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Role of Probiotic Strain *Lactobacillus acidophilus* in the Reversal of Gut Dysbiosis Induced Brain Cognitive Decline

Murugan Mukilan[1,2*], Mepully Thomas Antony Mathew[2], Siva Yaswanth[2], Vivekanandan Mallikarjun[2]

[1]Advanced Technology Development Centre, Indian Institute of Technology, Kharagpur 721 302, West Bengal, India
[2]Department of Biotechnology, Sri Ramakrishna College of Arts & Science, Coimbatore 641 006, Tamil Nadu, India

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Memory

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Enterotoxin

Cognitive impairment

ABSTRACT

In the central nervous system, bidirectional communication between the brain and gut results in memory formation due to synaptic plasticity changes. During a healthy state, oral balanced microflora plays a pivotal role in memory formation by inhibiting the enterotoxin level produced by infectious pathogens. In disease conditions, beneficial microbial dysbiosis may result in excess enterotoxin production. Further, excess enterotoxin secretion prevents beneficial bacteria's proliferation and impairs neurotransmitter precursor compounds' transport to the brain. Blockade of neurotransmitter precursor compounds may result in the development of memory loss. The present study stated the role of *Lactobacillus acidophilus* in recovering memory loss. Reversal of cognitive impairment is shown with the help of a three-step behavioural analysis, which consists of one pre-infusive behavioural analysis and two post-infusive behavioural analyses (phase 1 and 2). The pre-infusive analysis showed no cognitive impairment in an assimilated environment without any infusions. After oral microbial infusions, phase 1 of post-infusive behavioural analysis showed the presence of cognitive impairment in the experimental groups who received oral infusions. Formed cognitive impairment is reverted with the help of *L. acidophilus* oral infusion in phase 2 of post-infusive analysis. Comparative three-step behavioural analysis proved that *Pseudomonas aeuroginosa* induced cognitive impairment may revert to normal conditions with the help of *L. acidophilus*. The outcome of the present study proves that cognitive impairment developed due to poor oral hygiene can be treated with the help of probiotic microorganisms.

* Corresponding author
E-mail: mukilan@srcas.ac.in (Murugan Mukilan)

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1 Introduction

Learning and memory formation (LMF) plays a major role in developing cognitive functions during repeated exposure to a non-harmful/harmful stimulus (Bisaz et al. 2014; Abraham et al. 2019; Mukilan 2023). Repeated exposure to this stimulus results in brain neuronal circuit changes. Changes in brain neuronal circuits happen during short-term memory (STM) formation and long-term memory (LTM) formation due to LMF. In LMF, STM formation uses existing proteins for memory formation, which lasts 24 hours. Compared to STM, LTM formation employs a unique neuronal signaling pathway for the acquaintance of information and its storage in different brain regions for a long period (throughout the life span) (Abraham et al. 2019; Evans et al. 2021; Lin et al. 2021; Mukilan 2023). Formation of LTM uses RNA-dependent protein synthesis machinery along with pre and post-synaptic neurons. This LTM formation begins with repeated exposure to a particular defined stimulus for a limited time in subsequent days. As a result of exposure, pre-synaptic neurons (PrSN) release specific neurotransmitters into the synaptic cleft (SC). Released neurotransmitters bind to specific receptors of post-synaptic neurons (PoSN) and trigger the activation of cyclic adenosine monophosphate (cAMP) (Bai and Suzuki 2020; Lin et al. 2021). Further upregulated cAMP activates protein kinase A (PKA) and enzyme-regulated kinase 1/2 (ERK-1/2). Activated ERK-1/2 molecules may add phosphate molecules to the cAMP response element binding protein-1 (CREB-1) for its activation. Further phosphorylated and activated CREB-1 initiates the induction of immediate-early genes (especially *Egr-1*, *C-fos*, and *C-jun*) responsible for forming post-synaptic density proteins. Cumulative expression of these neuronal signaling proteins results in the LTM formation (Ganesh et al. 2010; Rajan et al. 2011; Ganesh et al. 2012; Mendez et al. 2015; Mukilan et al. 2015; Mukilan et al. 2018a; Thangaleela et al. 2018; Mukilan 2023). This formed LTM is majorly impaired due to stress, gut-microbiota dysbiosis, and poor oral hygiene (Evans et al. 2021; Lin et al. 2021; Dai et al. 2022; Mukilan 2023).

In healthy individuals, oral and intestinal microbiotas play a major role in regulating the homeostasis mechanism of an endocrine and neural system through the central nervous system (CNS) (Luca et al. 2018; Ma et al. 2019; Myers Jr et al. 2021). The homeostasis state of the host is majorly disturbed by chronic stress (CS)/pathogenic infection (PI). This CS increases corticotrophin-releasing hormones (CRH) in the bloodstream through the hypothalamic-pituitary-adrenal axis (HPA). Produced CRH released from the axon terminals may stimulate the production of endotoxins (ET) in the gut (Herman et al. 2016; Fung et al. 2017; Misiak et al. 2020; Sheng et al. 2021; Hinds and Sanchez 2022). These ETs regulate the secretion of neurotransmitter precursor compounds responsible for gut microbiota interaction with CNS (Strandwitz 2018; García-Cabrerizo et al. 2021; Salami 2021; Miri et al. 2023). Later on, secreted neurotransmitter precursor compounds are transported to the brain (CNS) from the gut via the vagus nerve. The PrSN uses transported precursor compounds to produce brain neurotransmitters like 5-hydroxytryptamine (5-HT). Synthesized 5-HT released into the SC results in the binding with PoSN and activates the CREB-mediated neuronal signaling pathway (CMNSP). Activated CMNSP may further result in the formation of LTM (Mukilan et al. 2015; Mukilan et al. 2018b; Thangaleela et al. 2018; Mukilan 2022; Mukilan 2023).

