Role of *Lactobacillus plantarum* MTCC1325 in membrane-bound transport ATPases system in Alzheimer’s disease-induced rat brain

Nimgampalle Mallikarjuna, Praveen Kukkarasapalli, Yellamma Kuna* 

Division of Neurobiology, Department of Zoology, Sri Venkateswara University, Tirupati, Andhra Pradesh, India

**Introduction**

Alzheimer’s disease (AD) is a neurodegenerative disorder, clinically characterized by memory dysfunction and progressive loss of cognition. No curative therapeutic or drug is available for the complete cure of this disease. The present study was aimed to evaluate the efficacy of *Lactobacillus plantarum* MTCC1325 in ATPases activity in the selected brain regions of rats induced with Alzheimer’s.

**Methods:** For the study, 48 healthy Wistar rats were divided into four groups: group I as control group, group II as AD model (AD induced by intraperitoneal injection of D-Galactose, 120 mg/kg body weight for 6 weeks), group III as normal control rats which were orally administered only with *L. plantarum* MTCC1325 for 60 days, and group IV where the AD-induced rats simultaneously received oral treatment of *L. plantarum* MTCC1325 (10ml/kg body weight, 12×10^8 CFU/mL) for 60 days. The well known membrane bound transport enzymes including Na⁺, K⁺-ATPases, Ca²⁺-ATPases, and Mg²⁺-ATPases were assayed in the selected brain regions of hippocampus and cerebral cortex in all four groups of rats at selected time intervals.

**Results:** Chronic injection of D-Galactose caused lipid peroxidation, oxidative stress, and mitochondrial dysfunction leading to the damage of neurons in the brain, finally bringing a significant decrease (-20%) in the brain total membrane bound ATPases over the controls. Contrary to this, treatment of AD-induced rats with *L. plantarum* MTCC1325 reverted all the constituents of ATPase enzymes to near normal levels within 30 days.

**Conclusion:** *Lactobacillus plantarum* MTCC1325 exerted a beneficial action on the entire ATPases system in AD-induced rat brain by delaying neurodegeneration.

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*Corresponding author: Kuna Yellamma, Email: yellamma55@gmail.com*

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Further, it maintains the membrane potential, cell volume, trans membrane flux of Ca\(^{2+}\), and excitatory neurotransmitters.\(^8\) Recent research findings demonstrated that frontal cortex and cerebellum regions of AD patient’s brain showed increased levels of Na\(^+\) and K\(^+\) ion concentrations and Amyloid beta 25-35 peptide in primary astrocytes which eventually led to the reduced levels of membrane transporter Na\(^+\), K\(^+\)-ATPases.\(^6\) This observation gives us a clue that elevation of ATPases-associated energy metabolism might help in controlling the AD progression to some extent.

Lactic acid bacteria (LAB), considered as GRAS (generally recognized as safe),\(^7\) are the best studied beneficial microorganisms for the prevention of diarrhea, lactose intolerance, treating ulcer, infectious diseases prevention, restoring the composition of gut microbiome and introducing beneficial functions to host through immune, neuro modulation.\(^8\) They also possess a wide variety of activities like anticancer, antioxidant, anti diabetic, antiobesity, and antihyperlipidemic effects.\(^9\) Recent studies have found that the L. plantarum versus pentosus showed protective effect against memory deficit in AD-induced mice by D-Galactose and scopolamine.\(^10\) Further, another study reported that L. Plantarum NDC75017 improves the learning and memory ability in aging rats.\(^11\) Previous reports proved that strong antioxidants increases the Na\(^+\), K\(^+\)-ATPases activities by decreasing AChE (Acetyl cholinesterase) levels and improves the cognition by enhancing cholinergic transmission.\(^12\)

All the above cited research findings prompted us to undertake the present work to investigate the potential therapeutic qualities of L. plantarum MTCC1325 on the functional activities of membrane-bound ATPases in the brain tissue of D-Galactose-induced AD model rats.

