Impact of Lactic acid bacteria (LAB) as probiotic against bacterial pathogen from Cyprinus carpio L.

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Abstract

The aim of the present study is to investigate the potential of lactic acid bacteria as a probiotic (biological dieases control). Six strains of lactic acid bacteria J1, J3, J4, J6, J7, J8 were isolated from intestines of the common carp Cyprinus carpio were compared as to the antagonistic activity against fish pathogen bacteria Aeromonas hydrophila, Pseudomonas luteola, Pseudomonas aeroginosa, Serratia rubidaeae and staph sp. Different method were used for measuring growth by optical density, agar well diffusion and cross-streak method also screened from bile salt and antibiotic tolerance. All strains of LAB showed different spectra of inhibition against pathogenic bacteria. The highest inhibition measured against Serratia rubidaeae and A. hydrophila, moderate inhibition occure against P. luteola and P. aeroginosa and lower inhibition to staph sp. Also culture supernatant free fluid of all LAB showed no antagonistic activity against pathogenic bacteria moreover all strain show resistance to all antibiotic sensitivity OA2A–P disc except J1, J3, J4 were sensitive to erythromycin in concentrate 60 mcg after incubation for 48 hr and only J1, J3, J4, J6 were exhibited tolerance reaction to bile salt at concentration not more than 3000 ppm the present study recommend to use lactic acid bacteria J6 as a good probiotic remedy to the fish culture in Iraq.

Abstract

تأثير بكتيريا حامض اللاكتيك LAB كمعززات حيوية ضد البكتريا المرضية في Cyprinus carpio L.

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الخلاصة

هدف الدراسة الحالية إلى اختيار قعدة بكتيريا حامض اللاكتيك كمعززات حيوية في السيطرة البيولوجية ضد البكتيريا المرضية. قُورنت ستة عزلات من بكتيريا حامض اللاكتيك J1, J3, J6, J4, J7, J8 فيما بينها والمعزولة من أمعاء إسمال الكارب الشائع (Cyprinus carpio) في فعاليتها التضادية ضد البكتيريا المرضية للأسماك Aeromonas Serratia rubidaeae, Pseudomonas aeroginosa, Pseudomonas luteola,hydrophila cross واذا القالفه معيار النمو باستعمال تقنية سيراميك agar well diffusion والانتشار بواسطة الانتشار بحفر الاركار O.D طريقة النمو التضادي التعاكسى– (Streak method)– stataع عن مقاومة البكتيريا البلببية للمضادات الحيوية والإملاح الصفاراء. أظهرت جميع عزلات البكتيريا البلببية مستويات تثبيط مضادات مختلفة ضد البكتيريا المرضية فكان أعلى تثبيط هو ضد بكتيريا Aeromonas hydrophil و Pseudomonas Serratia rubidaeae. أما التثبيط المتوسط فقد ظهر ضد بكتيريا Pseudomonas luteola و Pseudomonas aeroginosa宽度

