Emirates Journal of Food and Agriculture. 2021. 33(10): 805-814
doi: 10.9755/ejfa.2021.v33.i10.2771
http://www.ejfa.me/

RESEARCH ARTICLE

Inhibition biofilm formation and cells infection of *Salmonella* and *Staphylococcus* by extracts of potential probiotic strains: Application of mixture design

Najla Haddaji[1,2]*, Abdelkarim Mahdhi[2], Karima Nsibi[2], Nouha Boauli[1], Mouna Ghorbel[1], Olfa Bechambi[1], Nadia Leban[3], Ridha Mzoughi[2], Amina Bakhrouf[2]

[1]Department of Biology, Faculty of Sciences, University of Ha'il, Ha'il, Kingdom of Arabia Saudi, [2]Laboratory of Analysis, Treatment and Valorization of Pollutants of the Environment and Products (LATVPEP). Faculty of Pharmacy, University of Monastir, 5000, Tunisia, [3]Laboratory of Human Genome and Multifactorial diseases. Faculty of Pharmacy, University of Monastir, 5000. Tunisia

ABSTRACT

The mixture design approach, becoming increasingly common in several sciences, was used to find biological control treatment for pathogenic bacteria. The current study examined the effectiveness of a microbial extract consortium in inhibiting and eradicating biofilms as well as cell infections. There were three different combinations of probiotic strain extracts (*Bacillus* (A), *Lactobacillus* (B), and *Candida famata* (C)) used in this study. The levels of biofilm inhibition, eradication, and cell infection of *Salmonella* were improved where the consortiums were 25% (A) and 75% (B); 73% (A) and 27% (C); 66% (B) and 33% (C) respectively. The best effects of probiotic extract combinations on *Staphylococcus* biofilm formation were 27% (A) and 73% (B). However, anti-infective activity was obtained with a combination composed of 50% from each probiotic extract (B and C). Using a mixing design is shown to be an effective strategy for selecting the best combination of components, exploiting extracts under the optimal conditions, and conferring maximum protection against Gram-negative and Gram-positive pathogenic bacteria. Results indicated a positive effect of the different combinations on the ability to form biofilms and infect cells of the tested pathogenic strains.

Keywords: Probiotic; Pathogens; Mixture design; Biofilm; Cell infection

INTRODUCTION

Over the recent years, the emergence of antibiotic-resistant bacteria has continuously increased due to the misuse and overuse of antibiotics for human therapy and livestock production. Thereby, this led to increased antimicrobial resistance in diverse environments (Berendonk et al., 2015; Garbisu et al., 2018). The transmission of antibiotic-resistant bacteria in the environment can increase the prevalence of resistance determinants in the human microbiome (Leonard, Zhang, Balfour, Garside, & Gaze, 2015). However, the emergence of pathogenic bacteria responsible for animal and human infections is considered as a public health problem. In order to combat infectious diseases caused by pathogens, antibiotics must be avoided as well as the spread of antibiotic-resistant bacteria, progressive alternate approaches including probiotics, antibodies, and vaccines have shown promising results in trials that suggest the role of these alternatives as preventive or adjunct therapies (Aslam et al., 2018). Indeed, the use of probiotic was a promising approach to avoid the risk associated to pathogenic bacteria, guarantee a healthy environment and reduce the use of conventional antibiotics. Probiotics are live microorganisms which when are administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2001). The prevention of infections is the most effects studied of probiotics. Previously studies reported that *Lactobacillus casei* prevented *Salmonella* and *Escherichia coli* infections and dissemination to organs (Cano & Perdigón, 2003). The inhibition *in vivo* and *in vitro* conditions of the cellular apoptosis of macrophage infected with *Salmonella* by some probiotic strains has been also documented (Carlos Valdez, Rachid, Gobbato, & Perdigon, 2001; Gobbato, Maldonado Galdeano, & Perdigon, 2008). Probiotics can excrete their beneficial effect on host by producing metabolites which inhibit

***Corresponding author:**
Najla HADDAJI, Department of Biology, Faculty of Sciences, University of Ha'il, Ha'il, Kingdom of Arabia Saudi.
E-mail: najla_haddaji@yahoo.fr

Received: 04 June 2021; **Accepted:** 06 October 2021

the colonization or growth of pathogenic bacteria or by competing with them for resources such as nutrients or space (Balcázar & Rojas-Luna, 2007; Abdelkarim Mahdhi et al., 2012). Probiotic efficacy depends on some factors such as level and frequency of application (Gomez-Gil, Roque, & Turnbull, 2000). By increasing biofilm formation, beneficial bacteria contribute to increased bacterial resistance to heat, acidic environments, antimicrobials, and food preservatives (Xu, LEE, & Ahn, 2011). On the other hand, probiotic have the capacity to prevent cellular invasion by pathogens (Ben Slama, Kouidhi, Zmantar, Chaieb, & Bakhrouf, 2013). *Salmonella* and *staphylococcus* are among pathogenic bacteria frequently found in the animal and human infection diseases. They invade endothelial cells and have the ability to enter and penetrate the intestinal epithelium using a "zipper" mechanism like the use of bacterial surface ligand to engage host cell surface receptors and their adhesive ability to biotic surfaces. It has been demonstrated that probiotic properties differ among strains and depend from the environmental conditions and the nature of strains. Furthermore it is not possible to extrapolate and generalize the effects found for some tested strains to others (Perdigon, Galdeano, Valdez, & Medici, 2002). For this reason, the use of probiotic mixture was promising because it has been revealed that combination of different probiotic strains has benefit effects (Abdelkarim Mahdhi, Bahi, Mzah, & Bakhrouf, 2013). To better study the effect of a mix of probiotic strains, it is important to use the mixture design technique. This procedure is widely used for formulation in the chemical, pharmaceutical and food industries. This method not only estimates the relationship between formulation and performance through regression analysis in shorter experiment times, but also optimizes the component elements according to their target to determine the best ratio of ingredients (Harbi, Chaieb, Jabeur, Mahdouani, & Bakhrouf, 2010; Abdelkarim Mahdhi et al., 2013; Zhou, Liu, Huang, Dong, & Jiang, 2007).

In the present study, a mixture design approach was used to evaluate the effectiveness of three potential probiotic strains extracts to prevent biofilm formation and invasive ability of pathogenic bacteria in the search for effective biological treatments.

