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Antimicrobial metabolites from Probiotics, *Pleurotus ostreatus* and their co-cultures against foodborne pathogens isolated from ready-to-eat foods

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Abstract

Background The incidence of foodborne pathogens in ready- to-eat (RTE) can be attributed to various foodborne diseases. Most of the isolated microorganisms from RTE foods are resistant to common antibiotics and thus, resulted to treatment failure when commercially available antibiotics are administered. However, the secondary metabolites secreted by microorganisms can serve as alternative therapy that are reliable and safe. Secondary metabolites obtained from mono- and co-culture microorganisms can inhibit the growth of antibiotic-resistant microorganisms. Bioactive compounds in the secreted metabolites can be identified and utilized as sources of new antibiotics. In this study, antimicrobial activity of secondary metabolites from *Lactobacillus fermentum*, *Saccharomyces cerevisiae*, *Pleurotus ostreatus*, and their co-cultures were tested against foodborne pathogens isolated from RTE foods using agar well diffusion. The bioactive compounds in the metabolites were identified using gas chromatography-mass spectrometry.

Results From a total of 100 RTE foods examined, *Salmonella enterica*, *Shigella dysenteriae*, *Escherichia coli*, *Klebsiella pneumoniae* (subsp *ozaenae)*, *Pseudomonas fluorescens*, *Clostridium perfringes*, *Bacillus cereus*, *Listeria monocytogens*, and *Staphylococcus aureus*, *Penicillium chrysogenum*, *Aspergillus flavus*, and *Aspergillus niger* were isolated and displayed multiple antibiotic resistance. The secondary metabolites secreted by co-culture of *L. fermentum* + *P. ostreatus* + *S. cerevisiae*, and co-culture of *P. ostreatus* + *S. cerevisiae* have the highest ($P \leq 0.05$) zones of inhibition (23.70 mm) and (21.10 mm) against *E. coli*, respectively. Metabolites from mono-cultured *L. fermentum*, *P. ostreatus*, and *S. cerevisiae* showed zones of inhibition against indicator microorganisms with values ranging from 8.80 to 11.70 mm, 9.00 to 14.30 mm, and 9.30 to 13.00 mm, respectively. Some of the bioactive compounds found in the metabolites of co-cultured microorganisms were alpha-linolenic acid (25.71%), acetic acid 3-methylbutyl ester (13.83%), trans-squalene (12.39%), pentadecylic acid (11.68%), 3- phenyllactic acid (30.13%), linolelaidic acid methyl ester (15.63%), and 4-O-methylmannose (53.74%).

Conclusion RTE foods contain multiple antibiotics resistance pathogens. The pronounced antimicrobial activity of the secondary metabolites against microorganisms from RTE foods could be attributed to the presence of

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bioactive compounds in the metabolites. These metabolites can be exploited as alternative food preservatives, biopharmaceuticals and can be used towards better health delivering systems.

Keywords *Lactobacillus fermentum*, *Saccharomyces cerevisiae*, Prebiotic, Co-cultivation, Antimicrobial activity, Functional foods

Introduction

Foodborne pathogens cause different diseases leading to morbidities, mortalities and high financial burden on healthcare costs, particularly among under developing and developing countries (Bintsis 2017). World Health Organization (WHO) reveals that an estimated 600 million people are suffering from foodborne diseases, while up to 420,000 deaths has been recorded (WHO, 2022). The issue of food safety is of concern and progressively becoming a challenge to public health. Bacteria, fungi, viruses, parasites, and microbial toxins in foods are the biological threats attributed to acute and chronic foodborne diseases (Todd 2014). Combinations of antibiotics are often used for the treatment of foodborne diseases to achieve success. The application of antibiotic therapy for the treatments of foodborne diseases has tremendously increased in public health and often, habitually abused. Nowadays, such development is contributing to the emergence and occurrence of multiple antibiotic-resistant pathogens, leading to treatment failure, increase mortality as well as treatment costs, reduced infection control efficiency, and spread of resistant pathogens from hospital to communities (Hashempour-Baltork et al. 2019). Ready-to- eat (RTE) foods are considered food safety hazards, being major sources of foodborne pathogens to consumers (Abalkhail 2023). Contamination of RTE foods occurred through poor hygiene, the quality of cooking ingredients, inappropriate storage of foods for extended periods, and improper food preparation techniques, which had led to occurrence of wide range of foodborne bacteria, fungi, viruses, and protozoans (Bintsis 2017). The microorganisms isolated from RTE foods displayed a wide range of multiple antibiotic resistance, causing several foodborne disease outbreaks and thus, requires pressing attentions.

The genetic variation in bacterial populations, mutations that render antibiotics ineffective, plasmid exchange within the bacterial colony, resulting to proliferation of resistant gene and thus, contribute to multiple antibiotic resistance (Munita and Arias 2016). The presence of extended-spectrum antibiotic resistance genes in bacteria thwarts the selection of therapeutic agents and thus, increase the treatment failure witnessing in available antibiotics, and cause adverse clinical complications (Rajaei et al. 2021). However, exploitation of novel natural metabolites from microorganisms is required to discover and develop new antimicrobial agents as a strategy to suppress the emergence of multi-drug resistance foodborne pathogens.

