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Original article

Anti-salmonella properties of kefir yeast isolates: An in vitro screening for potential infection control

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ABSTRACT

The rise of antibiotic resistance has increased the need for alternative ways of preventing and treating enteropathogenic bacterial infection. Various probiotic bacteria have been used in animal and human. However, *Saccharomyces boulardii* is the only yeast currently used in humans as probiotic. There is scarce research conducted on yeast species commonly found in kefir despite its claimed potential preventative and curative effects. This work focused on adhesion properties, and antibacterial metabolites produced by *Kluyveromyces lactis* and *Saccharomyces unisporus* isolated from traditional kefir grains compared to *Saccharomyces boulardii* strains. Adhesion and sedimentation assay, slide agglutination, microscopy and turbidimetry assay were used to analyze adhesion of *Salmonella* Arizonae and *Salmonella* Typhimurium onto yeast cells. *Salmonella* growth inhibition due to the antimicrobial metabolites produced by yeasts in killer toxin medium was analyzed by slab on the lawn, turbidimetry, tube dilution and solid agar plating assays. Alcohol and antimicrobial proteins production by yeasts in killer toxin medium were analyzed using gas chromatography and shotgun proteomics, respectively. *Salmonella* adhered onto viable and non-viable yeast isolates cell wall. Adhesion was visualized using scanning electron microscope. Yeasts-fermented killer toxin medium showed *Salmonella* growth inhibition. The highest alcohol concentration detected was 1.55%, and proteins with known antimicrobial properties including cathelicidin, xanthine dehydrogenase, mucin-1, lactadherin, lactoperoxidase, serum amyloid A protein and lactotransferrin were detected in yeasts fermented killer medium. These proteins are suggested to be responsible for the observed growth inhibition effect of yeasts-fermented killer toxin medium. *Kluyveromyces lactis* and *Saccharomyces unisporus* have anti-salmonella effect comparable to *Saccharomyces boulardii* strains, and therefore have potential to control *Salmonella* infection.

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Abbreviations: WHO, World Health Organization; FAO, Food Agriculture Organization; ATCC, American type Culture Collection; YEPDA, Yeast Extract Peptone Dextrose Agar; YEPDB, Yeast Extract Peptone Dextrose Broth; PBS, Phosphate buffered saline; CFU, Colony Forming Unit; DSR, Desk Sputter Coater; HCL, Hydrochloric Acid; NaOH, Sodium hydroxide; KTM, Killer Toxin Cedium; CFS, Cell Free Supernatant; DTT, Dithiothreitol; LC-MS/MS, Liquid Chromatography with tandem mass spectrometry/Liquid Chromatography with tandem mass spectrometry; HPLC, High-performance liquid chromatography; mL, Milliliter; Min, Minute; h, Hour; RSLC, Rapid Separation Liquid Chromatography; AGC, Automatic Gain Control; SPSS, Statistical Package for the Social Sciences; IBM, International Business Machines; SD, Standard Deviation; LFQ, Label Free Quantitation; GIT, The gastrointestinal tract; DNA, Deoxyribonucleic Acid; RNA, Ribonucleic Acid; ATP, Adenosine triphosphate.

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1. Introduction

Salmonella resistance to current antibiotic drugs is rising at an alarming rate worldwide especially in Africa and Asia. For example in Malawi, the percentage of multi-drug resistant *Salmonella enterica* serovar Typhi increased from 7% in 2010 to 97% in 2014 (Feasey et al., 2015). This has raised the need for alternative prophylaxis and therapies. One of the ways of preventing and treating infectious diseases in human and animal is the use of probiotic microorganisms (Feye et al., 2019; Gut et al., 2018; Kogan & Kocher, 2007). Probiotics are defined by World Health Organization (WHO) as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002). *Lactobacillus*, *Bifidobacterium*, *Streptococcus thermophilus*, *Enterococcus*, *Leuconostoc* species, *Escherichia coli* Nissle 1917, *Bacillus* species and *Saccha-*

romyces boulardii are currently used as probiotics in humans against bacterial pathogens infection (Bakken, 2014; Bekar et al., 2011; Khodadad et al., 2013; Nami et al., 2015). Probiotics are commonly found in popular fermented functional foods such as yoghurt, milk, kefir, cheese, soybean, fruits, sourdough and vegetable products (Plessas et al., 2016; Prado et al., 2015; Priyodip et al., 2017; Saarela et al., 2000) or formulated into either lyophilized forms as capsules, powders or in aqueous solutions (Martins et al., 2009). These microorganisms exert antagonism against susceptible bacteria by production of antibacterial molecules, prevention of biofilm formation and pathogen invasiveness; adhesion of bacteria cells onto cell walls, and degradation of bacterial toxins among others (Gut et al., 2018).

Kefir drink is a probiotic product made from a kefir grain, a consortium of many microorganisms and exopolysaccharide, consumed in many parts of the world including the Caucasus Mountains of Russia, Europe, Asia, South and North America due to its health benefits conferred by the probiotic components (Garrote et al., 1997). Bacterial cultures of kefir include *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Streptococcus* genera whereas the yeast cultures include *Kluyveromyces*, *Candida*, *Saccharomyces* and *Pichia* (Plessas et al., 2016).

The aim of this study was to screen kefir yeast isolates for potential application in *Salmonella* infection control. The study hypothesized that yeasts isolated from kefir and *Saccharomyces boulardii* may show *Salmonella* binding capability, as well as growth inhibition due to production of antimicrobial metabolites. Specifically, *Salmonella enterica* serovar Arizonae (*S. Arizonae*) and *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) adhesion onto *Saccharomyces unisporus* ATCC 10612 (*S. unisporus*) and *Kluyveromyces lactis* var. *lactis* ATCC 56498 (*K. lactis*) as well as growth inhibition due to antibacterial metabolites were analyzed in an *in vitro* experiments in comparison to *Saccharomyces boulardii* strains {*Saccharomyces* var *boulardii* MYA-796 (SB48) and *Saccharomyces* var *boulardii* MYA-797 (SB49)}.

2. Materials and methods

2.1. Materials

Microbiological cultures including kefir yeast isolates (*S. unisporus* and *K. lactis*), *S. boulardii* strains (Victoria University culture collection), *S. Arizonae* ATCC 13314 and *S. Typhimurium* ATCC 14028 (In vitro Technologies, Melbourne, Australia). Microbiological media including yeast extract peptone dextrose agar (YEPDA), YEPD broth (YEPDB), nutrient agar, chloramphenicol drug (100 mg/L), and nutrient broth (Basingstoke, United Kingdom). Phosphate buffered saline (PBS), cycloheximide (0.1%), bile extract porcine (containing glycine & taurine conjugate of hyodeoxycholic acid), glycerol, citrate-phosphate buffer, 0.22 µm sterile filters (Sigma Aldrich, St. Louis, USA) and absolute ethanol (Rowe Scientific, Melbourne, Australia).

2.2. Preparation of *Salmonella* and yeast cultures

Salmonella and yeast cultures were prepared as described in a previous study (Tiago et al., 2012) with some modifications. Well-isolated *S. Arizonae* and *S. Typhimurium* colonies initially grown on nutrient agar and incubated at 37 °C for 24 h were inoculated into 10 mL nutrient broth and incubated at 37 °C for 24 h. One milliliter of each culture was serially diluted to approximately 10³ CFU/mL. Another set of *Salmonella* cultures with approximately 10⁹ CFU/mL (undiluted culture) was set aside. Well-isolated yeast colonies on YEPDA were picked and inoculated into 10 mL YEPDB and incubated at 25 °C for 24 h in a shaking incubator at 100 hor-

izontal strokes/min (Innova 4230 New Brunswick Scientific, Edison, NJ, USA). The yeast cultures with approximately 10⁸ CFU/mL were labelled accordingly. All microbial cultures were kept in a refrigerator at 4 °C prior to use when necessary.

2.3. Adhesion of *Salmonella* onto yeast cell wall

2.3.1. *Salmonella* adhesion onto yeast cell wall

Adhesion of *Salmonella* onto yeast cell wall was performed as previously described (Tiago et al., 2012) with some modifications. One milliliter of viable yeast culture broth (approximately 10⁸ CFU/mL) was added to a 15 mL centrifuge tube containing 0.5 mL of *Salmonella* culture (approximately 10⁹ CFU/mL). The bacteria-yeast mixture was vortexed for 30 s and incubated at 37 °C for 4 h. One hundred microliter was removed from the top and was serially diluted in 0.1% peptone water to 10⁻⁷. One milliliter of each dilution was added to a Petri dish and then 20 mL molten nutrient agar at 45 °C (supplemented with 0.1% cycloheximide to suppress yeasts growth) was added and mixed gently. Plates were incubated at 37 °C for 24 h. *S. Arizonae* and *S. Typhimurium* colonies were counted and expressed as log₁₀ CFU/mL. For the controls, 1 mL sterile YEPDB (containing no yeast cells) was added to 0.5 mL of each *Salmonella* serovars and treated as above.

To assess adhesion of *Salmonella* onto non-viable yeast cells, 10 mL of yeast cultures were heated at 100 °C for 5 min to obtain inactivated cells. Adhesion and sedimentation were performed as per the protocol for viable yeast cells described above.

Slide agglutination was performed as described in the literature (Perez-Sotelo et al., 2005) by inoculating 0.02 mL viable and non-viable yeast suspension with 0.01 mL *Salmonella* on microscopic slides. The two cultures were mixed using sterile inoculating loop before gentle rocking the slide. Agglutination was observed under illumination (Perez-Sotelo et al., 2005).

