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ANTIVIRAL AGENTS

Anti-influenza Activity of a *Bacillus subtilis* Probiotic Strain

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ABSTRACT Among *Bacillus* bacteria, *B. subtilis* is the species that produces the most antimicrobial compounds. In this study, we analyzed the activity of probiotic strain *B. subtilis* 3 against the influenza virus. The antiviral effect of this strain has been demonstrated *in vitro* and *in vivo*. A new peptide, P18, produced by the probiotic strain was isolated, purified, chemically synthesized, and characterized. Cytotoxicity studies demonstrated no toxic effect of P18 on Madin-Darby canine kidney (MDCK) cells, even at the highest concentration tested (100 μg/ml). Complete inhibition of the influenza virus *in vitro* was observed at concentrations of 12.5 to 100 μg/ml. The protective effect of P18 in mice was comparable to that of oseltamivir phosphate (Tamiflu). Further study will assess the potential of peptide P18 as an antiviral compound and as a promising candidate for the development of new antiviral vaccines.

KEYWORDS *Bacillus subtilis*, antiviral peptide, influenza virus, probiotics

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Probiotic bacteria attract much attention from scientists and physicians as important tools for correcting the microbiota and maintaining the health status of the host. Different mechanisms are known for the beneficial effects of probiotics, such as the production of antimicrobial substances (1, 2), an upregulation of immune response and downregulation of inflammatory response (3), stimulation of mucus secretion (4) and dendritic cell maturation (5), an improvement of gut mucosal barrier function, and modulation of host gene expression (6). Probiotic bacteria show efficacy in the treatment and prevention of different gastrointestinal conditions, including inflammatory bowel disease, irritable bowel syndrome, necrotizing enterocolitis (7), acute diarrhea (8), and antibiotic-associated diarrhea (9, 10). New applications of probiotics are focused on conditions influenced by altered gut microbiota, such as metabolic syndrome, obesity, atopic dermatitis, and mood disorders (11). Probiotic bacteria were tested for the prevention and treatment of viral infections. Different strains of *Bifidobacterium* and *Lactobacillus* demonstrated beneficial effects in treating rotavirus infection in animals and humans (12–14). Some orally administered probiotic bacteria stimulate respiratory immunity and increase resistance to viral respiratory tract infections. Infected mice receiving oral or intranasal treatment with *Lactobacillus* strains have reduced signs of influenza infection, lower virus titers in the lungs or nasal washings, increased body weight during infection, and increased survival (15). The effectiveness of probiotic bacteria in respiratory tract infections was confirmed in clinical trials in children, adults, and elderly individuals (15). Although animal studies and clinical trials demonstrate antiviral activities of specific probiotic bacteria, the mechanisms of these effects are unclear.

Our previous study showed beneficial effects of probiotic strain *Bacillus subtilis* 3 in the prevention and treatment of bacterial infections in animal models (16, 17) and in clinical trials (8, 9). The antibacterial activity of this probiotic strain was associated with

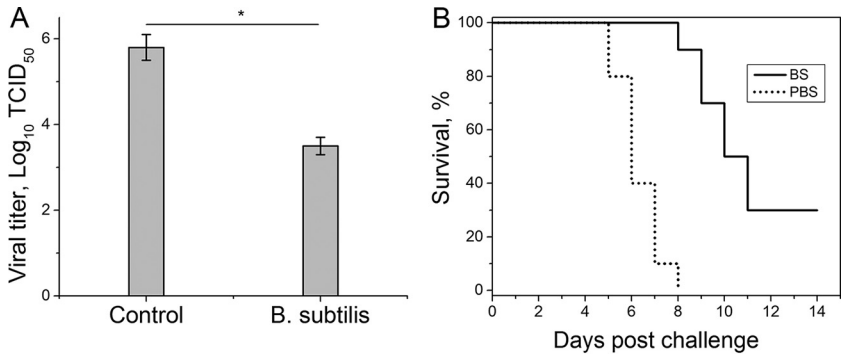

FIG 1 Antiviral activity of *B. subtilis in vitro* and *in vivo*. (A) MDCK cells were inoculated with the influenza virus following treatment with a *B. subtilis* strain (10^6 CFU per well). Viral titers were analyzed by titration in MDCK cells. *, $P < 0.05$. (B) Mice (10 per group) received a single dose of *B. subtilis* (10^7 CFU per mouse) or PBS by oral gavage. After 24 h, both groups of animals were infected intranasally with influenza virus. Survival was monitored for up 14 days postinfection.

the production of antibiotic aminocoumacin with a broad spectrum of pathogen suppression (18) and with the stimulation of immune resistance of the host (19). Previously, we found that some bacteria can produce peptides mimicking hemagglutinin of the influenza virus (20). The mimicking of proteins provides a way to find new therapeutic compounds for the treatment of pathogens (21). Bacteria of the *Bacillus* genus are considered as a promising source in the search for new inhibitory substances because of their capacity to produce a large number of antimicrobial peptides (22). This study aims to evaluate the antiviral activity of the *B. subtilis* 3 probiotic and to characterize the compounds responsible for this activity.