Lactic acid bacteria (LAB) notably referred to as probiotics, are capable of producing a variety of byproducts like bacteriocins, amines, short-chain fatty acids, vitamins, and exopolysaccharides due to their ability to utilize macromolecular substances. Most of these secondary metabolites have therapeutic values and thus, have expanded applications in the food, pharmaceutical, and agricultural sectors (Wang et al. 2021). Antimicrobial potentials of LAB are attributed to their ability to create a competitive environment and secrete inhibitory substances, which impede the action of food spoilage by pathogenic microorganisms (Ibrahim et al. 2021). *Saccharomyces cerevisiae* is a species of yeast, commonly used for various industrial applications and genetic studies due to its dynamic activities during fermentation such as resilience to adverse conditions of osmolarity, low pH, remarkable tolerance to high sugar concentrations, production of aromatic, volatile compounds, and enzymes with antimicrobial activities (Parapouli et al. 2020). Probiotic potentials of *S. cerevisiae* occur as results of extracellular protease, secretion of inhibitory proteins, stimulating immunoglobulin A, acquisition and elimination of secreted toxins, killer toxins, sulfur dioxide, and other secondary metabolites (Fakruddin et al. 2017). *S. cerevisiae* is a known probiotic yeast with preferred influences during winemaking, baking, and brewing since ancient times, and therefore, serve as a recommended probiotic for the prevention and treatment of antibiotic-related diarrhea, including *Clostridium difficile*-associated diarrhea (Czerucka and Rampal 2019). *Pleurotus ostreatus;* markedly known as a macrofungus and its fruiting body can be consumed by people all over the world due to its taste, flavour, nutritional values, and its pronounced bio-functionalities such antimicrobial, antidiabetic, anticholestrolic, antiarthritic, antioxidant, anticancer, and antiviral activities (Torres-Martínez et al. 2022). *P. ostreatus* act as prebiotic to enhance proliferation of gut microorganisms due to varying degree of secondary metabolites like phenolic compounds, terpenoids, and lectins (Törős et al. 2023).

Co-cultivation is a biological process that involved growing two or multiple microorganisms together to take advantage of their synergistic interactions to produce secondary metabolites and to increase the yields of the new metabolites (Rusu et al. 2023). Co-cultivation of suitable microorganisms has helped to identify and

develop new biotechnological substances for the production of enzymes, food additives, preservatives, antimicrobial substances, and microbial fuel cells (Bader et al. 2010; Peng et al. 2021). Utilization of co-cultures microorganisms appears to be advantageous over a single microorganism because of their potential for synergistic metabolic pathways of involved microbial strains (Canon et al. 2020). In co-cultures, a number of secondary metabolites are produced during co-cultivation hence, new substances of industrial interests have been discovered (Kapoore et al. 2022). Microbial co-cultures produce multiple and functional bio-compounds with antimicrobial activities, which can be used as safe alternative to combat the failure observing in some commercially available antibiotics, reported to be less effective against certain pathogenic microbes. This study therefore, aimed to examine the antimicrobial potentials of crude secondary metabolites secreted by *L. fermentum*, *S. cerevisiae*, *P. ostreatus*, and their co-cultures against pathogenic microorganisms isolated from RTE foods. The bioactive compounds in the metabolites secreted by the microorganisms were further identified using GC-MS.

Materials and methods

Chemicals, reagents, antibiotics, and culture media

Lysozyme and trypsin were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Phenol was gotten from Merck KGaA (Darmstadt, Germany). Bile salt, and NaCl were purchased from Acros Organics (Geel, Belgium, nutrient agar (HiMedia, India), Eosin Methylene Blue (EMB) agar, McConkey Agar (MCA), potato dextrose agar (PDA), yeast extract agar, De Man Rogosa and Sharpe (MRS) agar, the antibiotics of COT - cotrimoxazole 25 µg, CHL- chloramphenicol 30 µg, CFX- ciprofloxacin 10 µg, AMX- amoxicillin 25 µg, AUG- augmentin 30 µg, GEN - gentamycin 10 µg, NIT- nitrofurantoin 20 μg, ERY- erythromycin 10 µg and STR- streptomycin 30 µg were products of Oxoid, Basingstoke Hampshire, UK.

Source of *Pleurotus ostreatus*

Pleurotus ostreatus spawn was collected from the Federal Institute of Industrial Research Oshodi, Lagos. *P. ostreatus* was sub-cultured on PDA, and incubated at 25ºC for 5–6 days to maintain a pure strain of fungus mycelia.

