Fish and Shellfish Immunology 95 (2019) 606–616

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Oral administration of transgenic biosafe microorganism containing antimicrobial peptide enhances the survival of tilapia fry infected bacterial pathogen

Bing-Chang Lee[a], Chun-Wei Hung[b], Cheng-Yung Lin[a], Chen-Han Shih[c], Huai-Jen Tsai[a,*]

[a] Institute of Biomedical Sciences, Mackay Medical College, New Taipei City, Taiwan, ROC
[b] Institute of Molecular and Cellular Biology, National Taiwan University, Taipei, Taiwan, ROC
[c] Department of Biosystems Engineering, The University of Arizona, Tucson, AZ, USA

ARTICLE INFO

Keywords:
Lactoferricin
Electroporate
Transgenic strain
Tilapia
Antibiotics

ABSTRACT

To develop an alternative to conventional antibiotics used in the aquaculture and livestock industries, we employed *Bacillus subtilis,* considered a biosafe microorganism, to express the degradable antimicrobial peptide lactoferricin. An expression plasmid pP43-6LFBII-GFP, in which reporter GFP cDNA was fused downstream of lactoferricin cDNA driven by an endogenous constitutive P43 promoter was electroporated into *B. subtilis*, followed by regeneration and cultivation. The putative colonies harboring plasmids were primarily screened by PCR-amplification of lactoferricin cDNA. Four transformants which were stable inheritance of plasmid containing lactoferricin cDNA included strains T1, T4, T7 and T13. Based on Western blot and Southern blot analyses, we found that transgenic strains T1 and T13 not only highly expressed exogenous recombinant lactoferricin, but also exhibited more stable inheritance of plasmids with 931 and 647 copies per cell, respectively. In the antibacterial *in vitro* experiment, the bactericidal activity of each microliter of cell lysate from transgenic strains T1 and T13 (5×10^8 CFU) for *Escherichia coli* was equivalent to 56 and 53 ng of Ampicillin dosage, respectively, while for *Staphylococcus epidermidis*, the equivalency T1 and T13 was 154 and 130 ng of Ampicillin dosage, respectively. Equivalencies of bacterial activity for *Vibrio parahaemolyticus* and *Edwardsiella tarda* followed suit. In the antibacterial *in vivo* experiment, we oral-in-tube fed tilapia fry (*Oreochromis mossambicus* X *O. niloticus*) with cell lysate from transgenic strain T1 and T13 individually. After 1-h of incubation, we immersed these treated fish fry in a water tank containing *E. tarda* (5×10^{11} CFU) for a 5-hr bacterial challenge. After one month cultivation, an average survival rate of 63 and 67% was observed after having fed the fish fry with transgenic strains T1 and T13, respectively. However, the average survival rate of fish fry fed with *B. subtilis* WT strain and transgenic strain T19 without expressing recombinant lactoferricin reached only 5 and 9%, respectively. These data indicate that the survival of fish fry infected by the intestinal pathogen tested could be significantly enhanced by feeding transgenic *B. subtilis* containing antibacterial peptide. Therefore, we suggest that this strategy could be applied to both aquaculture and livestock industries to (i) reduce the dependency on conventional antibiotics during seasonal outbreaks and (ii) eliminate the problem of antibiotic resistance.

1. Introduction

Antibiotics have been used worldwide in medicine, agriculture and aquaculture. However, over the past two decades, increased overuse of antibiotics may have led to the creation of antibiotic-resistant pathogens, calling for the development of effective countermeasures [1]. Overuse of antibiotics may also result in undue influence on hosts. Reports on their safety during pregnancy are concerning, and the emergence of resistant strains is on the rise [2,3]. Unlike traditional antibiotics, natural cationic bovine lactoferrin is a defense peptide that exists in the host immune system, displaying both anti-microorganism and immunoregulation properties [4]. Therefore, bovine lactoferrin could serve as an alternative natural medicine to treat and prevent infections [5].

Bovine lactoferrin is a transferrin that belongs to a multifunctional glycoprotein family, consisting of approximately 700 amino acid residues with a molecular weight of 80 kDa. It is an antimicrobial iron-binding protein secreted in milk, saliva, tears and pancreatic secretion

* Corresponding author.
E-mail address: hjtsai@ntu.edu.tw (H.-J. Tsai).

https://doi.org/10.1016/j.fsi.2019.10.052
Received 9 September 2019; Received in revised form 16 October 2019; Accepted 27 October 2019
Available online 01 November 2019
1050-4648/

of cattle [4]. Bovine lactoferrin displays an acute function to reduce inflammatory response and regulate innate immune response to prevent septic shock and death [6–8]. Mice administered orally with bovine lactoferrin demonstrated a reduced chance of gastrointestinal infection [9]. Furthermore, Actor et al. reported that bovine lactoferrin could activate the host immune system, promoting the production of mature T-cell precursors and converting them into competent T helper cells to, in turn, induce B cells to differentiate into functional antigen-presenting cells [10]. Interestingly, both *in vitro* and *in vivo* analyses demonstrated that bovine lactoferrin can inhibit the growth of bacterial, fungal and viral pathogens [4,11,12]. The 17–41 amino acid residues at the N-terminus of bovine lactoferrin constitute a functional domain which exhibits antimicrobial activity, named lactoferricin [13].

Since lactoferricin is positively charged by Arginine and hydrophobic Tryptophan, it has a cationic and amphipathic property. Therefore, lactoferricin can effectively bind to bacteria, disrupt the target cell membrane, and alter membrane potential and pH gradients, resulting in impeding the growth of bacteria and fungi [14,15]. In addition, lactoferricin can enhance host immunity to prevent fungal infection [16]. Animal studies also demonstrated that lactoferrin in protecting mice against *Candida albicans* infection [17]. Interestingly, after oral administration of lactoferricin, bacterial and parasitic infections may be reduced and prevented in mice [18,19]. Taken together, it is well documented that lactoferricin peptide can serve as a highly potent therapeutic agent to treat and prevent pathogenic infections [20,21]. Bacteria, yeasts, and mammalian cell lines are common biomaterials used to produce exogenous proteins. To develop recombinant lactoferricin against pathogen-related infections through hosts, a recombinant lactoferrin was established *via* baking yeasts, and it successfully exhibited *in vitro* antimicrobial activity [22]. Similar to this strategy, a transgenic pig, which expresses recombinant lactoferrin, was developed, and transgenic milk-fed mice were protected against infection from gastrointestinal pathogens [23]. However, generation of cell lines, yeasts and transgenic organisms is time-consuming and costly. Although *Escherichia coli* is the most commonly used bacterium to produce recombinant proteins, it is an environmental pollutant [24]. Therefore, developing an environmental and economical biosafe microorganism with rapid growth for expression of recombinant lactoferricin remains a challenge.

Belonging to the *Bacillales* order, *B. subtilis* is a Gram-positive bacterium that is commonly found in soil and the gastrointestinal tract of ruminants and humans. Cells are rod-like in shape with length ranging from 4 to 10 μm and width from 0.25 to 1.0 μm [25]. This bacterium was originally named as *Vibrio subtilis* [26] and then was renamed as *B. subtilis* [27]. *B. subtilis* is considered a facultative anaerobe, yet it was further reclassified as an obligate aerobe [28]. Similar to other *Bacillus* members, *B. subtilis* can form endospores, enabling it to survive under extreme environmental conditions for a decade, such as low humanity, high salt concentration, extreme pH, radiation and chemical solvent [29]. *B. subtilis* is a nonpathogenic bacterium and generally recognized as a safe organism. It can secrete proteases, and it has been widely used in a variety of animal feed products [30]. Therefore, *B. subtilis* is an ideal biomaterial with which to produce recombinant bovine lactoferricin and overcome the drawbacks of other approaches, as noted above.

B. subtilis has been used as a host cell to express exogenous cellulose [31], yet the protease originated from it may degrade the exogenous proteins, resulting in a decrease in overall productivity. Therefore, the search for improved recombinant lactoferricin expression in *B. subtilis* continues, and this might be achieved by using a highly stable expression vector, potent promoter and optimization of culture medium. For example, a protease-deficient strain, *B. subtilis* WB800, has been created. It lacks proteases, such as phospholipase C, interleukin 3, and xylanase. Thus, overexpressed exogenous proteins cannot be degraded [32–35].

In this study, we employed *B. subtilis* WB800 as a host cell to produce recombinant bovine lactoferricin through transferred plasmids, and we demonstrated that the resistance of pathogenic infection of fish fry could be greatly enhanced by oral administration of this transformant as a feed supplement.

2. Materials and methods

2.1. Expression plasmid construction

To construct plasmid pP43-6LFBII-GFP to express heterologous lactoferricin in *B. subtilis*, we used pHY300PLK (TaKaRa) as a backbone, which served as a shuttle vector between *E. coli* and *B. subtilis* using genes resistant to ampicillin and tetracycline. The *B. subtilis* endogenous P43 promoter, which is a constitutive promoter of the cytidine deaminase gene [36], was cloned by using forward primer P43–P-BamHI-F 5′-GGATCCGATTCTTCAAAAGCTTCGTGCATG-3′ and reverse primer P43–P-SalI-R 5′-GTCGACGTGTACATTCCTCTCTTACCTATAATGGTACCG-3′ from genomic DNA extracted from *B. subtilis*. The PCR-amplified DNA fragment containing P43 promoter was digested with *Bam*HI and *Sal*I and then inserted into plasmid pHY300PLK cut with corresponding restriction sites. Reporter GFP cDNA was fused downstream of a sequence containing six tandem repeats of lactoferricin cDNA obtained from the construct reported by Lin et al. [37] and inserted into the above plasmid cut with *Xba*I and *Hin*dIII to construct the expression plasmid pP43-6LFBII-GFP.

