Int.J.Curr.Microbiol.App.Sci (2018) *7*(7): 4127-4134

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 7 Number 07 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.707.481

Study the Effect of Probiotic Bacteria Isolated From Foods on Pathogens

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Keywords

Probiotic strains, *Bacillus* sp., Antibacterial. Antibiotic, Gastrointestinal tract conditions

Article Info

Accepted:
28 February 2018
Available Online:
10 July 2018

A B S T R A C T

Eleven Bacillus strains were isolated from foods and evaluated their probiotic potential and safety. Then study the effect of selected strains against pathogens, and on the population of pathogens in reconstituted skim milk (RSM).Seven strains from *Bacillus sp.* (3) and *Bacillus subtilis* (4) were selected which were grow in low pH 1.0 to 5.0 and tolerant bile salt 0.3 to 2%, highly resistant to simulated gastrointestinal tract conditions, and showed antimicrobial activity against *Salmonella typhimurium* ATCC20231 and *Staphylococcus aureus* ATCC25923. They had antibiotic susceptibility against 6tested antibiotics. Also, it's exhibited non-hemolytic on sheep blood. Two pathogens were completely inhibited after 60 and 72hrs of incubation with selected probiotic strains in RSM. The results indicate that *Bacillus subtilis* or *Bacillus sp.* could be used as probiotic cultures for animal feeds and human, fermented vegetables, milk, meat product as well as to achieve biopreservation of dairy products in food industries.

Introduction

Food borne illness are major international health problems in the world wide and reduced the economic growth (WHO, 2007), although enhanced performance of effective legislative control on food processing procedures in industries. Food borne diarrheal diseases are causes of illness and death~2.2 million people annually and over than 200 types of illness transmitted by food (Mensah *et al.*, 2002 & Lynch *et al.*, 2006). These pathogens are *Listeria monocytogenes, Staphylococcus aureus, Escherichia coli, Salmonella typhi, Bacillus cereus,* yeasts and moulds. The use of naturally produced anti-microbial agents without any adverse effects on human health to inhibit the propagation of pathogenic microorganisms in foods, that is may be challenge the problems associated with food contamination.

The expression of "probiotics" was plagiaristic from the Greek word, it is meaning as "for life" (Reid *et al.*, 2003). After that, FAO/WHO (2006) were defined probiotics as: "Live microorganisms'' which when administered in sufficient amounts confer a beneficial health to host by improving its microbial balance in gut (Aslam and Qazi, 2010). Probiotic bacteria may be producing various Anti-microbial metabolites,

which include organic acids (lactic and acetic acids), and bacteriocins. The organic acids not only lower the pH, but they can also be toxic to the pathogens. The beneficial of probiotic bacteria are prevention and treatment of diseases in gastrointestinal disturbances, such as dysentery, diarrhoea, typhoid (Tambekar and Bhutada, 2010).

Lactobacillus, Bifidobacterium, Lactococcus, Leuconostoc, Bacillus and many others are integrated in the list of probiotics (Isolauri *et al.,* 2004). Probiotics are well recognized to possess specific properties such as; gastric juice and bile tolerance, adhesion to the epithelial cells of the intestine, survive in the gastrointestinal tract of humans and animals and improvement of the intestinal microbial balance (Ministry of Food and Drug Safety, 2015).

Probiotics bacteria, like common microorganisms, it may possess undesirable properties such as the advent of harmful biochemical and virulence factors. They are non-spore former, and may be destroyed in high bile concentrations of the duodenum and stomach acids. Therefore, large numbers from probiotics bacteria were require surviving in the gut anaerobic. To challenge this problem, many studies are in progress on the applicability of a new spore forming Bacilli probiotics, such as; *Bacillus coagulans, Bacillus subtilis, Bacillus licheniformis, Bacillus polymyxa, and Bacillus mesentricus*. They are non-pathogenic and naturally found in water and soil, ferment a large number of sugars, secrete protease, lipase and amylase enzymes, survive in the stomach and reach the intestine to germinate, and thermo stable.

