TOXICOLOGICAL ASSESSMENT OF *Lactobacillus plantarum* AS A PROBIOTIC STRAIN IN DARK CHOCOLATE

Nazaruddin Ramli*, Siti Radhiah Omar, Shubha Sri Ramakrishnan.
University Kebangsaan Malaysia, Faculty of Science and Technology,
School of Chemical Sciences and Food Technology, 43600, Bangi, Selangor, Malaysia.
*E-mail: naza@ukm.my

Abstract

This study was conducted to determine the toxicity effect of isolated Lactobacillus plantarum from cocoa bean fermentation on Sprague dawley rats. The toxicity effect of L. plantarum was tested using three types of doses which were 10^6 cfu/mL (low dose), 10^8 cfu/mL (medium dose), and 10^{10} cfu/mL (high dose) and compared with a control group. Each dose is then formulated in dark chocolate and was toxicologically assessed. For sub acute toxicity test, rats were weighed, and their volume of water intake was recorded weekly which last for 28 days followed by haematology test at the final day of the experiment being conducted. To ascertain the survival of L. plantarum in the gastrointestinal tract of the rats, Feces Restoration Analysis was done by serial dilution with 10^{-1} to 10^{-6} dilution factor on MRS agar. The sub-acute test showed neither toxicity effects nor death incidents occurred. There was no significant difference showed in any of the L. plantarum dosage blood parameter tests that were conducted at ($p<0.05$). For the platelet count, the rats treated with all dosages of L. plantarum for ALT, AST and ALP has a significant difference ($p<0.05$) when compared to control group (117 ± 11.57 IU/L). There was no significant reduction in urea and creatinine value respectively. Both medium and high concentrations of L. plantarum vividly declined as compared to the control group (41-58%) and the low dosage group for haematocrit. In conclusion, the different dosages of L. plantarum in dark chocolate proven no toxicity effect and are safe for consumption.

Keywords: dark chocolate, *Lactobacillus plantarum,* probiotic, *Sprague dawley,* toxicity.

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1. INTRODUCTION

Lactic acid bacteria (LAB) are gram-positive, rod-shaped, non-spore forming and fermentative bacteria that grow anaerobically (Holzapfel et al., 2001). This group of bacteria produces lactic acid as a minor product in carbohydrate fermentation processes. The largest group of lactic acid bacteria belongs to the genus *Lactobacillus*, which comprises more than 50 different species (Stiles and Holzapfel; Tannock, 1999). *Lactobacillus* species are found in the gut of humans and animals, but they may vary in number among animal species according to the age of the host or their location within the gut (Maaike et al., 2006). However, only a few *Lactobacillus* species contain representatives that reside in the human gut and are involved in both traditional and industrial food fermentations. Those species include *L. crispatus*, *L. gasseri*, and *L. plantarum* (Cataloluk and Gogebakan 2004). In this review, we will focus on *L. plantarum*, which has been used for centuries in human food preservation. *L. plantarum* is essential for good health, and fortunately it is an easy probiotic to incorporate into a diet. This species may exist in various fermentation products, such as meat (Italian fermented sausage and dry fermented sausage), vegetables (cocoa seed, olive, wain and cassava) and dairy products (Stilton cheese, ricotta forte and traditional feta cheese) (Vries et al., 2006).

L. plantarum was grouped into homo-fermentative lactic acid bacteria due to its production of lactic acid without alcohol (Battock and Azam-Ali, 1998). These species of bacteria were identified in human gastrointestinal tracts a long time ago, and they have the potential of lowering the risks of heart attack and an immobilized bowel system. In cocoa fermentation, a high volume of lactic acid bacteria has been reported, and the dominated species has been found to be *L.*

plantarum and *Acetobacter pasteurianus* (Camu et al., 2006). An isolated *L. plantarum* from ogi (fermented corn slurry) was studied to identify its probiotic nature (Oyetayo and Osho et al. 2004). Probiotics are defined as living microorganisms when administered in adequate amounts, confer a health benefit to the host. An ideal probiotic should be able to adhere to the intestinal epithelium and remain viable in the intestine (Boyle et al., 2006).