MATERIAL AND METHODS

Media, Culture, and bacterial strains

Two potential probiotic bacterial strains: *Lactobacillus plantarum (*Lp1) (Ben Slama et al., 2013), and *Bacillus* sp "HM117834" and yeast strain (*Candida famata*) (A Mahdhi, Hmila, Behi, & Bakhrouf, 2011) were used in this study. Probiotic properties of the tested strains have previously been assessed in the studies mentioned. Antibiotic effects and anti-biofilm activities were observed for these probiotic strains against pathogens. Pathogenic strains were *Staphylococcus aureus* ATCC 25923, and *Salmonella enterica serovar Typhimurium* ATCC 14028.

Strains were cultured at 37°C for 24 hours. Strains of *Bacillus*, *Salmonella*, and *Staphylococcus* were grown on nutrient broth agar (Difco), *Lactobacillus*, on MRS broth (Difco) for 24 hours at 37°C, and yeast, on BYPD medium.

Probiotic extract preparation and treatments

A strain of *Bacillus*, a strain of *Lactobacillus*, and a strain of the yeast *Candida famata* were cultivated separately in falcon tubes (45ml) according to optimal conditions. For each falcon tube, incubation at 37°C for 24 h was followed by centrifugation at 4000 rpm for 20 minutes. The supernatants were recovered and filtered through a sterile filter (0.2m) in a second sterile tube.

Treatments were: 1: 100% (A); 2: 100% (B); 3: 100% (C); 4: 50% (A) + 50% (B); 5: 50% (A) + 50% (C); 6: 50% (B) + 50% (C); 7: 1/3 (A) + 1/3 (B) + 1/3 (C); 8: 4/6 (A) + 1/6 (B) + 1/6 (C); 9: 1/6 (A) + 4/6 (B) + 1/6 (C); 10: 1/6 (A) + 1/6 (B) + 4/6 (C).

Biofilm inhibition assay of Probiotic Extracts

Various mixtures of probiotic extract were tested for their anti-biofilm activity in order to determine the best biofilm inhibition mixture. During the assays, 96-well polystyrene plates with flat bottoms were used. *Salmonella* and *staphylococcus* strains were grown in 5 ml tryptic soy broth supplemented with yeast extract (TSB-YE) at 37C for 24 h. An aliquot of 10 µl (10^6 UFC) was dispensed into each well of 96-well plates (Nunc, Roskilde, Denmark) containing probiotic extract supplemented with 2% glucose (w/v); glucose has previously been shown to play a role in biofilm formation (Haney, Trimble, Cheng, Vallé, & Hancock, 2018). Control wells contained only TSB-YE/ glucose (2% w/v). After that, the plates were incubated at 37°C for 24 hours to allow biofilm to form.

Using the method of Djordjevic et al., the crystal violet test was performed to measure biofilm formation following the incubation period (Djordjevic, Wiedmann, & McLandsborough, 2002). The results were expressed as percentage of biofilm inhibition (BI): $BI = [(OD_{\text{negative control}} - OD_{\text{Experimental}})/OD_{\text{negative control}}] \times 100$.

Biofilm eradication assay of Probiotic Extracts

As described previously, biofilms were allowed to grow for 24 h prior to the addition of the probiotic extract during biofilm eradication assays. A biofilm was formed when the plates were incubated overnight.

The media were removed from each well after 24h of incubation at 37°C and washed three times with sterile phosphate-buffered saline (PBS). Each well was filled with different mixtures of probiotic extracts. The crystal violet assay was performed after the plates had been incubated for 24 hours (Chaieb, Kouidhi, Jrah, Mahdouani, & Bakhrouf, 2011). Biofilm eradication (BE) percentages were calculated from the obtained results:

$$BE = [(OD_{\text{negative control}} - OD_{\text{Experimental}})/OD_{\text{negative control}}] \text{ X } 100$$

Cell culture and infection assay

The used epithelial cells lines were routinely maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a humidified 37°C atmosphere of 5% CO_2. One day prior to infection, 2.10^5 of cell were plated in T75 flasks. On the day of infection, cultured cells in the flask were counted. Bacteria, grown in nutrient broth for 18 h in the presence or absence of probiotic extracts, were collected by centrifugation (12000rpm, 5 min, 25°C), suspended in Dulbecco's phosphate buffered saline (DPBS), and added to the cells. Infection assay was performed at the multiplicity of infection (MOI) of 100:1 by centrifuging bacteria onto cells at 1000 rpm for 10 min. After 2 h of infection, the cells were washed three times with DPBS to remove non-adherent bacteria and then incubated (3 h, 37°C) in the DMEM medium containing 250 mg/ml of gentamicin, rinsed three times with 1 ml of DPBS, lysed with 0.1% Triton X-100 (15 min, 25°C). To determine the number of living intracellular bacteria, 20 µl of the lysate in ten-fold serial dilutions in PBS were added to LB agar plates, and the numbers of bacteria were reported as CFU (colony forming units) (Van Alphen, Burt, Veenendaal, Bleumink-Pluym, & Van Putten, 2012).

Experimental design and methods

To optimize the formulation of the probiotic strains extracts, we used the D-optional method in the mixture design, provided by the software MINITAB® 14. In this study, the proportion of each probiotic extract in the mixed starter is restricted to 0-100 %, in the form of $z_1+z_2+.+z_p = 1$, $0 \leq L_i \leq z_i \leq U_i \leq 1$, i= 1, 2,… p.

Statistical analysis

The effects of different probiotic extracts combinations on biofilm formation inhibition and eradication and invasive capacity of the tested pathogenic strains were analyzed using the MINITAB version 14 software to define the mixture providing optimal biofilm inhibition, eradication and cell infection. The statistical significance f, the ratio of the mean square variation due to regression and mean square residual error was tested using analysis of variance (ANOVA).

RESULTS

Model establishment

Through linear regression fitting, the regression models of the responses (Biofilm inhibition, biofilm eradication and cell infection) were established (Table 1).