2.2. Preparation and gene transfer of B. subtilis competent cells

B. subtilis WB800 strain was obtained from Liew et al. [38]. The procedure to produce competent cells of *B. subtilis* followed the protocol described by Meddeb-Mouelhi et al. [39] and Lu et al. [40], except that cells were harvested at OD_{600} = 2.3–2.4. For gene transfer, 100 μL of competent cells were added to 1 μL of plasmid pP43-6LFBII-GFP (50 μg/mL), chilled on ice for 20 min, followed by dispensing this mixture into a 1-mm cuvette for electroporation. Electroporation was performed in an electroporator (BIO-RAD GENE PULSER Xcell™, USA) under the conditions of 2,500 V, 25 μF, 4–5 ms and 200 Ω. After electroporation, cells were transferred to a glass tube, which contained 2 mL of recovery medium (0.5 M D-Sorbitol, 2.5% LB broth) [40], incubated at 37 °C for 3 h, plated on an LB plate containing tetracycline (20 μg/mL) and then incubated again at 37 °C for 18 h.

2.3. PCR detection and Western blot analysis

The existence of exogenous DNA in transgenic strains was determined by PCR using 10 μM of forward primer (5′-GGAATCATTGTCATTAGTTGGCTGG-3′) and 10 μM of reverse primer (5′-AAGCTTACGAACTCTCATCGAAGCGCACATTGAAG-3′). The PCR reaction in a total volume of 50 μl was performed with the GeneAmp PCR System 9700 (Thermo Fisher Scientific, USA) under a cycle of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 2 min for 35 cycles in total.

To analyze the total proteins contained in *B. subtilis* strains, we harvested one mL of cells grown at OD_{600} = 1, followed by centrifugation and resuspension in 60 μL 1 X sample buffer (10% SDS, 50% glycerol, 0.05 M DTT, 0.01 M EDTA, 0.05% bromophenol blue and 0.125 M Tris-HCl, pH 6.8; GeneMark). In total, 20 μL of supernatant were taken to perform protein analysis using SDS-PAGE on a 12% polyacrylamide gel to undergo separation at 120V (BIO-RAD Mini-PROTEAN Tetra System). After electrophoresis, the proteins were transferred (BIO-RAD Mini-PROTEAN II Cell) to a polyvinyl difluoride membrane at 100V for 80 min, followed by immunoblotting with a rabbit anti-GFP polyclonal antibody (LTK Bio-Laboratories). The immune-positive bands shown on the membrane were further detected using the ChemiDoc-it 815 luminometer (Bio-Rad, USA).

2.4. In vitro bactericidal agar plate assay

After 100 ml of LB agar were autoclaved, the dissolved agar was immediately subjected to a 55 °C water bath. After cool down, the test pathogen, such as *E. coli*, *S. epidermidis*, *V. parahaemolyticus* or *E. tarda*, grown at OD_{600} = 0.5, was separately added into the above unsolidified LB agar and then dispensed in plates. Each plate was filled with 15 ml of unsolidified LB agar to obtain agar plates having an equal thickness of agar layer. After these LB agar plates confluent with each pathogen were completely solidified, several wells with a 5-mm diameter were generated on each agar plate. Three millimeters of wild-type (WT) and transgenic strains of *B. subtilis* grown at OD_{600} = 1 were harvested by centrifugation, resuspended in 3 mL of synthetic gastric juice (150 mM HCl, 15 mM KCl) containing 10% pepsin at a working pH of 2.0, and incubated for 10 h in a 37 °C water bath. After incubation, the solution pH was readjusted to 7.0, centrifuged again, and the supernatant was saved. Thereafter, we took 40 μL from supernatant-containing bacterial extracts and added into each well of the agar plate, followed by measuring the inhibition zone after the agar plate with lawn confluent bacterial growth was cultured at 37 °C for 12 h. Ampicillin served as a positive control, and it was also used to obtain a regression equation based on the size inhibition zone versus the known concentrations.

2.5. In vivo antibacterial activity test

About 1000 outdoor tilapia fry (*Oreochromis mossambicus* X *O. niloticus*) with an average body length of 20 mm and weight of 0.425 g were sampled from Fang-Fung fish hatchery farm (Pingtong County, Taiwan) in August of 2018. These fish fry were adapted in the aquarium tank with the dimension of 30 X 30 × 35 cm^3 (36 L water) set in an indoor wet lab for 3 weeks. Then 30 fish fry with an average body length of 24.5 mm and weight of 0.625 g were selected and cultured in one aquarium tank. The fish samples were divided into four groups for each experiment. At the first trial, one experimental group of fish fry was fed with *B. subtilis* transgenic strain T13, while three groups served as control groups. One control group was fish fry fed with *B. subtilis* WT strain, another control group was fry fed with transgenic strain T19 which was transgenic without expressing recombinant lactoferricin, and the remainder control group was mock treatment that was fry immersed in a tank without any pathogen infection for 5 h. At the second trial, the experimental group was fish fry fed with transgenic strain T1, while three control groups were as same as those described at the first trial. Each group was performed in duplicate (two separated aquarium tanks). Thus, in total, 240 tilapia fry separately cultured in eight aquarium tanks were used to carry out for each trial in this study.

WT and transgenic strains T1, T13 and T19 of *B. subtilis* were cultured in water bath at 37 °C with shaking at 250 rpm/min for 10 h and measured the index of OD_{600}. When the index of OD_{600} was near 0.9, we measured the index at 10-min interval until OD_{600} = 1 was reached. We harvested cells, centrifuged, resuspended in 3 mL of synthetic gastric juice containing 10% pepsin (pH 2), and incubated for 10 h at 18–22 °C. Thereafter, fish fry were fed *via* the oral-in-tube method with 50 μL of cell extracts prepared from the above treated WT and transgenic bacterial strains. After oral-in-tube delivery, the treated fish fry were back cultured in an aquarium tank for 1 h before undergoing the pathogen challenge test.

Pathogenic organisms, such as *E. tarda*, were cultured until they reached 5 × 10^8 CFU/mL. The fish fry which had been fed with *B. subtilis* strains were collected and immersed in a small tank filled with 1000 mL of water containing *E. tarda* (5 × 10^{11} CFU) for 5 h at 22–24 °C. After challenge, these fish fry were conveyed to the original culture tank (36L). The daily survival rate of each tank was recorded until fish samples in the control group were all dead, the time when the experiment was completed.

2.6. Key Resources Table

Type	Resources	Source	Identifer
Antibody			
	rabbit anti-GFP polyclonal	LTK BioLaboratories	
Chemical			
	ampicillin	Sigma	Cat#A9393
	bromophenol blue	Sigma	Cat#B0126
	D-sorbitol	Sigma	Cat#85529
	DTT	Sigma	Cat#43816
	EDTA	Sigma	Cat#E9884
	glycerol	J.T.Backer	Cat#4043
	HCl	Aencore	Cat#SW081239
	tetracycline	BioBasicCanada	Cat#TB0504
	Tris-HCl	Sigma	Cat#TRIS-RD

3. Results

3.1. Screening B. subtilis transformants harboring the lactoferricin cDNA fragment

The expected molecular size of recombinant lactoferricin cDNA fragment after PCR amplification was 1.7 kb (Fig. 1A). The results showed that no DNA fragment was amplified from the WT control. However, a DNA fragment with 1.7 kb was found in some extent colonies after we screened from 1100 colonies grown on LB medium. As expected, the plasmids harbored by most transformants were lost

