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Evaluation of Probiotic potential of Novel candidate Enterobacter avium strain against Chick faecal borne *Alcaligenes faecalis*"

Vinothini V. M. Post Graduate Department of Microbiology, Standard Fireworks Rajaratnam College

Radhathirum alai-arasu, S. Assistant Professor Department of Microbiology, Standard Fireworks Rajaratnam College

ABSTRACT

Consumers and regulatory agencies have reduced or even eliminated the use of antibiotics in food producing animals. This created a need to find alternatives to maintain healthy and productive animals. Probiotics were employed as a good alternative treatment strategy through oral bacteriotherapy for curing many diseases of human and animals. The present work was performed to isolate and characterized properties of probiotic Enterococci. For this purpose the antagonistic activity of E. avium probiotic was evaluated which posses the ability to suppress growth of opportunistic pathogen A. faecalis associated with bacteremia and respiratory infections in chick. As the E. avium fed chick recovered and showed improved growth and body weight they offers a potential novel candidate for controlling infection in chick.

KEYWORDS : probiotics, *Enterococci*, antagonistic properties, *A. faecalis*

INTRODUCTION

The *Alcaligenes faecalis,* an opportunistic pathogen that potentiates viral and other bacterial infection to cause meningitis in new born, bacteremia in cancer patients and associated with pancreatic abscess and corneal ulcer. Some poults infected with, *A. faecalis* developed mild diarrhoea, had urate deposits around the cloaca, were cool to the touch and huddled, and had an odour of the droppings which was characteristic of increased corticosteroid activity. Reports have indicated that *Alcaligenes* species have also been associated with respiratory infections in the chicks as usually severe and often lethal, and optimal antibiotic therapy is not well established (Omoregie and Osagie, 2012; Mordi et al., 2013).

There is a growing demand for probiotic functional food and has been used in livestock for decades to decrease the risk of infectious diseases and promote growth performance. Lactic acid bacteria, especially *Lactobacillus* and *Enterococci* are the most commonly used microorganisms as probiotics have "Generally Recognized As Safe" (GRAS) status (Amraii et al, 2014; Menconi et al, 2014). Several strategies have been anticipated to identify novel probiotic strains. The properties of probiotic are strain-specific; the selection of strain directly depends on the type of pathogenic infection (Sheela et al, 2010; Gomes et al, 2010). Hence this study intended isolates the strain with high probiotic potentiality which may exist in traditional dairies. The criteria used for *in vitro* selection of probiotic bacteria, in food preparations, which allow them to be established in the intestinal tract, include Bile tolerance and gastric juice resistance, which enable them to survive and grow to do their impressive action in the gastro-intestinal tract (GIT).

MATERIALS AND METHODS

Isolation and identification of Probiotic and Pathogenic strains: The pathogenic strain was isolated from diseased chick fecal waste on MacConkey agar (Hi media, Mumbai, India) incubated aerobically at 37°C for 18 to 24 h. The curd samples were collected from college canteen, SFRC, Sivakasi was used for isolation of LAB with MRS medium (Hi media, Mumbai, India). Species identification was done by using 16S rDNA sequencing and BLAST analysis. ***Inhibition assays:*** For detection of antimicrobial activity, an agar spot test was used (Burkholder and Bhunia, 2009). Test cultures for lactobacilli were spotted (2-3 µl) on the surface of MRS agar and incubated for 24 h at 37° C to develop the spots. The agar plates were then overlaid with SS soft agar 0.75% (w/v) with pathogens. The plates were incubated at 37° C for 24 h. Zones of inhibition around the central spots were measured.

Bile salts stress resistance: The cultures of appropriate dilution were spread plated on to MRS agar and bile salt agar enriched with 1 % (wt/vol) of bile salt. The plates were incubated 37°C for 24h. After incubation, the viable bacterial count was determined by comparing the colonies grown on MRS agar with that of on MRS agar with bile salt 1 % (wt/vol) and surviving percentage was calculated (Ho et al, 2011).

