



Prophylactic properties of *Bacillus subtilis* in a bovine β -lactoglobulin sensitized mice model

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Abstract

Probiotics have gained considerable attention in recent years due to their biotechnological and immunomodulatory properties. The proteolytic activity and the protective effect of *Bacillus subtilis* isolated from Algerian goat milk preventing the development of allergic reactions were determined. On the base of SDS–PAGE analysis and the increase of detectable α -NH₂ groups, it can be concluded that the isolated isolate has high proteolytic activity. Oral administration of studied *Bacillus subtilis* DAF442 resulted in significantly lower titers of anti β -lactoglobulin IgG and IgE. This was correlated with a significant decrease in the anaphylactic response ex vivo as measured by reduced short circuit current (Isc) and epithelial conductance (G) and in vivo in mice. *Bacillus subtilis* DAF442 pretreatment promoted an important protection against changes in intestinal morphology induced by β -lactoglobulin. Hence it can be concluded that studied *Bacillus subtilis* strain with high proteolytic activity can modulate effectively the immune response and prevent allergic reactions against β -lactoglobulin.

Keywords Allergy · Bovine beta-lactoglobulin · Anaphylactic reaction · *Bacillus subtilis* · Proteolytic activity

Introduction

Over the last 150 years, accelerated social and environmental changes were triggered by the industrial revolution and altered profoundly the patterns of human activities, living standards, diet and the frequency and the gravity of infectious diseases. However, all these changes increased the severity of allergic disorders [1].

Cow milk is one of the first foods introduced into an infant diet and one of the most common causes of food allergy [2]. Virta et al. [3] have confirmed a significant association between cow milk allergy and pediatric inflammatory bowel disease.

Inflammation of the gut is associated with disruption of the gut microbiome [4–6]. According to Ivakhnenko and Nian'kovskii [7], the administration of probiotics to infants with atopic dermatitis and cow milk allergy can be a strategy applied in addition to the elimination diet and can be the fundamental treatment to alleviate clinical symptoms of the disease and to decrease gastrointestinal manifestations caused by cow milk allergy.

Most probiotic strains added to formula have been isolated from food or fecal infant microbiota. Although the use of probiotics is now recommended, rigorous scientific efforts

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are still required to validate specific strains with antiallergenic potential and lack of virulence factors for preventive and therapeutic applications [8].

Spore forming bacteria, such as *Bacillus* species, are interesting because their spores pass well by the acid barrier of the stomach and are stable for long periods in commercial and home made food products [9]. Bacilli, considered as gut commensals, have been used as probiotics for prophylaxis of human gastrointestinal disorders, to prevent recurrent respiratory infections or as the adjuncts during the ingestion of antibiotics [10, 11].

The number of studies using probiotics to select candidate strains able to prevent sensitization and cow milk allergy has increased over the past years, focusing mostly on their effects in animals. *Bacillus subtilis* has been identified as attractive hosts for the production of recombinant proteins and chemicals, which are widely used in food industries. Indeed, the incessant developments of fundamental and applied studies strengthen *Bacillus subtilis*' superior positions as the most dominant microbe in fermentation industries [12]. Engineered *Bacillus subtilis* strains have been used for the production of several commercial nutraceuticals and pharmaceuticals, including riboflavins [13] (poly- γ -glutamic acid [14, 15], 2,3-butanediol [16], and acetoin [17].

Until today, there are no results of studies on *Bacillus subtilis* in vivo effect on allergy against β -lactoglobulin (β -Lg).

In this context, the purpose of this article is to characterize a candidate *Bacillus subtilis* probiotic strain with prophylactic properties against cow milk allergy protein.

Materials and methods

The following products were purchased from Sigma-Aldrich (St Louis, MO, United States): Glycerol, Tris, sodium dodecyl sulfate (SDS) and Tween 80. The culture media MRS (de Man-Rogosa-Sharpe) was obtained from Biokar (Beauvais, France). Agar was purchased from Fisher Bioblock Scientific (Illkirch, France). Sodium chloride, sodium hydroxide and glycine were obtained from Panreac (Lyon, France). Acrylamide solution was obtained from Fisher Bioreagents (New Jersey, USA). 2-mercaptoethanol and Coomassie blue R250 were from Fluka (Germany). β -lactoglobulin, bovine serum albumin, sodium bicarbonate, goat anti-rabbit IgG peroxidase conjugate, OPD (diamine-orthophenylene), Tween 20, monobasic potassium phosphate, magnesium chloride, dihydrogen potassium phosphate, sodium chloride, potassium chloride, polyvinyl alcohol and hydrogen peroxide were purchased from Sigma (France). Alum [$\text{Al}(\text{OH})_3$], sulfuric acid (H_2SO_4) and calcium chloride were obtained from Merck (France). Materials and instruments from Labsystem and microtiter plate (Maxisorp; Nunc, Roskilde, Denmark) were

used for ELISA. All materials and instruments of Ussing chamber were purchased from Physiologic Instruments (San Diego, CA, USA).

