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Evaluation of partial characteristics of the strain *Enterococcus faecalis* P3 isolated from peacock feces *in vitro*

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Abstract

In this study, the potential probiotic properties such as NaCl tolerance, acid tolerance, simulated gastro-intestinal juice tolerance, adhesion ability to Hep-2 cells, antibiotic susceptibility and antimicrobial activity against selected pathogens, of the strain *E. faecalis* P3 identified in our group were evaluated *in vitro*. The results showed that *E. faecalis* P3 had tolerance to NaCl. The viability was kept higher than 8 log CFU/mL at 2-5 % concentration of NaCl during 24 h incubation. *E. faecalis* P3 grew well in acid condition (pH 1.8-6.2) for 24 h incubation. The viable numbers decreased with the increase of incubation time in simulated gastro-intestinal juices. The viable numbers were kept higher than 10 log CFU/mL in simulated gastric juice (SGJ) pH 2.5 after 2 h incubation. Furthermore, *E. faecalis* P3 was able to adhere to Hep-2 cells. The results of antibiotic susceptibility indicated *E. faecalis* P3 was sensitive to most of the clinically important antibiotics. *E. faecalis* P3 had good inhibition ability on *Staphylococcus aureus*. In conclusion, *E. faecalis* P3 appeared to be a good candidate for use as a probiotic agent in food or feed industry.

Keywords: Adhesion ability; antibiotic susceptibility; *E. faecalis*; tolerance.

1. Introduction

According to the currently adopted definition by FAO/WHO 2001, probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host". Many reports have indicated that probiotics can compete with, and suppress the growth of undesirable micro organisms in the colon and small intestine, and thus help to stabilize the digestive system (Pan *et al*., 2009; Guo *et al*., 2009; Ayeni *et al*., 2011). Other benefits of probiotics include prevention of intestinal infections, anti-tumor activities, and improvement of lactose utilization in human gut (Kailaspathy & Rybka, 1997; Guo *et al*., 2009). In order for a strain to be designed as a probiotic, it should have tolerance to acid, bile and enzymes in the gastro-intestinal tract, and also have the ability to adhere to intestinal surfaces (Piano *et al*., 2006; Pan *et al*., 2009; Guo *et al*., 2009). It has been demonstrated (Pan *et al.*, 2009) that survival ability and colonization in the gastro-intestinal tract are the main preconditions for probiotics to provide any beneficial effects after consumption.

In this paper, the partial characteristics such as NaCl tolerance, acid tolerance, simulated gastro-intestinal juice tolerance, adhesion ability, antibiotic susceptibility and antimicrobial activity against selected pathogens, of the strain *Enterococcus faecalis* P3 (*E. faecalis* P3) identified in our group were studied. It is the first report on the characteristics of *E. faecalis* isolated from peacock feces. All results obtained in this study will build a basis for its application in food or feed industry.

2. Materials and methods

2.1. The bacterial strain

The (*Enterococcus faecalis* P3) strain used in this study was isolated from fresh peacock fecal samples (Hangzhou Normal University, Zhejiang, China). Pure culture of *E. faecalis* P3 was stored at -80 °C in de Man Rogosa Sharp (MRS) broth (Oxoid) supplemented with 50 % glycerol.

2.2. NaCl tolerance of *E. faecalis* P3

The tolerance ability of *E. faecalis* P3 to NaCl was evaluated in MRS broth supplemented with NaCl at different concentrations (0.9, 2.0, 3.0, 5.0, 8.0 %). The initial bacterial suspension concentration for this study was approximately 10.0~11.0 log CFU/mL. The tolerance

ability of *E. faecalis* P3 was evaluated by measuring the growth at 37 °C after 24 h incubation. Each sample was serially diluted 10 times with saline solution and 100 µl aliquots were plated on MRS agar. Colonies were enumerated after incubation at 37 °C for 24 h.

2.3. Acid tolerance of *E. faecalis* P3

The ability of *E. faecalis* P3 to grow at different pH was evaluated in acidified MRS broth (pH 1.0, 2.0, 2.5, 3.0, 4.0, 5.0, 6.2). The initial bacterial suspension concentration for this study was approximately 10.0~11.0 log CFU/mL. pH tolerance of *E. faecalis* P3 was evaluated by measuring the growth at 37 °C after 24 h incubation. Afterwards, each sample was serially diluted 10 times with saline solution and 100 µl aliquots were plated on MRS agar. Colonies were enumerated after incubation at 37 °C for 24 h.

