JOURNAL OF PURE AND APPLIED MICROBIOLOGY, APRIL 2014. Vol. **8**(2), p. 1619-1625

Optimization of Culture Conditions of *Bacillus subtilis natto* and Preparation of Freeze-Dried Powders as a Potentially Novel Antithrombotic Probiotic

Mingfang He[1a], Kaijie Tang[1a], Bohua Wang[3], Maohua Qu[1], Liping Lin[1], Yu Zong Chen[2]* and Weiping Chen[1]*

[1]Key Lab of Agricultural Products Processing and Quality Control of Nanchang City, Jiangxi Agricultural University, Nanchang - 330 045, China.
[2]Department of Pharmacy, National University of Singapore - 117 543, Singapore.
[3]Hunan University of Arts and Science, Changde.

(Received: 11 October 2013; accepted: 24 December 2013)

Increasing interests in natural health-promoting products have led to extensive exploration of probiotic bacteria for beneficial effects against various disease conditions and health problems. Previously unexplored bacterial strains have been discovered to have beneficial effects not covered by existing probiotic products, which may be further developed into useful products. In this work, we aimed at developing a bacterial strain *Bacillus subtilis natto*, which has not been explored as a probiotic before, into a product for facilitating the prevention and treatment of thrombosis, a major cause of complications in hospital patients not yet covered by any existing probiotic products. We optimized its culture conditions and prepared its powder forms for optimally retaining its beneficial effects. Using *Bacillus subtilis natto* as the target strain, we determined the optimal additive composition of enrichment medium for the strain growth (yeast powder 0.7 %, peptone 0.5 %, starch 0.3 %, and NaCl 0.5 %) and culture condition (pH 7.0, temperature 37°C, culture time 20 h). The strain powder form was made vacuum freeze drying and found to be optimally retained at the additive composition of 10 % skim milk powder, 4 % glucose, 4.0 % glycerol, 2 % gelatin, 4.0 % sucrose, 3 % V_C, and 10.0 % trehalose in the single factor experiment. The protective effect of the strain powder was found to be optimal at the additive composition of 2 % glycerol, 15 % skim milk powder, 2 % gelatin, 12 % trehalose, as suggested by the L_9 (3^4) orthogonal test. Our work facilitates the future efforts in developing *Bacillus subtilis natto* into a *antithrombotic* probiotic.

Key words: *Bacillus subtilis natto* probiotics, Proliferation vacuum freeze drying, Protective agent *antithrombotic.*

Probiotic bacteria have been extensively explored as health-promoting products because of their favored characteristics over other natural products in safety, function, technology, and the ability to survive in gastric environment[1,2]. They have been developed for various disease conditions and health problems including lactose intolerance, acute gastroenteritis, food allergy, dermatitis, Crohn's disease, rheumatoid arthritis, diarrhea, high cholesterol, and cancers[3,4]. Some of the products have made to the market. The increasing interests in natural health-promoting products have led to intensifying efforts for the discovery and commercialization of probiotic bacterial strains with beneficial effects against disease conditions and health problems not yet covered by existing probiotic products[5,6]. There is a need to further develop some of these strains into useful products against the disease conditions

* To whom all correspondence should be addressed.
A these authors contributed equally to this work.
Tel.: +86 791 83813420; fax: +86 791 83813655;
E-mail: iaochen@163.com

and health problems affecting high number of patients and not yet covered by existing probiotic products.

As part of our on-going efforts in facilitating the development of some of these probiotic bacterial strains into useful products, we aimed at the development of a probiotic bacterial strain Bacillus subtilis natto as a potential product for the prevention or treatment of thrombosis. Bacillus subtilis natto is a strain of Bacillus subtilis from the bacillus genus first separated from Japanese traditional fermented soybean food—natto by SAWAMURA in 1906[7]. This strain is abundant in water-soluble V_K[8]. Apart from its well-known physiological functions such as the improvement of intestinal flora[9], *Bacillus subtilis natto* has been reported to produce antithrombotic as well as immune enhancement effects partly via its secretion of proteases (particularly alkaline proteinase) and natto kinase needed for the strain's growth and metabolism processes[10,11]. In particular, its antithrombotic effect arises from the activities of natto kinase in degrading fibrin in the crosslinked state, activating urokinase, and dissolving the plasminogen activator inhibitor[12,13] .

