Final Report of Completed Project

DBT Sanction Order No: BT/PR6180/FNS/20/612/2012

1. **Project Title:** "Effect of fermented papaya preparation in modulating neuroinflammation: implications in neurodegeneration"

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3. **Date of Sanction** : DBT Sanction Order No Date: BT/PR6180/FNS/20/612/2012 dated 21-3-2013

4. **Date of Completion: 3 years 6 months**

5. **Approved Cost: Rs. 37, 82, 400/-**

6. **Budget Released: Rs. 31,41,637/-**

7. **Approved Objectives of the Project:**

a. Establishment of neuroinflammatory model using Lipopolysaccharide (LPS)

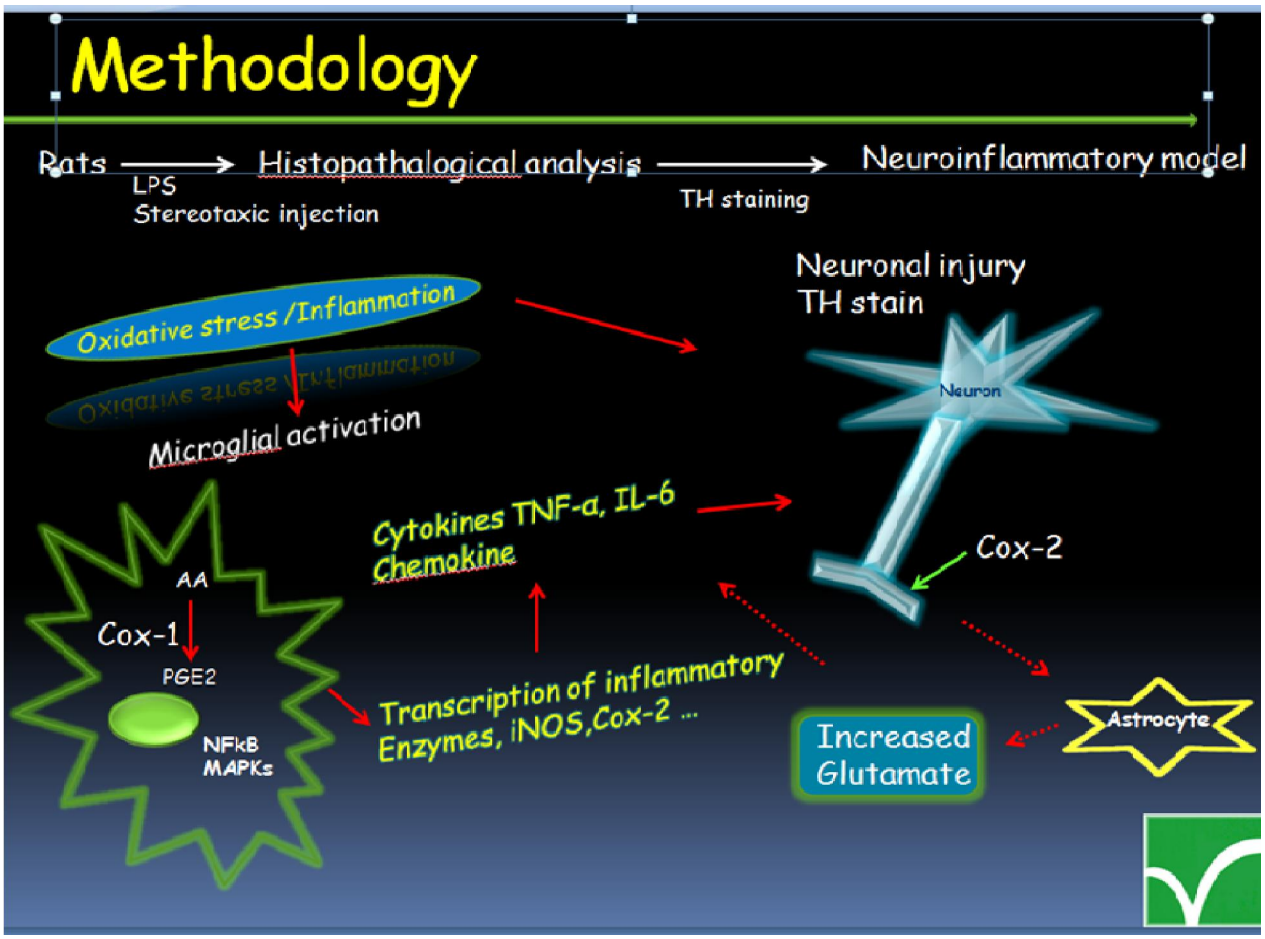
b. Nutritional profile of FPP fermented with L.plantarum, L.acidophilus and in combination.

c. Effect of FPP in reducing the neuroinflammatory response induced by LPS.

d. Evaluation of FPP as neuroprotectant in LPS induced neuroinflammatory model.

