Oral Administration of *Lactobacillus Acidophilus* Restores Nitric Oxide Level in Diabetic Rats


Biochemistry, Pharmaceutics and Industrial Pharmacy and Department of Home Economics, Faculty of Specific Education, Ain Shams University, Cairo, Egypt.

**Abstract:** Nitric oxide (NO) is an important regulator of many physiological and pathophysiological processes. Excessive formation of reactive oxygen metabolites (ROMs) is responsible for disruption of NO in diabetic. The objectives of this work was to formulate *Lactobacillus acidophilus* (LA) in a gastric resistance formulation and to investigate the effect of administration of LA alone or in combination with acarbose (AC) on plasma NO level and some oxidative stress parameters in diabetic rats. Granules containing LA with and without AC have been prepared using Eudragit L 100 as drug release retarding polymer. Colony forming unit (CFU) has been done after the granulation process and after placing in acidic medium to assure the viability of the LA. The rats were divided into control, diabetic, diabetic received AC, diabetic received LA and diabetics received AC plus LA groups. Fasting blood sugar (FBS), glycosylated hemoglobin (HbA1c %), triacylglycerol (TAG) level, Arylesterase (ARE) activity of paraoxonase enzyme, malondialdehyde (MDA) and plasma nitrate were measured. The results obtained from this study revealed that treatment with LA alone or in combination with AC significantly decrease the elevated FBS level, HbA1c %, plasma TAG and MDA compared to diabetic rats. The same treatment elevated the ARE activity compared to that of diabetic group. Administration of LA alone or with AC showed a significant elevation in plasma nitrate level compared with that of diabetic group. Finally we concluded that, LA and AC supplementation exert hypoglycemic, hypotriglyceridemic and antioxidant effects. These effects are responsible for restoration of NO level.

**Key words:** Diabetes, Probiotics, *Lactobacillus acidophilus*, Acarbose, Nitrate, Arylesterase activity, Malondialdehyde, Nitric oxide, Rats.

**INTRODUCTION**

Reactive oxygen metabolites (ROMs) such as superoxide, hydrogen peroxide and hydroxide play a crucial role in development of diabetic complications (Hunt et al., 1991). Nitric oxide (NO) is a mediator that regulates several physiological processes such as hormones releasing and the immune response (Lipton et al., 2001). NO bioactivity is low in diabetes mellitus, either due to increasing the destruction of NO by ROMs or decreasing NO production from L-arginine by the NO synthases (NOS) (Wolff et al., 1993; Pieper et al., 1998). NO has contradictory effect on diabetic animals, decreasing the release and/or the production of endogenous NO may lead to several diabetic complications (Contreras et al., 1997).

The activity of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase is altered under diabetic conditions (Tiedge et al., 1997). Paraoxonase-1 (PON1) is one of antioxidant enzymes and it has arylesterase (ARE) activity (Magdalena et al., 2009). This enzyme presents in the plasma and associate with high density lipoprotein and responsible for its antioxidant effect (Aviram et al., 1998). Also, it has ability to degrade lipid peroxides present in oxidized low density lipoprotein (Deakin et al., 2004; Laplaud et al., 1998). The activity of PON1 was found to be low in many diseases such as diabetes, atherosclerosis, hyperlipidemia and cardiovascular heart diseases (Mackness et al., 1991; Gan et al., 1991). It is believed that antioxidant treatment protects the function pancreatic b-cell from the damaging effect of ROMs (Kaneto et al., 1999).

