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SHORT COMMUNICATION

Antimicrobial activity of a meat-borne *Bacillus subtilis* strain against food pathogens

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Abstract The aim of this study was to test the antibacterial activity of *Bacillus subtilis* TR50 isolated from a traditional cured sausage against ten food-borne pathogenic bacteria. Most of the pathogens were sensitive to cell-free concentrated supernatant from TR50. The inhibitory activity was retained at pH values between 4 and 8, up to 80 °C for 30 min and after treatment with pepsin and trypsin, whereas it disappeared after chymotrypsin hydrolysis; lipase treatment abolished inhibitory activity only against one target strain. The ammonium sulphate precipitation of TR50 concentrated cell-free culture retained the most residual antibacterial activity unlike the ethyl acetate and acid fractions. All these results suggest that the antimicrobial compound(s) produced by TR50 could be of proteinaceous nature and require a lipid moiety for activity against the pathogens tested. This is the first report on the antimicrobial activity against food-borne pathogens of a *B. subtilis* strain occurring in a traditional sausage potentially useful for food safety improvement.

Keywords *Bacillus subtilis* · Food-borne pathogens · Antimicrobial compounds · Traditional sausage

Abbreviations

PDB/CFS	TR50 20-fold concentrated cell-free supernatant from PDB culture
PCB/CFS	TR50 20-fold concentrated cell-free supernatant from PCB medium culture
TSB/CFS	TR50 20-fold concentrated cell-free supernatant from TSB medium culture
MH/CFS	TR50 20-fold concentrated cell-free supernatant from MH medium culture
BHI/CFS	TR50 20-fold concentrated cell-free supernatant from BHI medium culture
MRS/CFS	TR50 20-fold concentrated cell-free supernatant from MRS medium culture
WF/CFS	TR50 20-fold concentrated cell-free supernatant from WF medium culture
7-CFS	TR50 20-fold concentrated cell-free supernatant from 7 days PDB medium culture

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Introduction

More than 250 food-borne diseases caused by bacteria, viruses and parasites, whose most common effects are nausea, vomiting, abdominal cramps and diarrhoea, have so far been described.

Every year nearly 10% of the worldwide population is affected by food-borne illnesses [1] even though fatal cases of food-borne diseases are a rare event in industrialized countries.

In recent years, campylobacteriosis, salmonellosis, listeriosis and clinical diseases associated with enterohaemorragic *Escherichia coli* strains represented the most common food-borne infections in the United States (Centre for Disease Control, http://www.cdc.gov).

As a consequence of treating food-borne gastrointestinal illnesses with antimicrobial agents, many food pathogens, such as *Campylobacter* spp., *Salmonella* spp. or *Listeria monocytogenes,* have acquired antibiotic resistances towards fluoroquinolones, ceftriaxone, ampicillin and gentamycin, respectively [2–4]. Continuing efforts are being made by research and pharmaceutical industry sectors in order to identify new molecules with antibiotic action which bypass the mechanisms of antibiotic resistance. The increasing incidence of some meat-borne pathogens such as *E. coli* O157, *L. monocytogenes, Salmonella typhimurium, Bacillus cereus and Aeromonas* spp. underlines the importance of controlling these microfloras in meat-based foods [5].

The *Bacillus* genus has been widely used for the production of antibiotics as well as several extracellular enzymes. One of the most interesting biotechnological applications of some *Bacillus* strains is their ability to prevent and control human gastrointestinal disorders [6]. Part of the beneficial effect claimed for *Bacillus* in probiotic and pharmaceutical preparations is associated with the production of some metabolites useful in improving intestinal microbial balance, as well as with the reduction in viable pathogen cells and their toxin production [7].

The species belonging to this *taxon* produce bacteriocins and bacteriocin-like inhibitory substances (BLIS) that exhibit a broad antimicrobial activity and prevent the polymerization of carbohydrates in peptidoglycan. In addition to bacteriocins, *Bacillus* produces cyclic peptides and lipopeptides that interact with membrane lipids to form a pore causing cellular leakage [8, 9].