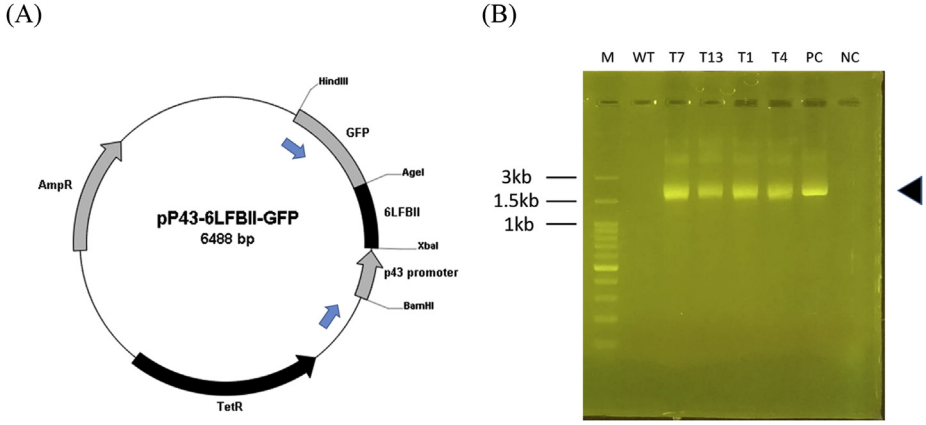
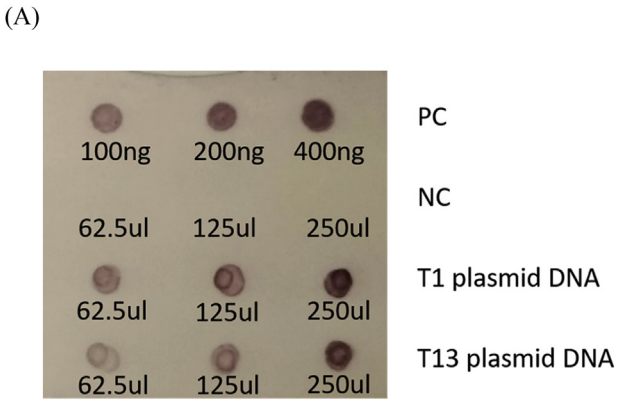
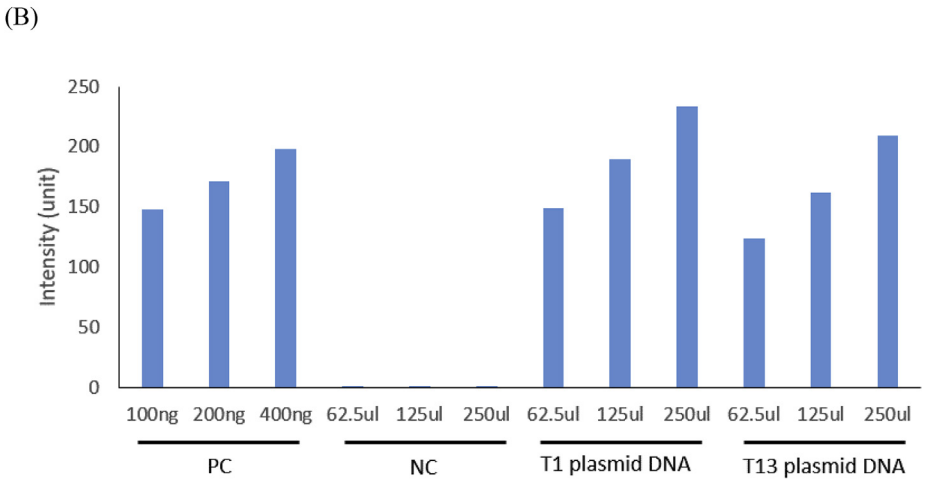


Fig. 1. Using PCR to detect the existence of plasmid containing lactoferricin cDNA in transformed *B. subtilis*. (A) Schematic map of plasmid pP43-6LFBII-GFP, in which the coding region of 6LFBII-GFP was driven by P43 constitutive promoter; 6LFBII represented six tandem repeat of cDNAs coding for six copies of recombinant lactoferricin, but two lactoferricin cDNAs were separated by one pepsin cutting site; GFP cDNA was a reporter gene and served as a positive control (PC); and AmpR and TetR represent Ampicillin and Tetracycline resistance genes, respectively. Primer pairs used to amplify the recombinant lactoferricin cDNA fragment were shown by short arrows. (B) The amplified PCR products were analyzed on agarose gel electrophoresis, as indicated by arrowhead, after DNAs were extracted from *B. subtilis* wild-type (WT) and transgenic strains T7, T13, T1 and T4, as indicated. In a parallel experiment, the PCR amplification of DNA extracted from the transgenic strain without adding templates served as a negative control (NC).





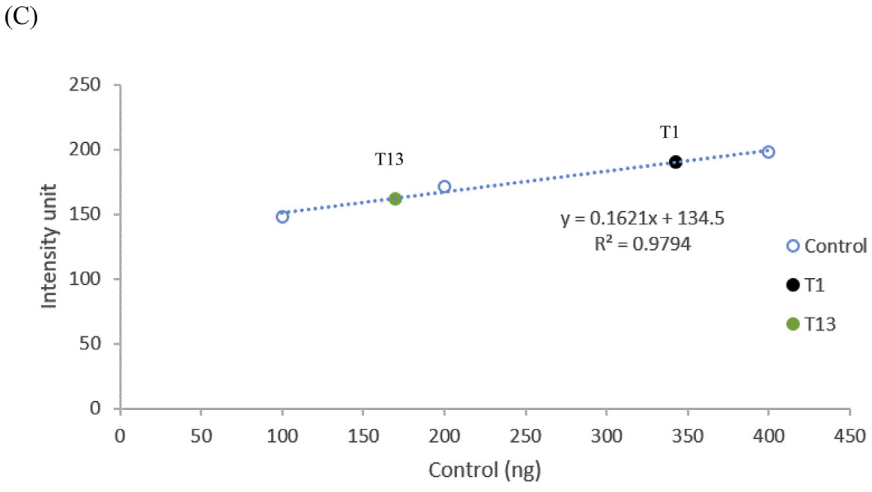


Fig. 2. Using dot blot analysis to determine the copy number of plasmid harbored by *B. subtilis* transgenic strains T1 and T13. (A) Three known amounts and copy numbers of plasmid pP43-6LFBII-GFP (100 ng, $1.43X10^{10}$ copies; 200 ng, $2.86X10^{10}$ copies and 400 ng, $5.71X10^{10}$ copies) were dotted on a nitrocellulose paper to serve as a positive control (PC) and then plotted as a standard curve to indicate the relationship between copy number of plasmid DNA and signal intensity shown on the nitrocellulose paper. Plasmids extracted from 62.5, 125 and 250 µl cell cultures of *B. subtilis* wild-type (WT) strain ($6.3X10^8$ CFU/ml) served as a negative control (NC). Plasmids extracted from the same three volumes of cell cultures of transgenic strains T1 ($4.2X10^8$ CFU/ml) and T13 ($3X10^8$ CFU/ml) were separately dotted on the same paper to determine the copy number of plasmid DNA based on comparing the relative signal intensity of known positive control. (B) Signal intensity unit was determined by VisionWorks® Life Science Software and presented by a histogram plot. (C) Based on the standard curve, the linear regression equation and R^2 were obtained. Thus, the copy number of plasmid pP43-6LFBII-GFP harbored by 125 µl of transgenic strains T1 and T13 was calculated.

gradually after several passages. Eventually, we obtained four *B. subtilis* transgenic strains T7, T13, T1 and T4 (Fig. 1B), in which the plasmids were transmitted in a stable manner.

3.2. Copy number of plasmid-containing transgenic B. subtilis strain

A 700-bp GFP fragment tagged with DIG served as a probe to hybridize the plasmid DNAs extracted from transgenic strains T1 and T13. A 100 ng DNA containing 1.43×10^{10} copies, 200 ng DNA containing 2.86×10^{10} copies and 400 ng DNA containing 5.71×10^{10} copies of plasmid pP43-6LFBII-GFP served as positive control (Fig. 2A). These three plasmid DNA concentrations exhibited the signal intensity units of 148, 171 and 198, respectively (Fig. 2B). No signal was detected from the plasmid-containing *B. subtilis* WT strain, suggesting that DIG-tagged GFP was a highly specific probe (Fig. 2A). We, therefore, extracted plasmids from 62.5, 125 and 250 µl of cultured transgenic *B. subtilis* T1 strain (4.2×10^8 CFU/ml) and T13 strain (3×10^8 CFU/ml) and performed dot blot analysis (Fig. 2A). As a result, the signal intensity of units 149, 190 and 233 was displayed in transgenic T1 strain, respectively, while signal intensity of units 124, 162 and 209 was displayed in strain T13, respectively (Fig. 2B). A regression equation $y = 0.1621x + 134.5$ ($R^2 = 0.9794$) was obtained by calculating the ratio between the

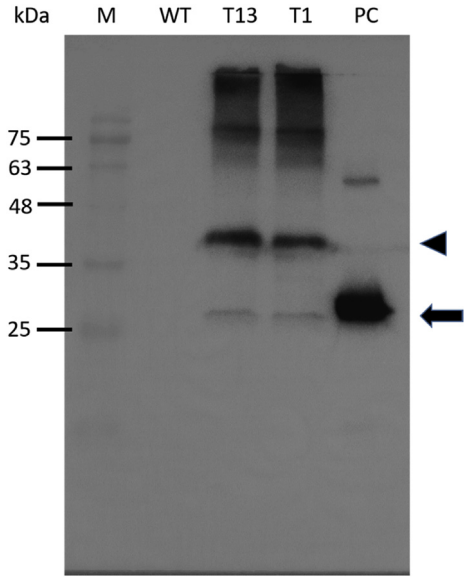


Fig. 3. Western blot analysis of proteins extracted from *B. subtilis*. Total proteins extracted from wild-type (WT) and transgenic strains T13 and T1 were separated on an SDS-PAGE, followed by Western blot analysis. The polyclonal antibody against GFP was used to detect recombinant lactoferricin fused with GFP reporter. Proteins extracted from *B. subtilis* WT strain served as a negative control. The recombinant protein lactoferricin fused with GFP (40 kDa) produced by transgenic strains was detected, as indicated by arrowhead. The pure GFP (30 kDa) served as a positive control (PC), as indicated by arrow.

signal intensity of dot plot analysis and the amount (ng) of plasmid pP43-6LFBII-GFP (Fig. 2C). Based on these results, plasmid pP43-6LFBII-GFP from a volume of 125 μl of T1 and T13 strains was equivalent to 342 and 170 ng of the positive control, respectively, indicating that plasmids extracted from T1 (5.25×10^7 CFU) and T13 (3.75×10^7 CFU) contained 4.89×10^{10} and 2.43×10^{10} copies, respectively. Therefore, the copy number of plasmids containing transgenic *B. subtilis* T1 and T13 strains was calculated as 931 and 647 copies per cell, respectively.