An imbalance in this CMNSP results in the development of cognitive memory impairment/neurodegeneration. Recent studies have proved that stress formation may result in the induction of impaired cognitive dysfunction along with gut/oral dysbiosis. Thus, the formed cognitive decline may be reversed with the intake of live probiotic microorganisms along with diet may block the release of endotoxins (Wong et al. 2018; Angelucci et al. 2019; Asl et al. 2019; Bermúdez-Humarán et al. 2019; Morshedi et al. 2020; Naomi et al. 2022). Earlier reports from our group showed that poor oral hygiene plays an important role in forming declined cognitive functions via the oral administration of microorganisms (Mukilan 2023). In the present study, we predicted that probiotic microorganisms may participate in reversing the microorganism-induced cognitive impairment by suppressing ET. Goldfish were subject to the three-step behavioural analysis with the infusions of isolates and probiotic microorganisms through the oral passage to test the hypothesis.

2 Materials and Methods

2.1 Study Animals and Home Tank Design

Commercially available naïve adult goldfish *Carassius auratus* (Mean length: 6.5 – 8 cm, and weight 6 – 15 g) were purchased from a local aquarium of P.N. Palayam, Coimbatore, Tamil Nadu, India. After purchase, animals were carefully examined for non-microbial colonization in the skin and housed in home tanks as groups (n = 6/group). The aquarium (glass rectangular tank with a length, breadth, and height of 42 X 30 X 21 inches) was provided with continuous air circulation, temperature (26 ± 2˚ C), and light: dark cycle (12: 12 hours). Commercial dry round food pellets (Taiyo Pet Products Pvt. Ltd., India) were provided twice a day (9.00 and 18.00 h) at their aquarium on alternative days, and the aquarium was routinely cleaned and replaced with pure water to maintain dissolved oxygen level and dust free environment. Fishes were identified according to their phenotypic/morphological differences. The study design and experimental protocol follow the institutional animal care guidelines of Sri Ramakrishna Institutions, Coimbatore, Tamil Nadu, India.

2.2 Study Design and Experimental Setup (ES)

The experimental chamber was separated into three different parts as per the need of the study. These three chambers include two feeding chambers (FC) and one central chamber (CC). Each FC had a LBH of 6 X 30 X 21 inches with a central opening, and CC had a LBH of 30 X 30 X 21 inches. In the two FCs, one FC is designated for positive (with food reward), and the other is for negative chambers (without food reward). The FC's central opening facilitates the fish's movement into the positive and negative reward chambers. Developed ES was used for pre-infusive and post-infusive behavioural analysis (Figure 1).

2.3 Behavioural study

2.3.1 Experimental Groups (EGs)

Fishes were randomly separated into four different groups. These are control (C), Group 1 (G - 1) received oral infusions of *Pseudomonas aeruginosa*, Group – 2 (G - 2) received oral infusions of *Bacillus subtilis*, and Group - 3 (G - 3) received oral infusions of *Escherichia coli* during post-infusive behavioural analysis (phase - 1). Infused groups (G - 1, 2, and 3) received oral infusions of *Lactobacillus acidophilus* after completion of phase - 1 post-infusive analysis.

2.3.2 Reward-Based Learning (RBL)

Reward-based learning used in this study was followed to understand the development of cognitive memory formation with the help of color cues. During the training phase, individuals were trained to use blue and red color cues at the opposite sides of the experimental chamber. FC with a blue color cue acts as a positive chamber with a food reward, and FC with a red color cue acts as a negative chamber without a food reward.

2.4 Microbial Cultures Used

Three isolates used in the present study (*P. aeruginosa*, *B. subtilis*, and *E. coli*) were availed on request from the PSG Institute of Medical Sciences & Research (PSG IMSR). Probiotic strain *L. acidophilus* (MTCC No. 10307) was received from MTCC, IMTECH, Chandigarh, Punjab, India.

2.5 Purity Confirmation and Oral Infusion Mixture Preparation

Pure culture was isolated on a nutrient agar medium with the help of simple streak and quadrant streak methods for the isolates and probiotic strains. Once the purity was confirmed, an individual probiotic colony (IPC) was selected from quadrant-streaked nutrient agar plates. Selected IPC again streaked on *Lactobacillus* MRS Agar plates for further confirmation. Later on, from the quadrant plates individual colonies were picked up and used for the arousal of overnight cultures of isolates and probiotic strains. Nutrient broth and MRS broth were used to grow the overnight culture. Grown overnight cultures were used to prepare the oral infusion mixture and phosphate buffer saline (PBS) in a ratio of 50:50 (Mukilan 2023).

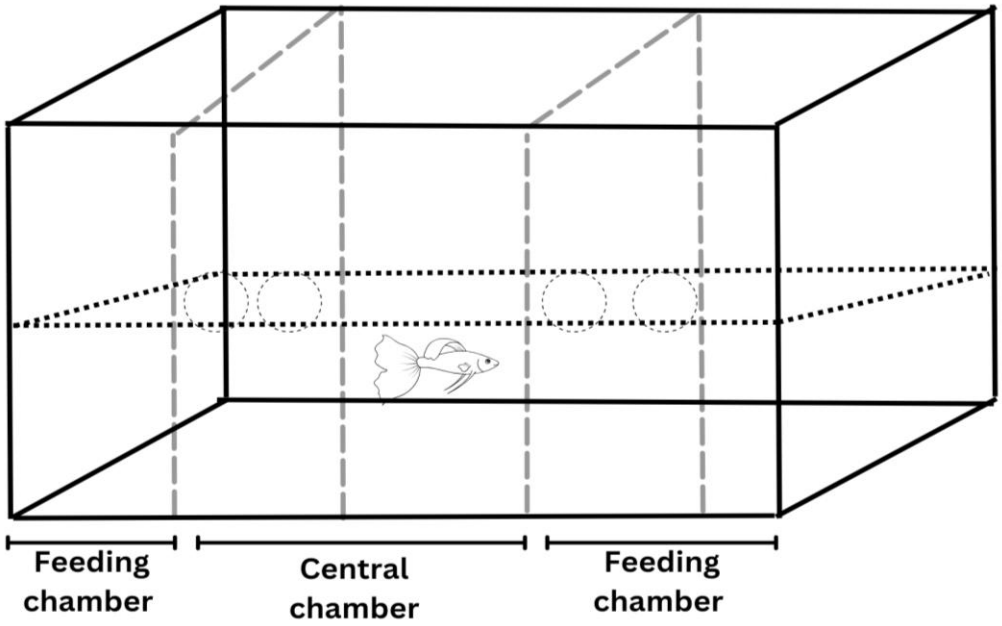

Figure 1 Diagrammatic representation of the experimental setup (ES) used to test cogntive functions development in the three-step behavioural analysis. Represented ES consists of one central chamber (CC) and two feeding chambers [right chamber (RC) and left chamber (LC)].