Table 1

Adhesion of *Salmonella* onto yeast cell wall: quantitative and qualitative analysis results.

	Yeast strains/ control	<i>S. Arizonae</i> Count (log ₁₀ CFU/ mL)	<i>S. Typhimurium</i> Count (log ₁₀ CFU/ mL)
Live yeast	Control (YEPD)	9.14 ± 0.05 ^b	9.20 ± 0.05 ^b
	SB48	8.69 ± 0.40 ⁺⁺	8.66 ± 0.14 ^{+++^a}
	SB49	8.63 ± 0.35 ⁺⁺⁺	8.82 ± 0.18 ^{+++^a}
	KL	8.88 ± 0.10 ⁺⁺⁺	8.81 ± 0.11 ^{+++^a}
	SU	8.97 ± 0.07 ⁺⁺⁺	8.84 ± 0.20 ^{+++^a}
Heat-killed yeast	Control (YEPD)	8.92 ± 0.04 ^b	8.93 ± 0.06 ^b
	SB48	8.67 ± 0.05 ⁺⁺	8.32 ± 0.17 ^{++^a}
	SB49	8.68 ± 0.07 ⁺⁺⁺	8.28 ± 0.19 ^{++^a}
	KL	8.68 ± 0.08 ⁺⁺⁺	8.69 ± 0.11 ^{++^a}
	SU	8.69 ± 0.09 ⁺⁺⁺	8.67 ± 0.09 ^{++^a}
Bile	Control (bilee)	9.04 ± 0.05 ^b	8.96 ± 0.07 ^b
	SB48	8.74 ± 0.10 ⁺⁺	8.68 ± 0.09 ⁺⁺
	SB49	8.72 ± 0.13 ⁺⁺⁺	8.86 ± 0.03 ⁺⁺⁺
	KL	7.72 ± 0.07 ⁺⁺⁺	8.88 ± 0.02 ⁺⁺⁺
	SU	8.78 ± 0.06 ⁺⁺⁺	8.67 ± 0.13 ⁺⁺⁺
pH 2.0, 4.0, 5.0 and 7.0, and 8.0 (live yeast)	SB48	++	+++
	SB49	++	+++
	KL	++	+++
	SU	++	+++

The experiments was performed twice and in triplicate and the values are reported as the mean plus standard deviation; + = very weak agglutination seen after 5 s of gentle rocking of slide; ++ medium level agglutination seen after 5 s of gentle rocking of slide; +++ = very strong instant agglutination seen after rocking of slide. Means with different superscript are significantly different (p < 0.05)

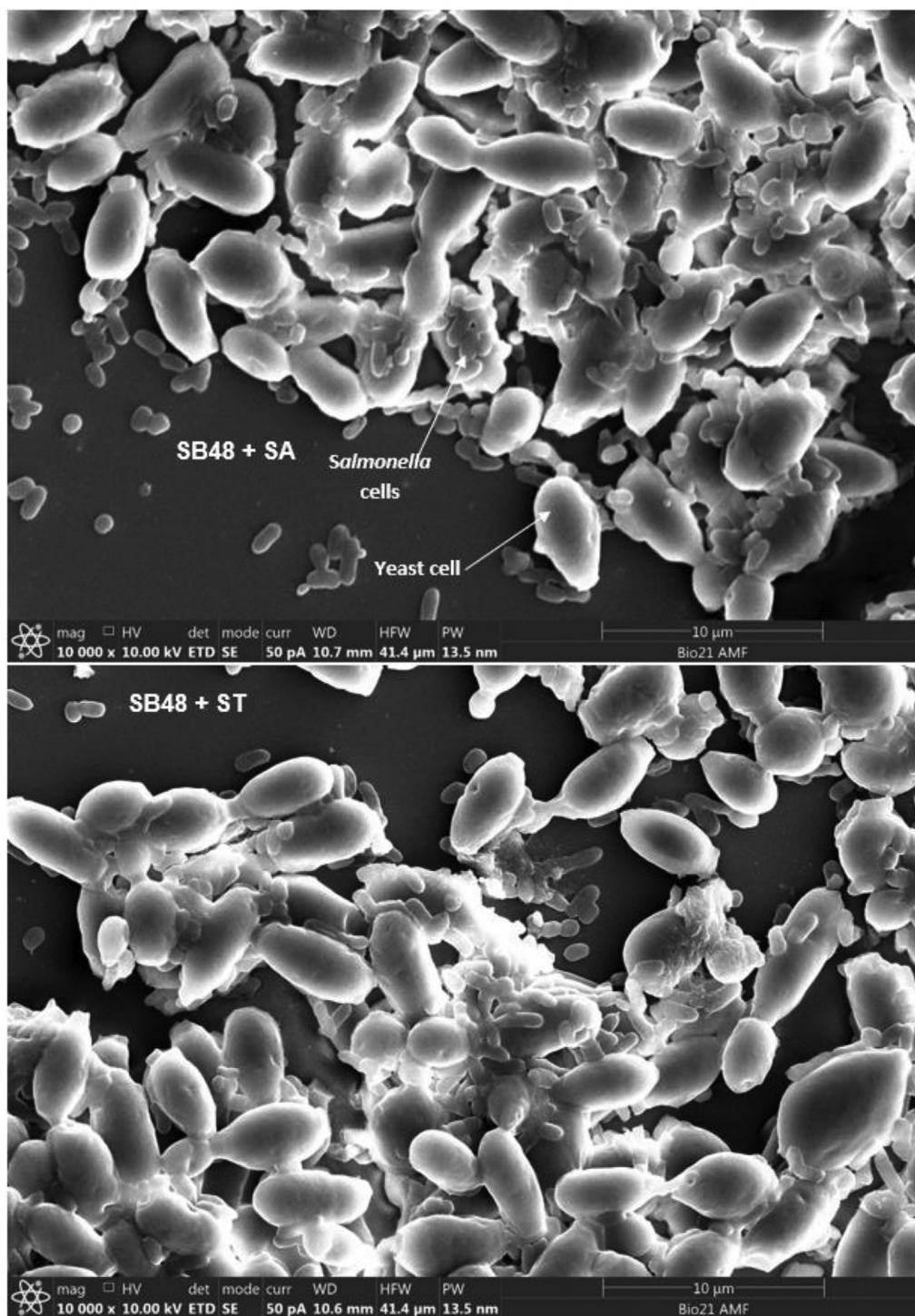


Fig. 1A. Adhesion of *Salmonella* servers onto SB48 cell walls observed using scanning electron microscope. SA = *S. Arizonae*; ST = *S. Typhimurium*.

Salmonella (0.01 mL, 10^9 CFU/mL) and yeast (10^8 CFU/mL 0.02 mL) were added to a clean microscope slide and mixed using sterile inoculating loop. The smear was allowed to air dry before fixing with 0.1% glutaraldehyde, washed three times with 1X PBS and allowed to air dry again. Smear on slides were dehydrated using classical dehydration process (Kiekens et al., 2019; Piroeva et al., 2013) with modification. Briefly, slides were soaked stepwise in 50%, 75% and absolute ethanol consequentially for 30 min at room temperature before drying at 37 °C for 1 h. The slides were then glued onto scanning electron microscope stubs and gold-

coated by sputtering for 5 min using a desk sputter coater DSR1 (Markham, Canada). The adhesion was qualitatively observed using a Teneo scanning electron microscope (Thermo Fisher Scientific, Hillsboro, USA). Adhesion of *Salmonella* onto both viable and heat-inactivated yeast cell wall was analyzed at magnification of 10,000 \times .

2.3.2. *Salmonella* growth in presence of inactivated yeast cells

Growth behaviour of *Salmonella* in presence of inactivated yeast cells was analyzed using turbidimetry assay. Six milliliters of yeast

