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ORIGINAL ARTICLE

Determination of an antimicrobial activity of *Weissella confusa*, *Lactobacillus fermentum*, and *Lactobacillus plantarum* against clinical pathogenic strains of *Escherichia coli* and *Staphylococcus aureus* in co-culture

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Abstract Lactic acid bacteria (LAB) have long been used to produce safe and high quality products as they are potential producers of a wide range of antimicrobial compounds that exert either narrow or wide spectrum antimicrobial activity towards spoilage or disease-causing organisms. The present investigation aimed to study the antimicrobial effect of three LAB strains, viz., *Lactobacillus plantarum* (86), *Lactobacillus fermentum* (AI2) and *Weissella confusa* (AI10), against two clinical pathogenic strains viz., *Escherichia coli* NG 502121 and *Staphylococcus aureus* AY 507047 in co-culture. Effects of change in inoculum size, and growth measurement at different time intervals were also studied. The pH and viable count were measured for initial as well as 24 h incubated samples. A significant ($P<0.05$) reduction (2–3 log cycles) in growth of both pathogens while co-cultured with LAB strains was observed. The nonsignificant ($P<0.05$) pH difference revealed the action of other metabolites apart from organic acids. LAB strains overruled the growth of *E. coli* and *S. aureus* within 10 and 6 h of the initial growth stage, respectively, compared to controls. These results led us to further characterize and purify the antimicrobial compound produced by the studied strains, so that they can be exploited in the production of safe foods with longer shelf life.

Keywords Co-culture · Lactic acid bacteria · Antimicrobial activity · Growth kinetics · Dose dependency

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Introduction

The gastrointestinal (GI) tracts of humans and animals comprise a complex microbial ecology, with associated positive and negative consequences (Charlier et al. 2009). *Escherichia coli* and *Staphylococcus aureus* play a fundamental role in enteric infections, and are the most common threatening agents in industry and medical science (Ballal and Shivananda 2002; Mohammedsaeed et al. 2014; Poppi et al. 2015). *E. coli* is an opportunistic pathogen that produces a potent enterotoxin and is a causative agent for GI tract disorders such as constipation, diarrhoea, etc., while *S. aureus* is the causative agent of a wide panel of infections ranging from superficial lesions to life-threatening septicaemia. It is also responsible for causing food poisoning through ingestion of either food contaminated with *S. aureus* or the enterotoxin produced (Le loir et al. 2003).

Lactic acid bacteria (LAB) are found as a dominant flora in various fermented foods, as well as constructing an essential part of natural ecosystems found in the GI tract and vagina (Sreekumar and Hosono 2000). The main activity of these bacteria is to ferment sugar, which results in the production of organic acids; the preservative effect in food is attributed primarily to organic acids followed by other antimicrobial compounds like H_2O_2, diacetyl, acetaldehyde, CO_2, fatty acids, exopolysaccharides (EPS), and proteinaceous compounds known as bacteriocins (Vermeiren et al. 2006;

Charlier et al. 2009). Combinations of these compounds can restrict the growth of pathogenic as well as food spoilage microorganisms, and hence LAB have been considered as multifunctional agents to provide food safety and stability along with natural fermentation. The antimicrobial potential of LAB has created new horizons in both the food industry and medical science. Many lactobacilli and lactococci species found to be inhibitory towards *E. coli* and *S. aureus* in vitro and in vivo (Laughton et al. 2006; Turner et al. 2007; Anas et al. 2008; Celine Delbes-Paus et al. 2010; Varma et al. 2010; Liu et al. 2011; Savino et al. 2011; Huang et al. 2015; Kumar and Kumar 2015). LAB strains isolated from healthy infant faeces ($n=95$) restricted the growth of several enteropathogenic bacteria i.e., ETEC H10407, *Yersinia enterocolitica ATCC 23715, Salmonella enteritidis*, *Shigella sonnei* ATCC 9290, H7 and *Shigella flexneri* ATCC 12022 (Davoodabadi et al. 2015). Over decades, research has been carried out to study the effects of live LAB and pathogen against each other in experimental co-culture. Co-culture is a system containing two distinct types of cells, and allowing the growth of both types of cells.