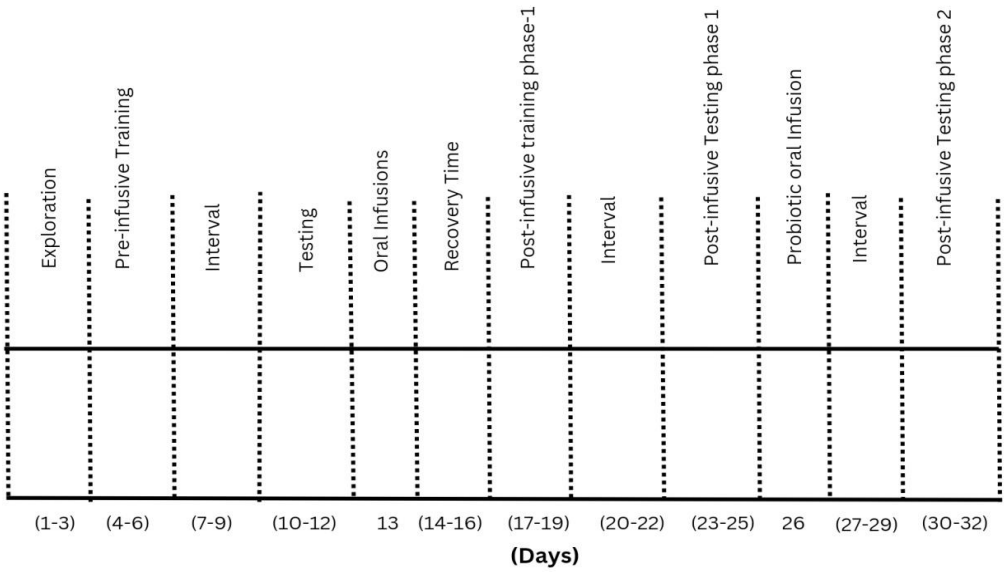

Figure 2 Line diagram showing the experimental time period of the three-step behavioural analysis used in this present study

2.6 Behavioural Analysis

Three-step behavioural analysis was designed for the study, consisting of one pre-infusive and two post-infusive analyses (Phases 1 and 2). The timeline followed for the behavioural study is shown in Figure 2.

2.6.1 Pre-infusive Behavioural Analysis (PrBA)

Experimental groups of EGs were allowed to stay in the aquarium to adapt to the laboratory conditions for 3 days during the habituation process. In the exploration phase, EGs were acquainted with the experimental setup for 3 days between days 1 – 3. Followed by exploration, training, and testing phase takes place in the ES in two different time intervals (4-6 days and 10-12 days). Further 72 hours of interval (3 days) was given between the training and testing phase for memory consolidation (Mukilan 2023).

2.6.2 Post-infusive Behavioural Analysis (PoBA)

Post-infusive behavioural analysis (PoBA) was carried out for the three EGs (Groups – 2, 3, and 4) after the completion of PrBA. On day 13th, oral infusion mixtures of isolates (*P. aeruginosa*, *E. coli*, and *B. subtilis*) were infused into respective EGs. Oral infusions were given into respective EGs with the help of an oral gauge. An interval period of 72 hours (Days 13 – 15) was given to infused EGs for their transportation to the gut (Mukilan 2023).

2.7 Statistical Analysis

Behavioural responses of four EGs (pre-infusive and post-infusive analysis) are plotted as a bar graphical representation with the help of KyPlot (Version 5.0).

3 Results

The present study employs a three-step behavioural analysis to prove a serene environment's role in forming cognitive memory. Three-step behavioural analysis showed the impact of a stress-free environment, oral infusions of isolates (*P. aeruginosa*, *B. subtilis*, and *E. coli*), and probiotic strain (*L. acidophilus*) on cognitive memory formation. Behavioural studies consist of one PrBA and two phases of PoBA (Phases 1 and 2).

3.1 Impact of stress-free habituated environment on cognition

Initially, the role of a stress-free environment was tested against cognitive learning and memory formation with the help of PrBA. PrBA consists of three different kinds of behavioural scores taken up from the exploration (Days 1-3), training (Days 4-6), and testing (Days 10-12) periods in the ES. In pre-infusive behavioural analysis, behavioural scores showed that animals spent more time in CC than the two FCs [LC and RC]. Exploration data revealed that animals were active in the ES. All experimental groups were allowed to explore the three chambers present in the ES with the help of brain navigation without color cues (Figure 3).

The training phase of the PrBA showed that all experimental groups (n = 6/group) tried to learn the presence of positive cues (blue color) based on reward-based learning. Behavioural scores of the training phase showed that animals learned about both positive and negative stimuli during the training period (Days 4 - 6). On day 4, the number of entries to the positive chamber was low compared to the negative chamber; later on, it got reversed due to learning of provided information/stimuli. Cumulative behavioural

scores showed a decrease in time spent in CC compared to the time spent in the FC. When comparing the time spent in each FC, RC has more attempts/time spent due to reward-based learning (Figure 4).

