

A LOCALLY SYNTHESIZED PROBIOTIC FOR MAINTAINING IMMUNE SYSTEM FUNCTIONS IN IMMUNOCOMPROMISED NZW RABBITS

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ABSTRACT

This study aimed to develop a novel probiotic, using microorganisms isolated and identified from rabbit's colon, for developing a nutritional supplement to maintain and treat the weakened immune system responses in immunocompromised animals. 30 NZW rabbits divided into three groups, the first served as a control, while the other two were induced with Azathioprine (20mg/kg/Day) for two weeks to make them immunocompromised, one of them treated with the probiotic and the other group has left to heal naturally, both were monitored for another two weeks as treatment or healing period. Results indicated that treatment with this probiotic enhanced effectively the

immunocompromised rabbits, an improvement of full blood count has indicated in the treated group ($5.42 \pm 2.20 \times 10^9$), whereas the non-treated group was ($3.20 \pm 0.59 \times 10^9$), compared to control group ($9.68 \pm 0.30 \times 10^9$). A significant decrease recorded in lymphocytes percentage of rabbits treated with probiotic (25%), and non-treated (20%) compared to control (66%). Monocytes count has been higher in immunocompromised groups, treated and non-treated (10% and 12% respectively) compared to control (5%). Neutrophils count showed less percentage in non-treated (3%) and treated (2%) compared to control group (23%). These findings declared that the daily uptake of this probiotic has improved significantly the specific and nonspecific immunity of impaired rabbits, caused by the immunosuppressive drug. The study implicated the use of this product could be a basis for further studies to

develop a promising commercial product for reducing medical complications caused by an immunosuppressant given to human patients or domesticated animals under certain conditions.

KEYWORDS: Rabbits, Immunosuppression, Probiotic, Flowcytometry.

INTRODUCTION

The maturation and the optimal development of the immune system since birth depend on the development and composition of the indigenous microbiota.^[1] The evidence on the correlation between the composition of the colonizing gut microbiota and variations in host's immunity has arisen years ago.^[2] Gut microbiota is a highly specialized host-specific assemblages of microbes whereby metabolic activity directly impacts human health and disease. They live in harmony with the gut of the host and help to maintain the integrity of the gastrointestinal tract.^[3] In consequence of that, the Intestinal resident bacteria may exert a dual function: stimulation of mucosal mechanisms of defense, and maintenance of the homeostasis of the immune response. Immunosuppression involves an act that reduces the activation or efficacy of the immune system's components occurs as, in most cases, an adverse reaction to treatment of other conditions.^[4] Immunosuppressants drugs are used to control severe manifestations of allergic, autoimmune, and transplant-related diseases. Many of currently available immunosuppressants were been developed to be used in oncology or organ transplantation.^[5,6] Probiotic refers to group of organisms, that are live microorganisms, when administered in adequate amounts can confer health benefit on the host and could positively manipulate the composition and the function of the gut microbiome.^[3] They can be found in dairy and nondairy products, which are usually consumed after the antibiotic therapy for some illnesses, capable to destroy the microbial flora present in the digestive tract (both the useful and the targeted harmful microbes). Regular consumption of food containing probiotic microorganisms is recommended to establish a positive balance of the population of useful or beneficial microbes in the intestinal flora.^[7] The genera of bacteria and fungi that have been employed for their probiotic properties are most commonly species of *Lactobacillus*,^[8,9] *Bifidobacterium*,^[10] *Streptococcus*,^[11] *Escherichia coli*,^[10] and species of the yeast genus of *Saccharomyces*.^[12] Genera of *Enterococcus*, and *Bacillus*, have also been studied but, however, particular concerns have been raised upon their safety properties.^[13] Probiotics come in many forms, including powders, tablets, capsules, and foods, such as yogurts and dairy drinks. The form of

taking them in does not matter as long as it contains enough organisms to grow in the intestines. The effective dose varies, from as little as 50 million to as many as 1 trillion live cells per dose.^[14,15]