The regression model equations are as follows:

For Staphylococcus:

$$Y_{\text{Biofilm inhibition}} = 6.48A + 63.27B + 56.11C + 118.88\,AB +$$

Table 1. Analysis of Variance for Salmonella Biofilm inhibition (I), Biofilm eradication (II) and Cell infection (III) (component proportions)

I. Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	1103.28	1103.279	220.656	2.02	0.258
Linear	2	579.44	877.080	438.540	4.01	0.111
Quadratic	3	523.84	523.843	174.614	1.60	0.323
Residual Error	4	437.55	437.552	109.388		
Total	9	1540.83				
II. Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**P**
Regression	5	307.668	307.668	61.534	2.02	0.257
Linear	2	272.756	229.828	114.914	3.78	0.120
Quadratic	3	34.911	34.911	11.637	0.38	0.772
Residual Error	4	121.648	121.648	30.412		
Total	9	429.315				
II. Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**P**
Regression	5	1.87832	1.878321	0.375664	7.17	0.040
Linear	2	1.04831	0.407028	0.203514	3.88	0.116
Quadratic	3	0.83001	0.830010	0.276670	5.28	0.071
Residual Error	4	0.20972	0.209719	0.052430		
Total	9	2.08804				

Table 2. Analysis of Variance for Staphylococcus Biofilm inhibition (I), Biofilm eradication (II) and Cell infection (III) (component proportions)

I. Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	2936.18	2936.18	587.24	1.56	0.343
Linear	2	1945.97	2104.29	1052.14	2.80	0.173
Quadratic	3	990.22	990.22	330.07	0.88	0.523
Residual Error	4	1501.85	1501.85	375.46		
Total	9	4438.04				
I. Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**P**
Regression	5	274.00	274.005	54.801	0.25	0.918
Linear	2	96.87	50.075	25.037	0.12	0.894
Quadratic	3	177.13	177.135	59.045	0.27	0.843
Residual Error	4	868.53	868.532	217.133		
Total	9	1142.54				
I. Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**P**
Regression	5	8.3616	8.36155	1.67231	1.50	0.358
Linear	2	0.7543	0.27238	0.13619	0.12	0.888
Quadratic	3	7.6072	7.60724	2.53575	2.28	0.222
Residual Error	4	4.4561	4.45609	1.11402		
Total	9	12.8176				

DF: Degrees of freedom; Seq SS: Sum of square; Adj SS: Sum of adjusted squares; Adj MS: Adjusted average squares F: F-ratio; P: P-value.

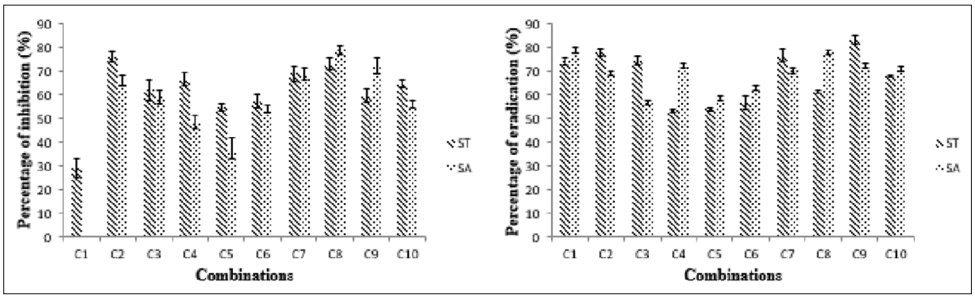

Fig 1: Percentage of biofilm (A) and biofilm cradication (B) of *Salmonella* and *Staphylococcus* after treatment with the different combinations. ST: *Salmonella enterica serovar Typhimurium;* SA: *Staphylococcus aureus.*

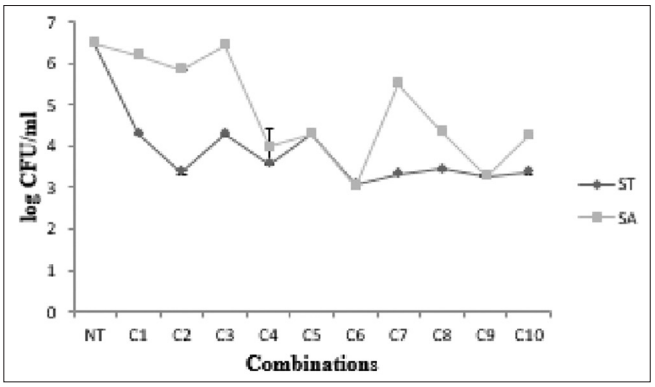

Fig 2: Number of intracellular bacteria after treatment with the different combination. ST: *Salmonella enterica serovar Typhimurium;* SA: *Staphylococcus aureus.*

74.39 AC + 3.41 BC

R^2 = 66.16%; P = 0.34

$$Y_{\text{Biofilm eradication}} = 70.67\ A + 79.80\ B + 72.82\ C + (-33.25)(AB) + (-42.28)(AC) + 25.43(BC)$$

R^2 = 23.98%; P = 0.91

$$Y_{\text{Cell infection}} = 6.01A + 5.58B + 6.28C + (-5.60)(AB) + (-5.05)(AC) + (-9.746.11)\ (BC)$$

R^2 = 65.23%; P = 0.35

For Salmonella

$$Y_{\text{Biofilm inhibition}} = 34.76A + 73.23B + 63.23C + 74.59\ AB + 55.10\ AC + (-41.64\ BC)$$

R^2 = 71.60%; P = 0.25

$$Y_{\text{Biofilm eradication}} = 78.41\ A + 68.62\ B + 58.05\ C + 4.84(AB) + (-14.86)(AC) + 20.40(BC)$$

R^2 = 71.47%; P = 0.26

$$Y_{\text{Cell infection}} = 4.24A + 3.46B + 4.17C + (-2.51)(AB) + (-0.37)(AC) + (-3.1711BC)$$