8. Progress made against targets including methodologies, discussions, etc.

Methodology:



1. Establishment of neuroinflammatory model using Lipopolysaccharide (LPS).

a) Male Wistar rats (3 months of age) can be challenged with LPS (1 mg/kg body weight, via the i.p. route) or can be delivered to ventricle through sterotaxic instrument. Rats were sacrificed at different time points (24 hrs, 3days, 7 and 15 days) brain will be removed after perfusing with formaldehyde brains were stored. Histopathological analysis can be done and extent of damage to hippocampus can be measured as tyrosine hydoxylase (TH) staining to establish neuroinflammation.

b) After establishing the extent damage at a particular time point important regions of brain like hippocampus, striatum and midbrain which are affected during neurodegeneration will be processed for RNA extraction. Regulation of important cytokines like TNF-α, IL-1, IL-6 and chemokine MCP-1 can be established by realtime PCR.

c) Immunohistochemistry will be done for expression studies of Cox-2 and iNOS to understand proinflammatory response. TH staining can be done to study the extent of damage to CA1, CA2 and CA3 regions of hippocampus.

2. Nutritional profile of FPP fermented with *L. plantarum, L.acidophilus* and in combination.

a) After fermenting papaya extract with different lactobacillus supernatant will be lyophilized and later dissolved in known amount of vehicle. HPLC analysis can be done for total sugars, proteins, lipids and vitamins.

b) Once the nutritional status is established certain amount of probiotic content is maintained as constant in all the batch of fermentation.

3. Effect of FPP in reducing the neuroinflammatory response induced by LPS.

a) Wistar rats are fed with fermented papaya preparation along with Probiotics for 6 weeks fecal samples will be collected every 4_{th} day to check the retention of Probiotics in the gut.

b) Fecal samples can be suspended in saline, centrifuged and supernatant is plated on MRS agar specific media for lactobacillus.

c) Rats will be induced with LPS after 6 weeks of feeding with fermented papaya preparation along with Probiotics; different regions of brain will be collected and evaluated for cytokine and chemokine regulation by real time PCR.

d) Total modulation in peripheral inflammation can be observed by measuring TNF- α in serum samples.

e) Proinflammatory enzymes Cox-2 and iNOS expression will be observed in brain after LPS insult in FPP fed rats.

4. Evaluation of FPP as neuroprotectant in LPS induced neuroinflammatory model.

a) Rats fed with FPP after LPS insult will be perfused with paraformaldehyde. Longitudinal section of brain at hippocampal region was taken.

b) Hippocampal sections can be stained for TH positive neurons to observed the extent of neuroprotection offered by fermented papaya preparation along with Probiotics

Discussion : Objectives achieved

1. Establishment of Neuroinflammatory model using Lipopolysaccharide (LPS)

Animal experiments were approved by the Institutional Animal Ethical Committee. Wistar rats (3 months of age) were challenged with LPS (1 mg/kg body weight, via the i.p. route). Rats were sacrificed at different time points at 3 days and 15 days and blood was collected. Serum was separated for cytokine analysis. Different brain regions like cortex, hippocampus, striatum and midbrain brain were collected. The tissues were processed for protein extraction and western blot was done for expression of tyrosine hydoxylase (TH) to study extent of damage to establish neuroinflammation. The perfused brain was processed for immunohistochemistry for glial fibrillary acidic protein (GFAP) in cortical region.

Materials and methods

Cytokine assays:

The serum TNF-α/ IL-1 was quantified by using ELISA kit (Thermo Scientific) as per the manufacturer instruction. The standard graph was obtained by plotting standard concentration (Y axis) versus corresponding TNF-α / IL-1 concentration (X axis). The amount of TNF-α/ IL-1 in each sample were determined using standard curve.

Western blot:

Tissue samples of different brain regions of control and LPS treated rats were homogenized, lysate was prepared and protein content was estimated. The lysate was separated on SDS-PAGE and transferred electrophorectically to a PVDF membrane. The membrane was probed with TH and Cox-2 anti body and developed later using NBT/BCIP as substrate.

Significant results:

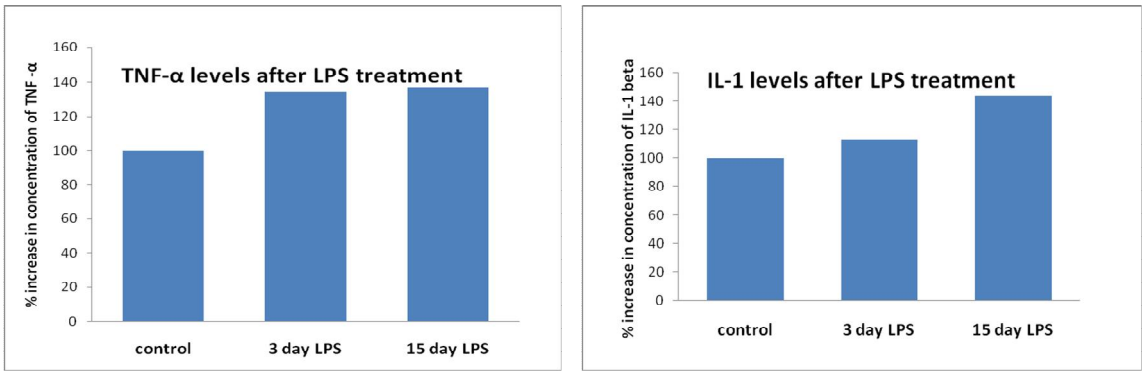


Figure. 1. Percentage increase in cytokine levels after LPS treatment for 3 and 15 days in Wistar rats.