2.4. Tolerance of *E. faecalis* P3 to simulated gastric juice

The simulated gastric juice (SGJ) used was composed of saline solution supplemented with pepsin (5.0 mg/mL). The saline solution was adjusted to pH 2.5 with HCl, and sterilized by autoclaving at 121 °C for 15 min. Each cell suspension was added to the simulated gastric juice pH 2.5. The initial bacterial suspension concentration for this study was approximately 10.0~11.0 log CFU/mL. After 1, 2, 3, 4, 5, 6 h of incubation, 1.0 mL sample taken from each solution was serially diluted with sterile saline solution. Appropriate dilutions were spread-plated on MRS agar and incubated at 37 °C for 24 h.

2.5. Tolerance of *E. faecalis* P3 to simulated intestinal juice

The simulated intestinal juices (SIJ) were prepared from saline solution with 10 mg/mL pancreatin (Sigma-Aldrich, Shanghai, China). Each cell suspension was added to the simulated intestinal juices pH 7.0. The initial bacterial suspension concentration for this study was approximately 10.0~11.0 log CFU/mL. After 1, 2, 3, 4, 5, 6 h of incubation, 1.0 mL of each solution was serially diluted with sterile saline solution. Appropriate dilutions were spread-plated on MRS agar and incubated at 37 °C for 24 h.

2.6. Inhibitory activity assay

The antimicrobial activity of *E. faecalis* P3 was studied using the agar diffusion test. Briefly, *E. faecalis* P3 was grown overnight in MRS broth at 37 °C. The culture was centrifuged and the supernatant was obtained. Indicator bacteria (*Staphylococcus aureus*, Type B *Salmonella parathyphi* and *Bacillus subtilis*) obtained from our laboratory, was spread onto the soft Luria-Bertani (LB) plate, and then 3 mm-diameter wells were punched into the surface using a sterile borer. Subsequently, 50 µl of the supernatant of *E. faecalis* P3 culture was added into each well on the plate and incubated at 37 °C for 20 h. The antibacterial activity was recorded as the inhibition zones around the well.

2.7. Adhesion capacity of *E. faecalis* P3

Small intestine epithelial cell lines Hep-2 of one-month-old chicken (Zhejiang University of Technology, Zhejiang, China) were used for this experiment. Hep-2 were grown in Dulbecco's modified eagles medium (DMEM) (Sigma-Aldrich, Shanghai, China) supplemented with 10 % heat inactivated foetal bovine serum, 100 U/mL Penicillin and 100 U/mL Streptomycin. The cell lines were maintained at 37 °C in 10 % CO_2 environment. The culture medium was replaced every 24 h. Mono-layers of Hep-2 cell lines were seeded at a concentration 4 $\times 10^4$ cells/mL after 15 days incubation.

The adhesion experiment was performed in a six-well tissue culture plate containing 15-day-old mono-layers of Hep-2 cells in each well. Before starting the adhesion trial, Hep-2 cells were washed two times with phosphate-buffered saline (PBS, pH 7.4). 2.0 mL of non-supplemented DMEM and 1.0 mL *E. faecalis* P3 solution were added into each well. The plates were incubated at 37 °C in 10 % CO_2 environment for 1 h. Then the mono-layers were washed five times with PBS to remove all non-adhered bacterial cells. The adhesion ability of *E. faecalis* P3 was quantified using the gram staining method (Sogaard *et al.*, 2007).

2.8. Antibiotic susceptibility of *E. faecalis* P3

Antibiotic susceptibility of *E. faecalis* P3 was examined by antibiotic disc assay. 100 µl of the strain culture was plated onto MRS agar. Antibiotic discs were impregnated onto the surface of MRS agar. The tested antibiotics were following: tetracycline, gentamycin, kanamycin, neomycin, chloromycetin, erythromycin, polymyxin B, streptomycin, ciprofloxacin, midecamycin, cafazolin, rifampicin, penicillin and ofloxacin. Antibiotics concentrations were in accordance with the recommendations of National Committee for Clinical Laboratory Standards (Lin *et al.*, 2007). Plates were incubated at 37 °C under anaerobic conditions for 24 h. Antibiotic susceptibility of *E. faecalis*

P3 was assessed by measuring the diameter of inhibition zones around the discs. Susceptibility was expressed in terms of resistant (R), moderately susceptible (MS) and susceptible (S). Sensitive strains showed inhibition zones that were larger than 15 mm in diameter. Inhibition zone of resistant strains was less than 10 mm. Moderate susceptible strains had inhibition zone in diameter between 10 and 15 mm.

