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Full Length Research Paper

Isolation and screening of lactic acid bacteria, *Lactococcus lactis* from *Clarias gariepinus* (African catfish) with potential use as probiotic in aquaculture

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In aquaculture probiotic feeding could play a crucial role in developing microbial control strategies, since disease outbreaks are recognized as important constraints to aquaculture production and the fear of antibiotic resistance. In this study, lactic acid bacteria (LAB) strains from the intestinal tissue of African catfish *Clarias griepinus* were successfully screened, characterized and identified. The isolates (S1#8, S1#9, S1#10, S1#18, S1#19 S1#20 and S1#21) shared common morphological characteristics of LAB such as non-sporulating, Gram positive cocci or cocco-bacilli shape. These were the dominating morphology found in the catfish, as compared to other types of LABs reported to be found in freshwater fishes. All of the 35 isolates have the ability to utilize lactose as part of their metabolism process and showed negative reactions towards catalase test. These isolates were also tested for antimicrobial activities using disc diffusion assay against indicator *Salmonella typhimurium* and *Escherichia coli*. Based on the partial 16S rRNA sequences, the selected LAB isolates belonged to a member of *Lactococcus lactis* with 98% DNA similarity. This strain can be used as probiotic in aquaculture feeding.

Key words: *Clarias gariepinus*, *Lactococcus lactis,* lactic acid bacteria (LAB), disc diffusion, bacteriocins.

INTRODUCTION

Research on lactic acid bacteria (LAB) has advanced greatly since the last decade due to its important roles in many diverse areas of food biotechnology, nutrition, health and safety. LABs are regarded as the major group of probiotic bacteria (Collins et al., 1998; Nousiainen and Setala, 1993). The use of probiotics in aquaculture is rather recent. Aquaculture is one of the fastest growing sectors in global food production; and Asia presently contributes to almost 90% of the total production (Rana, 1997). The African catfish (*Clarias gariepinus*) originating from Africa has been introduced and commercially cultured in Europe, South America and South East Asian countries (Marimuthu et al., 2010; Pillay and Kutty, 2005). It is an attractive freshwater fish species in Malaysia and other places due to its resistance to diseases and ability to grow at fast rate (Marimuthu et al., 2010). However, in Asia-Pacific region, aquaculture based disease outbreaks are the main productivity constraint that indirectly have impact on the socio-economy and sustainability of the producing community. Fish diseases are the major problem in fish farming industry, and bacterial infections are considered to be the major cause of mortality in fish (Gomez-Gil et al., 2000). In recent decade, prevention and control of the diseases have led to a substantial increase in the use of therapeutic medicines such as antibiotic. However, the use of antimicrobial agents as preventive measure posts significant risks since the evolution of antimicrobial r esistance among pathogenic

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Abbreviation: LAB, Lactic acid bacteria.

bacteria (Sahu et al., 2008).

Until recently, it has not been generally accepted that lactic acid bacteria are part of the native microbial flora in fish intestines (Pilet et al., 1995). Thus, supplementation of such bacteria in fish feed was regarded as futile. Nevertheless, LAB isolated from fishes inhibited the growth of fish pathogens such as *Vibrio anguillarum* and *Aeromonas salmonicida* (Gildberg et al., 1995). Therefore, increased understanding of probiotic use of LAB would lead to the development of natural antibiotic and reduce the dependency on chemical or drug uses in aquaculture (Subasinghe, 1997). The progress made in genetic and biochemical analyses of LAB is exploited to improve the aquaculture products so that it will be more acceptable to the consumers. Obviously, there is a need to search for LAB strain suitable for use as probiotic in aquaculture. The main strategy in the use of probiotics is to isolate intestinal bacteria with favourable properties from mature animals and incorporate these bacteria into the feeding of young and growing animals. This is due to the fact that these bacteria often produce bacteriocins and other chemical compounds that could inhibit the growth of pathogenic bacteria. Therefore, this project aims to screen and identify potential LAB from *C. gariepinus*, an African catfish in order to exploit them for further uses in aquaculture feeding.