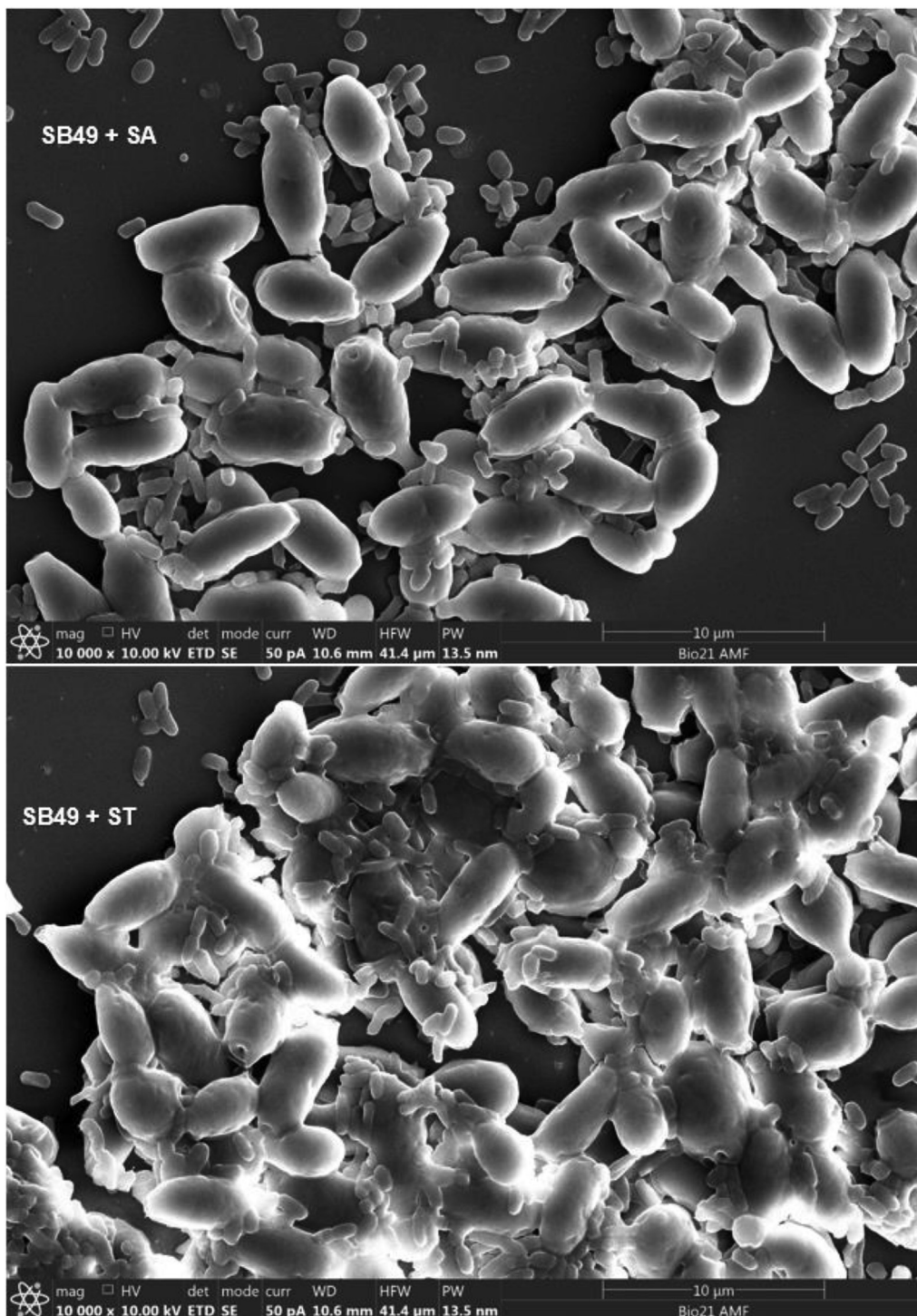


Fig. 1B. Adhesion of *Salmonella* servers onto SB49 cell walls observed using scanning electron microscope. SA = *S. Arizonae*; ST = *S. Typhimurium*.

(10^9 CFU/mL) and 3 mL of *Salmonella* (10^9 CFU/mL) cultures were centrifuged (Eppendorf 5810 R, Hamburg, Germany) at 4000g for 15 min at 4 °C and washed twice with 1X PBS respectively. The yeast pellets were re-suspended in 6 mL of 1X PBS whereas the *Salmonella* pellets were re-suspended in 3 mL 1X PBS and 3 mL nutrient broth (as source of nutrients for growth). One milliliter of the yeast suspension (10^2 CFU/mL) and 0.5 mL of the *Salmonella* suspension (10^3 CFU/mL) were added to a 15 mL centrifuge tube and vortexed for 20 s to mix. *Salmonella* cultures in 1X PBS and nutrient broth were used as control. All the tubes containing

cultures including controls were supplemented with 0.1% cycloheximide (4 mL/L) to inactivate yeasts. Prepared *Salmonella*-yeasts mixtures (0.2 mL) and controls were dispensed into flat-bottom 96 well microtiter plate (Biorad, California, USA). The optical densities were read at 0, 2, 4, 6, 12 and 24 h using microplate reader at 595 nm (Biorad, California, USA).

2.3.3. Factors affecting *Salmonella* adhesion onto yeast cell wall

The pH of yeast and *Salmonella* culture broths were adjusted separately to 2.0, 4.0, 5.0, 7.0, and 8.0 with either 1 M HCl or 1 M

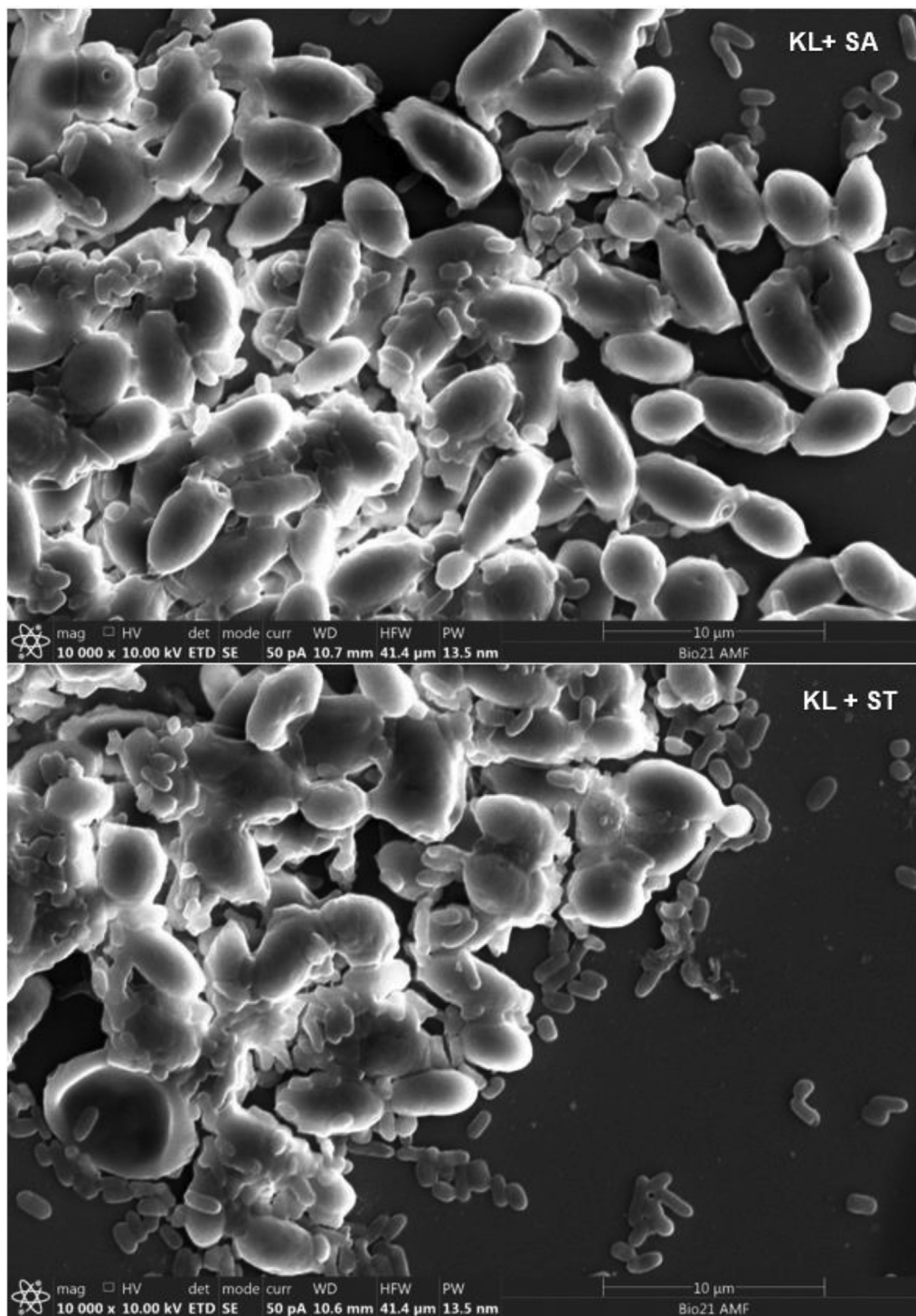


Fig. 1C. Adhesion of *Salmonella* servers onto KL cell walls observed using scanning electron microscope. SA = *S. Arizonae*; ST = *S. Typhimurium*, KL = *K. lactis*.

NaOH. *Salmonella* ($20 \mu\text{L}$, 10^9 CFU/mL) and yeast ($10 \mu\text{L}$, 10^8 CFU/mL) of same pH were added onto a clean microscope slide, mixed using a sterile inoculating loop, rocked gently and observed under illumination for agglutination as described in the literature (Perez-Sotelo et al., 2005). Both viable and non-viable yeasts were tested.

Effect of bile salt on *Salmonella* adhesion onto yeast cell wall was performed as described in the literature (Tiago et al., 2012) with some modifications. Briefly, 6 mL each of yeast

(10^8 CFU/mL) and *Salmonella* (10^9 CFU/mL) broths were centrifuged (Eppendorf 5810 R, Hamburg, Germany) separately at $4000g$ for 15 min at 4°C and washed twice with 1X PBS. The pellets were each re-suspended in 6 mL 1X PBS containing 3 g/L bile (supplemented with 0.1% cycloheximide 4 mL/L). For the control, *S. Arizonae* and *S. Typhimurium* prepared separately without yeast cells, were similarly treated as above. Qualitative and quantitative analysis were performed as described under adhesion and sedimentation assays. However, experimental conditions including time, temperature, initial culturing of yeast and *Salmonella* in bile-free

