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Synergistic activity of plant prebiotics and *Lactococcus lactis* KA-FF 1-4 to enhance vancomycin-resistant enterococci (VRE) growth inhibition

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Abstract

Synbiotics are products containing both probiotics and prebiotics, in which the prebiotic compound selectively favours the probiotic to provide synergistic activity for gut health promotion. Due to its potential probiotic effect, *Lactococcus lactis* KA-FF 1-4 showed inhibitory activity against vancomycin-resistant enterococci (VRE). Its synergistic activity with three commercial plant prebiotics namely inulin, fibersol-2, and XOS is presented in the present work. *In vitro* batch fermentation with each prebiotic supplement had no effect on the growth rate of *L. lactis* KA-FF 1-4. However, supplementation with either 2% w/v inulin or fibersol-2 dramatically enhanced anti-VRE activity. Further study was performed on the effect of various concentrations (0.5, 1, 2, and 5%) of inulin or fibersol-2 on anti-VRE activity. In co-culture, *L. lactis* KA-FF 1-4 with 1% inulin supplementation could apparently reduce the VRE concentration from log 4.0 to 2.7 CFU/mL after 12 h, while complete inhibition was observed with 2% fibersol-2 supplementation after 6 h. These results clearly showed the synergistic effect on VRE growth inhibition, which may serve as a guideline for the development of synbiotic formulations in the future.

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Introduction

Synbiotics are a combination of prebiotics and probiotics, which synergistically improves probiotic activity as compared to the activity of probiotics alone (Schrezenmeir and de Vrese, 2001; Pranckutė *et al.,* 2016). Most prebiotics are forms of dietary fibre that serves as a carbon source to support the growth and activity of probiotics, including bacteriocin production (Chen *et al.,* 2007; Lim *et al.,* 2011). However, most commercial synbiotics have not been formulated based on synergistic effects. Instead, synbiotics are often formulated based on shelf life, availability, cost, industrial performance, or convenience because of other marketing considerations. A few studies have reported the synergistic effects of synbiotic formulations (Audisio *et al.,* 2001; Saminathan *et al.,* 2011; Farinha *et al.,* 2015). The compatibility between the chosen prebiotics and probiotic strains and the prebiotic concentrations are important factors to consider for synergistic synbiotics (Kondepudi *et al.,* 2012).

Vancomycin-resistant enterococci are enterococci in human gut that have developed resistance to vancomycin. An increase in the number of patients with VRE infections has caused an increase in the use of broad-spectrum antibiotics and the transmission of VRE-associated genes. Probiotic therapy has been used to eradicate VRE overgrowth or colonisation in the human intestine (Manley *et al.,* 2007; Crouzet *et al.,* 2015). *Lactococcus lactis* M19 and *Pediococcus acidilactis* MM33 can produce Nisin Z and pediocin PA-1/AcH, and show anti-VRE activity in mice (Millette *et al.,* 2008). In contrast, in humans, administration of *Lactobacillus rhamnosus* GG in the form of gelatine capsules to comorbid patients could not reduce VRE in faeces. It was reported that the treatment dose and human gut environment should be considered (Doron *et al.,* 2015).

The potential probiotic *Lactococcus lactis* KA-FF 1-4 was isolated from fermented fish. This strain could produce bacteriocin, which showed inhibitory effects against a broad spectrum of food-borne pathogens, including VRE. Plupjeen *et al.* (2020) evaluated the co-culture of *L. lactis* KA-FF 1-4 (10^8 CFU/mL) and VRE at various concentrations (10^3 - 10^6 CFU/mL). The highest VRE concentration that was reduced to zero was 10^4 CFU/mL of *L. lactis* KA-FF 1-4. However, the growth of *L. lactis* KA-FF 1-4 was stable, and the anti-VRE activity was reduced to 3 - 6% in a human gut model that mimicked the gut microbiome. Therefore, prebiotics were shown to improve growth and inhibitory activity.

It is known that prebiotics are non-digestible fibres, such as resistant starch, polysaccharides, and oligosaccharides (Gibson *et al.,* 2004). Almost all of these plant fibres are extracted from natural sources.