Isolation of lactic acid bacteria from yoghurt

Ten local yoghurts were collected from different shops in Akure metropolis. One milliliter of the sample was homogenized with 10 mL of sterile water. Serial dilution was made up to 10^{-5}. Aliquot (0.1 µL) from (10^{-4} and 10^{-5}) was inoculated on MRS agar. Inoculated plates were then incubated at 37°C for 48–72 h in an anaerobic jar. Bacterial strains were identified after biochemical tests using Bergey's manual of determinative bacteriology (Holt et al. 1994). The probiotic characteristics of LAB were assessed by subjecting the isolates to different tolerance and tests like pH, bile salt, NaCl, haemolytic ability, lysozyme, phenol, hydrophobic and hemolytic activities using the methods described by Jose et al. (2015) and Reuben et al. (2019). One of the best probiotics- *L. fermentum* was selected and further characterized using molecular tools. The molecular characterization of *L. fermentum* isolated from yoghurt was done by polymerase chain reaction (PCR) to amplify the 16SrRNA gene of the bacterium using the forward primer 27F 5'AGA GTT TGA TCM TGG CTC AG3' and reverse primer 1492R-5' TAC GGY TAC CTT GTT ACG ACT T 3'. PCR products of 16 S rRNA amplification was sequenced in the GenBank database and strain was identified using nucleotide BLASTN search tool at http://www.ncbi.nlm.nih.gov/ BLAST. The sequenced gene in Gen bank revealed the isolates as *L. fermentum* CIP 102,980 with accession number NR 104927.1.

Isolation of *Saccharomyces cerevisiae* from palm wine

Palm wine was collected in sterile bottles from palm wine tappers in Akure metropolis. Serial dilution was made up to 10^{-5} dilution factor. About 0.1 mL from the corresponding dilutions (10^{-4} and 10^{-5}) were inoculated onto yeast extract agar. Inoculated plates were incubated at 37 °C for 72 h. *S. cerevisiae* strains were confirmed in accordance with standard methods described by Sharif et al. (2020). Pathogenicity tests like gelatin liquefaction, haemolysis, and DNase were carried out according to Olutiola et al. (1991). The isolate cannot produce gelatinase, DNase, and no haemolytic observed. The stress tolerance of yeast to temperature, sodium chloride, lysozyme, trypsin, pH, Bile salt, and probiotic activity were assessed using methods described by Kim et al. (2004) and Fakruddin et al. (2013). *S. cerevisiae* strain that portrayed the attributes of probiotic was selected for further study.

Microbiological analysis of ready-to-eat foods

A total of 100 RTE foods namely; fried rice, cooked beans, dough nut, sausage roll, and other hawk ready to eat foods were obtained from Akure metropolis to isolate food pathogens including bacteria and fungi. One gram of each food sample was mixed with 10 mL to prepare stock. Stock (1.0 mL) was diluted with 9 mL of sterile distilled water until 10^{-5} dilutions factor was obtained. Exactly 0.1 mL aliquot portions of the dilutions were spread onto triplicate sterile plates of nutrient agar, EMB MCA, and PDA. The plates were incubated at 37 °C for 24 h and 48 h for bacteria and fungi. After the incubation time, the different culture plates were examined for microbial growth. Colonies were sub cultured to obtained pure cultures, which stored on nutrient agar slants at 4ºC for further studies. The isolates were confirmed based on

cultural characteristics and biochemical tests in accordance to methods of Cappuccino and Sherman (1999) and Cheesbrough (2006). Bacteria were identified using Bergey's Manual of Determinative Bacteriology (Holt et al. 1994). The screened fungi from the food samples were identified based on taxonomic schemes and descriptions as described by Ainsworth et al. (1973).

Antibiotic sensitivity test

Antibiotic susceptibility tests were performed on the isolates using agar disc method as described by Clinical Laboratory Standard Institute (CLSI, 2017). The antibiotic disc was aseptically placed on the molten Mueller Hinton agar and allowed for 30 min to pre-diffuse. These plates were incubated for 18–24 h at 37ºC. Thereafter, the diameter of zones of inhibition were recorded and the results were interpreted using according the standard interpretative charts recommended by the Clinical Laboratory Science Institute (CLSI, 2017).

Metabolites from microorganisms and their co-cultures

The co-culture methods of Vinale et al. (2017) and Sun et al. (2021) was adopted with little modification. Briefly, *L. fermentum* was grown in MRS broth (Oxoid, UK) at 30ºC for 48 h, *S. cerevisiae* was grown in yeast malt broth at 25ºC for 48 h and *P. ostreatus* was grown in PD broth at 26±2ºC for 48 h. The co-culture of microorganisms was carried out by inoculating the two or three microorganisms (100 µL) of each in 250 mL Erlenmeyer flasks containing their growth media (50% v/v/v) and incubated in a shaker at 160 rpm at 27±1 °C, maintained for 7 days. The supernatant was obtained by centrifuging the mono or mixed cultures at 5,000 g for 10 min at -4 °C (HC-16 F, Jiangsu, China), and then subjected to extraction using ethyl acetate for 4 h. The mixture was membrane-filtered using a 0.22 µm Millipore filter, and the filtrate obtained was concentrated under reduced pressure.