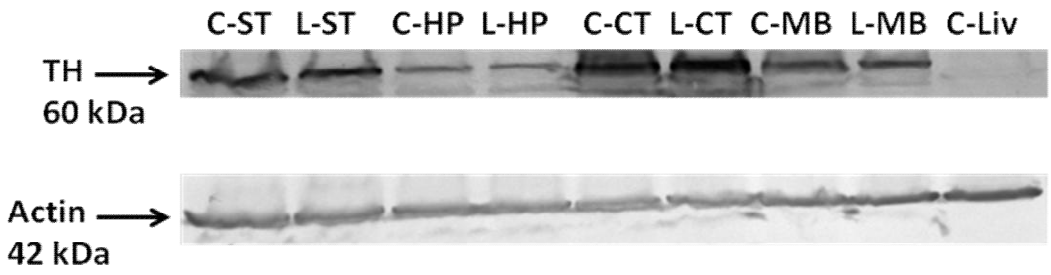


Figure. 2. Expression of Tyrosine hydroxylase (TH) protein indifferent regions of brain after LPS treatment for 15 days in Wistar rats.

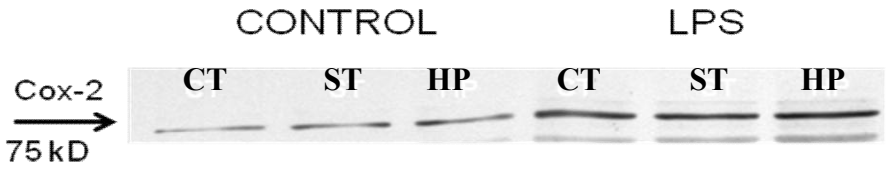


Figure. 3 . Cyclooxygenase -2 (Cox-2) protein expression in different regions of brain after LPS treatment for 15 days in Wistar rats.

Immunohistochemistry:

Rats treated with LPS and controls were perfused with paraformaldeyde and brains were dissected and stored in same solution. The perfused brains were cut into 20µ sections using vibrotome. Sections were processed and labeled with GFAP antibody conjugated to Cy3 showed increased expression of GFAP in cortical regions.

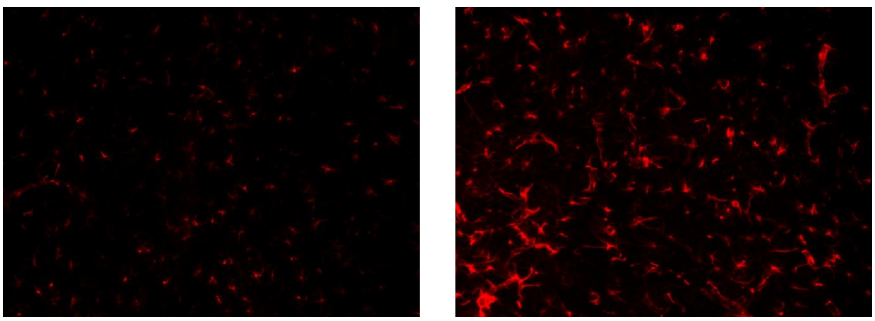


Figure. 4. Proliferation of glial fibrillary acidic protein in cortical regions of brain after LPS treatment for 15 days in rats (10X magnification).

Cytokine levels were measured after treating the rats with LPS for 15 days. We were able to see raise in TNF-α and IL-1 in serum samples indicating prolonged peripheral inflammation (Figure.1). It is evident that prolonged peripheral inflammation leads to neuro-inflammation which indicated by increased expression cycloxygenase-2 in brain regions may lead to neuronal death (Figure.3). Therefore we wanted to know whether neuro-inflammation has affected neurons in different brain regions taking example of tyrosine hydroxylase (TH) for dopamine neurons (Figure.2). We performed western blot analysis to observe the protein expression. We were able to demonstrate that prolonged exposure to LPS in rats decreased the TH expression in hippocampus, striatum and midbrain. However there was not much difference in cortex indicating few brain regions are more vulnerable to neuro-inflammation. The major marker for neuro-inflammation is glial activation. We could observe that there was significant increased glial activation in cortical region of rats treated with LPS for 15 days compared control (Figure.4).

1. Nutritional profile of FPP fermented with *L.plantarum, L.acidophilus* and in combination.

The study involves a novel process of fermentation of raw papaya fruit cultivar 'Solo' using Lactic acid bacteria (LAB) to develop Fermented Papaya Product (FPP) with enhanced nutritional value addition such as antioxidant activity, phenolic content and short chain fatty acids. We took up the scale up of papaya fermentation with 3L fermentation medium in NBS BioFlo 3000 bench top fermenter using fermenting organism viz. *Lactobacillus plantarum* NCIM 2912, *Lactobacillus acidophilus* NCIM 2909, and *Lactobacillus casie* NCIM 2737. The microbial co-culturing of *L. plantarum* and *L. acidophilus* were carried out in bench top fermenter for enhanced antioxidant activity and nutritive value addition compare to the individual strain as fermenting organism. The process involving fermentation of raw Papaya fruit extract supplemented with other media ingredients for 24 hours at bench top level fermenter was optimized for developing fermented papaya product with nutritional value addition.