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Inulins are polysaccharides consisting of D-fructose linked by β-2,1 glycosidic bond, and they have been shown to enhance the growth and antimicrobial activity of lactic acid bacteria (Kareem *et al.,* 2014; Farinha *et al.,* 2015; Mandal *et al.,* 2016). Fibersol-2 is a resistant maltodextrin that is composed of not only of α-1,4 and α-1,6 glycosidic bonds, but also α-1,2 and α-1,3 glycosidic linkages. The consumption of fibersol-2 tended to increase the *Bifidobacterium* population in faeces (Fastinger *et al.,* 2008). Xylo-oligosaccharides (XOS) are oligosaccharides containing β-1,4-linked xylose residues. XOS has been found to stimulate the growth of bifidobacteria (Aachary and Prapulla, 2011).

The present work aimed to enhance the anti-VRE effects of *L. lactis* KA-FF 1-4 by using plant prebiotic supplements. The effects of three commercial prebiotics, including inulin, XOS, and fibersol-2 on the growth and anti-VRE activity of *L. lactis* KA-FF 1-4 were investigated. In addition, the synergistic formulations of *L. lactis* KA-FF 1-4 and different prebiotic concentrations were studied.

Materials and methods

Microorganisms and culture

Lactococcus lactis KA-FF 1-4 as an antimicrobial producer was cultured in de Man Rogosa (MRS) (Difco, France) at 37°C for 18 h. The target VRE strain, *Enterococcus faecium* 426, was cultured in MRS with 128 µg/mL vancomycin at 37°C for 18 h.

Prebiotic preparation

Three commercial prebiotics, inulin (Orafti®GR, Belgium), xylo-oligosaccharide (XOS) (Shandong, China), and fibersol-2 (Matsutani, Japan) were completely dissolved in distilled water, and sterilised by using a 0.2 µm filter (Minisart filters, Sartorius AG, Germany). Each aliquot was further kept at 4°C until use.

Culture of L. lactis KA-FF 1-4 and VRE under prebiotic supplementation

One percent *L. lactis* KA-FF 1-4 at 10^6 - 10^7 CFU/mL or VRE at 10^7 CFU/mL was inoculated in 30 mL of modified basal medium (2.00 g/L peptone, 10.00 g/L yeast extract, 0.10 g/L NaCl, 0.04 g/L K_2HPO_4, 0.04 g/L KH_2PO_4, 0.01 g/L $MgSO_4$ $7H_2O$, 0.01 g/L $CaCl_2$ $6H_2O$, 2.00 g/L $NaHCO_3$, 2 mL Tween 80, 0.05 g/L hemin, 10 µL vitamin K_1, 0.50 g/L L-cysteine-HCl, and 0.05 g/L bile salts) (Onumpai *et al.,* 2011) with prebiotic supplementation at concentrations of 0.5, 1, 2, and 5%. The cell culture was incubated at 37°C for 24 h, and sampled at 3-h intervals to determine the cell concentrations of *L. lactis* KA-FF 1-4 and VRE by standard plate counting on MRS agar and bile esculin azide agar (modified, Himedia) with 128 mg/mL vancomycin, respectively. All measurements were performed in duplicate. The specific growth rate (μmax) was calculated during the exponential phase of growth using Eq. 1:

$$\mu max = (\ln x - \ln x_0) \times (t - t_0) \qquad \text{(Eq. 1)}$$

where, x and x_0 = number of viable cells at time t and t_0, respectively.

Preparation of rabbit polyclonal antibody against bacteriocin KAFF produced by L. lactis KA-FF 1-4, and its application to the quantification of the bacteriocin concentration by ELISA