**Corresponding Author:** E.I. Taha, Pharmaceutics and Industrial Pharmacy

Ehab I. Taha, Ph. D. Tel: 00966-567843885
Fax: 00966-14676295
E-mail: ehab71328@yahoo.com, eelbadawi@ksu.edu.sa
The term "probiotics" is used to describe the kind of non-pathogenic microorganisms that are used medicinally as LA (Alm et al., 1982; Duggan et al., 2002; Bengmark et al., 2005). The dietary supplementation with LA modulates immunity, release antioxidants and improve gastrointestinal functions (Madsen et al., 1999). LA may help in the maintenance of pH and protection against pathological changes in the colon (Salmi nen et al., 2001). Probiotics containing food has the ability to influence body functions in order to reduce the risk of many diseases (Sobko et al., 2005). Some strains of lactic acid bacteria such as LA produce NO in the intestinal lumen which can mediate the favorable effects of these bacteria (Sobko et al., 2005). α-glucosidase inhibitors such as acarbose (AC) inhibit the digestion of carbohydrates and used in the treatment of diabetes (Azuma et al., 2006, Hasegawa et al., 2008). Also, it exerts many beneficial effects such as improvement of fibrinolytic activity, decreased plasma triacylglycerol (TAG) and restore endothelial function in a rat model of type 2 diabetes (Azuma et al., 2006; Hasegawa et al., 2008). The aim of this work was to formulate LA in a stable gastric resistance formulation and to investigate its effect alone or in combination with AC on NO production and some oxidative stress status in diabetic rats.

**METHOD AND MATERIALS**

**Chemicals:**

Ellman’s reagent [5,5-dithio-bis(2-nitrobenzoic acid)], thiobarbituric acid (TBA), nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), nitrate reductase, streptozotocin (STZ), phenyl acetate, Tris- HCL, Calcium chloride, Potassium dihydrogen phosphate and 1,1,3,3-tetraethoxy-propane were purchased from Sigma Chemical Co., St. Louis, MO, USA. Eudragit L 100 was obtained from Rohm Pharma, GmbH, Germany. LA was obtained from Chris Hansen, Horsholm, Denmark. Mann-Rogosa-Sharpe (MRS) broth and peptone water were supplied by Merck, Darmstadt, Germany. Penassay seed agar was purchased from Difco, Detroit, USA. AC was provided from Alkan pharmaceutical company, Cairo, Egypt. Avicel PH 102 was obtained from FMC Corporation, Philadelphia, USA. All of the remaining chemicals are commercially available as analytical grade.

**Preparation of LA and AC formulations:**

Since LA is facultative anaerobes, great attention is devoted to formulation design and technological processes to guarantee and maintain the bacterial viability and stability in the final dosage form. LA has been formulated in granules using Eudragit L 100, pH dependent polymer, to assure its release in the intestine (Akhgari et al., 2006). The granules formed by this polymer are able to hydrate and gel very slowly, due to the high molecular weights and viscosity, the granules dissolve over a long time and provides a prolonged release of the active ingredient embedded in the polymeric matrix. An aqueous solution of LA (equivalent to 7.56 x 10^7 colony forming unit per ml (CFU/ml) was mixed with Eudragit L 100 (Toshiko et al., 2006). The wetted mass was forced through 355 μm sieve. The produces granules were allowed to dry at 30 °C in circulating air oven up to constant weight. AC was formulated into large tablets using Avicel PH 102. The prepared tablets were crushed into granules and forced through 355 μm sieve. A specific amount of rats’ diet was triturated using a mortar and pestle then enough water was added with continuous trituration to form slurry. LA and AC granules separately and in combination were added to the slurry so that each gram of rats’ diet containing LA equivalent to 7.56 x 10^7 CFU/ml and/or 80 mg AC. The drugs slurry mixtures were gently stirred to avoid crushing of the granules and allowed to dry in circulating hot air oven at 30 °C. After complete drying, the formed masses were removed and forced through 710 μm sieve.