Although some other *Bacillus subtilis* strains have been explored as probiotics for treating mild gastrointestinal disease like diarrhea and as nutritional supplement, Bacillus subtilis natto has not been explored as a probiotic before. We selected this strain for further development because of three reasons. The first is that one of its targeted disease condition, thrombosis, is a major cause of complications and occasional deaths in hospital patients[14] but is not covered by any existing probiotics. The second is that no *Bacillus subtilis* strain has been found to have harmful effects in the intestinal tract[15] and some of the strains have been explored as nutritional supplements and preventive products for mild gastrointestinal diseases like diarrhea[16,17] and as biocontrol agents for agriculture and aquaculture[18]. Therefore, it is highly likely that this particular strain may be developed into effective and safe antithrombotic product. The third is that, in addition to its antithrombotic effects, this particular strain has other health-beneficiary effects including the improvement of intestinal flora, lowering of cholesterol levels and blood pressure, activation of immune system and enhancement in immunity concurrent[19]. Hence it may be potentially developed into a product with multiple health benefits.

Our specific task was to optimize the culture conditions of *Bacillus subtilis natto* and prepare its powder forms for retaining optimal beneficial effects so as to facilitate its further development into a useful health-promoting product. We optimized the liquid proliferation medium and proliferation conditions of *Bacillus subtilis natto*, and subsequently investigated the effect of carbon source, nitrogen source, mineral salt, temperature, PH and time on the proliferation of *Bacillus subtilis natto* to find the optimal proliferation medium and proliferation conditions of *Bacillus subtilis natto*. We further tested the effect of additives of various compositions and at different concentrations on the survival of gelsiccation *Bacillus subtilis natto* so as to maximally reduce the death rate of thallus in freeze-drying process and increase the survival rate. The viable rate of Bacillus natto with the optimal additive compositions was evaluated with respect to that with other additive compositions to assess to what extent the viable rate can be optimally maintained for developing an effective probiotic product.

MATERIALS AND METHODS

Strains and chemicals

The *Bacillus subtilis natto strain* was purchased from China General Microbiological Culture Collection Center and preserved in the fridge with temperature below -18°C. The prepared mediums are: slant medium (beef extract 0.3 %, peptone 1 %, NaCl 0.5 %, agar 2 %, pH=7), seed medium (beef extract 0.3 %, peptone 1 %, NaCl 0.5 %, pH=7), carbon source basal medium for fermentation (yeast extract 3 g L^{-1}, peptone 10 g L^{-1}, NaCl 5 g L^{-1} pH 7.2-7.4), nitrogen source basal medium for fermentation: glucose 5g L^{-1}, NaCl 5g L^{-1} pH 7.2-7.4), and counting medium (beef extract 0.3 %, peptone 1 %, NaCl 0.5 %, pH=7). The instruments used in this work include DH 3600 electric heated incubators (Tianjin Taisite Instrument Co., Ltd.), centrifugal machine AnkeLXJ-a-B (Shanghai Anting Scientific Instrument Factory), table concentrator ZD-8802 (Jiangsu Taicang Experimental Equipment Co., Ltd.), TU1901 Double beam UV-visible

spectrophotometer (Beijing Presee General Instrument Co., Ltd.), and FD-1D-50 Vacuum freeze-drying machine (Beijing Boyikang Medical Instrument Co., Ltd.). After fermentation, we diluted the 1 mL fermentation broth to the level with the OD_{600} values kept within the range of 0.10 0.65. The same procedure was applied to the dilution of unvaccinated substrate as blank control. The OD_{600} was determined by using UV spectrophotometer.