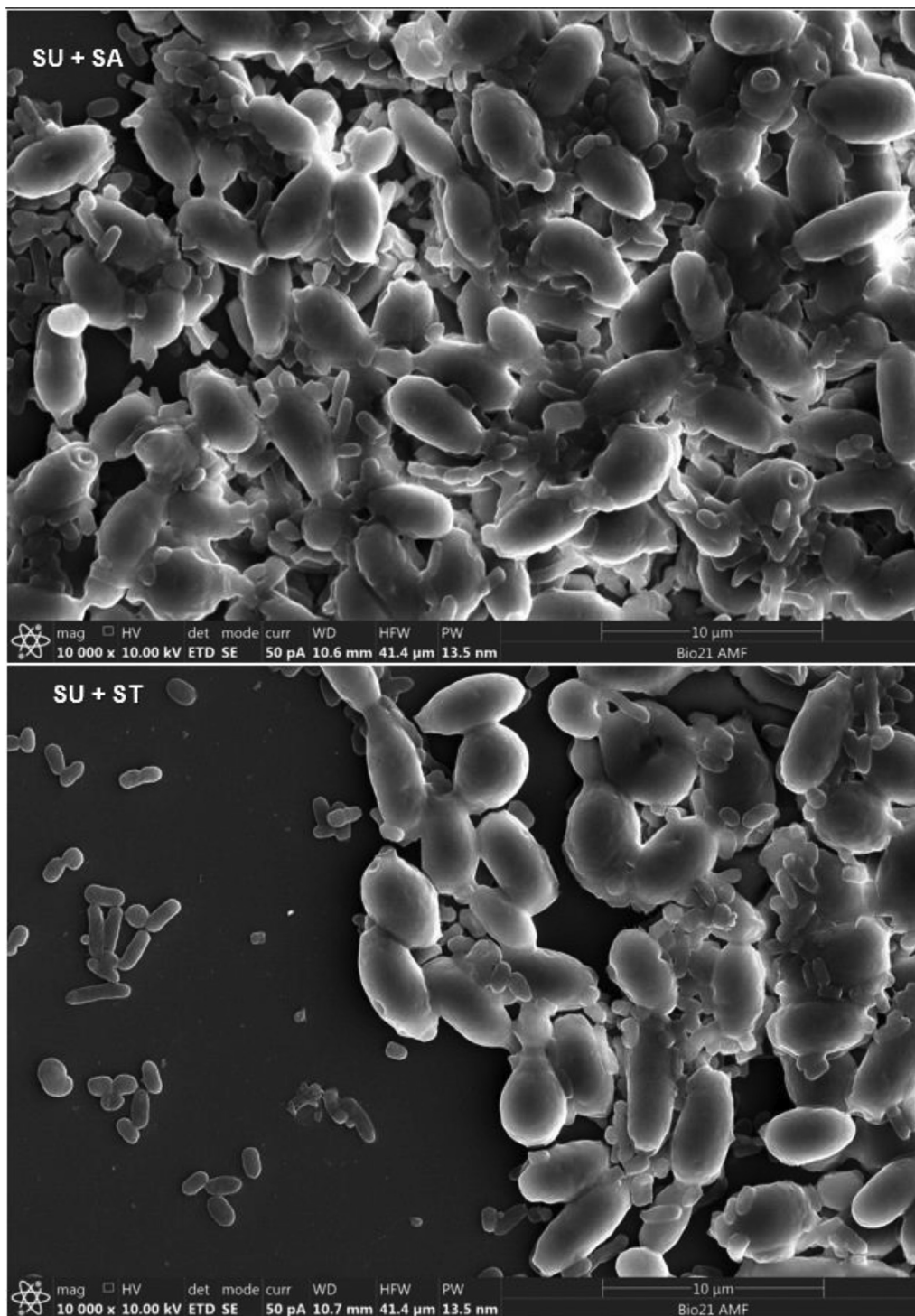


Fig. 1D. Adhesion of *Salmonella* servers onto SU cell walls observed using scanning electron microscope. SA = *S. Arizonae*; ST = *S. Typhimurium*, SU = *S. unisporus*.

media were maintained to reflect standardized simulated intestinal digestive system (Minekus et al., 2014).

2.4. *Salmonella* growth inhibition by yeast metabolites

2.4.1. *Salmonella* growth inhibition assay

Salmonella growth inhibition by yeast colonies was performed as described in the literature (Gut et al., 2019). Yeast colonies on YEPDA grown for 72 h at 25 °C were cut and placed onto Muller-Hinton agar (supplemented with 0.1% cycloheximide) initially

inoculated with 10^4 CFU/mL of *S. Arizonae* and *S. Typhimurium* using spread plate technique. The Muller-Hinton agar plates were incubated at 37 °C for 24 h. Plates were checked for growth inhibition indicated by complete or partial clearing around the yeast slabs.

Further experimentation to investigate *Salmonella* growth inhibition (Bajaj et al., 2013) was carried out with some modifications. Yeasts were grown in a killer toxin medium (KTM) composed of YEPDB plus glycerol (50 g/L), buffered at pH 5 using 50 mM citrate-phosphate buffer, and incubated at 25 °C under shaking

(180 rpm) for 24–48 h. KTM has been reported to enhance production of killer toxin by yeast species (Bajaj et al., 2013). Sampling for KTM cell free supernatant (KTM-CFS) was performed at 24 and 48 h. The yeast fermented KTM was centrifuged (Eppendorf 5810 R, Hamburg, Germany) at 4000g for 30 min at 4 °C and filter sterilized using 0.22 µm membrane filter.

KTM-CFS was analyzed for antibacterial potential using established turbidimetry assay (broth dilution) with some modifications (Balouiri et al., 2016). Briefly, 0.1 mL of 10^4 CFU/mL of *Salmonella* culture in fresh nutrient broth was dispensed into flat-bottom 96 well microtiter plate (Bio-Rad, California, USA) containing 0.1 mL of KTM-CFS. The optical density was read using microplate reader at 595 nm (Biorad, California, USA) at 0, 2, 4, 6, 12 and 24 h. Growth curves were constructed using a Microsoft Excel.

To determine if the effect of KTM-CFS on *Salmonella* demonstrated by turbidimetry assay is bacteriostatic or bactericidal, 5 mL of the KTM-CFS was inoculated with 0.5 mL of *Salmonella* cultures (approximately 10^3 CFU/mL). Sampling was performed at < 1, 30, 60, 90, 120 and 240 min by plating 0.1 mL onto nutrient agar, incubated in 37 °C for 24 h before counting colonies. For control, unfermented KTM was inoculated with *Salmonella* and tested along with the samples.

2.4.2. Determination of alcohol content in KTM and its effect on *Salmonella* species

Alcohol content of KTM-CFS was analyzed quantitatively by gas chromatography as described in a previous study (Nikolaou et al., 2017) with some modifications. Gas chromatography (Shimadzu, Kyoto, Japan) with SGE BP20 GC capillary column (12.0 m length, 0.22 mm inner diameter, 0.25 µm film thickness, Fisher Scientific, Hampton, USA) and flame ionization detector at 200 °C was used. Samples were filter sterilized through a 0.22 µm membrane filter (Sigma Aldrich, St. Louis, USA) and 5 µL was injected into the column. The oven temperature was set as 35 °C for 5 min, then increased to 200 °C at a rate of 10 °C/min. The injector temperature was maintained at 200 °C, with a split ratio of 50:1 and flow rate of 1.1/min. Analysis of the results were performed using a Lab Solution software (Shimadzu, Kyoto, Japan). Alcohol concentration was calculated using 6 point standards (0, 125, 250, 500, 750 and 1000 mM/L; standard curve ($R^2 > 0.99$) created with absolute ethanol.

To determine if the concentration of ethanol in yeast fermented KTM is responsible for *Salmonella* growth inhibition demonstrated by the turbidimetry assay, ethanol control (1, 1.5 and 2% in sterile distilled water) effect on *Salmonella* was investigated. Five milliliters of each control concentration was inoculated with 0.5 mL of *Salmonella* cultures (approximately 10^3 CFU/mL). Sampling time, plating, incubation and colony counting were performed as described under 2.4.1.

To determine if the growth inhibition was due to other volatile metabolites other than ethanol, KTM-CFS was heated in loosely capped centrifuge tubes at 60 °C for 10 min in a shaking incubator at 120 horizontal strokes per min. Five milliliters of the heat treated KTM-CFS was inoculated with 0.5 mL of *Salmonella* cultures (approximately 10^3 CFU/mL) prepared above. Sampling time, plating, incubation and colony counting were performed as described under 2.4.1.

2.4.3. Shotgun proteomics

Yeast-fermented KTM and unfermented KTM (control) samples were analyzed for proteins and peptides identification using shotgun proteomics. Samples were diluted to between 1 and 5 mg/mL total protein concentration, and approximately 10 µg/L total protein of each was buffer exchanged into 50 mM ammonium bicarbonate and the protein was reduced in 2.5 mM DTT at 95 °C for 5 min followed by alkylation with 10 mM chloroacetamide

for 30 min at ambient temperature. Trypsin was then added at the rate of 0.5 µg per 10 µg of protein and incubated at 37 °C overnight. All enzyme digests were analyzed by LC-MS/MS using the QExactive Plus mass spectrometer (Thermo Scientific, Bremen, Germany) coupled online with a RSLC nano HPLC (Ultimate 3000, Thermo Scientific, Bremen, Germany). Two hundred nanograms of sample was injected and concentrated on a 100 µm, 2 cm nanoviper pepmap100 trap column with 97.5% buffer A (0.1% Trifluoroacetic acid) at a flow rate of 15 min⁻¹. The peptides were then eluted and separated with a Thermo RSLC pepmap100, 75 µm × 50 cm, 100 Å pore size, reversed phase nano column with a 30 min gradient of 92.5% buffer A (0.1% formic acid) to 42.5% B (80% acetonitrile 0.1% formic acid), at a flow rate of 250 nL min⁻¹. The eluent was nebulised and ionised using the Thermo nano electrospray source with a distal coated fused silica emitter (New Objective, Woburn, MA, USA) with a capillary voltage of 1900 V. Peptides were selected for MS/MS analysis in full MS/dd-MS2 (TopN) mode with the following parameter settings: TopN 10, resolution 70000, MS/MS AGC target 5e5, 118 ms Max IT, NCE 27, 1.8 m/z isolation window, dynamic exclusion was set to 10 s. Results were analyzed using MaxQuant to obtain protein identifications and their respective label-free quantification values using in-house standard parameters. Data were normalized based on the assumption that the majority of proteins do not change between the different conditions. Protein identification numbers were also used to verify protein names using Uniprot database.