Antimicrobial activity of metabolites from microorganisms and their co-cultures

The antimicrobial activities of metabolites from *L. fermentum*, *S. cerevisiae*, *P. ostreatus*, and their co-cultures were tested against multiple antibiotics resistant microorganisms isolated from ready-to-eat foods using agar well diffusion method (Cheesbrough 2006). The pathogenic microorganisms (indicators) were activated in Mueller Hinton broth and incubated at 37 ºC for 18–24 h. The inoculum size was adjusted to McFarland standardized. Inoculum (50 µL) was spread on the surface of prepared Mueller Hinton agar plates using a glass spreader. Well of 6 mm in diameter were made per plate. The plates were incubated at 37 °C for 24 h for bacteria, while fungi were cultured on PDA and incubated at 25 °C for 48–72 h. Standard antimicrobial agent; 100 µg/mL of ciprofloxacin and ketoconazole were used as positive reference for the bacterial and fungal isolates, respectively. Antimicrobial activity was determined by measuring the diameter of inhibition zone around each well.

Gas chromatography - mass spectrometry elucidation of bioactive compounds in metabolites

The bioactive compounds in the metabolites from mono and co-cultured *L. fermentum*, *S. cerevisiae* and *P. ostreatus* mycelia were detected using Gas chromatography - mass spectrometry (GC-MS) by adopting methods of Shaker et al. (2022) with little modification. A varian 3800 gas chromatograph equipped with a Agilent MS capillary column (30 m × 0.25 mm i.d.) connected to a Varian 4000 mass spectrometer operating in the EI mode (70 eV; m/z 1–1000; source temperature 230 ºC and a quadruple temperature 150 °C) was used for the GC-MS analysis. The column temperature was initially maintained at 200 °C for 2 min, increased to 300 °C at 4 °C/min, and maintained for 20 min at 300 °C. The carrier gas was Nitrogen at a flow rate of 1.0 mL/min. The inlet temperature was maintained at 300 °C with a split ratio of 50:1. A sample volume of 1µL in chloroform was injected using a split mode, with the split ratio of 50:1. The mass spectrometer was set to scan in the range of m/z 1-1000 with electron impact (EI) mode of ionization. Runtime was 70 min using computer searches on a National Institute Standard and Technology (NIST) 14.0 library NIST Ver.2.1 MS data library and comparing the spectrum obtained through GC–MS compounds.

Statistical analysis

All experiment was performed in triplicate. The zones of inhibition were calculated using one-way analysis of variance and standard error±mean was used for comparison of means. Duncan Multiple range test was used to detect significant differences, set at the 5% level.

Results

Percentage resistance of isolates from RTE foods against common antibiotics

Table 1 shows the resistance patterns of isolated bacteria from RTE foods to antibiotics. About 80–90% of *Staphylococcus aureus* were resistant to nitrofurantoin, and erythromycin. *Escherichia coli* showed resistance of 33.3 to 100% against tested antibiotics. At least 46.2% of *Salmonella enterica* showed resistance to one or more antibiotics. *Shigella dysenteriae* showed resistance percent of 25 to 100% to all tested antibiotics. The percentage resistance of *Pseudomonas fluorescens* to more than one antibiotic was within 28. 6 to 100%.

Table 1 Percentage resistance (%) of bacteria isolated from RTE foods against commercial antibiotics

Microorganisms	*N*	COT	CHL	CFX	AMX	AUG	GEN	NIT	ERY	STR
Staphylococcus aureus	20	(14)70	(12)60	(5)16.7	(10)50	(15)75	(6)30	(18)90	(16)80	(8)40
Escherichia coli	15	(10)66.7	(6)40	(3)20	(10)66.7	(11)73.3	(10)66.7	(5)33.3	(12)80	(15)100
Salmonella enterica	13	(10)76.9	(10)76.9	(13)100	(9)69.2	(6)46.2	(5)38.5	(8)61.5	(12)92.3	(8)61.5
Shigella dysenteriae	8	(8)100	(6)75	(4)50	(2)25	(3)37.5	(5)62.5	(6)75	(6)75	(2)25
Pseudomonas fluorescens	7	(5)71.4	(3)42.9	(7)100	(2)28.6	(5)85.7	(3)42.9	(6)85.7	(7)100	(4)57.1
Klebsiella pneumoniae	6	(2)33.3	(4)66.7	(2)33.3	(3)50	(4)66.7	(2)33.3	(3)50	(5)83.3	(4)66.7
Bacillus cereus	5	(3)60	(5)100	(2)40	(0)0	(4)80	(3)60	(4)80	(3)60	(4)80
Listeria monocytogens	2	(2)100	(2)100	(1)50	(2)100	(1)50	(1)50	(2)100	(2)100	(1)50