RESULTS

Antiviral activity of *B. subtilis* 3 *in vitro* and *in vivo*. A previous cytotoxicity study showed no toxic effect of *B. subtilis* UCM B-5007 on Madin-Darby canine kidney (MDCK) cells at concentrations of 10^7 to 10^9 CFU ml^{-1} (data not shown). The incubation of the influenza virus with *B. subtilis* bacteria resulted in a significant inhibition of virus replication (Fig. 1A). The *B. subtilis* strain was also effective in preventing influenza infection in animals. Mice challenged with a lethal dose of influenza virus began to die on day 5 and all were dead on day 8 postchallenge. Pretreatment with *B. subtilis* protected 30% of the animals from a deadly infection (Fig. 1B).

Isolation and characterization of *B. subtilis* peptides. Extracted samples of peptides were fractionated by high-performance liquid chromatography (HPLC), and 20 fractions were obtained (Fig. 2A). Each fraction was analyzed by an enzyme-linked immunosorbent assay (ELISA) using antibodies against *B. subtilis* peptides. The highest activity of interaction with anti-mimetic peptide antibodies was found in fraction 11 (Fig. 2B). Further analysis of this fraction by electrophoresis showed the presence of three proteins with molecular masses of 55.1, 44.1, and 22.6 kDa (Fig. 2C). Fraction 3, with a molecular mass of 22.6 kDa and which expressed the highest activity with anti-*B. subtilis* peptide antibodies, was further analyzed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Fig. 3). The main proteins identified in this fraction are presented in Table 1. An analysis of the obtained protein sequences against the NCBI database revealed the full homologies of these proteins with known peptides (Table 1). One of the peptides, TVAAPSVFIFPPSDEQLK, was found to be a component of influenza A neutralizing antibody. Thus, this peptide was selected for chemical synthesis to assess its possible antiviral activity by *in vitro* and *in vivo* assays.

Characterization and validation of the chemically synthesized peptide TVAAP SVFIFPPSDEQLK. Peptide identification was confirmed by MALDI-TOF MS analysis (Fig. 4). Cytotoxicity studies demonstrated no toxic effect of this peptide, named P18, on MDCK cells (Fig. 5A), even at the highest concentration tested (100 μg/ml). No cell