Ascertain of *Bacillus subtilis natto* optimal medium component

The optimal medium components for *Bacillus subtilis natto* were determined as follows: The PH value of carbon source basal medium with 5 g L^{-1} lactose, sucrose, starch, glucose and trehalose respectively were adjusted to 7.0. The PH value of nitrogen basal medium with 10 g L^{-1} peptone, yeast powder beef extract, carbamide, NH4Cl and (NH4)2SO4 respectively was adjusted to 7.0, For inorganic salts, the PH value of basal medium with 5 g L^{-1} NaCl,K_2HPO_4,KH_2PO_4,$CaCl_2$ and $MgSO_4$ respectively was adjusted to 7.0. Each medium configuration was sterilized for 30 min at 121°C, inoculatesd into 2 ml seed solution and cultivated at bed temperature incubator under 200 r/min and at 37°C for 24 h. The OD_{600} values and the count of live *Bacillus subtilis natto* were measured for determining the optimal configurations. The $L_9(3^4)$ test on the optimization of proliferation medium for *Bacillus subtilis natto* was then conducted by the orthogonal experiment design (Table 1) on four medium components (yeast powder, peptone, starch, NaCl).

The optimal culture conditions for *Bacillus subtilis natto* were determined as follows: For finding the optimal proliferation medium, the pH value of the proliferation medium was adjusted to 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 respectively. Each medium configuration was sterilized for 30 min at 121°C, inoculated into 2 ml seed solution, and cultivated at bed temperature incubator under 200 r/min and at 37°C for 24 h. For finding the optimal temperature, the optimal proliferation medium was cultivated at bed temperature incubator under 200 r/min with the temperature set at 33°C, 35°C, 37°C, 39°C, 4°C respectively. For finding the optimal growth time, the optimal proliferation medium was cultivated at bed temperature incubator under 200r/min at the optimal temperature (determined in the previous step) for 0, 2, 4, 6, 8, 10, 12, 14, 16, 20, 22, 24h respectively. The OD_{600} values and the count of live *Bacillus subtilis natto* were measured for determining the optimal configurations.

The effect of different protective agent on the lyophilization viable organism rate of *Bacillus subtilis natto*

The procedure for selecting the bacterium power protectant for gelsiccation *Bacillus subtilis natto* is as follows: We first cultivated the Bacillus subtilis natto under optimal culture conditions in the initial stage of stable phase, followed by 3000 r/min centrifugation for 10 min and then the addition of different concentration and protective agent(3 % , 5 %, 10 % , 15 % , 18 % skim milk 1 %, 2 %, 3 %, 5 %, 7 % sucrose 1 %, 2 %, 3 %, 5 %, 7 % glucose 5 %, 8 %, 10 %, 12 %, 15 % trehalose, 0.5 %, 1.0 %, 1.5 %, 2.0 %, 2.5 % Gelatin 1 %, 2 %, 3 %, 4 %, 5 % glycero l 1 %, 2 %, 3 %, 4 %, 5 % VC) after washing the gathered thallus three times with normal saline to conduct single factor experiment for ascertain the effect of different protective agent on the viable organism of Bacillus subtilis natto. We subsequently chose the protective agent with high viable organism for the next optimizing process with orthogonal design to ascertain the optimum compomemts combination of protective agent. The orthogonal design parameters are given in Table 2.

RESULTS AND DISCUSSION

Ascertain of *Bacillus subtilis natto* optimal medium component

Our tests of the effects of five carbon sources glucose, sucrose, lactose, trehalose and starch on the proliferation of *Bacillus subtilis natto* showed that the highest OD_{600} (5.13) and the count of living *Bacillus subtilis natto* (8.8×10^9 cfu mL^{-1}) can be achieved when starch was used as the carbon source, followed by trehalose (OD_{600} was 4.59 and the count of living *Bacillus subtilis natto* was 5.60×10^9 cfu mL^{-1}) and sucrose (OD_{600} was 4.15 and the count of living *Bacillus subtilis natto* was 4.14×10^9 cfu mL^{-1}), with glucose as the least useful source (OD_{600} was 3.61 and the count of living *Bacillus subtilis natto* was 1.26×10^9 cfu mL^{-1}). Therefore, starch was selected as the carbon source for the proliferation medium. In testing the effects of six nitrogen sources yeast powder, peptone, beef extract, urea, NH_4Cl, and $NH_4{}_2SO_4$ on the proliferation of *Bacillus subtilis natto*, we found