Source of bacterial isolates

Algerian goat milk was used to isolate the studied *Bacillus subtilis* strain. Thirty-five milk samples were homogenized in sterile PBS pH 7.4 (in 1M: 0.136 M NaCl, 2.68 mM KCl, 1.76 mM KH_2PO_4 and 10.14 mM $\text{Na}_2\text{HPO}_4 \times 12 \text{ H}_2\text{O}$). One mL of each sample was submitted to a 10-fold serial dilution (also in physiological solution) (0.9% NaCl) and spread on the surface of de Man, Rogosa and Sharpe (MRS) agar plates. After incubation at 37 °C for 24 h, colonies were randomly selected and screened for their proteolytic activity.

Amplification and sequencing of 16S rDNA

Total DNA was extracted using the commercial kit Bacterial DNA (Omega bio-tek, USA), according to the manufacturer recommendations and was used as a template for 16S rDNA gene amplification. More details on the 16S rDNA procedure were reported by Dib et al. [18].

Proteolytic activity studied on UHT skim milk

Screening for proteolytic activity of the isolated *Bacillus subtilis* strain was performed using the UHT skim milk system from "Délisse". Collected strains were reactivated by inoculation (5%, v/v) into UHT skim milk and incubated overnight at 37 °C [19]. Controls were prepared by inoculation of equivalent volume of MRS broth in UHT skim milk. After 24 h, samples were taken and mixed with solubilization buffer for electrophoresis (50 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 3% 2-mercaptoethanol, 0.07% bromophenol blue) at a 1:10 volume ratio. Samples were heated at 100 °C for 3 min and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), according to Laemmli [20].

Measurement of the released α -NH₂ groups

The increase of the quantity of α -NH₂ terminal groups was determined at 0 h and after 24 h of fermentation, according to Doi et al. [21].

Experimental protocol

Animals and housing conditions

Thirty-nine female Balb/c mice (3–4 weeks old) were purchased from Pasteur Institute of Algiers (Algeria). The

animals were housed in polypropylene cages in controlled temperature (24 °C) and humidity (40–70%) conditions, and in a 12:12 h light–dark cycle. Mice were offered *ad libitum* tap water and a standard rodent pellets bovine milk-free diet (Bouzareah, Algiers). All animal experiments were started after 1 week of acclimation. All procedures were carried out in accordance with the current Algerian legislation covering the protection of animals.

Animals, oral gavage and sensitization protocol

The first group that received 0.3 mL of a saline solution (0.9% NaCl) for 18 days was used as a negative control ($n = 13$). This group did not receive any treatment. The second, third and fourth group ($n = 13$ each), corresponding to the alum group, positive control and the *Bacillus subtilis* DAF442 feeding group received, respectively, orally either 0.3 mL of saline solution (group 2, 3) or 0.3 mL of a pure culture of *Bacillus subtilis* DAF442 containing 10^8 CFU/mL (group 4) for 18 days (first period), according to Dib et al. [18]. At the end of the first period, the second group was immunized with 2 mg of alum [$\text{Al}(\text{OH})_3$] diluted in 0.1 mL of PBS. The third and fourth groups were immunized on days 0, 14, 21 and 28 by intraperitoneal injection with 10 μg of β -Lg (Sigma) adsorbed onto 2 mg of alum [$\text{Al}(\text{OH})_3$] in 0.1 mL of PBS [22]. On the 35th day the intestines and blood samples were collected for measure of electrophysiological parameters, intestinal villi length and immunological parameters.

Specific IgG and IgE antibodies responses

The potential protective impact of *Bacillus subtilis* was investigated in a Balb/c model of IgE-mediated hypersensitivity to cow milk [23, 24].