Bacillus species are Gram positive bacilli, produce heat resistant spores and wide spread in environment or many types of food. *B.subtilis* is not harmful to mammals, including humans, and is commercially important as producer of a high fine chemicals and enzymes and diverse amount of secondary metabolites like antibiotics, as well as heterologous proteins, antigens and vaccines (Stein, 2005; Bérdy,2005 &Valdez *et al.,* 2014]. *B.subtilis* grow efficiently with low-cost carbon and nitrogen sources, because its enzymes are very efficient breaking down a great variety of proteins, carbohydrates and lipids from animal and vegetable origin, into their constituent units (Ochoa, 2012). *B. subtilis* spores have the ability to resist extreme pH conditions, UV irradiation, high temperatures, solvents and long time periods of storage without refrigeration [Sonnenschein *et al.,* 1993]. In review, *B. subtilis* has adaptability of growth nutrients utilization, production high level of enzymes, secretion of antimicrobial compounds, develops in aerobic and anaerobic conditions, and *B.subtilis* is Generally Recognized As Safe (GRAS) by the Food and Drug Administration (FDA). Finally, *B. subtilis* in "theory" may be well measured as a ideal multifunctional probiotic bacterium for hosts (Cutting, 2011; Olmos *et al.,* 2011; Sorokulova, 2013; Huang *et al.,* 2013 & Olmos *et al.,* 2014).

The aim of this study to evaluate thepotential probiotics properties of Bacillus bacteria isolated from food, and their antibacterial effects against pathogenesis. Finally study the effect of co-culturing probiotic bacteria with pathogenic in skim milk.

Materials and Methods

Bacterial strains

Eleven bacterial strains (M01 –M11) were isolated under aerobic conditions from twelve traditional food products; further all strains were identified by partial 16SrRNA gene sequencing and phylogenetic analysis. All strains were identified by 97-100% identity

including *Bacillus circulans* (4), *Bacillus sp. (3)* and *Bacillus subtilis* (4) according to Abu Zaid *et al.,* (2015). Standard Biochemical tests such as gram reaction, spore former, motility, catalase reaction, oxidase activity, Esculin hydrolysis were performed following standard assessment according to (Cappuccino and Sherman, 1999).

Probiotic potential

pH tolerance

The isolates were inoculated into sterile Nutrient broth (NB) tubes of varying pH, i.e. 1, 2, 3, 4, and 5 incubated at 37°C for 24 hours. Then 0.1ml inoculums from each tube was poured Nutrient agar medium by pour plate method and incubated at 37°C for 48hrs. The growth of bacteria on agar was used to designate isolates as pH tolerant (Tambekar and Bhutada, 2010).

Bile salt tolerance

The medium with varying concentrations of bile salt (0.3, 0.5, 1.0, 2.0, 3.0 and 4.0%) was inoculated with each selected bacterial culture and incubated at 37°C for 48hrs. Then 0.1ml inoculums was transferred to nutrient agar by pour plate method and incubated at 37°C for 48hrs. The growth of bacteria on agar plate was used to designate isolates as bile salt tolerant (Tambekar and Bhutada, 2010).

Hemolytic activity

For testing haemolytic activity, isolates were streaked on Columbia agar plates; containing 5% (w/v) sheep blood according to Ghrairi *et al.,* (2008).

Antibiotic susceptibility

The selected Bacillus strains were evaluated for antibiotic susceptibility by using agar diffusion method on Mueller Hinton agar (Bauer, 1966). Antibiotics such as ampicillin (Am 10 U); penicillin (P 10 mg); nalidixic acid (NA, 30 µg); vancomycin (V, 30 µg); colistin (CS, 10 µg); tetracycline (T, 10 µg); Gentamicin (GM, 10 mg); Fusidic acid (FA, 10 U).All antibiotics were purchased from Bio-Rad, Laboratories, GmbH., Germany. Isolates culture suspension containing ~10^6cfu/mL of the 18 h culture at 37°C, then streaked on agar by a sterile cotton swab. The studied antibiotic discs were aseptically placed on the plates. The diameters of inhibition zone were calculated in mm under the colony counter after 24 hrs of incubation.

