INFLUENCE OF PROBIOTIC AND MEDICINAL PLANTS INCORPORATED IN ANIMAL FEED FORMULATIONS

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Abstract

Antibiotics used in animal feed to prevent various microbial diseases leads to antibiotic resistance. Such resistances will not only occur in pathogenic bacterial species but can also be transferred to humans during the consumption of animal products. Long term usages of antibiotics lead to suppress the natural immunity. Replacing of commercial antibiotics with probiotics, edible medicinal plant material could benefit the poultry animals health and consumers without any negative effects. *Sporolactobacillus laveolacticus* MTCC 7167, a spore forming bacteria were screened for its probiotic characteristics in sustaining at acidic and bile environments. Three medicinal plants such as *Tinospora cordifolia*, *Abutilon indicum*, *Holoptelea integrifolia* were selected and screened for their antimicrobial property to be used as a value added natural material incorporated in animal feed. The aim of this study was to develop an effective antimicrobial and probiotic animal feed with immune-modulatory potential using the agricultural wastes.

Keywords: Probiotics, animal feed, *Lactobacillus*. 
Introduction

Food safety relates to the preservation and welfare of animal and human health depending mainly on the feed components with consumer-safe. The chemically synthesized antibiotics and growth promoters were been out of use, since the trend has been changed from quantity to quality. With the growing concern on routine usage of antibiotics in animal it results in the utter ban of these antimicrobial growth promoters in many countries. It began the search for alternatives and renewed interest in feeding plant extracts and probiotics in modifying the microbial ecosystem of animal gut. The hazard on the existence of antibiotic leftovers in milk and meat products on using chemical promoters shown to have risky health effects, which leads to the ban for the use in animal feed applications(Yeo et al., 2016).

Plant as animal feed additive

Plant additives are used because of its natural component of feeds, they are non-hazardous and eco-friendly, cost effective, and acts as growth promoters. Herbal medicines possess much responsiveness as antibacterial drug source as they are regarded as time tested and harmless for animal and human usage and also towards the environment. Plant extracts are more likely interest due to their potent biological functions possessing antimicrobial, antiviral, antioxidant and inflammatory effects(Abdul et al., 2015). This overflowing property of plants lead to the possibility to use plant extracts in order to replace antibiotics in feed applications for improving animal health and performance (Amira, Sh. Soliman, 2018). The antimicrobial effects of plants happen by a number of mode of action by presence of wide range of chemical components as certain bioactive components helps in preventing the growth of virulent structures in bacteria (Liu et al., 2018).

Probiotic bacteria

Probiotics are live bacteria and yeasts that are good for our digestive system. Probiotics are often called as good and healthy bacteria as they keep the guts clean. Lactic acid bacteria (LAB) and Bacillus groups were widely being used as probiotic for farm animal feed manufacturing. Particularly, Lactobacillus acts as prime factor during early stages in gut microbiota enrichment during post-weaning, having advantages in gut colonizing (Jung & Park, 2012). LAB can lower the gut pH, benefits abdominal task, inhibit enteric pathogens and improves host immunity. Non spore-forming LAB are less tolerant to oxygen which makes it difficult in persistence during development, storage and delivery to gut. But in case of spore-forming bacteria, the spores were persistent in feed development, storage and during distribution (Lange et al., 2010).

Probiotic as animal feed additive

Probiotics are lively and supportive microorganism that when given to humans and animals, provide favourable functional effects in setting up intestinal microflora that is friendly to host object and aggressive to pathogens (Nelson et al., n.d.). Probiotics were switched for antibiotics to farm animals with the sole determination in preventing infections, to promote growth and to increase the feed conversion efficacy. It has been proven to balance the intestinal microbe in piglets after Lactobacilli incorporation, with precise decline in enteric bacterial counts in intestinal faeces. The feed additives were aimed to enhance immune response, reduce pathogen load in gut, stimulate beneficial gut microbes, and stimulate digestive functional roles (Lange et al., 2010).

Wastes to feed formulation

With the increasing demand of livestock as food for humans, interest over feed source of economic means has been developed. Corn Stover, an abundant crop with greater potential for development and application(Li et al., 2014). After harvest, the corn stalks were left on the field or burned in the field causing loss and life of the soil. Such stalks can be used as animal feed stuff of higher
fibrous content (Ayasan et al., 2019). The leftover vegetable waste which is of high protein content could be an intensive source for feed applications (Casewell et al., 2003).