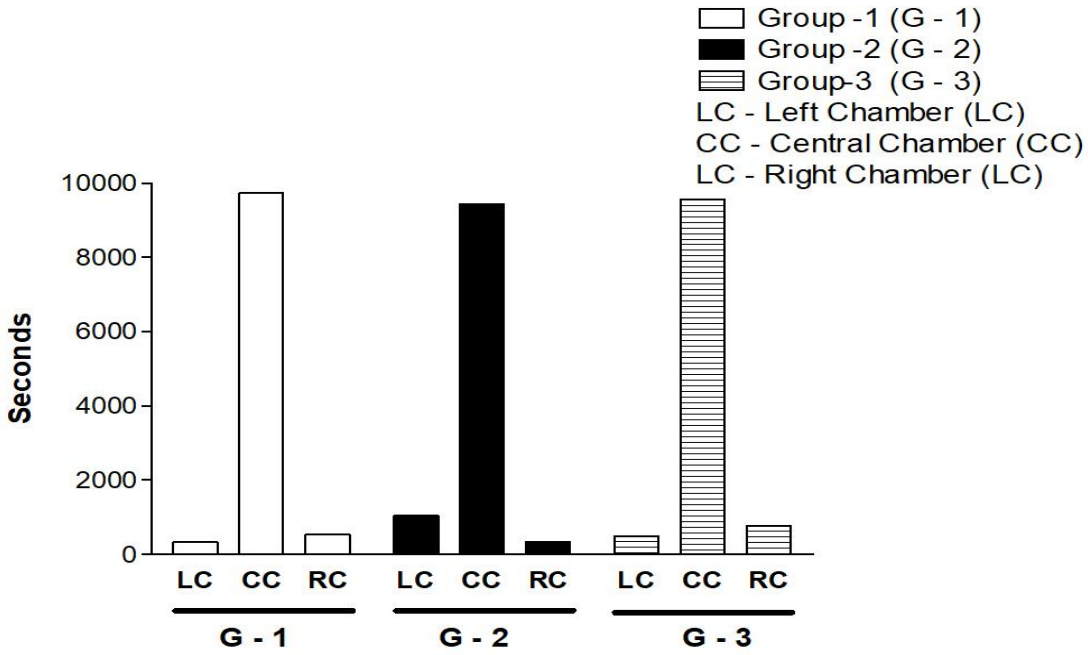

Figure 3 Behavioural scores of the exploration phase (Pre-infusive behavioural analysis) showed that animals spent more time in the central chamber (CC) compared to the left chamber (LC), and right chamber (RC). The outcome of the exploration phase stated that animals were active and tried to explore both LC and RC of the experimental setup (ES) in a serene habituated environment.

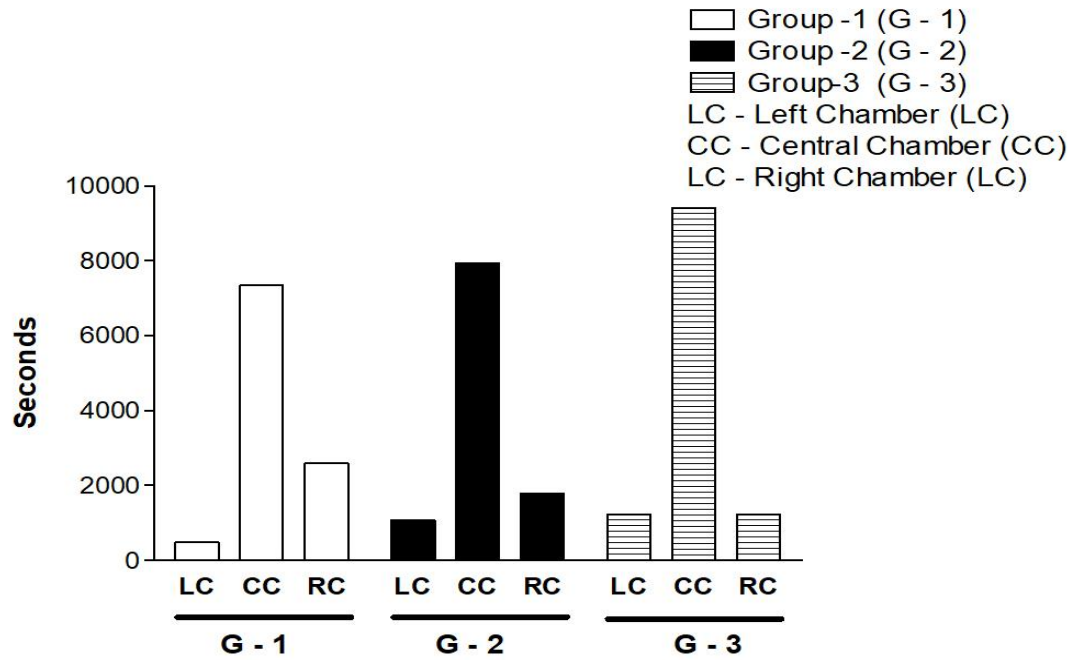

Figure 4 Training phase of the pre-infusive behavioural analysis showed that the experimental animals tried to learn the presence of positive cues based on reward-based learning. Behavioural scores of the training phase (pre-infusive analysis) showed that during the training period (Days 4 -6) animals learned about the positive and negative stimuli. During the training phase, time spent in the CC gradually decreased compared to the amount of time spent in the FC (LC, and RC).