MATERIALS AND METHODS

Digestive tracts from two catfishes (labeled as samples 1 and 2) were sterilely dissected and washed. The fish intestines were cut, weighed and homogenized in Man Rogossa Sharp (MRS) agar and vortexed. These were serially diluted (from 10^{-1} to 10^{-6} for two samples, respectively) and were pour plated on MRS agar. The plates were incubated anaerobically, at 30°C for 48 h. Single colonies developed were picked up for morphological, lactose utilization, gram reaction, catalase ad antimicrobial test, and further, genomic extraction for rRNA sequencing.

In lactose utilization test, the selected colonies were then streaked onto nutrient agar NA with the addition of lactose containing 0.005 g/L bromo-cresol purple, as a pH indicator dye. The plates were incubated at 30°C for 24 h. Isolates that were able to utilize lactose and produce acid were differentiated by the change of media color from violet to yellow. Catalase activity was tested by adding a drop of 30% hydrogen peroxide solution onto the cell smears. Positive reaction would be seen as bubbles or froths generated from the colonies, indicating a rapid production of oxygen gas. Only isolates that showed negative reaction were subjected to further identification test.

The antagonistic activity of the isolated LAB against *Salmonella typhimurium* and *Escherichia coli* were determined by using agar disc diffusion. LAB isolate was propagated in MRS broth medium and incubated anaerobically at 30°C for 48 h. Cell propagated in MRS broth was pipette onto 5 mm diameter filter paper disc (diameter of 5 mm, Whatman No.1) and dried for 10 min. About 500 µl of indicator organism, (either *S. typhimurium* or *E. coli*) was spread on the plates. The test was performed in triplicates. Then, the plates were incubated at 37°C for 24 h and the zone of inhibitions formed surrounding the disc was observed.

Following overnight incubation at 30°C in 10 ml MRS broth, genomic extraction was carried out using DNeasy Blood and Tissue kit according the manufacturer protocol (QIAgen, USA). A pair of 16S rRNA primer was designed and synthesized based on the published 16S rRNA sequence of LAB (Edwards et al., 1989). A forward primer (pA 5'- AGA GTT TGA TCC TGG CTC AG -3') and a reverse primer (pE 5'- CCG TCA ATT CCT TTG AGT TT -3') were purchased from 1st Base (Malaysia) Sdn. Bhd. The amplifications were performed with initial denaturation at 94°C for 4 min, and 29 cycles of denaturation at 94°C for 2 min; annealing at 55°C for 1 min and extension at 72°C. All DNA templates used were approximately 5 ng DNA.

Finally, the amplified polymerase chain reaction (PCR) products were analysed in a 1.0 (w/v) % agarose gel in 1x TAE buffer at 90 V for 65 min; and gels were visualized by using gel documentation system (Alpha Imager). The amplified DNA fragments were purified and sent to 1st Base for automated DNA sequencing (1st Base Malaysia, Sdn Bhd). The homology of the sequences were analyzed using the BLASTN programs and compared with the Genbank database at National Center for Biotechnology Information (NCBI) accessible on-line at http://www.ncbi.nlm.nih.gov/Genbank/

RESULTS AND DISCUSSION

About 60 isolates were selected for further lactic acid bacteria identification. Out of the 60 isolates, 11 isolates from sample 1 and 19 isolates from sample 2 were found to be positive in lactose utilization test and these isolates were mostly identified to be Gram positive cocci or coccobacilli (Figures 1b and c). In lactose utilization test, most isolates were able to convert lactose into lactic acid indicated by changes in the pH of the media color from purple to yellow (Figure 1a). All isolates showed negative catalase test (Table 1) due to the absence of catalase enzyme and therefore unable to decompose H_2O_2.