the highest OD_{600} (3.35) and the count of live *Bacillus subtilis natto* (1.64×10^9 cfu mL^{-1}) can be reached when yeast powder was used as the nitrogen source, and the corresponding values were significantly lower when various other extracts and inorganic molecules were used as nitrogen source. We therefore chose yeast powder as the nitrogen source for proliferation medium. Our study further showed that OD_{600} (4.03) and the count of live *Bacillus subtilis natto* (3.07×10^9 cfu mL^{-1}) are at the maximal values when $MgSO_4$ was used as the inorganic salt, followed by NaCl (OD_{600} was 3.88 and the count of live *Bacillus subtilis natto was* 2.58×10^9 cfu mL^{-1}), $CaCl_2$ (OD_{600} was 3.05 and the count of live *Bacillus subtilis natto was* 1.65×10^9 cfu mL^{-1}), K_2HPO_4 (OD_{600} was 2.80 and the count of live *Bacillus subtilis natto was* 1.43×10^9 cfu mL^{-1}) and KH_2PO_4 (OD_{600} was 2.11 and the count of live *Bacillus subtilis natto was* 0.93×10^9 cfu mL^{-1}). We therefore chose $MgSO_4$ as the inorganic salt for the proliferation medium.

The $L_9(3^4)$ tests (Table 3) and F tests (Table 4) on the proliferation medium for *Bacillus subtilis natto* showed that the respective level of yeast powder and NaCl have significance effects in protecting the count of live *Bacillus subtilis natto* and the effect between peptone and starch have no significant effect. The multiple comparisons among the tested experimental factors (LSD) further showed that there are substantial differences between $A_3B_1C_3D_2$ and $A_2B_3C_1D_2$, with $A_3B_1C_3D_2$ and $A_1B_1C_1D_1$, and $A_3B_1C_3D_2$ was found to have the optimal portfolio with the configuration of yeast 0.7 %, peptone 0.5 %, starch 0.3 %, and NaCl 0.5 %.

Ascertain of *Bacillus subtilis natto* optimum

Table1. Factors and levels of the $L_9(3^4)$ orthogonal experiments

level	Factor A Yeast powder %	Factor B peptone %	Factor C starch(%)	Factor D NaCl %
1	0.3	0.5	0.3	0.3
2	0.5	1	0.5	0.5
3	0.7	1.5	0.5	0.7

Table 2. Factors and levels of the L9(34) orthogonal experiments

level	Factor A glycerol %	Factor B skim milk %	Factor C gelatin (%)	Factor D trehalose %
1	2	5	1.5	8
2	3	10	2	10
3	4	15	2.5	12

Table 3. The orthogonal experiment results of *Bacteria natto* proliferation medium optimization

No.	yeast powder(A)% 1	peptone (B)% 2	starch (C)% 3	NaCl (D)% 4	growth OD	
					I	II
1	0.3	0.5	0.3	0.3	3.97	3.46
2	0.3	1.0	0.5	0.5	5.31	4.5
3	0.3	1.5	0.7	0.7	4.88	4.91
4	0.5	0.5	0.5	0.7	5.20	4.85
5	0.5	1.0	0.7	0.3	4.33	5.44
6	0.5	1.5	0.3	0.5	6.79	5.42
7	0.7	0.5	0.7	0.5	6.44	6.86
8	0.7	1.0	0.3	0.7	6.41	5.66
9	0.7	1.5	0.7	0.3	5.11	5.68

culture condition

Regarding to the effect of different inorganic salt on the proliferation of *Bacillus subtilis natto*, our study showed that OD_{600} and the count of live *Bacillus subtilis natto* are the highest values when pH =7.0, the next is 7.506.506.0, and are the lowest values when pH =8.0. Our study of the effect of temperature on the proliferation of *Bacillus subtilis natto* showed that OD_{600} and the count of living *Bacillus subtilis natto* are the highest when temperature is 37 °C, followed by 35 °C, 33 °C and 39 °C, with the minimum values reached at 31 °C. Therefore, the optimal pH and temperature were chosen as 7.0 and 37 °C respectively.