2.9. Statistical analysis

All the experiments were repeated at least three times, and the results were presented as mean values ± standard deviation (SD). Statistical analysis was performed using Origin 8.0 for Windows. Student's t test was used to compare the significant differences among values. Statistical significance was defined at $p < 0.05$.

3. Results and discussion

3.1. NaCl tolerance

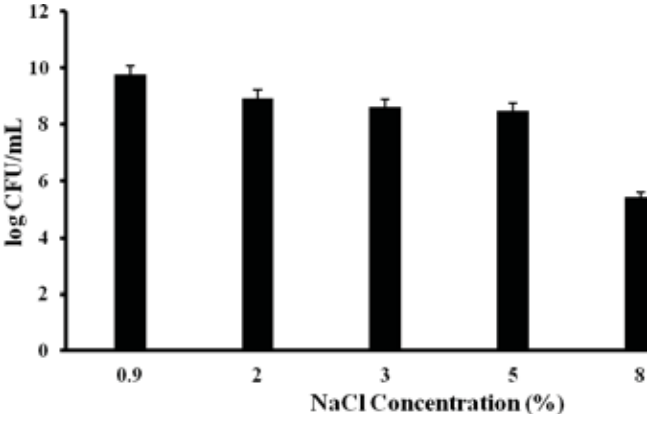

Fig. 1. NaCl tolerance of *E. faecalis* P3

The viability of *E. faecalis* P3 decreased significantly from 10.38 log CFU/mL at 0.9 % NaCl to 8.50 log CFU/mL at 5.0 % NaCl. The viability of *E. faecalis* P3 remained constant after increasing NaCl concentration from 2 to 5 %. The viability of *E. faecalis* P3 decreased to 6.29 log CFU/mL at 8 % NaCl (Figure 1). *E. faecalis* P3 in our present study showed good tolerant to NaCl. Many *enterococci* from processed foods with the tolerance to salinity environments have been reported (Giraffa, 2003; Jurkovic *et al*., 2006; Gome *et al*., 2008). Some other genera of lactic acid bacteria (LAB) also showed NaCl tolerance. Rui-Moyano *et al*. (2008) studied the effect of NaCl concentration on the viability of isolated LAB from dry Iberian fermented sausages. Under 4.0 % concentration of NaCl, some 57.5 % of strains isolated from M17 agar were able to grow adequately. Ayeni *et al*. (2011) found that strain *Weissellla confusa* 8 obtained from Nigerian traditional fermented dairy food was the most tolerant organism against NaCl. In MRS broth supplemented with 6.5 % NaCl, strain *W. confusa* 8 could grow well.

3.2. Acid tolerance

The survival ability of probiotics under acid environments is an important requirement. The stability of *E. faecalis* P3 under different pH conditions for 24 h incubation was examined (Figure 2). The viability of *E. faecalis* P3 decreased with the decrease of pH. The viable numbers of *E. faecalis* P3 decreased significantly ($p<0.05$) from 11.41 log CFU/mL at pH 6.2 to 9.57 log CFU/mL at pH 5.0 and 5.77 log CFU/mL at pH 4.0, respectively. Afterwards, the viability of *E. faecalis* P3 decreased with the decrease of pH. The viable *E. faecalis* P3 could be found even after 24 h incubation under low pH (pH 1.8 and 2.0). Therefore, *E. faecalis* P3 showed good acid tolerance. These results were in accordance with the work of Guerra *et al*. (2007). Generally, the acid tolerance varies greatly depending on the species and strains. Pan *et al*. (2009) investigated the acid tolerance of *Lactobacillus acidophilus* NIT. The results showed that the survival percentage was greater at pH 3 than that at pH 2 during the whole incubation period. The number of viable bacteria decreased with the increase of incubation time. The acid tolerance of probiotics has been linked to the induction of H^+-ATPase activity (Matsumoto *et al*., 2004; Ventura *et al*., 2004; Guo *et al*., 2009). In the present study, it was observed that *E. faecalis* P3 had the resistance ability to acidic conditions.

