receptors expressed by antigen-presenting cells. (B) The antigen-presenting cells will transport the ß-glucan molecule to the bone-marrow to start a cellular activation by neutrophils. (C) The antigen-presenting cells will also transport the ß-glucan molecule to other lymphoid organs to enhance interleukin and cytokine secretion. Abbreviations: CR3: complement receptor 3; CXCR3: CXC chemokine receptor 3; DC: dendritic cell; IFN-I: type 1 interferons, IFN-γ: interferon gamma; IL: interleukin; LacCer: lactosylceramide; MO: macrophage. [Source: de Graaf et al., 2018].

2.3.2. T HELPER 1 (TH1) AND T HELPER 2 (TH2) BALANCE REGULATION

This property has been studied mostly in fungal organisms that contain ß-glucans. When the ß-glucan is digested, the capability of macrophages to produce IL-2 and IL-12 and therefore, foster type 1 immunity response (Th1), is improved (Kidd, 2003). Th1 has a beneficial effect on fighting tumoral cells. Not only do ß-glucans promote Th1 immune response but, they also balance both Th1 and Th2 production. This can prevent autoimmune diseases and allergies (Nakashima et al., 2018).

2.3.3. ANTIOXIDANT PROPERTY

Some of the main antioxidant elements in barley linked to its fiber are arabinoxylans, phenolic acids and α-tocopherol (vitamin E). Those are natural antioxidants that act as free radical scavengers, antibacterial, antiviral, anti-inflammatory, anti-allergic and anti-thrombotic (Gupta et al., 2010). ß-glucans extracted from barley are also known to be very efficient scavengers of

reactive oxygen species (ROS), which may lead to the prevention of various diseases such as arteriosclerosis, cardiovascular and cerebral diseases, diabetes and cancer (Kofuji et al., 2012).

2.3.4. ANTITUMORAL PROPERTY

The antitumoral activity is mostly studied in ß-glucans from purified mushrooms (Nakashima et al., 2018). The union of a ß-glucan fragment with the lectin site of a CR3 expressed on a granulocyte will activate the I-domain making it possible for tumoral cells to bind with the iC3b subunit (*Figure 7)*. This bond will activate the granulocyte's phagocytosis property as well as its production of cytokines and interleukins to target and kill the tumoral cell. (Chan et al., 2009).

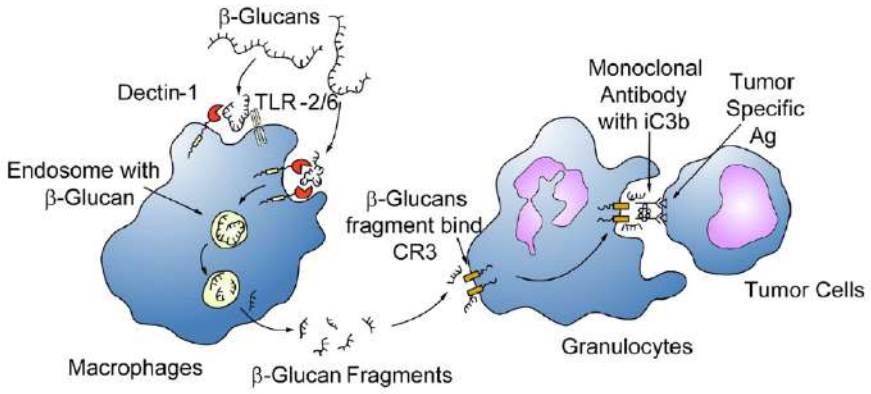

Figure 7. *Schematic representation of the ß-glucan uptake from the immune cells. Firstly, ß-glucans are bound to the Dectin-1 receptor from macrophages and fragmented inside this immune cell. After the smaller fragments of the ß-glucan are released, these are captured by and bound to the complement receptor 3 (CR3). This union will activate the immune system and therefore the phagocytosis of the tumoral cells. [Source: Chan et al., 2009]*

The immune response that ß-glucans generate has had a positive impact on some cancer patients, both in improving quality life of those patients, and their secondary effects from chemotherapy. The administration of ß-glucans showed an increase of multiple immune cell types, and therefore are considered an immune-potentiating adjuvant when combined with other treatments (de Graaff et al., 2018).

2.3.5. CHOLESTEROL-LOWERING PROPERTY

The activity of decreasing plasma cholesterol levels is related to the ß-glucans extracted from cereals such as oat and barley. It has been studied that ß-glucans affects low-density lipoprotein cholesterol (LDL), which is the main biomarker for cardiovascular diseases (Daou & Zhang, 2012). However, the levels of high-density lipoprotein cholesterol (HDL) are not altered. The property of reducing cholesterol levels is due to the ability of ß-glucans to act as dietary fiber and, therefore, capture bile acid micelles that contain fats (*Figure 8)* (Sima et al., 2018).

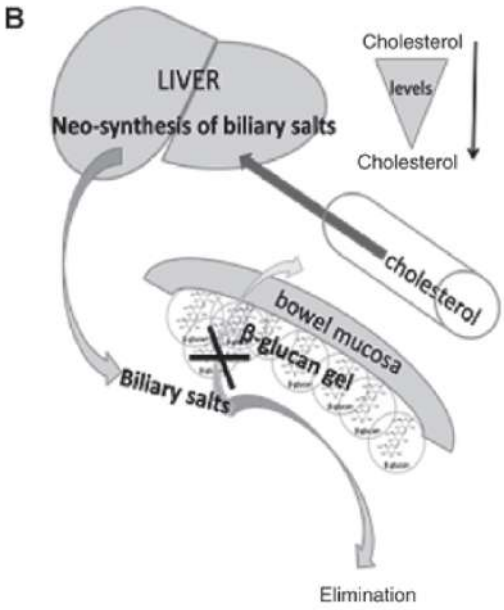

Figure 8. Schematic representation of how ß-glucans can inhibit resorption of bile acid micelles consequently reducing cholesterol levels in blood. [Source: Sima et al., 2018].

2.3.6. BENEFICIAL EFFECTS DUE TO ß-GLUCAN FERMENTATION IN THE COLON

ß-glucans from cereals such as oat and barley are fermented by microorganisms living in the colon. These microorganisms are responsible for transforming ß-glucans molecules into short-chain fatty acids (SCFAs) (Hughes et al., 2008). The most common SCFAs produced are propionic acid, acetic acid, and butyric acid. All of them can have an impact on the immune system as well as other benefits such as preventing obesity (Nakashima et al., 2018). The SCFAs are also responsible for regulating the growth of harmful microbiota from the colon and therefore maintaining a healthy gut environment (Hughes et al., 2008).

2.4. ANTI-VIRAL PROPERTIES OF ß-GLUCANS FOCUSED ON SARS-COV-2

It has been described that ß-glucans show promising anti-viral qualities that have a positive impact in upper respiratory tract infections (URTI). Nevertheless, it is still uncertain if those anti-viral responses are triggered by ß-glucans acting as a training agent in the trained innate immunity (TRIM) or they directly stimulate the immune cells (Geller & Yan, 2020).

Most of the studies related on this topic, worked with ß-glucans extracted from yeast. This is because it consists of a ß-1,3 glycosidic bond linked to a ß-1,6 glycosidic bond, which since recently (Arcidiacono et al., 2019), it was believed to be the only possible biological structure of ß-glucan able to modify the immune system.

