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Original Research

In vitro and Extracellular Assays Based Scoring System for Selection of Potential Probiotic Isolates with Antioxidant Property from Native Chicken

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Abstract

Probiotics have been used as a major alternative approach in the newly hatched chicks to colonize normal micro flora in the intestine. This study involves the characterization of antioxidant potential of probiotic isolates obtained from backyard chicken. Different probiotic isolates namely E. thailandicus, E. hirae, E. faecium, E. fecalis, E. durans, P. acidilactici, L. salivarius were identified by molecular methods using PCR amplification followed by sequencing. Extracellular assays viz DPPH and FRAP assays were performed for assessing the antioxidant potential of these isolates. In vitro assay was also performed in Caco2 and HepG2 cell lines to study the antioxidant changes induced by the probiotic isolates by profiling the antioxidant genes such as SOD (Superoxide dismutase) and GPx (Glutathione peroxidase) with GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) as control by real time PCR. An intricate scoring system was established for evaluating the probiotic isolates for their efficient antioxidant activity. The scoring system revealed that the isolates E. hirae-1, E. faecium-1, E. fecalis-3, P. acidilactici exhibited maximum potential with respect to antioxidant activity.

Keywords: Antioxidant, Broiler, In Vitro Assays, Native Chicken, Probiotics, Real Time PCR

Introduction

Poultry industry is one of major sector gained importance to boost the economic activity in many countries. In large scale rearing system, where poultry are exposed to various diseases and unfavourable stressful environmental conditions which result in serious economic losses. During recent decades, there is a substantial increase in the use of veterinary medicines for the prevention and control of animal diseases. However, the use of antimicrobial drugs as a preventive measure have been questioned due to the evolution of antimicrobial resistance among pathogenic bacteria apart from constituting a potential health hazard to consumer. Probiotics have been used as a major alternative approach in the newly hatched chicks to colonize normal micro flora in the intestine. The existence of complex microbial flora in the gastrointestinal tract of all warm-blooded animals is found to be effective in providing resistance to disease (Fuller, 1989). Isolation of lactic acid producing bacteria from native chicken and their application in commercial broiler has been attempted by earlier research workers. Probiotics have played many therapeutic roles by modulating immunity, lowering cholesterol level improving lactose tolerance and preventing some cancers (Kailasapathy, 2000).

Probiotics were reported to increase the body weight gain and feed efficiency and reduced mortality in broiler chickens (Panda *et al*., 2007). According to Kabir (2009) the most commonly used probiotic species are *Lactobacillus bulgaricus, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus helveticus, Lactobacillus lactis, Lactobacillus salivarius, Lactobacillus plantarum, Streptococcus thermophilus, Enterococcus faecium, Enterococcus faecalis, Bifidobacterium spp.* and *Escherichia coli*, and *except for lactobacillus bulgaricus* and *Streptococcus thermophilus*, all the remaining ones are intestinal strains. Dhama (2011) stated that probiotics could prevent early chick mortality, gastro intestinal disturbances like scouring, loss of appetite, improper digestion and poor absorption of nutrients and infectious conditions by fighting against pathogenic microbes especially the enteric pathogens. Thus, these can alleviate reduced production performance and prevent heavy economic loss to the poultry producers.

In vitro studies provide the first step in evaluating probiotics with specialized properties and these tests ensure that the microorganism is able to withstand the conditions of gastrointestinal tract and exert their functional properties (Pereira *et al*., 2018). It is imperative to note that the biological effects of probiotics are strain-specific and that the success of one strain cannot be extrapolated to another strain (Figueroa-González *et al.*). Probiotics are known to have many beneficial health effects, and their consumption shows that strain specific probiotics can present antioxidant activity and reduce damage caused by oxidation (Wang *et al*., 2017). Hence, the present study was designed to evaluate potential probiotic species based on their antioxidant property by *in vitro* characterisation.

Materials and Methods

Source of Samples and Processing

Native chicken reared in different types of backyard environments were selected for the present study. A total of 120 samples were collected from different regions of the digestive tract *viz.*, crop, duodenum, ileum, caecum, proventriculus, jejunum and fecal swabs by slaughtering them at poultry research station TANUVAS, as per standard protocol. They were then cultured in optimum temperature in MRS broth and single isolates were obtained post sequential sub-culturing. Twenty-six isolates were morphologically unique and were used for further studies.