Preparation of fermentation medium

The raw fruits of 'Solo' variety were washed with double distilled water several times followed by disinfection by wiping the surface with 95% ethanol. The fruits were sliced and pulverized in to slurry to obtain the extract in an electric juicer blender (Kenwood JE 720).

The clear extract obtained was used for preparation of fermentation medium**.** The media containing 1:1 ratio of papaya extract and sterile water constituted Sucrose (3.33%), Lactose monohydrate (0.66%), skimmed milk powder (10%) and $CaCO_3$ (0.66%). The pH of the medium was adjusted to 7.25 ± 0.05 with 5N NaOH and media was pasteurized at 70°C for 30 mins. Upon cooling, the fermentation medium was transferred to the sterile vessel and fermenting organism was introduced. Sampling was done at an interval of every 6 hours starting from 0 h throughout the fermentation. The antioxidant activity, change in biomass and pH of the fermented broth were checked. The samples were stored at -20° C till further analysis. The cell free supernatant was tested for antioxidant activity, total phenolics, and short and medium chain fatty acids.

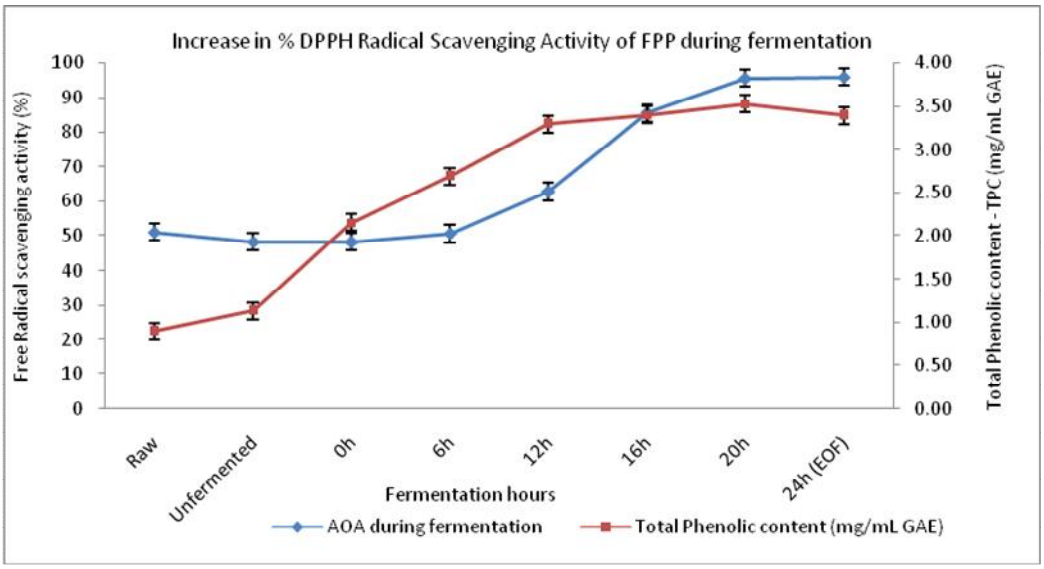


Figure.5. Increase in total phenolic content and percent free radical scavenging activity (DPPH free radical) during *L. plantarum* fermentation

Analysis of antioxidant activity and phenolic content

The antioxidant content in fermented product was assessed by scavenging the stable free radicals such as DPPH (2, 2'-Diphenyl-1-picryl hydrazyl) and ABTS (2,2-azinobis 3-ethylbenzothiazolin 6-sulfonic acid) using filtrate of fermented sample. The concentration of total phenolics in fermented product was determined using colorimetric method using Folin-Ciocalteu's reagent.

Estimation of vitamins and fatty acids (SCFA/MCFA) in fermented papaya product

The water soluble vitamins viz. ascorbic acid, nicotinic acid, pyridoxine, thiamine, folic acid and riboflavin were estimated by HPLC equipped with photo diode array detector using C18 Inertsil (ODS-3V; 5µm, 4.6 mm X 150 mm) column.

The fermented product was analyzed for presence of SCFA (short chain fatty acid) such as Butyric acid (4:0) and MCFA (medium chain fatty acid) like Caproic acid (6:0), Caprylic acid (8:0), Capric acid (10:0) and Lauric acid (C12:0). The fatty acids in fermented product were extracted and methylated to form respective fatty acid methyl esters (FAMEs) and analyzed in Gas chromatography equipped with Flame Ionization Detector (GC-FID).

Significant results:

Fermented papaya product (FPP)

The fermentation of papaya fruit extract using *L. plantarum*, *L. acidophilus* and *L. casie* were tested for the increase in antioxidant activity.

