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SHORT COMMUNICATION

The Isolation and Characterization of Coagulase-negative *Staphylococcus* spp. from Intestine of a Malaysian Rabbit *(Oryctolagus cuniculus)*

TENGKU HAZIYAMIN TENGKU ABDUL HAMID* & FARHANEEN AFZAL MAZLAN

Department of Biotechnology, Kulliyyah of Science, International Islamic University Malaysia, Kuantan Campus, Jalan Istana, Bandar Indera Mahkota, 25200, Kuantan, Malaysia

ABSTRACT

The isolation and characterization of lactic acid bacteria (LAB) from the intestine of *Oryctolagus cuniculus*, a domestic rabbit species in Malaysia is described. Fifty isolates from rabbit intestine were screened by biochemical tests. From 50 isolates, four were identified and shown to be catalase-positive, lactose positive and Gram-positive cocci. Antibacterial assays were carried out against *Escherichia coli* and *Salmonella enteritidis* as indicator bacteria. The samples exhibited antibacterial properties as indicated by zone of inhibitions. Three isolates were further subjected to 16S ribosomal RNA (rRNA) gene sequencing and analysis. Partial 16S rRNA sequencing results from these isolates showed high sequence similarity with coagulase negative *Staphylococcus* sp. This result showed that by screening of LAB from rabbit intestine, bacteria from the family *Staphylococcaceae* could be isolated and this could be potentially used as probiotics in rabbit feeding.

Keywords: Lactic acid bacteria, probiotic, 16S ribosomal RNA, coagulase-negative *Staphylococcus*.

Malaysia provides suitable environment for rearing domestic rabbit (*Oryctolagus cuniculus*) (Davis & Yogendran 2009), which could augment poultry or cattle in providing protein source for the country. However, there is lack of report on factors affecting domestic rabbit production in Malaysia. Growth rates of rabbits range from 10 to 20 g/day in the tropical regions, and this is lower compared to temperate countries 35 to 40 g/day (Samkol & Lukefahr 2008). The differences may be largely due to heat stress and quality of the diets (Lukefahr & Cheeke 1991). Most diseases of the gastrointestinal tract of domestic rabbit link to their diet and the disruption of finely balanced digestive microbiota (Irlbeck 2001; Cheeke 1987). Any disruption of the normal digestive process in rabbits can result in gastrointestinal disease. Even though primary gastrointestinal disease due to the presence of enteric pathogens is uncommon in rabbit, other organisms such as *Escherichia coli* and Rotavirus may also be involved (Jenkins 2000). Clostridia species are normal inhabitants of rabbit gastrointestinal tract which can proliferate under certain conditions to produce severe enteritis and in some cases, enterotoxemia (Jenkins 2000). In addition, *Staphylococcus* infection leads to substantial economic losses in the livestock industries (Corpa *et al*. 2009; Mørk *et al.* 2005), which also contribute to major loss in rabbit industry.

Probiotic feeding was proposed as a strategy to improve gut health and immune system in rabbit (Onbasilar 2008). Lactic acid bacteria (LAB) is regarded as a major group of probiotic bacteria (Collins *et al*. 1998). Salminen *et al*. (1999) have proposed the probiotics components of microbial cells that have a beneficial effect on the health and well-being of the host. Several lactobacilli, lactococci and bifidobacteria are health-benefiting bacteria (Rolfe 2000). LAB and other gut microbiota ferment various substrates such as lactose, biogenic amines and allergenic compounds into short-chain fatty acids and other organic acids and gases (Gibson & Fuller 2000; Jay 2000). Depending on the strain of LAB, different mechanisms were used to produce beneficial health impacts to the host. The aim of this study was to isolate and characterize LAB from the intestine of *Oryctolagus cuniculus*, a domestic rabbit in Malaysia.

Intestinal tissues from a dissected rabbit was chopped and rinsed with sterile ringer solution.

*Corresponding author: *haziyamin@iium.edu.my*

Solutions containing bacterial suspensions were serially diluted using De Man, Rogosa and Sharpe (MRS) broth and from each dilution, 100 µl suspension was spread uniformly on MRS agar and then incubated for 3 days at 30°C. Fifty single colonies were picked out using sterile toothpick and were used for morphology and biochemical analysis.

Lactose utilization test was carried out using MRS agar medium containing lactose and bromocresol purple as described by Thomas (Thomas 1973). Catalase test was used to detect the presence of catalase enzyme by applying dropwise 30% hydrogen peroxide solution on the bacterial suspension that had been spotted on microscopic slide. Gram staining was carried out to characterize the shape and type of the isolates. All colonies appeared coccoid morphology showing Gram positive (purple) staining properties (Figure 1). Out of 50 isolates tested, only four showed yellow zone formation from lactose fermentation on MRS media. The summary of biochemical tests used is shown in Table 1.