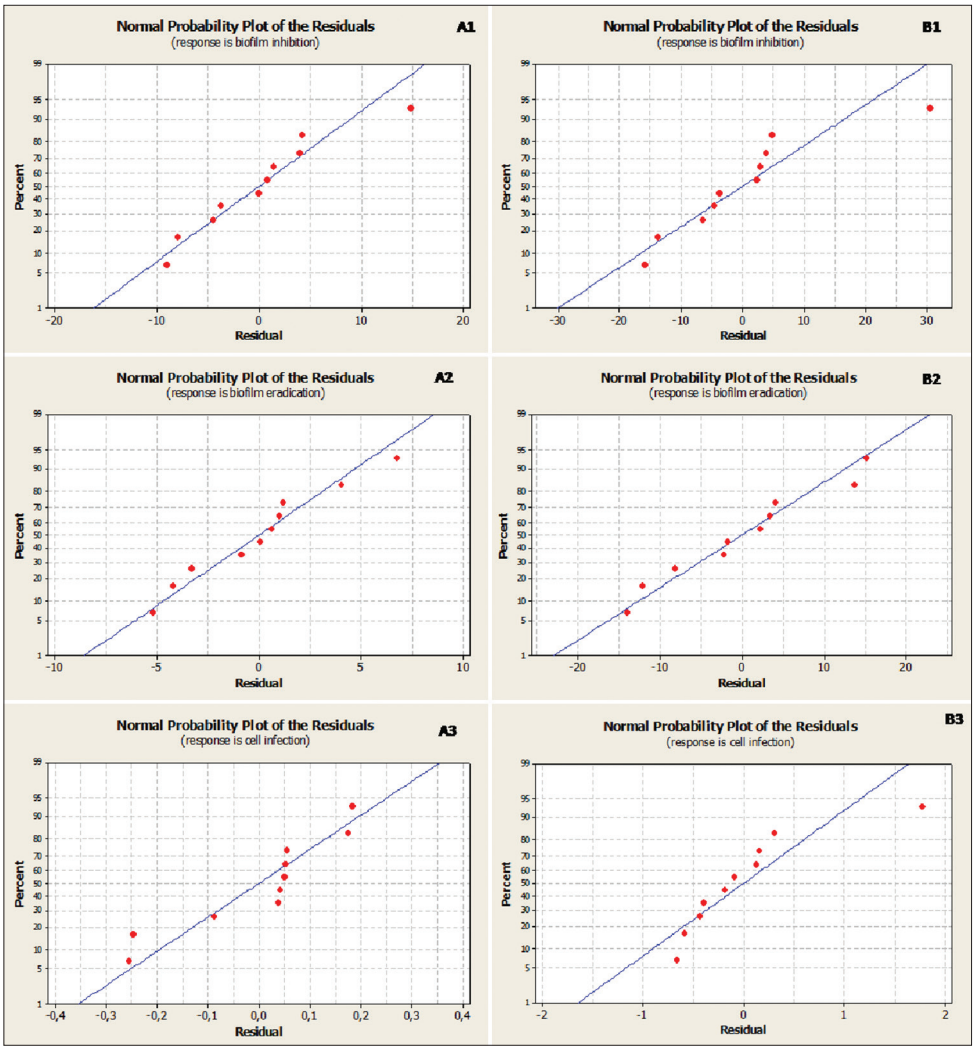

Fig 3: Normal probability plot of the residuals.A1: For biofilm inhibition of Salmonella, A2: For biofilm eradication Salmonella, A3: For cell infection of Salmonella. B1: For biofilm inhibition of *Staphylococcus.* B2: For biofilm eradication *Staphylococcus.* B3: Cell infection of *Staphylococcus.*

R^2 = 89.96%; P = 0.04

Where A: *Bacillus* extract, B: *Lactobacillus* extract and C: *Candida* extract.

According to the ANOVA (Table 1, 2), for example, the regression-fitted average squares for biofilm inhibition rate (1103.28) and the residual error-adjusted average squares (437.55) allowed calculation of the Fisher ratios (F-value) for assessing the statistical significance. The model F-value (2.02 and 7.17 for biofilm eradication and cell infection respectively) implies that most of the variation in the response can be explained by the regression equation.

The adjusted coefficient (R^2) reached 71% for biofilm eradication and 89% for cell infection, indicating that the quadratic models had a good fit with the target ratio formula.

The P-value for the obtained regression for the biofilm inhibition and eradication and cell infection was more than 0.1 and means consequently that at least one of the terms in the regression equation have a significant correlation with the response variable (e.g: P = 0.34 for *Staphylococcus* biofilm inhibition and P = 0.25 for *Salmonella* biofilm inhibition). The ANOVA test also provides a term for residual error, which measures the amount of variation in the response data left unexplained by the model.

Biofilm inhibition and eradication of *Salmonella* and *Staphylococcus*

The studied bacterial strains have been observed to form biofilms on abiotic surfaces. The conducted bioassays results, summarized in the Fig. 1, showed that the different mixtures of probiotic extracts had a significant impact on inhibiting biofilm formation of *Salmonella* and *Staphylococcus*, especially with treatments using combinations C2 and C8 (77.49 and 74.04% for *Salmonella* and 66.15 and 76.26%