Materials and methods

Chemicals

D-Galactose, TCA (trichloroacetic acid), ATP (adenosine triphosphate) and MRS (de Man, Rogosa and Sharpe) broth were procured from Hi-Media Laboratories (Mumbai, India). Nacl (sodium chloride), Mgcl\(_2\) (magnesium chloride), Kcl (potassium chloride), and H\(_2\)SO\(_4\) (sulphuric acid) were obtained from Qualigen Chemicals (Mumbai, India). ANSA (1-amino 2-naphthol 4-sulphuric acid), Tris buffer, ammonium molybdate, and Sodium bisulphate were purchased from CDH chemicals (New Delhi, India). Lactic Acid Bacteria: The pure culture of L. plantarum MTCC1325 was obtained from the IMTech Culture Collection Center, Chandigarh, India.

Experimental design

A total of 48 healthy adult male Wistar rats (3 months old, weighing 180±20 g) were procured from Sri Venkateswara Enterprisers, Bangalore and maintained in the laboratory in the controlled conditions of 28±2°C, 12 h light/12 h dark. The rats were fed with standard pellet diet and water ad libitum throughout the experiment period. The rats were divided into 4 groups, each consisting of six animals.

- **Group I** (control group): Rats were subcutaneously injected with saline (1 mL/kg body weight).
- **Group-II** (AD model group): Rats received D-galactose (120 mg/kg body weight) through intraperitoneal injection.
- **Group III** (LP: Lactobacillus Plantarum group): Rats were injected with saline for first six weeks and with L. plantarum from seventh week onwards (10 mL/kg body weight of rat; 12×10\(^8\) CFU/mL) for 60 days.
- **Group IV** (AD+LP [protective group]): Rats received D-Galactose (120 mg/kg body weight) for six weeks then treated with L. plantarum (10 mL/kg body weight of rat; 12×10\(^8\) CFU/mL) from seventh week onwards for another 60 days.

**Preparation of Lactobacillus plantarum culture for treatment**

Lactobacillus plantarum MTCC1325 was provisionally isolated and identified from the saukraut fermented food product by Stephenson and Rowatt in 1947 and it was found that this strain had the ability to produce Acetylcholine Neurotransmitter.\(^13\) A loop full of pure culture of L. Plantarum was inoculated in MRS broth and incubated at 30°C in orbital shaker until to get log phase. One milliliter of this active log phase culture was serially diluted up to 10\(^{-9}\) dilution in drinking water. The Dilution 10\(^8\) containing 12×10\(^6\) CFU/mL of L. plantarum MTCC1325 was selected for oral administration. Each group of rats were orally administered with L. plantarum MTCC1325 (10 mL/kg body weight) through stainless steel gastric gavage.\(^14\)

**Isolation of tissues**

After 6 weeks of AD induction, the animals were treated with L. plantarum for a period of 60 days. The animals were kept fasting for 12 h before sacrificing and on the selected days of experimentation (30th and 60th days), they were decapitated and the brain regions of hippocampus (HP) and cerebral cortex (CC) from each animal were kept fasting for 12 h before sacrificing and on the selected days of experimentation (30th and 60th days), they were decapitated and the brain regions of hippocampus (HP) and cerebral cortex (CC) from each animal were rapidly isolated, thoroughly washed with ice cold saline and then stored in -80°C for biochemical analysis.