Other antibiotics with low molecular weights and different chemical structures are produced by *Bacillus* strains such as bacilysocin, bacilysin and the amicoumacins [10, 11].

B. subtilis TR50, isolated in 2003 from the natural microflora of an artisanal sausage produced in Southern Italy [12], survived the sausage manufacturing and curing process. TR50, together with other *Bacillus* strains, produced high levels of proteolytic and lipolytic activities. Its technological properties suggest that it could play a role in the development of texture and organoleptic characteristics in this sausage. In addition, TR50 lacks genes codifying *B. cereus*'s toxins [13].

This paper reports on the characterization of antimicrobial activity of *B. subtilis* TR50 against ten of the most widespread food-borne bacteria.

Materials and methods

Microorganisms and culture conditions

Bacterial strain TR50, isolated from natural microflora of a traditional sausage produced in Southern Italy, was identified as *Bacillus subtilis*, as reported by Baruzzi et al. [12].

B. subtilis TR50 was inoculated in tryptone soya broth (TSB), containing 0.5% glucose and incubated overnight at 30 °C in an orbital shaker at 130 rpm, under aerobic conditions.

Bacterial cultures, used as indicator strains for assessing the inhibitory activity of TR50, were grown overnight under aerobic conditions, as shown in Table 1.

All cultures were maintained as frozen glycerol stocks at −20 °C until required. All media and supplements were purchased from Oxoid Ltd (Basingstoke, Hampshire, United Kingdom), except PDB, purchased from Voigt Global distribution Inc. (DIFCO, Lawrence, KS, USA).

Drugs and chemicals, where not otherwise specified, were from Sigma–Aldrich (St Louis, MO, USA).

TR50 growth and antibacterial compound production in different media

B. subtilis TR50 growth and antibacterial compound production were evaluated in seven different synthetic media after 3 days of incubation. Two millilitres of TR50 overnight culture in TSB were inoculated in 100 ml of the following synthetic media: plate count broth (PCB), brain heart infusion (BHI), Mueller Hilton (MH), tryptone soya broth (TSB), de Man, Rogosa and Sharpe (MRS), potato dextrose broth (PDB) and Fred Waksman Basic 77 broth (WF) [14]. The cultures were incubated for 3 days at 30 °C on a rotary shaker (175 rpm). Total counts, constituted by vegetative cells and spores, were assessed after 3 days of incubation by plating them onto PDA by the standard serial dilution method. Spore counts were obtained by plating microbial growth after heat treatment (10 min at 85 °C). The experiments were performed in triplicate.

One millilitre of cell-free supernatant from each culture was centrifuged (9,000 × *g* for 20 min at 4 °C), filtered with 0.22-μm syringe cellulose acetate filters (Whatman International Ltd, Springfield Mill, James Whatman Way, Maidstone, Kent, UK), freeze-dried and concentrated 20-fold with ultrapure MilliQ water (Millipore, Marlborough, MA, USA). These samples were designated as "name of medium"/CFS. All samples were stored at −80 °C. The concentrated supernatants were assayed for their antibacterial activity by the disc diffusion test, following the British Society for Antimicrobial Chemotherapy guidelines, recently updated [15]. Halos without growth around discs were digitally acquired as areas (mm^2) by the ChemiDoc System (Bio-Rad Laboratories S.r.l., Milan, Italy) and processed using the Quantity One 4.3.1 software (Bio-Rad). Each sample was tested in triplicate together with the respective uninoculated 20-fold concentrated culture medium. Based on the results obtained in the above-mentioned experiments, TR50 was grown in PDB medium for further analyses.