Previously, we studied three LAB strains, *Lactobacillus plantarum* (86), *Lactobacillus fermentum* (AI2), and *Weissella confusa* (AI10), for a series of features, such as acid and bile resistance, bile salt hydrolase (BSH) activity, EPS production, etc. (Patel et al. 2012, 2013a, b). The present study aimed to determine the antimicrobial effect of these three LAB strains against two highly virulent clinical strains through co-culture. Various parameters, such as the effect of change of inoculum size, on the survival of both groups of bacteria, and growth kinetics in the co-culture system, were also further analysed.

Materials and methods

Bacterial strains and culture conditions

Three LAB strains namely, *L. plantarum* (86), *L. fermentum* (AI2), and *W. confusa* (AI10) (GenBank accession numbers JN792454, JN792468, and JN792460) were obtained from the Dairy Microbiology Dept., SMC College of Dairy Science, Anand Agricultural University, Anand, Gujarat, India, while two clinical strains viz., *E. coli* NG 502121 and *S. aureus* AY 507047 were procured from the Biomedical Centrum (BMC), Lund, Sweden. During the study, de Man-Rogosa-Sharpe (MRS) broth and brain heart infusion (BHI) broth were used for the propagation of LAB and pathogens, respectively, at 37 °C. The stock cultures were prepared in glycerol (80 %) and preserved at −20 °C. All media and ingredients were purchased from Merck (Darmstadt, Germany).

Antimicrobial study by co-culture

Bacterial strains were individually propagated in their respective broth medium and incubated at 37 °C for 24 h followed by two successive transfers to PYG (with 1 % glucose) broth. The active cultures were centrifuged at 4000 rpm, 4 °C for 10 min to collect the cell pellet. The cells were washed twice with phosphate buffer (PB) (pH 7.2) and re-suspended in 2 mL PB to measured 1 OD_{520} (Bio-Rad, Hercules, CA). Then, each culture was inoculated at 1 OD in to different 10 mL PYG (1 % glucose) broth tubes, singly as well as in co-culture, and incubated at 37 °C for 24 h. Samples were removed at 0 h and 24 h for the determination of viable cell count and pH measurement, and a 1-mL aliquot of each system was used to prepare serial dilutions and then poured on the appropriate agar plates, i.e. if *L. fermentum* strain AI2 and *E. coli* were co-cultured, then MRS agar was used for *L. fermentum*, while MC agar was used for *E. coli*, and in case of *S. aureus*, Chromo agar was used. Plates were incubated at 37 °C for 24 h (pathogens) to 48 h (LAB) and colonies were counted. Each experiment was conducted in duplicate and repeated three times.

Dose dependency

The experiment was performed in a similar way as the co-culture experiment except that the inoculum size of LAB:pathogens was varied, e.g. 1:2, 2:1 and 1:10.

Growth measurements at different time intervals

Mono- and co-cultures were inoculated at 1 OD in a respective tube containing 20 mL sterile PYG (1 % glucose) broth followed by incubation at 37 °C. Every 6 h, samples were withdrawn and analysed for pH and viable count on selective media for LAB and pathogen strains. The colonies were counted after growth at 37 °C up to 24 h–48 h, and the $\log_{10}$ CFU was plotted against incubation period to prepare growth curves of individual strains and co-culture.

Statistical analysis

The results of three individual experiments were gathered to generate the mean ± standard deviation (SD). One way analysis of variance (ANOVA) was used to determine the significance by using Minitab at $P<0.05$.

Results and discussion

Before the co-culture experiments, various commercial media were tested to determine suitable media that would favour the growth of specific bacterium only, either LAB or pathogen. Of

these, MRS was found to selectively support the three lactobacilli strains, while Chromo agar and MC agar allowed proper growth of *S. aureus* and *E. coli*, respectively. PYG broth with 1 % glucose supported luxuriant growth of both groups of bacteria as compared to PYG broth with 0.5 % glucose.