Probiotic chocolate products are well known in foreign countries, but the studies of the toxicity effects of this healthy product has never been reported. Research involving probiotic has shown only the positive effects of these bacteria in clinical health care. For example, using *L. plantarum* PH04, (Nguyen et al., 2007) found that this bacterial strain has the potential to reduce cholesterol. Moreover, (Bernandeau et al., 2007) who used *L. rhamnosus* MA27/6B and *L. acidophilis* MA27/6R reported that both LAB bacteria were not pathogenic; they reported that the bacteria were not only safe to eat by animals, but they also acted as growth inducers. This study aimed to review the toxicity effect of *L. plantarum*, which was isolated from cocoa fermentation to observe *S. dawley* rat growth. This research was conducted based on the response of rats toward different levels of doses. A sub-acute toxicity test was performed, and a blood profile was analyzed to determine the toxicity levels of different doses.

2. MATERIALS AND METHODS

Bacterial, Chocolate and Cultivation *L. plantarum* was isolated from a fermented cocoa bean. The chocolate was supplied by the Barry Callebaut Company. The bacteria were then inoculated in 9 mL of sterile *Lactobacilli* MRS broth containing peptone and dextrose. The culture was incubated overnight at 37 °C before being stored at -80 ºC.

Serial Dilution and Dose Preparation A serial dilution with dilution factors of 10^{-1} to 10^{-8} of *L. plantarum* stock culture was performed for three types of ratios, which were 1:1, 1:20 and 1:300. For each ratio, nine test tubes containing 9 mL of sterile dilution water was prepared, and 1 mL of *L. plantarum* from the stock culture was added to the first test tube. For each ratio, 0.1 mL of dilution from 10^{-4} to 10^{-8} was spread on MRS agar and incubated overnight at 37 °C (Susan et al., 1983). The numbers of colonies formed were counted by an electronic cell counter using the following formula to obtain the total colony forming unit (cfu) for each ratio:

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$$N = \frac{C}{V(n1 + 0.1\ n2)d}$$

L. plantarum doses were prepared according to the previous serial dilution. The three concentrations of *L. plantarum* doses were obtained from the three ratios (1:1, 1:20, 1:300). The high dosage was obtained from the 1:1 ratio, the medium dosage was obtained from the 1:20 ratio, and the low dosage was obtained from the ratio of 1:300. Dark chocolate containing doses of *L. plantarum* were prepared for 28 days of experiment. Chocolates were melted and placed in a mixture machine for two hours until the temperature reached 33 ºC to stabilize the liquid. Melted chocolate was weighed at 99 g and mixed with 33 mL of diluted liquid according to the ratio. Chocolates were then divided into 3 g per portion.

Sub-acute Toxicity Test

A lethal dose (LD_{50}) was determined within the three prepared doses. About 20 adult males of *S. dawley* rats were divided into four groups. Each group represented the control, low, medium and high dosage phase. A pure dark chocolate without *L. plantarum* was given to the control experimental mice. The other three groups of mice were given the dark chocolate with the three level doses of *L. plantarum*. All of the mice were supplied depending on their body weights, and they were fed with a ratio of 8.6 g/kg. The weight changes and the volume of water taken by the rats were recorded.

Blood Profile Determination
A blood profile was determined by a haematology test (number of red blood cells, white blood cells, haemoglobin and platelets), a liver functional test involving enzyme alanine aminotransferase (ALT), aspartate aminotranferase (AST), alkaline phosphate (ALP) and a kidney test (urea and creatinine). Blood was taken through capillary reaction (Animal research, 2002) after 28 days of the experiment and submitted to a toxicology lab to be analyzed using the haematology analyzer Boule Medoric CA500-16 VET. Serum from the blood sample was obtained through the vena cava posterior. Blood was placed into an anti-coagulant tube and centrifuged at 2,500 x g for 15 minutes at 4 °C before being analyzed by the blood chemical analyzer Vitalab Selectra E (Norliza 2004).

