Journal of Experimental Biology and Agricultural Sciences, June - 2023; Volume – 11(3) page 581 – 592

Journal of Experimental Biology and Agricultural Sciences

http://www.jebas.org

ISSN No. 2320 – 8694

Impact of *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli* Oral Infusions on Cognitive Memory Decline in Mild Cognitive Impairment

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Received – March 07, 2023; Revision – May 29, 2023; Accepted – June 07, 2023
Available Online – June 30, 2023

DOI: http://dx.doi.org/10.18006/2023.11(3).581.592

KEYWORDS

Learning

Memory

Bacillus subtilis

Staphylococcus aureus

Escherichia coli

Pseudomonas aeruginosa

Cognitive decline

ABSTRACT

Synaptic plasticity is a result of changes in the neuronal circuits which may result in the formation of protein-dependent (long-term memory (LTM) formation) and protein-independent (short-term memory (STM) formation) memories. This STM formation is based on existing proteins, but LTM formation depends on RNA and protein synthesis within the neuronal cells. This RNA and protein synthesis may depend on stimulus exposure like odour, taste, and other environmental stimuli. The present study is aimed to show the impact of oral bacterial infusions on cognitive memory formation through pre and post-infusive behavioural analysis. The results of the study revealed that oral infusions of *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* result in impaired cognitive learning and memory formation. This impaired cognitive memory formation is shown with the help of two-step (pre and post-infusive) behavioural analysis. Pre-infusive behavioural study shows no decline in cognitive learning and memory formation before oral microbial infusions in a serene habituated environment. After oral microbial infusions, a post-infusive behavioural analysis may reveal a memory decline in the treated group. Comparative two-step behavioural analysis indicates that *P. aeruginosa* infusions strongly impact cognitive memory decline compared to the other three groups. This cognitive memory decline may happen due to the production of primary/secondary metabolites within the animal gut and their transportation to the CNS through the blood-brain barrier. The outcome of the present study states that poor oral hygiene plays a significant role in cognitive memory decline concerning mild cognitive impairment (MCI).

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Peer review under responsibility of Journal of Experimental Biology and Agricultural Sciences.

Production and Hosting by Horizon Publisher India [HPI] (http://www.horizonpublisherindia.in/).

1 Introduction

In brain cognition, long-term memory (LTM) formation plays a pivotal role in the survival of any organism (Kandel 2001; Bisaz et al. 2014). This LTM is formed by continuous exposure to any environmental and physiological stimulus for a limited/unlimited period. LTM formation is based on the RNA-dependent protein synthesis mechanism and is initiated by releasing neurotransmitters from the presynaptic neuron into the synaptic cleft. Released neurotransmitters may bind with their respective receptors present on the surface of postsynaptic neurons at the synaptic cleft (Davis and Squire 1984; Schafe and LeDoux 2000; Jones et al. 2001; Scharf et al. 2002; Abraham and Robins 2005; Igaz et al. 2006; Fonseca et al. 2006; Abraham and Williams 2008; Alberini 2008; Alberini 2009; Smart and Paoletti 2012; Evans et al. 2021; Lin et al. 2021). As a result of binding of neurotransmitters with the specific postsynaptic membrane receptors may result in the upregulation of cyclic adenosine monophosphate (cAMP). Increased level of cAMP further activates several downstream molecules like protein kinase A (PKA), enzyme-regulated kinase – 1/2 (ERK– 1/2), cAMP response element binding protein - 1 (CREB-1), Immediate early genes (IEG's), and postsynaptic density (PSD) proteins (Jones et al. 2001; Mohamed et al. 2005; Abraham and Williams 2008; Ganesh et al. 2010; Besnard et al. 2011; Ganesh et al. 2012; Philips et al. 2013; Nelson et al. 2013; Miyashita et al. 2018; Mukilan et al. 2018a, 2018b; Wang et al. 2019; Sharma and Singh 2020; Mukilan 2022). The Collective expression of these molecules will result in the formation of LTM in a stress-free environment (Ganesh et al. 2010; Besnard et al. 2011; Ganesh et al. 2012; Nelson et al. 2013; Miyashita et al. 2018; Mukilan et al. 2018a, 2018b; Wang et al. 2019; Sharma and Singh 2020; Karen et al. 2021; Mukilan 2022).