**Biochemical analysis**

HP and CC regions of brain tissue samples isolated from all the groups of experimental rats were used for the estimation of membrane bound transport ATPases using following methods. The total ATPases and Mg\(^{2+}\)-ATPases were estimated by the method of Tirri et al.\(^15\)

**Total ATPases**

One percent homogenates of different regions of rat brain were prepared in 0.25 M ice cold sucrose solution. The reaction mixture in a final volume of 2.6 mL contained 0.5 mL of tris buffer (0.13 M; pH 7.4), 0.4 mL of substrate ATP (4.0 μ moles), 0.5 mL of MgCl\(_2\) (0.05 M), 0.5 mL of NaCl (0.05 M), 0.5 mL of KCl (0.05 M), and 0.2 mL of homogenate. The contents were incubated at 37°C for 15 min and the reaction was terminated by adding 1.5 mL of...
Lactobacillus plantarum enhances ATPases activities in Alzheimer’s Disease

BioImpacts, 2016, 6(4), 203-209

The blank contained 2.0 mL TCA, 1.0 mL ammonium molybdate, and 4.0 mL ANSA. The enzyme activity was expressed as μ moles of inorganic phosphate formed/mg protein/hour.

Protein estimation
Protein content of the selected brain tissue regions was estimated by the method of Lowry et al.\textsuperscript{20} To 0.5 mL of crude homogenate, 1mL of 10% TCA was added and the samples were centrifuged at 1000 g for 15 min. Then supernatant was discarded and the residue was dissolved in 0.5 mL of 1 N NaOH. To this, 4 mL of alkaline copper reagent was added followed by 0.4 mL of folin-phenol reagent (1:1 folin-phenol reagent: H₂O). The color was measured at 600 nm in a UV-visible spectrophotometer against blank.

Statistical analysis
All the experiments were carried out in triplicate and expressed as mean ± SD. One-way ANOVA was used to test the significance of differences among four different groups followed by Tukey and Dunnet’s multiple range tests. Statistical analysis was performed using SPSS (version 20; IBM SPSS Inc., Chicago, IL, USA). The data were regarded as significantly different at p<0.05.

Results
Total ATPases
The results obtained in the present study clearly demonstrated that for 60 days the activity levels of total ATPases in the control rat brain were maximum in CC (44.65 µmoles of Pi formed/mg protein/hour) followed by HP (40.43 µmoles of Pi formed/mg protein/hour). Conversely, in AD model group, their levels were reduced significantly by -21.87% and -19.05% in CC and HP, respectively (Fig. 1). Interestingly, oral administration of \textit{L. plantarum} to AD-induced rats (protective group) showed a significant elevation in the activity levels of total ATPases by 3.44% in CC and 1.67% in HP, compared to AD model group.

10% TCA. The contents were centrifuged and the released inorganic phosphate was estimated in the supernatant fraction. Sodium-potassium ATPases can be obtained by subtracting the Mg\textsuperscript{2+}-ATPases from the total ATPases.

\textbf{Mg\textsuperscript{2+}-ATPases}

One percent homogenates of different regions of rat brain were prepared in 0.25 M ice-cold sucrose solution. The reaction mixture in a final volume of 1.6 mL contained 0.5 mL of tris buffer (0.13 M; pH 7.4), 0.4 mL of substrate ATP (4.0 μ moles), 0.5 mL of MgCl\textsubscript{2} (0.05 M), 10 μ moles of ouabain, and 0.2 mL of homogenate. The contents were incubated at 37°C for 15 min and the reaction was terminated by adding 1.5 mL of 10% TCA. Zero time controls were maintained by adding TCA prior to the addition of homogenate. The contents were centrifuged at 1000 X g for 15 min and the inorganic phosphate was estimated in the supernatant fraction.

\textbf{Ca\textsuperscript{2+}-ATPases}

Ca\textsuperscript{2+}-ATPase activity was determined by the method of Fritz and Hamrick\textsuperscript{17} as supported by Desaiah and Ho.\textsuperscript{18} The reaction mixture contained 135 mM imidazole HCl buffer (pH 7.5), 5 mM MgCl\textsubscript{2}, 0.05 mM CaCl\textsubscript{2}, 4 mM ATP, and 30-40 mg protein. The mixture was incubated at 37°C for 30 min and stopped by the addition of 0.1mL of 50% TCA. The inorganic phosphate (Pi) liberated was estimated. Mg\textsuperscript{2+}-ATPase activity was measured in the presence of 0.5 mM EDTA and the value subtracted from ATPase activity was expressed as μ moles of Pi formed/mg protein/hour.