Materials and method

Plant Materials

Collection of leaves

The leaves of Tinospora cordifolia, Abutilon indicum and Holoptelea integrifolia plants were collected from locals of Saravanampatti, Coimbatore, Tamil Nadu, India (11.0797° N, 76.9997° E). The heathy and unaffected leaves were picked, washed thoroughly with clean water.

Preparation of extracts

The collected leaves of each plant were dried under shade at room temperature for about a week and then the dried leaves were grinded finely. 10 g powder of each plant was immersed in 50 ml of hexane (1:5 w/v) for 24 hours and was extracted by cold percolation method. The extracted solution of each plant were filtered through a Buchner funnel using Whatman number 1 filter paper and then the filtrate was stored at 4° C. The remaining residues were again mixed with same volume of hexane and the extraction was repeated twice. The resulting residues of each plant were again extracted using chloroform, followed by methanol (100%) and water (sterile, autoclaved) in a sequential way similarly and then the residual pastes were stored at 4° C.

The stored extracts of hexane, chloroform and methanol of each plants were evaporated to dryness under room temperature till it acquires paste consistency. The resulting paste substances were collected and stored in a clean fresh tube. The extracts of water solvent of each plants were dried in hot air over at temperature of 40° C until dry and the paste substances were collected in the similar way.

Test organisms

The following bacteria and fungi were used for this experiment: Bacteria: Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus. Fungi: Candida albicans. Bacterial inoculums were organized by growing the cells in Mueller Hinton Broth (MHB) for 24 hrs at 37° C and then diluted with un-inoculated MHB until the cell count reach 0.6 ± 0.02 OD value. Fungal inoculums were prepared in Potato Dextrose Broth (PDB) at 27° C and then diluted with un-inoculated PDB until the cell count reach 0.6 ± 0.02 OD value.

Antimicrobial activity

The antibacterial and antifungal activity was carried out by disc diffusion method. For antibacterial activity, a loop full of bacterial culture was inoculated into MHB, the previous day. The overnight fresh bacterial culture of OD 0.6±0.02 were swabbed onto the solidified Mueller Hinton Agar (MHA) and dried for 10 minutes. The sterile discs were loaded with each plant extracts of similar 5 mg/ml concentration with respective solvents. The loaded discs were placed over the medium with Ampicillin (2 mcg) as positive and unloaded sterile disc as negative control. The plated were placed at 4° C of about 30 minutes for compound diffusion in plant samples and then incubated at 37° C for 24 hrs.

For antifungal activity, a loop full of fungal culture was inoculated into PDB, the previous day. The overnight fresh fungal culture of OD 0.6 ± 0.02 were swabbed onto the solidified Potato Dextrose Agar (PDA) and dried for 10 minutes. The loaded plant extract discs were placed over the medium with Clotrimazole (10 mcg) as positive and unloaded sterile disc as negative control. The plated were placed at 4° C of about 30 minutes for compound diffusion in plant samples and then incubated at 27° C for 24 hrs.
Probiotic Characterization

Strain and growth parameters

The strain used in this study was Sporolactobacillus laevolacticus MTCC 7167 were revived in Nutrient Broth at 35° C for 24 hrs. The strain was sub- cultured in MRS broth medium to be used for further purposes. The strain was checked for staining, catalase test and endospore test. For growth profiling, the fresh overnight culture of the strain was inoculated into MRS broth and the cell growth on hourly interval were analyzed using Spectrophotometer at 600 nm. The uninoculated MRS broths were used as blank for this analysis.

Growth at acidic pH

For acidic pH analysis, MRS broth with altered pH from 2 - 5 using 6 M HCl and 10 N NaOH were used. A single isolated colony of the strain was sub- cultured in MRS broth and incubated at 37° C overnight in the previous day. The fresh overnight culture was inoculated into pH altered MRS broth (1% v/v). The growth of the bacteria was measured using Spectrophotometer at 600 nm against pH unaltered MRS broth (pH 6.5) at respective timings. The experiment was carried out in triplicates inorder to find the mean value (Yeo et al., 2016).

Bile salt tolerance

For bile salt tolerance test, MRS broth with varying bile salt concentration of 0.1% - 0.6% was used. A loop full culture of S. laevolacticus was inoculated into MRS broth medium, the previous day. The fresh overnight culture was inoculated into MRS broth with varying bile salt concentration (1% v/v). The growth of the bacteria was measured using Spectrophotometer at 600 nm against un-inoculated MRS broth used as blank at respective timings (Anderson et al., 2010). The experiment was carried out in triplicates inorder to find the mean value.

