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Original Article

Isolation of facultative anaerobic bacterial pathogens from canned food and use of *Lactobacillus plantarum* as a bio-control agent

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ABSTRACT

Preserved foods can play a significant role in causing food poisoning when they are not handled, processed, or stored properly. **Objective:** To investigate facultative anaerobic foodborne bacterial pathogens from canned foods and to control their growth *Lactobacillus plantarum* was used as a bio-control agent. **Methods:** Different canned food samples were collected to isolate and identify facultative anaerobic bacterial pathogens. **Results:** Out of n=65 samples, n=13 samples cultured positive as facultative anaerobes. They were further confirmed with biochemical and molecular identifications as foodborne bacterial pathogens with a ratio of 62% *Escherichia coli*, 30% *Salmonella typhimurium*, and 8% *Vibrio cholerae*. During bio-control studies, the results revealed possible inhibition of facultative anaerobic bacterial pathogens by using purified compounds of *Lactobacillus plantarum*. **Conclusions:** The use of probiotics in canned foods requires careful consideration, as factors such as the specific strain, food matrix, processing conditions, and storage practices can influence its effectiveness.

INTRODUCTION

Preserved foods can be a potential source of food poisoning if they are not handled, processed, or stored properly. Several factors contribute to the risk of food poisoning from preserved foods like inadequate preservation methods, if foods are not preserved correctly through methods such as canning, pickling, fermenting, drying, or freezing, harmful microorganisms can survive and multiply. This can lead to food spoilage and an increased risk of foodborne illnesses [1]. During the preservation process, if the equipment, utensils, or water used are contaminated with harmful bacteria, viruses, or toxins, they can transfer to the food and cause food poisoning. Preserved foods must be stored at appropriate temperatures to maintain their quality and safety. If they are stored at temperatures that are too high or too low, bacteria can grow and produce toxins, leading to food poisoning when the food is consumed [2]. Heat treatment is a common preservation method used in canning and pasteurization to kill harmful microorganisms. If the food is not heated to the appropriate temperature or for the required duration, some bacteria may survive, leading to potential food poisoning when the food is consumed. Preserved foods can become contaminated with harmful microorganisms if they come into contact with raw or

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contaminated ingredients during preparation, handling, or storage [3]. While some microorganisms cause food spoilage and do not pose significant health risks, their presence can indicate improper preservation and handling practices, raising concerns about potential contamination with harmful pathogens. Low-acid canned foods (those with a pH greater than 4.6) must be heated to higher temperatures to destroy spores of *Clostridium botulinum*, a bacterium that produces botulinum toxin, which can cause botulism, a severe form of food poisoning. It is essential to follow proper food preservation techniques, adhere to food safety guidelines, and pay attention to storage conditions to reduce the risk of food poisoning from preserved foods [4]. Different bacterial infections caused by preserved foods are a type of foodborne illness that occurs when preserved or canned foods become contaminated with harmful bacteria, leading to infection when consumed. Properly preserved foods should be safe to eat, but if the preservation process is inadequate, or if there is post-processing contamination, harmful bacteria can survive and grow, posing a risk to consumers. Botulism is a severe and potentially life-threatening bacterial infection caused by the bacterium *Clostridium botulinum*. It can occur when low-acid preserved foods, such as improperly canned vegetables, honey, or fermented fish, are not heated to a temperature sufficient to destroy the spores of the bacterium. *C. botulinum* produces a potent neurotoxin that affects the nervous system, leading to symptoms such as blurred vision, difficulty swallowing, muscle weakness, and even paralysis [5]. *Clostridium perfringens* is commonly found in soil and the gastrointestinal tracts of animals and humans. It can cause food poisoning when preserved foods, especially meat and poultry dishes, are improperly cooked or reheated. The bacteria produce toxins that can cause abdominal cramps and diarrhoea. *Bacillus cereus* is found in soil and dust. It can cause food poisoning when contaminated preserved foods, especially rice and other grains, are stored at improper temperatures or for extended periods. *B. cereus* produces toxins that can lead to vomiting and diarrhoea. *Salmonella spp* is a group of bacteria commonly associated with raw or undercooked poultry, eggs, and unpasteurized dairy products. However, they can also contaminate canned or preserved foods if the preservation process is insufficient to kill them. Salmonella infection leads to symptoms such as diarrhea, abdominal cramps, fever, and vomiting [6]. Probiotics play a significant role in controlling foodborne pathogens in the gut by promoting a healthy balance of gut microbiota and providing a protective barrier against harmful bacteria. Probiotics are live microorganisms, primarily beneficial bacteria like *Lactobacillus* and *Bifidobacterium* strains, which, when consumed in adequate amounts, confer health benefits to the host. Probiotics can competitively exclude harmful pathogens by occupying adhesion sites on the intestinal lining [7]. This makes it challenging for pathogenic bacteria to attach and colonize the gut, reducing their ability to cause infections [8]. Many probiotic strains can produce antimicrobial compounds, such as bacteriocins and organic acids, which have the ability to inhibit the growth and activity of pathogenic bacteria in the gut [9]. They can influence the gut environment by producing lactic acid and other organic acids, which create a more acidic pH. This acidic environment is less favorable for the growth and survival of certain pathogenic bacteria. Probiotics help improve the integrity of the intestinal barrier, reducing its permeability and preventing the translocation of harmful pathogens from the gut into the bloodstream [10]. They can stimulate the gut-associated lymphoid tissue, promoting the production of antibodies and other immune factors. This enhances the body's immune response and helps to combat and eliminate foodborne pathogens. Some probiotic strains produce bacteriocins, which are proteinaceous compounds that target specific pathogens and inhibit their growth [11]. They can influence gut motility, reducing the time harmful bacteria spend in the gut, which limits their opportunity to cause infections. Probiotics have anti-inflammatory properties and can help reduce gut inflammation caused by pathogenic infections, promoting faster recovery.