Recent reports show that gut microbiota (GM) is essential for the synthesis of brain precursor neurotransmitter components, which will affect the synthesis and concentration of related neurotransmitter production in the brain (Gao et al. 2018; Ticinesi et al. 2018; Gao et al. 2019; Jameson et al. 2020; Chen et al. 2021; Dicks 2022). It is also reported that intestinal precursor neurotransmitter transportation pathways are involved either directly or indirectly with the cognitive functions of the brain (Ticinesi et al. 2018; Caspani and Swann 2019; Jameson et al. 2020; Chen et al. 2021; Dicks 2022). Neurotransmitters like serotonin (5-HT), dopamine (DA), and norepinephrine (NE) are converted from amino acid precursors, and it is released from presynaptic neuron into the synaptic cleft and binds with postsynaptic neuronal receptors (Yano et al. 2015; Agus et al. 2018; Luqman et al. 2018; Strandwitz 2018; van de Wouw et al. 2018; Sadaqat et al. 2021; Chen et al. 2021; Dicks 2022). One of the precursor molecules, short-chain fatty acids (SCFA), regulates this process. These SCFA molecules act as a crucial molecules for the signal transport between gut microorganisms and the brain (Franco-Robles and López 2015; van de Wouw et al. 2018; Heyck and Ibarra 2019; Hu et al. 2020; Markowiak-Kopeć and Ślízewska 2020; Verhaar et al. 2022; Neto et al. 2023). Recent studies showed gut microorganisms have an impact on the gut-brain axis (GBA) through the hypothalamic-pituitary-adrenal (HPA) axis (Murciano-Brea et al. 2021; Rosin et al. 2021). Some of the gut bacterial species may also regulate neurotransmitters like 5-HT, DA, noradrenaline (NA), and γ-aminobutyric acid (GABA) (Chen et al. 2017; Wong et al. 2018; O'Donnell et al. 2020).

In a normal/non-infected state, synthesized precursor neurochemical substances are transported from the enteric nervous system (ENS) to the central nervous system (CNS) through the vagus nerve for the synthesis of neurotransmitters in the brain (Cryan and Dinan 2012; Bauer et al. 2016; Baj et al. 2019; Giuffré et al. 2020; Orr et al. 2020; Sadaqat et al. 2021). Any imbalance in GM may result in impaired neurochemical signal transmission to the CNS and further results in the altered expression of 5-hydroxytryptamine (5-HT) receptor subunits in the hippocampus, which may lead to impaired cognition (Ganesh et al. 2010; Zhang and Stackman Jr 2015; Mukilan et al. 2018a; Bo et al. 2020; Liu et al. 2021; Roux et al. 2021; Li et al. 2022). The present study has made an initial attempt to show the impact of *P. aeruginosa*, *B. subtilis*, *S. aureus*, and *E. coli* oral infusions in cognitive memory formation with the help of reward-based learning based on pre and post-infusive training strategies in the naïve goldfish *Carassius auratus*.

2 Materials and Methods

2.1 Experimental Animals and Apparatus

Naïve goldfish (*Carassius auratus*) with a body length of 6.5 – 8 cm and 6 – 15 g weight were purchased from a local aquarium. Purchased fish were housed in rectangular tanks (serving as a home tank) having a length of 42 inches, breadth of 30 inches, and height of 21 inches. Fish were housed in groups of 8 (n = 8/group) at the home tank with sufficient continuous aeration at a standard temperature of 28 ± 2˚ C and 12 hours of light and dark cycle for maintaining a regular circadian rhythm. Commercially available round food pellets were purchased from the local market and used as a food source for the experimental fish. Fish were given enough food pellets twice daily to maintain their energy budget (6 g/fish). The home tank was minimized to debris free environment by cleaning and replacing the water available in the home tank on alternative days. Experimental protocols followed as per the institutional ethical guidelines of Sri Ramakrishna Institutions, Coimbatore.

2.2 Experimental Apparatus

An experimental glass tank of 42 X 30 X 21 inches was selected for the *C. auratus* experiment. The experimental glass tank was

divided into three compartments: one central chamber (CC) and two feeding chambers (one on the right side of CC and another on the left side). The central chamber was 30 X 30 X 21 inches and the feeding chamber (FC) was 6 X 30 X 21 inches.

2.3 Oral Infusion Mixture Preparation and its Infusion

All four isolates *P. aeruginosa*, *B. subtilis*, *S. aureus*, *E. coli* were availed from PSG Institute of Medical Sciences & Research, Coimbatore. All microorganisms were streaked on nutrient agar for their pure isolation. After confirming purity, colonies were selected from quadrant-streaked plates overnight in four 5 ml nutrient broth test tubes. The grown overnight culture was used to prepare an infusion mixture with saline/PBS for the oral route infusions in the *C. auratus* experimental groups. The formulated infused blend was orally infused into the experimental animal with 500 ml as a single dose. After infusions, experimental animals were maintained in a separate tank for their recovery and habituation for 24 hrs.

2.4 Behavioural Study

Behavioural analysis was carried out for all formulated five groups of experimental animals, i.e. Control (CON), *P. aeruginosa* infused group (T_1), *B. subtilis* infused group (T_2), *S. aureus* infused group (T_3), and *E. coli* infused group (T_4) through pre and post infusive behavioural studies.