3.3. Recombinant lactoferricin was produced in transformed strains

As shown in Fig. 3, no detectable band was recognized by antibody against GFP from the WT strain of *B. subtilis* (Fig. 3, lane WT). In contrast, a major positive hybridization band with approximate molecular mass of 40 kDa was detected in transgenic *B. subtilis* T13 and T1 strains (Fig. 3, lanes T13 and T1, as indicated by arrowhead), suggesting that the exogenous lactoferricin-GFP recombinant protein was expressed in transgenic strains T13 and T1. Nevertheless, we noticed that a minor positive band with 30 kDa was also shown on the gel (Fig. 3, lanes T13 and T1, as indicated by arrow), suggesting that the GFP reporter might somehow exist in the transformants.

3.4. Bactericidal activity against freshwater pathogens of recombinant lactoferricin produced by transgenic B. subtilis strains

As shown in Fig. 4A, no inhibition zone was observed in the cell extracts from WT strain and negative control groups (T19) added into the wells on agar plate with lawn confluent growth of pathogenic *E. coli*. However, an inhibition zone with diameters of 5.27 ± 0.20, 4.95 ± 0.17, 4.08 ± 0.07, and 5.17 ± 0.29 mm occurred in transgenic strains T1, T4, T7 and T13, respectively. Based on the size of the inhibition zone shown on the known concentrations of Ampicillin (2, 3 and 4 μg) (Fig. 4B), a regression equation of $y = 1.422x + 2.0767$ ($R^2 = 0.9979$) was obtained (Fig. 4C). Therefore, we calculated that the bactericidal efficacy of 40 μl cell lysates from transgenic T1, T4, T7 and T13 strains (3×10^8 CFU/ml) was equivalent to 2.24, 2.02, 1.41 and 2.13 μg of Ampicillin dosage, respectively. In other words, the bactericidal efficacy against *E. coli* of transgenic strain T1, T4, T7 and T13 at the above concentration was 56, 51, 36 and 53 ng of Ampicillin per μl, respectively.

Regarding of bactericidal activity against *S. epidermidis*, no inhibition zone was observed in cell extracts from either the WT strain or the T19 strain which was transgenic without expressing recombinant lactoferricin, and thus served as negative controls in agar diffusion assay (Fig. 4D). In contrast, cell extracts from the transgenic *B. subtilis* T13 exhibited an inhibition zone with a diameter of 6.16 ± 0.18 mm. In a parallel experiment, we found that an inhibition zone was not generated in cell extracts from T1 and T13 strains without first being treated with synthetic gastric juice, as indicated by T1' and T13', respectively, suggesting that the biological activity displayed in a single recombinant lactoferricin needs to be cleaved by pepsin to release it from a complex of six recombinant lactoferricin. This result was consistent with the gene construct we designed, in which six genes coding for six lactoferricin were interrupted by five pepsin cutting sites.

Based on the size of inhibition zone, as shown on the known concentrations of Ampicillin, a regression equation of $y = 0.3812x + 4.165$ ($R^2 = 0.9733$) was obtained (Fig. 4F). Therefore, we calculated that the bactericidal efficacy of the 40 μl cell lysate from strain T1 and T13 (3×10^8 CFU/ml) was equivalent to 6.18 and 5.23 μg of the Ampicillin dosage. In other words, the bactericidal efficacy against *S. epidermidis* of 1 μL from cell extracts produced by 3×10^5 CFU transgenic *B. subtilis* from strain T1 and T13 were equivalent to that of 154 and 130 ng Ampicillin, respectively.

3.5. Bactericidal activity against marine pathogens of recombinant lactoferricin produced by transgenic B. subtilis strain

Since marine pathogen *V. parahaemolyticus* is better able to resist Ampicillin, we used Tetracycline instead. As shown in Fig. 4G and H, no inhibition zone was observed in cell extracts from WT and a negative control transgenic strain T19 (control) in the agar diffusion assay, suggesting that WT and strain T19 (two negative controls) displayed no bactericidal activity against *V. parahaemolyticus*. In contrast, the cell extracts from transgenic *B. subtilis* T13 strain exhibited an inhibition zone with a diameter of 7.31 ± 0.15 mm. Similarly, an inhibition zone was not generated in cell extracts from strain T13 strain without synthetic gastric juice treatment, suggesting, again, that the biological activity displayed in a single recombinant lactoferricin needs to be cleaved from a six-fused recombinant lactoferricin by pepsin.

Based on the size of inhibition zone, as shown on the known concentrations of Tetracycline, a regression equation of $y = 2.882x + 4.3007$ ($R^2 = 0.9234$) was obtained (Fig. 4I). Therefore, we calculated that the bactericidal efficacy of the 40 μl cell lysate from strain T13 (3×10^8 CFU/ml) was equivalent to 1.04 μg of the Tetracycline dosage. In other words, the bactericidal efficacy against *V. parahaemolyticus* of 1 μL from cell extracts produced by 3×10^5 CFU transgenic *B. subtilis* T13 strain was equivalent that of 26 ng of Tetracycline.

As shown in Fig. 4J and K, no inhibition zone was observed in cell extracts from WT and a negative control transgenic strain T19 (control) in the agar diffusion assay, suggesting that WT and transgenic strain T19 (two negative controls) displayed no bactericidal activity against euryhaline pathogen *E. tarda*. In contrast, the cell extracts from strain T13 exhibited an inhibition zone with a diameter of 9.03 ± 0.15 mm. Similarly, no inhibition zone was generated in cell extracts from strain T13 without synthetic gastric juice treatment, suggesting yet again that the biological activity displayed in a single recombinant lactoferrincin needs to be cleaved by pepsin.

Based on the size of inhibition zone shown on the known concentrations of Ampicillin, a regression equation of $y = 0.84x + 7.9233$ ($R^2 = 0.9998$) was obtained (Fig. 4L). Therefore, we calculated that the bactericidal efficacy of an 80 μl cell lysate from strain T13