METHODOLOGY

Total of thirty (30) New Zealand White (NZW) rabbits, weighing 2100-2255g, obtained from the Experimental Animal Breeding and Research Centre (University Putra Malaysia), and selected with high accuracy and reconstructions, according to the “Institutional Animal Care and Use Guidelines” approved by the Animal Ethic Committee (University of Kuala Lumpur), were used in this study. They divided into three main groups of 10 animals each. Two experimental groups induced with Azathioprine (Imuran[®]) to be immunocompromised, while the third group left healthy to serve as normal control group. The animals were hosted in the Animal Holding Unit of the Faculty of Science and Mathematics (University Pendidikan Sultan Idris-UPSI), under controlled conditions (room temperature 25-30°C, humidity 70-80%, and automatic 12hr light-dark cycle). Prior to the experiment, all rabbits physically examined to ensure that they are free of infections or diseases, and with no deformities. They have been acclimatized for one week, and were fed with standard pellets and free accesses of drinking water (*at libitum*). All rabbits of the experimental groups were monitored during two different periods of time; the first period was for immunosuppression (two weeks) and the second period was for healing efficiency (two weeks). Samples of blood and of rabbit’s colon collected for microbial and immunological examinations, as well as, for probiotic preparation.

Immunosuppressant Drug

Azathioprine (Imuran[®]) is the chemical drug used for immunosuppression in this study, administrated orally to the rabbits using fed tube. It has been experimented by using different tests and doses using several pilot studies, prior starting the experiment, to come out with the most effective dose for NZW rabbits. We concluded that the dose of 20 mg/kg/Day for two week was the best dose to make these animals totally immunocompromised. The least doses given were needed a longer period to suppression, and higher doses used caused some side diseases, like leukemia, or death. The pilot studies were also important to obtain the safety and the effectiveness of the treatment we decided to test our synthetic probiotic. These studies were helpful to finalize the best effective number of doses, and the period of time needed for treatment as well, quantitatively and qualitatively.

THE MICROBIAL INVESTIGATION

Twisted and circular motion swabs of the target colon area have been collected. These swabs placed in sterile collection of transport tubes within 2 hours of animal sacrifice time. They sub-cultured directly on Blood Agar (BA), Nutrient Agar (NA), MacConkey Agar (MA), and Eosin Methylene Blue (EMB), and kept for incubation at 37°C for 24 hours under aerobic and anaerobic conditions (BD GasPack™ EZ gas generating systems). All isolates of colonies were tested for Gram-staining and culture plate colony morphology. Washing samples undergo with the serial dilution technique, and then, these serials were plated by using spreading technique. The plates incubated under aerobic and anaerobic condition at 37°C for 24 hours. The growing colonies were calculated to gain the colony forming unit per milliliter. Meanwhile, the remained washing samples kept and stored at 4°C. In most cases, the cultivated microorganisms were identified on the basis of colony morphology and Gram-staining results, only those microorganisms that have particular interest were identified further, up to genus and species level, using series of biochemical tests and API method.

Preparation of Probiotic

Picked up the most common bacterial colonies selected, from growth of control rabbits' samples, and transferred to MRS broth (de MAN, ROGOSA and SHARPE, Merck) series of dilutions to conduct selective enrichment, and then run further diagnostic tests in order to use it in the production of the probiotic. At the end of all these tests we have decided to choose among the selected and isolated species four of the most common bacterial strains to synthesize our probiotic. These strains are considered as well among the most commonly used in term of safety in certain commercial probiotics productions, which includes: *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium bifidum*, *Bifidobacterium longum*. These chosen bacterial strains were grown on nutrient agar and incubated in anaerobic incubator at 37°C for 48 hours. After that, each strain calculated by serial dilution. The two billion CFU per strain were used for the manufacture of the probiotic. These strains, with the chosen CFU amount, in the nutrient broth, were administrated to the rabbits after mixing with skim milk (as carrier material) using feeding tube through the mouth.