Bacteriocin KAFF produced by *L. lactis* KA-FF 1-4 was evaluated by enzyme-linked immunoassay (ELISA) with rabbit polyclonal antibody prepared from bacteriocin obtained from the National Laboratory Animal Centre Mahidol University, Salaya, Thailand. To prepare the antibody, bacteriocin KAFF was purified using hydrophobic interaction (Amberlite XAD 16), cation exchange chromatography (SP Sepharose®), and RP-HPLC (Resource RPC) methods. The purity and size of bacteriocin was confirmed by SDS-PAGE before used in antibody preparation. The ELISA method was modified based on the method of Leversee and Glatz (2001). The F-bottom 96-well plate (Greiner-one, USA) was coated overnight at 4°C with 100 µL of *L. lactis* KA-FF 1-4 cell-free supernatant. The coated plate wells were washed twice with phosphate-buffered saline containing 0.05% v/v Tween 20, pH 8.0 (PBST), blocked by adding 200 µL of PBST containing 1% w/v bovine serum albumin (blocking buffer) to each well, and the plate incubated at 37°C for 1 h. The plates were then washed twice with PBST. Primary antibodies (100 µL of primary antibody diluted 1:200 in blocking buffer) were added to the wells and incubated at 37°C for 1 h. Unbound antibodies were removed by washing with PBST three times. Then, 100 µL of goat anti-rabbit immunoglobulin G [IgG] horseradish peroxidase conjugate (KPL, Milford, USA) was used as a secondary antibody, which was diluted to 1:5000 in blocking buffer, added to each well, and further incubated for 30 min at 37°C. After the washing step, the bound antibodies were detected by a colorimetric reaction by adding 100 µL of *o*-phenylenediamine (KPL, Milford, USA) to each well, which was incubated at room temperature for 10 min in a dark room. The reaction was stopped by 0.1 N hydrochloric acid, and the absorbance (405 nm) of each well was read with a

Synergy HTX microplate reader (Biotek, Vermont, USA) with Biotek Gen 5 software. The bacteriocin standard was prepared at concentrations of 0.125, 0.25, and 1 ng/mL. The cell-free supernatant of *L. lactis* KA-FF 1-4 without non-prebiotic treatment served as a control.

Determination of anti-VRE activity

Anti-VRE activity was determined by a spot-on-lawn assay following the modified method of Batdorj *et al.* (2006). In brief, each cell-free supernatant (CFS) was adjusted to pH 5.5 using 0.1 M NaOH, and heated at 70°C for 30 min. A two-fold dilution of CFS was made in sterilised deionised water. Soft MRS agar medium (0.75% agar w/v) containing 10^6 CFU/mL VRE was placed over 1.5% w/v MRS agar. Ten microliters of each CFS was spotted onto the soft agar containing VRE, incubated at 37°C for 18 h, and the clear zone was observed. The anti-VRE activity was expressed as arbitrary units per millilitre (AU/mL) using Eq. 2:

$$AU/mL = (2^{(N-1)} \times 1{,}000) \times 10^{-1} \quad \text{(Eq. 2)}$$

where, N = highest dilution showing activity.

Co-culture of L. lactis KA-FF 1-4 and VRE

One percent *L. lactis* KA-FF 1-4 and VRE at concentrations of 10^8 and 10^4 - 10^5 CFU/mL, respectively, were co-cultured in modified basal medium with prebiotic treatment. The effect of prebiotics at concentrations of 0.5, 1, 2, and 5% was investigated. The mixed culture was incubated at 37°C for 24 h. The number of VRE was monitored by total plate counting using Bile Esculin Azide Agar (modified, Himedia) with 128 mg/mL vancomycin. The mixed culture without prebiotic served as the control.

Statistical analysis

All analyses were carried out based on the analysis of variance (ANOVA) test, and *p*-values below 0.05 ($p < 0.05$) were considered significant when using Duncan's test.

Results

The effect of three prebiotics on the growth of L. lactis KA-FF 1-4 and anti-VRE activity

L. lactis KA-FF 1-4 was cultured in the presence of three different prebiotic compounds, including 2% inulin, fibersol-2, and XOS to assess the effect of treatment with inulin, fibersol-2, and XOS, respectively, as compared to that of the control (without prebiotic addition). The initial concentration of viable cells in the prebiotic supplementation media was log 6.0 - 6.3 CFU/mL (Figure 1A). The concentration of *L. lactis* KA-FF 1-4 rapidly increased to log 8.7 - 9.0 CFU/mL after 6 h in the control and all prebiotic treatments. The specific growth rate of *L. lactis* KA-FF 1-4 after inulin, fibersol-2, and XOS treatments was 0.08 - 0.09/h, which was similar to that of the control. The cell concentration was stable after 6 h, except when cells were subjected to inulin treatment. The cell concentration decreased to log 8.2 - 8.0 CFU/mL ($p \leq 0.05$) after 6 h.