Assessment of Acid tolerance: From the culture of each strain grown overnight cells were centrifuged at 5000g for 10 min at 4°C. The pellets washed in sterile phosphate buffered saline (PBS) pH 7, and resuspended in PBS were further diluted 1/100 in PBS. Culture suspension (1 ml) containing approximately 10^9 cfu/ml LAB was transferred into 9 ml phosphate-buffered saline (PBS). The pH was adjusted to 1.0, 2.0 and 3.0 using 0.1 N HCl and was incubated at 37°C for 2 and 4h. After different time intervals the acid pH treated samples were inoculated into MRS broth, incubated for 18 h and bacterial growth was determined by measuring the optical density at 600 nm (OD_{600}). The values were compared with the control (pH 7) and represented as relative survival percentage.

Auto- and co aggregation assay: The ability of bacteria to auto aggregate and co aggregate was assessed according to the method described by Tareb et al. 2013. The OD600 of the bacterial suspensions was monitored at 3, 20 and 24 h. The OD600 in control tubes containing only the pathogen or the lactic acid bacterial strain respectively and of the mixed culture after different periods of incubation were expressed as a percentage of the total number of bacteria present.

Experiment with chick: The chicks (females, 45 days of age) were used in the present study between February and March, 2015. Two set of domestic chicks were selected for the invivo experiment were obtained from a local hatchery, housed and cared for experiments 1and 2. In experiment 1 poults were randomly challenged via oral gavage with approximately 10^6 cfu/poult of pathogenic strain VAF2 and placed in pen. In experiment 2 the poults were given orally only with ~10^6 cfu/ chick of *E. avium* VEA4 orally. The untreated chick reared for experiment 3 was taken as control. Feeds were given to the poults like rice, grains, etc. in 10 g in each per day. In all experiments chicks were cared and reared for the period of 40 d. From the days 10 of post infection, the faecal samples were analyzed for LAB and pathogenic strain using MRS and Maconkey selective plating (Karimi Torshizi et al, 2008).

RESULTS AND DISCUSSION

Despite of the advances in applied science, poultry diseases continue to cause a considerable economic burden; fortunately, some probiotic strains have been considered for their positive effects on certain infectious diseases and recently their use to prevent and treat has significantly augmented. Thus there is a clear interest in the identification and characterization of new candidate strains with

well-demonstrated probiotic properties. Of the 82 isolates obtained on MRS agar plates the isolate VEA4 showed observable antagonistic effects against the pathogen *A. faecalis* was selected for the experiment.

Identification of selected strain: The results of comparative 16 S rRNA gene analysis showed that the probiotic isolate exhibit 99 % similarity to *Enterococcus avium* where as pathogenic isolates exhibited 98 % homology with *Alcaligenes faecalis*. The 16 S rRNA gene sequences of the two strains were deposited in the GeneBank database under the accession numbers KR363182 (*E. avium* VEA4) and KR363183 (*Alcaligenes faecalis* VAF2) respectively. Similarly, studies have previously reported antibacterial property of *E. avium* against *Listeria monocytogenes* (Ehrmann et al, 2002; Audisio et al, 2005).

Invitro analysis of probiotic properties of E. avium VEA4: Undeniably, bile salts may constitute a deleterious factor preventing a strain from exerting its beneficial properties in vivo. The strain *E. avium* VEA4 exhibited above 70 % survivability at pH 3 for more than 2 h (Fig: 1). The tolerance to bile salt was enumerated as 84% with 42 $\times 10^{4}$ CFU/ml for 3 h. *Enterococcus* sp. M5aA and M5a have been able to resisted bile (38% to 96%) as well as low pH 3 - the surviving of enterococci in pH 3.0 have ranged from 2.0 up to 5.9 log10 cfu/mL for 3.0 h incubation at 37 °C (Szaboova et al, 2012). Autoaggregation increased as a function of time and was highest at the 20 h time point ranged between 80 and 85 % (Fig: 2.A). Coaggregation also increased as a function of time and was highest at the 24 h time point (above 90 %) (Fig: 2.B). The strain *Enterococcus avium* VEA4 exhibited markedly increased antibacterial profile and strong interaction with *Alcaligenes faecalis* VAF2. The time needed for significant aggregation was between 10 and 120 min. Interestingly, within the Enterococci, Weissella and pediococci, no, or only weak (≥ 90 min), autoaggregation ability was detectable, whereas aggregation within the lactobacilli was generally much more pronounced (Ehrmann et al, 2002). Furthermore, the coaggregation reduces the cell–cell distances between probiotics and pathogens, increasing the efficiency of antimicrobial metabolites produced by viable probiotics (organic acids, bacteriocins, etc.). The Coaggregative percentages of lactobacillus strains and pathogenic strains were above 90 %. When viable *Lb. rhamnosus* 3968 and *Lb. farciminis* 3969 bacteria were used, the percentage coaggregation was 61.2%±2.5 and 53.6%±2.9 respectively, compared to 59.7%±2.5 and 51.1%±2.6 respectively (Tareb et al, 2012).