2.4.1 Pre-infusive Behavioural studies

To examine the role of *P. aeruginosa*, *B. subtilis*, *S. aureus* and *E. coli* infusions on cognitive learning and memory formation, uninfused animal groups (n = 8/group) underwent behavioural analysis through exploration, learning and memory behavioural paradigms. Pre-infusive studies showed the normal behavioural responses of experimental groups in a habituated/ stress-free environment concerning positive/negative reward mechanisms.

2.4.1.1 Exploration

After habituation, animals were allowed to explore the experimental setup for three days to avoid stress during behavioural analysis.

2.4.1.2 Training

Animals were trained in an experimental setup with two feeding chambers. In each feeding chamber, one blue and red colored light was placed. The blue-light chamber acted as a positive reward place with a food pellet, and the red-light chamber acted as a negative reward place without a food pellet. Animals were introduced in the centre chamber at 0 minutes of the training phase. Further, animal movements were watched to know the time to reach the feeding chambers after introduction in the central chamber to know the learning state of each group. The positive reward was confirmed by the intake of food pellets by the animal.

2.4.1.3 Testing

Testing was done after three days of training, and this interval time was given to all experimental groups after pre and post-infusive training for proper memory consolidation in the brain. Both pre-infused and post-infused animal groups were tested for retrieving learned information through the reward mechanism used during the training paradigm.

2.4.2 Post-infusive Behavioural studies

After 24 hrs of infusions, the animal groups were trained to the positive/negative reward mechanism. Different experimental groups were tested for the retrieval of memory formation after 72 hrs of training in the experimental setup.

2.5 Data analysis

Behavioural scores of all experimental groups (exploration, training and testing) are plotted with KyPlot (version 1.0) for graphical representation.

3 Results

3.1 Effect of pre-infusive behavioural studies on cognitive memory formation

3.1.1 Exploration

Exploration data showed that, initially, all groups of animals spent more time in the CC during the exploration period of three days. On Day 1, animals spent more time in the central chamber due to the new environment/stress while the group 2 and 3 spent less time in either feeding chamber, but group 1 still needs to be exposed to the FC. Later on days 2 & 3, the time spent on CC is reduced, and a visit to both feeding chambers is increased due to the increased exploration skills acquired by the animal in the CC. These exploratory behavioural scores were plotted as a graph (Figure 1).

3.1.2 Training

Followed by the exploration studies, training was carried out for all experimental groups with the help of positive/negative reward mechanisms through red/blue visual cues. The training was given between days 4 - 6. During the period of training, animals came to know about food rewards-based learning by their entry into both of the feeding chambers. From their exploratory behaviour, animals learned about their visit to FC with blue color light having a reward and FC with red color light not having a reward. The behavioural scores of training showed that group 3 animals had more visits to the right chamber than groups 2 and 1 (Figure 2).

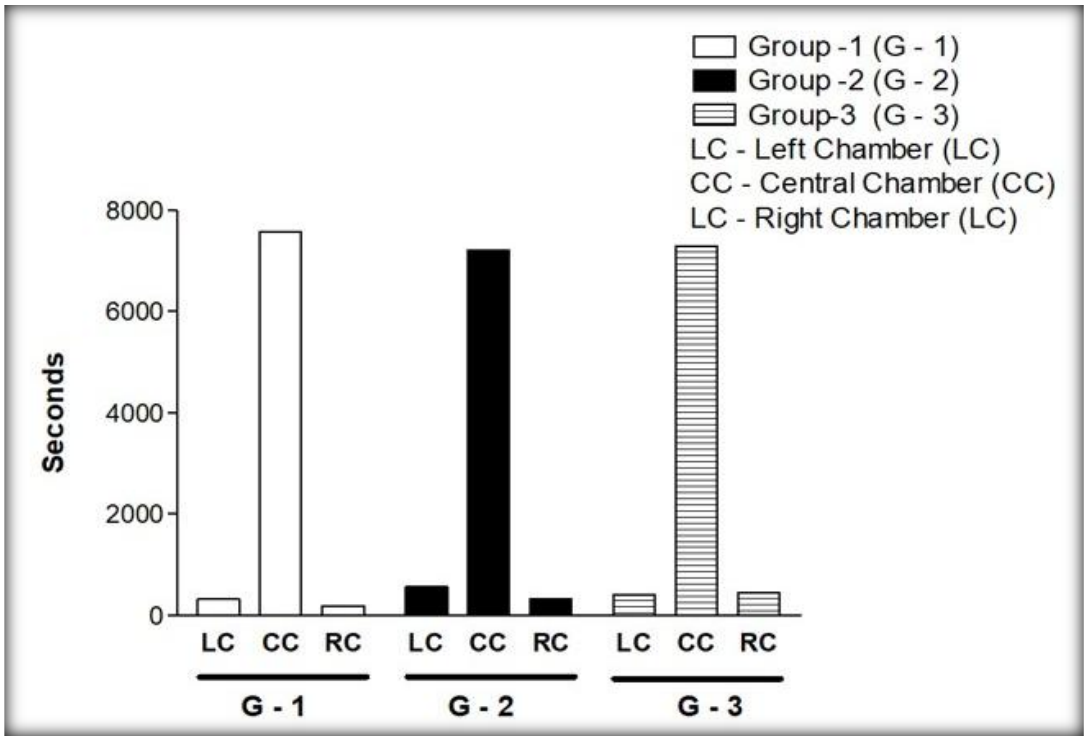

Figure 1 Exploration phase of pre-infusive analysis showed that animals were active and explored all the regions of the experimental setup in a stress-free manner