**Viability testing for LA formulation:**

Viability and stability of LA has been both a marketing and technological challenge for industrial producers. For the LA to be functional they have to be viable and in sufficient dosage levels (Galdeano et al., 2004). A prerequisite for any effect of ingested bacteria formulation is a successful implantation in the gastrointestinal tract. So bacteria must remain viable during gastric transit. Viability of LA cells in the formulation as well as its survival in acidic medium (after remove of the granules and washing it with saline) were counted, the results were expressed as absolute numbers of CFU/ml using a standard plate viable count technique.Mersch-Sundermann, V., 1998. Medizinische Mikrobiologie. Georg Thieme Verlag, Stuttgart.. One gram of LA granules was suspended into 10 ml of peptone water containing phosphate buffer (pH 6.8), 1 ml was taken and mixed with 9 ml sterile peptone water and ten-fold serial dilutions were made. Five levels spacing one logarithmic unit were investigated by pipetting 1 ml from each level in three parallel plates. Promptly 15 ml of penassay seed agar were added and mixed well. Content were allowed to solidify, inverted plates were incubated at 37 °C and examined daily for 3 days. Suitable dilutions were counted and the results.
were recorded (Hidetoshi et al., 2006). The loss of bacteria due to granulation was evaluated by calculation from numbers of viable cells after granulation process. Viability of LA in 0.04 M HCl was used to evaluate its resistance against acidic conditions at 37 °C for 2 h, using a dissolution tester (apparatus 2, USP XXIV).

Experimental Animals:
Male albino rats of Wistar strain weighing approximately 252–277 gm were used in this study. Before the experiment animals were fed on standard diet pellets which is satisfy for rodents. The diet pellets was containing 22.6 % protein, 53.8 % carbohydrate, 5.6 % fat, 6.6 %, mineral and vitamins mixture, and 3.3 % fibers, total: 356 kcal/100 g (El-Nasr Co. Abou-Zaabal, Egypt). All rats were trained to consume the standard diet pellets in 1 hour that was provided twice a day from 10:00 AM to 11:00 AM and from 4:00 PM to 5:00 PM.

The rats were acclimatized to the laboratory conditions for a period of 2 weeks. They were maintained at an ambient temperature of 25 ± 2 °C and 12/12 h of light/dark cycle. The rats were divided into 5 groups: normal control group was injected intra peritoneal with citrate buffer pH 4.5. Diabetes was induced in the rest of the groups by intra peritoneal injection of 50 mg/kg of STZ dissolved in citrate buffer pH 4.5 as described by (Nagai et al., 2003). Rats with fasting blood sugar (FBS) more than 200 mg/dl were considered diabetic. Control and diabetic groups were received the standard diet pellets containing no treatments while rats in one of other three diabetic groups were received the standard diet pellets mixed with AC granules equivalent to 80 mg/kg body weight (Azuma et al., 2006), the other diabetic group was received the standard diet pellets mixed with LA granules equivalent to 7.56 x 10^4 CFU/ml, and the last diabetic group was received the standard diet pellets mixed with LA granules equivalent to 7.56 x 10^4 CFU/ml and AC granules equivalent to 80 mg/kg body weight. Treatments were continued for two weeks after induction of diabetes. All studies were approved by the Institutional Animal Care and Use Committee of Faculty of Pharmacy, Al-Azhar University and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Blood Sample Collection:
After 14 days, blood samples were collected from the ocular vein of 12 h fasted and anesthetized rats, by exposure to diethyl ether in a glass jar. At the end of the experiment rats were euthanized by decapitation. The blood was poured into heparinized tubes. A fraction of whole blood samples were used for determination of HbA1c % while the remaining part was centrifuged at 4000 rpm for 10 min using type CT5, Germany centrifuge, the plasma was used for determining the levels of the biochemical parameters.

Biochemical Investigations:
1. Determination of FBS, glycosylated hemoglobin (HbA1c %) and TAG plasma levels:
FBS and TAG level were determined using the method described by Trinder and Wahlefeld (Trinder et al., 1969, Wahlefeld 1974). HbA1c % was estimated by using the cation-exchange resin method in which the whole blood was mixed with a hemolysing reagent to prepare a hemolysate and mixed with cation-exchange resin. The non-glycosylated hemoglobin binds to the resin leaving HbA1c % free in the supernatant. Percentage of HbA1c % was determined by measuring the absorbance at 415 nm using Jenway LTD, Felsted, Dunow, Essex, CM6 3LB, Model 6105 UV / VIS, England, spectrophotometer (Heinze et al., 1979).