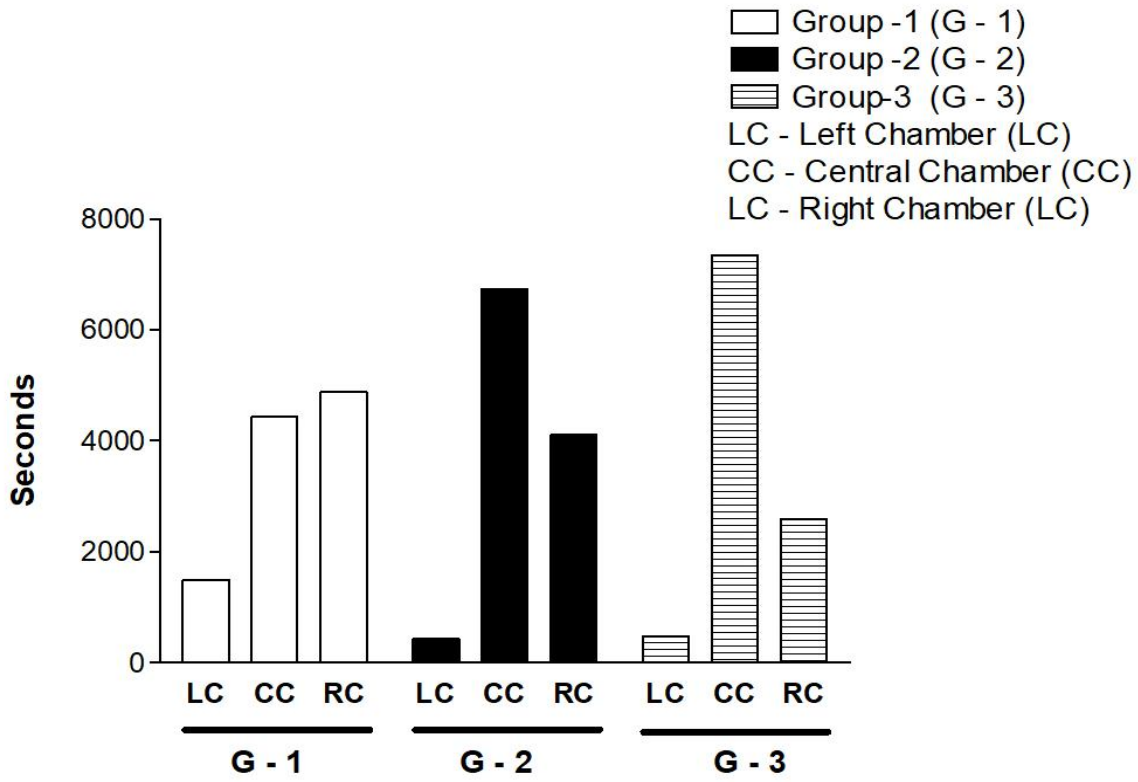

Figure 5 Pre-infusive testing phase showed that animals learned about the positive and negative stimuli; and were able to retrieve the learned information during the testing phase. Compared to the training phase, the identification of positive stimuli was high compared to the negative stimuli identification. Identification of negative stimuli was gradually decreased in the days – 2, and 3 of testing.

After the PrBA training phase, all experimental groups were given an interval period of three days. Three days of time interval is given to consolidate learned information in the brain. Testing was done between days 10 – 12 to test the retrieval of learned information. Behavioural responses of the testing phase showed that correct choices were high compared to the PrBA training period. Thus, it was proved that there was no hindrance in developing cognitive functions during pre-infusive behavioural studies (without any infusions) (Figure 5).

3.2 Role of *P. aeruginosa, B. subtilis,* and *E. coli* oral infusions on the development of cognitive impairment

Before PoBA, all desired microorganisms, including three isolates (*P. aeruginosa*, *B. subtilis*, and *E. coli*) and one probiotic strain (*L. acidophilus*), were streaked on the nutrient agar medium to confirm the purity of microorganisms used in the study by the use of simple and quadrant streaking method. For *Lactobacillus* strain confirmation, the individual colony was again streaked on the *Lactobacillus* MRS agar plates for double confirmation from the nutrient agar plate. After purity confirmation, three isolates were used to prepare an overnight culture with the specified volume of 5 ml (Figure 6). Later, overnight-grown cultures were mixed with PBS in the ratio of 50:50 as oral infusion mixtures. Prepared oral infusion mixtures were given to the animal with the help of an oral gauge on day 13. After infusions, an interval of 72 hours was given to transport oral infusion toward the gut of animals (Days 14 – 16).

PoBA Phase 1 analysis was done after the completion of interval time from day 17. In the first phase of PoBA, training (Days 17 – 19) and testing (Days 23-25) were done for three infused groups that received oral microbial infusions of *P. aeruginosa*, *B. subtilis*, and *E. coli*. Behavioural scores of the training period showed that oral microbial infusions do not impact the brain homeostasis mechanism (Figure 7). Followed by training, testing was done between days 23 – 25 which showed the impairment in the memory retrieval from brain regions. Behavioural scores of PoBA testing phase 1 showed increased response in CC and decreased response in RC. Decreased response in RC showed memory retrieval imbalance in the experimental group treated with *P. aeruginosa* (Figure 8). Memory retrieval may be impaired due to microbial metabolite transport from the gut to the brain through BBB. Excess amounts of enterotoxin present in this microbial metabolite may inhibit the growth and proliferation of beneficial bacteria. Inhibition of beneficial bacterial proliferation may decrease the synthesis of neurotransmitter precursor compounds responsible for the brain neurotransmitter synthesis. The least amount of neurotransmitter synthesis results in impaired LTM formation.