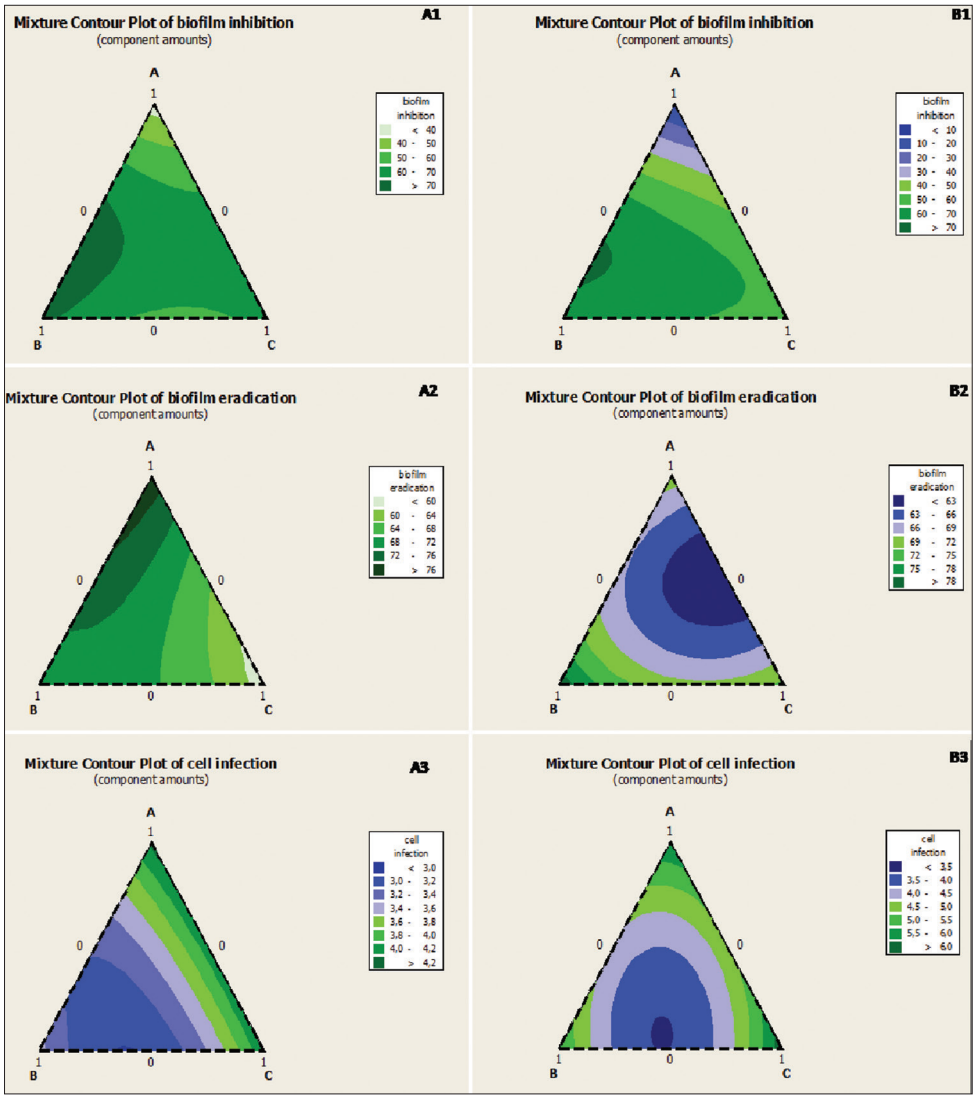

Fig 4: Mixture contour plots between the variables (A: *Bacillus* extract, B: *Lactobacillus* extract and C: *Candida* extract). A1, A2 and A3: *Salmonella* biofilm inhibition, biofilm eradication and cell infection rate responses respcetively. B, B2 and B3: *Staphylococcus* biofilm inhibition, biofilm eradication and cell infection rate responses respcetively.

for *Staphylococcus*). There is a good effect on eradication regardless of tested strain (gram-negative or positive) for different combinations (52% to 84%).

Interpretation of residual graph

The normal probability plot for the conducted experiments (biofilm inhibition and eradication) shows that the distribution of the residual value, defined as the difference between the predicted (model) and observed (experimental) values, fit a straight line, and the residual values are distributed normally on either sides of the line indicating that experimental points are reasonably aligned with the predicted value (Fig 3: A1, A2, B1, B2).

Effect of different combinations on biofilm formation

The mixture surface and contour plots between the probiotic extracts are shown in Figs. 4 A1, A2, B1, B2. The 3D-mixture surface plot describes individual and cumulative contributions of the 3 probiotic extracts on biofilm formation. The lines of the mixture contour plots predict the values of the biofilm inhibition rate response for different proportions of probiotic extracts (Fig 4). For *Salmonella*, the plot data indicated that inhibition of biofilm was higher when probiotic extract of *Bacillus* (A) and *Lactobacillus* (B) were used (Fig. 4 A1, A2). The plot demonstrate that biofilm eradication was more than 70% when probiotic extracts of A and C were used in the proportion of 73% from (A) and 23% from (C). Concerning the effect of the different combination on *Staphylococcus* biofilm formation, the plot demonstrate a high level of inhibition (>70%) when the probiotic extracts B and A were used. The better combination able to eradicate biofilm (>75%) was composed by the extracts B and C in the proportion of 50% from each extract.

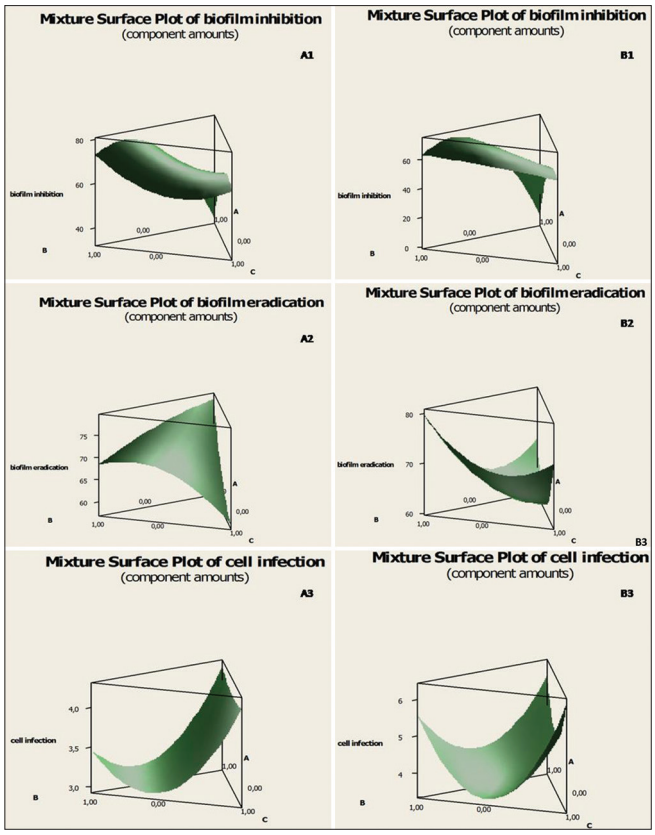

Fig 5: 3D surface plot of the biofilm inhibition and eradication and cell infection rates, between the variables (A: *Bacillus* extract, B: *Lactobacillus* extract and C: *Candida* extract). A1, A2 and A3: *Salmonella* biofilm inhibition, biofilm eradication and cell infection rate responses respcetively. B, B2 and B3: *Staphylococcus* biofilm inhibition, biofilm eradication and cell infection rate responses respcetively.

Effect on invasive ability of S*almonella* and *Staphylococcus*

The studied bacterial strains have the ability to infect cell culture. The results demonstrate that the used combination of the different probiotic extracts have a preventive affect against pathogenic bacteria especially with treatments using C6 and C9 combinations where the number of intracellular bacteria counted was minimum (Fig. 2).

Interpretation of residual graph

The normal probability plot for the experiments of cell infection revealed that the residual values are distributed normally on either sides of the line indicating that experimental points are reasonably aligned with the predicted value (Fig 3: A3, B3).

Effect of different combinations on cell infection ability

The responses of the different treatments with the combinations of probiotic extracts are presented as a mixture surface and contour plots (Figs. 4, 5). The 3D-mixture surface and contour plot indicate that prevention of pathogens infection was optimal where the probiotic extract of *Lactobacillus* (B) and that of the yeast (C) were used in the treatment of the both tasted pathogenic strains (*Salmonella* and *Staphylococcus*) (Figs. 4 and 5: A3, B3).