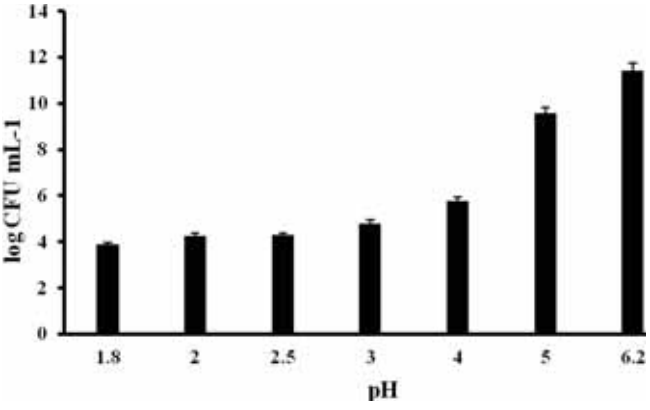

Fig. 2. Stability of *E. faecalis* P3 under different pH

3.3. SGJ tolerance

Low pH of the gastric juice can cause destruction of most of ingested probiotics and hence is one of the major challenges faced by probiotic cultures upon oral

administration. In this sense, the resistance to SGJ is an important selection criterion for probiotics. The viability of *E. faecalis* P3 decreased progressively in SGJ pH 2.5 under incubation time (Figure 3), but could survive at levels higher than 10 log CFU/mL after 2 h incubation. The SGJ tolerance of probiotics also depends on the species and strains. Neuno-Palop & Narbad (2011) investigated the survival of *E. faecalis* CP 58 in SGJ pH 3.0. The results showed that 42 % survival rate could be obtained in SGJ pH 3.0 after 90 min incubation. Bhardwaj *et al*. (2010) reported that the viable numbers of the tested strain *E. faecium* KH 24 were 7 log CFU/mL at SGJ pH 2.0 after 2 h incubation, but were completely destroyed at SGJ pH 1.0 after 2 h incubation. Thus, *E. faecalis* P3 had good SGJ tolerance.

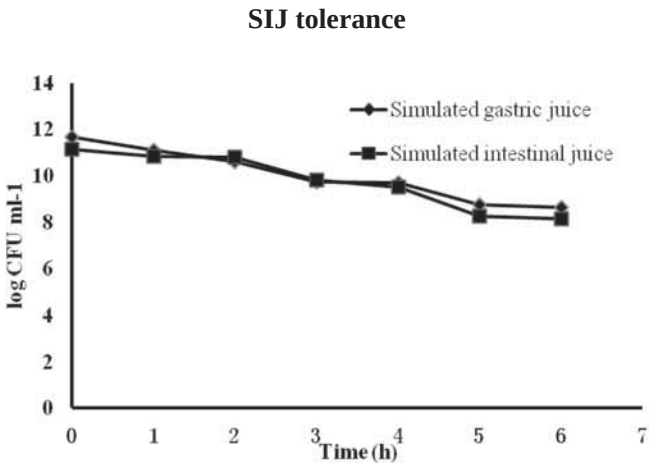

Fig. 3. Tolerance of *E. faecalis* P3 to simulated gastric juice (SGJ) pH 2.5 and simulated intestinal juice (SIJ)

The viability of *E. faecalis* P3 also decreased in SIJ with the increase of incubation time. The viability of *E. faecalis* P3 was reduced from the initial 11.14 log CFU/mL to 10.80 log CFU/mL after 2 h incubation. Viable numbers of higher than 8.0 log CFU/mL could be kept after 6 h incubation (Figure 3). Many studies with similar results of ours have been reported. Tan *et al*. (2013) found around 2 log CFU/mL of *E. faceium* YF5 was lose in SIJ pH 6.0 after 90 min incubation. Bao *et al*. (2010) found the addition of SIJ caused a further reduction in the viability of the tested strain from 8.36 log CFU/mL for 3 h incubation to 7.63 log CFU/mL for 6 h incubation. The SIJ tolerance also depends on the species and strains. Guo *et al*. (2009) found the viability of *L. acidophilus* NCFM (99.6 %), *L. rhamnosus* GG (99.2 %), *L. casei* Zhang (97.4 %) and *L. casei* Shirota (97.6 %) was kept after 4 h incubation in SIJ.