Handling, Restraint & Blood Collection

Removal of blood from the marginal ear vein or artery is one of the most common and least invasive methods of collecting blood from a rabbit. This technique can be used with all strains and for single and repeat samples. The use of the artery is normally common for larger volume samples or for arterial blood, but carries a greater risk of haematoma and bruising. The rabbit were placed in the rabbit restrainer and the hair removed from the ear using the clippers. A needle of 1 or 1.5 inches (20 to 22 gauges) used for blood collection. The rabbit's ear extended from the head to provide a flat surface and then the needle inserted into the artery. Once blood begins to flow, lower the ear and position the receptacle under the needle for blood collection. The central ear artery may give a relatively large volume of blood up to 15 ml. The blood was collected in 4 ml EDTA tube (BD Vacutainer), and 4 ml plain tube (BD vacutainer).

The Immunological Study

The level of immune status in blood was measured by Full Blood Count (FOC), and Standard panel of T cells (CD3), B cells (Mouse anti Rabbit IgM), Subset T Lymphocyte (Mouse anti Rabbit CD4, Mouse anti Rabbit CD8). In this study the Flowcytometer (BD FacsCanto II) used. The rabbit immunology kits includes the followings reagents: Antibody CD45 APC, Antibody CD14 PE, Antibody T-Lymphocyte FITC, Antibody B cells FITC, Antibody CD4 FITC, Antibody CD8 FITC, Secondary antibody APC conjugated, BD 1 x lysing Solution, and BD Trucount tube.

Full Blood Count Analysis (Beckman Coulter)

Most blood counts today include the FBC count and leukocyte differential count (LDC) that is, not just the total white blood cell (WBC) count but also the count of each WBC type, such as neutrophils, eosinophils, basophils, monocytes, and lymphocytes. The blood is well mixed (though not shaken) and placed on a rack in the analyzer. Blood counting machines aspirate a very small amount of the specimen through narrow tubing followed by an aperture and a laser flow cell. Laser eye sensors count the number of cells passing through the aperture, and can identify them, this is flowcytometry. The two main sensors used are light detectors and electrical impedance. The instrument measures the type of blood cell by analyzing data about the size and aspects of light as they pass through the cells (called front and side scatter) and measure different characteristics of the cells to categorize them.

Flowcytometer Canto Clinical II

Flowcytometry is a technology that is used to analyse the physical and chemical characteristics of particles in a fluid as it passes through at least one laser. Cell components are fluorescently labeled and then excited by the laser to emit light at varying wavelengths. The fluorescence can be measured to determine various properties of single particles, which are usually cells. Up to thousands of particles per second can be analyzed as they pass through the liquid stream. Examples of the properties measured include the particle's relative granularity, size and fluorescence intensity as well as its internal complexity. Flow Cytometer provides rapid analysis of multiple characteristics of single cells. The information obtained is both qualitative and quantitative. Contemporary flow cytometers are much smaller, less expensive, more user-friendly, and well suited for high-volume operation. Flowcytometry technique is used for immunophenotyping of a variety of specimens, including whole blood, bone marrow, serous cavity fluids, cerebrospinal fluid, urine, and solid tissues. Characteristics that can be measured include cell size, cytoplasmic complexity, DNA or RNA content, and a wide range of membrane-bound and intracellular proteins.

Data Analysis

The responses to the structured close-ended questions will be rated in percentages. The percentage of respondents for each alternative will be given and analysed. The data collected will be analysed using the computer software 'Statistical Package for Service Solution' (SPSS).

RESULTS

Colon microbial samples were taken, using sterile swabs and cultured on agar plates by streaking technique, and incubated in aerobic and anaerobic incubators at 37°C for 24 hrs. All plates were observed for the colony morphology and for microbial growth. Gram staining and serial dilution techniques used to obtain the results of the bacterial growth (Fig.1) and the bacterial concentration (Table 1) for the sample. The microbial growth isolation and identification results indicated that the majority of bacterial species recorded were Gram positive Bacilli and Gram negative Rods (mainly *Bacteroides spp.*, *Bacillus spp.*, and *Aeromonas spp.*).

Results indicated that probiotic treated rabbits showed an improvement of full blood count ($5.42 \pm 2.20 \times 10^9$), whereas, the non-treated group ($3.20 \pm 0.59 \times 10^9$), compared to control group ($9.68 \pm 0.30 \times 10^9$) (Fig.2).