Characterisation of Isolates by Molecular Method

The genomic DNA was isolated from all the twenty-six isolates using proteinase K and phenol: chloroform: Isoamyl-alcohol (25:24:1) method (Walter *et al*., 2000). The final DNA pellet was air dried and dissolved in 30 µl of nuclease free water and stored at -20°C until further use. PCR was carried out using the following already published primers with cycle conditions comprising of initial denaturation 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 53°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 5 min. The PCR amplicons were checked in 1.5% agarose gel electrophoresis along with 100 bp DNA molecular weight marker. The purified PCR products were sequenced using Genetic Analyzer 3130 (Applied Bio systems, USA) and were annotated. Out of 26 isolates. 11 isolates were selected for further studies as mentioned in Table 2.

In vitro Assay for Selection of Potential Probiotic Isolates with Antioxidant Property

The Caco2 cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 0.1 mM of nonessential amino acids, 0.1 g/mL of streptomycin, 10 mM of sodium bicarbonate, and 10% fetal bovine serum (FBS) in a Co_2 incubator (Eppendorf).

HepG2 cell line was maintained in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum (FBS) in a Co_2 incubator. The sub-cultured confluent cells were utilized for further assays. 10^5 probiotic bacteria were co-cultured (n=11 isolates) with approximately 10^6 cells per ml of CaCo2 and HepG2 and incubated for 24 hrs.

Quantitative Real-Time PCR

After 24hrs of incubation, both stimulated and unstimulated cell lines were utilized for total RNA extraction (Settivari *et al*., 2006) using Qiagen RNA extraction kit according to the manufacturer's instructions. The extracted RNA was quantified using Picodrop Spectrophotometer (Picodrop Ltd., United Kingdom) and it was subjected to cDNA synthesis using oligodT primers and iScript reverse transcriptase (Bio-Rad, India) according to the manufacturer's instructions. The primers used in real time PCR are given in the table.

Table 1: List of primers

Gene	Primer (5'-3')	Product Size (Bp)	Refrence
Glutathione peroxidase (GPx)	FP- TTGTAAACATCAGGGGCAAA	140	Yarru *et al*, 2009
	RP- TGGGCCAAGATCTTTCTGTAA		
Superoxide dismutase (SOD)	FP- AGGGGGTCATCCACTTCC	122	
	RP- CCCATTTGTGTTGTCTCCAA		
Glyceraldehyde-3-phosphate dehydrogenase	FP- CCTCTCTGGCAAAGTCCAAG	128	
	RP- CAACATCAAATGGGCAGATG		

The expression profiles of genes involved in antioxidant function (SOD, GPx) in all treatment groups were measured using the qRT-PCR technique. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous control gene in the qRT-PCR experiments. Thermal cycling was carried out in BioRadrealplex PCR with the respective annealing temperatures that were optimised for each gene. (50°C, 2 min; 95°C, 10 min; and 40 cycles at 95°C, 15 s; 60°C, 1 min). Each gene was measured in triplicate and the formation of single PCR products was confirmed using melting curves. Negative controls, which consisted of all of the components of the qRT- PCR mix except cDNA, were used for all the primers mentioned in the above table. The relative quantification of gene expression changes was recorded after normalizing for GAPDH gene expression computed by using the $2^{-\Delta\Delta C\ T}$ method.

Extra Cellular Assay for Assessing the Probiotics with Antioxidant Potential

The extra cellular assay as DPPH and FRAP assay were performed for assessing the probiotics with antioxidant potential.

DPPH Radical Scavenging Assay

The DPPH radical scavenging assay was performed according to Xing *et al.* (2015). Briefly, 1 mL of culture supernatant was mixed with 1 mL of DPPH solution (0.2 mM). The mixture was incubated in the dark at room temperature for 30 min. Distilled water was used for the control sample. The scavenged DPPH was then monitored by determining the absorbance at 517 nm. The radical scavenging activity was quantified using the following formula: DPPH radical scavenging activity (U/mL) = ABSC − ABSS/S × 100, where ABSC and ABSS are the absorbance of the control and the test samples at 517 nm respectively and S is the volume (mL) of the sample. The results are expressed as DPPH radical scavenging activity (U/mL).