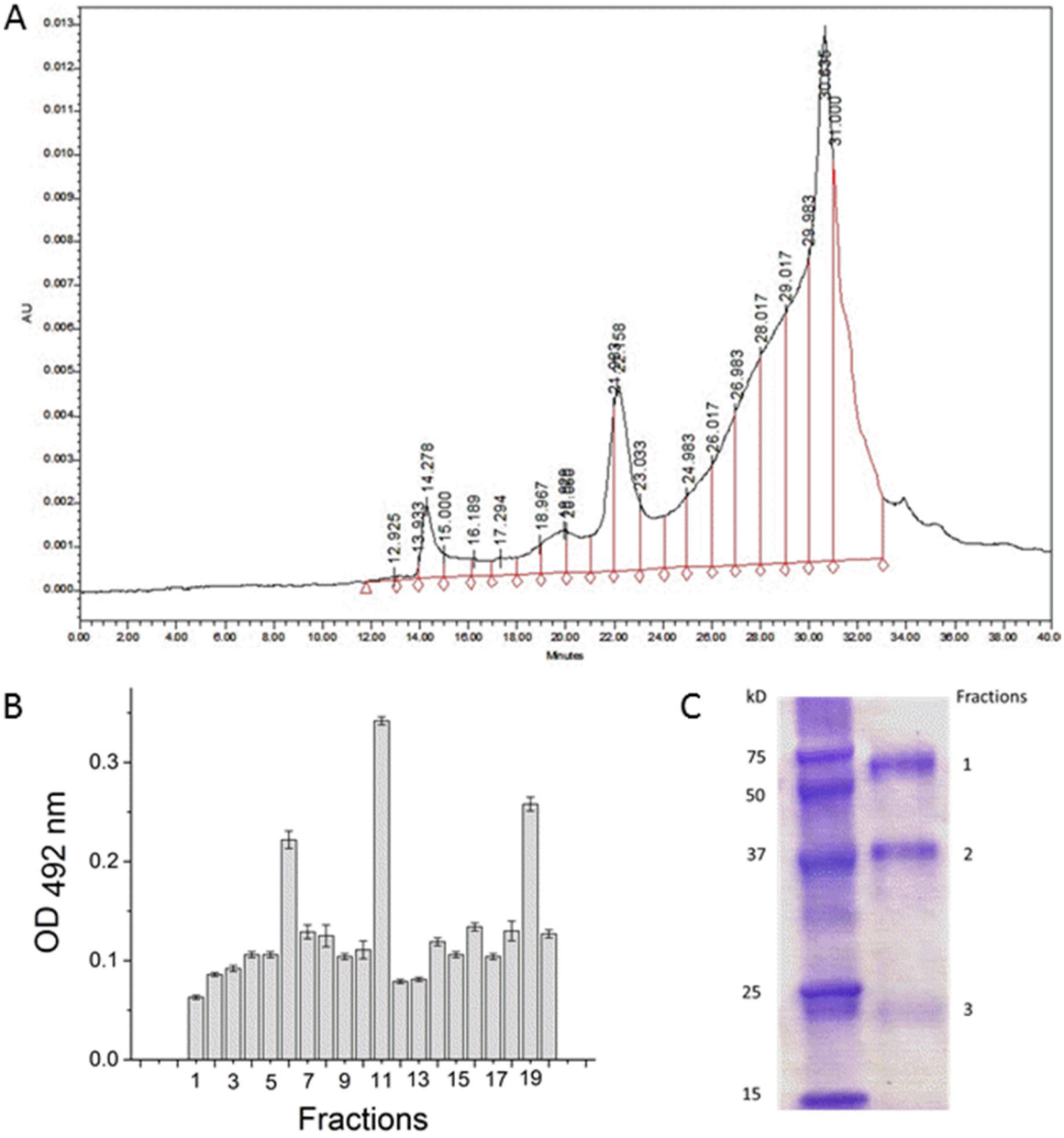

FIG 2 Isolation and characterization of *B. subtilis* peptides. (A) HPLC separation of the protein fractions from *B. subtilis* 3. Peptides (2 mg/ml) were applied to a TSKgel DEAE-5PW column and the fractions were collected by elution with NaCl solutions of increasing ionic strength (0.01 and 1 M) with 0.01 M Tris-HCl (pH 7.4). (B) Protein fraction interactions with antibodies to peptides from *B. subtilis* 3. Each collected peptide fraction was dried and analyzed by ELISA using antibodies against *B. subtilis* peptides. Fraction 11 showed the highest activity of interaction with antibodies. (C) Gel electrophoresis analysis of fraction 11. Homogeneity of the fraction was analyzed in 12% polyacrylamide together with molecular mass standards and stained with Coomassie blue.

degeneration or other morphological changes were found after microscopic analysis of the monolayers. Thus, for further experiments *in vitro*, the peptide was used in concentrations ranging from 3.1 to 100 μg/ml. Peptide P18 was evaluated for its ability to inhibit influenza virus A/FM/1/47 (H1N1) *in vitro*. Complete inhibition of the virus was observed at concentrations of 12.5 to 100 μg/ml (Fig. 5B). Peptide P18 significantly reduced the viral titer at a concentration of 6.2 μg/ml. Other tested concentrations of P18 (3.1 and 1.6 μg/ml) were not effective in virus inhibition.

Efficacy of P18 *in vivo*. The activity of the P18 peptide for the prevention and treatment of influenza infection was tested in mice. Based on the results of P18 efficacy against the influenza virus from *in vitro* studies, a concentration of 12.5 μg/ml was used

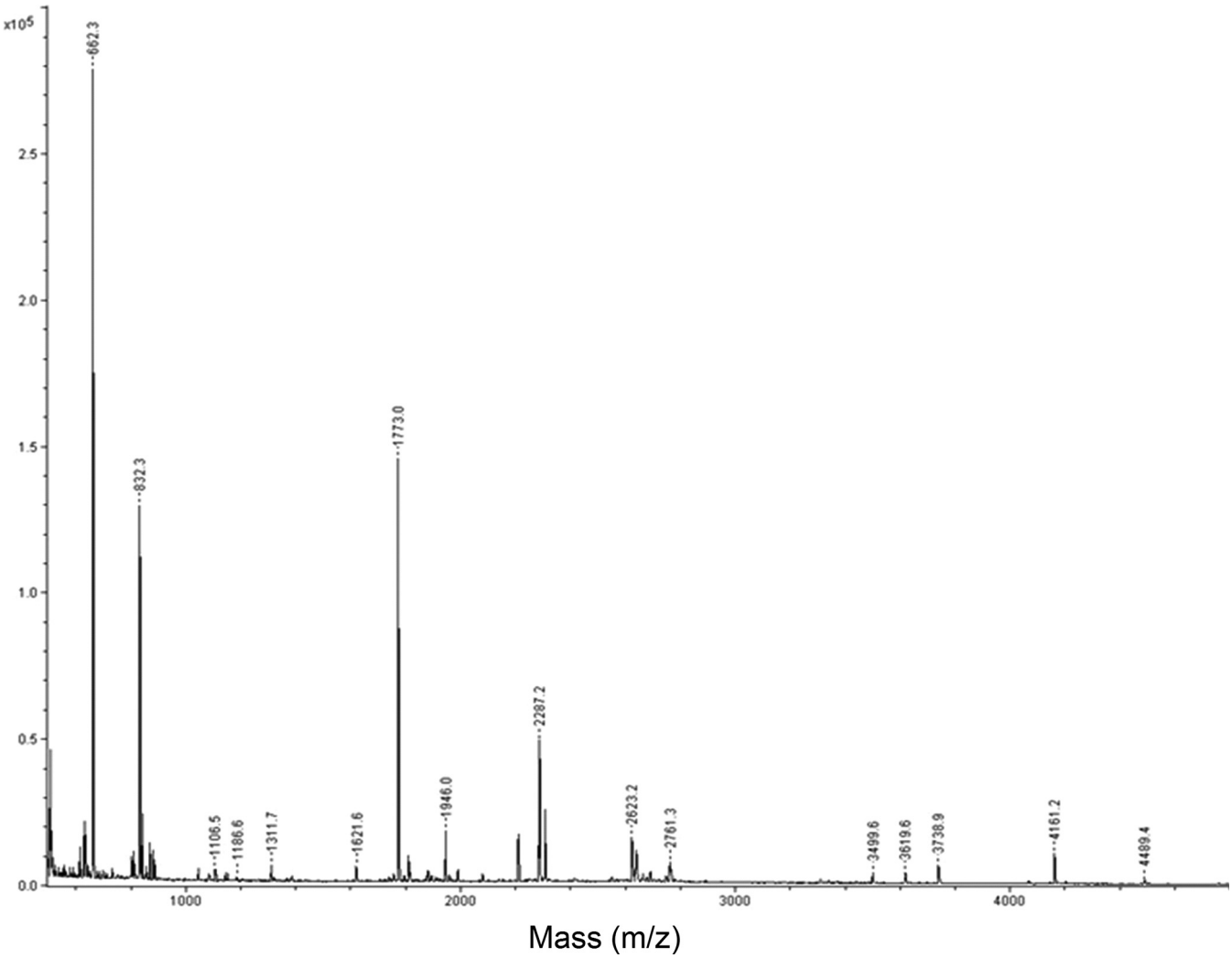

FIG 3 MALDI-TOF mass spectrum of fraction 3 (from Fig. 2C). Spectrum was acquired using the instrument in reflectron mode and calibrated using a standard peptide mixture.