The antibacterial assay of LAB was conducted by agar well diffusion method (Schillinger and Liicke, 1989). In this assay, *Escherichia coli* and *Salmonella enteritidis* were used as the indicator microorganisms. Aliquots of 80 µl streptomycin dispensed at 0.5 mg/ml per well were used as the positive control. These tests were carried out in triplicates. As shown in on Figure 2 and Table 2, the isolates also exhibited various degrees of inhibition against both Gram-negative indicators: (i) *E. coli* (9. 0-11.0 ± 0.1 mm); (ii) *S. enteritidis.* (12.0-14.0 ± 0.1 mm) and (iii) antibiotic control (14.0-22.0 ± 0.1 mm). Since the antagonistic properties of LAB were usually against their related species these isolates could be further tested for antagonistic activity against pathogenic species such as *S. aureus.* In animals, staphylococcal infections lead to substantial economic losses in the livestock industries (Corpa *et al.* 2009; Mørk *et al.* 2005) and this bacterium can affect rabbit (Okerman *et al.* 1984). Moreover, it has also been reported that the pathogenic strain Type 4 and 5 of *S. aureus* were isolated from rabbit (Poutrel & Sutra 1993).

Genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen 2010). PCR analyses were performed using 2 sets of universal primers for 16S ribosomal RNA gene. The first set (h1R) with forward primer sequence 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse 5'-GGT TAC CTT GTT ACG ACT T-3'. The second set (hpE) with forward sequence 5'- AGA GTT TGA TCC TGG CTC AG-3' and the reverse sequence 5'-CCG TCA ATT CCT TTG AGT TT-3' (purchased from 1st BASE Sdn Bhd, Malaysia). The PCR products were gel purified (Qiagen) and used as templates for further sequencing reactions (outsourced to sequencing service at 1st BASE Sdn Bhd, Malaysia). Basic Local Alignment Search Tool (BLAST) was used for similarity searches between sequences obtained. Sequences that show high similarity with the sample were collected from NCBI database for phylogenetic analysis and *Escherichia coli* was used as an outgroup. Phylogenetic analysis was done using software MEGA version 4 (Tamura 2007). Neibour-joining method was used to construct the phylogenetic tree.

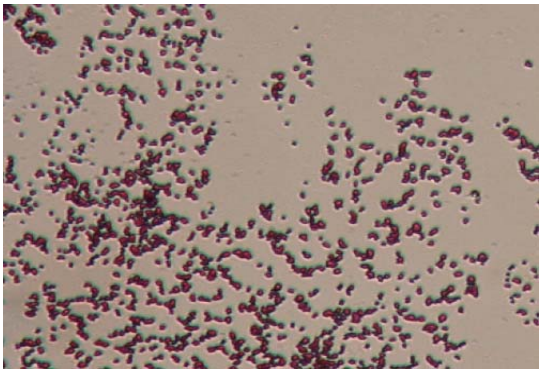

Figure 1. Morphology of lactic acid bacteria samples (1000x magnification).

Based on BLAST search results of partial rRNA sequences for the three samples a phylogenetic tree was constructed together with other homologous sequences determined (Figure 4). With a bootstrap value above 50%, these isolates show high sequence similarity (at least 98%) with the members from Staphylococceae family in the databank. Some of these microorganisms belong to the coagulase negative Staphylococci which include *S. pasteuri, S. piscifermentans, S. epidermidis* and *S. carnosus*. Coagulase negative staphylococci and lactic acid bacteria (LAB) are the most important microorganisms used as the starter cultures in meat fermentations (Bonomo 2009). Studies were conducted to assess the safety of coagulase negative staphylococci used in fermented dairy foods (Irlinger 2008), and their biodiversity were shown to be different from that of clinical isolates (Cotona *et al.* 2010).

Table 1. Gram staining and biochemical test of isolates from rabbit intestinal samples

Samples	Morphology	Gram Staining	Catalase	Lactose Utilization
F1.14	Coccus	purple	+	+
F1.5	Coccus	purple	+	+
F2.10	Coccus	purple	+	+
F2.14	Coccus	purple	+	+

Table 2. Disk diffusion assay of bacterial isolates against *E. coli* and *S. enteritidis*

Zone of Inhibition (Diameter in mm)		
Samples of LAB	*E. coli* (mm)	*S. enteritidis* (mm)
F1.14	11.5	14.0
F1.5	10.3	12.0
F2.10	9.8	12.4
F2.14	11.4	12.8
Positive control (Streptomycin)	14.0	22.0

Values are mean ± SD of triplicates test. The diameter of inhibition zones (mm) including well diameter of 6 mm.