β-haemolytic activity evaluation

The β-haemolytic activity, exhibited by *B. subtilis* TR50 cells, washed in phosphate saline buffer, as well as by

Table 1 Origin and incubation conditions of indicator strains

Reference strain	Medium	Conditions	Source
Bacillus cereus DSM4312	TSB	30 °C/60 rpm	Deutsche Sammlung von Mikroorganismen und Zellkulturen, Bramischweig, Germany
B. cereus DSM4313			
Aeromonas hydrophila DSM30187	NB	37 °C/150 rpm	
Listeria monocytogenes DSM20600	BHI	37 °C/150 rpm	
Yersinia enterocolitica DSM4780			
Enterococcus faecalis V583 (ATCC700802)	BHI	37 °C	American type culture collection, LGC standards, Middlesex, United Kingdom
Escherichia coli ATCC35401	NB	37 °C/150 rpm	
Helicobacter pylori ATCC43504			
Salmonella enterica ATCC13311			
Staphylococcus aureus NCTC8325	BHI	37 °C/150 rpm	National collection of type cultures, Salisbury, United Kingdom

20× cell-free supernatants from PDB, BHI and TSB (soaked onto sterile discs), was evaluated in triplicate on tryptone soya agar plates containing 5% defibrinated sheep blood, after incubation at 30 °C for 48 h. 20-fold concentrated uninoculated media were used as negative controls whereas *B. cereus* DSM4312 and *B. cereus* DSM4313 were used as positive controls. The presence of green or clear halos was related to haemolytic activity.

Growth and antimicrobial activity *of Bacillus subtilis* TR50 in PDB

B. subtilis TR50 cells, grown overnight in TSB at 30 °C, were used for inoculation in triplicate in 200 ml of PDB (2% of inoculum) contained in one-litre flasks. Flasks were incubated at 30 °C in an orbital shaker at 60 rpm up to 9 days. Three millilitres of microbial culture was withdrawn from each flask at a defined time in order to evaluate the microbial counts and antibacterial activity of each cell-free supernatant, as reported above in the paragraph "TR50 growth and antibacterial compound production in different media".

Stability to heat treatments, pH and enzymatic hydrolyses

The antibacterial activity of cell-free supernatant from 7 days of PDB culture, concentrated 20-fold (7-CFS), was evaluated after heat treatment, incubation at different pH values and extended hydrolysis with different enzymes. Tests were carried out in triplicate against the *A. hydrophila* DSM30187*, B.cereus* DSM4313 and *Staphylococcus aureus* NCTC8325 indicator strains by the disc diffusion assay described above.

The ability of 7-CFS to retain antimicrobial activity was assessed by incubating the sample in a water bath at different temperatures (40–100 °C) for 30 min. The stability of CFS to the sterilization process was tested by autoclave (120 °C for 30 min).

One millilitre of 7-CFS was freeze-dried and dissolved in the appropriate 100 mM sodium citrate/phosphate buffers in order to obtain pH values from 3.0 to 8.0 or dissolved in 100 mM Trizma base solution to reach pH 9.0 and 10.0. Samples were incubated at 37° C for 24 h.

The 7-CFS was treated with six different enzymes (pepsin, proteinase K, trypsin, chymotrypsin, lipase and proteinase E) for 24 h, as described by Sutyak et al. [16].

Appropriate buffers or media without 7-CFS were used as negative controls.

Extraction and concentration of inhibitory molecules

In order to develop a procedure for fast and efficient extraction and purification of active compounds from concentrated cell-free TR50 culture (7-CFS), three different extractions were carried out.

Two hundred millilitres of fivefold concentrated cell-free TR50 culture was extracted 3 times with an equal volume of ethyl acetate (HPLC grade, Carlo Erba Reagenti SpA, Rodano, Italy) according to the method of Han et al. [17], previously validated for the purification of iturin A and macrolactin A from *Bacillus* spp. The ethyl acetate extract was pooled and evaporated to dryness under vacuum at 40 °C on rotavapor (Postfach, Switzerland). The dried extract was dissolved in 50 ml of methanol to reach a final concentration of 20-fold (EA fraction) in comparison with the original volume of cell-free TR50 culture. The remaining water phase was heated at 40 °C for 30 min to remove ethyl acetate residues, freeze-dried and concentrated 20-fold with ultrapure MilliQ water (water phase fraction).