for animal treatments. Oseltamivir phosphate (Tamiflu) was used as a positive control in these experiments. All mice treated with phosphate-buffered saline (PBS) and infected with a virus exhibited clinical signs of infection (ruffled coat, hunched posture, slowed movement, shivering, labored breathing, anorexia, little to no movement, and paralysis and were moribund) and died on day 8 of the experiment (Fig. 6). The treatment of animals with P18 and oseltamivir phosphate before the infection (Fig. 6A)

TABLE 1 Characterization of proteins identified by MALDI-TOF MS

Molecular mass (kDa)	Peptide sequence	Source	Accession no.
17.73	SGTASVVCLLNNFYPR	Chain A, crystal structure of the non-neutralizing HIV antibody	3MNV_A (PDB)
18.11	DIQMTQSPSSLSASVGDR	Immunoglobulin kappa light chain	BAC01680.1 (GenBank)
19.46	TVAAPSVFIFPPSDEQLK	Chain H, structure of influenza A neutralizing antibody	3ZTJ_H (PDB)
22.87	VDNALQSGNSGESVTEQDSKDSTYSLSSTLTLSK	Chain L, structure of the antibody 7b2 that captures HIV-1 virions	4YDV_L (PDB)
37.38	VQWKVDNALQSGNSQESVTEQDSK	Chain L, crystal structure of broadly neutralizing antibody	4NM4_L (PDB)
41.61	EAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK	Chain L, crystal structure of highly potent anti-HIV antibody	3RPI_L (PDB)

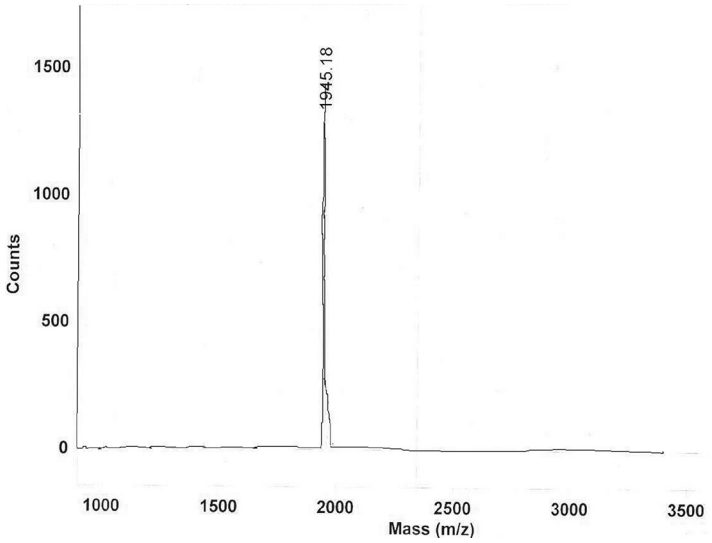

FIG 4 MALDI-TOF mass spectrum of the chemically synthesized peptide P18. Peptide TVAAPSVFIFPPSD EQLK (P18) was synthesized at the highest available purity (90%).

resulted in significant protection in comparison to the control (30% and 80%, respectively). A significant efficacy of P18 was observed in animals that were treated after virus inoculation (Fig. 6B). A single oral application of P18 protected 80% of mice; the rate of survival after oseltamivir phosphate treatment was 70%. None of the surviving animals showed any visible signs of influenza. The viral titers in the lungs were significantly lower in mice pretreated with oseltamivir phosphate than in the controls and those treated with P18 (Fig. 6C). However, the treatment of mice with P18 after infection was more effective in the elimination of the virus than oseltamivir phosphate (Fig. 6C).

DISCUSSION

Influenza is still a significant health problem that results in high morbidity and mortality in the United States and worldwide (23). The therapeutic approaches used for the prevention and treatment of influenza infection include amantadine, neuraminidase inhibitors (24), and vaccines (25). However, some of the adverse effects of the antiviral compounds used and the drawbacks of vaccination indicate the need for improved therapies and preventive treatment of influenza infection. Different alterna-