3.4. Antimicrobial activity

Anti-pathogen activity is one of important properties for probiotics. *E. faecalis* P3 showed the varying degrees of the inhibition ability against pathogen strains. *E. faecalis* P3 had higher inhibition ability to *Staphylococcus aureus*, when compared to the other pathogens (results not shown). The antimicrobial activity of LAB may be due to many factors such as the production of H_2O_2, organic acids and bacteriocin (Lin *et al*., 2007; Silvia & Nakaia, 2003; Kirtzalidou *et al*., 2011). In fact, the drop of pH arising from the production of lactic acid can be enough to inhibit the growth of certain strains. This is because the non-dissociated form of lactic acid triggers a lowering of the internal pH of the cell that causes a collapse in the electrochemical proton gradient in sensitive bacteria, hence having a bacteriostatic or bactericidal effect (Bhatia *et al*., 1989; Lin *et al*., 2007). Barrow *et al*. (1980) pointed out that the growth of most micro-organisms could be inhibited, when pH of the medium was below 4.5. In our study, the pH of the culture decreased to 4.3 after 24 h incubation. Linaje *et al*. (2004) showed that all the tested *enterococci* had inhibitory activity towards *Clostridium*, *Escherichia*, *Listeria*, and *Staphyloccoccus*, due to bacteriocins produced by these *enterococci*. The antibacterial mechanism of *E. faecalis* P3 *in vitro* needs to be studied in detail.

3.5. Adhesion ability

Adhesion ability to the intestinal mucosa is a prerequisite for the colonization of probiotics. Probiotics must adhere to the mucus layer to avoid being removed from the colon's peristalsis (Ripamonti *et al*., 2011; Guo *et al*., 2010; Tsai *et al*., 2008). Different intestinal cell lines (Ht-29, Caco-2, INT-407 and IPEC-J2) have been used as *in vitro* models to assess the adhesive properties of probiotics (Marcinakova *et al*., 2010; Ripamonti *et al*., 2011; Rebucci *et al*., 2007). Adhesion ability of probiotics (such as *Lactobacillus* and *Bifidobacerium*) can be measured by the gram staining (Tan *et al*., 2013). In our study, *E. faecalis* P3 was able to adhere to Hep-2 well (Figure 4). This result was in agreement with the observation of the adhesion ability of *E. faecalis* EE4 to human mucus (Strompfova *et al*., 2004). Cebrain *et al*. (2012) found that *E. faecalis* UGRA10 showed the adhesion ability of HeLa 229 cells with 21.65 % efficiency. Generally, the adhesion ability is not dependent on the bacterial species but is fairly strain specific (Klingberg *et al*., 2005). Laukova *et al*. (2004) examined the adhesion ability of enterococci from different ecosystems, and found that the isolates did

not prefer binding to mucus from the same source as from where they were isolated. Marcomalpva *et al.* (2010) and Rinkinen *et al*. (2003) also reported that the mucus adhesion properties of probiotics were more dependent on the type of probiotics than on the type of the host.

