Mol2Net-04, **2018**, BIOCHEMPHYS-01 (pages 1- x, *type of paper, doi: xxx-xxxx*
http://sciforum.net/conference/mol2net-4

SciForum
Mol2Net-04

Evaluation of bacteria of aquatic origin as fish probiotic in vitro

Mouna JLIDI [1,*], Asmahen Akremi[1], Adel HAJ BRAHIM[1], Houda HMANI[1], Lobna DAOUD[1], Manel BEN ALI[12], Nabil SOUISSI[3] , Samir BEJAR[1] and Mamdouh BEN ALI[12]

[1]Laboratory of Microbial Biotechnology and Engineering Enzymes (LBMIE), Center of Biotechnology of Sfax (CBS), University of Sfax, Road of Sidi Mansour km 6, PO Box 1177 Sfax 3018, Tunisia asmahen.akremi@gmail.com (Asmahen Akremi); adelhadjibrahim@gmail.com (Adel HAJ BRAHIM); houda_enis@yahoo.fr (Houda HMANI); lobna.daoudm@gmail.com (Lobna DAOUD) ; samir.bejar@cbs.rnrt.tn (Samir BEJAR);

[2]Astrum Biotech, Business incubator, Center of Biotechnology of Sfax (CBS), University of Sfax, Road of Sidi Mansour km 6, PO Box 1177 Sfax 3018, Tunisia manel.benali@gmail.com (Manel BEN ALI); mamdouh.benali@cbs.rnrt.tn (Mamdouh BEN ALI);

[3] Laboratoire de Biodiversité Marine, Institut National des Sciences et Technologies de la Mer, Centre de Sfax. Avenue Madagascar BP 1035-3018 Sfax Tunisia nabil.suissi@gmail.com (Nabil SOUISS)

***** Corresponding Author: E-Mail: jlidimanno@gmail.com
Tel.: +21621656587 Fax:

Received: / Accepted: / Published:

Abstract: The rapid development of aquaculture, its intensification, and the occurrence of fish health problems on farms push to develop alternative methods to antibiotics and chemotherapy for controlling fish diseases. Probiotics may provide a potential alternative method to protect fish from opportunistic and pathologic bacteria and promote a balanced environment. In this work, we have assessed the *in vitro* probiotic properties of twenty one bacteria from aquatic and fish origin, for their application in aquaculture. Selection was based on their antimicrobial activity (Bacteriocin) against fish pathogens and their *in vitro* safety assessment. This includes the evaluation of their haemolytic, proteolytic and mucinolytic activities, bile salt deconjugation ability and antibiotic susceptibility. Twelve of the twenty one bacteria isolated from several showed strong antibacterial activity against several pathogenic species such as *Lactococcus garvieae*, *Vibrio anguillarum*, *Vibrio harveyi*, *Aeromonas hydrophila* and *Aeromonas salmonicida*, and were taxonomically identified by partial 16S rDNA gene sequencing. The cell-free culture supernatants from cultures of these twelve strains were treated with proteinase K (10 mg/ml; 37ºC, 1h) and submitted to heat treatment (100ºC, 10 min), which showed that eleven strains exert extracellular antimicrobial activity against fish pathogens due to the production and secretion of thermo-stable antimicrobial peptides (i.e., Bacteriocins). The tested strains showed a great heterogeneity respect to their safety and antibiotic susceptibility.

Keywords: Aquaculture, pathogens, probiotics, screening bacteriocins.

1. Introduction

Aquaculture has become an increasingly industry which offers a high quality of animal protein and a significant economic importance. According to the Food and Agriculture Organisation (FAO) of the United Nations report, global aquaculture production has grown from 31.1% in 2004 to 44.1% of the total production of 73.8 million tonnes of fish produced in 2014 [1]. The major constraint being encountered in aquaculture is the outbreaks of infections-bacterial disease that results in the tremendous economic losses to the fish farming industry, many factors, including ambient stress, disease, and deterioration of environmental conditions; increase the risk of fish disease [2, 3]. Antibiotics and chemotherapeutics have been used as traditional methods to combat bacteria diseases. However they have discouraged due to their potential negative consequences, such as drug resistance, drug residue and environment pollution [4, 5]. Hence, it is important to search for environmentally friendly treatments; the application of probiotics has emerged as a promising alternative [6, 7] for controlling and combating the growth of pathogenic bacteria without any side effects [8, 9]. Probiotics are defined as live microorganisms that confer a beneficial effect on the host when administered in adequate amount [10, 11].