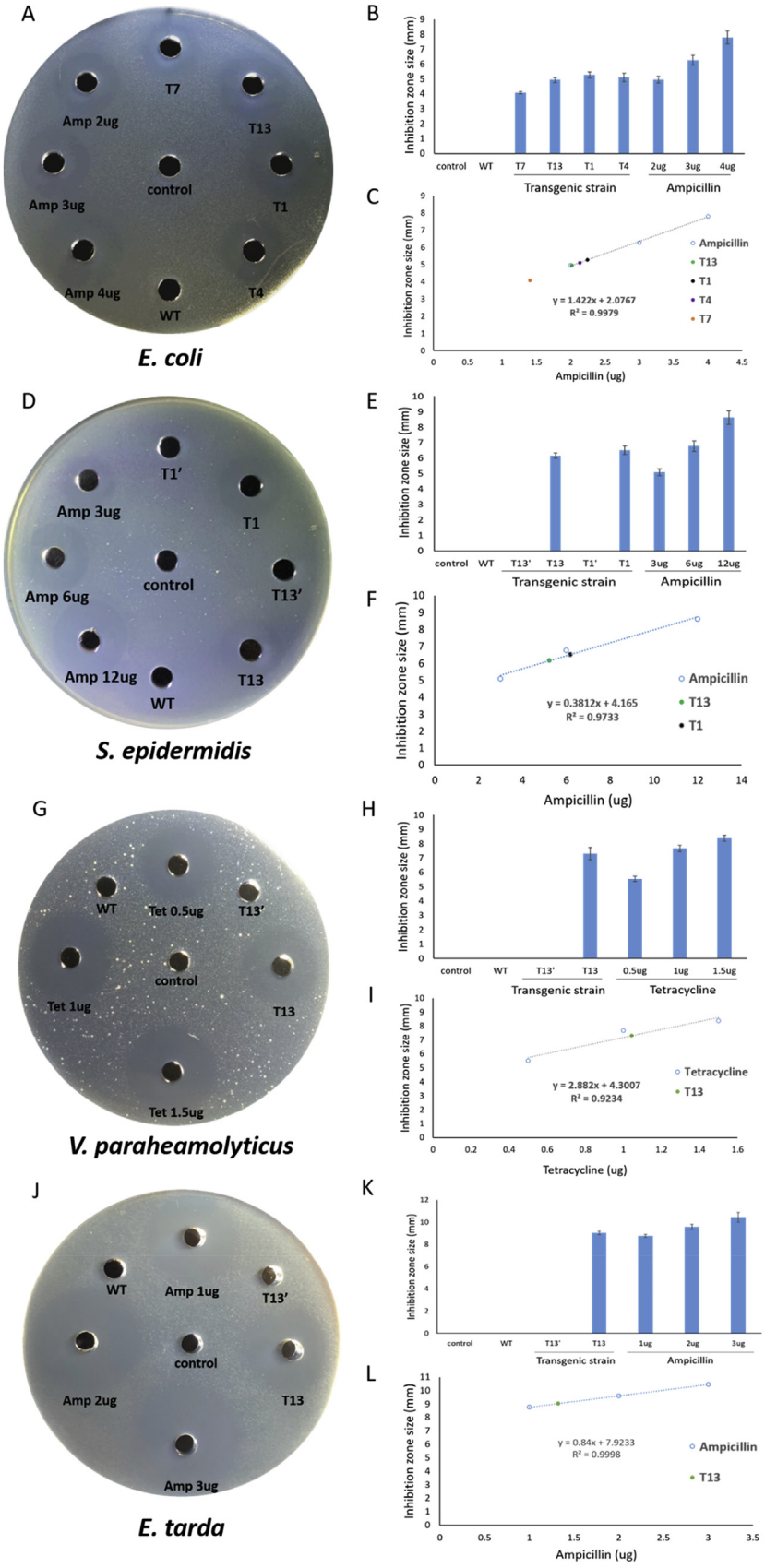


Fig. 4. Using an agar well diffusion assay to study the bactericidal efficacy of recombinant lactoferricin produced by *B. subtilis* transgenic strains against pathogens growth. The bactericidal activity of recombinant lactoferricin contained in the proteins extracted from five *B. subtilis* transgenic strains (T1, T4, T7, T13 and T19) were added into the well on agar plate with lawn confluent bacterial growth, such as *E. coli*, *S. epidermidis*, *V. parahaemolyticus* and *E. tarda,* as indicated. Three known concentrations of Ampicillin (Amp) or Tetracycline (Tet) were loaded on agar plate served as standard to define the relationship between bactericidal efficacy and size of inhibition zone (A, D, G, J). The extracts from wild-type strain (WT) and transgenic strain T19 (control) without expressing lactoferricin served as negative controls. Another negative control groups were T1′ and T13′, in which the extracts were obtained from lactoferricin-expressing transgenic strain T1 and T13 without synthetic gastric juice treatment (D, G, J). The experiment groups were the extracts from each transgenic strain expressing lactoferricin, as indicated, treated with synthetic gastric juice. Diameter size (mm) of each inhibition zone was measured and presented on a histogram plot (B, E, H, K). Data were averaged from six independent trials (n = 6). Based on the standard curve, the linear regression equation and R^2 were obtained (C, F, I, L).

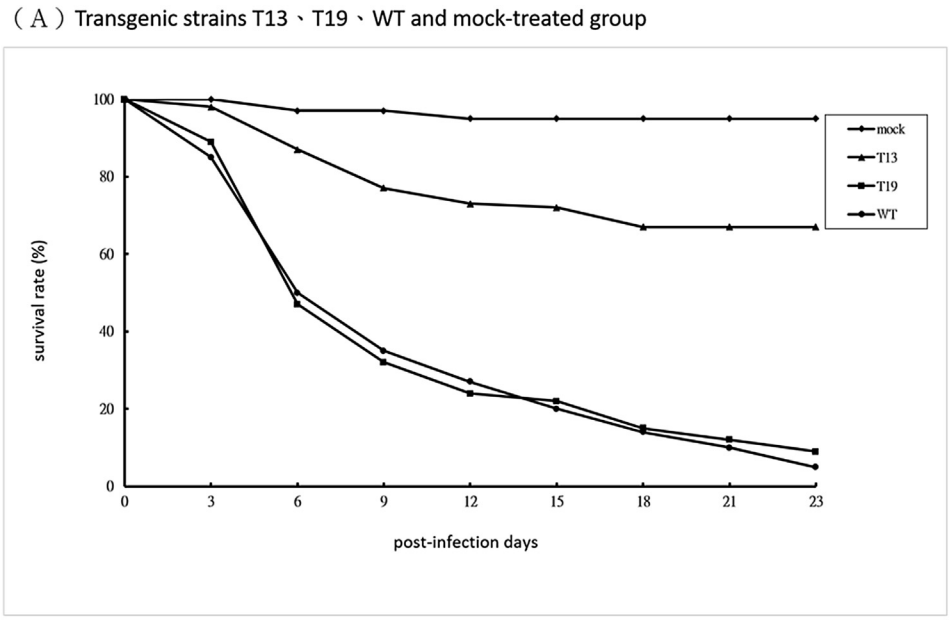


Fig. 5. Using *in vivo* study to determine the effect of bactericidal activity directed by recombinant lactoferricin produced by *vivo* strains to defend against pathogen infection of fish fry. study to determine the effect of bactericidal activity directed by recombinant lactoferricin produced by *B. subtilis* strains to defend against pathogen infection of fish fry. Tilapia fry fed with wild-type (WT) strain and transgenic strains T1, T13 and T19 were challenged with *E. tarda*. Transgenic T1 and T13 strains were transformed cells expressing recombinant lactoferricin, while WT strain and transgenic strain T19 which was a transformed cell containing exogenous DNA without expressing lactoferricin served as negative control groups. *Tilapia* fry immersed in freshwater without contaning *E. tarda* served as a mock group. Sample size for each assessment group was 30. The cumulative survival rate was recorded every three days since *tilapia* larvae (n = 30) were fed with *B. subtilis*, followed by immersion of water containing *E. tarda*. Data were presented as an average from two independent trials. (A) Transgenic strains T13 and T19, WT and mock groups; and (B) Transgenic strains T1, T19, WT and mock groups.

(3×10^8 CFU/ml) was equivalent to 1.32 μg of the Ampicillin dosage. In other words, bactericidal efficacy against *E. tarda* of 1 μL from cell extracts produced by 3×10^5 CFU transgenic *B. subtilis* strain T13 was equivalent to that of 33 ng of Ampicillin.

3.6. Fish fry fed with transgenic B. subtilis strain can prevent from pathogen infection

As shown in Fig. 5A, the average survival rate was not significantly different between *E. tarda*-infected tilapia fry fed *B. subtilis* WT strain and those fed the transgenic T19 strain which did not express lactoferricin. Nevertheless, non-infected tilapia cultured in freshwater served as mock group, in which fish fry were alive and healthy after immersion in water without pathogen for 5 h. However, the average survival rate of *E. tarda*-infected tilapia fry fed with the transgenic lactoferricin-expressing T13 strain was much higher than that of *E. tarda*-infected tilapia fry fed with WT strain, specifically, 67 versus 5% after infection for 23 days. We also noticed that all dead fish were suffering gastrointestinal symptoms of infection by *E. tarda* such as inflammation, internal bleeding and erosion of the entire abdominal cavity (Fig. 6).

Similar to the result shown in Fig. 5B, the rapid reduction of survival rate was observed in *E. tarda*-infected tilapia fry fed with *B. subtilis* WT and T19 strains (control groups). A total of 48% of fish samples remained at day 6 and 32% at day 9 post-infection, while the survival rate of *E. tarda*-infected tilapia fry fed with another transgenic T1 strain decreased slowly and remained at 63% until the end of the experiment. The average survival rate of *E. tarda*-infected tilapia fry fed the transgenic lactoferricin-expressing T1 strain was much higher than that of *E. tarda*-infected tilapia fry fed the WT strain, such as 63 versus 5% for 32 days after infection. Collectively, these results suggest that feeding tilapia fry lactoferricin-expressing transgenic strains mitigates the mortality caused by the symptoms of gastrointestinal disease during *E. tarda* infection, in turn resulting in the increase of survival rate by 58–62%, as compared with control groups.

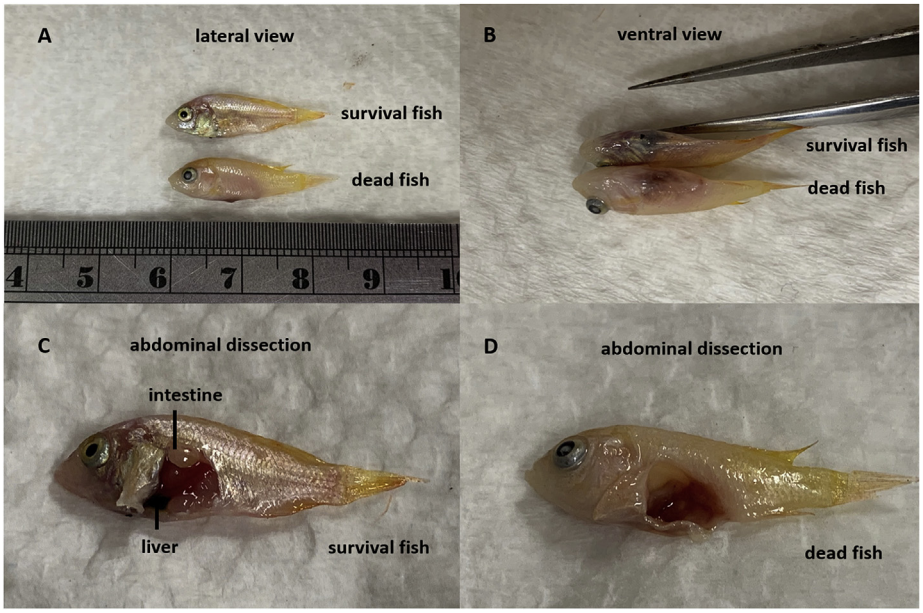


Fig. 6. Anatomical examination of the digestive tract of fish fry. After *in vivo* antibacterial activity test, the morphologies of survival and dead tilapia fry samples were examined from (A) lateral and (B) ventral view. Unlike survival healthy fish fry, symptoms such as swollen abdomen and internal bleeding were externally observed in the dead fish fry infected with pathogen *E. tarda.* After abdominal dissection, (C) the *E. tarda*-infected survival fish fry exhibited a normal intestine and liver in the abdominal cavity; while (D) the *E. tarda*-infected dead fish fry exhibited erosive bleeding symptom, resulting in the intestine and liver were hardly distinguished in the digestive tract.