N- Number of isolates, Antibiotic codes are defined under materials and methods, Digits inside and outside parenthesis are number of resistance isolates and their percentage resistance, respectively

Table 2 Zones of inhibition (mm) by microbial metabolites against foodborne pathogens

Organisms	L. fermentum	*P.* ostreatus	S. cerevisiae	L. fermentum + S. cerevisiae	L. fermentum + *P.* ostreatus	*P.* ostreatus + S. cerevisiae	L. fermentum + *P.* ostreatus + S. cerevisiae	Ciprofloxacin	Fluconazole
Salmonella enterica	11.70 ± 0.43^{a}	12.30 ± 0.03^{bc}	12.70 ± 0.13^{b}	15.00 ± 1.02^{a}	16.70 ± 1.03^{a}	13.70 ± 0.04^{e}	19.80 ± 2.10^{c}	22.30 ± 1.80^{c}	
Shigella dysenteriae	8.80 ± 0.00^{c}	10.70 ± 0.00^{d}	9.30 ± 0.00^{d}	10.10 ± 0.00^{c}	11.40 ± 0.60^{d}	13.30 ± 0.81^{e}	14.70 ± 1.11^{d}	21.30 ± 1.30^{b}	
Escherichia coli	9.00 ± 0.20^{c}	14.30 ± 0.53^{a}	11.00 ± 0.00^{bc}	14.30 ± 0.10^{a}	15.00 ± 0.00^{ab}	21.10 ± 1.45^{a}	23.70 ± 2.40^{a}	$e21.40 \pm 2.00^{b}$	
Klebsiella pneumoniae	9.00 ± 0.20^{c}	10.50 ± 0.00^{d}	13.00 ± 0.53^{a}	14.70 ± 0.00^{a}	12.70 ± 0.38^{c}	13.40 ± 0.31^{e}	15.00 ± 1.70^{de}	18.10 ± 1.13^{a}	
Pseudomonas fluorescens	9.30 ± 0.33^{c}	12.40 ± 0.20^{bc}	10.00 ± 0.00^{d}	15.00 ± 0.20^{a}	11.70 ± 0.00^{d}	15.70 ± 0.73^{d}	15.10 ± 0.90^{de}	24.10 ± 1.00^{c}	
Bacillus cereus	10.00 ± 0.00^{b}	11.30 ± 0.11^{c}	10.20 ± 0.30^{d}	14.30 ± 1.01^{a}	16.30 ± 0.33^{a}	17.10 ± 0.90^{c}	18.70 ± 0.55^{c}	24.10 ± 0.80^{c}	
Listeria monocytogens	9.00 ± 0.63^{c}	9.00 ± 0.20^{e}	10.00 ± 0.00^{d}	10.00 ± 0.00^{c}	13.70 ± 0.33^{c}	18.30 ± 1.50^{bc}	16.30 ± 0.49^{cd}	22.30 ± 0.81^{b}	
Staphylococcus aureus	10.00 ± 0.13^{b}	13.00 ± 0.13^{b}	11.00 ± 0.00^{bc}	15.10 ± 0.00^{a}	15.00 ± 0.00^{ab}	15.70 ± 1.11^{d}	20.30 ± 1.03^{b}	21.70 ± 0.33^{b}	
Fungi									
Penicillium chrysogenum	10.30 ± 0.11^{b}	9.30 ± 0.31^{e}	10.00 ± 0.03^{d}	14.70 ± 0.71^{a}	14.30 ± 0.33^{b}	19.20 ± 2.10^{b}	18.30 ± 0.03^{c}		21.30 ± 0.03^{a}
Aspergillus flavus	9.10 ± 0.10^{c}	9.10 ± 0.30^{e}	9.30 ± 0.80^{d}	12.00 ± 0.20^{b}	13.00 ± 0.00^{c}	16.00 ± 0.33^{c}	16.00 ± 0.50^{cd}		22.00 ± 0.40^{a}
Aspergillus niger	10.00 ± 0.00^{b}	12.00 ± 0.30^{bc}	13.00 ± 1.00^{a}	11.00 ± 0.00^{c}	15.30 ± 0.00^{ab}	20.00 ± 1.10^{b}	14.80 ± 0.63^{e}		21.70 ± 0.50^{a}

Data are represented as mean ± standard error ($n=3$). Values with the same superscript letter(s) along the same column are not significantly different ($p \leq 0.05$)

Antimicrobial effects of metabolites obtained from microorganisms and their co-cultures