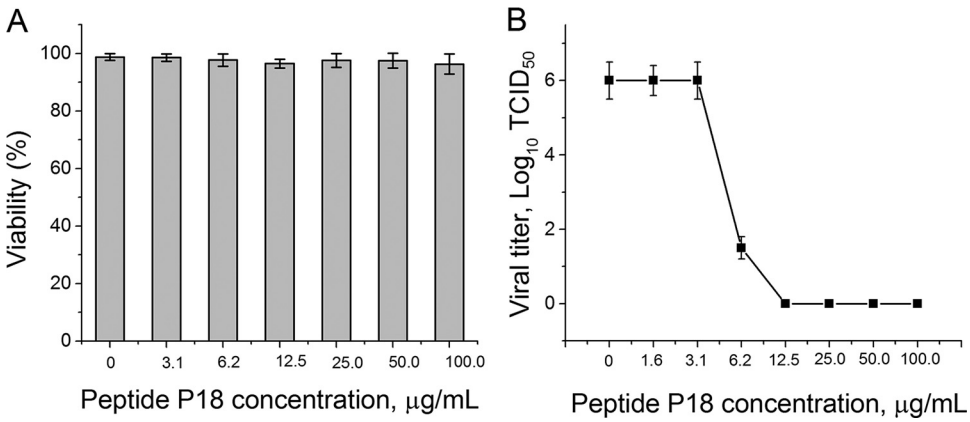

FIG 5 Characterization of peptide P18. (A) Cytotoxicity of P18 peptide was analyzed by MTT assay in MDCK cells. Viability of cells in the control wells (no peptide added) was scored as 100%. Other samples were normalized to this value. (B) Monolayers of MDCK cells were infected with the influenza virus. After 1 h of incubation, serial dilutions of peptide P18 were added to the wells and no peptide was added to the control wells. Viral titers were analyzed 3 days postinfection by titration in MDCK cells.

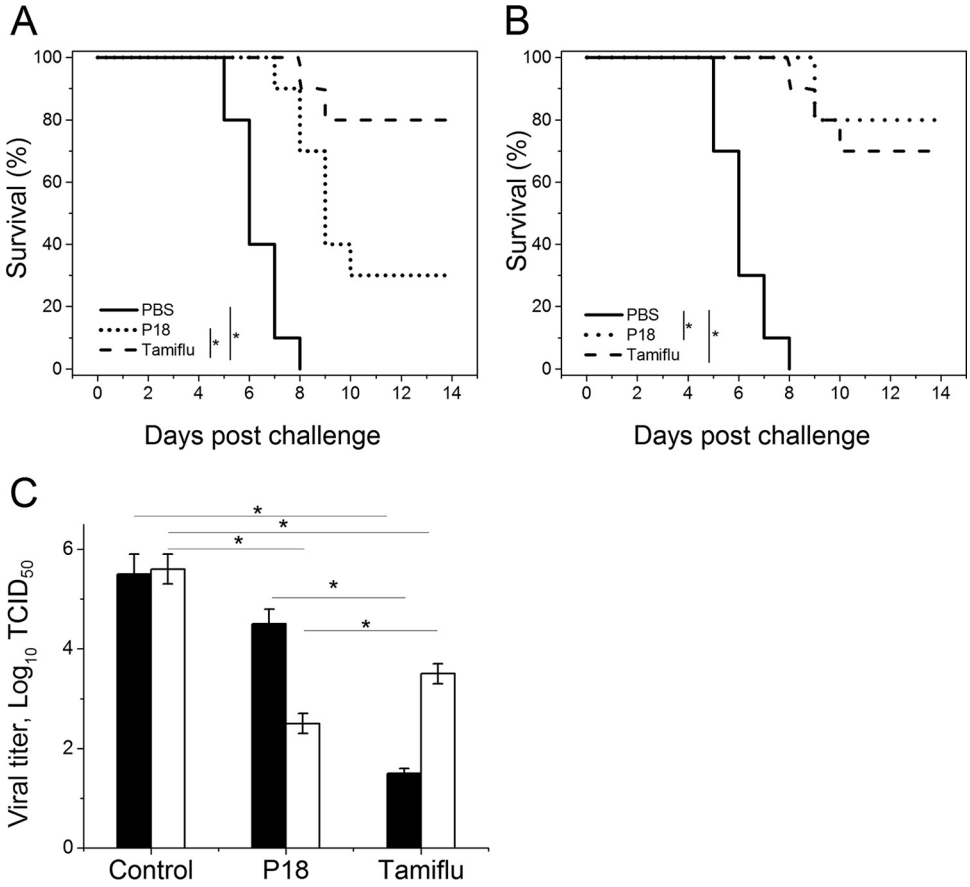

FIG 6 Efficacy of peptide P18 *in vivo*. Mice were treated with PBS, P18, or oseltamivir phosphate before infection with influenza virus (A) or after infection (B). On day 4 postinfection, the lungs from three mice in each group before infection (solid bars) and postinfection (open bars) were removed, and viral titers were evaluated in each supernatant by $TCID_{50}$ analysis in MDCK cells (C). *, $P < 0.05$.

tives have been tested to combat the influenza virus. For example, China's Ministry of Health recommended using extracts from some natural herbs that have beneficial immunomodulatory effects (26). More and more scientific data suggest that probiotic bacteria can be effectively used to decrease the risk or duration of influenza symptoms. The beneficial effects of *Lactobacillus* and *Bifidobacterium* strains have been shown in animal models and in clinical trials (15).

Our study was aimed to evaluate the antiviral efficacy of a *B. subtilis* probiotic strain. The bacteria significantly inhibited influenza virus replication *in vitro* and increased the survival rate of mice challenged with the virus after a single dose of probiotic bacteria. The protection of mice against the influenza virus by oral pretreatment with *Lactobacillus rhamnosus* M21 was reported by Song et al. (27). The authors orally treated animals with lactobacilli for 2 weeks before challenging them with a lethal dose of virus. In another study, a 2-week treatment using killed spores of *B. subtilis* PY79 protected mice from influenza infection (28). The rate of protection with a single dose of live *B. subtilis* 3 cells was comparable with results presented by Song et al. (27) for *L. rhamnosus* M21.