Effect of simulated gastrointestinal conditions

Simulated gastrointestinal conditions were designed according to Zago *et al.,* (2011). The growth of isolates at pH 2.0 in the presence of 1% lysozyme, 1% trypsin, and 0.3% bile slat in Tryptic Soy Broth TSB was used to designate isolates as simulated gastrointestinal conditions.

Antimicrobial activity

For detection of antagonism activity, an agar spot test was used (Mezaini *et al.,* 2009). Overnight cultures, on NB medium, of the strains to be tested for production of antimicrobial compound were centrifuged (10 minutes at 15000 g, 4°C). Cell-free supernatants were filtered across cellulose acetate filter (0.2 µm) to remove residual cells. An overnight culture (37°C) of the target strain was diluted in sterile Mueller-Hinton Medium, and 1 mL of 10^6CFU were spread on solid Mueller-Hinton medium. The Petri dishes were dried for 10 minutes. Cork borer was used to punch one hole, 4.1 mm in diameter. Nutrient agar was used to seal the bottom of holes. Samples (30 µL) of filtered cell-free supernatants were transferred on the

agar plate. The target strains used in this study are *Salmonella typhimurium* ATCC20231, and *Staphylococcus aureus* ATCC25923.

Effect of co-culturing probiotic bacteria with pathogenic.

This method was used to study the effect of probiotic bacteria on the population of pathogenic in reconstituted skim milk (RSM).

A 9 mL of RSM was inoculated with1 mL overnight culture of probiotic bacteria and 0.1 mL of pathogenic. Inoculated RSM medium was mixed well and incubated at 37°Cfor 0, 12, 24, 36, 48, 60, 72 hrs. Following incubation, the population of spoilage and pathogenic bacteria was counted on nutrient agar (Denkova *et al.,* 2013).

Statistical analysis

The data obtained from treatments were analyzed by one-way ANOVA using 'Proc Mixed' (SAS 8.2, Cary, NC, USA). In all cases, the level of statistical significance was of P <0.05.SAS program was used to statistical analyzed (SAS 2001), LSD means comparisons were conducted with the Duncan option in SAS.

Results and Discussion

Eleven bacterial strains (M1_M11) were isolated from foods under aerobic condition characterized by biochemical conditions then identified by partial 16SrRNA gene sequencing and phylogenetic analysis by Abu Zaid *et al.,* (2015). Eleven strains were identified by 97-100% identity including *Bacillus circulans* (4), *Bacillus subtilis* (4),Other isolates were identified by 85-92% identity; therefore these species are considered as new ones and named: Bacillus sp. (M07, M08, M10).Cutting, (2011) identified Bacillus strains as probiotic, they were *B. subtilis, B.coagulans, B. cereus* and *B. clausii.* Recently, Manhar *et al., (*2015) was also introduced *B. amyloliquefaciens* as potential probiotics. Probiotic Bacillus strains can produce spores under insensitive conditions such as high heat, low pH, dry, and worn out nutrients, which bacterial cells cannot stay alive, and remain viable till exposed to more good conditions. This provides many opportunities for probiotic *Bacillus sp.* over non-spore forming bacteria such as *Lactobacillus,* and *Bifido* bacterium strains. Spore forming probiotic Bacillus strains are widely used as nutritional supplements in humans, increase shelf life of dairy products and as growth promoters in animals and fish (Cutting, 2011). *Bacillus subtilis* and *Bacillus sp.* strains showed significant differences antimicrobial effect against two tested pathogenic strains, but *Bacillus circulans strains* exhibited no activity against pathogens as shown in Table 1. On the basis of maximum zone scored by M6 against *Salmonella typhimurium* and *Staphylococcus aureus* were 28, and 25 mm respectively.