β -Lg-specific IgG and IgE levels were determined by a colorimetric Enzyme-Linked Immunosorbent Assay (ELISA) as described previously [18, 25].

Ex vivo study in Ussing chamber

Tissue preparation

Mice were anesthetized with sodium pentothal (5 mg/kg) and the intestinal segments were taken out and placed in ice-cold oxygenated Ringer solution (in mM: Na^+ 140 mM, K^+ 5.2, Ca^{++} 1.2, Mg^{++} 1.2 mM, Cl^- 120 mM, 25 mM, HPO_4 2.4 mM, H_2PO_4 0.4 mM). The jejunum was then opened along the mesenteric border.

Ussing chamber assay

The small intestine was washed to remove the intestinal contents and immediately mounted as sheets in Ussing chambers (exposed area 0.2 cm^2) as described previously [25].

Histological analysis

On the sacrifice day, the fragments of jejunum were fixed in 10 mM formalin, embedded in paraffin, cut into 3–5 μm and stained with haematoxylin and eosin for light microscopic examination. The measurement of the length of the jejunum villi was performed using an optical microscope equipped with a micrometer. Villi length was expressed in μm .

Assessment of clinical anaphylactic reactions

As described in a previous report [26], the hypersensitivity response was evaluated based on a scoring system. Thirty minutes after mice were challenged intraperitoneally with β -Lg (1 mg/mL) the hypersensitivity response was scored as follows: (0) no symptoms, (1) scratching nose and mouth, (2) swelling around eyes and mouth; pilo erection; reduced activity; higher breathing rate, (3) shortness of breath; activity after stimulation, (4) no activity after stimulation; shivering; muscle contractions, and (5) death by anaphylactic shock.

Statistical analysis

All data are expressed as mean $\pm$ standard error (SE). Statistical analysis was performed using Student's *t* tests or analysis of variance (ANOVA). *p* values < 0.05 were considered statistically significant, and *n* represents the number of independent experiments performed.

Results and discussion

Screening for proteolytic activity in UHT skim milk

SDS–PAGE profile showed that our isolate was able to hydrolyze milk proteins. Therefore, the proteolytic isolate with highest activity was selected (Fig. 1). The 16S rDNA amplification and sequencing indicated that the selected isolate presented 99% identity with the 16S rDNA sequences reported for *Bacillus subtilis* strains in GenBank database, our isolate was named *Bacillus subtilis* DAF442.

Measurement of the released α -NH₂ groups

After 24 h, *Bacillus subtilis* DAF442 degraded proteins as demonstrated by the increase in α -NH₂ groups

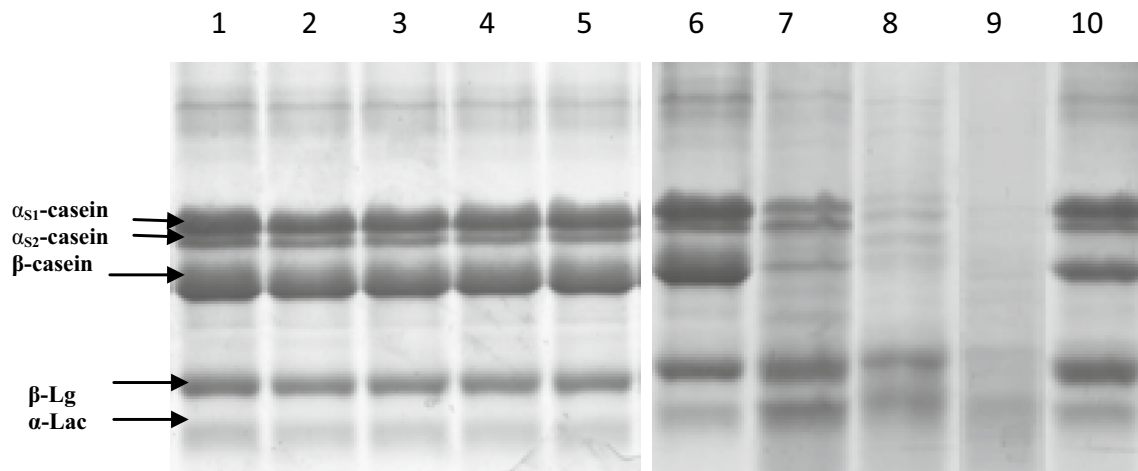


Fig. 1 SDS–PAGE of UHT skim milk treated with different LAB for 0 and 24 h at 37 °C. Lanes 1 and 6: control samples (skim milk without the proteolytic strain) incubated for 0 h and 24 h, respectively; lanes 2 and 7: skim milk incubated in the presence of strain 1 (*Enterococcus faecium*1) for 0 h and 24 h, respectively; lanes 3 and 8: skim