The Growth behavior of *Bacillus subtilis natto* obtained by cultivating *Bacillus subtilis natto* under the optimal conditions and the OD_{600} values of the samples taken every 2 h are presented with Fig.1, from which one can find that *Bacillus*

Table 4. The anova table of *Bacteria natto* culture medium optimization experiment

Source of variation	SS	df	MS	F	F0.05	F0.01
interblock	0.1217	1	0.1217	0.4041	5.32	11.26
A	7.2536	2	3.6268	12.0447**	4.46	8.56
B	0.3999	2	0.2	0.664	4.46	8.56
C	0.4126	2	0.2063	0.6852	4.46	8.56
D	4.7132	2	2.3566	7.8263**	4.46	8.56
error	2.4089	8	0.3011			
Total variation	15.31	17				

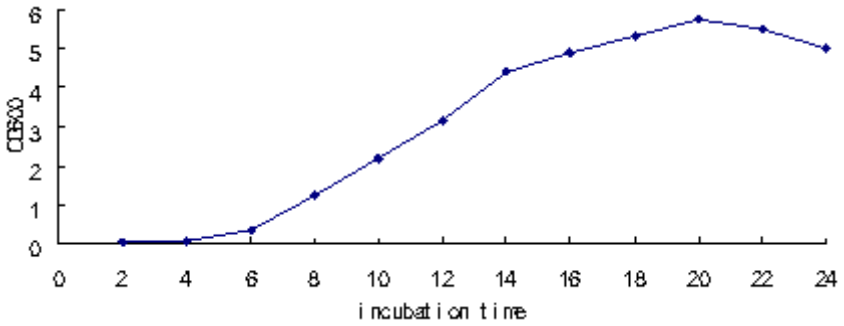

Fig. 1. The growth curve of *Bacillus natto*

Table 5. Multiple comparison between each experimental factor (LSD)

Combination	
$A_3B_1C_3D_2$	6.65 ± 0.30^{aA}
$A_2B_3C_1D_2$	6.11 ± 0.97^{abA}
$A_3B_2C_1D_3$	6.04 ± 0.53^{abA}
$A_3B_3C_2D_1$	5.40 ± 0.40^{abAB}
$A_2B_1C_2D_3$	5.03 ± 0.25^{bAB}
$A_1B_2C_2D_2$	4.91 ± 0.57^{bAB}
$A_1B_3C_3D_3$	4.90 ± 0.02^{bAB}
$A_2B_2C_3D_1$	4.89 ± 0.78^{bcAB}
$A_1B_1C_1D_1$	3.63 ± 0.23^{cB}

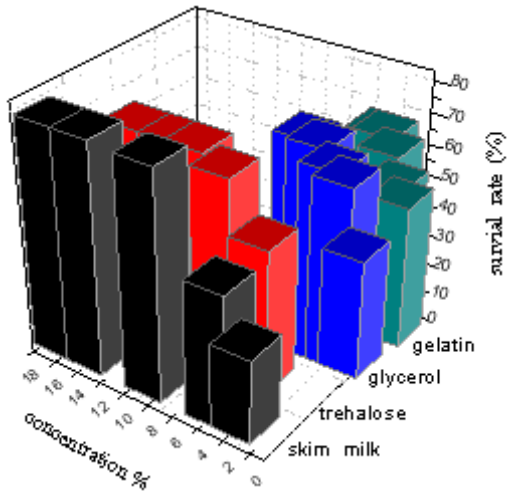

Fig.2. Freeze-dried *Bacillus natto* survival rate with different protective agent at different concentrations