The same finding Patel *et al.,* (2009) reported that, the probiotic *Bacillus* strain DET6 isolated from food wastes inhibited growth of Salmonella *typhi* and *E. coli*. Mahdhi *et al.,* (2012) selected probiotic *Bacillus* strains had an antibacterial activity against Gram-positive and Gram-negative bacteria with diameter of the inhibition zones range between 12 and 18.6 mm. Lee *et al.,* (2017) isolated and identified three probiotic *Bacillus* strains from Korean traditional soy sauces, and showed antimicrobial activity against *Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus and Escherichia coli.* On the other hand, *B. amyloliquefaciens* did not show any affect against *B. cereus, Yersinia enterocolitica* and *Salmonella typhimurium,* but inhibited the growth of *L. monocytogenes* and *K. pneumoniae* reported by Manhar *et al.,* (2015).

Probiotic potential of selected spore former Bacillus strains pH and Bile salt tolerance were shown in Table 2. In this study, *Bacillus subtilis* and *Bacillus sp.* strains were able to grow in pH from 1.0 to 5.0 and survive in bile salt concentrations from 0.3 to 2%. *Bacillus circulan* strains were lost their tolerance to low acidity at pH 1.0, 2.0 and 3.0and all bile salt concentrations. On other hand, probiotic bacteria isolated from baobab (maari) fermented seeds could tolerate bile salt concentration of 0.3%, and able to survive at pH 2.5 (Kabore *et al.,* 2012). Tambekar and Bhutada (2010) isolated probiotic, which tolerance to acid (pH 2.0) and bile salt at 2.0%.Tolerance to bile salts is a requirement for colonization and metabolic activity of bacteria in the small intestine of the host (Havenaar *et al.,* 1992). This will help probiotic bacteria to reach the small intestine and colon and contribute in balancing the intestinal microflora (Tambekar and Bhutada, 2010).

Antibiotic susceptibility test

The antibiotic resistances of the probiotic Bacillus strains were tested by using two groups of antibiotics categorized bytheir mechanisms. The first one were cell wall inhibitors including ampicillin, penicillin G and vancomycin, and second group were protein synthesis inhibitors including gentamicin and tetracycline (Argyri *et al.,* 2013). *Bacillus subtilis* and *Bacillus sp* strains were scored multi drug resistance against 6 type of antibiotics. Antibiotic susceptibility is measured to be the best probiotic characteristic (Patel *et al.,* 2009). In this study *Bacillus subtilis* and *Bacillus sp* strains also showedthe highest resistance to ampicillin and penicillin G. This is parallel to the reports by Manhar *et al.,* (2015). They reported that *B. amyloliquefaciens* AMS1 were resistant to ampicillin and penicillin and probiotic *Bacillussp*.

Hemolytic activity

One of the main criteria needed to be satisfied by a probiotic organism is to be non-pathogenic (Ljungh and Wadström, 2006). In general, it can be noticed that no strain observed complete hydrolysis (β hemolysis) on blood sheep agar. Other isolated strains 7 out of 11 observed no hemolytic activity (γ hemolysis). *Bacillus circulan* strains displayed greenish-brown color (α hemolysis) around their colonies grown on blood sheep agar Table 4. This study was comparable to probiotic *B. polyfermenticus CJ6*, it was observed no hemolytic activity (Jung & Chang, 2012). Probiotic *Bacillus sp*. BCNU9028 formed a green zone that indicatesα -hemolysis. *B. cereus*produces a clear zone that indicatesβ -hemolysis (Jung *et al.,* 2012). No-hemolysis and α-hemolysis are considered to be safe and β -hemolysis was considered harmful (Shin *et al.,* 2012). β -hemolysis is an implication that bacteria havecytotoxic phospholipases (Sorokulova *et al.,* 2008). The hemolytic factor decreases the amount of hemoglobin available as an iron source for the host (Seker, 2010).