Feces Restoration Analysis About 25 g of mouse feces were collected and soaked in 225 mL of peptone water before overnight storage at 4 ºC. A serial dilution using peptone water was done for dilution factor 10^{-1} to 10^{-6} and spread on MRS agar. Colonies that formed were counted (Wang et al., 2007).

3. RESULTS AND DISCUSSION

Subacute Toxicity Test
No cases involving death were recorded during the experiment, which proved that the three levels of doses were not LD_{50}. No obvious changes in mouse behavior, daily activity, or physiology were observed.

ALT, AST and ALP Levels
The concentration of ALT and ASP in the blood vessels will increase if any abnormalities in liver function occur. For the four types of behaviors tested, it was found that levels of ALT increased in all groups compared to the normal range of an adult *S. dawley*, which is 25-35 IU/L (Table 1) (Grad 2007). The concentration of ALT for the control mice was 117 ± 11.57 IU/L, whereas the concentration for the low dosage was 166.90 ± 23.06 IU/L.

Table 1 Clinical Adult *S. dawley* Pathology Reference (Gad 2007)

Test	Units	Male	Female
Blood cell count	X10^6/µ	6.7 - 9	5.7 -9
Hemoglobin	g/dl	13 -17	11 -17
Hematocrite	%	41 -58	39 - 55
Corpuscular Minimum Volume	Fl	55 - 65	55 - 65
Hemoglobin Min corpuscular	Pg	16-22	17 -22
Hemoglobin Min corpuscular concentration	%	28 -34	28 - 34
Platelet count	X10^6/µl	700 - 1500	700 - 1500
Protrombin time	Sec	12 -17	12 -18
Half time trombo plastine	Sec	17 - 27	17 - 27
White Blood Cell count	X10^3/µl	3.0 -14.5	2 - 11.5
Segmented neutrophil	X10^3/µl	0.3 - 3	0.01 -2
Neutrophile line	X10^3/µl	0 - 0	0 - 0
Lymphocytes	X10^3/µl	3 - 12	1.0 - 10
Monocytes	X10^3/µl	0 - 0.5	0 -0.03
Eosinophile	X10^3/µl	0- 0.03	0 -0.03
Basophile	X10^3/µl	0 -0	0 -0
Nucleated red blood cell count	/100 WBC	0 - 2	0 - 2
Glucose	mg/dl	70 - 125	70 -120
Total protein	g/dl	5.6 - 7.1	5.5 -7.3
Albumin	g/dl	3.9 - 4.9	4. - 5.2
Globulin	g/dl	1.5 - 2.3	1.4 -2
Cholesterol	mg/dl	42 - 90	45 -100
Triglyceride	mg/dl	30 -90	15 - 40
Urea nitrogen	mg/dl	10 -16	10-19
Creatinine	mg/dl	0.5 -0.8	0.5 -0.8

Table 1 - continuation

Total bilirubin	mg/dl	0 - 0.2	0 - 0.2
Aspartate amino transferase	IU/L	60 - 300	80 -250
Alanine amino transferase	IU/L	25 - 55	25 -50
Alkaline phosphatase	IU/L	85 - 245	60 -110
γ-Glutamyl transferase	IU/L	0 - 1	0 - 1
Creatinine kinase	IU/L	244 - 254	241-254
Calcium	mg/dl	8.5 -10.5	8.5 -10.2
Inorganic Phosphate	mg/dl	6 - 9.5	6 - 9
Sodium	mmol/L	139 - 155	139 -55
Potassium	mmol/L	4.4 – 5.7	4 - 5.5
Chloride	mmol/L	100 – 115	100 –113

For the medium and high doses, the concentrations of ALT decreased as compared to the low dose groups of mice, which were 160.90 ± 23.06 and 139 ± 15.30 IU/L, respectively. No toxicity levels were shown with respect to AST and ALP in the blood because the results obtained were still in the range of normal *S. dawley* adult rats, which are around 60-300 IU/L and 85-245 IU/L, respectively (Fig. 1).