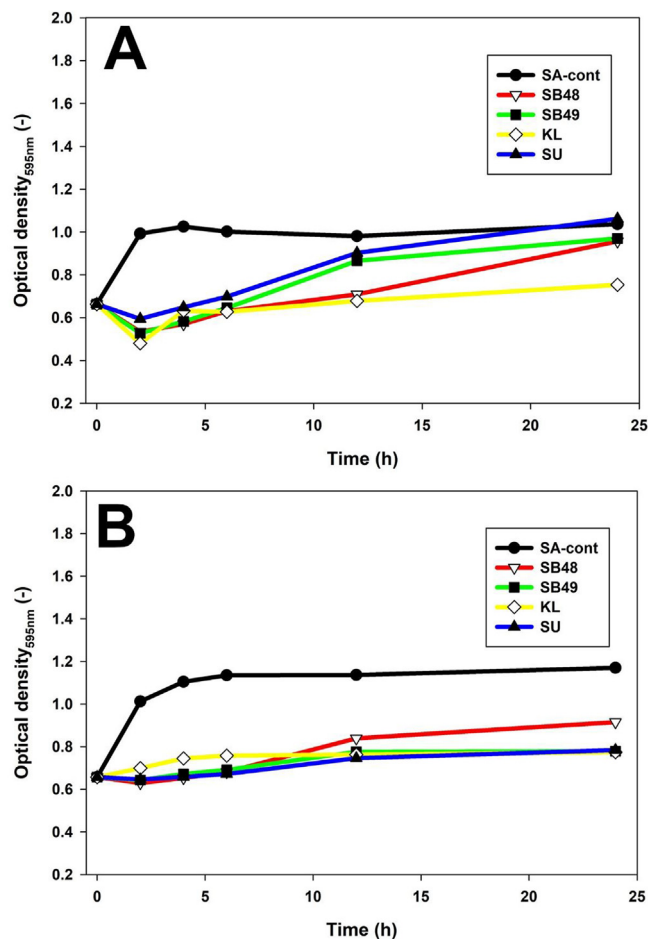


Fig. 2. *Salmonella* real time growth analysis in presence of cycloheximide inactivated yeast cells. A, *S. Arizonae*; B, *S. Typhimurium*.

2.5. Statistical analysis

Experiments were performed in triplicates and subsampled. Results were expressed as the mean ± standard deviation (SD). Means differences were statistically analyzed using Student's *t*-test. Mean differences were considered significant at $p < 0.05$. Statistical analysis was performed using SPSS version 26 Statistical software (IBM, New York, USA). Growth curves were constructed using Microsoft Excel 2016 (Microsoft, Washington, United States). The means for all the experiment followed a normal distribution. For shotgun proteomics, statistical analysis was performed using Perseus. The LFQ data was converted to \log_2 scale, samples were grouped by conditions and missing values were imputed based on normal distributions after all proteins were eliminated that had 2 or less valid values. Protein fold-changes were calculated and their significance was determined using a two-sided *T*-test with error-corrected *P*-values.

3. Results

3.1. Adhesion of *Salmonella* onto yeast cells

Adherence of *S. Arizonae* and *S. Typhimurium* onto viable and non-viable yeast cells was qualitatively and quantitatively analyzed (Table 1). Colony count of the two *Salmonella* serovars after sedimentation with *K. lactis*, *S. unisporus* and *S. boulardii* strains were lower ($p < 0.05$) compared to the controls for both viable

and non-viable yeast cells. Agglutination on the slides which is an indication of aggregation (between *Salmonella* and yeast cells) was also observed in all cases (Table 1). Figs. 1A, 1B, 1C and 1D show clear adherence of *Salmonella* onto viable yeast strains observed using a scanning electron microscope. Observed adhesions of *Salmonella* onto non-viable kefir yeast isolates and *S. boulardii* strain (data not shown) appeared similar to that onto viable yeasts. Scanning electron microscope results provide clear visual evidence that *Salmonella* count reduced after treatment with yeast, likely caused by attachment to yeast cells and subsequent settling at the bottom of the tubes.

The growth behavior of *Salmonella* in presence of inactivated yeast cells was determined at different time points using optical density (Fig. 2). Growth rate of *S. Arizonae* was lower in all yeast cells compared to the control ($p < 0.05$) until 12th h (Fig. 2). On the other hand, *S. Typhimurium* had its growth significantly suppressed by all yeasts compared to the control ($P < 0.05$) until the experiment was stopped at 24 h (Fig. 2).

3.2. Factors affecting *Salmonella* adhesion onto yeast cell walls

The effect of pH on adhesion was investigated qualitatively at pH 2, 4, 5, 7 and 8 (Table 1). The gastrointestinal tract (GIT) pH ranged from 3 to 7, with the stomach being more acidic (Minekus et al., 2014). There was no difference in observable agglutination with all the pH levels, which indicated that pH did not interfere with adhesion.

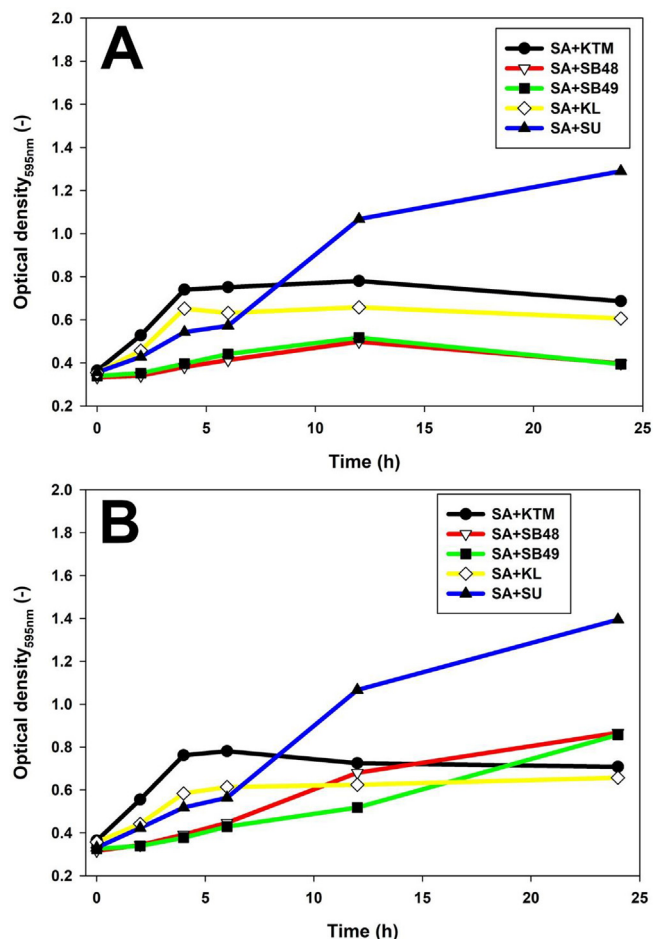


Fig. 3. *S. Arizonae* growth inhibition by yeast fermented KTM using optical turbidimetry assay. A, KTM fermented for 24 h; B, KTM fermented for 48 h.

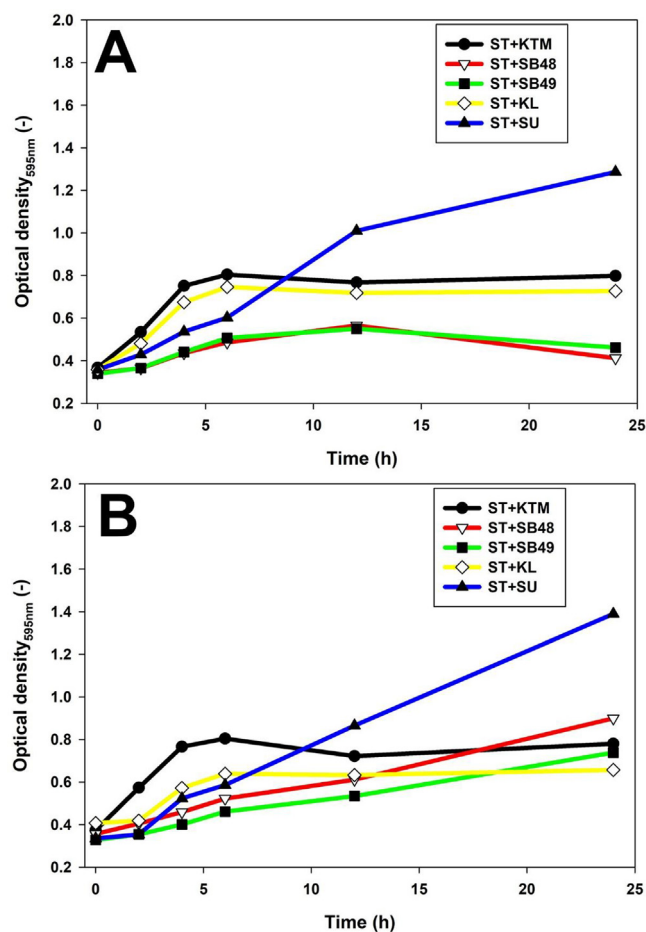


Fig. 4. *S. Typhimurium* growth inhibition by yeast fermented KTM using optical turbidimetry assay. A, KTM fermented for 24 h; B, KTM fermented for 48 h.