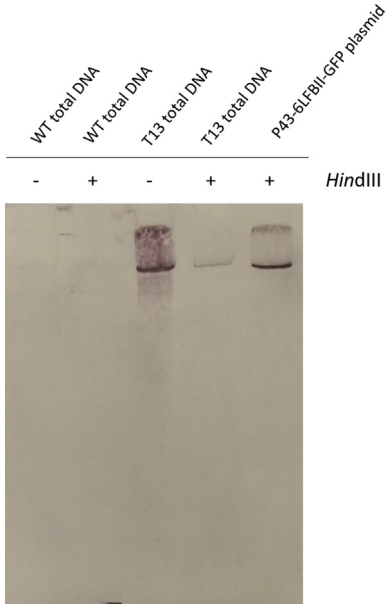


Fig. 7. Detection of the transferred plasmids within the total DNAs extracted from transgenic strain T13. The total DNAs, including genome and plasmid DNAs, were extracted from wild-type (WT) and T13 strains to determine the status of plasmids transferred in host cells of *B. subtilis*. The Southern pattern of extracted DNA samples without treated with restriction enzyme (−) was compared with that of extracted DNA samples cut with *Hin*dIII (+). Plasmid pP43-6LFBII-GFP cut with *Hin*dIII served as positive control. No positive signal with a molecular weight higher than pP43-6LFBII-GFP, suggesting that the plasmids containing lactoferricin cDNA transferred into *B. subtilis* transgenic strain T13 might not be integrated into the genome of host cells. Rather, the extant plasmids in transgenic strain were in an independent genetic element manner in the cytoplasm.

4. Discussion

4.1. Stability of circular plasmids harbored by B. subtilis transformants

Plasmid pP43-6LFBII-GFP, which was used to perform gene transfer into *B. subtilis* host cells through electrophoresis, was prepared in circular form. We found no evidence showing that the plasmids harbored in all transgenic strains screened in this study were inserted into the chromosome of host cells (Fig. 7). This suggested that heterologous plasmids extant in *B. subtilis* were in extrachromosomal circular form with a copy number of 647–931 per cell. However, we did find that transgenic strain T19 was PCR-positive, but Western blot-negative, indicating, in turn, that some transformants could not express recombinant lactoferricin. We reasoned that T19 might have modified, or partially deleted, the lactoferricin cDNA segment of the transferred plasmid pP43-6LFBII-GFP, resulting in failed production of lactoferricin. Therefore, it is absolutely necessary to use Western blot analysis to further check whether the target protein is produced by transformants.

When we detected the cell extracts from transgenic strain T7, Western blot analysis showed many different sizes of GFP bands between 25 and 48 kDa (Fig. 8). Although they produced recombinant lactoferricin, this target protein could have been unstable by partial degradation for some unknown reasons. However, a single GFP-positive band with a molecular weight of 40 kDa, corresponding to that of recombinant lactoferricin fused with GFP reporter, was detected in the cell extracts from transgenic strains T1 and T13 (Fig. 3), suggesting that the recombinant lactoferricin expressed in these two transformants was more stable since heterologous proteins were not at all, or just slightly,

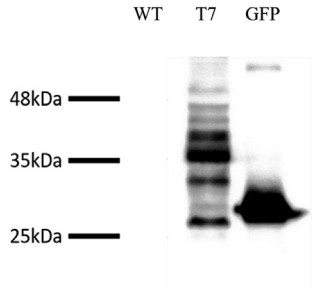


Fig. 8. Using Western blot analysis to detect the recombinant lactoferricin fused with GFP reporter produced by *B. subtilis* transgenic strain T7. The total proteins extracted from wild-type (WT) and transgenic strain T7 were performed Western blot analysis to detect the production of recombinant lactoferricin fused GFP using antibody against GFP. The purified GFP protein produced by *E. coli* served as a positive control. No positive signal was detected in the *B. subtilis* wild type strain. In contrast, several GFP-positive bands ranging from 25 to 48 kDa were observed in the proteins extracted from transgenic strain T7.

cleaved by endogenous enzyme of host cells. Therefore, large-scale screening to find stable transgenic strains among *B. subtilis* transformants is also indispensable for further study and industrial applications.

We then extracted plasmids from transgenic strains T1 and T13. These strains were not only those with the highest expression of foreign lactoferricin with no degradation, but also the most stable inheritance of harbored plasmids until the 22nd passage when we subcultured colonies every three or four weeks. The line of evidence suggested that T1 and T13 are two transformants exhibiting more stable inheritance of the transferred plasmids. Unexpectedly, we found that the plasmids harbored by T1 and T13 only consisted of three tandem repeats of lactoferricin cDNA, which is a truncated form of six tandem repeats of lactoferricin cDNA in the original plasmid pP43-6LFBII-GFP used to perform electrophoresis. Yet, no transformants having stable and high expression of lactoferricin, but containing six copies of lactoferricin cDNA, were found. Based on these results, we speculate that three copies of lactoferricin cDNA in the construct might have been the optimal gene dosage for *B. subtilis* WB800 to produce exogenous antibacterial peptide, such as lactoferricin, which does not reach a quantitative level sufficient to cause toxic effect on recipients.

4.2. Oral administration of transgenic strains could prevent fish fry from pathogenic infection

Since transgenic *B. subtilis* strains T1 and T13 not only possessed a relatively better bactericidal efficacy against *E. coli* but also they contained a considering stable inheritance of plasmids, they were further used for *in vivo* tests. It was hypothesized in this work that tandem repeats of recombinant lactoferricin produced by transgenic strains passing through the gastrointestinal tract of tilapia might be cleaved and released functional lactoferricin that would kill the intestinal pathogen, in turn decreasing the mortality rate of pathogen-infected fish. Indeed, this hypothesis was strongly supported by the *in vivo* antibacterial activity experiment performed in this study. It has been reported that *E. tarda* invades and then proliferates in the intestine of tilapia through oral infection. After infection in the digestive tract of the fish, it may continuously enter liver via the hepatic portal vein or invades kidneys and other tissues via systemic circulation, causing lesions and necrosis of organs such as liver and kidney [41]. *E. tarda* may also invade adjacent torso tissues to cause ulcers and perforations. The infected tilapia eventually floats on the water surface from swelling of the abdominal cavity. In this study, these symptoms of *E. tarda* were exactly phenocopied in the control tilapia fry immersed in the water tank containing *E. tarda* WT strain, causing an average mortality rate of 95% (120 fry in total). In contrast, average mortality rates of 37 and 33% were observed in the fry groups fed with the transgenic *B. subtilis* T1 and T13 strains (Fig. 5), respectively, for one month after *E. tarda* infection, significantly improving the average survival rate. Meanwhile, the mock control group immersed in the water tank for 5 h without pathogen challenge, the similarity of effect made it possible to rule out that the mortality of fish fry may result from immersion stress. Therefore, in this study, we have demonstrated that oral administration of transgenic *B. subtilis* producing recombinant lactoferricin could effectively protect fish fry from infection of *E. tarda*.

Moreover, the *B. subtilis* transgenic T1 and T13 strains we generated could potentially be applied to the aquaculture and livestock industries because these strains can be directly mixed in feedstuff as a food supplement. Alternatively, these transgenic strains can be freeze dried and combined with other materials to form a novel antimicrobial animal feed. Oral administration of this antimicrobial feedstuff can also improve the defense capability of host organisms against pathogen infection in the gastrointestinal tract, leading to the increase of survival rate and reducing the costs.

4.3. The application of recombinant lactoferricin could reduce the use of antibiotics

To reduce infection from such common euryhaline pathogen *E. tarda* and marine pathogen *V. paraheamolyticus*, in large-scale production settings, various antibiotics must be used to prevent cultured fish and farm animals from being infected. However, long-term treatment of antibiotics may cause the bacterial pathogens to gain resistance. Therefore, new antibiotics, or multiple antibiotics, must be used to inhibit the growth of these antibiotic-resistant mutant microorganisms. Moreover, even if nonpathogenic microorganisms develop antibiotic-resistant genes, existing resistance determinants can spread to other microbes, eventually infecting other organisms, or even humans [42]. The agar-well diffusion assay employed in this study demonstrated that the extracts from the transgenic strains containing the pepsin cleaved-recombinant lactoferricin could inhibit the growth of *E. coli, S. epidermidis*, *V. parahaemolyticus* and *E. tarda*, indicating that this recombinant protein is a broad-spectrum antibiotic agent. This evidence was supported by Lin et al. [37] who demonstrated that the antimicrobial resistance of *E. tarda* infected-fish can be improved by feeding zebrafish eggs containing lactoferricin. Thus, it could be used in both aquaculture and livestock industries as a supplement to reduce the dependence on conventional antibiotics, particularly during seasonal outbreaks.

Hardi et al. [43] reported that the antibacterial and immunostimulant abilities of plant extracts were able to prevent from *Aeromonas hydrophila* and *Pseudomonas fluorescens* infection on tilapia (*O. niloticus Linn)*. Deepika et al. [44] found that the combination of phenolic compounds including flavonoids and antibiotics can be used as a possible synergistic antimicrobial modulator against multi-drug resistant pathogens. Moreover, Amphan et al. [45] established an optimal feeding dosage of β-glucan for tilapia to increase disease resistance. Obviously, the large-scale preparation of plant extract, phenolic compounds or β-glucan to prevent tilapia from pathogens infection is tedious and costly. In contrast, it is relatively easier and more economic to prepare this biosafe microorganism *B. subtilis* in large-scale. Specifically, the extraction of recombinant lactoferricin produced by *B. subtilis* is absolutely not necessary since the intact cells of transgenic strain can be directly added into fish meal as a supplementary feedstuff to prevent fish from pathogen infection.