According to several studies, yeast ß-glucan was able to decrease the severity of the symptoms caused by URTI and it also reduced the systolic and diastolic blood pressure (Geller & Yan,

2020). As both respiratory symptoms and high blood pressure are described as symptoms caused by COVID-19 infection (Huang et al., 2020), ß-glucan could be considered as a supplement treatment for this illness. Moreover, ß-glucan also improved the production of IL-1ß, TNF-alpha and IFN-gamma, which encourage antibody production against viruses (Geller & Yan, 2020).

2.4.1. ß-GLUCAN AND IMMUNOSTIMULANT PROPERTIES IN SARS-COV-2 INFECTIONS

As mentioned before, ß-glucan has immunostimulant properties that can directly enhance certain immune responses. It has been proved that daily administration of ß-glucan for six weeks can increase the production and number of B-cells, consequently intensifying the levels of IgA in saliva and the respiratory tract (McFarlin et al., 2013). This could be associated in having a stronger protection towards URTIs, both bacteria and virus related.

2.4.2. ß-GLUCAN AND TRIM IN SARS-COV-2 INFECTIONS

ß-glucans are also believed to be able to stimulate the trained innate immunity (TRIM) inducing myelopoiesis and therefore the production and secretion of myeloid progenitors from the bone marrow (*Figure 9)* (Mitroulis et al., 2018; Netea et al., 2020). Thereby protecting against a secondary challenge by systemic inflammation caused by a certain pathogen. So, ß-glucans are able to activate TRIM proteins, which function as regulatory factors for more complicated immune activities regarding the defense of the host against pathogens (Yang et al., 2020). Some animal studies proved ß-glucan administration is able to protect against pathogen fungi and bacteria, as well as certain viruses such as rotavirus (Geller & Yan, 2020; Kim et al., 2011; Netea et al., 2020).

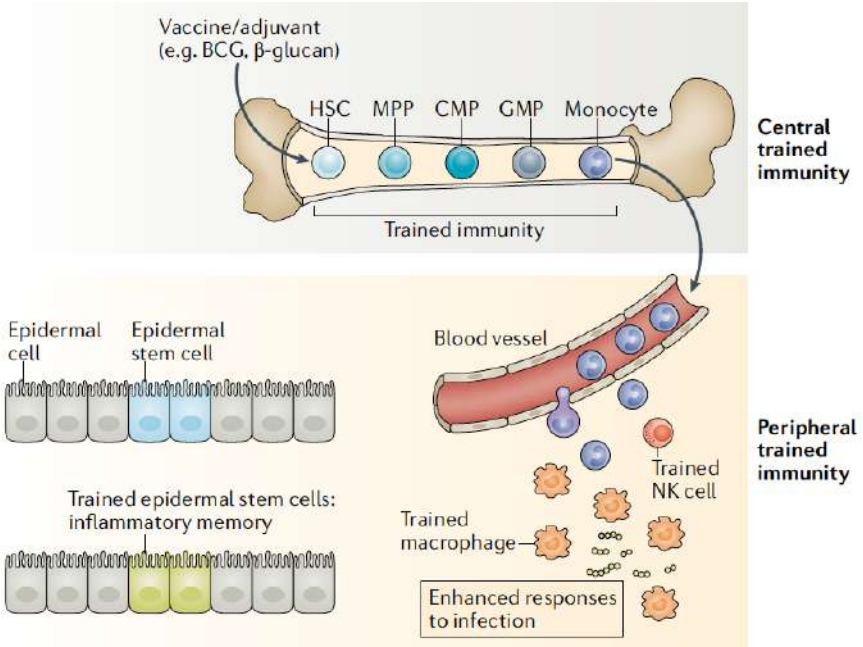

Figure 9. Central and peripheral TRIM enhanced by ß-glucan molecules transported by macrophages into the bone marrow where the trained immunity will be potentiated the hematopoietic stem cells (HSCs). The HSCs will eventually divide and differentiate into myeloid and lymphoid cell types of cells. The monocytes derived from trained HSCs migrate through blood vessels to peripheral organs where they will boost immune functions against pathogens, resulting in the liberation of cytokines, as well as interleukins. Abbreviations: BCG: Bacillus Calmette-Guérin; CMP: common myeloid progenitor, GMP: granulocyte-macrophage progenitor; MPP: multipotent progenitor. [Source: Netea et al., 2020].

3. HYPOTHESIS

Previous studies have shown that the intake of barley-based food products rich in bioactive compounds have beneficial effects such as modulating the immune system and boosting the antioxidant activity. It is for that reason that this R+D project wants to develop new barley-based food products rich in bioactive compounds, whose intake as part of a balanced diet could have beneficial effects on boosting the immune system.

Given that information, the following hypothesis are formulated:

- H_0 (null hypothesis) = A barley-based diet does not have significant modifications in mice's immune response compared to the control groups.
- H_a (alternative hypothesis) = A barley-based diet has significant modifications in mice's immune response compared to the control groups.

4. OBJECTIVES

The objective of this *Treball Final de Grau* is to **design and carry out a pilot study** regarding a R+D project that aims to analyze the immunological effects of orally administered whole barley grain as whole food matrix, pearled grain, and external pearling fractions to mice. The aim is for it to act as a supplemental treatment against SARS-CoV-2.

Moreover, the pilot study has the following sub-objectives:

1. To execute a pilot study using the experimental design proposed by previous studies.

2. To prove that the average daily gain and mice's behavior does not change depending on the diet.

3. To try out some techniques of the experimental model suggested by former studies regarding the evaluation of some lymphoid organs.

4. To identify points where improvement is possible to meliorate the large-scale project investigation.

5. MATERIAL AND METHODS

The following experimental study is approved by the Ethics Committee and therefore, it is carried out in an ethical manner in accordance with the Spanish and international laws. It also based on the EUs Directive 2010/63/EU on the protection of animals used for scientific purposes.

5.1. ANIMALS AND EXPERIMENTAL PROCEDURE

A group of 30 Balb/cB&J mice between 35 and 41 days old (15 males and 15 females) from Charles River Laboratories© (Barcelona, Spain) were kept in the animal facilities at the University of Lleida. The mice were randomly distributed in three different groups in such a way that 10 animals ended up in each group: 5 males and 5 females in each *(Table 1)*.

Table 1. *Distribution of the groups taking part in the experiment. Abbreviations: RC = control diet supplemented white rice; SD = standard chow diet.*

Group name	Sex	Diet
G1M	Male	RC
G2M	Male	SD
G3M	Male	RC
G1F	Female	RC
G2F	Female	SD
G3F	Female	RC

The experimental procedure is represented in *Figure 10.* Animals were provided with water and fed *ad libitum*. Each group was given a different diet during the entire six-week duration of the experiment. Those are the following: standard chow diet (SD) was fed to groups G2M and G2F, and control diet supplemented with white rice (RC) was fed to groups G1M, G3M, G1F and G3F.