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Various authors have shown that lactic acid bacteria are a part of the normal intestinal flora of fish (1). Most of the evidence comes from salmonid species like Arctic charr (Salvelinus alpinus) and Atlantic salmon (Salmo salar). (2) described the presence of lactic acid bacteria, including Lactobacillus in the intestines of various fish species at larval, fry and fingerling stages inhabiting ponds. However, it was discussed that some human activities like artificial feeding in ponds would have had an effect on the bacteria composition and load in some fish like carp (Cyprinus carpio) which showed the highest content of lactic acid bacteria in the intestines Intensive aqua farming accompanies several disease problems often due to opportunistic pathogens as evident from general aquaculture. High stocking densities, high food inputs and other organic loads stimulate the selection and proliferation of opportunistic bacteria (4). Due to this negative balance of the microbial community in rearing water as well as in fish gut, the aqua culturists often face mass mortality of their stocks. The use of antibiotics and chemotherapy remains the method of choice as disease control strategy. The abuse of chemotherapeutics, especially antibiotics has resulted in development of multiple antibiotic resistant bacteria (5) Increased concern about antibiotic resistant microorganisms has led to several alternatives including the use of non-pathogenic microorganisms as probiotic (6). Probiotic concept has been widely applied for health promoting in farm animals, pets and aquatic animals (7, 8). Probiotics are usually defined as live microbial feed supplements which beneficially affects on the host animal by improving its intestinal microbial balance (9). Based on this definition, probiotics may include microbial adjuncts that prevent pathogens from proliferating in the intestinal tract (10). Lactic acid bacteria (LAB) are among the most important probiotic microorganism typically associated with gastrointestinal tract whereas they exercise beneficial effects. (11) suggested that probiotic bacteria would be found to be useful not only as food but also as biological controllers of fish disease and activators of nutrient regeneration. In the biological control in aquaculture emerge and since then the research effort has continually increased Bacillus sp. in often antagonistic against other fresh water fish pathogenic bacteria (12,13,14) was reported to the similar experiments have shown that the inoculation of some probiotic strains, mainly lactic acid bacteria, increase fish survival after being challenged with fish pathogens. (15) Showed inhibition of Vibrio. vulnificus by LAB and stimulation of the non-specific immune response resulting in resistance to disease in the prawn fed on LAB incorporated diets. Selection of probiotic strains is achieved by screening procedures for several characteristics in vitro, such as inhibitory activities against several fish pathogens and gastric and intestinal secretions (16). In the present study we compared the antimicrobial activity of probiotic bacteria (LAB) J1, J3, J4, J6, J7, J8 which isolated in previous study by (17) from gastrointestinal tract of common carp fish Cyprinus carpio against pathogenic bacteria using different methods such as growth monitored by measuring the optical density (O.D), cross streaking and well diffusion method.

Introduction
Materials and Methods

- **Chemicals and media:** Analytical grade chemicals and dyes were obtained from Al-Kindi company for production of veterinary vaccines and drugs, bacteriological media were obtained from oxoide UK which include set of biochemical media, blood agar base, Nutrient agar, Nutrient broth tryptic soy agar, gas generating Kit and sense test disk from lamb GT. Manchester; England. MRS media were obtained from Himedia, and bile salt were supplied by sigma.

- **Bacterial strains:** bacterial strains includes:

1. *Pseudomonas aeruginosa* were obtained from central health laboratory/ Baghdad ministry of health.

2. *Aeromonas hydrophila, Staph sp. Serratia rubidaeae, Pseudomonas luteola* were obtained from the laboratory of fish pathology veterinary college of Baghdad University. All bacterial strains were cultivated in 10 ml of Nutrient broth. Bacterial cultivation was performed at 30°C for 20 h. Approximately 1 ml of bacterial culture was transferred to 9 ml of liquid medium and incubated at 30°C for another 18 h, cell concentration was then adjusted to obtain final concentration of 10^6 CFU/ml for determination of antibacterial activity.

3. lactic acid bacteria J1, J3, J4, J6, J7, J8 isolated from gastrointestinal tract of common carp fish in previous study by (17). Before use LAB strains were activated in MRS broth (18).

- **Bile Tolerance:** The modified method of (19) was used to determine bile tolerance of selected LAB. Before testing for bile tolerance, LAB strains were grown at 30°C for 24 hour in MRS broth without bile. One ml of the culture broth was poured on to MRS agar with bile salt concentrations of 2000, 3000 and 4000 ppm. Bacterial growth was determined after incubation at 30°C for 48 hour.

- **Bacteriocin assay:** The isolates of lactic acid bacteria were propagated in MRS broth and incubated at 30°C for 48 hours. Cells were separated by centrifugation at 5000 rpm for 10 minutes. The clear supernatants obtained were treated as follows:

1. Clear free fluid supernatants without any treatment (CFF).

2. Nutrelizing clear free fluid supernatants by adjusted to pH 6.5-7.0 with 2N NaOH (NCFF). Cell free supernatant was passed through 0.22μm membrane filter and evaluated for antimicrobial activity by agar well diffusion method (20).