Bile salt (0.3%) had no effect on adhesion of *Salmonella* onto *K. lactis*, *S. unisporus*, and *S. boulardii* strains (Table 1). Slide agglutination in presence of bile did not interfere with adhesion of *Salmonella* onto yeast cell walls with exception of *K. lactis*, which showed weak clumping on slide agglutination.

3.3. *Salmonella* growth inhibition by yeast antimicrobial metabolites

3.3.1. *Salmonella* growth inhibition by yeasts metabolites

Potential presence of anti-*Salmonella* molecules produced by yeast was investigated using slab on the lawn assay, and growth inhibition was not observed (data not shown). The effect of potentially antimicrobial metabolites in KTM-CFS was assessed (Figs. 3 & 4) using a turbidimetry assay. *Salmonella* growth was suppressed by *S. unisporus* KTM-CFS in the first 5 h, but started to grow rapidly thereafter, compared to the control. However, *K. lactis* exhibited growth rate suppression of *S. Typhimurium* at all-time points analyzed. Forty-eight hour fermentation of KTM with *K. lactis* showed stronger growth inhibition of *Salmonella* compared to 24 h (Figs. 3 & 4). *S. boulardii* strains showed significant growth rate reduction of *Salmonella* compared to kefir yeast isolates (Figs. 3 & 4). Fig. 5 showed that *Salmonella* count remained constant without significant decrease or increase in yeast-fermented KTM, which indicated growth inhibition effect.

3.3.2. Assessment of alcohol content in KTM

To confirm the presence of alcohol in KTM-CFS, GC analysis was performed with data shown in Table 2. Fermentation of KTM with

Table 2
Analysis of alcohol content in fermented KTM using GC.

Yeast strain	Alcohol concentration (% V/V)	
	24 h KTM fermentation	48 h KTM fermentation
SB48	1.55 ± 0.16 ^a	0.64 ± 0.01 ^a
SB49	1.42 ± 0.01 ^a	0.43 ± 0.04 ^a
KL	1.30 ± 0.06 ^a	1.21 ± 0.17 ^a
SU	1.46 ± 0.01 ^a	1.32 ± 0.07 ^a
KTM blank	0.08 ± 0.05 ^b	0.08 ± 0.05 ^b

Means with different superscript are significantly different (p < 0.05)

S. boulardii showed significantly higher concentration of alcohol after 24 h compared to that at 48 h. The concentration of alcohol produced by kefir yeast isolates in KTM-CFS from 48 h fermentation was also lower than that at 24 h (Table 2). Experimentation to determine if alcohol in yeasts-fermented KTM is responsible for growth inhibition above showed no effect on *Salmonella*, possibly due to low concentration (data not shown). Heat treatment of the fermented KTM-CFS to remove potential volatile antimicrobial compounds did not have effect on *Salmonella* growth for *K. lactis*, and *S. boulardii* strains. However, *S. unisporus* fermented KTM-CFS growth enhancing effect on *S. arizonae* was lost (Fig. 6).

3.3.3. Assessment of antimicrobial proteins in KTM

Production of proteins with potential antimicrobial properties in yeasts-fermented KTM was assessed using shotgun proteomics. Proteins shown in Table 3 were produced in substantial quantities

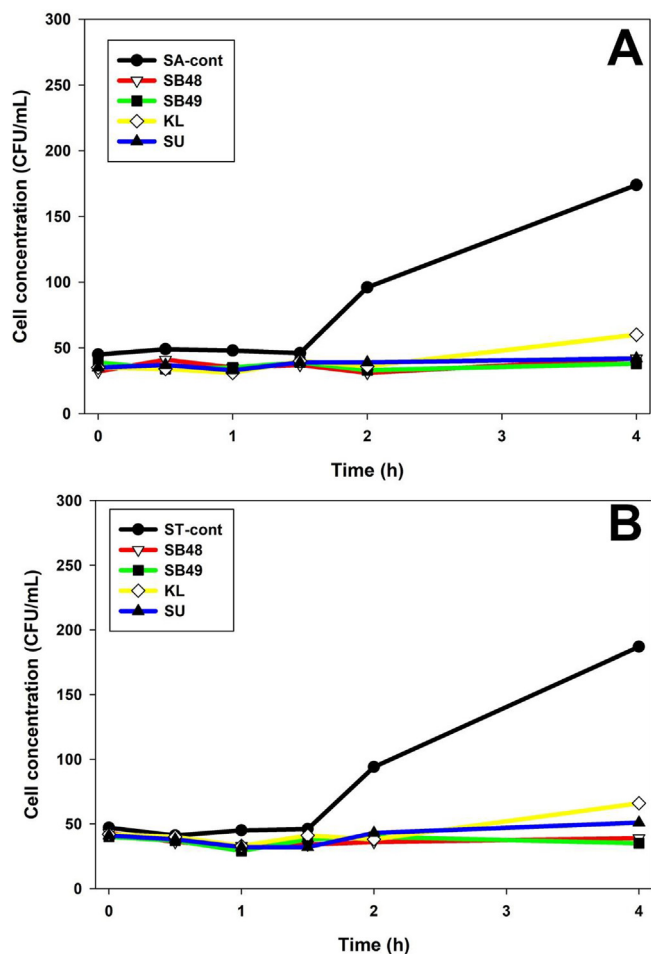


Fig. 5. Determination of bacteriostatic and bactericidal effect of KTM on *Salmonella* using colony forming unit counting assay. A: *S. Arizonae*; B: *S. Typhimurium*.

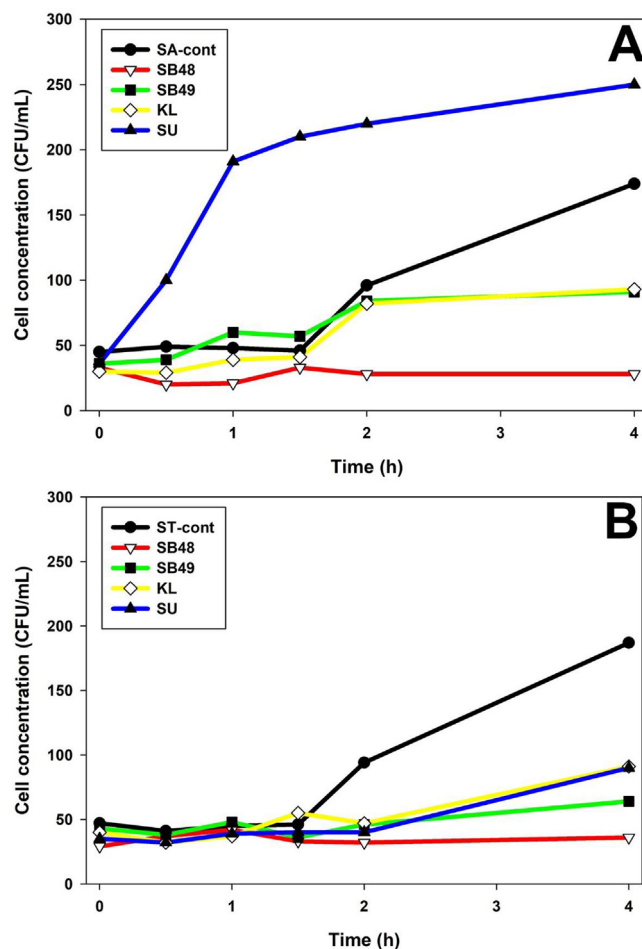


Fig. 6. Determination of bacteriostatic and bactericidal effect of heat treated KTM on *Salmonella* using colony forming unit counting assay. A: *S. Arizonae*; B: *S. Typhimurium*.

Table 3
Shotgun proteomic analysis of yeast fermented KTM.