milk incubated in the presence of strain 2 (*Enterococcus faecium*2) for 0 h and 24 h, respectively; lanes 4 and 9: skim milk incubated in the presence of *Bacillus subtilis* DAF442 for 0 h and 24 h, respectively; lanes 5 and 10: skim milk incubated in the presence of strain 3 (*Enterococcus durans*)

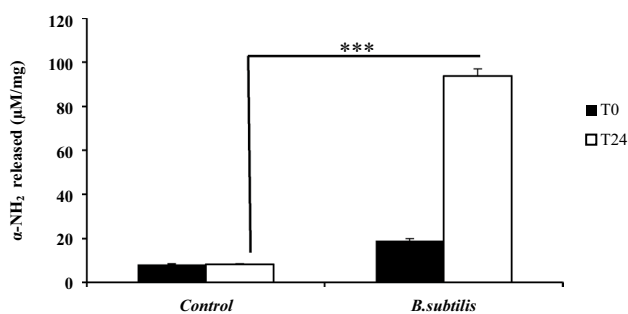


Fig. 2 Measurement of α -NH₂ groups released in cow milk samples after incubation at 45 °C (for 0 h and 24 h) in the presence of proteolytic strain *Bacillus subtilis* DAF442. Results are mean $\pm$ SE per group. The statistical significance of comparisons between the groups was assessed using the Student's *t* test. ****p* < 0.001 in the Student's *t*-test, *Bacillus subtilis* at T 24 h vs *Bacillus subtilis* at T0

(93.7 ± 3.17 μ M/mg) compared to fermented milk at T0 (19.02 ± 0.93 μ M/mg) and UHT skim milk at T24 (control) (8.24 ± 0.34 μ M/mg) (Fig. 2) (*p* < 0.001). These results agree with what was observed in SDS–PAGE. The increase in the α -NH₂ groups found in our study is due to the ability of *Bacillus subtilis* DAF442 to produce a wide range of enzymes. Indeed, these strains are the most important producers of commercial extracellular enzymes [27], including proteases with technologic use such as subtilisin. These proteases are able to intensively hydrolyze proteins [28]. Hence they have many various industrial applications [29, 30]. It has been reported that the production of proteases by *B. subtilis* strain 38 was dependent on bacterial growth [31]. Gaustevora et al. [32], found

that a highest level of extracellular protease activity was observed after 24 h incubation period.

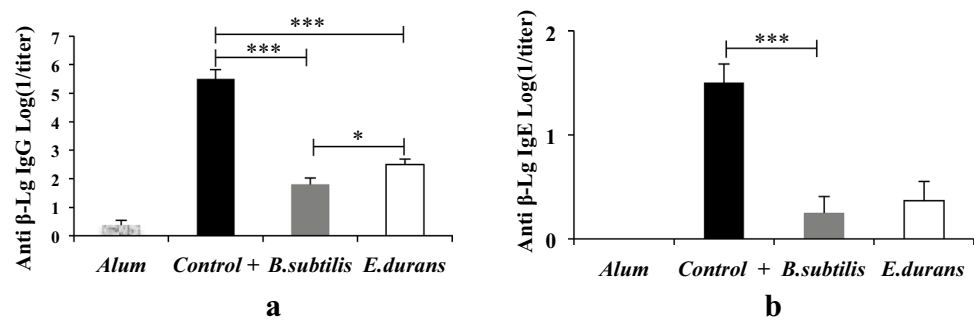
Bacillus genus gained importance on industrial scale [33]. Indeed, the use of *B. subtilis* in the food industry is recommended by the US FDA as one of the GRAS (Generally Recognized As Safe) organisms [34, 35].

Effect of *Bacillus subtilis* administration on IgG and IgE anti- β -Lg levels

As shown in Fig. 3, a significant decrease in serum titers of β -Lg-specific IgG and IgE was observed in the *Bacillus subtilis* DAF442 feeding group (*p* < 0.001), as compared with β -Lg-sensitized group (positive control). These results agree well with the study of Zhou et al. [36], which showed that oral administration of recombinant *Bacillus subtilis* spores significantly increased peanut specific IgA and decreased peanut specific IgE. Additionally, Huang et al. [37], indicated that *Bacillus* strains could stimulate systemic and intestinal IFN- γ production in mice. This ProTh1 cytokine plays a role in the host defense against several infectious diseases, including viral infections and has a variety of immune functions, such as stimulation of macrophages and natural killer cells [38, 39].