NaCl tolerance

For NaCl salt tolerance test, MRS broth with varying NaCl concentration of 1% - 6% was used. A loop full culture of S. laevolacticus was inoculated into MRS broth medium, the previous day. The fresh overnight culture was inoculated into MRS broth with varying bile salt concentration (1% v/v). The growth of the bacteria was measured using Spectrophotometer at 600 nm against un-inoculated MRS broth used as blank at respective timings (Anderson et al., 2010). The experiment was carried out in triplicates inorder to find the mean value.

Cell Surface Hydrophobicity

The overall adhesion capacity can be determined by evaluating the affinity of bacteria to a hydrocarbon solvent. For the cell surface hydrophobicity, a loop full of S. laevolacticus was inoculated into MRS broth medium in the previous day. The overnight fresh culture were centrifuged at 5000 rpm for 15 minutes and the pellet was collected. The pellet was washed twice with PBS (1X; pH 7.4) and then re-suspended in PBS to get the OD value around 0.6 ± 0.02. The initial absorbance at 600 nm was taken and the solvents such as hexane, chloroform and ethyl acetate (1:1 v/v) were added to the each culture tube by vortexing. After the phase separation, the OD for the upper aqueous phase were alone measured and calculated using the equation,

\[
\text{Hydrophobicity} = \frac{(\text{Ab initial} - \text{Ab final})}{\text{Ab initial}} \times 100
\]

Where Ab initial represents initial absorption before adding hydrocarbon sources and Ab final represents final absorption after phase separation with hydrocarbons (Manhar et al., 2016).
Aggregation test

Aggregation is the preliminary step in adhesion process that allows bacteria to form a barrier to prevent undesirable bacteria invasion. For aggregation assay, a loop full of S. laevolacticus and E. coli culture were inoculated into MRS and MHB medium in the previous day. The overnight fresh cultures were centrifuged at 5000 rpm for 15 mins and the pellet was collected. The pellet from both cultures were washed twice with PBS (1X; pH 7.4) and then re-suspended in PBS to get the OD value around 0.6 ± 0.02. The absorbance was measured after incubation of 24 hrs at 30° C and was performed in triplicates (Manhar et al., 2016). Auto- aggregation percentage was expressed using the equation,

Auto-aggregation (%) = 1 - (At/ A0) × 100

Where At = absorbance at 24 h time interval and A0 = absorbance at 0 h.

separation, the OD for the upper aqueous phase were alone measured and calculated using the equation,

Hydrophobicity = (Ab initial – Ab final)/ Ab initial × 100

Where Ab initial represents initial absorption before adding hydrocarbon sources and Ab final represents final absorption after phase separation with hydrocarbons.

Antagonistic activity

The antimicrobial activity of S. laevolacticus was performed by agar well diffusion method using the test pathogens such as Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Candida albicans. The pathogenic cultures were pre-inoculated into MHB for bacterial and PDB for fungal culture, a day before. The fresh overnight culture of the pathogen of OD value 0.6 ± 0.02 were swabbed over the respective agar medium and kept it dry for 10 minutes. A well of 6 mm were placed onto the agar medium to load the culture to be analyzed. The overnight fresh culture of S. laevolacticus was centrifuged at 5000 rpm for 15 minutes and the supernatant were loaded into the well. Ampicillin (2 mcg) for bacterial culture and Clotrimazole (10 mcg) for fungal culture were used as positive control. The plates were kept in the incubator at 37 °C for bacterial culture and 27 °C for fungal culture and analyzed for the zone in the very next day(Yeo et al., 2016).

Gastro-Intestinal Digestion Adaptability

The fresh culture of S. laevolacticus were subjected to in vitro Gastro Intestinal environmental conditions and checked for their growth and adaptability in such conditions. The culture was pre-inoculated into MRS broth medium, a day before. The cells of overnight fresh culture were centrifuged at 5000 rpm for 15 minutes and the pellets were collected. The pellets were then washed twice with freshly Prepared PBS (1X; pH 7.4) and then re-suspended in MRS broth (pH 3.0) for about 2 hours. The suspension is again washed twice and re-suspended in MRS broth with 0.3% bile salt for about 3 hours. The resulting suspension is then introduced into broth with 0.3% bile salt and spread onto MRS agar + 0.3% bile salt plates (Santini et al., n.d.) (Yeo et al., 2016).