Chick experiment

Treatment with pathogen in the drinking water caused a significant observable diarrheal stool in the chick with reduction in growth (Higgins et al, 2010; Shabani et al, 2012). In experiment 1, oral administration of *E. avium* VEA4 showed rapid recovery. In experiment 2, chick fed only with E. *avium* VEA4 had rapid growth and weight gain compared to untreated control chick (Table no.1). For example feed supplementation with the probiotic *E. faecium* NCIMB 10415 for piglets has been found to reduce pathogenic gut microorganisms (Carmen et al, 2013).

CONCLUSION

Thus the research conducted in our laboratory has elucidated an identification of efficient candidate probiotic organism. Interestingly strong antagonistic activity against pathogen and other characteristics such as acid and bile tolerance of *Enterococcus avium* VEA4 suggest their ability to cure the infection caused by *Alcaligenes faecalis* VAF2 in chick. The probiotic treatment significantly increased body weight and displayed a growth-promoting effect. Therefore, these products might be promising alternatives for antibiotic growth promoters in animal feed. They should be studied further to conclude the nature of bio therapeutic agents and also investigated for other probiotic bioactivities for human health benefits.

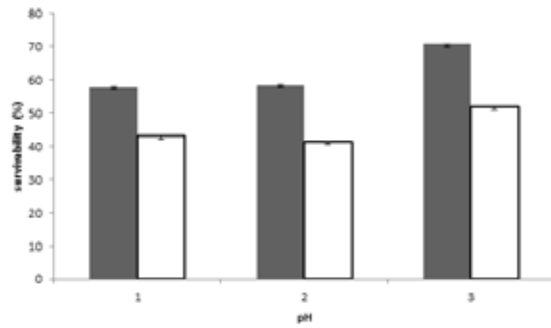
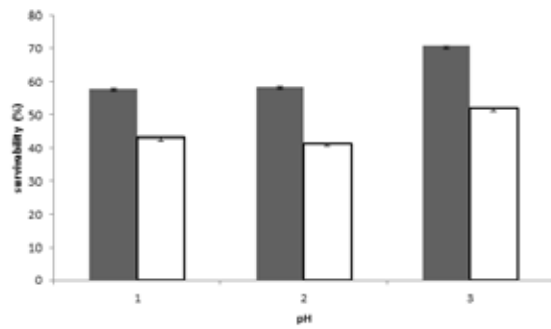
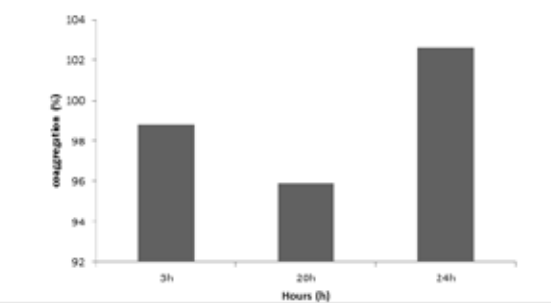


Figure No. 1: Acid tolerance of *Enterococcus avium* VL4. The percentage of cells resisting acid pH were determined by maintaining the cells at pH 2 (■) and 4 (□). At different time interval the optical density at 600 nm (OD_{600}) were measured and the value were compared with the control (pH 7).



[A]



[B]

Figure No. 2: [A] Autoaggregation and [B] Coaggregation percentages as a function of time for *E. avium* VEA4 was determined by spectrophotometric assay

Table No. 1 Effect of *E. avium* VEA4 based probiotic diet on body weight in chick

S.No	Treatment	Body Weight (g/bird)				
		0 d	10 d	20 d	30 d	40 d
1.	T-1	450±11	670±22	750±12	965±13	1100±18
2.	T-2	600±13	725±18	895±14	950±8	1150±16
3.	Control	500±14	625±16	730±11	860±9	927±14

Enterococcus-based diet was used on three different treatments (0-40 d) in replicates.

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