Ferric Reducing Antioxidant Power (FRAP) Assay

The reducing power of LL95 was tested by the FRAP assay according to Han *et al*. (2017) with minor modifications. Briefly, 750 µL of FRAP solution was heated to 37 ˚ C in a water bath. Then, 25 µL of sample and 75 µL of ultrapure water were added. The mixture was placed in the dark for 5 min and the absorbance was measured at 593 nm. A standard curve was prepared with 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM $FeSO_4.7H_2O$. The FRAP values of samples were calculated according to the standard curve. The results were expressed as the equivalent amount of $FeSO_4$ (mM).

Results and Discussion

Characterisation of Isolates by Molecular Method

The PCR product was run on a 1.5 % agarose gel to visualize the amplicons. The amplicons were then sequenced and the isolates were identified by BLAST analysis.

Table 2: List of isolates screened post molecular screening

Probiotic name	Number of isolates identified	Number of isolates taken for further studies
E. thailandicus	2	1
E.hirae	3	2
E.faecium	5	2
E.fecalis	3	3
E.durans	2	1
P.acidilactici	3	1
L. salivarius	8	1

***In vitro* Characterization of Potential Probiotic Isolates**

Characterisation by DPPH Assay

The optical density was read in triplicates and the readings expressed as ascorbic acid equivalents and the results are depicted in Table 3.

Ferric Reducing Antioxidant Power (FRAP) Assay

The optical density was read in triplicates and the readings expressed as ascorbic acid equivalents and the results are depicted in Table 3. Backyard chickens are reported to have better gut flora than handfed chicken (Huang *et al.*, 2004).

Table 3: The observed values of the assays involved in screening of potential probiotic isolates

Name of Isolate	Fold increase				Ascorbic acid equivalent	
	GPX-Hepg2	GPX-Caco2	SOD-HepG2	SOD-Caco2	DPPH	FRAP
E. thailandicus	1.28	0.17	0.3	1.36	64	52
E.hirae-1	1.35	1.94	2.95	22.63	428	52
E.hirae-2	0.55	1.45	2.89	8.42	555	61
E.faecium-1	1.59	1.89	0.75	8.06	389	29
E.faecium-2	0.41	1.28	3.95	3.6	261	33
E.fecalis-1	1.9	1.41	0.59	8.59	96	54
E.fecalis-2	0.56	1.61	3.89	15.29	501	32
E.fecalis-3	0.53	1.34	3.64	17.13	116	33
E.durans	0.51	1.02	0.53	4.51	657	33
P.acidilactici	1.71	1.95	7.05	24.19	583	37
L. salivarius	1.63	1.34	1.93	2.59	178	38

The above values are means of the respective assays

Hence, the supplementation of these microbiota from backyard chicken to hand fed broilers are presumed to improve their performance. In view of this presumption bacterial isolates were collected from backyard chicken and screened for their antioxidant potential both at intercellular through *in vitro* assays and extracellular by antioxidant assays.

Molecular methods are more reliable as the biochemical methods lack repeatability and are inconsistent. Recent comparative study between biochemical profile using API CH 50 carbohydrate fermentation test and multiplex PCR technique confirmed that the use of biochemical methods does not appear to be appropriate for the identification and study of lactobacilli, since the failure rate with the former method was high compared to molecular biology techniques (Brolazo *et al.,* 2011). Hence molecular methods of identification by PCR amplification and sequencing are adopted in this study for identification of probiotic isolates. The relative mRNA expression levels of different antioxidant enzymes of probiotic treated culture comparison to control culture were calculated using $2^{-\Delta\Delta Ct}$ method and the results were interpreted in Table 3, 4 and 5.