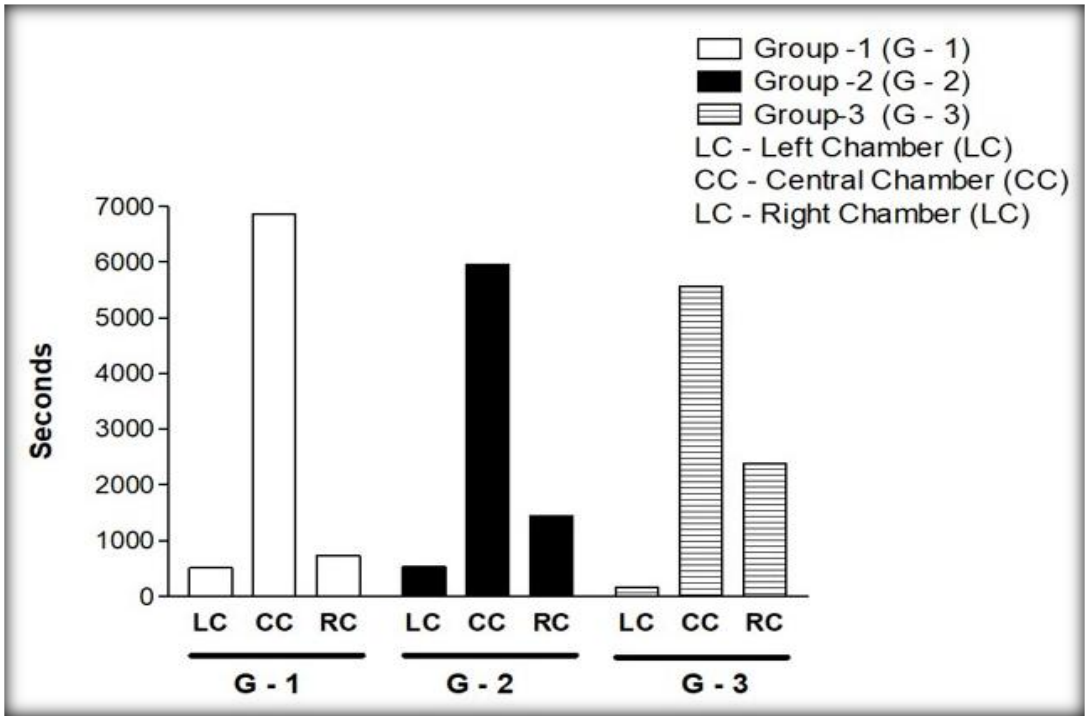

Figure 2 The Training phase of pre-infusive analysis showed that animals learned the task on subsequent experimental days (Days 4 – 6). The number of attempts was increased from the first day of training till the third day. Time spent in the central, right, and left chamber is given in the bar diagram, which shows an increased response in reward-based learning, and experimental animals learned about reward-based colour cues.