   The antagonistic effects of the culture supernatants of bacteriocin producing *Lactobacillus* were tested on various indicator organisms on Nutrient agar. All cultures were grown aerobically at 30°C for 48 hours. Inhibition zones around the wells were measured.

- **Inhibitory effect by agar well diffusion method:** The inhibitory effects of *Lactobacillus* strains on indicator organisms were carried by agar well diffusion assay. Petri dishes with nutrient agar that were previously inoculated with 0.1 ml of 24 hours old nutrient broth culture of individual test bacteria were poured. Once solidified, Petri dishes were stored for 2 hours at 4°C. Four wells of 5 mm diameter were made and filled with 50μl of culture supernatant. The inoculated plates were kept at 4°C for 2 hours and then incubated at 30°C for 24 hours. Inhibition zones around the wells were measured (20).

- **Inhibitory effect by O.D. measuring growth method:** According to (21) Five ml replicates of nutrient broth were individually supplemented with 2 ml of each of the
individual LAB strain supernatants. The each tubes were then inoculated with freshly grown culture of each indicator pathogenic bacteria respectively and incubated at 28 ± 2°C for 48 hr and growth was recorded by measuring the optical density at 540 nm. Control tubes comprised of nutrient broth inoculated with indicator pathogenic bacteria respectively

- **Inhibitory effect by cross – streak method:** All the three LAB strains were streaked on Tryptone Soya Agar (TSA) plates containing 1.0% sodium chloride and incubated at 28 ± 2°C for 48 hr. Freshly grown culture of five pathogen bacteria was streaked perpendicular to this growth and after incubation at 28 ± 2°C observed for antagonism according to (15).

- **Antibiogram of LAB isolates:** The isolates were inoculated into MRS broth individually and incubated for 24 hr about 25 ml of MRS agar was seeded with the cultures of LAB isolates10⁶ CFU/ml mixed well. Poured in to sterile petriplates and stored at 4 c° for 1hr to solidify the media (OA21-P) antibiotics in a single ring were placed up side down pressed on the top of the agar plates and kept again at 4 c° for 1hr the plate were incubated at 30 C° for 24 hr and 48 hr resistance was defined as the absence of a growth inhibition zone around the discs.

**Results and Discussion**

- **Bile salt tolerance:** Six strain of LAB were isolated from gastrointestinal tract of common carp fish (cyprinus carpio) were tested for their ability to grow at bile sult of 2000, 3000, 4000 ppm in order to bile tolerant strains. Only J1, J3, J4, J6 strain were able to grow in MRS agar supplemented with 2000 and 3000 ppm bile salt while all strains were sensitive to grow in MRS ager supplemented with 4000 ppm bile salt (Table 1).

This is similar to the result obtained by (22) with the strains of Pediococcus acidilactici (P2), Lactobacillus curvatus (RM 10) and Lactobacillus sake (L2) were the most resistant to 3000 ppm bile salt at pH 6 (23) reported that the bile salt tolerance of the Lactobacillus strains were able to grow in MRS agar supplemented with 3000 ppm bile salt. It has been reported that certain strains of Lactobacillus are able to reduce this detergent effect by their ability to hydrolyze bile salt by bile salt hydrolase enzyme (BSH) (22), which are then readily excreted from the GI-tract (24). This particular enzyme decreases bile solubility and thus weakening its detergent effect.

**Table (1) Bile salt tolerance of lactic acid bacteria isolated from common carp fish**

<table>
<thead>
<tr>
<th>Bile Salt Concentration (ppm)</th>
<th>Lactic acid bacteria strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>J1</td>
</tr>
<tr>
<td>2000</td>
<td>+</td>
</tr>
<tr>
<td>3000</td>
<td>+</td>
</tr>
<tr>
<td>4000</td>
<td>-</td>
</tr>
</tbody>
</table>