Most importantly, lactoferricin is an environmentally safe bactericidal agent since it is a biodegradable peptide that can be easily decomposed. Also, it does not accumulate in the environment, thus reducing the frequency of resistance in microbes and the amount of antibiotics used in fish pond and animal farms. Additionally, Situmorang et al. [46] established a tilapia larvae model system used as a research tool to illustrate the effects and modes-of-action of probiotics. In this study, the *Bacillus* species is considered not only as a safe microorganism but also as a probiotic microbe since administration of *Bacillus* species as a food supplement can enhance immune response [47,48] and growth rate [47] in fish. It has been also reported zebrafish fed with lactoferrin supplements could improve its immune activity against *E. tarda* [49]. Recently, the application of probiotic *Bacillus* species is overwhelming in aquaculture industry [50]. Therefore, we conclude that the transgenic strains derived from *B. subtilis* generated in this study might be more versatile and highly potentially useful in industry applications.

5. Conclusion

After gene transfer of *Bacillus subtilis,* considered a safe microorganism, large-scale screening of putative transformants was performed. Two transgenic strains T1 and T13, which not only enabled to express the exogenous recombinant antimicrobial peptide, but also exhibited stable inheritance of plasmids, were confirmed. In the antibacterial *in vitro* experiment, the bactericidal activities of transgenic

strains equivalent to conventional antibiotics for common fish pathogens such as *E. coli, S. epidermidis*, *V. parahaemolyticus* and *E. tarda* were determined. In the antibacterial *in vivo* experiment, we also demonstrated that the survival rate of fish fry infected by the intestinal pathogen tested could be greatly enhanced by feeding transgenic strains T1 and T13 separately.

Funding

This work was partially supported by the Ministry of Science and Technology, Taiwan, ROC, under grant numbers 107-2622-B-715-001-CC1. This work was also partially supported by Mackay Medical College, Taiwan, ROC, under grant number RD1071B05.

Acknowledgements

We thank Dr. Sui-Lam Wong, Department of Biological Sciences, University of Calgary, Canada, for providing *Bacillus subtilis* WB800 strain. We were grateful for the support from Prof. Jin-Chuan Sheu, Dr. Hsiao-Ching Nien and Mr. Spencer Lee, the Liver Disease Prevention and Treatment Research Foundation, Taiwan. We also thank Mr. Jian-Cheng Wu, Fang- Fung fish farm, Taiwan, for providing tilapia fry.

References

[1] E. Moreno, G. Prats, M. Sabate, T. Perez, J.R. Johnson, A. Andreu, Quinolone, fluoroquinolone and trimethoprim/sulfamethoxazole resistance in relation to virulence determinants and phylogenetic background among uropathogenic *Escherichia coli*, J. Antimicrob. Chemother. 57 (2) (2006) 204–211, https://doi.org/10.1093/jac/dki468.

[2] B.M. de Tejada, Antibiotic use and misuse during pregnancy and delivery: benefits and risks, Int. J. Environ. Res. Public Health 11 (8) (2014) 7993–8009, https://doi.org/10.3390/ijerph110807993.

[3] B. Li, T.J. Webster, Bacteria antibiotic resistance: new challenges and opportunities for implant-associated orthopaedic infection, J. Orthop. Res. 36 (1) (2018) 22–32, https://doi.org/10.1002/jor.23656.

[4] M. Tomita, H. Wakaobayashi, K. Yamauchi, S. Teraguchi, H. Hayasawa, Bovin lactoferrin and lactoferricin derived from milk: production and applications, Biochem. Cell Biol. 80 (1) (2002) 109–112.

[5] C.C. Yen, C.J. Shen, W.H. Hsu, Y.H. Chang, H.T. Lin, H.L. Chen, C.M. Chen, Lactoferrin: an iron-binding antimicrobial protein against *Escherichia coli* infection, Biometals 24 (4) (2011) 585–594, https://doi.org/10.1007/s10534-011-9423-8.

[6] M.L. Kruzel, M. Zimecki, Lactoferrin and immunologic dissonance: clinical implications, Arch. Immunol. Ther. Exp. 50 (6) (2002) 399–410.

[7] T.H. Lee, S.J. Kim, Y.M. Han, D.Y. Yu, C.S. Lee, Y.J. Choi, H.B. Moon, M.G. Baik, K.K. Lee, Matrix attachment region sequences enhanced the expression frequency of a whey acidic protein/human lactoferrin fusion gene in the mammary gland of transgenic mice, Mol. Cells 8 (5) (1998) 530–536.

[8] M. Zimecki, J. Artym, G. Chodaczek, M. Kocieba, M.L. Kruzel, Protective effects of lactoferrin in *Escherichia coli*-induced bacteremia in mice: relationship to reduced serum TNF alpha level and increased turnover of neutrophils, Inflamm. Res. 53 (7) (2004) 292–296, https://doi.org/10.1007/s00011-004-1257-1.

[9] S. Teraguchi, K. Shin, K. Ozawa, S. Nakamura, Y. Fukuwatari, S. Tsuyuki, H. Namihira, S. Shimamura, Bacteriostatic effect of orally administered bovine lactoferrin on proliferation of *Clostridum* species in the gut of mice fed bovine milk, Appl. Environ. Microbiol. 61 (8) (1995) 501–506.

[10] J.K. Actor, S.A. Hwang, M.L. Kruzel, Lactoferrin as a natural immune modulator, Curr. Pharmaceut. Des. 15 (17) (2009) 1956–1973, https://doi.org/10.2174/138161209788453202.

[11] P. Valenti, G. Antonini, Lactoferrin: an important host defence against microbial and viral attack, Cell. Mol. Life Sci. 62 (22) (2005) 2576–2587, https://doi.org/10.1007/s00018-005-5372-0.

[12] S.A. Gonzalez-Chavez, S. Arevalo-Gallegos, Q. Rascon-Cruz, Lactoferrin: structure, function and applications, Int. J. Antimicrob. Agents 33 (4) (2009) 301 e1–8, https://doi.org/10.1016/j.ijantimicag.2008.07.020.

[13] W. Bellamy, M. Takase, H. Wakabayashi, K. Kawase, M. Tomita, Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin, J. Appl. Bacteriol. 73 (6) (1992) 472–479, https://doi.org/10.1111/j.1365-2672.1992.tb05007.x.

[14] K. Yamauchi, M. Tomita, T.J. Giehl, R.T. Ellison 3rd, Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment, Infect. Immun. 61 (2) (1993) 719–728.

[15] O. Aguilera, H. Ostolaza, L.M. Quirós, J.F. Fierro, Permeabilizing action of an antimicrobial lactoferricin-derived peptide on bacterial and artificial membranes, FEBS Lett. 462 (3) (1999) 273–277, https://doi.org/10.1016/s0014-5793(99)01545-8.

[16] K.E. Fernandes, D.A. Carter, The antifungal activity of lactoferrin and its derived peptides: mechanism of action and synergy with drugs against fungal pathogens, Front. Microboil. 18 (2017), https://doi.org/10.3389/fmicb.2017.00002.

[17] K. Velliyagounder, W. Alsaedi, W. Alabdulmohsen, K. Markowitz, D.H. Fine, Oral lactoferrin protects against experimental candiasis in mice, J. Appl. Microbiol. 118 (1) (2015) 212–221, https://doi.org/10.1111/jam.12666.

[18] T. Isamida, T. Tanaka, Y. Omata, K. Yamauchi, K. Shimazaki, A. Saito, Protective effect of lactoferricin against *Toxoplasma gondii* infection in mice, J. Vet. Med. Sci. 60 (2) (1998) 241–244, https://doi.org/10.1292/jvms.60.241.

[19] L.A. Haversen, I. Engberg, L. Baltzer, G. Dolphin, L.A. Hanson, I. Mattsby-Baltzer, Human lactoferrin and peptides derived from a surface-exposed helical region reduce experimental *Escherichia coli* urinary tract infection in mice, Infect. Immun. 68 (10) (2000) 5816–5823, https://doi.org/10.1128/iai.68.10.5816-5823.2000.

[20] W. Bellamy, M. Takase, K. Yamauchi, H. Wakabayashi, K. Kawase, M. Tomita, Identification of the bactericidal domain of lactoferrin, Biochim. Biophys. Acta, Protein Struct. Mol. 1211 (1–2) (1992) 130–136, https://doi.org/10.1016/0167-4838(92)90346-f.

[21] N. Orsi, The antimicrobial activity of lactoferrin: current status and perspectives, Biometals 17 (3) (2004) 189–196.

[22] H.L. Chen, Y.W. Lai, C.C. Yen, Y.Y. Lin, C.Y. Lu, S.H. Yang, T.C. Tsai, Y.Y. Lin, C.W. Lin, C.M. Chen, Production of recombinant porcine lactoferrin exhibiting antibacterial activity in methylotrophic yeast, *Pichia pastoris*, J. Mol. Microbiol. Biotechnol. 8 (3) (2004) 141–149, https://doi.org/10.1159/000085787.