By disc diffusion assay, a few of the isolated LAB bacteria demonstrated a clear bacteriocidal effect against Gram negative pathogens *S. typhimurium* and *E. coli* (Table 1). Only isolates S1#1, S2#8 and S2#25 showed inhibitory zones of at least 10 mm diameter on *S. typhimurium*. Isolates S1#9, S1#10, S1#18, S1#19 and S1#20 showed inhibition of at least 10 mm size on *E. coli* indicator bacteria (Figure 2). The inhibitory action of LAB is mainly due to accumulation of main primary metabolites such as lactic and acetic acids, ethanol, carbon dioxide; or antimicrobial compounds such as formic, benzoic and acids, hydrogen peroxide, diacetyl, acetoin and bacteriocins. LAB has also shown to possess inhibitory activities due to the bactericidal effect of protease sensitive bacteriocins (Jack et al., 1995). By producing these compounds, probiotic microorganisms could survive the adverse condition in gastrointestinal tract (El-Naggar, 2004). Further test using the supernatant solutions, isolates S1#8, S1#9, S1#10, S1#18, S1#19 and S1#20 gave inhibition of variable in size (between 5.6 to 10.6 mm) on *S. typhimurium*. Supernatant from isolates S1#8, S1# 9, S1# 10 and S1#19 gave inhibition of sizes (between 8.0 to 9.3 mm) on *E. coli.* These results are also shown in Table 3.

From Figures 3a and b, bands of 1.5 kb in size corres-

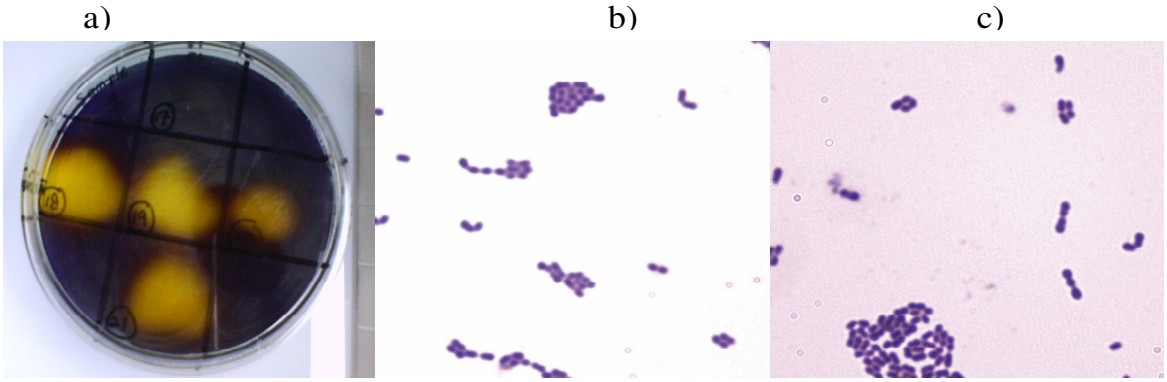

Figure 1. a) Colonies producing lactic acid lowered the pH causing the purple colour to change to yellow in nutrient agar media containing lactose and bromo-cresol, a pH indicating dye. b) Gram staining observation under light microscope (Nikon, 1000x magnification) showed that all 30 isolates were Gram-positve cocci and c) were in cocco-bacilli shape.

Table 1. Gram staining characteristics of the examined LAB.

Isolate	Gram Stain	Shape	Catalase (+) or (-)	Lactose utilisation	Antagonist test*	
					S. thyphi	*E. coli*
S1 #8	+	Coccus	-	+	-	-
S1 #9	+	Coccobacillus	-	+	-	+
S1 #10	+	Coccus	-	+	-	+
S1 #12	+	Coccus	-	+	-	-
S1 #16	+	Coccus	-	+	-	-
S1 #18	+	Coccus	-	+	-	++
S1 #19	+	Coccus	-	+	-	+
S1 #20	+	Coccus	-	+	-	-
S1 #21	+	Coccus	-	+	+	-
S1 #22	+	Coccus	-	+	-	-
S1 #23	+	Coccus	-	+	-	-
S2 #1	+	Coccus	-	+	-	-
S2 #2	+	Coccus	-	+	-	-
S2 #3	+	Coccus	-	+	-	-
S2 #4	+	Coccus	-	+	-	-
S2 #5	+	Coccus	-	+	-	-
S2 #6	+	Coccobacillus	-	+	-	-
S2 #7	+	Coccus	-	+	-	-
S2 #8	+	Coccobacillus	-	+	+	-
S2 #9	+	Coccus	-	+	-	-
S2 #10	+	Coccus	-	+	-	-
S2 #11	+	Coccus	-	+	-	-
S2 #12	+	Coccus	-	+	-	-
S2 #17	+	Coccus	-	+	-	-
S2 #20	+	Coccus	-	+	-	-
S2 #21	+	Coccus	-	+	-	-
S2 #22	+	Coccus	-	+	-	-
S2 #23	+	Coccus	-	+	-	-
S2 #24	+	Coccus	-	+	-	-
S2 #25	+	Coccus	-	+	+	-

*'+' Indicates at least 10 mm diameter inhibition.