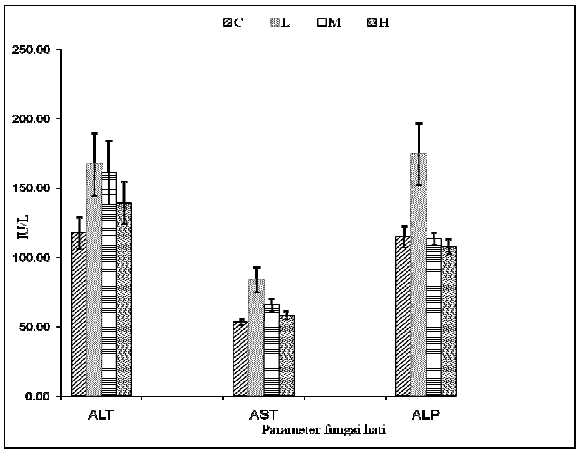

Fig. 1. Value of toxicity level for liver function parameter

C = control; *L* = low dose-10^6 cfu/ml of *L. plantarum*; *M* = medium dose-10^8 cfu/ml of *L. plantarum*; *H* = high dose-10^{10} cfu/ml of *L. plantarum*

Doses with *L. plantarum* were significant ($p < 0.05$) as compared to the control group for all three parameters (ALT, AST and ALP). For the ALT concentration, three types of doses showed significant changes ($p < 0.05$), which show the possibility of liver toxicity. The use of LAB bacteria in food caused different levels of hepatocellular damage, which can be identified through the secretion of certain enzymes in the blood. This can be corroborated by previous studies that used different types of *Lactobacillus* (*L. rhamnosus* and *L. rhamnosus* + *L. plantarum)*. Previous research showed that the level of ALT decreased in comparison to control groups (Adawi et al., 2001). This opposes our result because hepatocellular damage may also be caused by other factors, such as changes in the number of intestinal micro-flora, which can produce constipation.

Toxicity Effects on Urea and Creatinine.

The control experimental mice gave the highest value for the urea nitrogen test. The value was 46.48 ± 1.11 mmol/L, while the second highest value was obtained from the groups of mice that were treated with the low dose of *L. plantarum*. Both groups of medium and high dosages gave almost similar results, which were 41.85 ± 1.64 mmol/L and 41.98 ± 1.73 mmol/L, respectively, and no significant reduction in value ($p < 0.05$) was shown. For the creatinine analysis test, no significant difference ($p < 0.05$) was shown between control experimental mice and treated mice with dosages of *L. plantarum*. The results obtained for the control group, the low dose, medium dose and high dose were 4.44 ± 0.25 mmol/L, 5.55 ± 0.58 mmol/L, 4.59 ± 0.40 mmol/L and 4.91 ± 0.37 mmol/L, respectively (Fig. 2).

Nitrogen urea is an indicator of kidney damage. A high secretion of a catabolite product, such as protein within the kidney, will lead to an increase in nitrogen urea levels. Creatinine will act as an indicator for acute renal failure where there is an abrupt or rapid decline in renal filtration function.

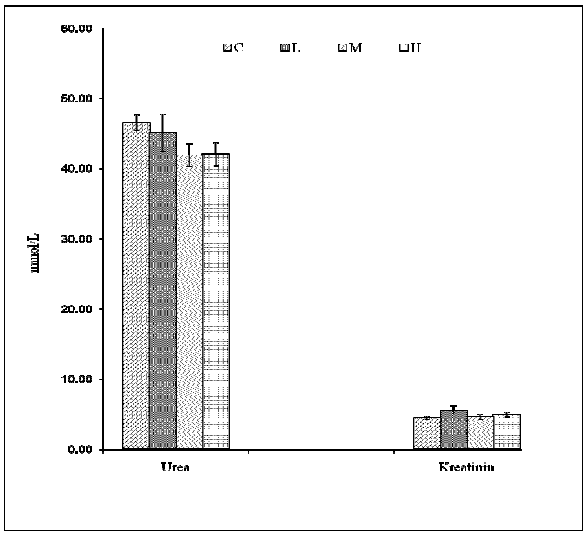

Fig. 2. Value of toxicity level for kidney parameter
C = control; *L* = low dose-10^6 cfu/ml of *L. plantarum*; *M* = medium dose-10^8 cfu/ml of *L. plantarum*; *H* = high dose-10^{10} cfu/ml of *L. plantarum*.