Probiotics are applied for enhancing the immune response and stress tolerance, as well as improving feed digestion and upgrading water quality by degrading or absorbing the waste. Probiotic beneficial actions include also antagonism to pathogens by secreting of inhibitory substance such as bacteriocins, enzymes, hydrogen peroxide and organic acids as well as colonization or adhesion properties, etc [6, 13, 14, 15].

In this present study, we have assessed the *in vitro* probiotic properties of twenty one bacteria from aquatic and fish origin, for their application in aquaculture. Selection was based on their antimicrobial activity (Bacteriocin) against fish pathogens and their *in vitro* safety assessment. This includes the evaluation of their haemolytic, proteolytic and mucinolytic activities, bile salt deconjugation ability and antibiotic susceptibility.

2. Results and Discussion

A total of 21 bacteria were isolated and purified from different biotopes (sea water and gut fish and shrimp). All the strains are a facultative anaerobe, motile, and have Gram-positive rods. They were positive in oxidase activity and catalase activity. Table 1 and fig 1 represents the zone of inhibition (in mm) against selected pathogenic bacteria directly with cross streak test. Isolated strains have shown heterogeneity activity at gram+ and gram– pathogenic bacteria. Antimicrobial effect was assayed using the agar diffusion test to filtered cell-free culture at Table 2. 12 strains were found that inhibit the growth of at least one of the 8 aquaculture pathogens tested. Awide range of inhibitory spectrum was found. The strongest inhibition was found in strains S7, which showed antimicrobial effect against majority of indicators studied. To have an idea about the natures of the activity (proteinase k, heat treatment. acidity of supernatant) has been shown at table 3. Eleven strains exert extracellular antimicrobial activity against fish pathogens due to production of bacteriocins .Nine of them produce thermoresistant bacteriocins and the rest produce thermolabils bacteriocins

The majority of the isolates have proteolysis activities on LB agar medium with 1% (*w/v*) skim milk. Only the isolates S7 have no haemolysis and mucin activities shown in Table 4. 11 strains were evaluated for its resistance to a panel of antibiotics, including those highlighted by the European Food Safety Authority (EFSA 2012) .The antibiotic resistance profile of selected strains are listed in Table 5 which indicates that all strains are sensitive to all selected antibiotics as suggested by EFSA.

Followed by BLAST analysis, the partial 16S rRNA gene sequence of 12 strains showed 99.9% similarity to various members of the Bacillus genus .such as. *B. subtilis*, *B pumilus* and *B. amyloliquefaciens*…To confirm the result, we use the mass spectrometry MALDI-TOF which identify microorganisms by using protein at IRYCIS (Instituto Ramón y Cajal deInvestigación Sanitaria).

We found finaly the strains (P42, C210, Cm, CJ3, Sp, S15) are *B.subtilus.* While (P32, CD6, C60, Z2, cont ,S7)are *B.pumilus.*

.