In co-culture, the LAB strains showed a significant ($P<0.05$) reduction in growth of *E. coli* compared to growth in mono-culture. Initially, the pH of the medium was set to neutral (6.8–7.0) in PYG broth. Viable counts of different LAB strains ranged from 7.71 to 8.01 log CFU/mL, whereas the *E. coli* count was 8.26 log CFU/mL initially, i.e. at 0 h. After 24 h incubation, the cell population had increased to 1 log cycle/ in co-culture and mono-culture tubes, but in the tube containing *E. coli* co-cultured with lactobacilli strains, the cell count was found to be decreased by 2 log CFU/mL (Fig. 1). Many lactobacillus strains have been proven to possess an antibacterial activity against coliforms in co-culture (Savino et al. 2011; Deng et al. 2015; Davoodabadi et al. 2015). Fooks and Gibson (2002) reported a 6 log cycle reduction for *E. coli* growth when co-cultured with *L. plantarum* in the presence of fructo-oligosaccharide (FOS). In another study, several bacteriocin-producing LAB species, characterized as *L. lactis*, *L. plantarum*, *L. casei*, *L. acidophilus* and *L. fermentum* showed potential antibacterial activity towards various clinical indicator and type cultures such as *B. subtilis* NCTC8236, *E. coli* V157, NCTC11560, *B. licheniformis* CIS26, *P. aeruginosa* CIS23, *K. pneumoniae* CIS29V and *K. aerogenes* CIS55 (Ogunse et al. 2007). No significant difference was observed between pH values of 24 h incubated mono-cultures and co-culture, which suggests that inhibition of *E. coli* cannot be directly correlated with the decrease in pH. On the other hand, the growth of all LAB strains was not influenced by the presence and growth of *E. coli*, which is in accordance with the studies of Yun et al. (2009) and Drago et al. (1997). The results probably indicate a phenomenon of invasion and/or competition between LAB and pathogens for nutrients after a certain time period (Tambekar and Bhutada 2010).

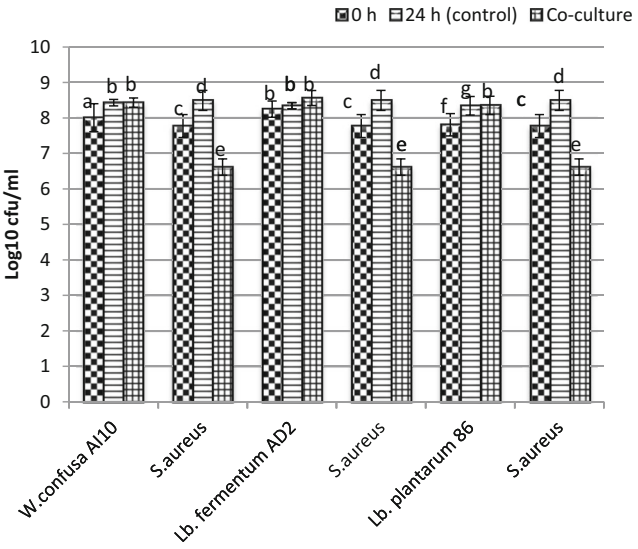

Fig. 2 Co-culture between LAB strains and pathogenic strain *Staphylococcus aureus* AY 507047. Values with a different lower case letter in each column and each set of treatments differ significantly at the 5 % level

The viable counts of *W. confusa* (strain AI10), *L. plantarum* (strain 86), and *L. fermentum* (strain AI2), was 8.01, 7.81, and 8.25 log CFU/mL when co-cultured with *S. aureus* (7.77 log CFU/mL) at an initial pH of 6.8. Growth of *S. aureus* was also suppressed significantly ($P<0.05$) by the presence of all three LAB strains. During co-culture of *S. aureus*, after 24 h incubation, the same inhibitory pattern was found as for *E. coli* (Fig. 2). A non-significant ($P<0.05$)

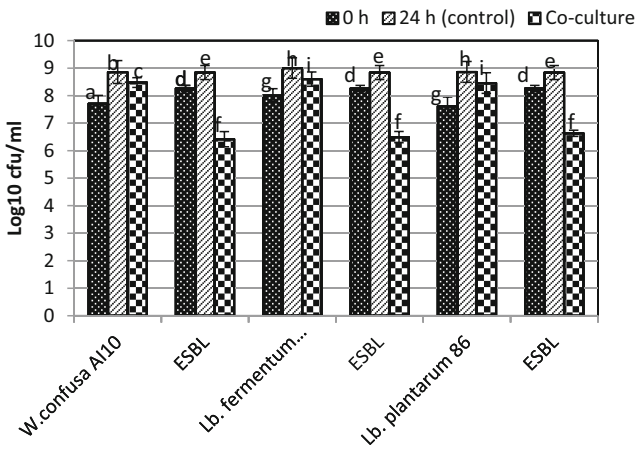

Fig. 1 Co-culture between lactic acid bacteria (LAB) strains and pathogenic strain *Escherichia coli* NG 502121. Values with a different lower case letter in each column and each set of treatments differ significantly at the 5 % level