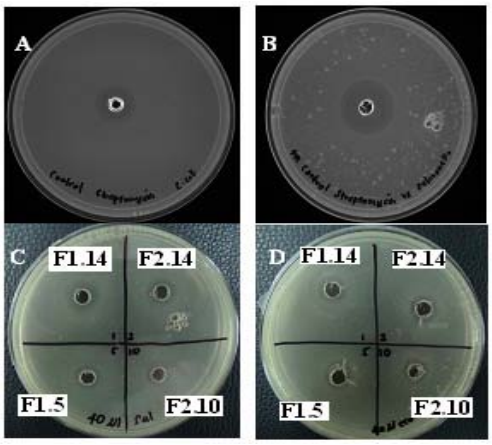

Figure 2. Agar well diffusion test showing zones of inhibitions (arrows). The nutrient agar (NA) plates containing control antibiotic Streptomycin against A). *E. coli* and B). *S. enteritidis*, The other plates show zones of inhibition for rabbit intestinal isolates against C). *E. coli* , and D). *S. enteritidis*, respectively.

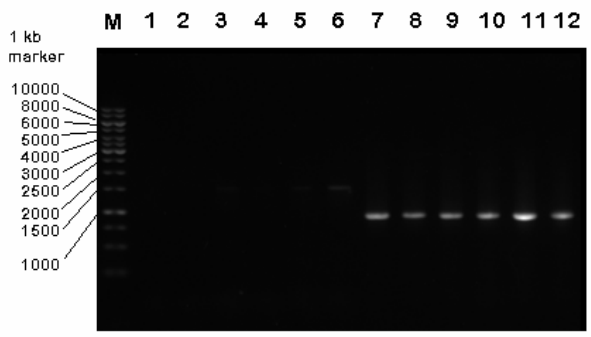

Figure 3. The 16S ribosomal RNA gene for samples F1.14, F2.14 and F2.10 were amplified using different sets of primer; h1R and hpE. The lane L is 1 kb marker DNA Ladder (Fermentas). The 1000bp product was obtained by using h1R primer for samples F1.14 (lane 1), F2 14 (lane 2) and F2.10 (lane 3). Lane 4-6 are the duplicate reactions using primer h1R. The 1500bp product was obtained by using primer hpE for samples F1.14 (lane 7), F2 14 (lane 8) and F2.10 (lane 9). Lane 10-12 are the duplicate reactions using this primer.

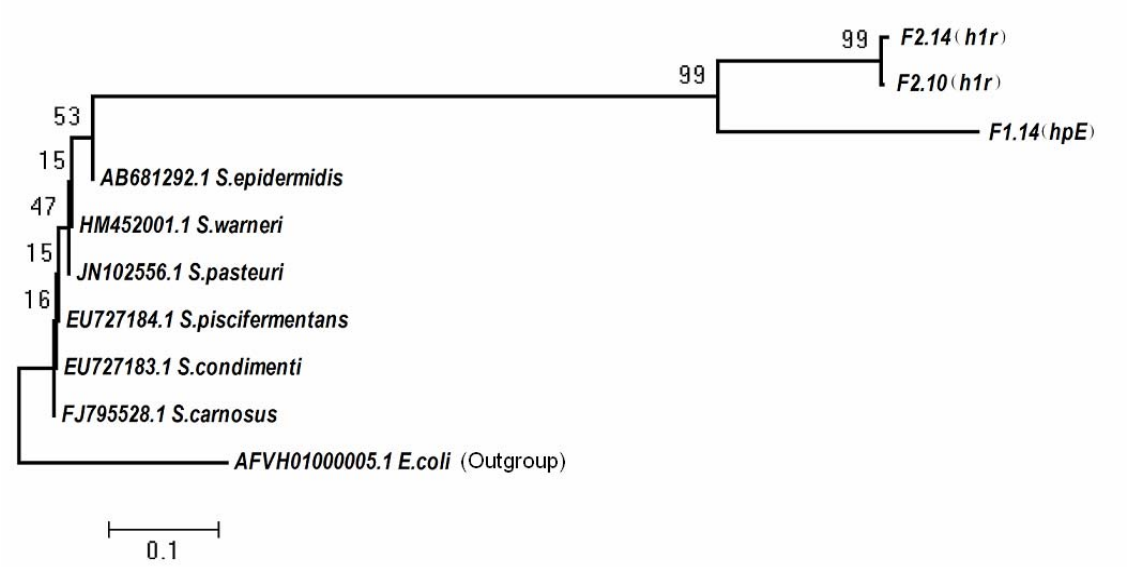

Figure 4. Phylogenetic tree shows relations between rRNA sequences for isolates (F1.14, F2.14 and F2.10) with other selected representatives from Staphylococcus genus of high similarities. Also shown is the accession number for each sequence used.

These isolates were catalase-positive, Gram positive cocci and exhibit antibacterial properties against *E. coli* and *S. enteritidis.* The partial DNA sequences revealed that they possess significant similarity with coagulase negative Staphylococci which are mainly clustered with *S. pasteuri, S. warneri, S. epidermidis, S. carnosus and S. piscifermentans.* Even though, some of these species have been implicated as pathogenic microorganisms, they may also be isolated from fermented meat or fish products; which they exhibit desirable probiotic properties. As some members of *Staphylococcaceae* family are safe and with the probiotic properties, these isolates could be useful antagonist against pathogenic staphylococcus in rabbit.

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