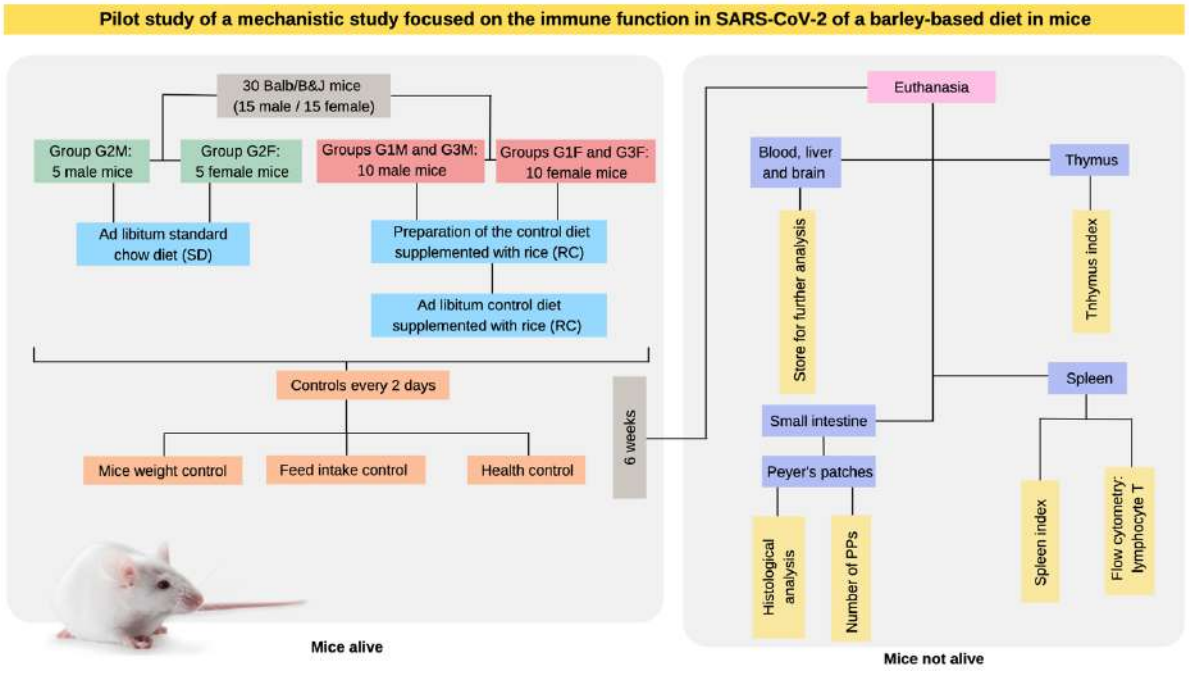

Figure 10. Schematic representation of the experimental design of the Treball Final de Grau: "Pilot study of a mechanistic study focused on the immune function in SARS-CoV-2 of a barley-based diet in mice.

During these six weeks, food intake and body weight were monitored every two days and a health evaluation for each mouse was carried out. After these six weeks, mice were euthanized, and organ samples were collected. Blood, the left liver lobe, and the brain were gathered and stored for further analysis not done in this pilot study. Thymus and spleen were weighed to calculate their indexes. The spleen was kept to later do a flow cytometry technique. Finally, all Peyer's patches (PPs) were counted and collected to histologically evaluate them.

5.1.1. MICE'S CAGES PREPARATION

Mice were kept in CM500-type cages during the whole experiment. At first, Safe Select® shaving was used as substratum, but on day 5 that shaving was changed for Alamo Temblón® shaving, as the first one generated dust and this could negatively impact the mice's wellbeing.

All mice were provided with environmental enrichment objects such as cardboard tubes and paper tissues to enhance the natural behavior of mice. Because male mice kept showing signs of distress due to their aggressive fights, other objects were added to the cage on day 10: a cardboard igloo and two wood trunks (*Figure 11)*.

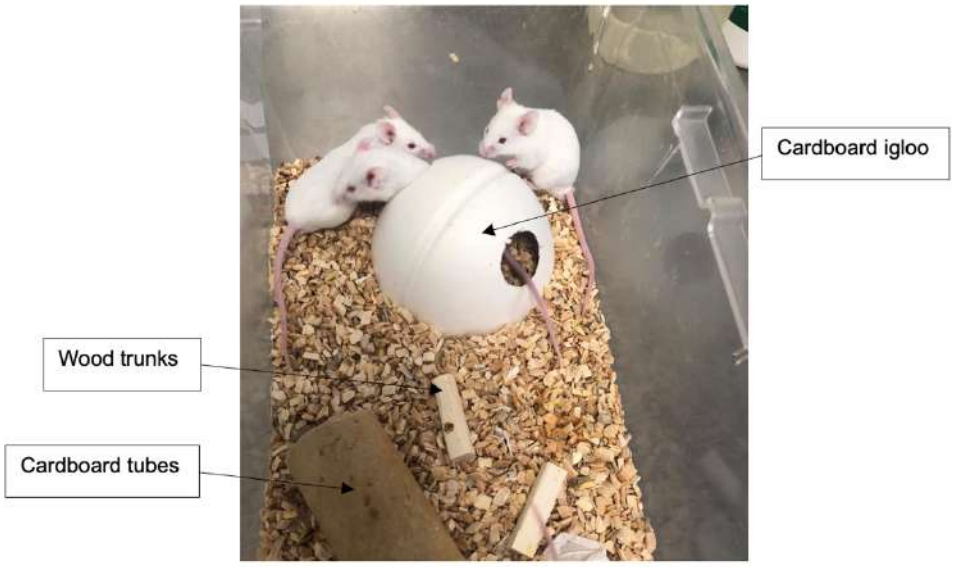

Figure 11. Picture of the environmental enrichment material for mice used during the experiment.

5.1.2. DIET PREPERATION

Diet 1. Standard chow feed supplemented with rice:

A diet supplemented with rice is used as one of the control groups as that cereal does not have ß-glucans in its composition and therefore, can be used to determine the role cereals' fiber plays in the modulation of immunity. That diet was fed to groups G1M, G3M, G1F and G3F.

To prepare 2.30 kg of the control diet supplemented with rice we needed two main ingredients: 1.7 kg of standard chow feed and 600 gr of rice semolina. The rice semolina was first grinded with *Moulinex® la Moulinette AD5601*. When having had 600 gr of grinded rice semolina, we mixed it with the 1.7 kg of standard chow feed. For each kilogram of mixed feed, a whole liter of doubly distilled water was added and homogenized. After creating a smooth paste, it was introduced inside a silicone pistol to form long barrels what would be cut smaller afterwards (*Figure 12)*. The feed was frozen at -20 ºC for several hours and then at -80 ºC. The product was then lyophilized on a IYOBETA 15 TELSTAR Lyophilizer (Terrassa, Spain) for 48 hours and afterwards kept at -20 ºC.

Figure 12. *Feed supplemented with white rice used to feed the mice.*

Diet 2. Standard chow feed:

The second control group was fed with standard chow feed, specifically Teklad Global® 14% Protein Rodent Maintenance Diet with the following composition expressed in *Table 2* (whole nutrient composition in *Annex 1).* No treatments or additional ingredients were added in that diet.