\textbf{Estimation of inorganic phosphates}

Inorganic phosphates were estimated by the method of Fiske and Subba Row.\textsuperscript{19} To 1.0 mL of the above supernatant, 1.0 mL of ammonium molybdate solution (2.5 g in 100 mL of 10 N H\textsubscript{2}So\textsubscript{4}) was added followed by 0.4 mL of ANSA (1-amino 2-naphthol 4-sulphuric acid) and allowed to react for 5 min. The blue color formed was read at 600 nm in a spectrophotometer against a reagent blank.

Protein content of the selected brain tissue regions was estimated by the method of Lowry et al.\textsuperscript{20} To 0.5 mL of crude homogenate, 1mL of 10% TCA was added and the samples were centrifuged at 1000 g for 15 min. Then supernatant was discarded and the residue was dissolved in 0.5 mL of 1 N NaOH. To this, 4 mL of alkaline copper reagent was added followed by 0.4 mL of folin-phenol reagent (1:1 folin-phenol reagent: H₂O). The color was measured at 600 nm in a UV-visible spectrophotometer against blank.

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\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{Effect of \textit{Lactobacillus plantarum} MTCC1325 on the activity levels of total ATPases in hippocampus and cerebral cortex regions of control and experimental groups of rats on 30th and 60th days. *Significant difference at p < 0.05 as compared with the control group. #Significant difference at p < 0.05 as compared with the AD model group. HP - Hippocampus; CC- Cerebral cortex; AD – Alziemer’s disease; LP - L. plantarum; ADLP - Alzheimers disease + L. plantarum.}
\end{figure}
Na\textsuperscript{+}, K\textsuperscript{-} ATPase

Similar to total ATPases, comparison of the levels of Na\textsuperscript{+}, K\textsuperscript{-} ATPases in the CC and HP regions of AD model rat brain showed that they were relatively lower by -15.24\% and -17.33\%, respectively, compared to the control group (Fig. 2). However, similar to that of the total ATPases, oral simultaneous administration of \textit{L. plantarum} MTCC1325 to AD-induced rats for 60 days, caused a significant enhancement in the levels of Na\textsuperscript{+}, K\textsuperscript{-} ATPases (HP: -2.25\% and CC: 0.55\%) as compared with AD model group, thereby revealing the positive effect of \textit{L. plantarum} in the restoration of AD-induced effects close to control levels.

Mg\textsuperscript{2+} - ATPases

As similar to that of Na\textsuperscript{+}, K\textsuperscript{-} ATPases, the activity levels of Mg\textsuperscript{2+}-ATPases were also significantly reduced in HP (-27.89\%) and CC (-31.91\%) regions of AD model group rat brains when compared to control (Fig. 3). However, prolonged (60 days) administration of \textit{L. plantarum} to AD-induced group rats showed a significant increase in the Mg\textsuperscript{2+}-ATPases activity levels (HP: 10.78\% and CC: -12.29\%) compared to the AD model group.

Ca\textsuperscript{2+} - ATPases

Similar to the other ATPases, the Ca\textsuperscript{2+} - ATPases activity levels were significantly reduced in HP (-20.63\%) and CC (-19.37\%) regions of AD model rat brain when compared to the control groups (Fig. 4). Conversely, a significant increase in Ca\textsuperscript{2+} - ATPases activity levels (HP: 20.90\% and CC: 20.09\%) were observed on the simultaneous treatment of AD-induced rats with \textit{Lactobacillus plantarum} MTCC1325 for 60 days.