Two hundred millilitres of 7-CFS was acidified to pH 2.0, by adding 3 N HCl and precipitated at 4 °C according to the instructions of Kim et al. [18] for lipopeptides. The resulting pellet was suspended in 10 ml of 25 mM phosphate buffer pH 7 (pH 2 fraction).

Ten millilitres of 7-CFS was also precipitated with ammonium sulphate at 55% saturation, as described by Zheng and Slavik [19] for bacteriocins. The pellet was suspended in the same buffer (AS fraction).

The three fractions (EA, pH 2 and AS) were stored at −20 °C.

The 7-CFS and the three above-mentioned fractions were evaluated in triplicate trials for their inhibitory activity against the indicator strains listed in Table 1 by the disc diffusion assay.

Results and discussion

The experiments described in this paper were designed to achieve a preliminary understanding of the production, antimicrobial activity and chemical nature of compounds by *B. subtilis* TR50. The production of these compounds was tested in seven different media (BHI, MH, MRS, PCB, PDB, TSB and WF), and the resulting cultures were checked for their inhibitory activity against the food-borne pathogens listed in Table 1.

After 3 days of growth, *B. subtilis* TR50 total counts in TSB, BHI and PDB broths were made up almost entirely by spores (6.0 log CFU/ml, 5.1 log CFU/ml and 4.9 log CFU/ml, respectively), whereas higher ratios of total viable cells/spores were found in MH, WF and PCB broth cultures, suggesting a prolongation of generation time. Very low cell and spore loads were found in MRS broth unlike high biomass concentration displayed by *B. cereus* 8A under the same conditions [20].

The cell-free filtrates TSB/CFS and BHI/CFS showed antibacterial activity against all ten food-borne pathogenic strains, whereas PDB/CFS inhibited all pathogens except *Enterococcus faecalis* V583. Furthermore, the cell-free filtrates WF/CFS and MH/CFS inhibited at high extent only *Helicobacter pylori* ATCC43504 (inhibition halos 286 and 126 mm^2, on average, respectively) and *L. monocytogenes* DSM20600 (halos 226 and 126 mm^2, on average, respectively); by contrast, PCB/CFS inhibited mostly *Yersinia enterocolitica* DSM4780 and *H. pylori* ATCC43504 (95 mm^2 inhibition halos, on average). As expected, no halos were produced by MRS/CFS on lawn of all indicator strains.

Our results indicate that the antibacterial activity of TR50 was greatly influenced by the cultivation media, as previously reported [20, 21]. Furthermore, although the highest viable cell counts of TR50 were found after 3 days of cultivation in MH, PCB and WF media, few pathogenic strains were inhibited by the respective cell-free filtrates of these cultures. On the other hand, BHI, PDB and TSB media showed higher TR50 spore counts and a stronger antibacterial activity. Other Authors related the production of heat-resistant spores by *B. subtilis* 168 to the increase in antibacterial activity [10]. Moreover, it was reported that different media and culture conditions may induce the production of different active compounds affecting the final antimicrobial activity [20–22].

BHI/CFS, TSB/CFS and PDB/CFS were also tested for β-haemolytic activity onto defibrinated sheep blood agar plates in order to exclude the possibility that in these media, TR50 could produce virulence factors. Both *B. subtilis* TR50 cells and concentrated cell-free supernatants of the above media did not show any halos related to haemolytic activity.

Although concentrated TSB/CFS and BHI/CFS gave high antibacterial activities, the PDB medium, previously used for the production of antimicrobial compounds by *Bacillus* strains [23], was preferred in the subsequent analyses of characterization of TR50 antimicrobial activity due to its simple chemical formulation (low peptide content).