Previously, we found that some bacteria can produce peptides mimicking hemagglutinin of the influenza virus (20). Thus, we decided to analyze whether the mechanism of *B. subtilis* 3 antiviral activity is associated with the production of antiviral peptides. Mimetic peptides were isolated from the culture medium after 24-h growth of the probiotic strain. HPLC analysis revealed 20 fractions that were further tested with specific antibodies. The fraction with the highest activity was analyzed by MALDI-TOF MS. Only one peptide from the six identified was found to be a component of influenza

A neutralizing antibody. This peptide (P18) was chemically synthesized and tested *in vitro* and in animals. Complete inhibition of virus *in vitro* was observed at concentrations of 12.5 to 100 μg/ml. *Bacillus* bacteria are one of the largest producers of antimicrobials. More than 795 different antibiotics, mostly of a peptidic nature, were identified from these bacteria (29). These peptide antibiotics have a wide spectrum of activity, and some of them demonstrated antiviral activity. Thus, Torres et al. (30) characterized the virucidal effect of the bacteriocin subtilosin, produced by *B. amyloliquefaciens*, against herpes simplex virus. Surfactin and biosurfactants produced by *B. subtilis* inhibit a broad spectrum of viruses, including Semliki Forest virus, herpes simplex virus, suid herpesvirus, vesicular stomatitis virus, simian immunodeficiency virus, feline calicivirus, and murine encephalomyocarditis virus (31). The authors postulated that the antiviral action of these compounds was due to a physicochemical interaction of the membrane-active surfactant with the virus lipid membrane.

High antiviral *in vitro* activity of surfactin and fengycin was also confirmed by other scientists (32). The testing of antiviral compounds produced by *Bacillus* bacteria have only been performed *in vitro*.

We studied the efficacy of synthesized peptide P18 for prophylaxis and treatment of influenza infection in animals. Pretreatment of mice with P18 resulted in a significant improvement in survival rate, but oseltamivir phosphate was more effective, showing protection in 80% of mice. The therapeutic efficacy of P18 was highly pronounced compared with that of oseltamivir phosphate activity (in 80% and 70% of mice protection, accordingly). A novel 20-amino-acid peptide (EB) derived from the signal sequence of fibroblast growth factor 4 demonstrated a protective effect in mice after intranasal treatment 6 h postinoculation with influenza virus (33). Intranasal administration of EB peptide resulted in a significant delay in mortality and clinical signs in treated mice, but all mice were dead on day 11 postinfection. An increased survival of mice infected with influenza virus was found after intravenous treatment with a recombinant human serum albumin-thioredoxin 1 fusion protein (HSA-Trx) (34). The tested protein had no effect on pulmonary virus replication in influenza-infected mice. The authors assumed that the therapeutic value of HSA-Trx results from inhibiting inflammatory cell responses and suppressing the overproduction of nitric oxide (NO) in the lungs. In our experiments, pretreatment and posttreatment of mice with peptide P18 significantly decreased influenza virus titers in the lungs of mice. The results obtained demonstrated that peptide P18 inhibits the replication of influenza virus *in vitro* and *in vivo*. To our knowledge, it is the first report about the isolation and identification of a *Bacillus* peptide with strong activity against the influenza virus. Peptide P18 has complete homology with the structure of influenza A neutralizing antibody. We can speculate that the mechanism of antiviral activity of this peptide is in its similarity to neutralizing antibodies. This is confirmed by the results of *in vivo* testing, where P18 showed a higher protective effect for animals in the treatment mode of application. The study of molecular mimicry provides a novel concept for the development of new antiviral drugs and vaccine development (35).

In summary, we showed the activity of probiotic strain *B. subtilis* 3 against the influenza virus *in vitro* and in animals. A new peptide, P18, produced by the probiotic strain was isolated and characterized. P18 inhibited the influenza virus *in vitro*, and its protective effect in mice was comparable with that of oseltamivir phosphate. Further study will assess the potential of peptide P18 as an antiviral compound and as a promising candidate for the development of new antiviral vaccines.

MATERIALS AND METHODS

Ethics statement. All animal procedures were approved by the internal review board of the Gromashevsky Institute of Epidemiology and Infectious Diseases, National Academy of Medical Sciences of Ukraine, Kiev, Ukraine.

Animals. Four- to 6-week-old BALB/c mice (BMS, Kiev, UA) were used. The animals were housed under specific-pathogen-free conditions and were acclimatized for 2 days to the room temperature

(21 ± 1°C), humidity (50% ± 5%), and lighting (12-h day/12-h night), with free access to water and food.

Cell cultures. Madin-Darby canine kidney (MDCK) cells were obtained from the Ivanovsky Institute of Virology (Moscow, Russia). MDCK cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (Corning, Manassas, VA, USA) at 37°C in an incubator with 5% CO_2.

Microorganisms. *Bacillus subtilis* 3 (UCM B-5007) was obtained from the Ukrainian Collection of Microorganisms (Kiev, Ukraine) and propagated on nutrient agar (HiMedia, Mumbai, India). Influenza virus A/FM/1/47 (H1N1) was obtained from the Ivanovsky Institute of Virology (Moscow, Russia) and adapted to mice by serial lung passage. Influenza virus was propagated in MDCK cells. After 72 h, cells infected with virus were harvested and stored at −80°C. Viral titers were determined by 50% tissue culture infective dose ($TCID_{50}$) analysis. The influenza virus was titrated in mice prior to use. The doses necessary to achieve 50% mortality (LD_{50}) 10 days after infection were determined after intranasal infection of 20 BALB/c mice per group.