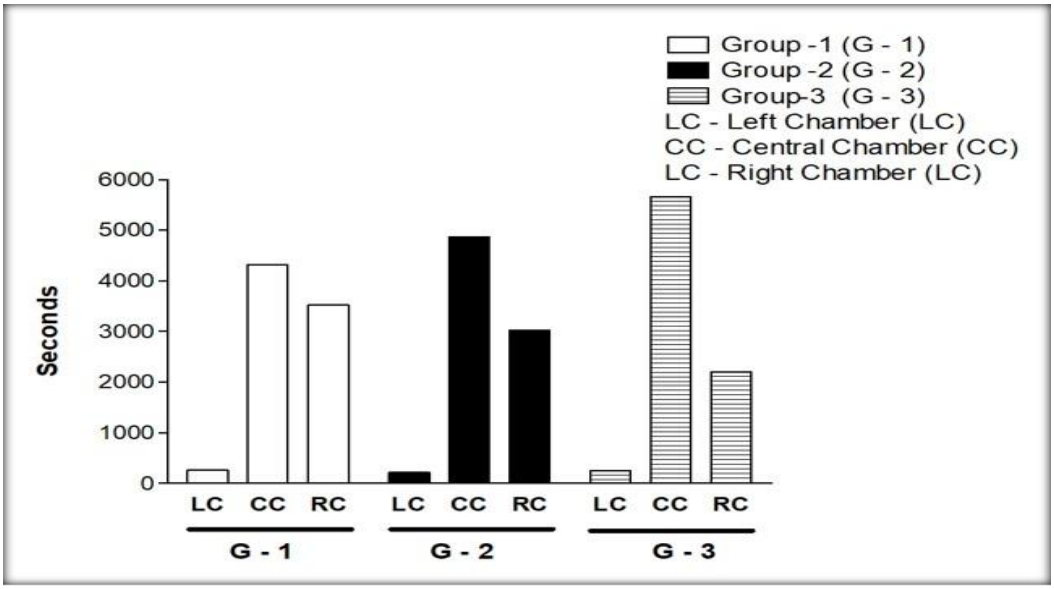

Figure 3 The testing phase of pre-infusive analysis showed that animals learned about the task and retrieved the learned information. The number of correct choices was high compared number of wrong choices.

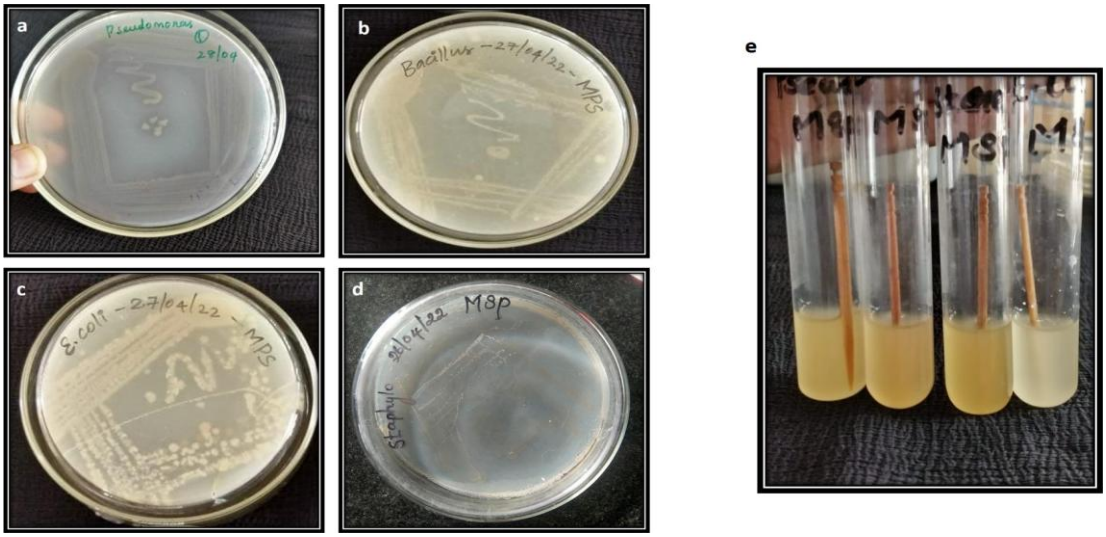

Figure 4 Representative photographs showing the quality of pure culture used in the study. All four microorganisms *P. aeruginosa*, *B. subtilis*, *S. aureus*, and *E. coli* were grown in the nutrient agar medium to check the purity of the culture with the help of the quadrant streaking method.

3.1.3 Testing

After three days of intervals, memory was tested for the three groups between days 10 - 12, with the same experimental setup having red and blue visual cues. Behavioural scores showed that memory retrieval was responsible for making the correct choice. Blotted behavioural scores showed that all trained individuals have an increased number of correct and fewer incorrect visits. It showed that stored information was retrieved from the brain during training. The behavioural scores of testing's showed that there was a greater number of correct responses in all the groups. It was also reported that group - 1 retrieved stored information efficiently through neuronal plasticity development to groups 2 and 3 (Figure 3).

3.2 Impact of oral infusions on cognitive memory formation

Pure culture of all four microorganisms (*P. aeruginosa*, *B. subtilis*, *S. aureus* and *E. coli*) were grown overnight in 5 ml of nutrient broth and mixed with saline/PBS in an appropriate ratio, and these were infused into the oral passage of the animal with an oral gauge (Figure 4). After oral infusions, animals were given three days

(Days 13 - 15) for proper movement of infusion towards the gut of the experimental animals. From Day 16, post-infusive behavioural analysis was done with the help of training and retention behavioural scores.