Creatinine is a product of the breakdown of creatinine phosphate in the muscles, and it is usually produced at fairly constant rates. It is filtered out of the blood by the kidneys, and, when the renal function fails to act normally, the concentration of creatinine in serum will increase. The renal function may stop for hours, days or even weeks. From the data of the experiment, we can say that *L. plantarum* supplied with chocolate does not interfere with renal function.

Haematology Test

The number of red blood cells (RBC), haematocrit (HCT), white blood cells (WBC), haemoglobin (HGB) and platelets (PLT) were recorded (Table 2). Even though an inconsistent number of red blood cells were obtained each week, the counted cells in all of the experimental mice were still within the normal range for adult *S. dawley*, which is about 6.7 – 9.0 x 10^6/µ. As for the white blood cells, the numbers of cells that were counted in all mice were also within the value of 3.0 – 14.5 x 10^3/µ, which is normal for rats. Haemoglobin analysis showed that all of the values obtained were in the normal range of 13.0 – 17.0 g/dl. For haematocrit, only the control experimental mice showed values within the normal range for adult *S. dawley*, which is 41-58 %; no significant decrease ($p < 0.05$) was shown. A significant difference was only shown in the platelet analysis for medium and high concentrations of *L. plantarum*, where the value for both decreased dramatically as compared to the control group and the low dosage group.

A red blood cell count is normally used to indicate anaemia and polycythaemia. Anaemia is a decrease in the normal number of red blood cells, or less than the normal quantity of haemoglobin in the blood, while polycythaemia is a disease in which the proportion of the blood volume that is occupied by red blood cells increases due to an increase in the mass of red blood cells or a decrease in the volume of plasma. A white blood cell count is essential in the detection of myelopoiesis and the identification of leukaemia. Other research on white blood cells also has been carried out to determine the relationship between the white blood cell count and fatal and non-fatal coronary heart disease (CHD) and cancer mortality and its relationship to cigarette smoke (Richard et al., 1985). The analysis of haematocrit is used to determine whether humans or animals suffer from haemolytic anaemia or polycythaemia. Haematocrit is the percentage of the total volume of red blood cells in the body. Patients with haematocrit levels less than 30 % show significantly higher risks of all-cause (12 to 33 %) and cause-specific death as compared with patients with haematocrit levels in the 30 to 33% range (Jennie et al., 1999).

Haemoglobin also plays an important role in the detection of anaemia and polycythaemia by measuring its level in blood. Haemoglobin is the iron-containing oxygen transport metallo-protein in the red blood cells of vertebrates; it acts as a buffer when carbon dioxide is formed during the respiration process (Dominguez et al., 1981). Platelets are counted to identify purpura or petechiae disease, leukaemia and chemotherapy for malignant disease, where the decrease of plate number is recorded. Purpura is the appearance of red and purple discoloration on the skin due to bleeding under the skin. Purpura measures

0.3-1 cm, whereas petechiae measures less than 3 mm, and ecchymoses is greater than 1 cm (Mitchell et al., 2007). The reasons for a decrease in the platelet number in this experiment may due to the insufficient of the coagulant agent in tube during the blood sample collection or an indication of the symptoms of purpura and petechiae disease in mice caused by the *L. plantarum* dosage.

Body Weight and Water Intake

The body weight of the experimental rats increased during the 28 days of the experiment. The highest rate occurred in rats treated with the medium dosage of *L. plantarum*, which was 16.26 g/week, followed by rats treated with the high dosage, which was 14.56 g/week. Rats treated with the low dosage of *L. plantarum* showed less increases in the rate of body weight gain (Fig. 3). *L. plantarum* can cause increases in the weight of the liver whether the animal is alive or dead (Bloskma et al., 1979). This may explain why the high rate is only shown by the rats that were treated with the high and medium dosages.