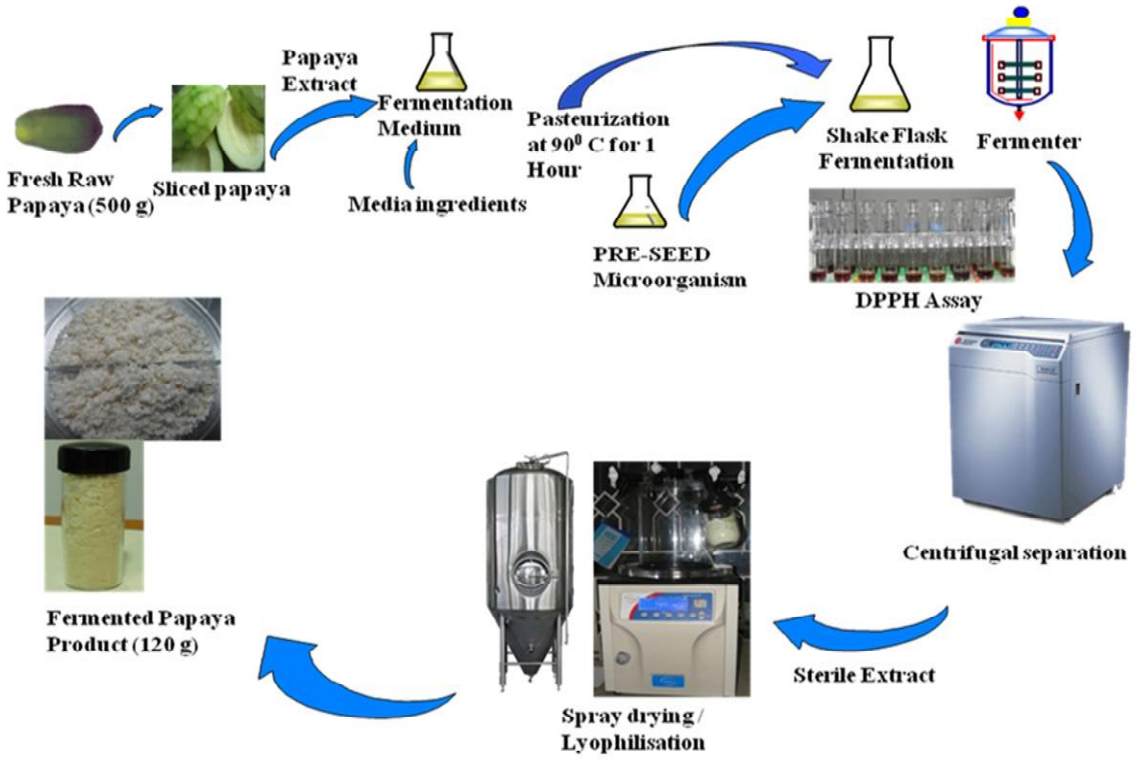
Parameter	***FPP - L. plantarum***	***FPP - L. acidophilus***	***FPP - L. casie***
Antioxidant activity - DPPH radical scavenging activity (%)	95.8	64.41	80.28
Antioxidant activity - ABTS radical scavenging activity (%)	94.08	70.28	79.15
Total phenolic content (mg/ml)	3.31	1.58	2.15
Water soluble vitamins (mg/L)			
Ascorbic acid	NIL	NIL	NIL
Nicotinic acid	6.09	NIL	0.366
Pyridoxine	0.63	NIL	NIL
Thiamine	7.01	4.44	1.8
Folic acid	2.1	0.1	1.62
Riboflavin	4.03	7.9	2.24
Nutrient analysis (values in %)			
Total Soluble Sugar	10.94		
Protein	0.67		
Total Ash	7.19		
Calcium	2.34		
Inorganic Phosphate	0.22		
Magnesium	0.17		

Table 1. Antioxidant activity, total phenolics content and water soluble vitamins in fermented papaya product (FPP) developed by *L. plantarum*, *L. acidophilus* and *L. casie*.

The nutrient analysis containing total soluble sugar, and other micronutrients in *L. plantarum* fermented papaya product

The fermented product of *Lactobacillus plantarum* NCIM 2912 exhibited momentous increase in antioxidant activity (95.8%) assessed by DPPH radical scavenging activity compare to the fermented papaya product developed by *L. acidophilus* (64.41%) and *L. casie* (80.28%). The antioxidant activity developed during 24 hours of fermentation was 95.80% and 94.08% by DPPH and ABTS method respectively in the *L. plantarum* fermented papaya product. Further the total phenolic content measured in *L. plantarum* fermented product was found to be 3.31 mg/mL GAE (Gallic acid equivalents) where as the fermented product of *L. acidophilus* and *L. casie* were found to be 1.58 mg/mL GAE and 2.15 mg/mL GAE respectively (Table 1). The *L. plantarum* shown significant increase during fermentation in antioxidant activity and phenolic content in fermented product (Figure 4).

The fermented papaya product developed by *L. plantarum* was analyzed by GC-FID (Gas chromatography – Flame ionization detector) for short and medium chain fatty acids. We observed 15.72%, 73.33%, 80.34% and 73.39% increase in butyric acid, valeric acid, caproic acid, and caprylic acid respectively upon fermentation.