To understand the impact of infusions on cognitive memory formation, infused animal groups were trained in the experimental setup to understand whether infusions impact cognitive memory formation. During post-infusive behavioural training, there was an enhanced impact on correct decision-making in all experimental groups between 16 - 19 days. After training, testing was done at three different time intervals (Days 23 - 26). Observed behavioural scores showed a gradual decrease in making correct decisions (Figure –5 a, b). After 17 days of oral infusion, post-infusion analysis

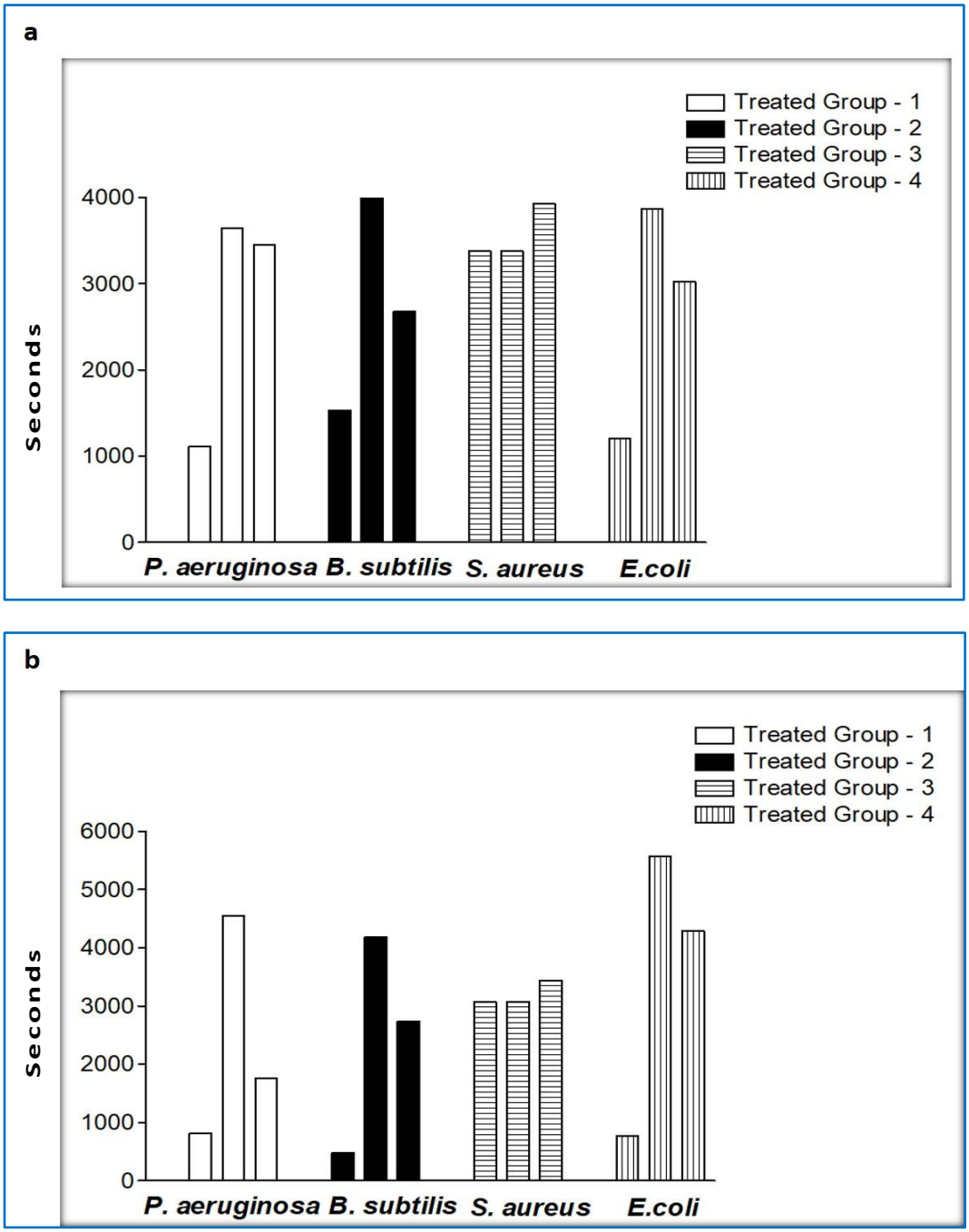

Figure 5 Post-infusive analysis showed that oral infusions did not impair its homeostasis mechanism. Scores of the training phase (5 a) provoked no hindrance in the learning abilities compared to the testing phase (5 b).

was done again to check whether these microorganisms have a more prolonged impact on cognitive memory formation. Surprisingly, obtained results showed that there might be a recovery in the testing phase, which offers a gradual increase in memory formation after pre-infusive analysis (Figure 6 a, b). Obtained results strongly concluded that cognitive decline might happen due to the infused microorganisms' production of primary or secondary metabolite for a limited time in the experimental groups. This study reports that increased *P. aeruginosa*, *B. subtilis*, *S. aureus*, and *E. coli* in the gut impact brain cognition.