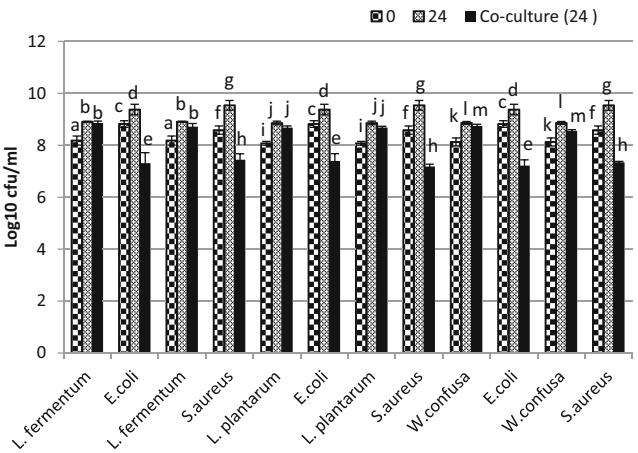

Fig. 3 Dose dependency (1:2) between LAB strains and pathogenic strains *E. coli* NG 502121 and *S. aureus* AY 507047 in co-culture experiments. Values with different lower case letters in each column and each set of treatments differ significantly at the 5 % level

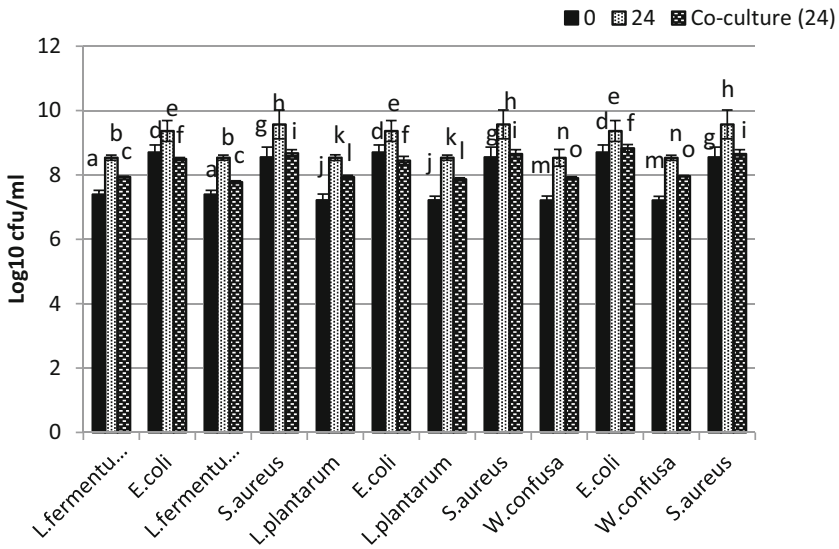

Fig. 4 Dose dependency (1:10) between LAB strains and pathogenic strains *E. coli* NG 502121 and *S. aureus* AY 507047 in co-culture experiments. Values with different lower case letters in each column and each set of treatments differ significantly at the 5 % level

difference in pH was seen among mono- and co-cultures after 24 h of incubation. *S. aureus* is reported to grow between pH values 4.6 and 10, with optimal growth at a pH value close to neutral, which allows the effect of other inhibitory metabolites to be exploited (Charlier et al. 2009; Celine Delbes-Paus et al. 2010). Notably, *L. plantarum* and *L. fermentum* are proven potential LAB candidates having antagonistic activity towards *S. aureus* (Nawaz et al. 2009; Tambekar and Bhutada 2010). Karska-Wysocki et al. (2010) demonstrated that a mixture of *L. acidophilus* and *L. casei* inhibited the growth of methicillin-resistant *S. aureus* to below detection level (99 %) in a co-culture system, in both agar and liquid medium. Mohammedsaeed et al. (2014) reported that the presence of *L. rhamnosus* GG potentially reduces the toxic effects of *S. aureus* on epidermal keratinocytes by two probable mechanisms, i.e. growth inhibition and decreased bacterial adhesion.