SB48	SB49	KL	SU
40S ribosomal protein S14-A	40S ribosomal protein S14-A	Fructose-bisphosphate	60 kDa chaperonin
40S ribosomal protein S14-B	40S ribosomal protein S14-B	aldolase	60S ribosomal protein L20-B
40S ribosomal protein S29	60S ribosomal protein L20-B	Lactadherin*	60S ribosomal protein L20-A
60 kDa chaperonin	60S ribosomal protein L20-A	40S ribosomal protein S14-A	60S ribosomal protein L21
60S ribosomal protein L13	60S ribosomal protein L36	40S ribosomal protein S14-B	60S ribosomal protein L28
60S ribosomal protein L20-B	Actin cytoplasmic 1	40S ribosomal protein S24	60S ribosomal protein L34
60S ribosomal protein L20-A	Adenosylhomocysteinase	40S ribosomal protein S29	60S ribosomal protein L36
60S ribosomal protein L28	Alcohol dehydrogenase 2	60 kDa chaperonin	60S ribosomal protein L8
60S ribosomal protein L34	Alcohol dehydrogenase 1	60S ribosomal protein L13	Actin, cytoplasmic 1
60S ribosomal protein L35a	ATP-dependent molecular chaperone	60S ribosomal protein L14	Alcohol dehydrogenase 2
60S ribosomal protein L35a	HSC82;ATP-dependent molecular	60S ribosomal protein L20-B	Alcohol dehydrogenase 1
60S ribosomal protein L36	chaperone HSP82	60S ribosomal protein L20-A	ATP-dependent molecular chaperone HSC82; ATP-
60S ribosomal protein L37a	Cathelicidin-2*	60S ribosomal protein L21	dependent molecular chaperone HSP82
60S ribosomal protein L8	Cathelicidin-6*	60S ribosomal protein L28	Broad substrate specificity ATP-binding cassette
Actin, cytoplasmic 1	Cathelicidin-7*	60S ribosomal protein L34	transporter ABCG2 ATP-binding cassette sub-family
Acyl-CoA-binding protein	Cysteine-rich secretory protein 2	60S ribosomal protein L34-A	G member 2
Adenosylhomocysteinase	Elongation factor 2	60S ribosomal protein L34-B	Calmodulin
Alcohol dehydrogenase 2	Enolase 1	60S ribosomal protein L35a	Cathelicidin-2*
Alcohol dehydrogenase 1	Enolase 2	60S ribosomal protein L36	Cathelicidin-6*
ATP synthase subunit alpha	Fructose-bisphosphate aldolase	60S ribosomal protein L37a	Cathelicidin-7*
mitochondrial	Glucan 1,3-beta-glucosidase I/II	60S ribosomal protein L8	CD59 glycoprotein
ATP-dependent molecular	Glyceraldehyde-3-phosphate	Actin, cytoplasmic 1	Cystatin E/M
chaperone HSC82	dehydrogenase 2 Glyceraldehyde-3-	Acyl-CoA-binding protein	Cystatin-C
ATP-dependent molecular	phosphate dehydrogenase 3	Adenosylhomocysteinase	Cysteine-rich secretory protein 2
chaperone HSP82	Heat shock protein SSB2	Alcohol dehydrogenase 2	Endoplasmic reticulum chaperone BiP
Broad substrate specificity	Heat shock protein SSB1	Alcohol dehydrogenase 1	Enolase 1
ATP-binding cassette	Histatherin	ATP synthase subunit alpha	Enolase 2
transporter ABCG	Immunoglobulin J chain	mitochondrial	Eukaryotic initiation factor 4A-I
ATP-binding cassette sub-	Inositol polyphosphate-5-phosphatase E	ATP synthase subunit beta	Fatty acid-binding protein, heart
family G member 2	Keratin 24	mitochondrial	Folate receptor alpha,
Butyrophilin subfamily 1	NPC intracellular cholesterol transporter 2	ATP-dependent molecular	Folate receptor 2Fructose-bisphosphate aldolase
member A1	Nucleobindin 2	chaperone HSC82;	Glucan 1,3-beta-glucosidase I/II
Calmodulin	Peptidyl-prolyl cis-trans isomerase	ATP-dependent molecular	Glycoprotein 2
Cathelicidin-2*	Pyruvate kinase 1	chaperone HSP82	Heat shock protein SSB2
cathelicidin-4*	Secretoglobulin family 1D member	Beta-1,4-galactosyltransferase	Heat shock protein SSB1
cathelicidin-6*	Selenoprotein M	1	Heterogeneous nuclear ribonucleoproteins A2/B1
cathelicidin-7*	Translationally-controlled tumor protein	Beta-2-microglobulin	Histatherin
CD59 glycoprotein	homolog	Broad substrate specificity	Immunoglobulin J chain
CD9 antigen	Transthyretin	ATP-binding cassette	Inositol polyphosphate-5-phosphatase E
Cystatin E/M	Triosephosphate isomerase	transporter ABCG2	Keratin 24
Cysteine-rich secretory	Ubiquitin-like protein SMT3	Butyrophilin subfamily 1	Lactadherin
protein 2	Uncharacterized protein	member A1	Lactoperoxidase
Elongation factor 2	Uncharacterized protein	Calmodulin	Lipoprotein lipase
Endoplasmic reticulum	Vacuolar protein sorting-associated	Cathelicidin-1*	Monocyte differentiation antigen CD14
chaperone BiP	protein 53 homolog	Cathelicidin-2*	Mucin-1*
Enolase 1		Cathelicidin-4*	NPC intracellular cholesterol transporter 2
Enolase 2		Cathelicidin-6*	Nucleobindin 1
Enoyl-CoA hydratase,		Cathelicidin-7*	Nucleobindin 2
mitochondrial		CD59 glycoprotein	Parathyroid hormone-related protein
Eukaryotic initiation factor		CD9 antigen	Peptidyl-prolyl cis-trans isomerase A
4A-I Eukaryotic initiation		Cellular repressor of E1A	Polymeric immunoglobulin receptor
factor 4A-II		stimulated genes 1	Prostaglandin-H2 D-isomerase
Eukaryotic translation		Collagen alpha-2(I) chain	Pyruvate kinase 1
initiation factor 2 subunit		Cystatin domain	Secretoglobulin family 1D member
gamma		Cystatin E/M	Selenoprotein M
Fatty acid-binding protein		Cystatin-C	SET nuclear oncogene
heart		Cysteine-rich secretory protein	Solute carrier family 38 member 10
FGG protein		2	TGOLN2 protein
Fibrinogen gamma-B chain		Elongation factor 2	Translationally-controlled tumor protein homolog
Folate receptor alpha		Endoplasmic reticulum	Transthyretin
Fructose-bisphosphate		chaperone BiP	U6 snRNA-associated Sm-like protein LSM4
aldolase		Enolase 1	Ubiquitin-40S ribosomal protein S27a Ubiquitin-60S
Glucan 1,3-beta-glucosidase		Enolase 2	ribosomal protein L40 Polyubiquitin-B
I/II		Enoyl-CoA hydratase,	Polyubiquitin-C
Glyceraldehyde-3-		mitochondrial	Ubiquitin-like protein SMT3
phosphate dehydrogenase 2		Eukaryotic initiation factor 4A-	Uncharacterized protein
Glyceraldehyde-3-		I Eukaryotic initiation factor	Uncharacterized protein
phosphate dehydrogenase 3		4A-II	Uncharacterized protein
Glycoprotein 2		Eukaryotic translation	Vacuolar protein sorting-associated protein 53
Heat shock protein SSB2;		initiation factor 2 subunit	homolog
Heat shock protein SSB1		gamma	WAP four-disulfide core domain 2
Heterogeneous nuclear		Fatty acid-binding protein	Xanthine dehydrogenase/oxidase
ribonucleoproteins A2/B1		heart	

Table 3
Shotgun proteomic analysis of yeast fermented KTM.

SB48	SB49	KL	SU
Histatherin		FGG protein	
Ig-like domain-containing protein		Fibroblast growth factor-binding protein 1	
Immunoglobulin J chain		Folate receptor alpha	
Inositol polyphosphate-5-phosphatase E		Glucan 1,3-beta-glucosidase I/II	
Keratin 24		Glyceraldehyde-3-phosphate dehydrogenase	
Lactadherin*		Glyceraldehyde-3-phosphate dehydrogenase 2	
Lactoperoxidase*		Glyceraldehyde-3-phosphate dehydrogenase 3	
Lactotransferrin*		Glycoprotein 2	
Lipoprotein lipase		Glycosylation-dependent cell adhesion molecule 1	
Monocyte differentiation antigen CD14		Granulin precursor	
Mucin-1*		Heat shock 70 kDa protein 1A	
NPC intracellular cholesterol transporter 2		Heat shock protein SSB2	
Nucleobindin 2		Heat shock protein SSB1	
Nucleobindin-1		Helix-destabilizing protein	
Parathyroid hormone-related protein		Heterogeneous nuclear ribonucleoprotein K	
Peptidyl-prolyl cis-trans isomerase		Heterogeneous nuclear ribonucleoproteins A2/B1	
Peptidyl-prolyl cis-trans isomerase A		HHIP like 2	
Perilipin-2		Histatherin	
Platelet glycoprotein 4		Ig-like domain-containing protein	
Polymeric immunoglobulin receptor		Immunoglobulin J chain	
Prostaglandin-H2 D-isomerase		Inorganic pyrophosphatase	
Protein BMH1		Inositol polyphosphate-5-phosphatase E	
Protein BMH2		Keratin 24	
Ribosomal protein L21e		Lactoperoxidase*	
60S ribosomal protein L21		Lactotransferrin*	
Secretoglobin family 1D member		Lipoprotein lipase	
Selenoprotein M		Monocyte differentiation antigen CD14	
Serum amyloid A protein*		Mucin-1*	
SET nuclear oncogene		Myosin heavy chain 9	
snRNA-associated Sm-like protein LSm4		NPC intracellular cholesterol transporter 2	
Sodium-dependent phosphate transport protein 2B		Nucleobindin 2	
Solute carrier family 38 member 10		Parathyroid hormone-related protein	
Sulfhydryl oxidase		Peptidyl-prolyl cis-trans isomerase	
TGOLN2 protein		Peptidyl-prolyl cis-trans isomerase A	
Translationally-controlled tumor protein homolog		Perilipin-2	
Transthyretin		Platelet glycoprotein 4	
Triosephosphate isomerase		Polymeric immunoglobulin receptor	
Ubiquitin-40S ribosomal protein S27a		Prostaglandin-H2 D-isomerase	
Ubiquitin-60S ribosomal protein L40		Pyruvate kinase 1	
Polyubiquitin-B, Polyubiquitin-C		Ribonuclease pancreatic	
Ubiquitin-like protein SMT3		Ribosomal protein L37	
Uncharacterized protein		Secretoglobin family 1D member	
Uncharacterized protein		Selenoprotein M	
Uncharacterized protein		SET nuclear oncogene	
Vacuolar protein sorting-associated protein 53 homolog		Sodium-dependent phosphate transport protein 2B	
WAP four-disulfide core domain 2		Solute carrier family 38 member 10	
Xanthine dehydrogenase/oxidase*		Sulfhydryl oxidase	
Zinc-alpha-2-glycoprotein		TGOLN2 protein	
		Translationally-controlled tumor protein homolog	
		Transthyretin	
		Triosephosphate isomerase	
		U6 snRNA-associated Sm-like protein LSm4	
		Ubiquitin thioesterase	
		Ubiquitin-60S ribosomal protein L40, Polyubiquitin-C, Polyubiquitin-B	
		Ubiquitin-60S ribosomal protein L40 Ubiquitin-40S ribosomal protein S31	
		Polyubiquitin	
		Ubiquitin	
		Ubiquitin-like protein SMT3	
		Uncharacterized protein	
		Uncharacterized protein	
		Uncharacterized protein	
		Uncharacterized protein	
		Vacuolar protein sorting-associated protein 53 homolog	
		WAP four-disulfide core domain 2	
		Xanthine dehydrogenase/oxidase*	
		Zinc-alpha-2-glycoprotein	

Key: * = protein with known antimicrobial properties.

in yeasts-fermented KTM, but present in trace amount or absent in KTM blank.