In order to maximize antimicrobial metabolite(s) concentration, *B. subtilis* TR50 was incubated in PDB at 30 °C for 9 days. After the early phase of adaptation, total cell counts reached 7.6 log CFU/ml at day three remaining stable up to the end of the experiment. Spore counts increased steadily till the seventh day when total cell loads were made up almost exclusively of spores, accounting for 7.3 log CFU/ml (Fig. 1a), value also recorded on day nine.

The inhibitory activity of PDB/CFS filtrates arising from TR50 cultures in PDB throughout 7 days was also evaluated against the target bacteria listed in Table 1. Overall, two different inhibition patterns (A and B) were observed. In pattern A, including *S. aureus* NCTC8325, *Y. enterocolitica* DSM4780, *E. coli* ATCC35401 and *A. hydrophila* DSM30187, the areas of the inhibition halos increased depending on the duration of TR50 incubation, whereas the remaining indicator strains, belonging to the pattern B, displayed inhibition halos that were stable over the time. A representative example of each inhibition pattern is shown in Fig. 1b.

The relationship between inhibitory activity and the increase in spore counts has previously been demonstrated for many antibiotics from *Bacillus* spp. [10, 21, 22], although other antimicrobial *Bacillus* metabolites drew inhibitions similar to TR50 pattern B against their target strains [24, 25].

The results of inhibitory activities recorded over 9 days of incubation led us to carry out a preliminary characterization of the nature of the active compounds released by *B. subtilis* TR50 after 7 days of growth in PDB (7-CFS).

The residual antibacterial activity of 7-CFS after heat treatment, incubation at different pH values and enzymes is

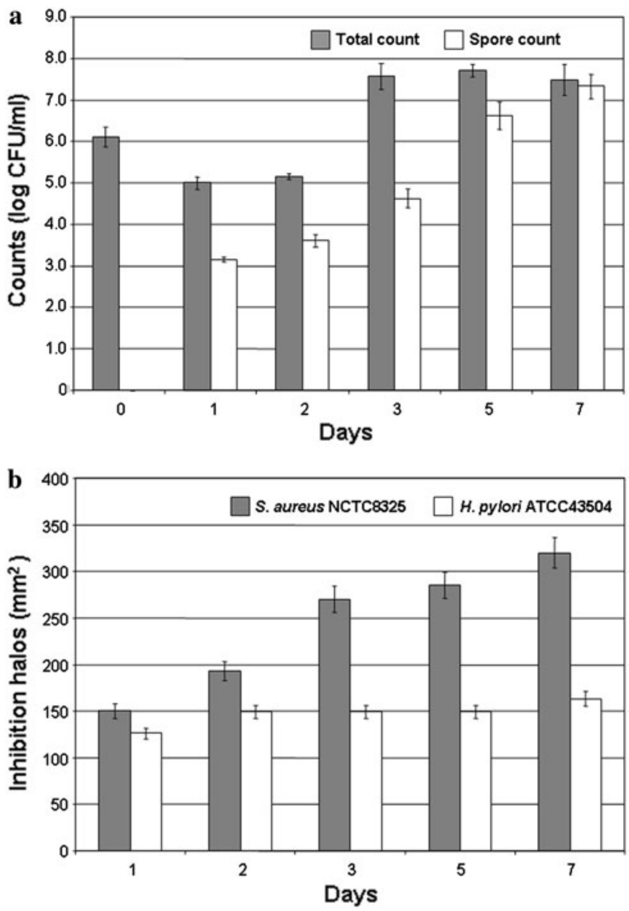

Fig. 1 *B. subtilis* TR50 grown in PDB at 30 °C (60 rpm; **a**) and antibacterial activity of its 20-fold concentrated filtrates against *S. aureus* NCTC6325 (pattern A) and *H. pylori* ATCC43504 (pattern B) thorough 7 days (**b**), **a** *bars* represent means (± standard deviation, $N = 3$) of total cell and spore counts, respectively, **b** the values represent the means (± standard deviation, $N = 3$) of halo areas calculated by subtracting the disc surface area