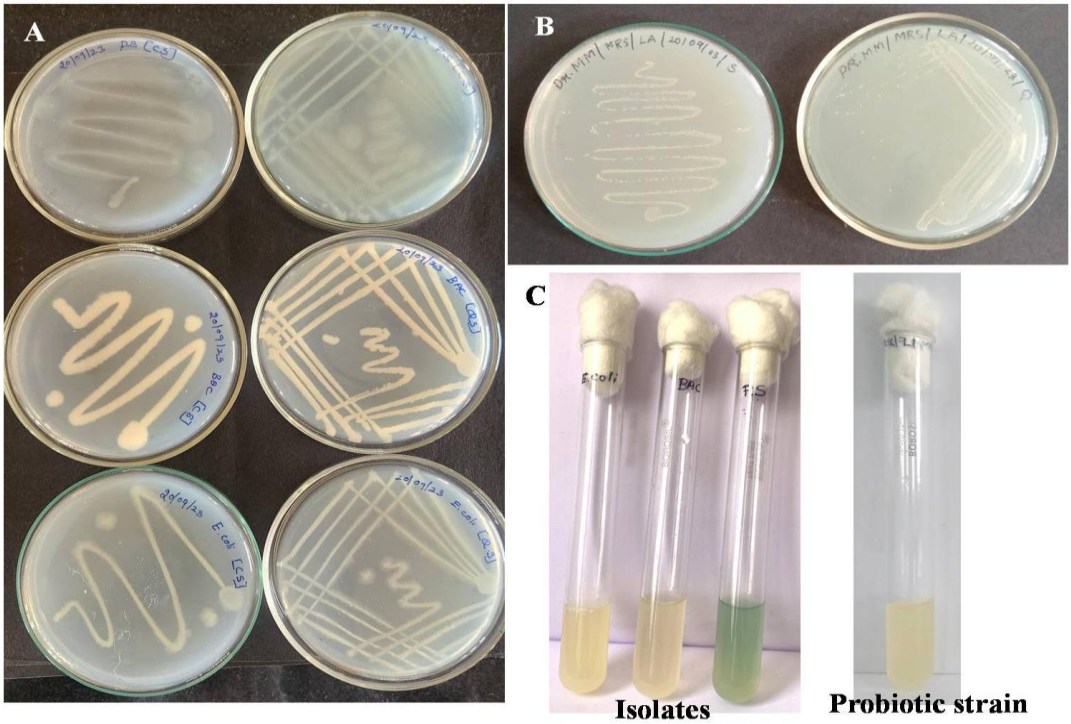

Figure 6 Representative plate photographs showing the purity of isolates and probiotic strain used in the present study. All four microorganisms [Three isolates (*P. aeruginosa*, *B. subtilis*, and *E.coli*) and one probiotic strain (*L. acidophilus*)] were streaked in nutrient agar medium by the way of simple and quadrant streaking method. For lactobacillus strain confirmation, an individual colony of lactobacillus was streaked on lactobacillus MRS medium for its double confirmation from the nutrient agar plate.

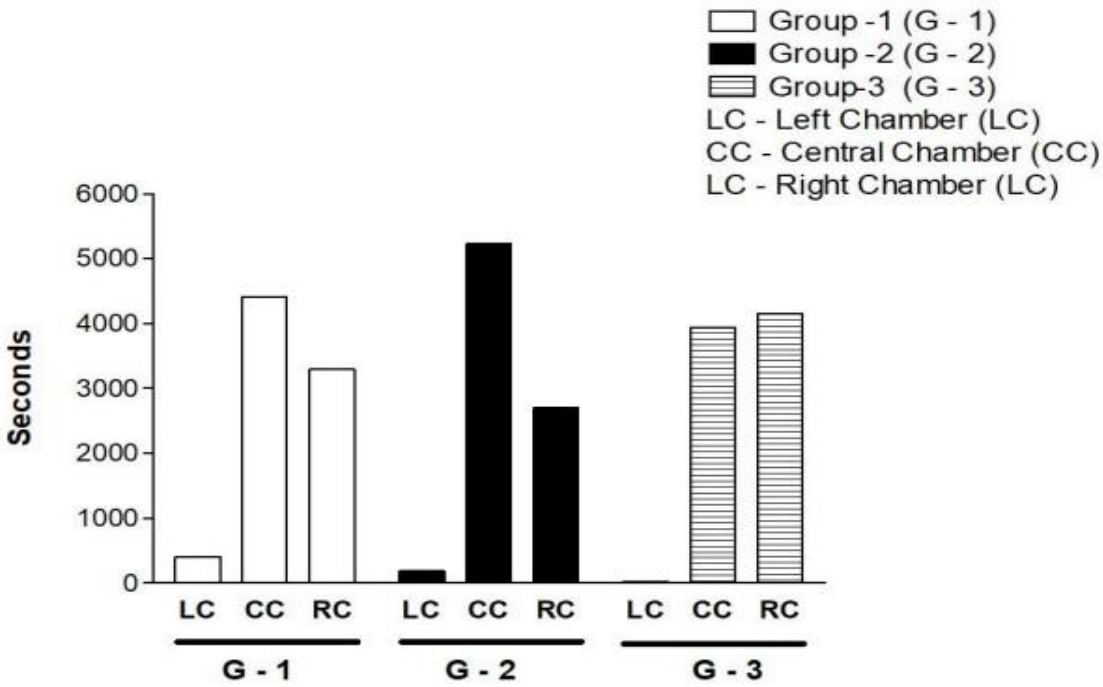

Figure 7 The training phase of post-infusive bahavioural analysis (phase -1) showed that oral infusions of *P. aeruginosa*, *B. subtilis*, and *E.coli* do not have an impact on the animal learning abilities. Behavioural scores of the training period showed that there is no imbalance in homeostasis mechanisms due to oral infusions of isolates.

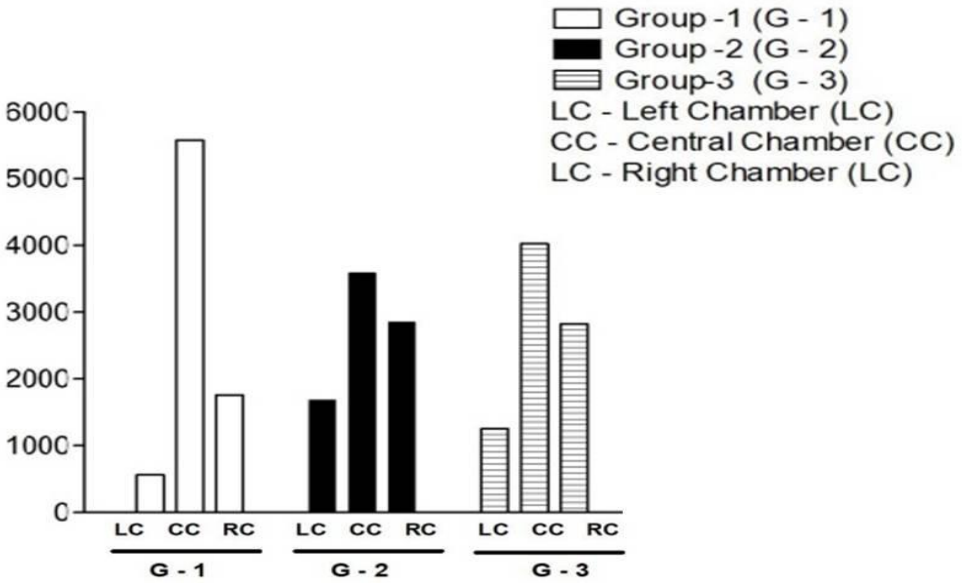

Figure 8 The testing phase of post-infusive behavioural analysis (phase -1) showed that oral infusions of *P. aeruginosa*, *B. subtilis*, and *E.coli* have an impact on information processing and memory retrieval in the experimental groups.