Peptide isolation. *B. subtilis* strain UCM B-5007 was cultivated in nutrient broth (HiMedia, Mumbai, India) on a rotary shaker (400 rpm) at 37°C for 24 h. After centrifugation at 4,000 × *g* for 15 min at 4°C, 200 ml of the supernatant was treated with 96% ethanol at a ratio of 1:1.5 for 20 h at 4°C. The obtained sample was centrifuged at 5,000 × *g* for 15 min and the supernatant was discarded. The pellet was resuspended in 40 ml of 60% ethanol, centrifuged at 5,000 × *g* for 15 min, and washed with PBS. This procedure was repeated three times. The probe in PBS was heated at 100°C for 10 min in a water bath and then centrifuged at 5,000 × *g* for 15 min. The protein content in supernatants was analyzed by a Lowry assay. Supernatants were used as the peptides for the immunization of animals and further purification.

Antibodies against *B. subtilis* peptides. Mice were immunized with isolated peptides by a subcutaneous injection of 100 μg in 0.1 ml of PBS with a subsequent boost injection after 4 weeks. Total IgG immunoglobulins were purified by protein A Sepharose affinity chromatography (36).

Purification of peptides. Peptides were analyzed and purified by Beckman System Gold HPLC (Beckman Coulter GmbH, Krefeld, Germany). Peptides (2 mg/ml) were applied to a TSKgel DEAE-5PW column (7.5 mm by 75 mm). The fractions were collected by elution with NaCl solutions of increasing ionic strength (0.01 and 1 M) with 0.01 M Tris-HCl (pH 7.4). Each collected peptide fraction was dried, and the most active fractions were identified by ELISA using antibodies against *B. subtilis* peptides. The most active fraction was analyzed in 12% polyacrylamide together with molecular mass standards and stained with Coomassie blue, as described elsewhere (37).

In-gel enzymatic digestion was performed according to published protocols (38, 39). Briefly, protein bands were excised from the gel, stained with Coomassie blue, and cut into cubes (1 mm by 1 mm). The gel cubes were destained by incubating with 100 μl of 40% acetonitrile in 0.05 M ammonium bicarbonate for 30 min at 37°C with occasional vortexing. Pure acetonitrile (500 μl) was added to the samples, and they were incubated at room temperature until destaining was complete. The acetonitrile was removed and gel pieces were dried. The dried gel pieces were covered with digestion buffer (12 μg/ml of trypsin in 0.05 M ammonium bicarbonate) and allowed to proceed 12 h at 37°C. The extraction buffer (50% [vol/vol] acetonitrile, 1% [vol/vol] trifluoroacetic acid [TFA], 49% H_2O) was added to achieve the approximate ratio of 1:2 between volumes of the digest and the extraction, and samples were incubated for 20 min at room temperature.

Sample preparation for matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) was performed using the dried-droplet method according to a published protocol (40). Briefly, 1 μl of the sample was pipetted to the sample plate, mixed with 0.3 μl of matrix, and air dried. The matrix solution was prepared by mixing a saturated solution of 2,5-dihydroxybenzoic acid in 50:50 H_2O to acetonitrile with 0.1% TFA.

Determination of amino acid sequence of the purified peptide. The molecular mass and the amino acid sequence of the purified peptide was determined with MALDI-TOF MS (Ultraflex II, Bruker, Germany) as described elsewhere (40). Spectra were acquired using the instrument in reflectron mode and calibrated using a standard peptide mixture.

Database search and peptide identification. The identification of peptides was performed using a Mascot program (Matrix Science, USA) searching against the NCBI database.

Peptide synthesis. The selected peptide sequence TVAAPSVFIFPPSDEQLK was synthesized by Metabion GmbH (Planegg, Germany) at the highest available purity (90%) using an automated synthesizer (Applied Biosystems 433A). The synthesizer was programmed for a standard fluorenylmethyloxycarbonyl (Fmoc)-based solid-phase peptide synthesis protocol. After the completion of the synthesis, the peptide was cleaved from the resin with trifluoroacetic acid, purified by reversed phase HPLC, and analyzed by MALDI mass spectrometry.

ELISA. Wells of a 96-well ELISA dish were coated overnight at 4°C with protein fractions (2 μg/ml in bicarbonate-carbonate buffer, pH 9.6), were blocked with sterile fat-free milk for 1 h at room temperature, were reacted with anti-peptide antibodies for 1 h at room temperature, and were washed 4 times with buffer (PBS plus 0.05% Tween 20). Goat anti-mouse IgG (gamma) antibody (human serum adsorbed and peroxidase labeled; Kirkegaard &Perry Laboratories, Gaithersburg, MD) was added and incubated 30 min at 37°C, and the dish was washed 6 times and tetramethylbenzidine (TMB) and hydrogen peroxide in citrate buffer (pH 5.0) was added. The reaction was stopped by 2 M H_2SO_4. The optical density was measured at 492/630 nm. All samples were tested in triplicates.