Table 4: Score key for the observed values

GPX-Hepg2		GPX-Caco2		SOD-HepG2		SOD-Caco2		DPPH		FRAP	
Range	**Score**	**Range**	**Score**	**Range**	**Score**	**Range**	**Score**	**Range**	**Score**	**Range**	**Score**
<0.5	1	<0.5	1	<2	1	<6	1	<175	1	<15	1
0.5-1	2	0.5-1	2	2.0-4.0	2	6.0-12.0	2	175-350	2	16-30	2
1.01-1.5	3	1.01-1.5	3	4.01-6	3	12.01-18.0	3	351-525	3	31-45	3
>1.5	4	>1.5	4	>6	4	>18	4	>525	4	>45	4

Table 5: Scoring assigned based on the scoring key given in Table 4

Name of Isolates	GPX-Hepg2	GPX Caco2	SOD HepG2	SOD Caco2	DPPH	FRAP	Total score
E. thailandicus	3	1	1	1	1	4	11
E.hirae-1	3	4	2	4	3	4	20
E.hirae-2	2	3	2	2	4	4	17
E.faecium-1	4	4	1	2	3	2	16
E.faecium-2	1	3	2	1	2	3	12
E.fecalis-1	4	3	1	2	1	4	15
E.fecalis-2	2	4	2	3	3	3	17
E.fecalis-3	2	3	2	3	1	3	14
E.durans	2	3	1	1	4	3	14
P.acidilactici	4	4	4	4	4	3	23
L. salivarius	4	3	1	1	2	3	14

The health promoting properties of probiotic are suggested to be strain dependant and therefore strain identification and characterization are very important (Drago *et al*., 2010). Antioxidant enzymes, such as CAT within the peroxisomes and cytosolic GPx, are involved in the conversion of hydrogen peroxide, a powerful and potentially harmful oxidizing agent, into water and molecular oxygen (Liska, 1998). Hydrogen peroxide is unstable and forms a hydroxyl free radical; its break-down on the inner mitochondrial membrane and within the mitochondrial matrix causes site-specific damage of critical matrix targets. The decomposition of hydrogen peroxide outside the mitochondria may cause damage to the outer mitochondrial membrane itself, which suggests that the breakdown of hydrogen peroxide to the hydroxyl radical is highly detrimental. Superoxide dismutase catalyzes the conversion of superoxide anions to hydrogen peroxide and is one of the primary enzymatic defences against ROS. In this study the expression levels of the antioxidant enzymes GPX and SOD were elevated correlating with the study of Yarru *et al.* (2009)

In order to normalise the varying range of observations between different systems used in this study for screening the probiotics a scoring system was derived in reference to Manjari *et al.* (2019). The extracellular antioxidant properties of the isolates were given scoring depending upon the range of the ascorbic acid equivalents.

Conclusion

According to the scores obtained by the *in vitro* assays and the antioxidant assays a consortium was formulated with the best of isolates *viz. E. hirae-1, E. faecium-1, E. fecalis-3, P. acidilactici.* This consortium shall be utilized for further studies including experimental trial in chicken to find the efficacy in broilers as feed supplement for better understanding of the molecular mechanisms of probiotic action. The probiotics with antioxidant properties improve the quality of meat. In conclusion, the present *in vitro* study demonstrated that there was sufficient evidence of the isolated strains having specific antioxidant effects which could be utilized for better efficiency in commercial hand fed birds.

Conflict of Interests

There is no conflict of interest.