Table 2 depicts zones of inhibition by metabolites from microorganisms against indicator microorganisms. The zones of inhibition observed reflect the antimicrobial effect of metabolites from monoculture and co-culture of *L. fermentum*, *P. ostreatus*, and *S. cerevisiae.* The metabolites have varying zones of inhibition against all the pathogenic microorganisms. The metabolites obtained from the co-culture of *L. fermentum*, *P. ostreatus*, and *S. cerevisiae* displayed zones of inhibition against all the microorganisms with 8.80 to 11.70 mm, 9.00 to 14.30 mm, and 9.30 to 13.00 mm, respectively. The metabolites from *L. fermentum*+*S. cerevisiae* have similar ($P=0.05$) zones of inhibition that are within 14.30 mm to 15.00 mm against *Salmonella enterica*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas fluorescens Bacillus cereus*, *Staphylococcus aureus*, and *Penicillium chrysogenum*. Metabolites from *L. fermentum*+*P. ostreatus* exhibited similar ($p=0.05$) zones of inhibition against *Listeria*

monocytogens, *B. cereus*, and *S. enterica*. The metabolites obtained from the co-culture of *P. ostreatus*+*S. cerevisiae* as well as *L. fermentum*+*P. ostreatus*+*S. cerevisiae* showed the highest inhibitory zone of 21.10 mm, and 23.70 mm against *E. coli*. The Findings of Serna-Cock et al. (2019) reiterated that, crude extracts of metabolites from co-cultures of *Lactobacillus brevis*, *Lactobacillus plantarum*, and *Weissella cibaria* demonstrated pronounced antimicrobial activity against *L. monocytogenes*.

Bioactive compounds in the metabolites of *Lactobacillus fermentum*, *Saccharomyces cerevisiae*, *Pleurotus ostreatus* and their co-cultures

The GC-MS chromatograms elucidation of bio-compounds in metabolites obtained from monoculture of *L. fermentum*, *P. ostreatus* or *S. cerevisiae* respectively showed seventeen (17) peaks, fifteen (15) peaks, and thirteen (13) peaks as shown in (Fig. S1 to S3 see supplementary file). Fig. S4 and S5 in supplementary file show GC-MS chromatograms of bioactive compounds in metabolites from co-culture of *P. ostreatus*+*S. cerevisiae* with twenty-two (22) peaks and co-culture of *L. fermentum*+*P. ostreatus*+*S. cerevisiae*, having twenty-seven (27) peaks. The bioactive constituents in the metabolites of mono-cultured *L. fermentum* are shown in Table S1. Some of the bioactive compounds (%) are 3-phenyllactic acid (30.13), methyl caprate (13.00), n-heptadecoic acid (8.64), E-2-Tetradecen-1-ol (4.97), 3,11-tetradecadien-1-ol (8.25), 11,14,17-Eicosatrienoic acid (4.02), palmitic acid (3.55), 1,3-butylene diacetate (3.50), 13-tetradece-11-yn-1-ol (1.71), 2-hexadecanoyl glycerol (3.32), and supraene (2.41). The bio-compounds in the metabolites of monocultured *P. ostreatus* are shown in Table S2. The most prevailing bioactive compound is linolelaidic acid methyl ester with peak area of 15.63%, followed by beta-hydroxyethyl methacrylate (12.80%), methyl elaidate (12.13%), 2-isopentyloxirane (12.08%), palmitic acid, desipramine (8.82%), methyl ester (5.78%) and others. The bioactive chemical constituents in the secondary metabolites of monocultured *S. cerevisiae* are shown in Table S3. 4-O-methylmannose was predominant compound with peak area 53.74%. Other bioactive compounds includes; neopentyl acetate (33.95%), squalene (2.71%), beta-linalool (1.66%), alpha-limonene diepoxide (2.04%), and others. The bioactive constituents in the metabolites from co-cultured *P. ostreatus*+*S. cerevisiae* are shown in Table S4. The bioactive ingredient in metabolites are alpha-linolenic acid having the highest peak area of 25.71%, while trans-squalene is 12.39%. otjer bioactive compounds are pentadecylic acid (11.68%), phytol (8.68%), stearic acid (7.06%), E-2-tetradecen-1-ol (5.62%), 9,12,15-octadecatrien-1-ol (5.20%), margaric acid (3.90%) and others.

Table S5 shows the bioactive compounds in the metabolites obtained from co-cultured *L. fermentum*+*P. ostreatus*+*S. cerevisiae*. GCMS identified the presence of acetic acid 3-methylbutyl ester (13.83%), alpha-methyl mannofuranoside (12.75%), caproic acid (10.l5%), trans-squalene (3.69%), 8-hydroxylinalool (5.37%), alpha-citrylidene ethanol (4.39%), isogeraniol (3.18%), isovaleric acid (2.02%) and others (Table S5).