The result of cell infection surface optimization response clearly indicates that the maximum of cell infection inhibition was obtained for *Salmonella* when the mixture composition was 66 % of the *Lactobacillus* extract (B) and 33% of that of the yeast *Candida* (C) (Fig. 6: T6). For *Staphylococcus*, an anti-infective activity was obtained with an optimal combination composed by 50% from each probiotic extracts (B and C) (Fig. 6: T6).

DISCUSSION

The results of this study showed that isolated probiotic organism extracts could inhibit and eradicate biofilms as well as pathogenic bacteria's infectious properties when used separately. However, the use of multispecies extract is necessary to enhance this inhibition because of the synergic and additional activities of these extracts. In previous study, probiotic strains such as *Lactobacillus* have been demonstrated to inhibit biofilm production (Ben Slama et al., 2013) and to improve rearing water quality and thereby have beneficial effects on reared organisms (Fdhila et al., 2017). Also different molecules have been tested and the results showed a positive effect on biofilm like the Glucomannan, it was shown that it is a successful anti-adhesive molecule; it exhibited a stronger inhibitory effect on adhesion of *Vibrio splendidus* in infected *Crassostrea gigas* (Fdhila et al., 2016). In addition, Chaieb et al. (Chaieb, Kouidhi, Jrah, Mahdouani, & Bakhrouf, 2011), demonstrated that thymoquinon, an active principle of *Nigella sativa* prevent bacterial biofilm formation. This study did not examine the mechanism of action in detail. In contrast, other researchers have demonstrated that physicochemical surface alterations have caused the effect on biofilm because of the action of groups II capsules in some microorganisms, which prevent biofilm formation by Gram-positive and Gram-negative bacteria alike (Valle et al., 2006). A polysaccharide extracted from a *Bacillus licheniformis* strain associated with the marine organism *Spongia officinalis* has shown to be effective in reducing initial adhesion and biofilm formation (Sayem et al., 2011). Many bacterial strains can produce enzymes that degrade matrix polysaccharides, including *Actinobacillus actinomycetemcomitans*, which produces dispersin B that breaks down the poly-N-acetylglucosamine (PNAG), a polysaccharide that is a key component to many bacterial extracellular matrixes (Kaplan, Ragunath, Ramasubbu, & Fine, 2003). However, Polysaccharides are ubiquitously present on the cell surface of *Lactobacilli* and are considered to contribute to the species- and strain-specific probiotic effects that are typically observed (Lee et al., 2016).

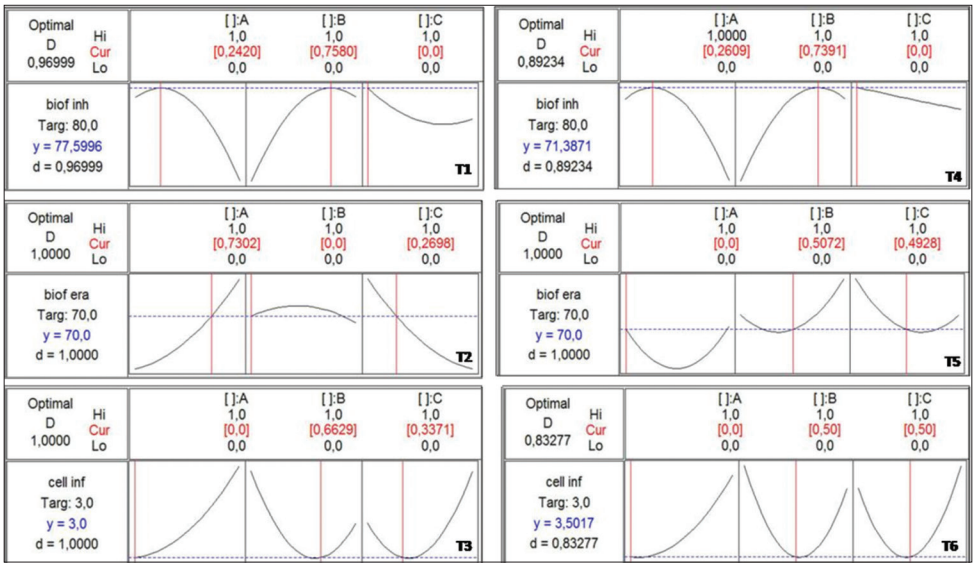

Fig 6: Optimization plot to confirm the experimental results. T1, T2 and T3: For *Salmonella* biofilm inhibition, biofilm eradication and cell infection rate responses respcetively. T4, T5 and T6: *Staphylococcus* biofilm inhibition, biofilm eradication and cell infection rate responses respcetively.

The β-hexosaminidase, is a matrix-degrading enzyme which, can effectively interfere with and disperse pre-existing biofilms of *S. epidermidis* by degrading its polysaccharide, as well as biofilms of some other Gram-positive and Gram-negative bacteria (Kaplan, Ragunath, Velliyagounder, Fine, & Ramasubbu, 2004).

On the other hand, probiotics can ameliorate protection of their host by producing metabolites which inhibit the colonization or growth of pathogens or by competing with them for resources such as nutrients or space (Balcázar & Rojas-Luna, 2007). Competition between bacteria (e.g. competition experiments between *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens*) for the adhesion site is one of the simplest strategies for avoiding initial colonization and biofilm development (An, Danhorn, Fuqua, & Parsek, 2006). The combined inhibitory effect and the variation in inhibition or eradication can be explained by producing enzymes capable of degrading the extracellular matrix, which inhibits biofilm formation and at the same time removes pre-existing biofilms. In addition, it may be a matter of the composition of the matrix of the biofilm (Iwase et al., 2010).