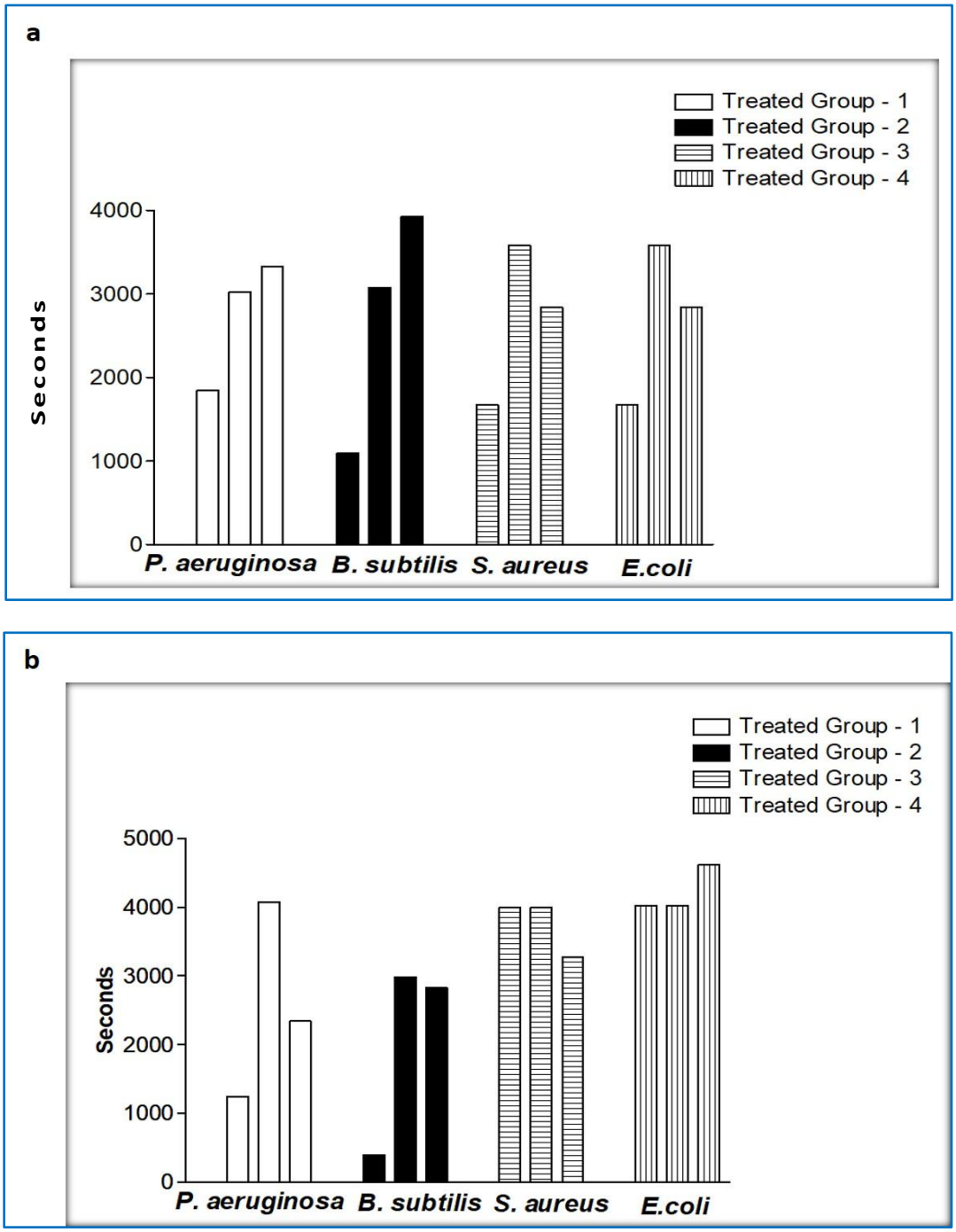

Figure 6 Post-infusive analysis showed that oral infusions do not have a prolonged impact on memory formation. Scores of the training phase (Fig. 6 a) proved that microbial colonization does not impact cognitive learning and memory formation. Obtained scores state that *P. aeruginosa*, *B. subtilis*, *S. aureus*, and *E. coli* oral infusions may impact cognitive memory formation for a short period.