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References

1. AFRC, R. F. (1989). Probiotics in man and animals. *Journal of Applied Bacteriology*, *66*(5), 365-378.
2. Brolazo, E. M., Leite, D. S., Tiba, M. R., Villarroel, M., Marconi, C., & Simoes, J. A. (2011). Correlation between API 50 CH and multiplex polymerase chain reaction for the identification of vaginal lactobacilli in isolates. *Brazilian Journal of Microbiology*, *42*(1), 225-232.
3. Charteris, W. P., Kelly, P. M., Morelli, L., & Collins, J. K. (1998). Ingredient selection criteria for probiotic microorganisms in functional dairy foods. *International Journal of Dairy Technology*, *51*(4), 123-136.
4. deMelo Pereira, G. V., de Oliveira Coelho, B., Júnior, A. I. M., Thomaz-Soccol, V., & Soccol, C. R. (2018). How to select a probiotic? A review and update of methods and criteria. *Biotechnology Advances*, *36*(8), 2060-2076.
5. Dhama, K., Verma, V., Sawant, P. M., Tiwari, R., Vaid, R. K., & Chauhan, R. S. (2011). Applications of probiotics in poultry: Enhancing immunity and beneficial effects on production performances and health-A review. *Journal of Immunology and Immunopathology*, *13*(1), 1-19.
6. Drago, L., Nicola, L., Iemoli, E., Banfi, G., & De Vecchi, E. (2010). Strain-dependent release of cytokines modulated by *Lactobacillus salivarius* human isolates in an in vitro model. *BMC Research Notes*, *3*(1), 1-5.
7. Figueroa-González, I., Quijano, G., Ramirez, G., & Cruz-Guerrero, A. (2011). Probiotics and prebiotics—perspectives and challenges. *Journal of the Science of Food and Agriculture*, *91*(8), 1341-1348.
8. Han, Q., Kong, B., Chen, Q., Sun, F., & Zhang, H. (2017). In vitro comparison of probiotic properties of lactic acid bacteria isolated from Harbin dry sausages and selected probiotics. *Journal of Functional Foods*, *32*, 391-400.
9. Huang, Y., & Adams, M. C. (2004). In vitro assessment of the upper gastrointestinal tolerance of potential probiotic dairy propioni bacteria. *International Journal of Food Microbiology*, *91*(3), 253-260.
10. Kabir, S. (2009). Effect of probiotics on broiler meat quality. *African Journal of Biotechnology*, *8*(15).
11. Kailasapathy, K., & Chin, J. (2000). Survival and therapeutic potential of probiotic organisms with reference to Lactobacillus acidophilus and Bifidobacterium spp. *Immunology and Cell Biology*, *78*(1), 80-88.
12. Liska, D. J. (1998). The detoxification enzyme systems. *Altern Med Rev*, *3*(3), 187-198.
13. Manjari, K. D., Divya, G., Saravanan, T. S., & Parthiban, M. (2019). In vitro selection of potential immunomodulatory multispecies lactic acid bacteria in broilers. *Indian Journal of Animal Research*, *53*(5), 655-660.
14. Maragkoudakis, P. A., Zoumpopoulou, G., Miaris, C., Kalantzopoulos, G., Pot, B., & Tsakalidou, E. (2006). Probiotic potential of *Lactobacillus* strains isolated from dairy products. *International Dairy Journal*, *16*(3), 189-199.
15. Panda, A. K., Rama Rao, S. S., Raju, M. V., & Sharma, S. S. (2008). Effect of probiotic (*Lactobacillus sporogenes*) feeding on egg production and quality, yolk cholesterol and humoral immune response of White Leghorn layer breeders. *Journal of the Science of Food and Agriculture*, *88*(1), 43-47.

16. Settivari, R. S., Bhusari, S., Evans, T., Eichen, P. A., Hearne, L. B., Antoniou, E., &Spiers, D. E. (2006). Genomic analysis of the impact of fescue toxicosis on hepatic function. *Journal of Animal Science*, *84*(5), 1279-1294.
17. Surai, P. F. (2002). *Natural antioxidants in avian nutrition and reproduction* (pp. 5-9). Nottingham: Nottingham University Press.
18. Walter, J., Tannock, G. W., Tilsala-Timisjarvi, A., Rodtong, S., Loach, D. M., Munro, K., & Alatossava, T. (2000). Detection and identification of gastrointestinal lactobacillus species by using denaturing gradient gel electrophoresis and species-specific PCR primers. *Applied and Environmental Microbiology*, *66*(1), 297-303.
19. Wang, Y., Wu, Y., Wang, Y., Xu, H., Mei, X., Yu, D., & Li, W. (2017). Antioxidant properties of probiotic bacteria. *Nutrients*, *9*(5), 521.
20. Xing, J., Wang, G., Zhang, Q., Liu, X., Gu, Z., Zhang, H., & Chen, W. (2015). Determining antioxidant activities of lactobacilli cell-free supernatants by cellular antioxidant assay: a comparison with traditional methods. *PLoS One*, *10*(3), e0119058.
21. Yarru, L. P., Settivari, R. S., Gowda, N. K. S., Antoniou, E., Ledoux, D. R., & Rottinghaus, G. E. (2009). Effects of turmeric (*Curcuma longa*) on the expression of hepatic genes associated with biotransformation, antioxidant, and immune systems in broiler chicks fed aflatoxin. *Poultry Science*, *88*(12), 2620-2627.

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