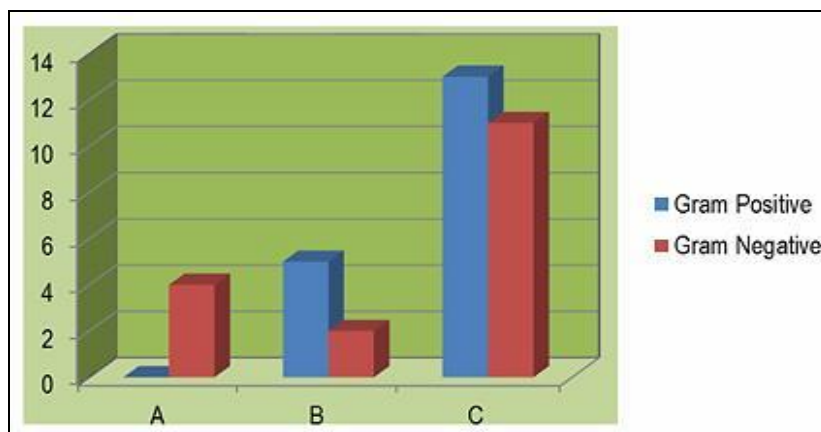


Figure 1: Gram Staining Results of isolated colonies according to the tested groups

Table 1: Distribution of the obtained microbiota in the three groups studied

Group	Gram Stain (per colony)		Bacterial Calculation (CFU/mL)
	Gram +ve	Gram -ve	
A	0	4	1.58x10 ⁸
B	5	2	5.0x10 ⁶
C	13	11	4.0x10 ⁶

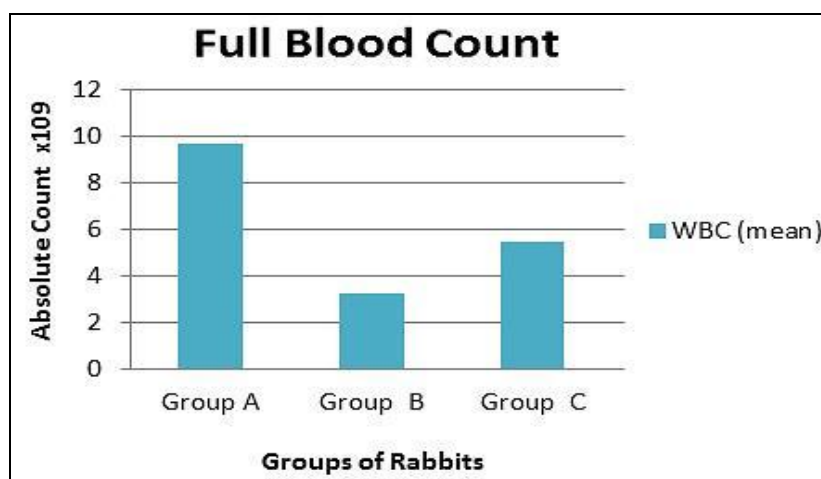


Figure 2: Comparison of means (\pm SD) among the experimental groups

The mean of WBC count has decreased in the immunocompromised group, while increased in probiotic treated group. The differential blood count (differential leukocytes count) of the probiotic treated rabbits (Group C) showed increase in lymphocytes and monocyte counts compared to non-treated (Group B) and control (Group A). Lymphocytes of the probiotic treated group scored a higher percentage (25%) compared to non-treated group (20%). Monocytes count has been higher in immunocompromised rabbits groups B and C (10% and 12% respectively) compared to control group (5%). While neutrophils count showed less

percentage in immunocompromised non-treated group B (3%) and immunocompromised probiotic treated group C (2%) compared to control group A (23%). These results indicated that lymphocytes play the major role in adaptive immunity, since rabbits have been induced to severe decrement in their immunity (totally immunocompromised) (Fig.3).