(+) tolerance     (-) sensitive

- **Antibacterial activity against fish pathogens:** The antagonism of the six LAB strain J1, J3, J4, J6, J7, J8 was ascertained by as test tube well as by well diffusion and cross–streaking on TSA plates. Inhibitory effect by O. D. test tube nutrient broth containing cell free fluid supernatants (cff) of the six strains of LAB respectively failed to record turbidity after inoculating with the pathogen (Aeromonas hydrophila, Pseudomonas luteola, Pseudomonas leutela, Serratia rubidaeae , Staph .sp.) and incubating for 30 h
implying inhibition (Fig. 1, 2, 3, 4, 5) Turbidity measured as optical density was obtained in all the control tubes while tubes containing the LAB supernatants recorded low optical densities of the value of zero time. The culture media (supernatant) were used which showed highest inhibition effects of Bacillus sp. may be due to production of antibiotics bacteriocins, Lysozymes, proteases and hydrogen peroxide and the alternative of pH values by the production of organic acid (25).

Fig. (1) Optical density of Aeromonas hydrophila (in test tube) in vitro containing six strains of LAB

Fig. (2) Optical density of Pseudomonas luteola (in test tube) in vitro containing six strains of LAB
Fig. (3) Optical density of *Pseudomonas aeruginosa* (in test tube) *in vitro* containing six strains of LAB

Fig. (4) Optical density of *Serratia rubidaeae* (in test tube) *in vitro* containing six strains of LAB
Inhibitory effect by well diffusion and cross-streak method: Six strains of LAB were assayed for the ability to inhibit growth of (Aeromonas hydrophila, Pseudomonas luteola, Pseudomonas aeruginosa, Serratia rubidaeae, Staph .sp.) by agar well diffusion method culture supernatants free fluid (cff) of LAB strains J1, J3, J4, J6, J7, J8 exhibited varying degree of inhibition activity against indicator (pathogen microorganism).

All LAB strain were exhibited highest antibacterial activity against Serratia rubidaeae Aeromonas hydrophila respectively. Similar result were reported by (26) they showed the activity against A. hydrophila of 19 LAB strains including Carnobacterium piscicola and Lactobacillus planatarum. The addition of freeze-dried Carnobacterium, divergens to compound feed did not improve the resistance of salmon fry challenged against pathogenic A. hydrophila (27). However, a similar dietary addition reduced the mortality rate of Atlantic cod fry when challenged against Vibrio anguillarum (28). (29) found The inhibition zone 8 mm was observed on the plates inoculated with Aeromonas hydrophila by Bacillus sp. The moderate antimicrobial activity were against Pseudomonas luteola.

Table (2) Diameter of inhibition zone (mm) caused by antimicrobial activity of LAB strains against pathogen microorganisms

<table>
<thead>
<tr>
<th>LAB strain</th>
<th>Aeromonas hydrophila</th>
<th>Pseudomonas luteola</th>
<th>Pseudomonas aeruginosa</th>
<th>Serratia rubidaeae</th>
<th>Staph .sp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFF</td>
<td>CFBH</td>
<td>CFF</td>
<td>CFBH</td>
<td>CFF</td>
</tr>
<tr>
<td>J1</td>
<td>20 mm</td>
<td>nil</td>
<td>12 mm</td>
<td>nil</td>
<td>10 mm</td>
</tr>
<tr>
<td>J3</td>
<td>12 mm</td>
<td>nil</td>
<td>10 mm</td>
<td>nil</td>
<td>9 mm</td>
</tr>
<tr>
<td>J4</td>
<td>20 mm</td>
<td>nil</td>
<td>13 mm</td>
<td>nil</td>
<td>11 mm</td>
</tr>
<tr>
<td>J6</td>
<td>15 mm</td>
<td>nil</td>
<td>10 mm</td>
<td>nil</td>
<td>9 mm</td>
</tr>
<tr>
<td>J7</td>
<td>8 mm</td>
<td>nil</td>
<td>7 mm</td>
<td>nil</td>
<td>6 mm</td>
</tr>
<tr>
<td>J8</td>
<td>10 mm</td>
<td>nil</td>
<td>9 mm</td>
<td>nil</td>
<td>8 mm</td>
</tr>
<tr>
<td>Chloramphenicol disc 30 mcg</td>
<td>25 mm</td>
<td>18 mm</td>
<td>16 mm</td>
<td>30 mm</td>
<td>nil</td>
</tr>
</tbody>
</table>