Table 1.direct antibacterial activity

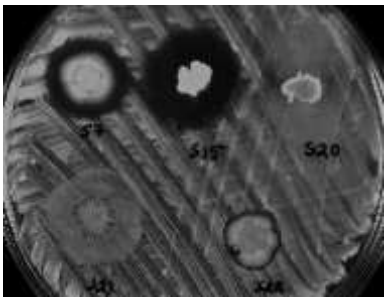
Diameter of the inhibition zone															
Indicators	AH	AS	YR	CM	LG1	LG2	PP	PD	VF	VS	VH 1	VH 2	VP	VV	VA
P42	+	++	-	++	++	++	+++	+++	++++	-	++++	++	+++	+++	++++
P32	+	++	+	-	-	-	+++	++	+++	-	+	+	-	+	++++
C210	-	++	-	-	++	-	++	++	+++	-	++	-	-	+++	++++
C211	-	++	-	-	+	-	++	++	+++	-	+++	-	+	+++	++++
Cm	++	-	-		+	-	+++	++	+++	-	++	++	+	++	++++
Cont	-	-	-	-	+	-	+++	++	+++	-	+++	++	++	+++	++++
C60	-	-	-	-	++	-	+++	+	+++	-	+	+		+	+++
CD6	+	++	-	+	++	-	+++	+++	+++	-	+++	++	+++	+++	++++
CJ3	-	-	-	-	++	-	++	+	+++	-	-	+	-	+	++++
III	+	++	-	-	+	-	+++	+	+++	-	++	++	++	++	++++
Z1	-	++	-	-	+	-	+++	+	+++	-	+	+	++	++	++++
Z2	+	++	-	-	++	-	+++	++	++	-	++	++	-	++	+++
Sp	++	++	-	-	+	-	+++	++	+++	-	++	+	++	++	+++
HB1	+	+	++	-	+	-	-	-	+	-	-	+	+	-	+++
HB2	+	++	+	-	+	-	+++	++	++	-	+	+	-	++	+++
S7	++	+	++	-	+	-	++	++	++	-	-	+	++	++	+++
S15	+	++	-	+	++	++	+++	++	+++	-	++	++	++	++	+++
S17	+	+	-	-	++	-	+++	++	+++	-	+	+	++	+	+++
S20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++
S21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Symbols for the diameter of the inhibition zone '-'=zone inhibition <5 mm;'+'= zone inhibition between 5 and 10 mm; '++'= zone inhibition 11-15mm; '+++'= zone inhibition 16-24mm ; '++++'= zone inhibition≥25mm .

AH, *Aeromonas hydrophila* LMG 5734; AS, *A. salmonicida* LMG *894*; YR, *Yersinia ruckeri* LMG3279; LG1, *Lactococus garvieae* JIP29-99; LG2, *L. garvieae* CECT 5807; PP, *Photobacterum damselea damselea* ; PD, *P. damselea piscicida*; VF, *Vibrio fischerie*; VS, *V. spendidus;*VH1, *V.harveyi* Lg 48/01; *VH2, V.harveyi* Lg 26/01; VP, *V.parahemolitus* ; VV, *V. vulnificus*; VA, *V. anguillarum*;

Figure 1. Antibacterial activity against: (a) *V.harveyi* Lg 26/01, (b) *A. salmonicida* LMG *894and (c) Vibrio anguillarum*

a **b** **c**



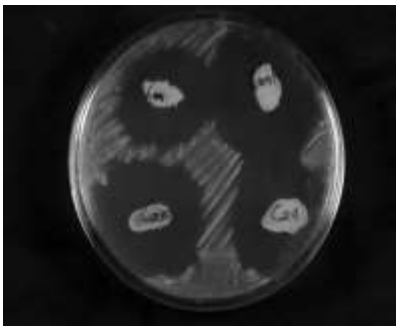


Table2.direct antibacterial activity

Indicators	AH	AS	YR	LG1	LG2	VH	VA	PD
P42	-	-	-	++	+	+	+++	++
P32	-	-	-	+	+	-	+++	-
C210	-	-	-	+	++	-	+++	-
Cm	-	-	-	+	+	-	+++	-
CD6	-	-	-	++	-	-	+++	+
C60	-	-	-	-	-	-	++	-
CJ3	-	-	-	-	-	-	++	-
Z2	-	-	-	+	++	-	+++	-
Sp	-	-	-	-	-	-	-	-
Cont	-	-	-	+	++	-	+++	-
S7	-	-	+	++	++	+	+++	++
S15	-	-	-	++	-	-	+++	++

Symbols for the diameter of the inhibition zone '-'=zone inhibition <5 mm;'+'= zone inhibition between 5 and 10 mm; '++'= zone inhibition 11-15mm; '+++'= zone inhibition 16-24mm ; '++++'= zone inhibition≥25mm .

AH, *Aeromonas hydrophila* LMG 5734; AS, *A. salmonicida* LMG *894*; YR, *Yersinia ruckeri* LMG3279; LG1, *Lactococus garvieae* JIP29-99; LG2, *L. garvieae* CECT 5807; *VH2*, *V.harveyi* Lg 26/01; PD, *Pedicoccus damnosus* GECT 4768;