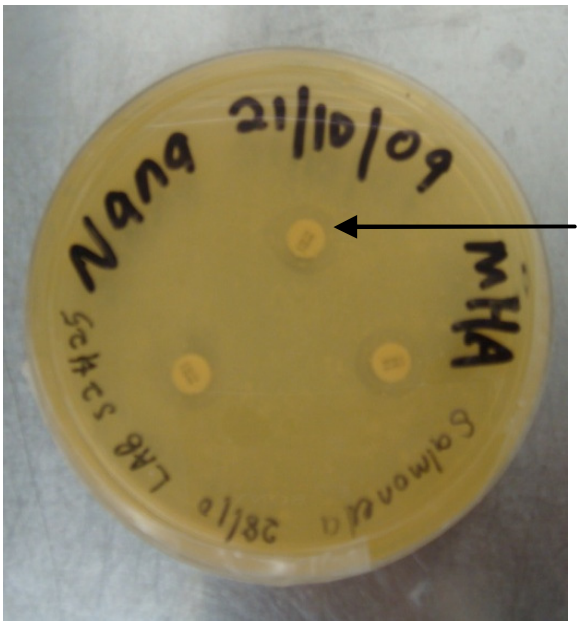

Figure 2. Positive antagonistic effect of LAB colonies against *S. typhimurium* and killing of the bacterial cells. Arrow shows zone of inhibition.

Table 2. NCBI blast results with *L. lactis* strain in Genebank database that show high percentage homology with rRNA sequences of LAB strain from catfish.

No.	Highest hit	E Value	Percentage homology (%)
S1#8	*Lactococcus lactis subsp. lactis*	0	99
S1#9	Lactoc *Lactococcus lactis subsp. lactis*	0	99
S1#10	*Lactococcus lactis subsp. lactis*	1e-132	99
S1#18	*Lactococcus lactis subsp. lactis*	0	98
S1#19	*Lactococcus lactis subsp. lactis*	5e-172	100
S1#21	*Lactococcus lactis subsp. lactis*	0	98
S2# 8	*Lactococcus lactis subsp. lactis*	0	97
S2#25	*Lactococcus lactis subsp. lactis*	0	99

ponding to the expected size of the 16S rRNA genes were amplified. While the DNA sequences from the other strains failed to be amplified, partial ribosomal DNA sequences of S1#8, S1#9, S1#10, S1#18, S1#19 S1#20 and S1#21 showed high homology (>97%) as compared to the 16S rRNA sequences from Genebank database. Table 2 summarizes these results and all isolates show the highest homology with *Lactococcus lactis.* These results are in accordance with the results obtained in the morphological and biochemical tests carried out (Table 1). However, report on the presence of *Lactoccoccus* sp. in freshwater fishes has been very few (Maugin and Novel, 1994, Nair and Surendran, 2005). Unlike *Carnobacterium* or *Vagococcus*, the species from other LAB groups are considered to be uncommon in aquatic environments (Stiles and Holzapfel, 1997; Ringø and Gatesoupe, 1998). Even though the presence of LAB in *Lactobacillus* or *lactococcus* family in freshwater fishes is rather 'atypical', there were reports on the presences of this type of LAB in freshwater fishes and prawns. Among LAB, *L. lactis* represents one of the important species, widely known for the production of nisin. Nisin is a ribosomally synthesized antimicrobial peptide used for the preservation of canned foods and dairy products (Delves-Broughton et al., 1996). Inhibitory activities (Table 3) from the supernatant of the LAB strains toward the two indicator strains could have originated from the soluble protein, however further confirmation were required to confirm its presence. Since the streptococcal infection has also become a problem in fishes, the *L. lactis* can be a good candidate for antagonistic strain for streptococcal infection. Studies were carried out to enhance the use of *L. lactis* as a probiotic in aquaculture. For instance, the presence of *L. lactis* were shown to pro-