4. Discussion

Adhesion is defined as a process whereby cells attach to surfaces of each other with the aid of adhesins (Brückner & Mösch, 2012). The adherence of *Salmonella* and other enteric bacterial pathogens onto yeast cell wall is postulated to be due to specific binding of type 1 fimbriae on bacteria cell with mannan oligosaccharides on yeast cell wall (Gut et al., 2018). Moreover, non-specific adhesion mechanisms including electrostatic and hydrophobic attachment between bacteria and yeasts have been reported (Adegbola & Old, 1985; Pérez-Sotelo et al., 2005; Tiago et al., 2012).

Adhesion of the two *Salmonella* serovars onto kefir yeast isolates and *S. boulardii* strains (Table 1, Fig. 1A-D) are consistent with a previous study in which Gram negative enteric bacteria (*Escherichia coli* and *Enterobacter aerogenes*) were bound to kefir yeast isolates and *S. boulardii* strains (Gut et al., 2019). Furthermore, similar results showed that enteropathogenic bacteria including *S. Typhimurium* (ATCC 14028) adhered onto viable and non-viable yeast cells (França et al., 2015; Martins et al., 2010; Posadas et al., 2017). The growth trend of *Salmonella* in the presence of inactivated yeasts (Fig. 2) was likely due to *Salmonella* binding to yeast cells and subsequent sedimentation. Bacteria cells in tight auto-aggregation have been reported to show no cell division (Hassani et al., 2009). This therefore resulted in reduced *Salmonella* population, as was indicated in a less turbid medium compared to the control. Both kefir yeast isolates and *S. boulardii* strains were found to survive well in simulated GIT conditions (Gut et al., 2019). Yeasts survival and proliferation may lead to higher numbers in GIT due to potential growth, increasing the capacity to bind *Salmonella* and subsequent shedding in feces. Furthermore, the advantage of yeasts is the fact that they are not affected by drugs targeting infectious bacteria such as *Salmonella*, making them suitable candidates for complementary therapy with antibiotics. For example chloramphenicol is an antibiotic typically used in *Salmonella* treatment (Gut et al., 2018) and works against bacteria by binding to ribosomes and blocking protein synthesis but does not affect yeasts (Das & Patra, 2017; Gut et al., 2019). The current study also established that non-viable yeast cells were as effective in *Salmonella* attachment as live yeast cells. This may have critical therapeutic or prophylactic application advantage as viable yeast cells have been associated with at least 100 fungemia cases (Gut et al., 2018). For example, the use of viable yeast cells in immunocompromised people or those with gastrointestinal disease has been reported to pose serious threat of fungemia (Kelesidis & Pothoulakis, 2012). Use of non-viable kefir yeast either prophylactically or as a complementary therapy for *Salmonella* infection may be a better choice for people with GIT diseases or compromised immunity.

Effect of pH on adhesion of *Salmonella* onto yeast cell walls (Table 1) was in line with a previous research (Tiago et al., 2012), which reported that pH between 4 and 8 had no effect on bacteria attachment onto yeast cell wall. Adhesion of *Salmonella* onto yeast cell wall at GIT pH is important because it is where invasion of pathogen and gastroenteritis occur. Therefore, if *Salmonella* could attach to yeast cells in the GIT under acidic condition, invasion may be prevented. Furthermore, bile salt has high surface activity (Attili et al., 1986) and reduces surface hydrophobicity (Tiago et al., 2012), therefore may prevent adhesion. However, 0.3% bile salt did not prevent adhesion in this study (Table 1) which correlated with previous reports (Gómez et al., 2002; Guglielmetti et al., 2009; Tiago et al., 2012). Adherence in presence of bile is important since

it will not interfere with potential prophylactic or therapeutic application in GIT.

Growth inhibitory properties of yeast against bacteria have been previously proposed to include production of ethanol and other antibacterial metabolites such as killer toxins (Bajaj et al., 2013; Muccilli & Restuccia, 2015). Many mechanisms of probiotics including yeasts against susceptible microbial cells have been proposed and involve destabilization of the cell membrane, cell lysis, degradation of nucleic acid, inhibition of protein synthesis and binding onto yeasts (Gut et al., 2021).

Lack of *Salmonella* growth inhibition by yeast colonies correlated with a previous study on *S. boulardii* effect on enteric bacterial growth (Rajkowska et al., 2012). However, a study reported that killer toxin produced by bakery *Saccharomyces* showed growth inhibition of *Escherichia coli* and *S. Typhimurium* as a result of cell membrane destruction (Alsoufi & Aziz, 2017). Therefore further experimentation involving use of KTM was required. Stronger growth inhibition exhibited by KTM fermented with *K. lactis* for 48 h (Figs. 3 and 4) may be due to accumulation of antibacterial metabolites produced by *K. lactis* when fermentation time was prolonged, consistent with a previous report in which *K. lactis* and *Kluyveromyces marxinus* showed antagonistic effect on *Salmonella* Paratyphi B, *S. Typhimurium* ATCC 14028 and *Salmonella* Enteritidis when fermentation time was extended by downregulating chromosomal *sopD* gene (Ceugniz et al., 2017). *Salmonella* growth promotion by *S. unisporus* KTM-CFS observed in this study after the initial 5 h (Figs. 3 and 4) was likely due to the loss of potential antibacterial molecules coupled with possible presence of growth factors such as amino acids and vitamins released by yeasts (Bechtner et al., 2019; Gut et al., 2021; Stadie et al., 2013). This growth promoting effect of *S. unisporus* on *S. Arizonae* was lost likely due to heat inactivation or evaporation of some volatiles (Fig. 6). However, *Salmonella* population generally remained stable in the presence of yeast-fermented KTM due to its bacteriostatic effect compared to the control which showed significant increase (Fig. 5).

Studies have confirmed production of alcohol by *Kluyveromyces* and *Saccharomyces* species commonly isolated from kefir (Ho et al., 2012; Magalhães et al., 2010). Concentration of alcohol produced by kefir yeast isolates in Table 2, was consistent with reported alcohol concentrations in the literature (Magalhães et al., 2010; Nuñez, 2016). Furthermore, production of alcohol in KTM fermented with *S. boulardii* is consistent with a previous study in which this yeast strain was used in a beer production (Mulero-Cerezo et al., 2019). The differences in concentration between 24 and 48 h fermentation could be due to the attainment of stationary growth phase resulting in constant metabolic activities at 24 h (Mulero-Cerezo et al., 2019), and the likelihood of loss due to evaporation during fermentation. Lack of bacteriostatic effect by 2% ethanol control showed that ethanol in the KTM may not be responsible for bacteriostatic effect observed, an indication of presence of other potential antimicrobial metabolites. Suppression of *Salmonella* growth by KTM fermented with SB48, SB49, *K. lactis* and *S. unisporus* after evaporation suggested presence of non-volatile molecules, possibly antimicrobial proteins which showed anti-salmonella activities. Antimicrobial proteins produced by yeasts (*Saccharomyces* and *Kluyveromyces* species) have been documented to be effective against bacteria (Branco et al., 2017; Rima et al., 2012; Al-Sahlaney et al., 2020; Hasan et al., 2019; Liu et al., 2018). The shotgun proteomics analysis of KTM-CFS confirmed the presence of such proteins which have been shown to have antimicrobial properties. Previous studies have reported antimicrobial activity of cathelicidin (Xia et al., 2015), xanthine dehydrogenase (Okamura et al., 2018), lactotransferrin and mucin-1 (Gut et al., 2018), lactadherin (Sabha et al., 2018), lactoperoxidase (Bafort et al., 2014) and serum amyloid A protein (Kagan et al.,

2012). The mechanisms of these proteins against susceptible bacteria have been reported to involve DNA, RNA, ATP synthesis, or protein synthesis inhibition, as well as disruption in membrane and ionic potential of the cell membrane (Biadała et al., 2020). The proteins marked with asterisk in Table 3 may have inhibited *Salmonella* growth in the current study however, further studies are needed.

5. Conclusion

Kefir yeast isolates obtained from traditional kefir grains showed comparable anti-salmonella effect to that of *S. boulardii* with respect to adhesion as well as growth inhibition due to antimicrobial metabolites production. Shot-gun proteomics analysis showed presence of cathelicidin, xanthine dehydrogenase, mucin-1, lactadherin, lactoperoxidase, serum amyloid A protein and lactotransferrin in yeast fermented killer toxin medium which have anti-bacterial properties. These proteins in KTM may be responsible for bacteriostatic effect observed in this study. *K. lactis* and *S. unisporus* have potential to be used prophylactically and therapeutically in control of *Salmonella* infection. However, further studies involving cell lines, animals as well as human trials are needed to prove these kefir isolates efficacy in prevention and treatment of *Salmonella* infection.

Credit authorship contribution statement

Abraham Majak Gut: Writing – original draft, Writing – review & editing. **Todor Vasiljevic:** Supervision, Writing – review & editing. **Thomas Yeager:** Supervision, Writing – review & editing. **Osaana N. Donkor:** Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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