Table 3.Nature of antibacterial activity against *L. garvieae* CECT 5807

Strains	SB	SC	SHT	SpH	CPK	SPK
P42	+	+++	++	+	++	+
P32	+	+++	+++	+	++	+
C210	+	++	++	+	++	-
Cm	+	++	++	+	++	+
CD6	++	+++	+	++	++	+
C60	-	+	+	-	+	-
CJ3	-	+	+	-	+	-
Z2	+	++	++	+	++	-
Sp	-	+	-	-	-	-
Cont	+	++	++	+	+	-
S7	++	+++	+	++	++	++
S15	++	+++	+	+	+++	++

SB: supernatant without any treatment , **SC**: supernatant concentrated 10 times ,**SpH**: pH neutralization treatment, **SHT**: heat treatment at 100 ° C to concentrated ssupernatant, **Cpk**: control proteolytic treatment, **Spk**: proteolytic enzyme treatment to concentrated supernatant

'-'=zone inhibition <5 mm;'+'= zone inhibition between 5 and 10 mm;

'++'= zone inhibition 11-15mm; '+++'= zone inhibition 16-24mm ; '++++'= zone inhibition≥25mm

Table 4: Extracellular enzymes production for safety assessment test in vitro

Strains	Proteolytic activity	Hemolytic activity	Mucinolytic activity
P42	+	α	-
P32	+	β	-
CD6	+	α	-
C60	-	β	-
C210	+	β	-
Cm	+	β	+
CJ3	-	β	-
Z2	+	β	+
Sp	+	β (weak)	-
Cont	+	β	-
S7	+	-	-
S15	+	α	-

Table 5: Antibiotics susceptibility test.

Antibiotics	Cut-off values (mg/l)	strains											
		P42	P32	C210	Cm	CD6	C60	CJ3	Z2	Sp	Cont	S7	S15
Ampicillin	n.r	S	S	S	S	S	S	S	S	S	S	S	S
Vancomycin	4	S	S	S	S	S	S	S	S	S	S	S	S
Gentamycin	4	S	S	S	S	S	S	S	S	S	S	S	S
kanamycin	8	S	S	S	S	S	S	S	S	S	S	S	S
Streptomycin	8	S	S	S	S	S	S	S	S	S	S	S	S
Erythromycin	4	S	S	S	S	S	S	S	S	S	S	S	S
Clindamycin	4	S	S	S	S	S	S	S	S	S	S	S	S
Tetracycline	8	S	S	S	S	S	S	S	S	S	S	S	S
chlromphenicol	8	S	S	S	S	S	S	S	S	S	S	S	S

n.r'=not required; 'S'= sensitive; 'R'= resistante

3. Materials and Methods

Bacteria used at this study were originally isolated from different biotopes: the sea water and the intestines of fish and shrimp (*Sparus aurata,Sardina pilchardus* and *Panaeus kerathurus*) of Sfax, Tunisia between May 2014 and avril 2016. All the strains were cultured in TSA (Oxoid) supplemented with NaCl (1.5%, w/v; Panreac Quimica S.A.U, Barcelona, Spain) at 28°C. The antimicrobial activity of putative probiotic bacteria was examined against indicator fish pathogens by colony overlay method [16, 17]**.**The extracellular antimicrobial activity of cell-free supernatants was determined using an agar well diffusion test (ADT) [18].

The isolated strains which presented antagonistic activity were characterized by determining of

colony morphology, motility, gram staining and the production of catalase and oxidase.

The haemolytic activity was screened as follows: *Bacillus sp.* strains previously cultured in TSB with 1.5%NaCl were streaked on horse blood agar plates (BioMérieux, Marcy l'Etoile, France) .After incubation at 28°C for 1-2 days. The presence of clear zones of hydrolysis around the colonies revealed β-hemolysin [19].

To degrade gastric mucin, we used from mucin porcine stomach type III (Sigma-Aldrich) was and agar incorporated into medium B without glucose at concentrations of 0.5 and 1.5% (w/v), respectively. Briefly, 10 µL of cultures grown in TSB broth with 1.5% salt was spotted into the surface of medium B with mucin. The plates were anaerobically (AnaeroGen, Oxoid) incubated at 30ºC for 72 h. After incubation, the plates were stained with a mixture of 0.1% (w/v) amido black (Merck KGaA, Darmstadt, Germany) in 3.5 mol/L acetic acid for 30 min and then washed with 1.2 mol/L acetic acid (Merck KGaA). The presence of a discolored zone around the colony was considered as a positive result. A fresh fecal slurry of a healthy adult cow was used as positive control.