To select the probiotics strains with high probiotic potential, simulate gastrointestinal conditions by identified strains were grow in the presence bile salt, lysozyme, trypsin at low pH. If the tested strains have ability to grow under these environmental stresses, these bacterial strains can tolerant, adhere, colonize and grow after transit from the stomach to small intestinal. In the present study, all isolated strains were studied in the presence of 1.0% lysozyme, 1.0% trypsin and 0.3% bile slat at pH 2.0. Most studied strains 7 out of 11 were could be grow under simulate gastrointestinal conditions.

In conclusion, the effect of trypsin and lysozyme might be avoided due to not fitting pH, where these enzymes could be inactivated

at low temperature and optimal pH 7.5-8.5 Barrett & McDonald (1980). Lysozyme activity was significantly decreased at pH 6.2 or below (Davies *et al.,* 1969). After this explanation, the most effective factors in this simulated gastrointestinal were bile salt and pH. As described above, the obtained results demonstrated that, bile slat and low pH induced adherent and biofilm formation due to increasing cell hydrophobicity (Zago *et al.,* 2011; Ambalam *et al.,* 2012; Nostro *et al.,* 2012).

Experimental results, given in Fig 1, and Fig 2, were shown that the effect of probiotic Bacillus strainson the population of pathogens in reconstituted skim milk (RSM). It depends on the variety of the pathogen as well as of the types of Bacillus probiotic strains. The agent of *Salmonella* was sensitive to the presence of *Bacillus subtilis* and *Bacillus sp* strains during all the period of incubation; it was complete inhibition up to the 60th hrs. *Staphylococcus aureus* was more resistant in the presence of *Bacillus subtilis* and *Bacillus sp*, it completely inhibited after 72 hrs of incubation period. *Bacillus circulan strains* have a similar behavior of control as shown from results and spoilage all samples after 48 hours of incubation periods. The same finding by Denkova *et al.,* (2013) concluded that *E.coli*, and *Salmonella* were sensitive to the presence of probiotic bacteria and during joint cultivation complete inhibition up to the 60th to the 72nd hour of incubation. On the other contrasting, *Staphylococcus aureus* showed more resistant to the presence of probiotic bacteria in the medium, but this pathogen was sensitive to *Bif. Bifidum* strain only.

In conclusion, seven strains (4 *Bacillus subtilis* and 3 *Bacillus sp.*) showed no hemolytic activity (γ hemolysis), antibacterial activity, tolerated low pH 2.0, bile salt up to 0.3%; viable under stimulated gastrointestinal tract (SGIT) stress. These strains are strongly suggested to use as probiotics and can be measured as generally recognized as safe (GRAS) bacteria. The other four strains from *Bacillus circulans* have ability to incomplete hemolysis (α hemolysis) and losted one of the important criteria (acid and bile tolerance). Therefore its need to advance work to assess their safety to use as probiotics. It could be concluded on the combine of different probiotic strains in order to complete the inhibition growth of bacteria and spores. As a result of the conducted studies strains of *Bacillus subtilis* and *Bacillus sp.* had high antimicrobial activity, it's important for the application of these probiotic strains in foods to extend the shelf life. Bacillus probiotics strains were used for the production of functional foods (yogurt; bioyogurt; bread with extended shelf-life; raw-dried meat products; cheeses; organic preservation of cosmetic creams).

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How to cite this article:

Abeer Abu Zaid. 2018. Study the Effect of Probiotic Bacteria Isolated From Foods on Pathogens. *Int.J.Curr.Microbiol.App.Sci.* 7(07): 4127-4134.
doi: https://doi.org/10.20546/ijcmas.2018.707.481