Discussion

In the present study, we evaluated the efficiency of \textit{L. plantarum} MTCC1325 on the activity levels of membrane bound transport ATPases including Na\textsuperscript{+}, K\textsuperscript{-} ATPases, Mg\textsuperscript{2+}-ATPases, and Ca\textsuperscript{2+} - ATPases in the HP and CC regions of D-Galactose-induced Alzheimer’s disease male Wistar rats brain. From our results, it was obvious that AD induction by the chronic injection of D-Galactose reduced total AT-
Lactobacillus plantarum enhances ATPases activities in Alzheimer’s Disease

BioImpacts, 2016, 6(4), 203-209

Pases activities in the selected regions (HP and CC) of rat brain when compared to the normal control group. This observation derives strong support from earlier reports which proved that long-term exposure of rats to D-Galactose impaired the activities of mitochondrial enzymes, membrane permeability, and ATP production. In the present experiment, it was also observed that Na⁺, K⁺-ATPases were high in protective group (D-galactose + L. plantarum) compared to the normal control group. Further, the remaining ATPases including Mg²⁺-ATPases and Ca²⁺-ATPases showed similar levels when AD-induced rats were treated with L. Plantarum for 30 days; but 60 days treatment showed significantly increased activity levels of ATPases in protective group compared to the normal control and AD model groups. An interesting finding in the present study was that oral administration of L. plantarum could effectively reverse the D-Galactose-induced AD effect in all experimental groups of rats. The results clearly demonstrated that AD-induced rats simultaneously treated with L. plantarum showed increasing activity levels of ATPases in CC region followed by HP region of the rat brain compared to AD model group. A very recent report by Rai et al. showed that the rats fed with fish visceral waste through lactic acid fermentation by P. acidilactici NCIM5368 enhanced the Na⁺, K⁺-ATPases in brain. This study lends strong support and reiterates our present observations on all types of ATPases. Furthermore, several clinical trial findings established that treatment of irritable bowel syndrome patients with lactobacillus strain (Lactobacillus plantarum 299v) showed significant improvement from symptoms.

ATP is continuously required by membrane ATPases to maintain ionic homeostasis across the membrane, lipid asymmetry, and cell to cell communication. In general, the Na⁺, K⁺-ATPases play an important role in learning and memory, which further establish that an impairment in the function of these membrane ATPases leads to the initiation of AD pathogenesis. The oxidative stress and dysfunction of redox-related enzymes disrupt energy metabolism in the brain, eventually resulting in the reduction of energy utilization in the AD brain. Thus, Na⁺, K⁺-ATPases play a major role in the augmentation of neural pathogenesis in Alzheimer’s disease. Furthermore, it has been described that the stimulators and therapeutics involved in the enhancement of Na⁺, K⁺-ATPase activities in AD are considered as potent neuroprotective agents against AD.

Our present findings are justified on the assumption that the supplementation of rat diet with L. plantarum might cause the enhancement of membrane bound transport ATPases activities which is in turn associated with its antioxidant properties. Further, L. plantarum may produce a variety of beneficial secondary metabolites which are involved in various biochemical pathways of the brain through the enteric nervous system (Gut-brain axis) and thus protect the neurons by stabilizing the structural and functional integrity of the biological membranes through the regulation of ionic concentration gradient. However, further research is required on higher mammalian experimental models such as rabbits, owl monkeys, vervet monkeys, squirrel monkeys, and so on to better understand the possible role of Lactobacillus strains in the protection against neurodegenerative diseases.

**Conclusion**

Based on the results of the present study, it can be concluded that the supplementation with L. plantarum MTCC1325 significantly improved the activities of the membrane transport ATPases system in the selected brain regions of AD-induced group. Further, it was revealed that L. plantarum MTCC1325 protects the neurons by stabilizing the structural and functional integrity of the biological membranes through the regulation of ionic concentration gradient by its antioxidant properties.

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**Competing interests**

The authors declare no conflict of interest regarding the publication of this paper.

**Ethical approval**

The research work was carried out and the experimental rats were maintained according to the guidelines of Institutional Animal Ethical Committee (Resolution No. 34/20122013/(i)/a/ CPCSEA/IAEC/ SVU/KY dt.01.07.2012).

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