Table 2. *Teklad Global 14% Protein Rodent Maintenance Diet composition used to feed groups G2M and G2F.*

Macronutrients		
Crude Protein	%	14.3
Fat (ether extract) [a]	%	4.0
Carbohydrate (available) [b]	%	48.0
Crude Fiber	%	4.1
Neutral Detergent Fiber [c]	%	18.0
Ash	%	4.7
Energy Density [d]	kcal/g (kJ/g)	2.9 (12.1)
Calories from Protein	%	20
Calories from Fat	%	13
Calories from Carbohydrate	%	67

5.1.3. HEALTH CONTROL

Mice's wellbeing was controlled every two days, at the same time mice and feed weight were determined.

There are some predisposed conditions associated with Balb/c strain that also had to be taken into consideration. They are the following (Burkholder et al., 2012):

- Male aggressions
- Heart ventricular mineralization
- Corneal opacities
- Conjunctivitis
- Blepharitis
- Periorbital abscesses
- Age-related hearing loss

As the mice were only kept alive for six weeks, most of these conditions might be irrelevant except the male aggression which might lead to fight wounds, pain and distress that could have a negative impact on the feed consumption as well as on the mice. Fight wounds are normally presented on the rump, hips, tail and/or genital region welfare (Burkholder et al., 2012). Some fight wounds might lead in limb injuries. In this case, the veterinarian would determine the proper treatment or solution to the problem.

Other observations that were taken into consideration:

- Dis: pain and/or distress. Includes squinted eyes, contracted skin around nose and ears pulled back.
    - Dis1: mild or anticipated pain and distress. Not well groomed; awkward gait; slightly hunched; looks at wound or pulls away when area touched; mildly agitated; BC=2.
    - Dis2: moderate pain and distress. Rough hair coat; dirty incision; squinted eyes; moves slowly; walks hunched and/or slowly; depressed or moderately agitated; slight dehydration; pruritic; restless; uncomfortable; not eating or drinking; BC=2.
    - Dis3: severe pain and distress. Very rough hair coat; eyes sunken (severe dehydration); slow to move or non-responsive when coaxed; hunched; large abdominal mass; dyspnea; self-mutilating; violent reaction to stimuli or when approached; BC=1.

- FW: fight wounds. Specify the area of the wound and the gravity according to 1 to 3 scale.
    - FW1 = small and/or superficial wound
    - FW2 = more than two deep wounds
    - FW3 = severe wounds

- D: diarrhea. Liquid faeces when the animal is picked up or seen in the cage bedding.
- A: anorexia. Lack of faeces in a cage that has not been cleaned. No evidence of mice chewing on the chow. Mice appear too thin or dehydrated.
- RHC: rough hair coat.

- ED: ear dermatitis. Crusty lesions on and below the ear associated with loss of an ear tag.
- BCS: body condition.
    - BC1 = mouse is emaciated. Skeletal structure extremely prominent, little or no flesh cover. Vertebrae distinctly segmented.
    - BC2 = mouse is underconditioned. Segmentation of vertebral column evident. Dorsal pelvic bones are readily palpable.
    - BC3 = mouse is well-conditioned. Vertebrae and dorsal pelvis not prominent; palpable with slight pressure.
    - BC4 = mouse is over-conditioned. Spine is a continuous column. Vertebrae palpable with only firm pressure.
    - BC5 = mouse is obese. Mouse is smooth and bulky. Bone structure disappears under flesh and subcutaneous fat.

5.2. EUTHANASIA PROCEDURE AND SAMPLE COLLECTION

Mice were euthanized after 6 weeks of starting the experiment. Fasting was not necessary before the euthanasia. First, they were anesthetized with isoflurane 4% for induction and isoflurane 2% for maintenance (ISOFLOR 250 ml). Blood was collected in tubes (serum Gel S-Monovette) directly from the heart (*Figure 13)*. It was determined that more than 0.2 ml of blood had to be extracted from each individual. Blood samples were centrifuged at 4 ºC, 3500 rpm, for 15 minutes. Mice were opened from the low part of the abdomen to the upper part of the thorax entrance. The right heart atrium was cut, and sodium chloride (0.9%) was perfused from the left ventricle to remove the remaining blood from the tissues (*Figure 14)*. Once the mouse was dead, the sample collection started.

The thymus and the spleen were collected to determine their weight and indexes using the following equation: *spleen and thymus indexes = spleen or thymus weight / body x 100* (Lei et al., 2015). After being weighed, they were stored at -80 ºC. The small intestine was also removed from the animal to determine the number of Peyer's patches. And the descending colon was cut from the large intestine to be afterwards evaluated. The left lobe from the liver and the brain were also collected. All organs' samples were each one of them divided to store at -80 ºC in liquid nitrogen and the other part was fixed in 10% (v/v) formalin for a minimum of 24 hours.

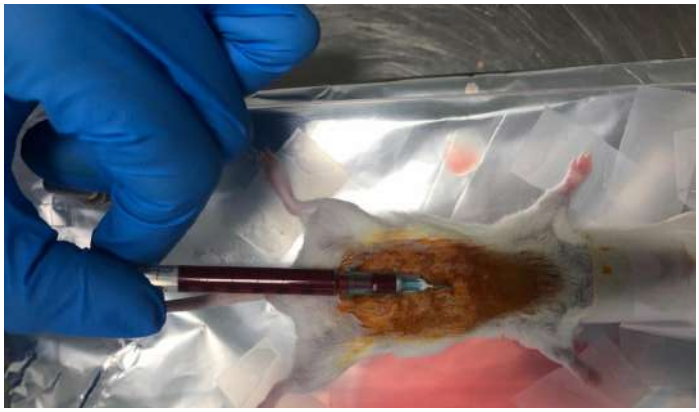

Figure 13. Blood collection procedure extracted directly from the heart while the mouse was anesthetized with isoflurane 2%.

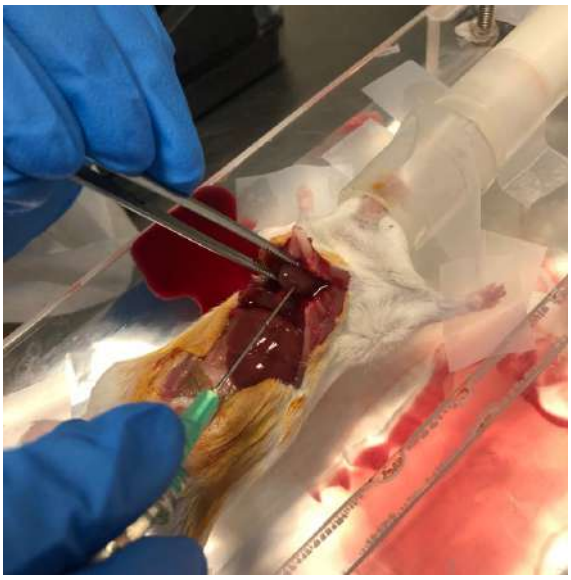

Figure 14. Euthanasia procedure using the perfusion method. The right atrium from the heart was cut, and then chloride sodium 0,9% was infused into the left ventricle to eliminate the remaining blood in the tissues and euthanize the mouse.