2. Determination of oxidative stress parameters:
A lipid peroxidation product, MDA, formed in the plasma was estimated by TBA method as described by (Fraga et al., 1988). The color developed was measured spectrophotometrically at 532 nm, 1,1,3,3 tetraethoxypropane was used as the standard. ARE activity of PON1 was measured using phenyl acetate as substrate (Haagen and Brock, 1992). The enzyme activity was calculated from the molar absorbivity coefficient of the produced phenol (1310M^-1 cm^-1).

3. Plasma Nitrate level:
NO was determined as stable end product (nitrate) in the plasma by nitrate reductase method as described by Bories and Bories (Bories and Bories, 1995). Briefly, 500 μl of 0.1 M phosphate buffer pH 7.5, 100 μl of FAD 0.2 mmol, 10 μl of 12 mmol NADPH, 200 μl of plasma and 80 μl of 500 U/L nitrate reductase were mixed together. The mixture was incubated at 25 °C in dark place for 45 minutes. The absorbance was measured at 340 nm against plasma blank in which all reagents are present except nitrate reductase which was replaced by distilled water.
The data were expressed as means ± SD. Analysis of results was performed using one-way ANOVA, followed by Tukey-Kramer test for multiple comparisons. Statistical significance was accepted at P < 0.001 using GraphPad Prism 4 program.

RESULTS AND DISCUSSION

The prepared LA granules showed resistance against acidic conditions when placed in 0.04 M HCl at 37 °C for 2 hours which assure that these granules would release its content only in the intestine. The viability of LA in the formulation after granulation process and as well as after placing in acidic medium for 2 hours showed insignificant loss in LA viable count which is 7.31 x 10^7 CFU/ml after granulation process and 7.24 x 10^7 CFU/ml after removal from acidic medium. This indicates a good microbiological stability of the prepared formulation. The results of the present study indicated that diabetic rats significantly show increase in plasma FBS level compared with control group. The treatment with LA and AC separately or in combination caused significant decrease in FBS compared with diabetic group. The treatment also revealed significant reduction in HbA1c % in studied groups compared with diabetic one. The results also show that the HbA1c % was still significantly higher in AC and LA treated groups when compared with control one (Table 1). These results are coping with a previous study which demonstrated that oral supplementation of probiotics significantly decreased blood glucose levels (Matsuzaki et al., 1997). Other studies reported that feeding of probiotics to diabetic animal model inhibited the destruction of pancreatic β-cells (Matsuzaki et al., 1997). There is another study stated that feeding of probiotics during progression of diabetes is significantly decreases the elevated blood glucose level (Tabuchi et al., 2003). LA may improve the blood sugar level through inhibiting the production of several mediators such as ROMs and cytokine which are responsible for the destruction of pancreatic cells. In addition it may lower blood sager level via decrease the intestinal absorption of carbohydrates through enhancing the AC effect. Diabetic group showed significant high level of plasma TAG compared with the control group. Treatments with both LA and AC caused significant reduction in TAG level compared with diabetic group. The combination between LA and AC tend to normalize the TAG level near to the control one (Table 1). This result agrees with several studies which demonstrated that LA and AC exert hypotriglyceridemic effect (Azuma et al., 2006; Hasegawa et al., 2008; Tabuchi et al., 2003). Lipoprotein lipase (LPL) is responsible for metabolism of TAG consequently normalize its plasma level. (Thirunavukkarasu et al., 2003) postulated that oxidative stress is associated with lowered (LPL) activity. Also, the same author has reported that co-administration of antioxidants lowered plasma TAG level due to its effect on LPL activity. Therefore, hypotriglyceridemic effect of LA and AC may be related to the initiation of lipases activity, decreasing intestinal absorption of lipids or increasing lipid catabolism and/or antioxidants activity. In the present study, the oxidative stress was evidenced by significant elevation of MDA and reduction of ARE activity of PON1 as a result of increase of ROMs production. Induction of diabetes in rats causes significant elevation of MDA level compared with control group. Treatment with LA and AC caused significant reduction in MDA level compared with diabetic group [Figure 1]. Also in the present work, ARE activity of PON1 was significantly lower in diabetic group than the control one. On the other hand treatments with LA and LA plus AC ameliorate ARE activity compared to diabetic group [Figure 2]. These results support the idea that probiotics possess protective effects against oxidative stress (Seher et al., 2007). These findings are in accordance with the study of (Hariom et al., 2007) who reported that probiotics supplementation improve antioxidants capacity in the liver and pancreatic tissues of diabetic rats. There are some reports stated that antioxidant therapies have been used for the reduction of diabetes and related complications.