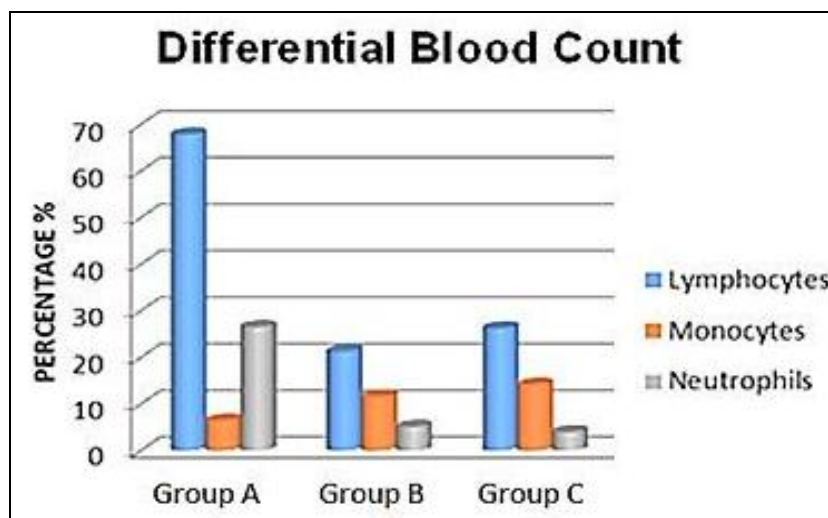


Figure 3: Comparison of the cellular immunity by groups of rabbits

The statistical analysis of the data showed a significant increase ($p \leq 0.05$) in WBC and monocytes counts of the immunocompromised groups, whereas significant decrease in lymphocyte count and insignificant decrease in neutrophils count, compared to control group (Table 2).

Table 2: data analysis of cellular immunocytes of the four groups studied

Variable	F	P value
WBC	21.32	0.000*
Lymphocyte	10.52	0.000*
Monocytes	74.13	0.000*
Neutrophils	3.007	0.061

* Significant at $P \leq 0.05$

Flowcytometry results (Standard T Panel analysis) showed populations of T lymphocyte, neutrophils, monocyte, B cells, CD4+ and CD8+ in the forward scatter and side scatter. Probiotic treated group showed an increment in cell percentage of CD4 (24%), lymphocytes (23%), neutrophils (19.8%) and B Cells (18.9%) compared to control rabbits, as CD4 (10.6%) and neutrophils (13.3%). The rest of cells populations only showed less than 10% (which are undetectable by the flowcytometer) (Fig.4).

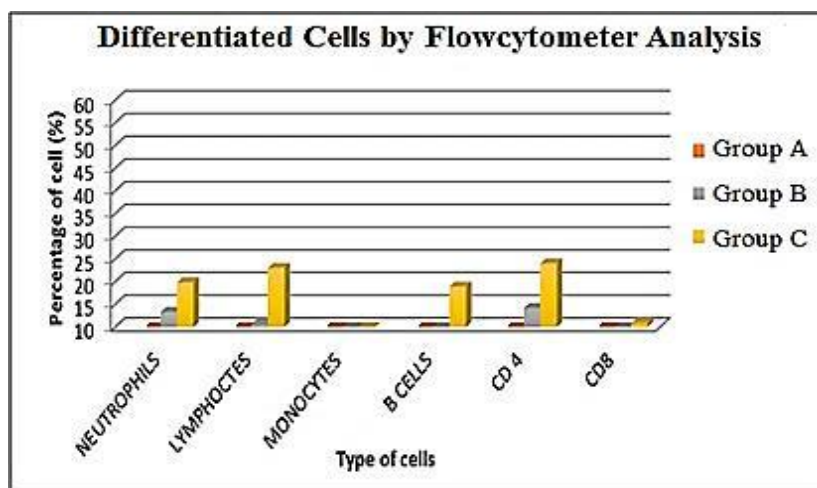


Figure 4: Differentiated cells by flowcytometer showing the positive populations of neutrophils, lymphocytes, monocytes, monocytes, B cells, CD4+ & CD8+