The amount of bacteriocin produced from treatment with inulin and fibersol-2 was 3.8 - 4.3 ng/μL, which was not significantly different from that of the control (3.7 - 4.2 ng/μL) (Figure 1B). However, this did not appear to be the case for XOS treatment.

The inhibitory activity against VRE was determined as shown in Figure 1C. VRE inhibitory activity of 100 - 300 AU/mL was detected only in the inulin and fibersol-2 treatment groups, but not in the control group. The maximum activity was observed

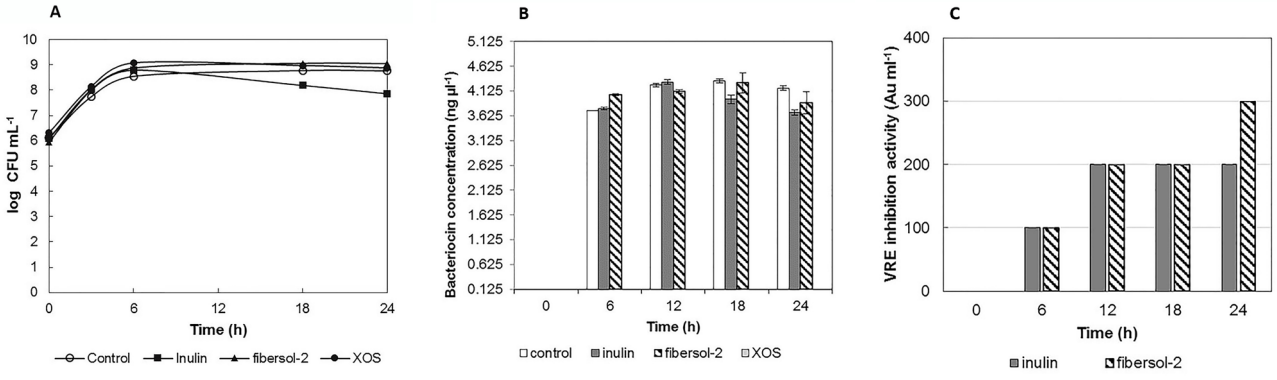

Figure 1. Time course of the fermentation of *Lactococcus lactis* KA-FF 1-4 in basal medium supplemented with 2% w/v inulin, fibersol-2, and XOS at 37°C for 24 h. The growth of *L. lactis* KA-FF 1-4 cultured in basal medium was used as a control. Cell concentration (A), bacteriocin production (B), and VRE inhibition activity (C).

in the stationary phase of cell growth after 12 - 24 h. It seemed that both inulin and fibersol-2 promoted both bacteriocin production and anti-VRE activity, while the control exhibited only bacteriocin production.

The effect of prebiotics on the growth of VRE

The growth of VRE in the presence of different prebiotics was monitored and compared to that of the control (Figure 2). The results revealed that the growth curve of VRE in the presence of prebiotic treatment was similar to that of the control. The concentration of VRE increased from log 7.0 to 8.8 - 9.0 CFU/mL after 3 h, and was stable. However, the concentration of VRE after inulin treatment decreased to log 8.0 CFU/mL at 18 and 24 h.

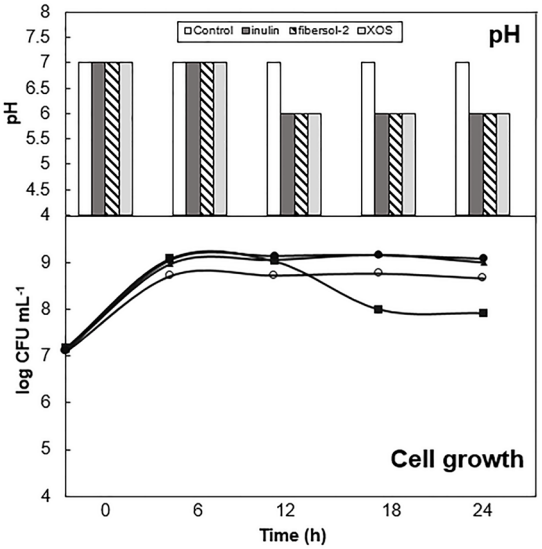

Figure 2. The growth of VRE (line graph) and pH value (bar graph) in basal medium supplemented with 2% w/v inulin (square), fibersol-2 (triangle), and XOS (black circle). The growth of VRE cultured in basal medium was used as a control (white circle).