METHODS

Samples of different canned foods were collected from the supermarkets of Haripur city, K.P.K, Pakistan. About 65 samples of canned food which included 5 Chicken peas, 2 Sweet corn, 5 White kidney beans, 2 Baked beans, 8 Green peas, 6 Coconut milk, 4 Baby corn, 2 Light meat tuma flakes in water, 3 Foul madammas, 3 Golden sweet whole kernel corn, 9 Fruit cocktail, 6 Pineapple, 2 Plain green olives, 2 Pitted black olives, and 6 Tomato paste. To ensure sterility, all of the equipment were autoclaved, and the samples were cultured in relevant media by providing relevant conditions to isolate the appropriate bacteria. Different types of biochemical tests and gram microscopy were performed to initial screening and to confirm facultative anaerobic foodborne bacterial strains further processed for molecular identifications. Bacterial DNA extraction were performed from biochemically identified colonies after that which processed for conventional PCR. During PCR the entire volume of PCR reaction was 25µl in microtube, which included 2 µl of extracted DNA sample, 5 µl of master mix, 1 µl of forward and 1µl of reverse primer in each sample, and 16 µl of deionized water. PCR amplifications were performed by providing required

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conditions to targeting invA gene for identification of *Salmonella typhimurium*, ompW for *V. cholerae* and uspA gene for *E. coli* as shown in Table 1. The amplified PCR product was run on 2% of agarose gel electrophoresis and relevant bends were visualized according to their size under UV trans-illuminator.

Table 1: List of primers used for specie identification targeting specific genes

Species name	Target	Primer Sequence	Size
Salmonella typhimurium	*invA*	***Forward*** *5'- GTG AAA TTA TCG CCA CGT TCG GGC AA -3'*	284 bps
		Reverse *5'-TCA TCG CAC CGT CAA AGG AAC C-3'*	
Vibrio cholerae	*ompW*	***Forward*** *5'-CACCAAGAAGGTGACTTTATTGTG-3'*	588 bps
		Reverse *5'-GAACTTATAACCACCCGCG-3'*	
Escherichia coli	*ompW*	***Forward*** *5'-CACCAAGAAGGTGACTTTATTGTG-3'*	856 bps
		Reverse *5'-GAACTTATAACCACCCGCG-3'*	

Lactobacillus plantarum were used as a bio-control agent to inhibit the growth of identified foodborne facultative bacterial pathogens. *Lactobacillus plantarum* inoculated in MRS broth and incubated anaerobically in the CO_2 incubator at 37℃ for 24 hours. After incubation the suspension were centrifuged at 4000 x g for 10 mints, obtained cell free culture suspension (CFCS) using sterilized syringe filter. Antimicrobial activity of cell free culture suspension (CFCS) of *Lactobacillus plantarum* as bio-control agent were checked by well diffusion method against identified foodborne facultative anaerobic bacterial pathogens. Target bacteria were inoculated on individual MHA plates and wells were created which sealed with 20µl of media agar then each well filled with 100µl CFCS to check their antimicrobial activity against test organism. The test plates were incubated at 37°C and zones of inhibition were observed after 24 hours and measured with scale.

RESULTS

A total of n=65 selected canned food samples were collected and processed, out of the total n=33 selected samples of canned food showed bacterial growth. After applying both aerobic and anaerobic (CO_2 jar) conditions to each bacterial inoculated plate, n=13 selected canned food samples showed growth of facultative anaerobic bacterial pathogens (Figure 1). Culture based identification of total bacterial isolates across selected canned food were confiremd with gram microscopy, biochemical and molecular identification. Facultative anaerobic bacterial pathogens identified and confirmed as n=1*Vibrio cholera* which showed gram negative comma shaped and yellow colonies on TCBS agar plate, n=8 *E.coli* shown gram negative rods with smooth pink colour colonies on MacConkey agar, and n=4 *Salmonella typhimurium* showed gram negative rods with black pigmented colonies on SS agar. All facultative pathogenic isolates were also confirmed by gram microscopy and biochemical identifications shown in Table 2 and molecular based confirmation is shown in Figure 2.