shown in Table 2. Unlikely other Authors, that used a single target strain for antimicrobial compound characterization [21, 26], we tested treated 7-CFS against one gram-negative and one gram-positive strains displaying the inhibition pattern A and one gram-positive endospore-forming bacterium grouped under the pattern B. So far to our knowledge, no studies checked the residual activity of *Bacillus* active compounds after physical–chemical treatments against the indicator strains shown in Table 2.

Hydrolysis of 7-CFS by means of chymotrypsin and proteinase K dramatically reduced its antimicrobial activity against all the tested strains suggesting the possible proteinaceous nature of compound(s) produced by *B. subtilis* TR50. Moreover, lipase completely reduced the activity of TR50 supernatant only against *A. hydrophila* DSM30187 and this result leads to suppose that the antibacterial compound(s) could have a lipid moiety involved in its antibacterial activity as previously reported by Yakimov et al. [27] for the lipopetide lichenysin A. Since the treatment with lipase modified at different extents the antibacterial activities of CFS against the three indicator strains used, we assume that CFS contains at least two different compounds. This hypothesis is also strengthened by the finding of two inhibition patterns (Fig. 1b).

Other Authors reported the simultaneous production of different antimicrobial compounds by the same *B. subtilis* strain in which peptide antibiotics represent the predominant class [14, 19, 24]. The slight loss of activity against *S. aureus* NCTC8325 displayed by 7-CFS, exposed to lipase, could indicate that compounds inhibiting this strain do not present lipid moiety necessary for its biological activity, as previously reported by Tabbene et al. [25] for methicillin-resistant *S. aureus* strains. Furthermore, the relative resistance of *S. aureus* NCTC8325 to 7-CFS treated with pepsin and pronase E could be due to the presence of N- and/or C-terminally blocked peptides in one of the possible compounds released by TR50 or to the presence of uncommon amino acids [28].

At pH values between 3 and 8, 7-CFS retained almost 50% of its antimicrobial activity, expressing the highest inhibitory activity at pH 7; outside that range, inhibitory activity was reduced (Table 2) in accordance with other results obtained in *B. licheniformis* by Mendo et al. [29]. The residual activity after heat treatment resulted to be temperature dependent. The antibacterial activity was rather stable when TR50 cell-free supernatant was incubated up to 60 °C for 30 min, whereas it reduced after treatment at temperatures higher than 80 °C (Table 2). Bizani and Brandelli [28] reported that the lipopeptides (fengycin, iturins ecc.,) also resulted scarcely stable to treatments at high temperature and pH values.

Following the hypothesis of the occurrence of some antimicrobial compounds in 7-CFS, three different techniques, widely used for purifying antimicrobials from *Bacillus* spp. [17–19], were applied for the extraction of metabolites produced by TR50.

Table 3 shows the residual activity of the fractions (EA, AS and pH 2), obtained by the extraction of 7-CFS with ethyl acetate, ammonium sulphate and acidification to pH 2, respectively.

The EA fraction retained on average only 33% of the activity shown by 7-CFS, whereas the respective water phase resulted completely inactive (Table 3).

The pH 2 fraction produced a weak antimicrobial activity only against *B. cereus* DSM4312 and *B. cereus* DSM4313, although several Authors have used acidification by HCl for the extraction of surfactins active against many gram-positive and gram-negative bacteria [30].