The fermented product developed by *L. plantarum* exhibited high antioxidant activity, phenolic content, considerable amount of water soluble vitamins and short and medium chain fatty acids in comparison to the fermented product developed by *L. acidophilus* and *L. casie*. Based on these facts the *L. plantarum* fermented papaya product will be taken up for further studies in animal model. The process involving fermentation of raw Papaya fruit extract supplemented with other media ingredients for 24 hours at bench top level fermenter was optimized for developing fermented papaya product with nutritional value addition. The microbial co culturing of *L. plantarum* and *L. acidophilus* in fermentation of papaya was carried out to develop fermented papaya product. This was to increase the antioxidant activity, phenolic content and other value addition such as increase in vitamins and SCFA/MCFA (short and medium chain fatty acids). The change in microbial population during fermentation and the fermenting organisms were differentiated by colony morphology in MRS agar and microscopic examination followed by gram staining (Figure 6). But it was revealed that the co culturing of both the organisms was failed and in fermenter *L. plantarum* was dominating after 6 hours of fermentation. This may be due to the metabolic path way of these two organisms as *L. plantarum* preferably stands to be hetero fermentative where as *L. acidophilus* is homo fermentative. Further to the co culturing fermentation for developing fermented papaya product, shake flask experiments are is in progress using *L. plantarum* and *L. casie* as fermenting organism. The same experiment is under progress at bench top fermenter level.

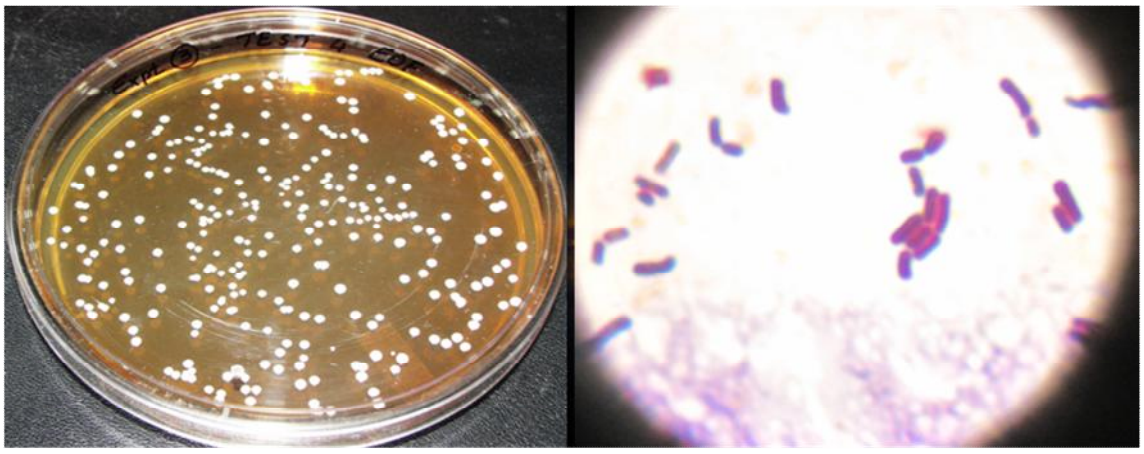


Figure 6. *Lactobacillus plantarum* grown on MRS agar and gram staining image of the bacteria (observed under 100X zoom resolution)

2. Effect of FPP in reducing the Neuroinflammatory response induced by LPS.

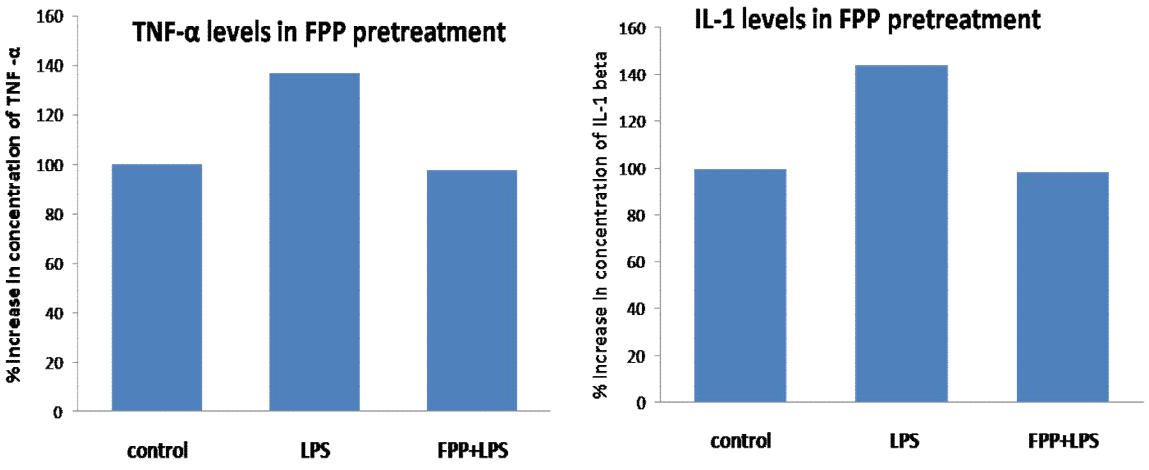


Figure. 7. Decreased cytokine levels after FPP pretreatment followed by LPS treatment for 15 days in Wistar rats.