VRE is a lactic acid-producing bacterium. Therefore, the pH value of the VRE culture was monitored (Figure 2). The pH value in the presence of all prebiotic supplements dropped to 6 after cultured for 6 h. However, the pH value was stable at 7 in the control. VRE could utiliSe all of the prebiotics to produce acid. However, there was no effect on the growth of VRE. Only inulin treatment reduced the concentration of VRE at the stationary phase even though the pH level was similar.

The effect of the inulin concentration on the growth of L. lactis KA-FF 1-4 and anti-VRE activity in co-culture

Inhibitory activity against VRE was observed in inulin supplementation conditions. Therefore, the influence of 0.5, 1, 2, and 5% inulin on growth and anti-VRE activity was further investigated (Figure 3). The concentration of *L. lactis* KA-FF 1-4 and VRE alone in the presence of all treatments increased to log 8.9 - 9.3 CFU/mL (Figure 3A) and log 8.8 - 9.0 CFU/mL (Figure 3B) after 3 and 12 h, respectively. Then, the cell concentration was stable for 24 h. The inulin concentration had no effect on the growth curve of *L. lactis* KA-FF 1-4 and VRE.

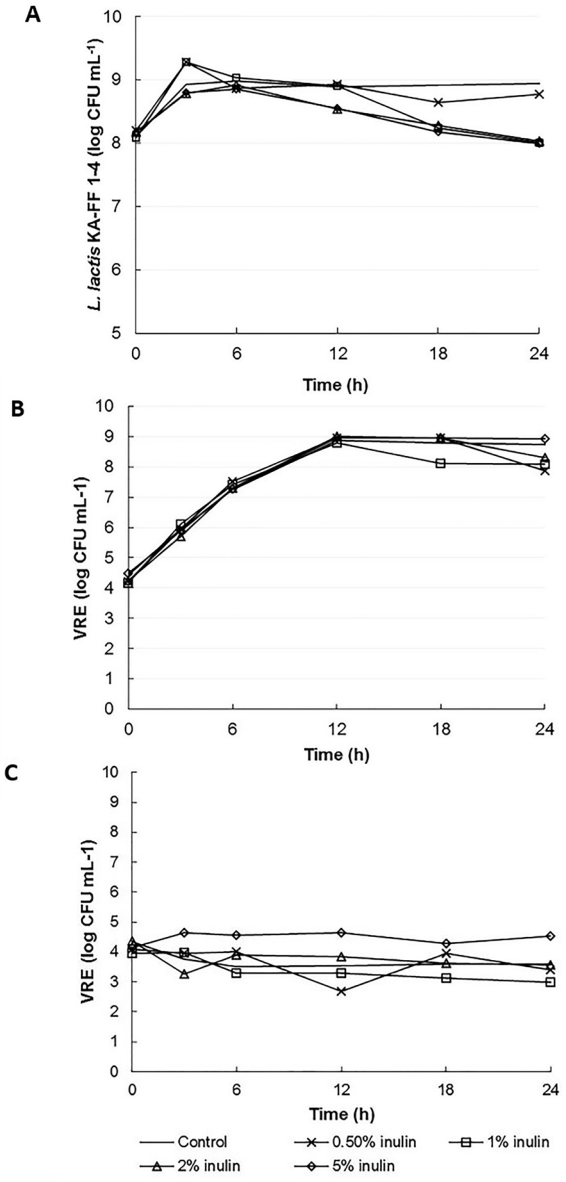

Figure 3. The influence of inulin at various concentrations on the culture of *L. lactis* KA-FF 1-4 (A), VRE alone (B), and the co-culture (C).