Plasma nitrate level as index of NO production was significantly low in diabetic group compared with control one. The treatment with LA or AC tends to normalize plasma nitrate level compare with control group. There is significant elevation in nitrate level in LA treated group compared with diabetic group (Figure 3). Treatment with LA plus AC showed significant elevation in plasma nitrate level compared with diabetic group. These results are in harmony with several observations reported that LA bacteria produce NO (Natas et al., 2009; Lamine et al., 2004; Xu and Verstraete, 2001). LA produces NO either by nitrate reduction or acid dependent mechanism. All together, this led to local NO generation in the intestinal lumen which may be diffused into tissues and mediate some of the beneficial effects of LA. The oxidative stress induced by hyperglycemia may be responsible for decrease NO in diabetic group. Increased the ROMs and decrease antioxidants may be responsible for decrease the NO production. Antioxidants have also shown to directly stimulate activity of NO synthase by increasing the availability of cofactors, which are required for NO synthesis (Chen et al., 2001).
Fig. 1: Mean ± SD of plasma MDA level in studied group at P value < 0.001.
   a: Significant different from control group.
   b: Significant different from diabetic group.

Fig. 2: Mean ± SD of plasma ARE activity in studied group at P value < 0.001.
   a: Significant different from control group.
   b: Significant different from diabetic group.

Fig. 3: Mean ± SD of plasma nitrate level in studied group at P value < 0.001.
   a: Significant different from control group.
   b: Significant different from diabetic group.

Table 1: Effect of different treatments on mean plasma FBS, HbA1c and TAG in studied groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Diabetic</th>
<th>AC</th>
<th>LA</th>
<th>AC plus LA</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBS mg/dl</td>
<td>87.20 ± 9.14</td>
<td>207.0 ± 10.9 *</td>
<td>121.2 ± 6.56 * *</td>
<td>132.4 ± 11.02 * *</td>
<td>110.2 ± 6.93 * *</td>
</tr>
<tr>
<td>HbA1c %</td>
<td>2.64 ± 0.47</td>
<td>7.70 ± 0.49 *</td>
<td>4.88 ± 0.54 * *</td>
<td>4.40 ± 0.69 * *</td>
<td>3.36 ± 0.37 * *</td>
</tr>
<tr>
<td>TAG mg/dl</td>
<td>59.37 ± 6.76</td>
<td>126.5 ± 8.17 * *</td>
<td>90.85 ± 6.96 * *</td>
<td>66.80 ± 3.82 * *</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD. of mean of 6 rats in each group at P value < 0.001.
   a: Significant from control
   b: Significant from diabetes

In conclusion, supplementation of food with LA alone or in combination with AC exerts the hypoglycemic and hypotriglyceridemic effect. These effects may be responsible for decreasing the glycemic index of the food. LA and AC may also exert antioxidant effect through significant increase in ARE activity of PON1 and decrease MDA level. These effects may be responsible for restoration of NOS activity and hence restore NO level.
REFERENCES