The serum TNF-α and IL-1 was measured using ELISA kit (Thermo Scientific) as per the manufacturer instruction. The standard graph was obtained by plotting standard concentration (Y axis) versus corresponding TNF-α / IL-1 concentration (X axis). The amount of TNF-α/ IL-1 in each sample were determined using standard curve. We found that the increased cytokines were attenuated by pretreatment of FPP showing decreased peripheral inflammation.

Western blot Result of rats treated with FPP followed by LPS for Cyclooxygenase -2 (Cox-2) protein expression in Hippocampus region of brain:

Tissue samples of different brain regions of control and LPS treated rats were homogenized, lysate was prepared and protein content was estimated. The lysate was separated on SDS-PAGE and transferred electrophorectically to a PVDF membrane. The membrane was probed with Cox-2 anti body and developed later using NBT/BCIP as substrate.

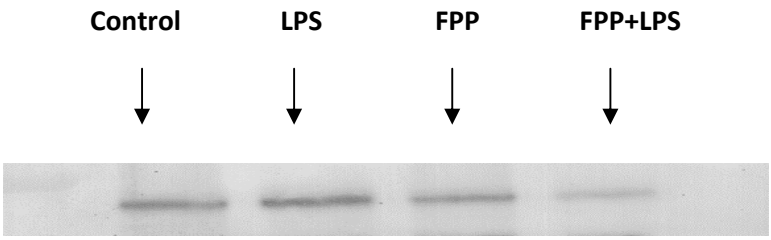


Figure.8. Decrease in level of Cox-2 protein expression after treating with FPP followed by LPS

4. Evaluation of FPP as Neuroprotectant in LPS induced Neuroinflammatory model.

Immunohistochemistry:

Rats were pretreated with FPP followed by LPS injections for 15 days were perfused with paraformaldehyde and brains were dissected and stored in same solution. The perfused brains were cut into 20µ sections using vibrotome. Sections were processed and labeled with GFAP antibody conjugated to Cy3 showed decreased expression of GFAP in cortical regions after FPP pretreatment (Figure.8).

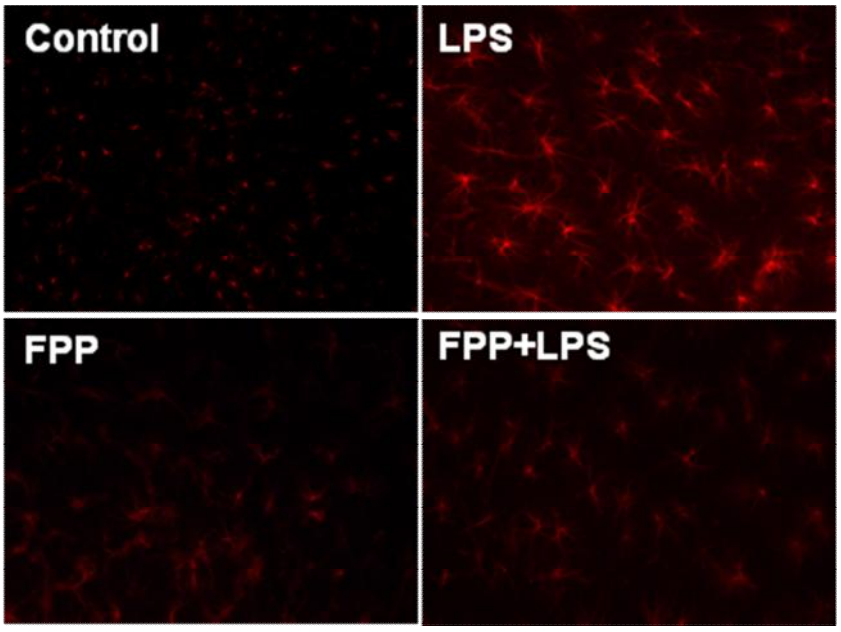


Figure. 8. Proliferation of glial fibrillary acidic protein was reduced in cortical regions of brain with pretreatment of FPP followed by LPS treatment for 15 days in rats (20X magnification).

Fermented papaya preparation (FPP) was prepared using all the three probiotic strains of *L. plantarum*, *L. acidophilus* and *L. casie.* Though *L. plantarum* exhibited high antioxidant activity, phenolic content, considerable amount of water soluble vitamins and short and medium chain fatty acids in comparison to the fermented product developed by other two strains. We also tried co culturing of *L. plantarum* and *L. acidophilus* as well as *L. plantarum* and *L. casie.* There was no increase in antioxidant activity, phenolic content and nutritive values in FPP at the end of fermentation even by 6 hrs only *L. plantarum* was dominating. Further FPP prepared from *L. plantarum* was evaluated in LPS induced

Western blot Result of rats treated with FPP followed by LPS for Tyrosine hydroxylase (TH) protein expression in Hippocampus region of brain :

Tissue samples of different brain regions of control and LPS treated rats were homogenized, lysate was prepared and protein content was estimated. The lysate was separated on SDS-PAGE and transferred electrophorectically to a PVDF membrane. The membrane was probed with TH anti body and developed later using NBT/BCIP as substrate.