Table 6. The results of $L_9(3^4)$ orthogonal experiment

No.	A(glycerol %)	B(skim milk %)	C(gelatin %)	D(trehalose %)	survival rate
1	2	5	1.5	8	0.2793
2	3	5	2	10	0.4197
3	4	5	2.5	12	0.4063
4	2	10	2.5	10	0.7214
5	3	10	1.5	12	0.6719
6	4	10	2	8	0.4766
7	2	15	2	12	0.826
8	3	15	2.5	8	0.4088
9	4	15	1.5	10	0.7627
K1	1.8149	1.0989	1.7105	1.1523	
K2	1.4949	1.8563	1.7115	1.8937	
K3	1.6391	1.9937	1.5269	1.9029	
k1	0.605	0.3663	0.5702	0.3841	
k2	0.4983	0.6188	0.5705	0.6312	
k3	0.5464	0.6646	0.509	0.6343	
R	0.1067	0.2983	0.0615	0.2502	

subtilis natto was under the growth adaptation period from 0~ 6 h, the logarithmic phase from 6~ 14 h, the growth stable phase from 14~ 20 h, and eventually reached the decline phase after 20 h. We selected the thallus in logarithmic phase as seed solution because of the exuberant multiplication capacity with high concentration and short incubation period, based on which the optimal growth age was determined to be 12 h. Around this optimal age, the viable count directly affects the quality of probiotic powder. *Bacillus subtilis natto* in the growth stable phase produced high number of spores, the viable count reached the highest value with a stable state, and the culture became stabilized. Therefore, the optimal harvest time was chosen as 20 h.

The effect of different protective agent on the lyophilization viable organism rate of *Bacillus subtilis natto*

We measured the freeze-dried Bacillus natto survival rate at different concentration of skim milk, trehalose, glycerol, gelatin, sucrose, glucose and VC, respectively. As can be seen in Fig. 2. (The results of sucrose, glucose and VC did not show), the optimal *Bacillus subtilis natto* survival rate can be reached at the concentration of 15 % skim milk, 10 % trehalose, 5 % glycerol, 2 % gelatin, with the corresponding largest survival rate of at 77 %, 71 %, 72 % and 69 % respectively. Single factor analysis showed that the skim milk, glycerol, trehalose and gelatin significantly affect the lyophilization viable organism rate of *Bacillus subtilis natto*. Orthogonal experiments (Table 6) further showed the differences of the factors in affecting the lyophilization viable organism rate of *Bacillus subtilis natto*. The sort order is B>D>A>C from large to small of the lyophilization viable organism rate, the optimal portfolio concentration is $A_1B_3C_2D_3$: glycerol 2 %, skim milk 15 %, gelatin 2 %, trehalose 12 %.

CONCLUSION

We were able to optimize culture conditions and prepare the powder forms for optimally retaining the beneficial effects of *Bacillus subtilis natto*, which facilitates the further development of *Bacillus subtilis natto*, into a potentially useful health-promoting probiotic product with a novel antithrombotic activity. The optimal survival rate of the powder forms may be maintained at the level of up to 77 %, which enables the potential probiotic product to produce its activities at sufficiently potent levels.

REFERENCES

1. Kanmani P, Satish, Kumar R, Yuvaraj N, Paari KA, Pattukumar V , Arul V, Probiotics and its functionally valuable products-a review. *Critical Reviews in Food Science and Nutrition*, 2013; **53**: 641-58.