5.3. FLOW CYTOMETRY ANALYSIS ON THE SPLEEN

The spleen of each mouse was collected and preserved at -80 ºC in liquid nitrogen. Spleens were submerged in 2-3 ml PBS on ice and crushed between the two sterile slides to collect the splenocytes. The splenocytes were placed in a 15 ml falcon tube and let rest to allow the remains of the capsule and big pieces of tissue to settle to the bottom of the tube. The single cell suspension was then centrifuged 400 xg for 5 minutes. Splenocytes were resuspended in 900 µL of sterile MQ water for a few seconds and 100 µL of PBS 10x were rapidly added to the tube to lye the red blood cells. Again, cells were centrifuged during five minutes at 400 xg and resuspended in pBS +5% FBS. After, cells were counted using the "Luna-II™ Automated Cell Counter" machine (*Figure 15).*

Figure 15. Luna-II™ Automated Cell Counter machine used to accurately count cells from the spleen.

No cells were counted with "Luna-II™ Automated Cell Counter" and therefore, it was not possible to continue with the procedure.

5.4. HISTOLOGICAL ANALYSIS

A simple Peyer's patch from the duodenum, jejunum and ileum was collected and preserved in formalin between 24 and 48 hours. The Peyer's patches were put in histological *cassettes* inside a "*Vogel V0-5-8100*" workstation. The processing was performed on the tissue processor "*Myr STP 120"* in which the samples were dehydrated by immersion in increasing concentrations of alcohol. The samples were then immersed in liquid paraffin in a *"Myr EC 500"* inclusion apparatus to facilitate the cutting with the "*ThermoScientific HM325"* rotating microtome, which is calibrated to a thickness of 5 μm. The cuts were stained with a hematoxylin-eosin stain and mounted on the coverslip. The histological preparations were studied with an "*Olympus BX50"* optical microscope equipped with an Olympus SC50 camera interfaced with cell Sens Entry® Olympus software.

5.5. STATISTICAL ANALYSIS

Different statistical analysis systems were used for each parameter evaluated:

- The average daily gain (ADG) was evaluated with R studio using an analysis of variance (ANOVA test). Results were considered significant at $p<0.05$ with a level of confidence of the 95%.

- Mice's health was evaluated using a Fisher test. The different qualitative variables and their abbreviation are specified in *Section 5.1.3*.

- The thymus and splenic indexes, as well as the number of PPs, were analyzed through R studio to describe differences between groups using an analysis of variance (ANOVA test). Results were considered significant at $p<0.05$ with a level of confidence of the 95%.

6. RESULTS AND DISCUSSION

6.1. EFFECTS OF THE DIET ON MICE'S AVERAGE DAILY GAIN

In this part of the experiment, mice and feed were weighed every two days to evaluate the mice's growth and the feed intake during the whole process.

There are already some studies, such as Lei et al. (2015), that performed a similar experimental procedure, but did not add a second control group as this case to evaluate barley's fiber properties on the immune system. In this pilot study, a group fed following a diet supplemented with rice was added to determine the fiber's activity on mice. Therefore, it was important to take mice's weight and growth into consideration during the experiment as each given diet must provide an optimum growth to establish a correct body weight and health. Also, if mice were to eat less than the control group, the subsequent statistical analysis regarding the immune effect of the diet would be biased. It is for this reason that the average daily gain (ADG) was calculated.

To prove that all groups ate statistically similar quantities of feed, the ADG from each mouse was calculated using the following formula: *((Final weight – Initial Weight) / Days of the experiment)*. The average daily gain from each group is shown in *Table 3* and *Figure 14*.

Table 3. *Average daily gain (ADG) and its standard deviation from each group used in the experiment.*

Group	ADG
G1M	0.15 ± 0.04
G2M	0.20 ± 0.02
G3M	0.20 ± 0.02
G1F	0.13 ± 0.02
G2F	0.13 ± 0.02
G3F	0.12 ± 0.02

As seen in *Figure 16*, ADG differed significantly between males and females except G1M that did not vary from the female groups. The different diets did not modify mice's feed intake and therefore, their growth during the experiment.

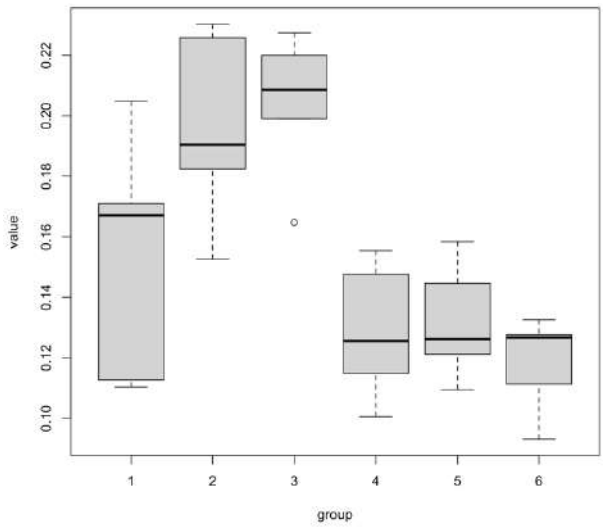

Figure 16. Effects of the diet on the average daily gain (ADG) per group of mice, being 1=G1M; 2=G2M; 3=G3M; 4=G1F; 5=G2F; 6=G3F.

As all groups showed similar results in matters of average daily gain. The standard chow feed was evaluated to determine their exact nutritional value and it was seen that the fiber content from that feed was too similar to the one from the feed supplemented with rice semolina. For this reason, it has been purposed to change the fiber composition of the standard chow diet to differ it from the second control group (the group fed a diet supplemented with white rice).

6.2. HEALTH EVALUATION

Mice's health was evaluated every two days during the weighing. Mice's wellbeing is important in this study proposed so as not to interfere in mice's feed intake and their hypothetical improvement in the immune system. The findings in the evaluation were noted down following what was considered in *Section 5.1.3* and different Fisher Tests with the program JMP Pro 16 for "*Fight wounds 1*", *"Fight wounds 2"* and *"Rough hair coat"* as qualitative variables were done. As shown in *Table 4*, all health abnormalities in male mice did not significantly differ from each group. Because of that, it can be said that the results in the other analysis are not biased by their health problems.

Table 4. P-values obtained with the Fisher Test of different health evaluation variables.

Variable	Prob ≤ P
Fight wounds 1	0.08
Fight wounds 2	0.26
Rough hair coat	0.07

The findings in male's health are represented in *Figure 17*. All males from every group showed at least one health disorder during the experiment, which was rough hair coat. G2M proportionally experienced less health disorders compared to the other two groups. Moreover, the clinical signs G2M's mice experienced were less serious. G3M's mice had multiple fighting wounds, mostly in the tail. Even, G1M's mice had not as many fighting wounds as G3M, more frequently exhibited rough hair coat.

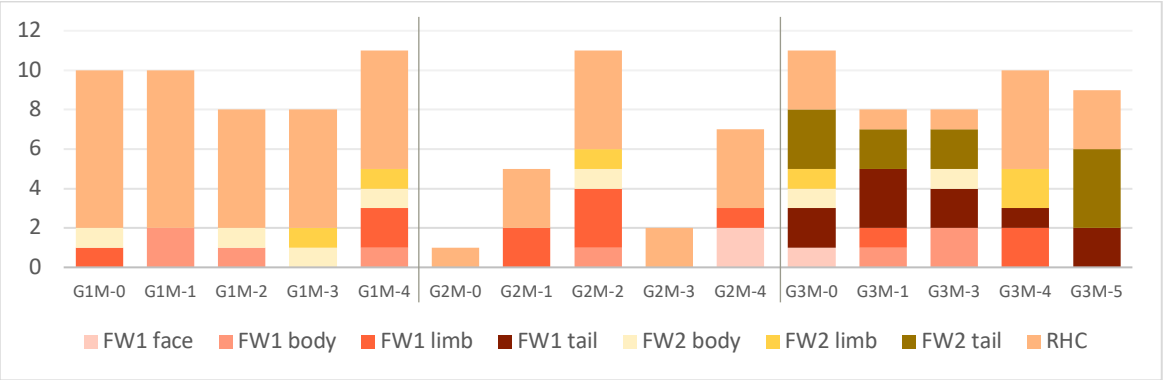

Figure 17. Findings observed in male mice during their health evaluation that was done every two days during six weeks.