4 Discussion

In brain cognition, oral hygiene, and the GBA will play a major role in the formation of LTM through the enteric nervous system (ENS) by its interconnection (Ribeiro et al. 2012; Martande et al. 2014; Shoemark and Allens 2015; Orr et al. 2020; Sadaqat et al. 2021). The ENS transports gut-secreted precursor chemical components to the central nervous system (CNS) for the synthesis of neurotransmitters like 5-HT, DA, NE, etc. (Strandwitz 2018; van de Wouw et al. 2018; Agus et al. 2018; Sadaqat et al. 2021; Dicks 2022). Synthesized neurotransmitters are released into the synaptic cleft by the presynaptic neuron, and it binds to the specific receptors of the postsynaptic neuron and resulting in the activation of molecules like adenyl cyclase (AC), extracellular regulated kinase-1/2 (ERK-1/2), protein kinase A (PKA), cAMP response element binding protein (CREB), immediate early gene (IEG) cascade and postsynaptic density proteins (PSD). Apart from these molecules, some microRNAs (miR) like miR-132/148a may also critically regulate LTM formation through the activation of down-stream molecules (Ganesh et al. 2010; Ganesh et al. 2012; Mukilan et al. 2018a, 2018b; Mukilan 2022). These signalling molecules will be expressed in a required balanced state during stimulus exposure in the development of LTM through extracellular regulated kinase neuronal signalling pathway (ERK-NSP) (Ganesh et al. 2010; Ganesh et al. 2012; Mukilan et al. 2018a, 2018b; Rajan 2021; Mukilan 2022). Formed LTM may be affected by environmental pollution, lifestyle, microbial colonization, pathogenic infection, etc (Byzitter et al. 2012; Zhang et al., 2020; Vinay et al. 2021; Da et al. 2023).

Recently, it was reported that pathogenic bacterial infections might contribute to neurodegenerative disorders, and these reports showed that there is an interlink between oral/gut dysbiosis and cognitive decline (Balin et al. 1998; Gérard et al. 2006; Lahner et al. 2009; Huang et al. 2018; McGee et al. 2018; Lotz et al. 2021; Gao et al. 2020; Orr et al. 2020; Da et al. 2023). Other than gut dysbiosis, increased pathogenic bacterial infections in the oral cavity may also lead to reduced cognitive memory formation in neurodegenerative disorders like AD, PD, and mild cognitive impairment (MCI) (Balin et al. 1998; Gérard et al. 2006; Lahner et al. 2009; Jahn 2013; Huang et al. 2018; McGee et al. 2018; Lotz et al. 2021; Ryder 2022; Da et al. 2023). Compared to PD, and MCI, decreased learning and memory formation were more prevalent in AD patients. This may happen due to high colonization of *Porphyromonas gingivalis* and other periodontitic bacteria in the oral cavity (Murman 2015; Dominy et al. 2019; Olsen 2021; Seymour and Zhang 2022; Cammann et al. 2023; Liu et al. 2023). Increased *P. gingivalis* further travelled from the oral cavity to the gut, resulting in gut dysbiosis. As a result of gut dysbiosis, a reduced amount of neurotransmitter precursor chemical components were transported from ENS to CNS (Dominy et al. 2019; Chen et al. 2021; Narengaowa et al. 2021; Cammann et al. 2023). Transported precursor components were responsible for synthesizing and releasing fewer neurotransmitters in the synaptic cleft. Later on, decreased amount of neurotransmitter released results in reduced expression of neuronal signalling molecules involved in LTM formation (Ganesh et al. 2012; Mukilan et al. 2018a, 2018b; Rajan 2021; Vinay et al. 2021; Mukilan 2022). For the first time, it was shown that other than periodontic microorganisms, *P. aeruginosa*, *B. subtilis*, *S. aureus*, and *E. coli* may also involve in the formation of impaired cognitive memory. Future studies may address the role of specific microbial components responsible for short-term memory decline in mild cognitive impairment (MCI).

Conclusion

The present study showed the impact of *P. aeruginosa*, *B. subtilis*, *S. aureus*, and E. *coli* oral infusions on cognitive memory formation. The study's outcome proved that poor oral hygiene caused by microbial colonization plays a significant role in cognitive memory decline in MCI. Experimental results demonstrated that proper oral hygiene is important in cognitive memory development, supported by the pre-infusive analysis. Compared to the pre-infusive analysis, the post-infusive analysis showed that oral microbial infections result in cognitive memory decline. From the post-infusive analysis, it is validated that poor oral hygiene results in cognitive memory impairment. This impaired cognitive memory may develop due to the transport of microbial primary or secondary metabolites from the gut to the brain through the blood-brain barrier. Thus, the current research finding stated the impact of oral and gut dysbiosis was pivotal for cognitive memory decline in MCI.

Acknowledgements

Author thanks the reviewers for the revision comments and suggestions for improving the manuscript. MM sincerely thank the Indian Institute of Technology, Kharagpur, India, for the Institutional Post-Doctoral Fellowship. This research work is also supported by the SEED MONEY grant sanctioned to MM by the Sri Ramakrishna College of Arts & Science (SRCAS), Coimbatore. Department of Biotechnology, Sri Ramakrishna College of Arts & Science (SRCAS), Coimbatore was supported by the DST-FIST PG College Level – A Program (SR/FST/COLLEGE-/2022/1203).

Author Contributions

MM contributed to developing the research idea and its design, prosecution, manuscript preparation, and manuscript revision. The author agrees with the data presented in this research manuscript.

Conflicts of Interest

The author declares no conflict of interest to express.

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