Animal feed preparation

The raw material of maize products was collected directly from the farm lands and the vegetable wastes from household regions. The collected materials were shade-dried for about a week until dry and minced coarsely. These raw materials were mixed in a proportional way to ensure the feed homogeneity with reduced water content for long time storage quality (Martin et al., 2016). The three medicinal plants along with lyophilized probiotic culture were then added to the existing raw materials and were pelleted.
RESULTS AND DISCUSSION

Plant materials

Extraction yield

Fresh 10g of leaves of *Tinospora cordifolia*, *Abutilon indicum* and *Holoptelea integrifolia* yielded 1.6%, 0.2%, 0.2% of hexane extracts; 1.4%, 0.2%, 0.6% of chloroform extracts; 3.7%, 4.1%, 5.8% of methanolic extracts; 6.9%, 1.3%, 4.5% of aqueous extracts, respectively.

Antimicrobial activity

The antimicrobial activity was analysed for Hexane, Chloroform, Methanol and Aqueous extracts of *Tinospora cordifolia*, *Abutilon indicum* and *Holoptelea integrifolia* plants using pathogenic organisms. The maximum activity was achieved for hexane and methanolic extracts among chloroform and no activity were observed for aqueous extracts. The plant extracts shown higher activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* than *Escherichia coli*, thus resulting in very lesser activity against microbial flora.

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<th>Test Strains</th>
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*H - hexane; C - chloroform; M - methanol; W - water

![Figure 1: Extraction yield](image)

![Figure 2: Antimicrobial activity of plant extracts](image)

Probiotic Characterization

Strain and growth parameters

The strain of *S. laevolacticus* possesses gram-positive, catalase negative and endospore forming as shown in Figure 4.5. The growth profile of *S. laevolacticus* were found to be at stationary phase during 24 hr time, the culture at 24 hr can be used for further analysis. The doubling time as observed from the growth time were found to be of 1 hr.
Growth at acidic pH

The strain *S. laevolacticus* were found to be decreased at pH 1 in 24 hrs of growth but at pH 2 and 3, the strain was found to be sustaining. From pH 4, the strain growth can be observed and it were improving at increasing pH. The sustainability at acidic pH without decline shows a positive result to be used as probiotic additive.

![Acid Tolerance Graph](image)

**Figure 3: Acid tolerance test**

Salt tolerance

The tolerance with bile and NaCl salt were shown in Graphs 4.4 & 4.5. At various concentrations of salts, the strains growth was observed with respective to no salt concentrations. The tolerating potential of strain showed a positive result for probiotic characteristics.

![NaCl Tolerance Graph](image)

**Figure 4: Salt tolerance test**

Hydrophobicity

![Hydrophobicity Graph](image)

**Figure 5: Hydrophobicity**

Cell surface hydrophobicity

The strain exerts higher hydrophobicity, electron donor and electron acceptor property of bacterial cell surface. The result showed that about 65% hydrophobicity; 35% electron donor; 64% electron acceptor property making it as a feasible probiotic for consumption.

Aggregation test
The aggregation test result shows that the combined value gets increased than the strain alone resulting in better aggregation with microbial flora.

![Figure 6: Aggregation test]

**Antagonistic activity**

The strain exerted better antimicrobial activity against *Pseudomonas aeruginosa, Staphylococcus aureus* and with a minimum activity against *Escherichia coli* and no activity against *Candida albicans*.

![Figure 7: Pelleted form of animal feed]

**Gastro-Intestinal digestion adaptability**

The strain when subjected to intestinal environment, it thrived at those conditions instead of taking it as a stress factor. This ensures for the ability of strain as probiotic for consumption.

**Animal feed preparation**

The raw materials of corn stalk and Stover along with medicinal plant leaves of *Tinospora cordifolia, Abutilon indicum, Holoptelea integrifolia* and vegetable wastes were grinded together to form a pellet for animal feed. Further, the pellets are to be analysed for its nutritional analysis by NIR studies.

**Conclusion**

Keeping farm animals in good physical state is essential to attain safe animal product. As the world is enduring advancement, novel diseases were evolving in animals and humans by constant usage of antibiotics and antimicrobial growth inducers. Enormous researches have been led to evaluate the influence of feed ingredients and feed additives on the aspects of gut health and development to improve growth while diminishing the use of antibiotics and other expensive ingredients for feed. Now, it is the need of time to work more expansively on the medicinal plants and probiotics for the better concern of mankind.

**References**


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