Indeed, it was shown that probiotics administration dramatically alters the gut microenvironment by promoting a change in the local microflora and in cytokine secretion, and can potentially modulate the Toll-like receptors and the proteoglycan recognition proteins of enterocytes, leading to the activation of dendritic cells and a Th1 response. The resulting stimulation of Th1 cytokines can suppress Th2 responses [40, 41].

Fig. 3 Effect of *Bacillus subtilis* DAF442 administration on IgG (a) and IgE (b) titers of mice, in response to β -Lg sensitization. Data are mean $\pm$ SE (standard error) with $n = 8$ (***) $p < 0.001$



Ex vivo Ussing chamber experiments

The Ussing chamber is one of several techniques used to assess the effects of bacteria on ion channels and barrier function [42]. Our results regarding this subject are presented in Fig. 4. The challenge with β -Lg induced an important increase in *I*_{sc} in the isolated jejunal segment of the positive control in comparison with the results observed in the negative control (Fig. 4a). The *I*_{sc} increased significantly after 4 min of β -Lg challenge ($75.95 \pm 12.62 \mu\text{A cm}^{-2}$). The elevation in *I*_{sc} was associated with a significant increase of the epithelium conductance of jejunal segment of the positive control ($29.07 \pm 5.03 \text{ mmho cm}^{-2}$) (Fig. 4b). In contrast, the baseline short-circuit current and conductance values observed in the intestines of negative control and *Bacillus subtilis* DAF442-fed groups, after a serosal β -Lg challenge, remained unchanged (*I*_{sc}: $63.12 \pm 10.36 \mu\text{A cm}^{-2}$; G: $19.47 \pm 2.97 \text{ mmho cm}^{-2}$, respectively). Our previous studies demonstrated that trans epithelial Cl^- was the ion involved in the secretory response of mice small intestine to antigen challenge [24]. Saïdi et al. [43] and El Mecherfi et al. [44], suggested that a local anaphylactic reaction occurs when β -Lg interacts as an allergen with the intestinal immune system of β -Lg-sensitized mice. It has been reported that mast cell mediators such as histamine and prostaglandins can act *via* specific receptors on the intestinal epithelium to initiate the secretion of chloride ions [45].

The inhibitory effect of probiotics on mast cells degranulation was likely responsible for the decrease of the local anaphylactic reaction. *L. casei* DN-114 001/CNCMI-1518 was observed to provide protection from anaphylaxis and arthritis by inhibiting mast cell and basophil degranulation in murine models of acute allergic and autoimmune inflammation [46]. It has been reported that *Bifidobacterium longum* broke down histamine [47]. Authors of this study suggested that this probiotic up regulated genes that create tight junction molecules, which are the molecules that hold the cells of our intestinal lining together, preventing the development of “leaky gut”. Interestingly, Oksaharju et al. [48], showed that the expression of the gene encoding HRH4 (a histamine receptor 4) was down regulated by GG