Bacterial DNA was extracted using the InstaGene Matrix resin (Bio-Red Laboratories Inc: Hercules CA USA).Strains were taxonomically identified by DNA sequencing of the PCR amplified genes encoding the 16S rRNA subunit(16S rDNA

. Strains were cultured in medium (pH=7.0) containing % w/v- peptone0.5,yeast extract 0.3, skim milk 2.5 ml and agar 1.6 were prepared and allowed to cool.

Single colonies of each strain were spotted. The plates were kept in 28 °C for 24 h. the protease production was observed in terms of clear zone[20].

The minimum inhibitory concentrations (MICs) of the strains to 10 antibiotics were determined by a broth microdilution test [21] The tested antibiotics were ampicillin (0.12–8 µg/mL), vancomycin(0.5–32µg/mL), gentamicin (2–128 µg/mL), kanamycin (4–256 µg/mL), streptomycin (4–256 µg/mL), erythromycin (0.12–8 µg/mL), clindamycin (0.25–16 µg/mL), tetracycline (0.5–32 µg/mL)and chloramphenicol (1–64µg/mL). Individual colonies were suspended in a sterile glass tube containing 5 mL salinesolution (0.85% NaCl) to a turbidity of one in the McFarland Scale (*ca.*, 3 × 108 cfu/mL) and subsequently 1,000-fold diluted in LSM broth, consisting of Iso-Sensitest (IST) (Oxoid) and MRS broth (IST/MRS, 9:1; pH 6.7). A volume of 50 μ L of the diluted suspensions was added to each microplate well containing 50 μ L volume of LSM broth with the respective antibiotic concentration. The plates were sealed with a transparent cover tape, and after incubation at 37 ºC for 18 h, MICs were established as the lowest antibiotic concentration inhibiting bacterial growth, and interpreted according to the breakpoints adopted by the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) in relation to the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance [22]. Strains showing MICs higher than the respective breakpoint were considered as resistant. *Enterococcus faecalis* CECT795 and *Staphylococcus aureus* CECT794 were used for quality control.

4. Conclusions

Twelve of twenty one strains from several biotopes (P42, P32, C210, Cm, CD6, C60, CJ3, Z2, Sp, Cont, S7 and S15) showed a strong antibacterial activity against fish pathogenic species. Moreover eleven strains exert extracellular antimicrobial activity against fish pathogens due to the production of bacteriocins. All selected strains are sensitives to antibiotics but Only S7 showed the safety assessment (absence hemolysis and Mucin activities).

Given their antimicrobial (Bacteriocin) activity against fish pathogens and their safety, *Bacillus pumilus* S7 may be considered as potential fish probiotics, and their effectiveness will be further tested in vivo.

Acknowledgments
This study was supported by a grant from the Ministry of Science and Technology (MOST) 106-2313-B-020-007-, Taiwan.
The authors would like to thank Miguel A. Morinigo at the department of microbiology , faculty of science University of Malaga and Luis Miguel Cintas at the faculty of veterinary university of Completense Madrid ,Spain, for providing the optimal cultural the pathogenic strains .

Conflicts of Interest

State any potential conflicts of interest here or "The authors declare no conflict of interest".