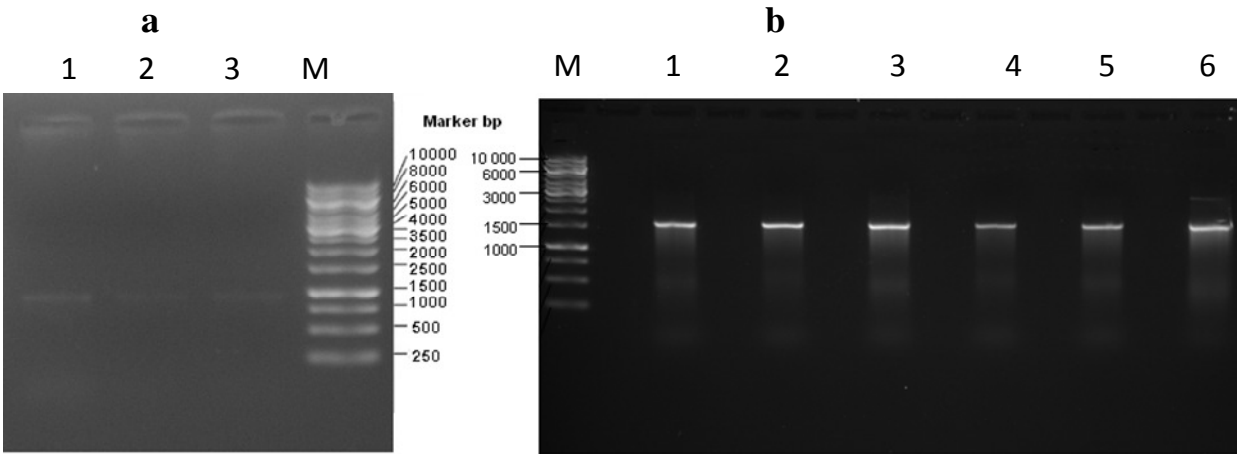

Figure 3. a) Lane M, Gene Ruler™ DNA Ladder Mix (Fermentas); lane 1, 5 µl of PCR amplified product from genomic DNA for S1#21; lane 2, S2 #8; lane 3, S2#25. b, Lane M, Gene Ruler™ DNA Ladder Mix (Fermentas); lane 1, 5 µl of PCR amplified product from genomic DNA for S1#8; lane 2, S1# 9; lane 3, S1#10; lane 4, S1#18; lane 5, S1#19; lane 6, S1#21. The control sample showed no amplification (result not shown).

Table 3. Inhibitory activities of supernatant from LAB strain against *S. typhimurium* and *E. coli.*

Cell free supernatant sample	*Salmonella typhimurium*	*Escherichia coli*
S1# 9	10.6	9.3
S1#10	5.6	8.0
S1#18	7.6	7.6
S1#19	9.4	-
S1#21	9.3	8.0
S2#25	8.0	-

* in +/- 0.1 mm

mote the growth rate of rotifers (Harzevili, 1998; Planas et al., 2004) and inhibit the fish pathogen, *Aeromonas hydrphila* in tilapia (Zhou et al., 2010).

Conclusion

From this study, LAB strains from the intestinal tissue of African catfish *Clarias garepinus* were successfully screened, characterized and identified. These isolates shared common morphological characteristics of LAB such as non-sporulating, Gram positive cocci or cocco-bacilli shape. These were the dominating morphologies found in the African catfish, as compared to other reported LABs commonly found in freshwater fishes. All the 35 isolates have the ability to utilize lactose as part of their metabolism process and showed negative reactions towards catalase test. Some of these strains have the capability to inhibit the pathogenic strains *S. typhimurium* and *E. coli* which are common human pathogen. Based on partial 16S rRNA sequences, the majority of the identified species (S1#9, S1#10, S1#18, S1#19 S2#8 and S1#21) belonged to the family *L. lactis,* a common strain in food industries which is however rare in aquaculture. Based on this features, this isolate could potentially be a useful probiotic strain for African catfish feeding or for other related formulation in aquaculture feeding.

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