Discussion

The RTE foods contain strains of multiple antibiotic microorganisms. The findings of Bantawa et al. (2019) revealed the antibiotic resistance pattern of *Staphylococcus aureus*, *Escherichia coli*, *Salmonella*, *Shigella* and *Vibrio* sp. isolated from animal foods to amoxicillin, tetracycline, chloramphenicol and nalidixic acid, cefotaxime with values ranging from 11 to 100%. In a similar manner, the findings of Karikari et al. (2022) indicated that *E. coli* and *Salmonella* sp. isolated from ready-to eat food displayed multiple antibiotic resistance as a result of extended-spectrum β-lactamase. Most of the ready to eat foods contain resistance microorganisms. Ogidi et al. (2016) revealed that, microorganisms isolated from ready-to-eat foods showed significant resistance to commonly used antibiotics; amoxicillin, chloramphenicol, ciprofloxacin, cotrimoxazole, gentamicin, tetracycline, nalidixic acid, and streptomycin. Rajaei et al. (2021) revealed the phenotypic and genotypic of antibiotic resistance bacteria isolated from raw kebab and hamburger. The exposure of microorganisms in foods to antibiotics as well as co-existence of non-resistance bacteria with multiple resistance bacteria may increase the emergence of resistance bacteria. Reservoirs of antibiotic-resistant bacteria coexist with resistance genes; mobile elements such as plasmids, transposons and integrons, which are able to transfer between bacterial cells, promote the acquisition and spread of resistance genes (Munita and Arias 2016).

The production of secondary metabolites by novel microbes-derived natural products is a promising strategy to curb antibiotic resistance. Larger number of microorganisms can be co-cultivated to produce numerous natural products with structural diversities and significant bioactivities (Boruta 2021; Caudal et al. 2022). *L. fermentum*, *Lactobacillus planetarium*, *Lactobacillus acidophilus*, *Lactobacillus reuteri*, *Lactobacillus rhaminous*, *Lactobacillus licheniformis*, *Streptococcus thermophiles*, *Lactococcus lactis*, *Bifidobacterium* spp., *S. cerevisiae* are beneficial probiotics that can inhibit pathogen microorganisms with various antimicrobial substances and with other therapeutic potentials and thus, useful in pharmaceuticals and food industries (Zommiti et al. 2020). *Saccharomyces* strains present a inhibitory effect against many gastrointestinal pathogens such as *Salmonella typhimurium*, *Shigella flexneri*, enteropathogenic and enterohaemorrhagic *Escherichia coli* strains, *Vibrio cholerae*, *Rotavirus*, *Helicobacter pylori*, *C. albicans* to treat

relapses of *Clostridium difficile* infection (Sen and Mansell 2020; Ryabtseva et al. 2023). *Pleurotus* species has a broad spectrum of antibacterial activity against *E. coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *B. subtilis*, and *Streptococcus faecalis* with varying degrees of inhibitory zones (Gashaw et al. 2020. Studies of Fadahunsi and Olubodun (2021) disclosed the antagonistic potentials of *S. cerevisiae* and other yeasts against food-borne pathogens namely; *Salmonella* sp., *Campylobacter jejuni*, *Listeria monocytogenes* and *Vibrio cholera.* The findings of Roussel et al. (2018) revealed that, probiotic yeast; *S. cerevisiae* CNCM I-3856 exercised an anti-infectious activity against a human enterotoxigenic *Escherichia coli* strain through multi-targeted approaches like inhibition of bacterial growth, toxin production, reduction of bacterial adhesion to mucins and intestinal epithelial cells, and suppression of ETEC-induced inflammation. The antibacterial and antifungal capabilities of *S. cerevisiae* against *B. subtilis*, *S. aureus*, *E. faecalis*, *S. typhi*, *S. flexneri*, *K. pneumonia*, *P. vulgaris*, *E. coli*, *V. cholera*, *P. aeruginosa*, *Aspergillus* spp., *P. chrysogenum* and *R. oryzae* was reported in the findings of Fakruddin et al. (2017). *S. cerevisiae* exhibited antimicrobial activity against diarrhea, acute and chronic gastrointestinal diseases causing microorganisms, act as a potential biotherapeutic agent for the treatment of diarrhea and colitis, modulate immune responses against acute necrotizing pancreatitis, giardiasis, autoimmune encephalomyelitis, and Crohn's disease (Abid et al. 2022). The antibacterial mechanisms of probiotics are due to their interdependence with pathogens, production of biosurfactants that inhibit pathogen adherence, decreased pH level, competition for nutrients, production of hydrogen peroxide (H_2O_2), lactic acid, diacetyl and small heat-stable inhibitory peptides (bacteriocins), which has led to the inhibition of several pathogens and thus, make them importance for various biotechnological applications (Bishnoi et al. 2012; Syal and Vohra 2013; Fakruddin et al. 2017). The secondary metabolites secreted by these microorganisms can be bactericidal or bacteriostatic substances, which need to be exploited as a strategy to mitigate multiple antibiotics resistant pathogens. Diversities of bacteria and yeast strains secret immense number of antimicrobials secondary metabolites that can be developed into a new, promising, cost-effective and medicinal benefits products (Mullis et al. 2019).