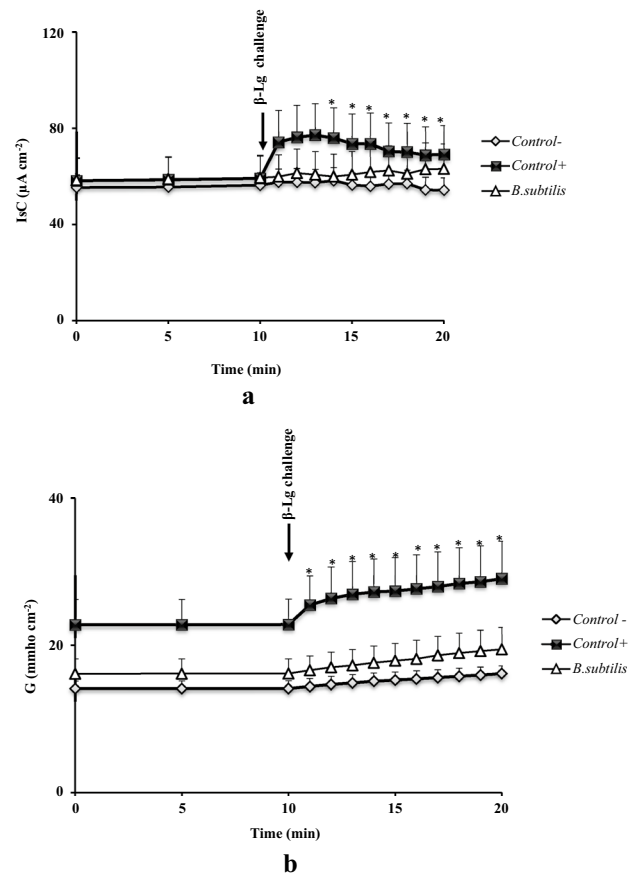


Fig. 4 Effect of mice intestine challenge with bovine β -Lg on time course of short-circuit current (*I*_{sc}) (a) and transepithelial conductance (*G*_t) (b) measured in Ussing chamber. Control-: negative control; Control+: β -Lg-sensitized mice challenged to β -Lg; *B. subtilis* DAF442: β -Lg-sensitized mice that have been challenged with β -Lg. β -Lg (60 $\mu\text{g/mL}$) were added to the serosal buffer of chambers containing tissues 10 min after stabilization phase. Values represent mean $\pm$ SE (standard error) (* $p < 0.05$ compared with C-group; $n = 8$ mice per group)

and Lc705, suggesting another inhibitory function for these probiotics in mast cell activation in the context of allergy.

Animal models of colitis are associated with alterations in colonic fluid and electrolyte transport, *I*_{sc}, and ion

transporter expression and these alterations appear to be modulated by probiotics [49, 50].

Histology

Changes in intestinal morphology were examined by light microscopy. The morphological appearance of the intestinal mucosa section in the negative control (control-)—long thin villi with an unstratified epithelium composed of tall cells with a flat striatum containing regular nuclei at the base and corresponding to the enterocytes is presented in Fig. 5a. In contrast, the villi of positive control mice suffered a very pronounced atrophy. At the level of the chorion, inflammatory infiltration is very intense in this group. However, the appearance of the intestinal mucosa of *Bacillus subtilis* DAF442-fed mice showed good protective effect on the intestinal villi and reduced villi damage.

As shown in Fig. 6, the length of villi in *Bacillus subtilis* DAF442-fed mice was significantly bigger than that in the positive control ($p < 0.001$). In contrast, the average length of villi of the negative control group ($54.66 \pm 0.87 \mu\text{m}$) and the group of mice fed with *Bacillus subtilis* DAF442 ($52.73 \pm 0.79 \mu\text{m}$) was basically similar.

Our results agree with those of Sanchez et al. [51], who showed that probiotic *Bacillus* strain maintained mucosal integrity and stimulated the systemic or mucosal immune responses. *Bacillus* species have been shown to enhance epithelial gut barrier functions [52, 53] and stimulate cytokine [53, 54] and sIgA in humans [55].