Very few studies regarding antagonistic activity of *Weissella* spp. have been reported. In the current study, *W. confusa* exerts strong inhibitory activity against two clinical strains. Patra et al. (2011) demonstrated the inhibition of *S. aureus*, *E. coli*, *Salmonella enterica*, and *Bacillus subtilis* by *L. plantarum*, *L. fermentum* and *Weissella* spp. The bacteriocin from *Weissella paramesenteroides* showed profound antimicrobial activity against foodborne pathogens and spoilage organisms (Pal and Ramana 2010). The pH results from the 24-h incubated experimental cultures supports the hypothesis that they produce antibacterial compounds other than organic acids. The inhibitory action of LAB is attributed to the production of various organic acids, H_2O_2, fatty acids, antimicrobial peptide or bacteriocins (Tambekar and Bhutada 2010).

Dose dependency

During dose dependency experiments, all LAB strains exhibited significant inhibitory action ($P < 0.05$) towards growth of pathogens despite doubling of pathogen inoculum size (Fig. 3). The counts (CFU) of different LAB strains in co-culture and mono-culture were almost the same, which indicated that the pathogen did not have any inhibitory activity against LAB. The doubling of inoculum size of *L. plantarum* and *L. fermentum* showed 3 log cycle reductions in the growth of both the pathogens. This could be attributed to the production of higher amounts of antimicrobial agents. *W. confusa* strain AI10 was still able to decrease the growth of pathogens

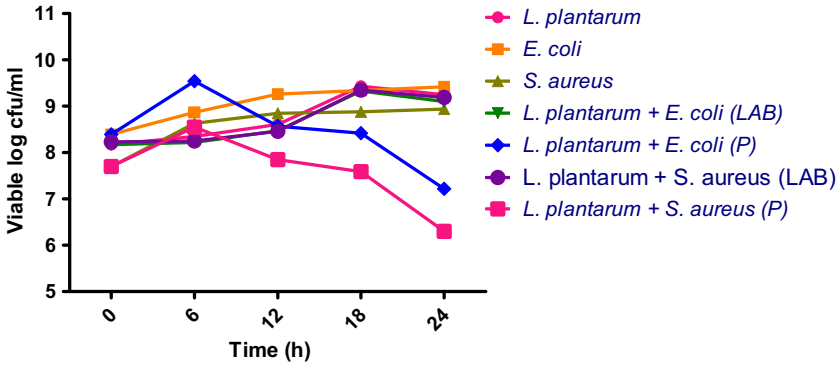

Fig. 5 Growth behavior of *Lactobacillus plantarum* 86 when grown in the presence of *E. coli* NG 502121 and *S. aureus* AY 507047

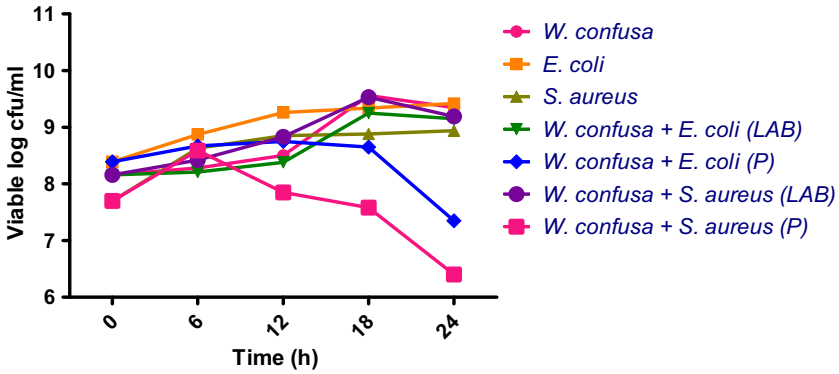

Fig. 6 Growth behaviour of *Weissella confusa* AI10 when grown in the presence of *E. coli* NG 502121 and *S. aureus* AY 507047

when the inoculum size of the pathogenic strains increased by ten times (Fig. 4). Robin et al. (2011) studied the antibacterial activity of *W. confusa* against *C. jejuni* using agar gel diffusion and co-culture techniques, but could not find any clear antagonism. However, in our study, LAB inhibited the growth of both clinical strains used to 1 log cycle (Fig. 4). These results reinforce the conclusion that very strong substance(s) are produced during growth of LAB co-cultured with pathogens. Similarly, Drago et al. (1997) studied co-culture between Lactobacilli strains and enteropathogens, either by incubating Lactobacilli simultaneously or after grown overnight with pathogens, and also reported significant inhibition of *E. coli* and *Salmonella enteritidis* under both conditions. Generally, LAB are considered more effective against closely related species; however, certain Gram-negative bacteria, e.g. *E. coli* are also found to be susceptible towards some bacteriocins (Gong et al. 2010). The change in inoculum size, ranging from a 1:1 to a 1:10 LAB:pathogen ratio illustrated the efficacy and concentration of antibacterial compounds produced during growth.