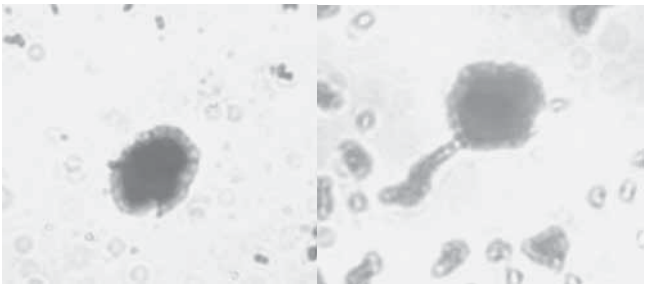

Fig. 4. Photographs showing adhesion ability of *E. faecalis* P3 to Hep-2 cells

3.6. Antibiotics tolerance

It is extremely important to know the resistance ability to antibiotics in probiotics potentially used for food and/or therapeutic applications. The antibiotics susceptibility profiles of *E. faecalis* P3 were shown in Table 1. The strain was susceptible to penicillin, chloromycetin, ciprofloxacin, cafazolin, rifampicin, neomycin, erythromycin, kanamycin, ofloxacin, midecamycin and tetracycline, and was resistant to polymyxin B. This strain also showed moderate susceptibility to streptomycin and gentamicin. Generally, the antibiotic tolerance is dependent on bacterial species (Abriouel *et al*., 2008; Nueno-Palop & Narbad, 2011; Hussain & Ashfaq, 2009). Nueno-Palop & Narbad (2011) found that the susceptibility of *E. faecalis* CP58 to tetracycline, rifampicin and erythromycin, and was resistant to kanamycin and chloramphenicol. Cebrian *et al*. (2012) found that *E. faecalis* UGRA 10 was susceptible to most of the clinically relevant antibiotics, although it was resistant to low levels of gentamicin, tobramycin, amikacin and clindamicin. Earlier, *E. faecium* KH 24 was resistant against amikacin, cefuroxime and cephalothin (Gupta & Malik, 2007; Bhardwaj *et al*., 2010). In this study, it is desirable that *E. faecalis* P3 was sensitive to most studied antibiotics, thereby showing low risk of having antibiotic resistance gene.

Table 1. Antibiotic susceptibility profiles of *E. faecalis* P3

Antibiotics	Inhibition diameter zone (mm)	Sensitivity
Polymyxin B	0.0	R
Tetracycline	39	S
Midecamycin	25	S
Ofloxacin	30	S
Kanamycin	13	S
Erythromicin	17	S
Neomycin	20	S
Rifampicin	16	S
Gentamycin	10	MR
Cafazolin	23	S
Ciprofloxacin	21	S
Streptomycin	10	MR
Chloromycetin	20	S
Penicillin	17	S

4. Conclusion

In this paper, the strain *E. faecalis* P3 isolated from peacock feces had tolerance to NaCl, low pH, simulated gastro-intestinal juice. It was susceptible to most studied antibiotics. It also could be adherent to Hep-2 cells well and exhibited antimicrobial activity against selected pathogens. *E. faecalis* P3 with the promising probiotic properties is a good candidate for further investigation to elucidate its potential health benefits and its application in food or feed industry.

5. Acknowledgement

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2016R423075) and Hangzhou Science and Technology Development Plan (20150432B03).

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تقييم جزئي في المختبر لخصائص *E. faecalis* P3 من سلالة المكورات المعوية المعزولة من البراز الطاووس

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خلاصة

في هذه الدراسة قمنا بتقييم مختبري للخصائص المحتملة لسلالة *E. faecalis* P3 المؤيدة للحياة (بروبيوتيك) مثل القدرة على تحمل كلوريد الصوديوم وتحمل الحموضة و تحمل محاكاة عصائر المعدة والأمعاء والقدرة على التصاق خلايا Hep-2 و الحساسية للمضادات الحيوية والنشاط المضاد للميكروبات لبعض مسببات الأمراض. أظهرت النتائج أن *faecalis* P3 لها القدرة على تحمل كلوريد الصوديوم حيث استمرت قابليتها على الحياة والنمو عند درجة اعلى من CFU/mL 8 log عند تركيز 5-2 % من كلوريد الصوديوم خلال 24 ساعة من الحضانة. نمت *E. faecalis* P3 جيدا في وسط حمضي (درجة الحموضة 1.8-6.2) خلال مدة 24 ساعة حضانة. تناقصت أعداد الخلايا الحية مع زيادة فترة الحضانة في الوسط المحاكي للعصائر الهضمية. بينما بقيت اعداد الخلايا الحية أعلى من CFU/mL 10 log في وسط محاكاة عصير المعدة عند الرقم الهيدروجيني 2.5 بعد 2 ساعة حضانة. وعلاوة على ذلك، كانت *E. faecalis* P3 قادرة على الاتصاق لخلايا Hep-2. وأشارت نتائج الحساسية للمضادات الحيوية ان *E. faecalis* P3 حساسة لمعظم المضادات الحيوية السريرية الهامة. كان ل *E. faecalis* P3 قدرة تثبيط جيدة على المكورات من نوع Staphylococcus aureus . وفي الختام، يبدو أن *E. faecalis* P3 تشكل مرشحا جيدا للاستخدام كعامل مؤيد للحياة (بروبيوتيك) في صناعة الأغذية أو الأعلاف.

كلمات البحث: قدرة الالتصاق، الحساسية للمضادات الحيوية، *E. faecalis* P3، القدرة على التحمل.