2. Foligné B, Daniel C, Pot B, Probiotics from research to market: the possibilities, risks and challenges. *Current Opinion in Microbiology,* **16**: 284-92.
3. Foolad N, Brezinski EA, Chase EP, Armstrong A W, Effect of nutrient supplementation on atopic dermatitis in children: a systematic review of probiotics, prebiotics, formula, and fatty acids. *JAMA Dermatology,* 2011;**149**: 350-5.
4. Eigenmann PA, Evidence of preventive effect of probiotics and prebiotics for infantile eczema. *Current Opinion in Allergy Clinical Immunology*, 2013; **13**: 426-31.
5. Chattha K S, Vlasova A N, Kandasamy S, *et al* Divergent Immunomodulating Effects of Probiotics on T Cell Responses to Oral Attenuated Human Rotavirus Vaccine and Virulent Human Rotavirus Infection in a Neonatal Gnotobiotic Piglet Disease Model. *The Journal of Immunology*, 2013; **191**(5): 2446-2456.
6. Bordoni A, Amaretti A, Leonardi A, Boschetti E, Danesi F, Matteuzzi D, Roncaglia L, Raimondi, S, and Rossi M , Cholesterol-lowering probiotics: in vitro selection and in vivo testing of bifidobacteria. *Applied Microbiology and Biotechnology*, 2013; **97**: 8273-8281.
7. Samanya M, Yamauchi K, Histological alterations of intestinal villi in chickens fed dried Bacillus subtili var. natto. Comparative Biochemistry and Physiology-Part A: *Molecular & Integrative Physiology*, 2002; **133**:95-104.
8. Yanagisawa Y, Sumi H, Natto Bacillus Contains a Large Amount of Water-Soluble Vitamin K (Menaquinone 7). *Journal of Food Biochemistry* , 2005; **29**: 267-277.
9. Milner M, Makise K, Natto and its active ingredient nattokinase: A potent and safe thrombolytic agent. *Alternative & Complementary Therapies,* 2002; **8**:157-164.
10. Yokota T, Hattori T, Ohishi H, *et al*, The effect of antioxidant-containing fraction from fermented soybean food on atherosclerosis development in cholesterol-fed rabbits. LWT-*Food Science and Technology,* 1996; **29**:751-755.
11. Tsukamoto Y, Ichise H, Yamaguchi M, Prolonged Intake of Dietary Fermented Soybeans (Natto) with the Reinforced Vitamin K~2 (Menaquinone-7) Enhances Circulating gamma-Carboxylated Osteocalcin Concentration in Normal Individuals. *Journal of health science,* 2000; **46**: 317-321.
12. Fujita M, Ohnishi K., Takaoka S, *et al* Antihypertensive effects of continuous oral administration of nattokinase and its fragments in spontaneously hypertensive rats. *Biological and Pharmaceutical Bulletin;*2011; **34**: 1696-1701.
13. Shirole T, Sharma SL, Jagtap AG, PP100—Potential of nattokinase as an antithrombotic & fibrinolytic agent. *Clinical Therapeutics,* 2013: 47-47.
14. Hatzinikolaou-Kotsakou E, Kartasis Z, Tziakas D, Stakos D, Hotidis A, Chalikias G, Bourikas G, Hatseras D I , Clotting state after cardioversion of atrial fibrillation: a haemostasis index could detect the relationship with the arrhythmia duration. *Thrombosis Journal,* 2005: **3**:2.
15. Fujiwara K, Yamazaki M, Abe H *et al* Effect of Bacillus subtilis var. natto fermented soybean on growth performance, microbial activity in the caeca and cytokine gene expression of domestic meat type chickens. *The Journal of Poultry Science,* 2009; **46**:116-122.
16. Hong HA, Duc LH, Cutting SM, The use of bacterial spore formers as probiotics. *FEMS Microbiology Reviews,* 2005: **29**:813-835.
17. Senesi S, Ricca E, Henriques AO, *et al*, *Bacillus* spores as probiotic products for human use. Bacterial Spore Formers: Probiotics and *Emerging Applications,* 2004: 131-141.
18. Nagórska K, Bikowski M, Obuchowski M, Multicellular behaviour and production of a wide variety of toxic substances support usage of Bacillus subtilis as a powerful biocontrol agent. *Acte Biochimca Polonice,* 2007; **54**: 495-508.
19. Sun P, Wang J Q, Zhang H T, Effects of Bacillus subtilis natto on performance and immune function of preweaning calves. *Journal of Dairy Science,* 2010; **93**:5851-5855.