Table 2 Factors affecting antimicrobial activity of *Bacillus subtilis* TR50 against *B. cereus* DSM4313, *S. aureus* NCTC8325 and *A. hydrophila* DSM30187

Treatment	Residual activity (%)[a]		
	B. cereus DSM4313	*S. aureus* NCTC8325	*A. hydrophila* DSM30187
Enzymes			
Lipase	19	62	0
Trypsin	18	41	41
Proteinase K	19	21	19
Chymotrypsin	5	5	0
Pepsin	18	75	40
Pronase E	5	58	22
Heat			
40 °C/30 min	92	100	68
60 °C/30 min	85	65	93
80 °C/30 min	72	65	57
100 °C/30 min	45	30	0
120 °C/30 min	25	0	0
pH			
3	80	70	75
4	80	100	70
5	100	100	75
6	100	100	90
7	100	85	100
8	80	50	60
9	80	0	40
10	80	0	20

[a] Residual activity, based on average ratio among inhibition areas, compared with antimicrobial activity before treatment

The AS fraction was active against eight out of ten indicator strains, preserving on average 62% of its initial inhibitory activity (Table 3). This finding is in accordance with the results of Zheng and Slavik [19], reporting that the inhibitory activity of a lipopeptide from *B. subtilis* 22 was maintained by concentrating its cell-free supernatant 100-fold after ammonium sulphate precipitation. These Authors supposed the presence of different inhibitory substances only partially evidenced by SDS–PAGE and isoelectric focusing gel electrophoresis. The sensitivity of *Y. enterocolitica* DSM4780 against different fractions of 7-CFS also supports the hypothesis of a co-occurrence of more active compounds produced by *B. subtilis* TR50.

In conclusion, this is the first report on the antimicrobial activity against ten widespread food-borne pathogens of a *B. subtilis* strain occurring in a traditional sausage. This finding confirms the importance of preserving food microbial biodiversity. The antibacterial activity seemed to be related to at least two antimicrobial compounds that showed no haemolytic activity and for whose identification studies are in course. TR50 metabolites were stable at different heat and enzyme treatments and active over a wide pH range, thus suggesting they could be applied during processing for food safety improvement.

The ability of *B. subtilis* to shift its growth habit from aerobic to anaerobic in the absence of oxygen but in the presence of nitrate [31] allows *B. subtilis* strains to grow under the microaerophilic conditions present in cured meat foods in which nitrates are intentionally added.

The technological traits (proteolysis and lipolysis) previously demonstrated for TR50 [12] and its broad

Table 3 Inhibitory activity of fractions obtained by ethyl acetate (EA) extraction and ammonium sulphate (AS) and acid (pH 2) precipitations

	7-CFS	EA fraction		AS fraction		pH 2 fraction	
	Halos (mm^2)	Halos (mm^2)	Recovery[a] (%)	Halos (mm^2)	Recovery[a] (%)	Halos (mm^2)	Recovery[a] (%)
Gram positives							
B. cereus DSM4312	40 ± 2	22 ± 1	55	32 ± 2	79	12 ± 1	30
B. cereus DSM4313	48 ± 2	18 ± 1	37	35 ± 2	74	10 ± 0	21
L. monocytogenes DSM20600	79 ± 2	30 ± 1	38	61 ± 2	78	0	0
S. aureus NCTC8325	312 ± 25	0	0	181 ± 7	58	0	0
Gram negatives							
A. hydrophila DSM30187	200 ± 8	43 ± 2	22	130 ± 8	65	0	0
E. coli ATCC35401	126 ± 9	0	0	53 ± 4	42	0	0
H. pylori ATCC43504	158 ± 9	43 ± 3	27	53 ± 3	33	0	0
Salm. enterica ATCC13311	43 ± 2	0	0	12 ± 1	28	0	0
Y. enterocolitica DSM4780	210 ± 11	45 ± 3	21	0	0	0	0

The values represent means ± standard deviation ($N = 3$) of inhibition halos calculated by subtracting the disc surface area

[a] Recovery: per cent average ratio between antimicrobial activity of fractions and that of unextracted 7-CFS

antibacterial activity make *B. subtilis* TR50 a novel candidate for improving the quality and safety of fermented meat products.

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