DISCUSSION

Probiotics have been among the recent subjects of numerous scientific studies and publications demonstrating their therapeutic effectiveness on both systemic and gastrointestinal tract. Recently, probiotic microbes have gained more attention for their direct and indirect inhibitory effects on cancer cells initiation and progression. Several studies have shown tumor suppressing properties for certain *lactobacillus* strains,^[16,17,18] such anti-tumor effects assumed to be exerted via different mechanisms, for example inhibition of pathogens colonization,^[19] induction of immune system,^[20] direct cytotoxic effects on cancer cells,^[17,18] anti-mutagenic effects,^[21] and modulation of carcinogens metabolism and prevention of DNA from oxidative damage.^[22] The antifungal activities of *Lactobacillus* stains have demonstrated in previous works as well.^[23,24] Other previous studies^[25,26,27,28,29,30] have claimed that probiotic microbes are capable to exert improvements to the immune functions those damaged by different immunosuppressive agents. It has been assumed that lactobacilli and their different strains could modulate innate and adoptive immune system, such as modulation of function of T cells, dendritic cells, and macrophages, as well as, cytokine production.^[31]

Rabbits are kind of typical herbivores whose diets contain large amounts of cellulose, which is decomposed by intestine microorganisms. So the gastrointestinal tract of rabbit is a complex ecosystem that often invades with a variety of bacterial community, which provide additional components to the host but these invading bacteria may affect the host biology.

The disorder of intestine bacteria can be an important reason for high diarrhea, morbidity, and mortality.^[32]

It is known that probiotic activity could be related to genera, species, or strains of included microorganisms. An approach in probiotic application could be the use of mixtures of strain belonging to different genera or species.^[33] Although the intestinal microbiota is complex and the role of most of the bacteria in providing benefit to the host is not clear yet, bacterial species of the genera *Lactobacillus* and *Bifidobacterium* have been shown to supply protection against enteric infections. Moreover, these species are considered to be safe strains in probiotics preparations (as related to their pathogenicity and their commensalism relationships with other microbial flora and pathogenic microbes in the colon) and also they are among the potential bacterial strains commonly used in commercial probiotics (as related to their resistance to low pH and bile, adhesion to epithelial cells, and antimicrobial activity).

However, not all probiotic strains have the same mechanisms of action and each has characteristics suitable for the product application.^[23] In rabbit digestive tract, lactobacilli didn't represent a high part of microbial flora compared to other bacterial strains. Different lactobacilli found in the gastrointestinal tract are concerned with the balance of microbiota and it has been widely studied due to their health-promoting properties.^[34] Their effects on intestinal microbiota in terms of protection include competition for adhesion sites with pathogenic microorganisms and antimicrobial substance production, such as organic acids, lactic acid, carbon dioxide, and bacteriocins.^[35] In addition, the regular use of probiotic appears to prevent certain gastrointestinal disorders, such as lactose intolerance. Other lactobacilli strains have the ability to produce hydrogen peroxide (H₂O₂), which can be toxic to microorganisms that do not produce catalase.^[36]

CONCLUSION

It has been well established long ago that resident microbiota in the gastrointestinal tract is able to provide resistance to disease and restore the balance of resident microbiota. However, imbalances in the microbial components can promote the growth of opportunistic microorganisms. In this regard, it is believed that probiotic bacteria can provide an alternative strategy for the prevention and treatment of infectious diseases and to boost immune system responses, instead of using chemical medications or antibiotics those normally come with unwanted side effects. In addition, the use of probiotics and their natural metabolic compounds, can be a substitute in various pharmaceutical industries.

Importantly, this study provides useful information on the effectiveness of treatment of the impaired immune system (tested on immunocompromised NZW rabbits), as a model of human disease, using a synthetic probiotic, developed from rabbits own colonal microbes. The obtained results of this study could be used as a reference to design new generations of very hoping immunenutritional supplements that can lead in future to develop an effective product to help mainly groups of human patients whom under surgery, trauma, burns and injury by enhancing their immunity and maintaining their body immune system. For Human Immunodeficiency Virus (HIV) or Acquired Immune Deficiency Syndrome (AIDS), cancer and chemotherapy patients, these kinds of nutrients will support their immune functions, since they are at immunodeficiency state. However, more research is still required to spread and improve actual knowledge about these natural additives, to understand their mechanisms of action in animals and to achieve higher effectively on the health and production of rabbits. There is the need to look for viable alternatives that could enhance the natural defence mechanisms of animals and reduce the massive use of antibiotics.

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