It must be noted that male mice continuously showed low signs of distress during the experiment. Females did not exhibit any abnormality in their health during the experiment. Balb/c mouse males are normally aggressive and are one of the main mouse strains that develop fight wounds during the experiments (Burkholder et al., 2012). However, Balb/c is also one of the main strains widely used for immunology research and for this reason it was believed to be a good fit for the study proposed. It was intended to put an end to the matter of aggressiveness increasing the number of environmental enrichment objects and changing the shaving into another less dusty one. Moreover, it was attempted to manipulate the mice the minimum number of times, therefore, leveraging the moment we weighted them to clean the cages. It was noticed that the tail bites decreased, but male mice carried on fighting. A solution to that issue would have been separating the dominant male that started those fights into another cage. However, because it was necessary to determine the feed intake and their growth as a group, this option was ruled out. For further studies with a similar matter, it would be appropriate to maintain the group established in the mice supplier laboratory and use multiple environmental enrichment object since the beginning of the study.

Five mice per cage were used for the pilot study, whereas in the large-scale investigation it is established to have six mice per cage (60 animals in total) as former studies did (Lei et al., 2015). It has been seen that working with five animals per cage (50 animals in total) could have beneficial effects regarding male's aggressiveness and it will also encourage the European guidelines to reduce animals used in research.

6.3. EVALUATION OF SOME LYMPHOID ORGANS

6.3.1. EFFECTS ON THE DIET ON SPLEEN AND THYMUS

In this part of the study, the spleen and thymus' indexes were evaluated to see if those indexes differed significantly between groups and sex. The indexes were calculated after weighing both organs and using the following formula: *spleen and thymus indexes = spleen or thymus weight / body x 100*. The average indexes from each group are shown in *Table 5*. The indexes were evaluated through R studio using an ANOVA test, stating a level of confidence of 0.95.

Table 5. Effects of the diet on spleen and thymus indexes in mice.

Group	Spleen index (%)	Thymus index (%)
G1M	0.350 ± 0.04	0.094 ± 0.01
G2M	0.288 ± 0.01	0.179 ± 0.04
G3M	0.295 ± 0.05	0.119 ± 0.06
G1F	0.406 ± 0.02	0.208 ± 0.03
G2F	0.421 ± 0.02	0.268 ± 0.04
G3F	0.497 ± 0.08	0.257 ± 0.06

The results of the splenic indexes are illustrated in *Figure 18.* Between male groups, there are no significant differences (*p>0.05)*. However, all groups vary significantly from G3F. In addition, G2M and G3M also differ from G1F and G2F.

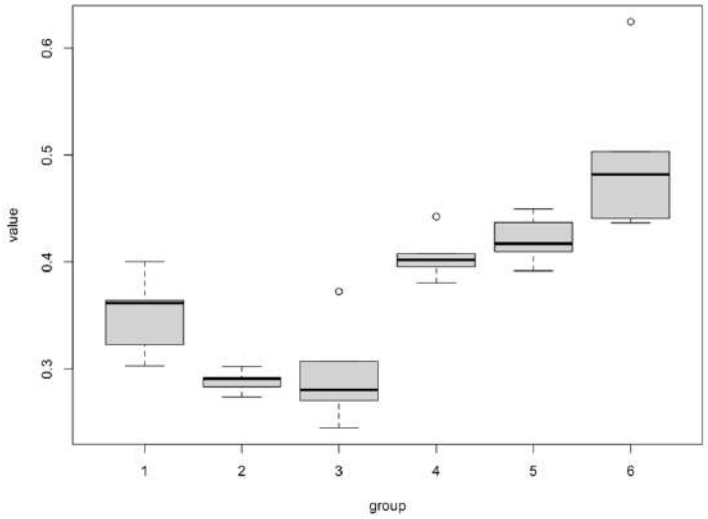

Figure 18. *Representation of the effects of the diet on splenic indexes per group of mice, being 1=G1M; 2=G2M; 3=G3M; 4=G1F; 5=G2F; 6=G3F.*

According to the male groups, the thymus index comparison between G1M and G2M showed significant differences (p<0.05); however, mice from G3M, which ate the same diet as G1M, did not significantly differ from G2M. All female groups exhibited no significant variations between groups from the same sex. G1M differed significantly from all groups except G3M, whether G3M, varied only from the female groups. G2M and G2F were both fed a diet supplemented with white rice but showed significant different thymus indexes results. The results are represented in *Figure 19*.

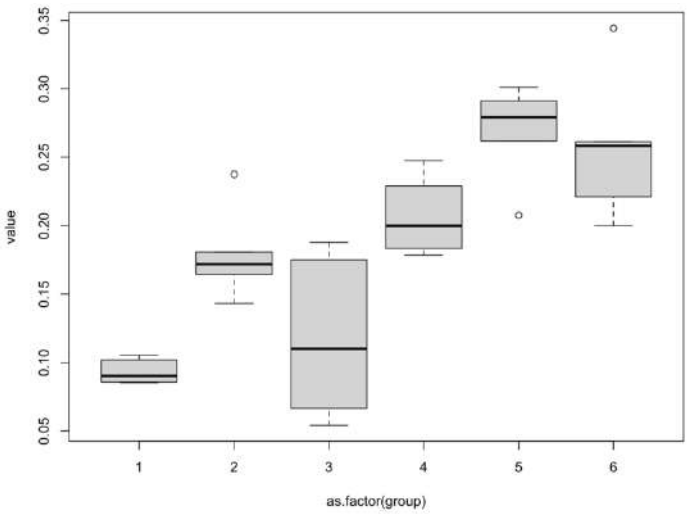

Figure 19. *Representation of the effects of the diet on thymus indexes per group of mice, being 1=G1M; 2=G2M; 3=G3M; 4=G1F; 5=G2F; 6=G3F.*

Thymus and splenic indexes differed significantly from certain groups. Nevertheless, it cannot be concluded that the groups fed with a diet supplemented with white rice have significant modifications in their lymphoid organs compared to the group fed a standard chow diet because the variations did not follow a pattern. As the same person did the thymus and spleen's extraction, the sampling error is low, and because of that it is not likely to be the responsible of these variations. As a result of these findings, it cannot be said the diet influenced the thymus and spleen's parameters.

The spleen was also collected and preserved at -80 ºC in liquid nitrogen to do a **flow cytometry analysis**. However, because the spleen was frozen, cells viability decreased drastically, and it was not able to count them. For this reason, the flow cytometry analysis was not carried out successfully. The large-scale investigation should do the analysis right after the euthanasia of the mice or preserve the spleen in paraformaldehyde for no more than 24 hours and then keep the spleen in a tube with PBS.