References and Notes

1. FAO the State of World Fisheries and Aquaculture 2016. Contributing to Food Security and Nutrition for All, FAO,2016; http:// www.fao.org/3/a-i5555e.pdf
2. BentzonTilia, M; Sonnenschein, E.C ; Gram, L. Monitoring and managing microbes in aquaculture towards a sustainable industry. Microb. Biotechnol, 2016;576–584
3. Wang, Y.B; Li, J.R; Lin, J; Probiotics in aquaculture: challenges and outlook. Aquaculture, 2008; 281, 1–4.
4. Alcaide, E; Blasco, M.D; Esteve, C.Occurrence of drug-resistant bacteria in two European eel farms. Appl. Environ. Microbiol.2005; 71, 3348–3350.
5. Sarter, S; Kha Nguyen, H.N; Hung, L.T; Lazard, J; Montet, D. Antibiotic resistance in gram-negative bacteria isolated from farmed catfish. Food Control, 2007; 18, 1391–1396.
6. Balcázar, J.L; de Blas, I; Ruiz-Zarzuela, I; Cunningham, D; Vendrell, D; Múzquiz, J.L. The role of probiotics in aquaculture. Vet. Microbiol, 2006a; 114, 173–186.
7. Balcázar, J.L ; Decamp, O; Vendrell, D ; de Blas, I ; Ruiz-Zarzuela, I. Health and nutritional properties of probiotics in fish and shellfish. Microb. Ecol. Health Dis, 2006b; 18,65–70.
8. Rinkinen, M; Jalava, K; Westermarck, E; Salminen, S; Ouwehand, AC.Interaction between probiotic lactic acid bacteria and canine enteric pathogens: a risk factor for intestinal Enterococcus faecium colonization. Vet. Microbiol, 2003; 92, 111–119.
9. Magnadottir, B. Immunological control of fish diseases. Mar. Biotechnol, 2010; 12,361–379.
10. Pineiro, M; Stanton, C. Probiotic bacteria: Legislative framework requirements to evidence basis. J. Nutr, 2007; 137, 850S–853S.
11. Food and Agriculture Organization (FAO)/World Health Organization (WHO). Guidelines for the Evaluation of Probiotics in Food; Food and Agriculture Organization of the United Nations and World Health Organization Working Group Report; Food and Agriculture Organization: Rome, Italy, 2002.
12. Verschuere, L; Rombaut, G; Sorgeloos, P; Verstraete,W. Probiotic bacteria as biological control agents in aquaculture. Microbiol. Mol. Biol. Rev, **2000**; 64, 655–671.
13. Cruz, P.M; Ibáñez, A.L; Hermosillo, O.AM; Saad, HCR. Use of probiotics in aquaculture. ISRN Microbiol, 2012; 84-91.
14. Huynh, T.G; Shiu, Y.L; Nguyen, TP; Truong, QP; Chen, JC; Liu, CH. Current applications, selection, and possible mechanisms of actions of synbiotics in improving the growth and health status in aquaculture: A review. Fish Shellfish Immunol, 2017; 64, 367–382.
15. Akhter, N; Wu, B; Memon, A.M; Mohsin, M. Probiotics and prebiotics associated with aquaculture: a review. Fish Shellfish Immunol, 2015; 45, 733–741.
16. Barbosa, TM; Serra, CR; La Ragione, RM; Woodward, MJ; Henriques, AO. Screening for Bacillus Isolates in the Broiler Gastrointestinal Tract.Applied and Environmental Microbiology, 2005; 71,968-978.
17. Seghouani, H; Garcia-Rangel, CE; Füller, J;Gauthier, J; Derome, N .Walleye Autochthonous Bacteria as Promising Probiotic Candidates against *Flavobacterium columnare*. Frontiers Microbiology, 2017; 8, 1349. doi: 10.3389/fmicb.2017.01349.
18. Cintas, L. M;Rodríguez, JM; Fernández, MF; Sletten, K.; Nes, IF; Hernández, PE; Holo, H. Isolation and characterization of pediocin L50, a new bacteriocin from Pediococcus acidilactici with a broad inhibitory spectrum. Appl. Environ. Microbiol, 1995; 61:2643–8

19. Araújo, C;Muñoz-Atienza, E; Nahuelquín, Y; Poeta, P; Igrejas, G; Hernández, PE; Herranz, C; Cintas, LM. Inhibition of fish pathogens by the microbiota from rainbow trout (Oncorhynchus mykiss, Walbaum) and rearing environment. Anaerobe, 2014, 32, 7–14.
20. Aarti, C; Khusro, A; Varghese, R; Arasu, MV; Agastian, P; AbdullahAl-Dhabi, N; Ilavenil, S;Choi, Kc. In vitro studies on probiotic and antioxidant properties of Lactobacillus brevis strain LAP2 isolated from Hentak, a fermented fish product of North-East India. J food science and technology, 2017; 86, 438-446
21. Klare, I; Konstabel, C;Müller-Bertling, S; Reissbrodt, R; Huys, G; Vancanneyt, M; Swings, J; Goossens, H; Witte, W. Evaluation of new broth media for microdilution antibiotic susceptibility testing of Lactobacilli, Pediococci, Lactococci, and Bifidobacteria. Appl. Environ. Microbiol, 2005; 71, 8982–6.
22. EFSA.Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA J, 2012; 10: 2740–2749.