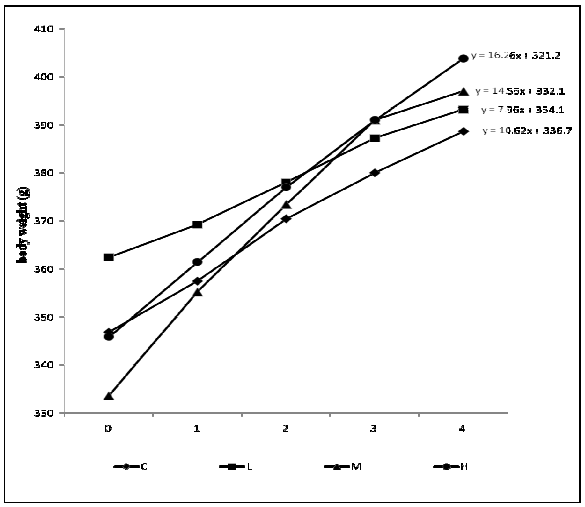

Fig. 3. Value of body weight for rats after 28 days experiment
C =control; *L* = low dose-10^6 cfu/ml of *L. plantarum*; *M* = medium dose-10^8 cfu/ml of *L. plantarum*; *H* = high dose-10^{10}cfu/ml of *L. plantarum*.

Other research using different species of *Lactobacillus (L. rhamnosus)* found that test mice showed a great increase in body weight when treated with 10^8 cfu/mL (Bernardeau et al., 2002). This experiment proved that a suitable dosage of live probiotics using *L. plantarum* is between 10^8 -10^{10} cfu/mL. The volume of water taken by the rats was not influenced by the presence of *L. plantarum* in their bodies; there was no consistent increase or decrease in their intake (Fig. 4). Several factors can be used to support this result. The first is the type of food used. The sweet and bitter flavors of dark chocolate will overcome the taste of *L. plantarum* in the chocolate. This can be proven through an experiment using *L. plantarum* UL4 only as the food. The water and pellet food taken by rats decreased due to the dreadful taste (Foo et al., 2003).

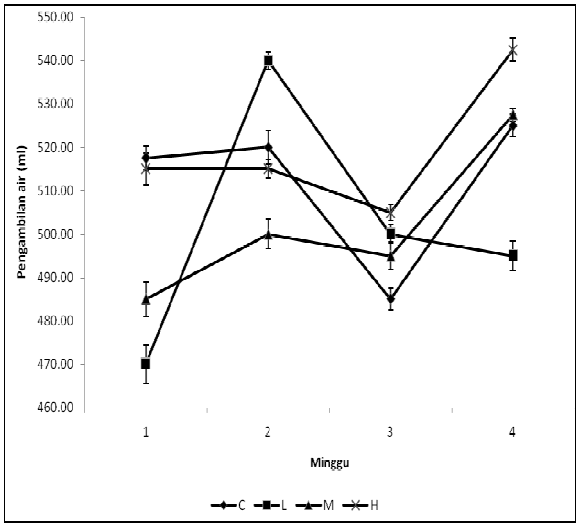

Fig. 4. Value of water intake by rats
C =control; *L* = low dose-10^6 cfu/ml of *L. plantarum*; *M* = medium dose-10^8 cfu/ml of *L. plantarum*; *H* = high dose-10^{10}cfu/ml of *L. plantarum.*

Feces Analysis

Mice feces were collected prior to the experiment to ensure the existence of *L. plantarum* in the gastrointestinal tract of the mice. It was found that *L. plantarum* was already present before the experiment, and the colony that was counted after the experiment cannot assure the exact amount of the given *L. plantarum* within the rats. Thus, the analyses were not done during the experiment. The colonization of *Enterobacteriacea* in the colon is influenced by the *Lactobacillus* spp., where

the number of colonization's is reduced (Adawi et al., 2001).

The isolated *L. plantarum* from the local fermented cocoa bean can be employed in chocolate product. No evidence of toxicity was detected for the subacute toxicity, haematology and biochemistry analysis tests, except for the level of ALT and the number of platelets harm towards the experimental mice. Although, the result of this research shows that the chocolate with *L. plantarum* can increase the weight of the experimental mice, but no dead case was reported. It can be concluded that no safety concerns were identified and has potential to be used in food industry.

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