6.3.2. EFFECTS OF THE DIET ON THE PEYER'S PATCHES

The **number of Peyer's patches** (PPs) were evaluated through R studio using an ANOVA test, stating a level of confidence of 0.95. The number of PPs are shown in *Table 6.*

Table 6. *Number of Peyer's patches found in the duodenum, jejunum, and ileum from each mouse.*

PEN	Mice nº	Duodenum	Jejunum	Ileum	Total
G1M	0	5	2	2	9
	1	2	2	4	8
	2	5	2	4	11
	3	5	3	2	10
	4	3	1	3	7
G2M	0	3	2	2	7
	1	5	0	3	8
	2	5	2	2	9
	3	3	1	2	6
	4	3	2	3	8
G3M	0	4	1	3	8
	1	2	2	1	5
	3	5	1	2	8
	4	4	2	3	9
	5	4	3	3	10
G1F	0	3	2	2	7
	1	4	2	3	9
	2	5	2	3	10
	3	3	1	1	5
	4	3	3	3	9
G2F	0	2	3	2	7
	1	2	2	1	5
	2	4	2	4	10
	3	3	3	2	8
	4	5	3	2	10
G3F	0	3	3	2	8
	1	5	3	1	9
	2	2	1	1	4
	3	3	1	1	5
	4	4	1	2	7
	Total	109	58	69	

The PPs are organized in follicles which contain multiple immune cells such as B cells, T cells and macrophages. PPs play an important role on the relationship between innate and adaptative

immunity in the gut, being able to identify antigens by their pathogen recognition receptors (PPRs) and generate a cascade of antibody production and cytokines and interleukins secretion (Jung et al., 2010; Panneerselvam & Vaqar, 2022). It is considered normal for a mouse to have between five to ten macroscopically visible Peyer's patches along the small intestine (from the proximal duodenum until the distal ileum). In this case, all groups showed similar numbers of PPs and their numbers mostly stayed inside the interval proposed by some authors (De Jesus et al., 2013). The duodenum had slightly more PPs than the rest of the small intestine in all groups. Male mice had between 5 and 11 PPs, and female mice had between 4 and 10. None of the groups differed significantly from the others according to the number of Peyer's patches. The results are expressed in *Figure 20.*

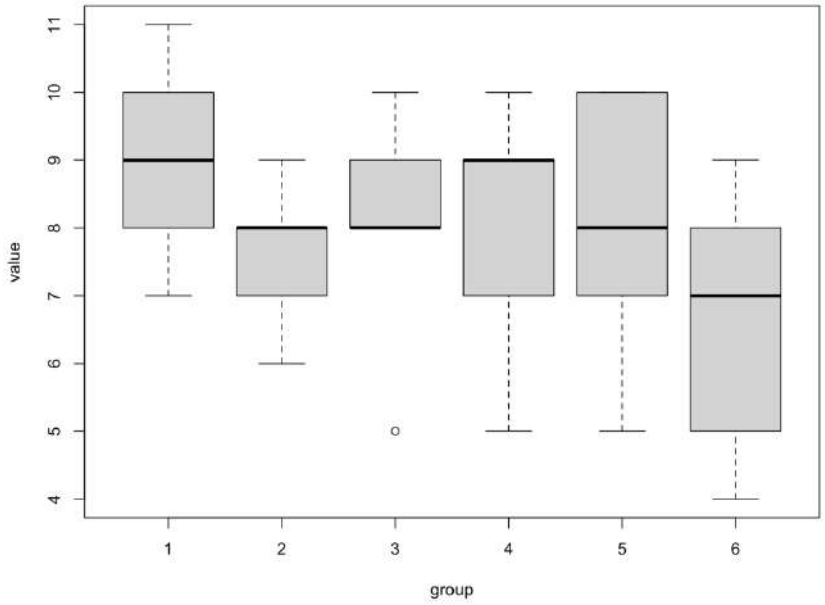

Figure 20. *Representation of the effects of the diet on number of Peyer's patches of each group and sex, being 1=G1M; 2=G2M; 3=G3M; 4=G1F; 5=G2F; 6=G3F.*

The PPs were stored in *cassettes* to do a histological analysis and later an immunohistochemistry determination of macrophages, and dendritic cells. However, the latest part did not take place in the pilot study. Two different types of tissue staining were done: firstly, the haematoxylin and eosin staining (H&E) and secondly, the Periodic acid-Schiff (PAS) staining.

H&E staining worked correctly (*Figure 20),* nonetheless, the PAS staining did not stain properly because the colouring agent was expired (*Figure 21)*.

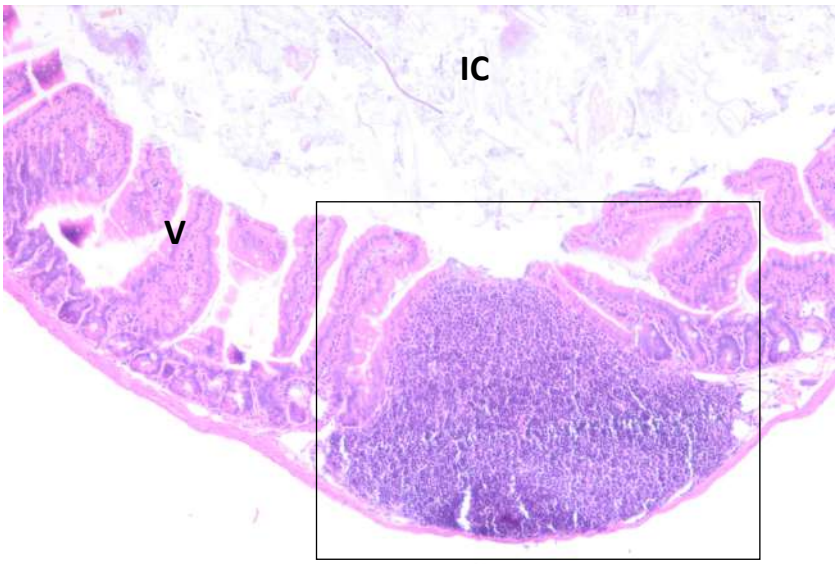

Figure 21. H&E-stained Peyer's patch (square) from the jejunum of a mice from group G3M. Abbreviations: V: villous; IC: intestinal content.

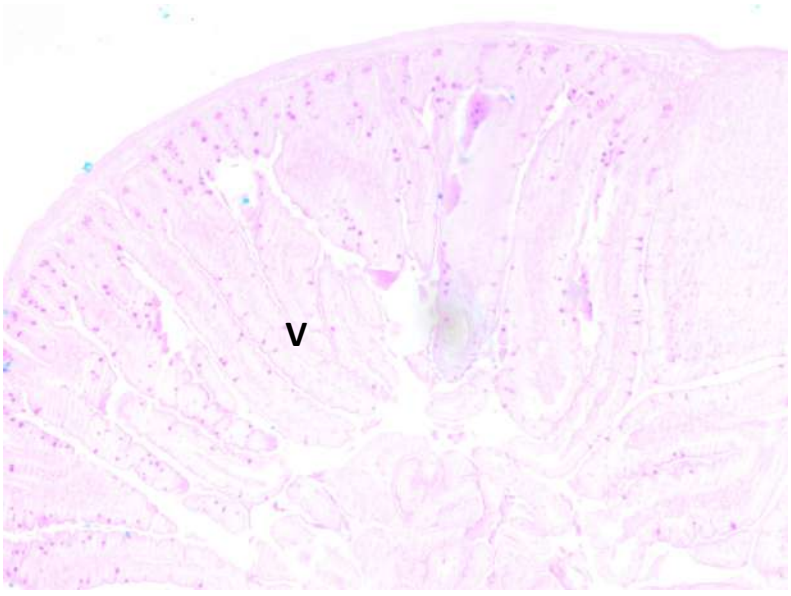

Figure 22. PAS-stained Intestinal epithelia from the duodenum of a mice from group G2F. The staining did not correctly exhibit due to the expiration of the coloring agent. Abbreviations: V: villous.