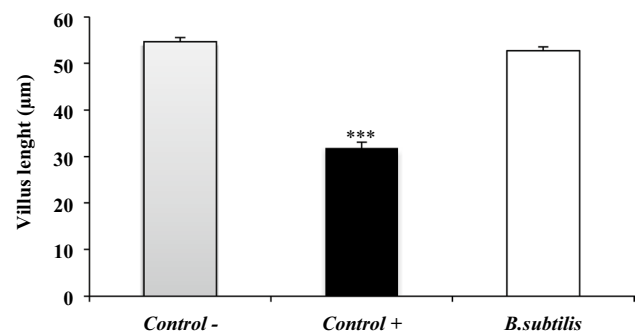


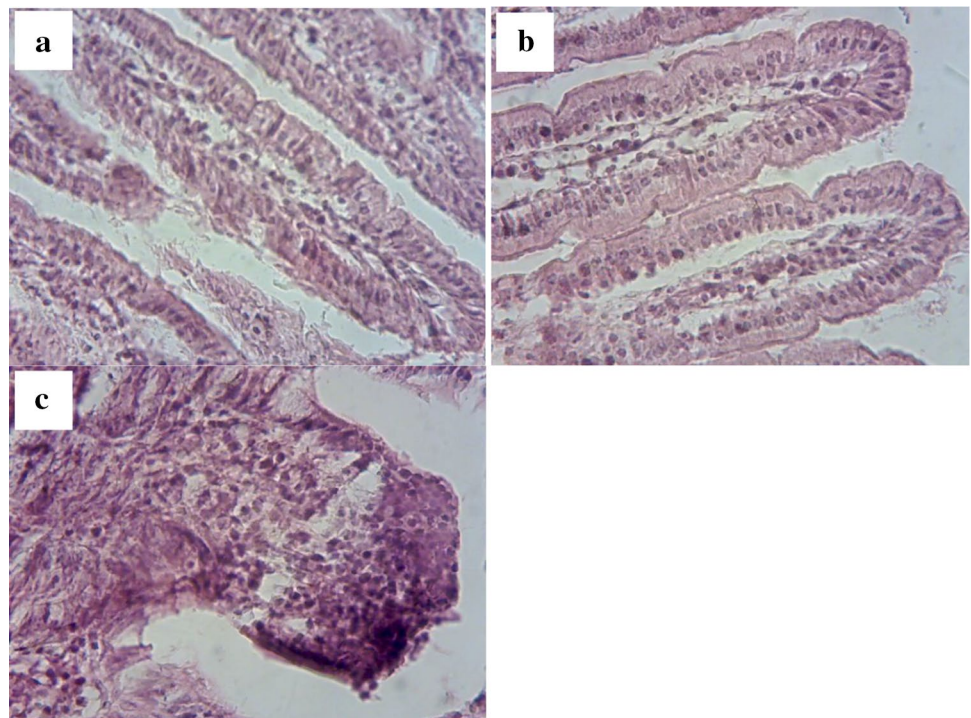
Fig. 6 Bacterium ingestion and β -Lg sensitization effect on villus length in jejunum fragments in experimental groups compared to non sensitized mice (control-). Values represent mean $\pm$ SE (standard error) (***) $p < 0.001$ compared with C-group; $n = 8$ mice per group

Assessment of clinical anaphylactic reactions

As determined by the scoring system described previously, our results showed that challenging mice with β -Lg produce a pronounced allergic reaction. However, the clinical manifestations of the *Bacillus subtilis* DAF442-fed group were significantly less important than those from the β -Lg-sensitized group (Fig. 7).

Several groups reported that select probiotic strains of *B. subtilis* relieved the symptoms associated with antibiotic-associated diarrhea and irritable bowel syndrome in human patients. However, the mechanism of the protection was not well established [56, 57]. Recently, the World

Fig. 5 Light microscopy ($\times 40$) showing intestinal villi stained with hematoxylineosin. Jejunal tissues were obtained from negative control (a), *B. subtilis* DAF442 (b) and β -Lg-sensitized mice (c), $n = 8$ mice per group



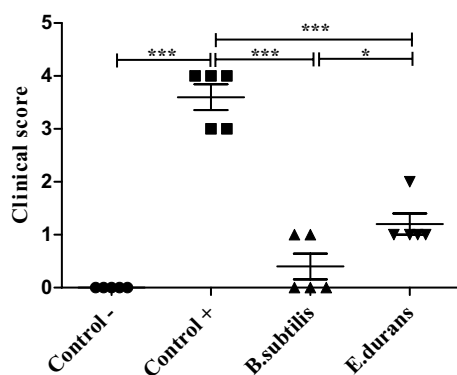


Fig. 7 Clinical scores for hypersensitivity reactions responses in control, *B. subtilis* DAF442, *E. durans* and β -Lg-sensitized mice after intraperitoneal challenge with β -Lg ($n=5$). Scoring system: 0, no symptoms; 1, scratching and rubbing around the nose and head; 2, puffiness around the eyes; pillar erect, reduced activity, and/or decreased activity with decreased activity with increased respiratory rate; 3, wheezing, labored respiration, cyanosis around the mouth and the tail; 4, no activity after prodding, or tremor and convulsion; 5, death

Allergy Organization stressed that there was a likely net benefit from using probiotics resulting primarily from prevention of eczema during pregnancy, breastfeeding and in infants at high risk of developing allergy [58].

Conclusion

The obtained results showed clearly that *B. subtilis* strain could modulate the systemic immune responses and intestinal functions preventing or managing allergic diseases in mice and support the use of probiotics as anti-allergic therapies in humans.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements The experiments described in this study comply with the current Algerian legislation covering the protection of animals.

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