Growth determination

The growth behaviour of LAB and pathogens in mono- and co-culture was measured at different time intervals as demonstrated in Figs. 5, 6 and 7. In both mono- and co-culture tubes, the lag phase for *L. plantarum* (strain 86) and *W. confusa* (strain AI10) was about 3–4 h, whereas the pathogenic strains grew rapidly (Figs. 5, 6). A decrease in the growth of pathogens in co-culture was observed with LAB strains growing during an incubation of 6–12 h, with growth of the pathogen being reduced by almost 1 to 2 log cycles within 18 h of incubation as compared to the control. Exceptionally, in co-culture of *L. plantarum* (strain 86) with enteropathogenic *E. coli*, *E. coli* showed more rapid growth than the control, which may be indicative of a synergetic role for *L. plantarum* (Fig. 5). The effect of LAB strains on the growth of *S. aureus* was significantly more marked. Growth of *E. coli* in co-culture ceased after 10 h of incubation, whereas in control cultures, it was continuing to grow until 18 h. The pH of PYG broth containing LAB and *E. coli* in co-culture dropped to 4.7 within the first 6 h of incubation (and to 4.9 in the case of *S. aureus*), while after 12 h of incubation the pH fell to 4.5–4.6 in all other tubes. The inhibition observed for pathogens during early stationary phase in co-culture could be because of the metabolic end products produced by LAB strains and depletion of nutrients. Occurrence of early stationary phase for *E. coli* could be correlated with density-dependent inhibition and a direct cell-to-cell contact that induces a cell to switch to an adaptive response and bacteriocin synthesis (Aoki et al. 2005; Ruiz-Barba et al. 2010). These latter authors also hypothesized sensing of specific bacteria by *Lactobacillus* spp. as an environmental inducer to switch on a particular adaptive response and in turn synthesize bacteriocins. Pinto et al.

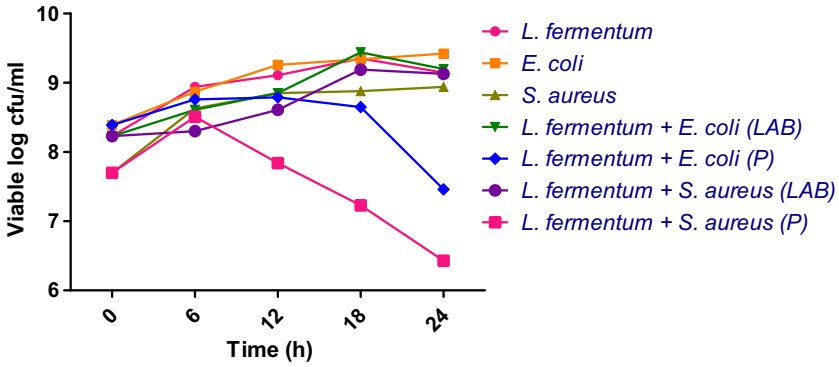

Fig. 7 Growth behaviour of *Lactobacillus fermentum* AI2 when grown in the presence of *E. coli* NG 502121 and *S. aureus* AY 507047

(2006) stated that the inhibitory activity of *L. plantarum*, isolated from traditional African fermented milk products as well as human intestine, was attributed to the production of organic acids and hydrogen peroxide. In contrast, Powell et al. (2007) showed production of a 3.5-kDa bacteriocin by a kefir isolate *L. plantarum* ST8KF, which was found to be active against lactobacilli and *L. innocua*.

Conclusions

Inactivation of pathogens and spoilage-causing organisms is of high concern in order to provide safe food products. LAB, including novel species of genus *Weissella*, show the potential to inhibit pathogens. Pronounced antibacterial activity of *Lactobacillus* and *Weissella* strains was observed against *E. coli* and *S. aureus*. The effect was pH independent and hence further detailed study on characterization of the antimicrobial compound(s) produced by these LAB, and their application in food models will be interesting.

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