6.4. POINTS OF IMPROVEMENT

Some points of improvement were noted to meliorate the large-scale investigation. These refinements were the following:

- To reduce the total number of mice used in the experiment from 60 to 50 individuals.

- To maintain the group established in the mice supplier laboratory instead of randomly distribute the mice on the first day of arrival.

- To use multiple environmental enrichment objects such as a cardboard igloo and wood trunks to prevent fighting.

- To reduce the fiber composition of the standard chow diet to differ it from the second control group (the group fed a diet supplemented with white rice).

- To do the flow cytometry analysis of the spleen right after its extraction.

7. CONCLUSIONS

Taking into consideration the results obtained in this pilot study (*section 6)* regarding a R+D project that aims to analyze the immunological effects of orally administered barley to mice to act as a supplemental treatment against SARS-CoV-2, the following conclusions can be reached according to the objectives established at the beginning of this project (*section 3):*

1. The main goal to design and carry out a pilot study has been achieved successfully.

2. Both diets did not influence neither the average daily gain nor mice's behavior.

3. Some techniques of the experimental model suggested were tried out on the thymus, the spleen, and the Peyer's patches, but there were no differences between diets.

4. Some points of improvement were noted to meliorate the large-scale investigation.

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ANNEX

Annex 1. Nutritional composition and characteristics of the standard chow feed fed to mice.

2014

ENVIGO

Teklad Global 14% Protein Rodent Maintenance Diet

Product Description- 2014 is a fixed formula, non-autoclavable diet manufactured with high quality ingredients and designed to promote longevity and normal body weight in rodents. 2014 does not contain alfalfa or soybean meal, thus minimizing the occurrence of natural phytoestrogens. Typical isoflavone concentrations (daidzein + genistein aglycone equivalents) range from non-detectable to 20 mg/kg. Exclusion of alfalfa reduces chlorophyll, improving optical imaging clarity. Absence of animal protein and fish meal minimizes the presence of nitrosamines. **Also available certified (2014C) and irradiated (2914). For autoclavable diet, refer to 2014S (Sterilizable).**

Ingredients (in descending order of inclusion)- Wheat middlings, ground wheat, ground corn, corn gluten meal, calcium carbonate, soybean oil, dicalcium phosphate, iodized salt, L-lysine, vitamin E acetate, DL-methionine, magnesium oxide, choline chloride, manganous oxide, ferrous sulfate, menadione sodium bisulfite complex (source of vitamin K activity), zinc oxide, copper sulfate, niacin, calcium pantothenate, calcium iodate, pyridoxine hydrochloride, riboflavin, thiamin mononitrate, vitamin A acetate, vitamin B_{12} supplement, folic acid, cobalt carbonate, biotin, vitamin D_3 supplement.

Standard Product Form: Pellet

Macronutrients		
Crude Protein	%	14.3
Fat (ether extract) [a]	%	4.0
Carbohydrate (available) [b]	%	48.0
Crude Fiber	%	4.1
Neutral Detergent Fiber [c]	%	18.0
Ash	%	4.7
Energy Density [d]	kcal/g (kJ/g)	2.9 (12.1)
Calories from Protein	%	20
Calories from Fat	%	13
Calories from Carbohydrate	%	67
Minerals		
Calcium	%	0.7
Phosphorus	%	0.6
Non-Phytate Phosphorus	%	0.3
Sodium	%	0.1
Potassium	%	0.6
Chloride	%	0.3
Magnesium	%	0.2
Zinc	mg/kg	70
Manganese	mg/kg	100
Copper	mg/kg	15
Iodine	mg/kg	6
Iron	mg/kg	175
Selenium	mg/kg	0.23
Amino Acids		
Aspartic Acid	%	0.9
Glutamic Acid	%	2.9
Alanine	%	0.9
Glycine	%	0.7
Threonine	%	0.5
Proline	%	1.2
Serine	%	0.7
Leucine	%	1.4
Isoleucine	%	0.6
Valine	%	0.7
Phenylalanine	%	0.7
Tyrosine	%	0.4
Methionine	%	0.3
Cystine	%	0.3
Lysine	%	0.7
Histidine	%	0.4
Arginine	%	0.8
Tryptophan	%	0.2

Vitamins		
Vitamin A [e, f]	IU/g	6.0
Vitamin D_3 [e, g]	IU/g	0.6
Vitamin E	IU/kg	120
Vitamin K_3 (menadione)	mg/kg	20
Vitamin B_1 (thiamin)	mg/kg	12
Vitamin B_2 (riboflavin)	mg/kg	6
Niacin (nicotinic acid)	mg/kg	54
Vitamin B_6 (pyridoxine)	mg/kg	10
Pantothenic Acid	mg/kg	17
Vitamin B_{12} (cyanocobalamin)	mg/kg	0.03
Biotin	mg/kg	0.26
Folate	mg/kg	2
Choline	mg/kg	1030
Fatty Acids		
C16:0 Palmitic	%	0.5
C18:0 Stearic	%	0.1
C18:1ω9 Oleic	%	0.7
C18:2ω6 Linoleic	%	2.0
C18:3ω3 Linolenic	%	0.1
Total Saturated	%	0.6
Total Monounsaturated	%	0.7
Total Polyunsaturated	%	2.1
Other		
Cholesterol	mg/kg	--

[a] Ether extract is used to measure fat in pelleted diets, while an acid hydrolysis method is required to recover fat in extruded diets. Compared to ether extract, the fat value for acid hydrolysis will be approximately 1% point higher.

[b] Carbohydrate (available) is calculated by subtracting neutral detergent fiber from total carbohydrates.

[c] Neutral detergent fiber is an estimate of insoluble fiber, including cellulose, hemicellulose, and lignin. Crude fiber methodology underestimates total fiber.

[d] Energy density is a calculated estimate of *metabolizable energy* based on the Atwater factors assigning 4 kcal/g to protein, 9 kcal/g to fat, and 4 kcal/g to available carbohydrate.

[e] Indicates added amount but does not account for contribution from other ingredients.

[f] 1 IU vitamin A = 0.3 µg retinol

[g] 1 IU vitamin D = 25 ng cholecalciferol

For nutrients not listed, insufficient data is available to quantify.

Nutrient data represent the best information available, calculated from published values and direct analytical testing of raw materials and finished product. Nutrient values may vary due to the natural variations in the ingredients, analysis, and effects of processing.

Teklad Diets are designed and manufactured for research purposes only.