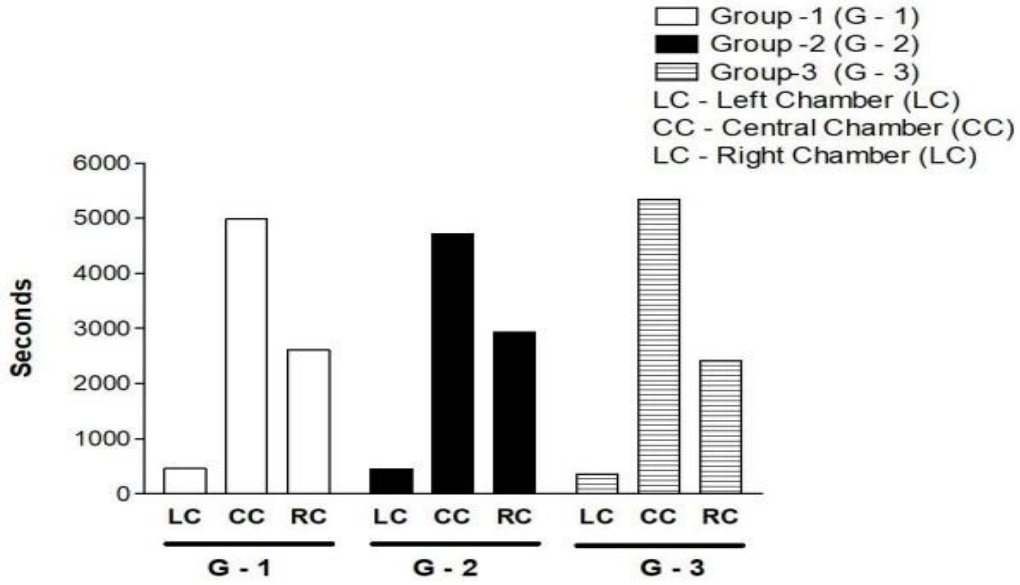

Figure 9 Post-infusive testing phase - 2 showed that probiotic infusion (*L. acidophilus*) reversed the oral infusions induced cognitive impairment. During testing, the number of correct responses was high compared to wrong responses, which shows the recovery of cognitive impairment.

3.3 Reversal of microbial metabolite-induced cognitive impairment using the probiotic strain (*L. acidophilus*)

To identify the role of *L. acidophilus* in the reversal of cognitive impairment, PoBA Phase -2 was carried out between days 30 – 32. Experimental behavioural scores proved that probiotics reversed microbial metabolite-induced cognitive impairment (LTM impairment). In the Phase -2 testing, the number of responses towards the correct responses increased like the Phase -1 training. Other than that, the number of wrong choices also concerned PrBA testing. The resulting outcome showed that the number of correct and wrong choices resembled the same as the testing phase of a stress-free environment (PrBA). Thus, from the PoBA Phase 2 testing study, this study proved that the probiotic strain (*L. acidophilus*) reversed the *P. aeruginosa*-induced cognitive impairment (Figure 9).

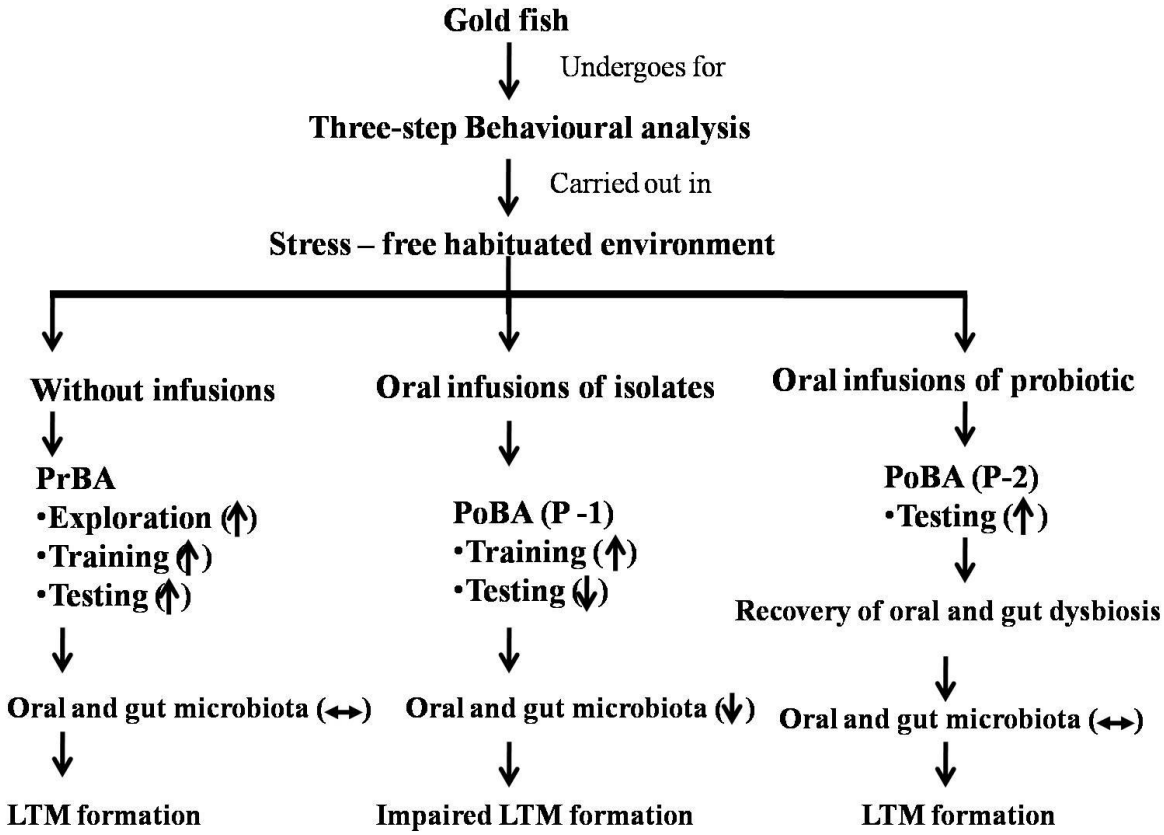

Figure 10 Flow-diagram showing the role of oral and gut microbiota in the development of LTM formation