[23] C.C. Yen, C.Y. Lim, K.Y. Chong, T.C. Tsai, C.J. Shen, M.F. Lim, C.Y. Su, H.L. Chen, C.M. Chen, Lactoferrin as a natural regimen for selective decontamination of the digestive tract: recombinant porcine lactoferrin expressed in the milk of transgenic mice protects neonates from pathogenic challenge in the gastrointestinal tract, J. Infect. Dis. 199 (4) (2009) 590–598, https://doi.org/10.1086/596212.

[24] M.A. Rasmussen, T.A. Casey, Environmental and food safety aspects of *Esxherichia coli* O157:H7 infections in cattle, Crit. Rev. Microbiol. 27 (2) (2001) 57–73, https://doi.org/10.1093/nar/27.17.3567.

[25] A.C. Yu, J.F. Loo, S. Yu, S.K. Kong, T.F. Chan, Monitoring bacterial growth using tunable resistive pulse sensing with a pore-based technique, Appl. Microbiol. Biotechnol. 98 (2) (2014) 855–862, https://doi.org/10.1007/s00253-013-5377-9.

[26] C.G. Ehrenberg, Dritter Beitrag zur Erkenntniss grosser Organisation in der Richtung des Kleinsten Raumes, Abhandlungen der Königlichen Akademie der Wissenschaften Berlin, (1833) 145–336.

[27] F. Cohn, Untersuchungen über Bakterien, Beitr Biol. Pflanz. 1 (1872) 127–224 http://www.biodiversitylibrary.org/item/27353#page/287/mode/1up.

[28] M.M. Nakano, P. Zuber, Anaerobic growth of a "strict aerobe" (*Bacillus subtilis*), Annu. Rev. Microbiol. 52 (1998) 165–190, https://doi.org/10.1146/annurev.micro.52.1.165.

[29] I.S. Tan, K.S. Ramamurthi, Spore formation in *Bacillus subtilis*, Environ Microboil Rep 6 (3) (2014) 212–225, https://doi.org/10.1111/1758-2229.12130.

[30] F.J. Contesini, R.R. Melo, H.H. Sato, An overview of *Bacillus* proteases: from production to application, Crit. Rev. Biotechnol. 38 (3) (2018) 321–334, https://doi.org/10.1080/07388551.2017.1354354.

[31] S. Romero, E. Merino, F. Bolívar, G. Gosset, A. Martinez, Metabolic engineering of *Bacillus subtilis* for ethanol production: lactate dehydrogenase plays a key role in fermentative metabolism, Appl. Environ. Microbiol. 73 (16) (2007) 5190–5198, https://doi.org/10.1128/AEM.00625-07.

[32] S.C. Wu, J.C. Yeung, Y. Duan, R. Ye, S.J. Szarka, H.R. Habibi, S.L. Wong, Functional production and characterization of a fibrin-specific single-chain antibody fragment from *Bacillus subtilis*: effects of molecular chaperones and a wall-bound protease on antibody fragment production, Appl. Environ. Microbiol. 68 (7) (2002) 3261–3269, https://doi.org/10.1128/aem.68.7.3261-3269.2002.

[33] L. Westers, D.S. Dijkstra, H. Westers, J.M. van Dijl, W.J. Quax, Secretion of functional human interleukin-3 from *Bacillus subtilis*, J. Biotechnol. 123 (2) (2006) 211–224, https://doi.org/10.1016/j.jbiotec.2005.11.007.

[34] M.A. Durban, J. Silbersack, T. Schweder, F. Schauer, U.T. Bornscheuer, High level expression of a recombinant phospholipase C from *Bacillus cereus* in *Bacillus subtilis*, Appl. Microbiol. Biotechnol. 74 (3) (2007) 634–639, https://doi.org/10.1007/s00253-006-0712-z.

[35] N.D. Phuong, Y.S. Jeong, T. Selvaraj, S.K. Kim, Y.H. Kim, K.H. Jung, J. Kim, H.D. Yun, S.L. Wong, J.K. Lee, H. Kim, Production of XynX, a large multimodular protein of *Thermoanaerobacterium sp.*, by protease-deficient *Bacillus subtilis* strains, Appl. Biochem. Biotechnol. 168 (2) (2012) 375–382, https://doi.org/10.1007/s12010-012-9781-x.

[36] P.Z. Wang, R.H. Doi, Overlapping promoters transcribed by B*acillus subtilis* sigma 55 and sigma 37 RNA polymerase holoenzymes during growth and stationary phase, J. Biol. Chem. 259 (13) (1984) 8619–8625.

[37] C.Y. Lin, P.H. Yang, C.L. Kao, H.I. Huang, H.J. Tsai, Transgenic zebrafish eggs containing bactericidal peptide is a novel food supplement enhancing resistance to pathogenic infection of fish, Fish Shellfish Immunol. 28 (3) (2010) 419–427, https://doi.org/10.1016/j.fsi.2009.11.019.

[38] P.X. Liew, C.L. Wang, S.L. Wong, Functional characterization and localization of a *Bacillus subtilis* sortase and its substrate and use of this sortase system to covalently anchor a heterologous protein to the *B. subtilis* cell wall for surface display, J. Bacteriol. 194 (1) (2012) 161–175, https://doi.org/10.1128/JB.05711-11.

[39] F. Meddeb-Mouelhi, C. Dulcey, M. Beauregard, High transformation efficiency of *Bacillus subtilis* with integrative DNA using glycine betaine as osmoprotectant, Anal. Biochem. 424 (2) (2012) 127–129, https://doi.org/10.1016/j.ab.2012.01.032.

[40] Y.P. Lu, C. Zhang, F.X. Lv, X.M. Bie, Z.X. Lu, Study on the electro-transformation conditions of improving transformation efficiency for *Bacillus subtilis*, Lett. Appl. Microbiol. 55 (1) (2012) 9–14, https://doi.org/10.1111/j.1472-765X.2012.03249.x.

[41] S.B. Park, T. Aoki, T.S. Jung, Pathogenesis of and strategies for preventing *Edwardsiella tarda* infection in fish, Vet. Res. 43 (2012) 67, https://doi.org/10.

1186/1297-9716-43-67.
[42] J. Davies, D. Davies, Origins and evolution of antibiotic resistance, Microbiol. Mol. Biol. Rev. 73 (3) (2010) 417–433, https://doi.org/10.1128/mmbr.00016-10.
[43] E.H. Hardi, R.A. Nugroho, I.W. Kusuma, W. Suwinarti, A. Sudaryono, R. Rostika, Born herbal plant extracts as a natural medication for prophylaxis and treatment of *Aeromonas hydrophila* and *Pseudomonas fluorescens* infection in tilapia (*Oreochromis niloticus*), F1000Res. 7 (2019) 1847, https://doi.org/10.12688/f1000research.16902.2.
[44] M.S. Deepika, R. Thangam, T.S. Vijayakumar, R. Sasirekha, R.T.V. Vimala, S. Sivasubramanian, S. Arun, M.D. Babu, R. Thirumurugan, Antibacterial synergy between rutin florfenicol enhances therapeutic spectrum against drug resistant *Aeromonas hydrophila*, Microb. Pathog. 135 (2019) 103612, https://doi.org/10.1016/j.micpath.2019.103612.
[45] S. Aemphan, S. Unaiak, C. Printrakoon, N. Areechon, Feeding-regimen of β-glucan to enhance innate immunity and disease resistance of *Nile* tilapia, *Orochromis niloticus Linn.*, against *Aeromonas hydrophila* and *Flavobacterium columnare*, Fish Shellfish Imminol. 87 (2019) 120–128, https://doi.org/10.1016/j.fsi.2018.12.062.
[46] M.L. Situmorang, K. Dierckens, F.T. Mlingi, B. Van Delsen, P. Bossier, Development of a bacterial challenge test for gnotobiotic N*ile t*ilapia *Oreochromis niloticus* larvae, Dis. Aquat. Org. 109 (1) (2014) 23–33, https://doi.org/10.3354/dao02721.
[47] J.H. Cha, S. Rahimnejad, S.Y. Yang, K.W. Kim, K.J. Lee, Evaluations of *Bacillus spp.* as dietary additives on growth performance, innate immunity and disease resistance of olive flounder (*Paralichthys olivaceus*) against *streptococcus iniae* and as water additives, Aquaculture 402–403 (2013) 50–57, https://doi.org/10.1016/j.aquaculture.2013.03.030.
[48] Y. Yi, Z. Zhang, F. Zhao, H. Liu, L. Yu, J. Zha, G. Wang, Probiotic potential of *Bacillus velezensis* JW: antimicrobial activity against fish pathogenic bacteria and immune enhancement effects on *Carassius auratus*, Fish Shellfish Immunol. 78 (2018) 322–330, https://doi.org/10.1016/j.fsi.2018.04.055.
[49] P.E. Ulloa, C.J. Solis, J.F. De la Paz, T.G. Alaurent, M. Caruffo, A.J. Hernandez, P. Dantagnan, C.G. Feijoo, Lactoferrin decreases the intestinal inflammation triggered by a soybean meal-based diet in zebrafish, J. Immunol. Res. 2016 (2016) 1639720, https://doi.org/10.1155/2016/1639720.
[50] F.K.A. Kuebutornye, E.E. Abarike, Y. Lu, A review on the application of *Bacillus* as probiotic in aquqculture, Fish Shellfish Immunol. 87 (2019) 820–828, https://doi.org/10.1016/j.fsi.2019.02.010.