LAB are capable of producing organic acids, bacteriocins, and other secondary metabolites. The findings of Gabriel et al. (2021) revealed that *L. plantarum* secreted plantaricin, benzeneacetic, and 3-phenyllactic acids and were identified by GCMS. *L. fermentum* produced 3-phenyllactic [2-Hydroxy-3-Phenyl propionic acid (PLA)], which is an organic acid produced by some microorganisms. It is a regular metabolite LAB and is used in the food industry. PLA is known to have antimicrobial activity against fungi and bacteria (Liu et al. 2017; Ning et al. 2017) and could be used as natural bio-control agent to extend the shelf-life of foods (Schnürer and Magnusson 2005; Schwenninger et al. 2008). Metabolite of mono-cultured *P. ostreatus* contain linolelaidic acid methyl ester, its byproducts are known to employ different biological effects like regulators of macrophage differentiation and atherogenesis anticancer activity (Vangaveti et al. 2010; Yuan et al. 2010; Tavakoli et al. 2013). Hamad et al. (2022) revealed antimicrobial, cytotoxic, immunomodulatory, and antioxidant activities of *P. ostreatus*. The researchers attributed the bioactivities to secondary metabolites like ethyl iso-allocholate, 3(2 H)-furanone, dihydro-2,2-dimethyl-5-phenyl (11.23%), amphetamine, acetic acid, [(benzoyl amino)oxy] or Benzadox, 7,8-epoxylanostan-11-ol,3-acetoxy, toosendanin, flavone 4′-OH,5-OH,7-DI-O-Glucoside, 1,3,2-dioxaborolane,2,4,diethyl, benzaldehyde, 4-(dimethylamino), pentacosan, tetraacetyl-D-xylonic nitrile, hexadecane, 2-butenoic acid,2-methyl-2(acetyloxy)-1,1a,2,3,4,6,7,10,11,11a-decahydro-7,1,2-hexadecanol, and phytophylene identified by GCMS.

Metabolites from monoculture of *S. cerevisiae* contained 4-O-methylmannose, which has been proven to be a secondary messenger important in human metabolism, especially in the glycosylation of certain proteins (Freeze and Sharma 2010; Giordano et al. 2011). The bioactive constituents present in the metabolites of co-cultured *P. ostreatus* and *S. cerevisiae* show the presence of alpha-linolenic acid (ALA), which has been proven to contribute to antimicrobial activity of some bacteria, fungi and the green algae against pathogenic microorganisms (McGaw et al. 2002; Ogidi et al. 2015). The bioactive compounds in the metabolites of co-cultured *L. fermentum*+*P. ostreatus*+*S. cerevisiae* showed acetic acid, 3-methylbutyl ester and others. Sun et al. (2021) revealed A total of 25 newly biosynthesized metabolites from the co-culture of *Aspergillus sydowii* and *Bacillus subtilis*. The findings of Hamed et al. (2024) revealed that co-culture strategy of *Aspergillus* sp. CO2 and *Bacillus* sp. COBZ21 increase their antimicrobial activity against *Escherichia coli* ATCC 25,922, *Staphylococcus aureus* NRRLB-767, and *Candida albicans* ATCC 10,231. Microorganisms remain the alternative and common sources of natural products for production of modern drug molecules. Metabolites from co-cultured microorganisms can be exploited and used for the treatment of many diseases and illnesses.

Conclusion

Ready-to-eat foods serve as potential vehicles of clinically relevant foodborne pathogens with multiple antibiotic resistance. The foodborne diseases caused by antibiotic-resistant microorganisms among different countries is not yet adequately controlled and thus, led to different health challenges. In this study, array of foodborne pathogens were isolated from RTE foods, the

occurrence of such microorganisms in RTE and their multidrug resistant required urgent attentions. Hence, antagonistic activity of mono and co-culture of *L. fermentum*, *S. cerevisiae*, and *P. ostreatus* was established against the pathogenic bacteria and fungi isolated from RTE foods. The metabolites from monoculture, and co-culture of *L. fermentum*, *P. ostreatus*, and *S. cerevisiae* inhibited the growth of indicator microorganisms from RTE foods due to potent secondary metabolites. The bioactive compounds identified with GC-MS in metabolites from mono and co-culture *L. fermentum*, *S. cerevisiae*, and *P. ostreatus* can be harnessed for the production of novel antimicrobial agents, which will reduce the emergence of multidrug resistance by microorganisms. The study revealed that the co-culture microorganisms could produce new bioactive compounds and increase the yield of existing metabolites. Co-culture of two or more microbes will yield unique advantages over the failure of commercially available antibiotics and to curb the multiple antibiotic-resistant displayed microorganisms. The metabolites secreted by studied *L. fermentum*, *S. cerevisiae*, and *P. ostreatus* can be further exploited as natural preservatives. Further study would reveals the uses of the microbial metabolites as preventive agents to minimize spoilage of foods.

Supplementary Information

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Supplementary Material 1

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Author contributions
OCO and BJA conceived and designed the research work. OEB, OCO, BJA carried out the experimental study. OEB and OCO analysed data. OCO and BJA supervised the work. OCO drafted the manuscript. All authors revised the manuscript. Authors read and approved the final manuscript.

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The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Ethics approval and consent to participate
Not applicable.

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Competing interests
None.

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