4 Discussion

In the gut-brain axis, gut microbiota plays (GM) a major role in the regulation of neurotransmitter production (Ma et al. 2019; Misiak et al. 2020; Sheng et al. 2021; Hinds and Sanchez 2022; Mukilan 2023). This GM consists of microorganisms like bacteria, viruses, and fungi and these microbiotas may control intestinal hydrogen ion concentration (pH), and inhibit the growth of infectious pathogens (IP) in the gut (Jiang et al. 2017; Kaczmarek et al. 2017; Gentile and Weir 2018; Hillemacher et al. 2018; Savin et al. 2018; Ng et al. 2023). Growth inhibition of IP results in the development of a healthy state (HS) via probiotic treatment. Further, HS will show the mutual exchange and regulation of neurotransmitter precursor compounds (NPC) and enterotoxin (ET) (Aponte et al. 2020; Piatek et al. 2020; Raheem et al. 2021; Shandilya et al. 2022; Mazziotta et al. 2023). In the brain, these transported NPC from the gut used for the synthesis of neurotransmitters like serotonin (5-HT), γ-aminobutryic acid (GABA), dopamine (DA), and noradrenaline (NA) (Chen et al. 2017; O'Donnell et al. 2020; Shandilya et al. 2022; Mazziotta et al. 2023). These synthesized neurotransmitters may positively regulate the calcium influx (CI), adenyl cyclase (AC), PKA, and cAMP levels in a stress-free environment. Regulation of these abovementioned molecules results in the phosphorylation of CREB followed by the activation of IEGs and PSD proteins (Mukilan et al. 2015; Mukilan et al. 2018a; Thangaleela et al. 2018; Mukilan 2023). However, an imbalance in the regulation and phosphorylation of these neuronal signaling molecules may result in cognitive memory decline. Formed cognitive memory decline was a result of reduced synaptic plasticity formation (Ganesh et al. 2012; Preethi et al. 2012; Mukilan et al. 2015; Ortega-Martínez 2015; Sen et al. 2017; Mukilan et al. 2018b; Thangaleela et al. 2018; Mukilan 2023).

This memory impairment may occur due to oral/gut dysbiosis. These oral and gut dysbiosis shows an increase in pathogenic microorganisms and a decrease in beneficial microorganisms (Orr et al. 2020; Sarkar et al. 2020; Lotz et al. 2021; Park and Wu 2022; Da et al. 2023; Jemimah et al. 2023). Recently, it was reported that oral infusions of *P. aeruginosa*, *B. subtilis*, *S. aureus*, and *E. coli* impact cognitive memory development. Recent reports showed that probiotic microorganisms may be a potential agent to increase synaptic plasticity development by regulating ET. During cognitive impairment, there is a reduced expression of neuronal signaling molecules due to the transport of an excess amount of ET from the enteric nervous system to the central nervous system (Aponte et al. 2020; Piatek et al. 2020; Raheem et al. 2021; Shandilya et al. 2022;

Mazziotta et al. 2023). This condition may be reversed with the help of short-chain fatty acids (SCFA). Production of SCFA may regulated by the probiotic organisms present in the gut. These SCFAs will interact with the ET produced by the IP. As a result of interaction, there is a regulation of infectious pathogens and ET levels through the blood-brain barrier (Daliri et al. 2018; Asadpoor et al. 2021; Ma et al. 2022; Qian et al. 2022; Ney et al. 2023). Regulation of ET results in the reduction of memory impairment through the expression recovery of neuronal signaling molecules involved in CMNSP (Ganesh et al. 2012; Preethi et al. 2012; Mukilan et al. 2015; Ortega-Martínez 2015; Sen et al. 2017; Mukilan et al. 2018a; Thangaleela et al. 2018; Mukilan 2023). The present study tried to elucidate the effect of probiotic microorganisms against the reversal of cognitive impairment. Initially, cognitive impairment was induced by the oral infusion of periodontic microorganisms, like *P. aeruginosa*, *B. subtilis*, and *E.coli*. Later, reversals of cognitive impairments were proved by infusing probiotic microorganisms into the impaired experimental groups. Ongoing studies in our lab may identify the impact of ET on cognitive memory formation through its transportation via the blood-brain barrier (BBB).

Conclusion

The present study proved probiotic microorganisms' role in retrieving induced cognitive memory decline through oral administration. The study's outcome showed that improper oral hygiene caused by microbiota imbalance can be reverted back into normal conditions with the help of probiotic supplementations. Experimental results also showed that enterotoxin produced by infectious pathogens may inhibit the formation of neurotransmitter precursor compounds and block its transportation from the gut to the brain through the blood-brain barrier. Three-step behavioural analysis showed impaired memory development occurred in the infused groups compared to the control initially. Later on, impaired cognition was reversed with the oral infusions of probiotics. Retrieval of impaired cognition may happen due to the transmission of microbial precursor compounds from the gut to the brain through the blood-brain barrier (BBB). Thus, the present study proved the role of probiotic oral microbial infusions on the development of cognitive memory. The overall outcome of the study showed that improper oral hygiene played a crucial factor in the development of cognitive dysfunction through the gut-brain axis.

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Author Contributions

MM performed the conceptualization, research design, funding acquisition, original investigation, draft preparation, review and editing of the manuscript. MTAM, SY, and VM did the experiment performance and data collection.

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