The VRE growth inhibitory activity was measured in co-cultures of *L. lactis* KA-FF 1-4 and VRE (Figure 3C). The concentration of VRE in the control (without prebiotic addition) was stable at log 3.5 - 4.3 CFU/mL after 24 h. Interestingly, the concentration of VRE in the presence of the 1%

inulin treatment decreased to 2.7 - 3.2 log CFU/mL after 12 h. Additionally, anti-VRE activity in the presence of 0.5, 2, and 5% inulin supplementation was similar to that in the control. The inulin concentration had no effect on the growth of VRE.

The effect of the fibersol-2 concentration on the growth of L. lactis KA-FF 1-4 and anti-VRE activity

The effects of 0.5, 1, 2, and 5% fibersol-2 on the growth and VRE growth inhibitory activity of *L. lactis* KA-FF 1-4 alone, VRE alone, and the co-culture were investigated (Figure 4). It was found that the maximum concentration of *L. lactis* KA-FF 1-4 was log 9 CFU/mL after 3 h, and the growth dynamics of all treatments were similar (Figure 4A). It was indicated that the amount of fibersol-2 had no effect on the growth of *L. lactis* KA-FF 1-4. However, the growth rate of VRE after 6 h of 1, 2, and 5% fibersol-2 treatment increased as compared to that of the control (Figure 4B). However, an enhancement effect was not observed with 0.5% fibersol-2 supplementation fibersol-2 treatment group and the control group (Figure 4C) as compared to VRE alone (Figure 4B). The VRE cell concentration was stable at log 3.5 - 4 CFU/mL in the control and 5% fibersol-2 treatment groups, while the VRE cell number in the 0.5, 1, and 2% fibersol-2 treatment groups was < 10 CFU/mL at 15, 21, and 6 h, respectively. The results indicated that treatment with 2% fibersol-2 was optimal in enhancing the anti-VRE activity of *L. lactis* KA-FF 1-4.

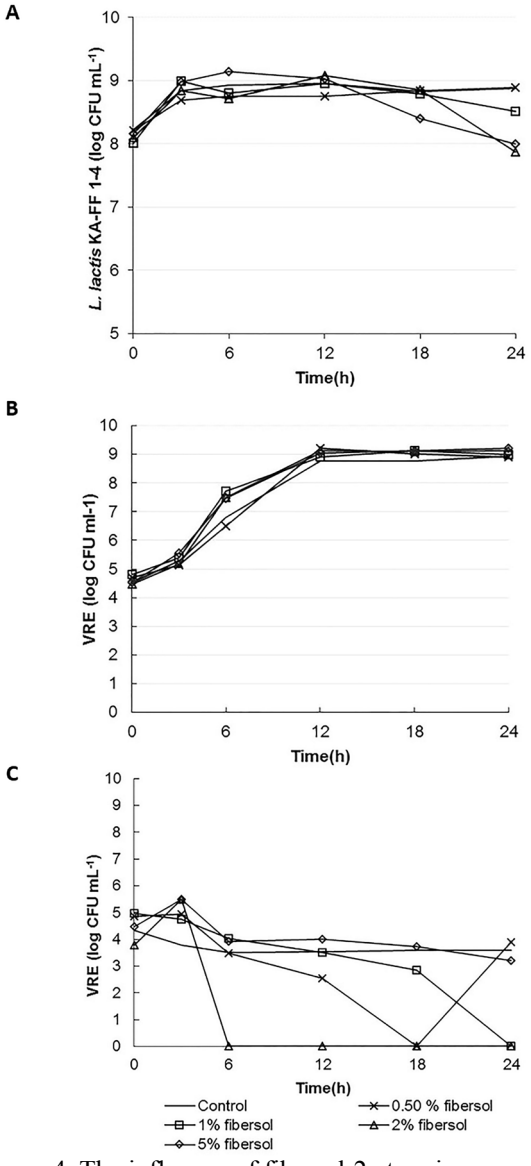

Figure 4. The influence of fibersol-2 at various concentrations on the culture of *L. lactis* KA-FF 1-4 (A), VRE alone (B), and the co-culture (C).