In this study, the invasion characteristics of pathogenic strains treated by probiotic extracts were determined. A reduction of invasion was demonstrated associated to the use of multispecies probiotic extracts. The observed effect on invasion can be correlated to the effect of the different combination of extracts on the motility of *Salmonella* that was reduced (data not shown). Other studies have described the direct effects *in vitro* of some compounds such as carvacrol (Burt, 2004; Inamuco et al., 2012). The earlier studies ported on some pathogenic bacteria like *Salmonella* and *Camplylobacter*, clearly shown a correlation between motility and invasion and that motility of pathogens can be affected using natural compounds like carvacrol (Shah et al., 2011; Van Alphen et al., 2012). Between other virulence factors implicated in the pathogenicity of several bacteria we find the outer membrane proteins (including PEB1, JlpA, MOMP and CadF) (Jin et al., 2001) the phase variable capsule (Bachtiar, Coloe, & Fry, 2007), and lipopolysaccharide (Guerry et al., 2002). On the other hand, it has been demonstrated that colonization of epithelial cells *in vitro* was reduced when bacteria lack flagella or expressing a non-motile flagellum (Grant, Konkel, Cieplak Jr, & Tompkins, 1993). Probiotic microorganisms can acted on cells of the innate and adaptive immune response. The continuous probiotic administration diminished the neutrophil infiltration with the consequent diminution of intestinal inflammation; activated the macrophage phagocytic activity (De LeBlanc, Castillo, & Perdigon, 2010). The inhibition of the cellular apoptosis of macrophage infected with *Salmonella* induced by certain *lactobacillus* bacteria has been also demonstrated *in vitro* and *in vivo* (Gobbato et al., 2008). We can suggest that the inhibition of biofilm formation can be correlated to the decrease of the infective capacity of pathogens. In fact, to enter inside, the cell host bacteria need to adhere to the cell and exert their effect. Here, it has been shown that the invasion of the host cells by some pathogens such as *salmonella* requires Focal Adhesion Kinase and the scaffolding protein p130Cas (Shi & Casanova, 2006).

CONCLUSION

In this study, it was observed that probiotic extracts significantly reduced the formation of biofilms and the

level of pathogen infections in cells as a result of their administration. The application of mixture design, which is fast and effective, also enables the exploitation of these probiotic extracts under optimal conditions and confers maximum protection against pathogens. This can be of great benefit to both human and animal health sectors based on the data obtained in this exploratory study. To get an exact molecular mechanism of action of the selected combinations, it would be very interesting to use other studies using host gene expression analysis. The discovery and development of new molecules with broad-spectrum activity against biofilm formation and infection associated with pathogenic bacteria may be enhanced by further research on such compounds.

ACKNOWLEDGEMENT

This research has been funded by Scientific research Deanship at University of Ha'il – Saudi Arabia through project number RG-191248.

REFERENCES

An, D., T. Danhorn, C. Fuqua and M. R. Parsek. 2006. Quorum sensing and motility mediate interactions between *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* in biofilm cocultures. Proc. Natl. Acad. Sci. 103: 3828-3833.

Aslam, B., W. Wang, M. I. Arshad, M. Khurshid, S. Muzammil, M. H. Rasool and M. U. Qamar. 2018. Antibiotic resistance: A rundown of a global crisis. Infect. Drug Resist. 11: 1645.

Bachtiar, B. M., P. J. Coloe and B. N. Fry. 2007. Knockout mutagenesis of the kpsE gene of *Campylobacter jejuni* 81116 and its involvement in bacterium-host interactions. FEMS Immunol. Med. Microbiol. 49: 149-154.

Balcázar, J. L. and T. Rojas-Luna. 2007. Inhibitory activity of probiotic *Bacillus subtilis* UTM 126 against *Vibrio* species confers protection against vibriosis in juvenile shrimp (*Litopenaeus vannamei*). Curr. Microbiol. 55: 409-412.

Ben Slama, R., B. Kouidhi, T. Zmantar, K. Chaieb and A. Bakhrouf. 2013. Anti-listerial and anti-biofilm activities of potential probiotic *L. actobacillus* strains isolated from Tunisian traditional fermented food. J. Food Saf. 33: 8-16.

Berendonk, T. U., C. M. Manaia, C. Merlin, D. Fatta-Kassinos, E. Cytryn, F. Walsh and M. N. Pons. 2015. Tackling antibiotic resistance: The environmental framework. Nat. Rev. Microbiol. 13: 310-317.

Burt, S. 2004. Essential oils: Their antibacterial properties and potential applications in foods a review. Int. J. Food Microbiol. 94: 223-253.

Cano, P. G. and G. Perdigón. 2003. Probiotics induce resistance to enteropathogens in a re-nourished mouse model. J. Dairy Res. 70: 433.

Carlos Valdez, J., M. Rachid, N. Gobbato and G. Perdigon. 2001. Lactic acid bacteria induce apoptosis inhibition in *Salmonella* Typhimurium infected macrophages. Food Agric. Immunol. 13: 189-197.

Chaieb, K., B. Kouidhi, H. Jrah, K. Mahdouani and A. Bakhrouf. 2011. Antibacterial activity of thymoquinone, an active principle of *Nigella sativa* and its potency to prevent bacterial biofilm formation. BMC Complement. Altern. Med. 11: 1-6.

De LeBlanc, A. D. M., N. A. Castillo and G. Perdigon. 2010. Anti-infective mechanisms induced by a probiotic *Lactobacillus* strain against *Salmonella enterica* serovar Typhimurium infection. Int. J. Food Microbiol. 138: 223-231.

Djordjevic, D., M. Wiedmann and L. McLandsborough. 2002. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. Appl. Environ. Microbiol. 68: 2950-2958.

FAO/WHO (2001) Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Acid Bacteria. Report of a Joint FAO/WHO Expert Consultation, Córdoba, Argentina. Available From: http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf?ua=1.

Fdhila, K., H. Haddaji, I. Chakroun, A. Dhiaf, M. E. E. Macherki, B. Khouildi and H. Marzougui. 2017. Culture conditions improvement of *Crassostrea gigas* using a potential probiotic *Bacillus* sp strain. Microbial Pathog. 110: 654-658.

Fdhila, K., N. Haddaji, I. Chakroun, M. E. E. Macherki, F. Nefzi and A. Bakhrouf. 2016. Glucomannan's protective effect on the virulence of *Vibrio splendidus* in pacific oyster. Fish Shellfish Immunol. 56: 410-416.

Garbisu, C., O. Garaiyurrebaso, A. Lanzén, I. Álvarez-Rodríguez, L. Arana, F. Blanco and I. Alkorta. 2018. Mobile genetic elements and antibiotic resistance in mine soil amended with organic wastes. Sci. Total Environ. 621: 725-733.

Gobbato, N., C. M. Galdeano and G. Perdigon. 2008. Study of some of the mechanisms involved in the prevention against *Salmonella enteritidis* serovar Typhimurium infection by lactic acid bacteria. Food Agric. Immunol. 19: 11-23.