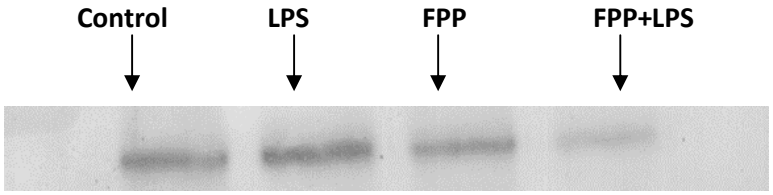


Figure.9. Decrease in level of TH protein expression after treating with FPP followed by LPS

9. Results:

a) Established of neuroinflammatory model using Lipopolysaccharide (LPS)

b) Nutritional profile of FPP fermented with L.plantarum, L.acidophilus and in combination: The present work is focused on evaluating Fermented Papaya Preparation (FPP) developed in house at VMSRF for neuroprotection. We developed FPP using three different strains of

Lactobacillus namely *L. plantarum*, *L. acidophilus* and *L. casie.* However *L. plantarum* exhibited high antioxidant activity, phenolic content, considerable amount of water soluble vitamins and short and medium chain fatty acids. The co culturing of *L. plantarum* and *L. acidophilus* as well as *L. plantarum* and *L. casie* for increment in antioxidant activity and phenolic content did not give any fruitful results.

c) Effect of FPP in reducing the neuroinflammatory response induced by LPS: We have established neuroinflammatory animal model to evaluate FPP. There was increased expression of GFAP and decrease in expression of Tyrosine Hydroxylase level in hippocampus compared to cortex.

d) . Evaluation of FPP as neuroprotectant in LPS induced neuroinflammatory model.

pretreated with FPP followed by LPS there was considerable decrease in GFAP expression in cortex and decrease in TNF-α and IL-1 levels in serum of these rats indicating FPP was able to attenuate neuroinflammation.

10. Summary:

The project work is focused on evaluating Fermented Papaya Preparation (FPP) developed in house at VMSRF for neuroprotection. Such dietary origin FPP developed by *L. plantarum* mediated fermentation of papaya enriched with nutrient and probiotics can be a holistic natural antioxidant to manage imbalance of complex redox state. FPP was prepared using all the three probiotic strains of *L. plantarum*, *L. acidophilus* and *L. casie.* However *L. plantarum* exhibited high antioxidant activity, phenolic content, considerable amount of water soluble vitamins and short and medium chain fatty acids. The co culturing of *L. plantarum* and *L. acidophilus* failed due to the overcome of *L. plantarum* during fermentation.

To establish neuroinflammatory animal model to evaluate FPP, we treated rats with LPS for 15 days and able to see glial activation in cortical regions by immunohistochemistry. We observed decrease in expression of Tyrosine Hydroxylase level in hippocampus, striatum and midbrain compared to cortex. The result indicated that neuroinflammation is affecting dopamine rich regions. There was increase in TNF-α and IL-1 levels in serum of these rats indicating peripheral chronic inflammation.

11. Any new product/process developed:

The Fermented Papaya Preparation (FPP) developed in house at VMSRF for neuroprotection. Such dietary origin FPP developed by *L. plantarum* mediated fermentation of papaya enriched with nutrient and probiotics can be a holistic natural antioxidant to manage imbalance of complex redox state. FPP was prepared using all the three probiotic strains of *L. plantarum*, *L. acidophilus* and *L. casie.* However *L. plantarum* exhibited high antioxidant activity, phenolic content, considerable amount of water soluble vitamins and short and medium chain fatty acids. Further when these animals were pretreated with FPP followed by LPS there was considerable decrease in GFAP expression in cortex and decrease in TNF-α and IL-1 levels in serum of these rats indicating FPP was able to attenuate neuroinflammation.

12. Any new lead:

The novel process of fermentation of raw papaya fruit cultivar 'Solo' using Lactic acid bacteria (LAB) to developed Fermented Papaya Product (FPP) with enhanced nutritional value addition such as antioxidant activity, phenolic content and short chain fatty acids. We took up the scale up of papaya fermentation using fermenting organism viz. *Lactobacillus plantarum* NCIM 2912, *Lactobacillus acidophilus* NCIM 2909, and *Lactobacillus casie* NCIM 2737. The microbial co-culturing of *L. plantarum* and *L. acidophilus* were carried out in bench top fermenter for enhanced antioxidant activity and nutritive value addition compare to the individual strain as fermenting organism. The process involving fermentation of raw Papaya fruit extract supplemented with other media ingredients for 24 hours at bench top level fermenter was optimized for developing fermented papaya product with nutritional value addition. The result indicated that neuroinflammation is affecting dopamine rich regions. There was increase in TNF-α and IL-1 levels in serum of these rats indicating peripheral chronic inflammation.

13. Any technology developed:

The technology for the fermentation of raw papaya fruit cultivar using Lactic acid bacteria (LAB) was developed Fermented Papaya Product (FPP) with enhanced nutritional value addition such as antioxidant activity, phenolic content and short chain fatty acids. The

process involving fermentation of raw Papaya fruit extract supplemented with other media ingredients optimized for developing fermented papaya product with nutritional value addition.

14. Any patent taken: Nil

15. Publications from project work. NIL

16.Financial progress. Viz. equipments purchased, manpower, copies of UCs/SEs of various financial year, etc.

ANNEXURE 1 - Attached