Cytotoxicity test. The cytotoxicity of the P18 peptide was analyzed by an MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay on MDCK cells in 96-well plates. Cells were seeded

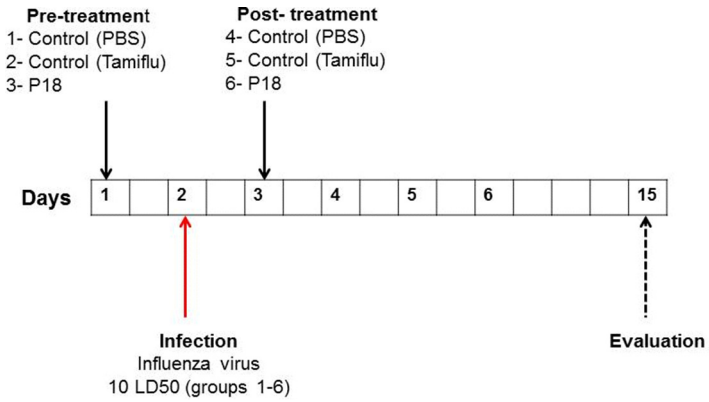

FIG 7 Experimental design. Animals were allocated to six groups (13 mice in each group): (i) control, PBS pretreatment; (ii) control, oseltamivir phosphate pretreatment; (iii) P18 pretreatment; (iv) control, PBS posttreatment; (v) control, oseltamivir phosphate posttreatment; (vi) P18 posttreatment. One day before the infection with virus, PBS pretreatment control mice received 0.2 ml PBS *per os*; oseltamivir phosphate pretreatment control mice received oseltamivir phosphate (1 mg/kg) by gavage. P18 pretreatment animals received P18 (0.1 mg/kg) by oral gavage. Mice from the posttreatment groups were infected with influenza virus and, after 24 h, were treated with PBS, oseltamivir phosphate, or P18.

at 1×10^4 cells per well and incubated for 24 h at 37°C in a humidified 5% CO_2 incubator. Increasing amounts of peptide (3.1 to 100 μg/ml) were added to each well. No peptide was added to the control wells. After 5 days of treatment with the peptide, MTT (25 μl at 5 mg/ml in PBS) was added to each well and the plate was incubated for 3 h at 37°C in a humidified chamber. After the incubation, the MTT solution was removed to stop the reaction and 150 μl of dimethyl sulfoxide was added to each well. The optical density was measured at 570 nm using a microplate reader (Bio-Tek, Winooski, VT). The viability of cells in the control wells was scored as 100%. Other samples were normalized to this value. To confirm the MTT results, the monolayers were also observed microscopically to estimate rounding and other morphological changes in comparison to those of control cells.

Antiviral activity *in vitro*. Monolayers of MDCK cells in 96-well plates were washed three times with DMEM containing 2 μg/ml of tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK) trypsin and were infected with the influenza virus (100 IU/well). After 1 h of incubation at room temperature, nonadsorbed virus was discarded and medium without serum was added to each well. Serial dilutions of peptide or the *B. subtilis* strain (10^6 CFU per well) were added to the wells and incubated at 37°C for 3 days in the incubator with 5% CO_2. After incubating, viral titers in the medium were analyzed by titration in MDCK cells.

Antiviral activity *in vivo*. (i) *B. subtilis* UCM B-5007. Two groups of mice (10 mice in each group) were used in this study. One group received a single dose of *B. subtilis* (10^7 CFU in 0.1 ml PBS per mouse) by oral gavage, while mice of the second group received 0.1 ml PBS per mouse. After 24 h, animals from both groups were infected intranasally with influenza virus (25 μl for each nostril, $10LD_{50}$). Survival rates were monitored for up to 14 days postinoculation.

(ii) Peptide P18. Animals were allocated into six groups (13 mice in each group): (i) control, PBS pretreatment; (ii) control, oseltamivir phosphate pretreatment; (iii) P18 pretreatment; (iv) control, PBS posttreatment; (v) control, oseltamivir phosphate posttreatment; (vi) P18 posttreatment. One day before the infection with virus, mice in the PBS pretreatment control group received 0.2 ml PBS *per os*, and mice in the oseltamivir phosphate pretreatment control group received oseltamivir phosphate (1 mg/kg) by gavage. Animals in the P18 pretreatment group received P18 (0.1 mg/kg) by oral gavage. Mice from the posttreatment groups were infected with influenza virus and, after 24 h, were treated with PBS, oseltamivir phosphate, or P18 (Fig. 7). To induce the infection, mice were lightly anesthetized by isoflurane inhalation and intranasally inoculated with influenza virus (25 μl for each nostril, $10LD_{50}$). Mice were observed for mortality for 14 days. On day 15, mice were euthanized by CO_2 asphyxiation. On day 4 postinfection, the lungs from three mice in each group were removed, weighed, and homogenized in cold DMEM. The homogenates were centrifuged at 3,200 $\times$ *g* for 5 min at 4°C, and viral titers were evaluated in each supernatant by $TCID_{50}$ analysis in MDCK cells.

Statistical analysis. All results are presented as means and standard deviations. The differences between groups were analyzed by two-sample *t* tests or one-way analysis of variance (ANOVA) followed by Bonferroni's correction. Survival analysis was performed using Kaplan-Meier curves and a log-rank test. The significance level was set at 0.05 to define statistical significance. Statistical calculations and graph plotting were being carried out using Microcal Origin version 9.0 (Northampton, MA) and 2010 Microsoft Excel.

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