Discussion

In the present work, the formulation of a synbiotic with inhibitory effects on the growth of VRE was investigated by measuring the synergistic inhibitory activity between *L. lactis* KA-FF 1-4 and commercial prebiotic compounds including inulin, fibersol-2, and XOS. However, the inhibitory activity against VRE occurred only as a result of inulin and fibersol-2 supplementation. Correspondingly, the production of bacteriocin was observed after inulin and fibersol-2 supplementation (Figure 1). It should be noted that inulin and fibersol-2 showed synergistic activity with *L. lactis* KA-FF 1-4 by enhancing bacteriocin production. However, XOS had no effect on bacteriocin production. It was reported that the production of bacteriocin by lactic acid bacteria is dependent on media conditions, including carbon sources (Tomás *et al.,* 2010; Sabo *et al.,* 2019). Meanwhile, bacteriocin production without prebiotics was also shown by ELISA, whereas anti-VRE activity was not observed in the spot-on-lawn assay. This might have occurred because the bacteriocin was in inactive form (Perez *et al.,* 2018). Brink *et al.* (2006) stated that nutrients play a key role in the production and activity of antimicrobial substances.

Prebiotic compounds may specifically increase the levels of beneficial microorganisms, and should not promote the growth of pathogenic bacteria (Fooks and Gibson 2002; Rousseau *et al.,* 2005; Nunpan *et al.,* 2019). Our results revealed that the prebiotic compounds did not enhance the growth of VRE. The growth curve of VRE after prebiotic treatment was similar to that of VRE without prebiotic treatment. Based on our results, it was suggested that inulin and fibersol-2 were appropriate for use in combination with *L. lactis* KA-FF 1-4 for VRE inhibition.

The concentration of the prebiotic was an important factor for the synergistic formulation.

Nunpan *et al. (*2019) reported that 3, 4, and 5% galacto-oligosaccharide (GOS) and fructo-oligosaccharide (FOS) could enhance the activity of *Lactobacillus acidophilus* to inhibit *Streptococcus mutans* growth. Furthermore, the growth rate of *L acidophilus* was not affected by all concentrations of GOS and FOS. The effect of either inulin or fibersol-2 on VRE in the present work is in line with these findings. In addition, the inhibition ratio of the pathogen after mixed culture with *L. acidophilus* and *L. paracasei* supplemented with 0.5 and 1% FOS significantly increased as compared to that observed when it was cultured without prebiotics (Lim *et al.,* 2011). The effects of 1, 2, and 5% FOS supplemented with *Lactobacillus* strains on antimicrobial activity were reported. Only 2% FOS supplementation showed inhibitory activity (Muñoz *et al.,* 2012). These studies suggested that the effect of the prebiotic concentration on inhibitory activity was dependent on the species. Thus, optimisation of the prebiotic concentration to enhance synergistic activity needs to be considered.

In the non-prebiotic treatment group, *L. lactis* KA-FF 1-4 could reduce the VRE concentration from 10^9 to 10^4 CFU/mL, which is consistent with the findings observed for both 5% inulin and fibersol-2. However, the VRE concentration in the presence of 0.5, 1, and 2% inulin decreased to 10^3 CFU/mL. Furthermore, the VRE concentration was reduced to zero in the presence of 0.5, 1, and 2% fibersol-2 supplemented with *L. lactis* KA-FF 1-4. The shortest time needed to reduce the concentration of VRE to zero was observed after 2% fibersol-2 supplementation for 6 h. Moreover, the excess or dearth of prebiotic affected synergistic activity. An excess concentration did not produce better results. Among the synergistic formulations, *L. lactis* KA-FF 1-4 with 2% fibersol-2 was the best formulation for application in VRE inhibition in the future.

Conclusion

Only inulin and fibersol-2 had synergistic activity with *L. lactis* KA-FF for vancomycin-resistant enterococci (VRE) growth inhibition. Moreover, 2% fibersol-2 supplementation was shown to strongly reduce the concentration of VRE to zero in the shortest amount of time. To further enhance the synergistic activity, strain-specific prebiotic types and prebiotic concentrations should be used.

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