Note: CFF = culture supernatant, NCCF = culture supernatant adjusted to pH 6.5-7.0 with 1M NaOH.
Which, *Pseudomonas aeruginosa* spoil food at low temperatures as a result of its lipolytic and proteolytic activity (30). Control of *P. aeruginosa* by bacteriocin activity of (31) has been reported that *P. aeruginosa* controlled by bacteriocin activity of *L. casei* and *L. plantarum*. The low antimicrobial activity was against *Staph. sp*. There are many reports about the antimicrobial activity of LAB most are against Gram positive bacteria (32) was reported to the isolated *Lactobacillus* sp., both homofermenters and heterofermenters, were able to inhibit the human and fish pathogens by acid production when using a high glucose concentration. A few strains also inhibited both gram-positive and gram-negative fish and human pathogens with low (0.2%) concentration of glucose in the medium. Inhibitory activities of these strains have been usually detected against related species such as *Staphylococcus aureus, Clostridium* and other fish pathogenic bacteria (33).

![Image of inhibition zone](image.png)

**Fig. (6) Diameter of inhibition zone (mm) caused by antimicrobial activity of LAB strains against pathogen microorganisms**

Neutralized culture supernatants (CFBH) of all strains exhibited no inhibition (Table 2, Fig. 6). The inhibitory effect of LAB may be due to acid or the bacitracin-like substances or combination of both (34). (35) have revealed that no correlation was found between bacitracin activity, lactic acid and hydrogen peroxide production. They reported that *Lactobacillus* strains 228, 345 and 431 produced H2O2 but did not demonstrate any inhibitory effect. Similar results were obtained (36) showed all strains of LAB may produce H2O2 but did not show any inhibitory effect.

- **Antibiogram of LAB activity:** Behavior of all LAB strains J1, J3, J4, J6, J7 and J8 were resistance to all antibiotic sensitivity OA2A-p disc except the J1, J3, J4 were sensitive to erythromycin in concentration 60 mcg after incubation for 48 hr (Table 3).
Table (3) Antibiogram of LAB isolates determined by antibiotic sensitivity
OA2A – PDisc

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Concentration (mg)</th>
<th>J1</th>
<th>J3</th>
<th>J4</th>
<th>J6</th>
<th>J7</th>
<th>J8</th>
<th>J1</th>
<th>J3</th>
<th>J4</th>
<th>J6</th>
<th>J7</th>
<th>J8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin</td>
<td>60 mcg</td>
<td>R</td>
<td>+</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>15 mcg</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Colistinsulphide</td>
<td>150 mcg</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Penicillin</td>
<td>2 unit</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>1000 mcg</td>
<td>R</td>
<td>+</td>
<td>+</td>
<td>R</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>R</td>
<td>+</td>
<td>R</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>5 mcg</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
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</table>

R resistance
+ zone inhibition diameter > 1 cm
++ zone inhibition diameter > 2 cm
+++ zone inhibition diameter > 3 cm

Beside the production of antimicrobial substances, bile salt tolerance and antibiotic resistant the great variety of mechanisms have been proposed for the action of probiotics. Competition for adhesion receptors in the intestine, competition for nutrients and immune stimulation. Further investigations on these lines would throw more light into the actual mechanism of probiotic action in aquaculture. It was concluded that lactic acid bacteria do offer ample scope as probiotics showing antagonism towards pathogenic bacteria. As these probions were able to suppress pathogen growth in vitro and in vivo, it can be hypothesized that they have ability to colonize the gastrointestinal tract of prawn, which however, merits further confirmation. Consequently, they may prove to be suitable candidates for oral administration to farm fish, in commercial ventures to improve health and protect them against infection.

References