Gomez-Gil, B., A. Roque and J. F. Turnbull. 2000. The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. Aquaculture, 191(1-3), 259-270.

Grant, C., M. E. Konkel, W. Jr. Cieplak and L. S. Tompkins. 1993. Role of flagella in adherence, internalization, and translocation of *Campylobacter jejuni* in nonpolarized and polarized epithelial cell cultures. Infect. Immun. 61: 1764-1771.

Guerry, P., C. M. Szymanski, M. M. Prendergast, T. E. Hickey, C. P. Ewing, D. L. Pattarini and A. P. Moran. 2002. Phase variation of *Campylobacter jejuni* 81-176 lipooligosaccharide affects ganglioside mimicry and invasiveness *in vitro*. Infect. Immun. 70: 787-793.

Haney, E. F., M. J. Trimble, J. T. Cheng, Q. Vallé and R. E. Hancock. 2018. Critical assessment of methods to quantify biofilm growth and evaluate antibiofilm activity of host defence peptides. Biomolecules. 8: 29.

Harbi, B., K., Chaieb, C. Jabeur, K. Mahdouani and A. Bakhrouf. 2010. PCR detection of nitrite reductase genes (nir K and nir S) and use of active consortia of constructed ternary adherent staphylococcal cultures via mixture design for a denitrification process. World J. Microbiol. Biotechnol. 26: 473-480.

Inamuco, J., A. K. Veenendaal, S. A. Burt, J. A. Post, J. L. Tjeerdsma-van Bokhoven, H. P. Haagsman and E. J. Veldhuizen. 2012. Sub-lethal levels of carvacrol reduce *Salmonella* Typhimurium motility and invasion of porcine epithelial cells. Vet. Microbiol. 157:200-207.

Iwase, T., Y. Uehara, H. Shinji, A. Tajima, H. Seo, K. Takada and Y. Mizunoe. 2010. *Staphylococcus epidermidis* Esp inhibits *Staphylococcus aureus* biofilm formation and nasal colonization. Nature. 465: 346-349.

Jin, S., A. Joe, J. Lynett, E. K. Hani, P. Sherman and V. L. Chan. 2001. JlpA, a novel surface-exposed lipoprotein specific to *Campylobacter jejuni*, mediates adherence to host epithelial cells. Mol. Microbiol. 39: 1225-1236.

Kaplan, J. B., C. Ragunath, N. Ramasubbu and D. H. Fine. 2003. Detachment of *Actinobacillus actinomycetemcomitans* biofilm cells by an endogenous β-hexosaminidase activity. J. Bacteriol. 185: 4693-4698.

Kaplan, J. B., C. Ragunath, K. Velliyagounder, D. H. Fine and N. Ramasubbu. 2004. Enzymatic detachment of *Staphylococcus epidermidis* biofilms. Antimicrob. Agents Chemother. 48: 2633-2636.

Lee, I. C., G. Caggianiello, I. I. van Swam, N. Taverne, M. Meijerink, P. A. Bron and M. Kleerebezem. 2016. Strain-specific features of extracellular polysaccharides and their impact on *Lactobacillus plantarum*-host interactions. Appl. Environ. Microbiol. 82: 3959.

Leonard, A. F., L. Zhang, A. J. Balfour, R. Garside and W. H. Gaze. 2015. Human recreational exposure to antibiotic resistant bacteria in coastal bathing waters. Environ. Int. 82: 92-100.

Mahdhi, A., A. Bahi, D. Mzah and A. Bakhrouf. 2013. Use of mixture design to construct a consortium of date palm (*Phoenix dactylifera* L.) fruit extract and potentially probiotic *Bacillus* strain to confer protection against vibriosis in Artemia culture. J. Sci. Food Agric. 93: 3850-3855.

Mahdhi, A., M. A. Esteban, Z. Hmila, K. Bekir, F. Kamoun, A. Bakhrouf and B. Krifi. 2012. Survival and retention of the probiotic properties of *Bacillus* sp. strains under marine stress starvation conditions and their potential use as a probiotic in Artemia culture. Res. Vet. Sci. 93: 1151-1159.

Mahdhi, A., Z. Hmila, A. Behi and A. Bakhrouf. 2011. Preliminary characterization of the probiotic properties of *Candida famata* and *Geobacillus thermoleovorans*. Iran. J. Microbiol. 3: 129.

Perdigon, G., C. M. Galdeano, J. Valdez and M. Medici. 2002. Interaction of lactic acid bacteria with the gut immune system. Eur. J. Clin. Nutr. 56: S21-S26.

Sayem, S. A., E. Manzo, L. Ciavatta, A. Tramice, A. Cordone, A. Zanfardino and M. Varcamonti. 2011. Anti-biofilm activity of an exopolysaccharide from a sponge-associated strain of *Bacillus licheniformis*. Microb. Cell Factories. 10: 1-12.

Shah, D. H., X. Zhou, T. Addwebi, M. A. Davis, L. Orfe, D. R. Call and T. E. Besser. 2011. Cell invasion of poultry-associated *Salmonella enterica* serovar Enteritidis isolates is associated with pathogenicity, motility and proteins secreted by the Type III secretion system. Microbiology. 157 Pt 5: 1428-1445.

Shi, J. and J. E. Casanova. 2006. Invasion of host cells by *Salmonella* Typhimurium requires focal adhesion kinase and p130Cas. Mol. Biol. Cell. 17: 4698-4708.

Valle, J., S. Da Re, N. Henry, T. Fontaine, D. Balestrino, P. Latour-Lambert and J. M. Ghigo. 2006. Broad-spectrum biofilm inhibition by a secreted bacterial polysaccharide. Proceedings of the National Academy of Sciences, 103(33), 12558-12563.

Van Alphen, L. B., S. A. Burt, A. K. Veenendaal, N. M. Bleumink-Pluym and J. P. Van Putten. 2012. The natural antimicrobial carvacrol inhibits *Campylobacter jejuni* motility and infection of epithelial cells. PLoS One. 7: e45343.

Xu, H., H. Y. Lee and J. Ahn. 2011. Characteristics of biofilm formation by selected foodborne pathogens. J. Food Saf. 31: 91-97.

Zhou, J. Z., X. L. Liu, K. H. Huang, M. S. Dong and H. H. Jiang. 2007. Application of the mixture design to design the formulation of pure cultures in tibetan kefir. Agric. Sci. China. 6: 1383-1389.