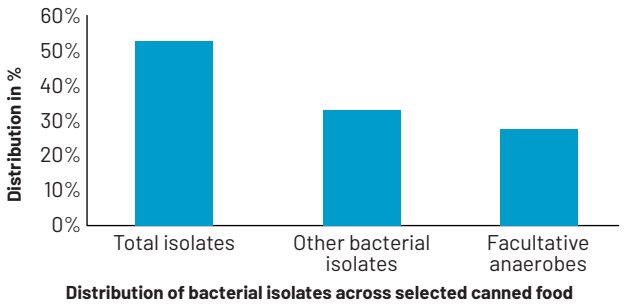

Figure 1: Culture based isolations of facultative anaerobes and other bacteria in selected canned food

Table 2: Interpretations of biochemical test of culture based identified facultative anaerobic bacteria

Sample code	Source	Colony Appearance	Gram Microscopy	Biochemical Test					
				Catalase	TSI	Simmons Citrate	MR	Indole test	Oxidase
S7	Green peas	Black colonies	GNR	+	Black H_2S production	_	+	_	_
S13	Tomato paste	Black colonies	GNR	+	Black H_2S production	_	+	_	_
S18	Fruit cocktail	Black colonies	GNR	+	Black H_2S production	_	+	_	_
S27	Green peas	Black colonies	GNR	+	Black H_2S production	_	+	_	_
S2	Chicken peas	Smooth pink colonies	GNR	+	red to yellow and gas at bottom	_	+	+	_
S4	Coconut milk	Smooth pink colonies	GNR	+	Red to yellow with gas at bottom	_	+	+	_
S5	Chicken peas	Smooth pink colonies	GNR	+	Red to yellow with gas at bottom	_	+	+	_
S6	Tomato paste	Smooth pink colonies	GNR	+	red to yellow with gas at bottom	_	+	+	_
S17	Coconut milk	Smooth pink colonies	GNR	+	red to yellow with gas at bottom	_	+	+	_
S19	Tomato paste	Smooth pink colonies	GNR	+	red to yellow with gas at bottom	_	+	+	_
S21	Green peas	Smooth pink colonies	GNR	+	red to yellow with gas at bottom	_	+	+	_
S24	Fruit cocktail	Smooth pink colonies	GNR	+	red to yellow with gas at bottom	_	+	+	_
S14	Green peas	Yellow colonies	GNR Cocco-bacillus	+	-	+	_	+	+

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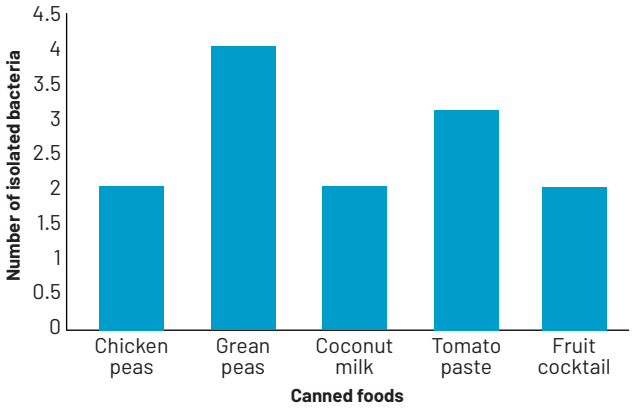

Figure 2: Distribution of identified facultative anaerobic bacterial foodborne pathogens different canned foods

After biochemical identifications n=13 facultative anaerobic foodborne bacterial pathogens were identified at molecular level, n=8 *Escherichia coli* were identified targeting uspA gene yields a product size of 856 bps, n=4 *Salmonella typhimurium* were identified targeting invA gene yields a product size of 284 bps, n=1 *Vibrio cholerae* were identified targeting ompW gene yields a product size of 588 bps as shown in Figure 3. Frequency of identified facultative anaerobic foodborne bacterial pathogens from selected canned food shown in and distribution of identified facultative anaerobic bacterial foodborne pathogens across selected canned food is shown in Figure 4 and Table 3

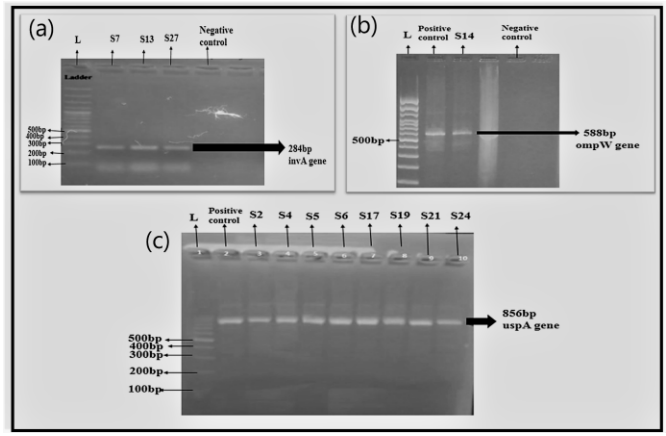

Figure 3: (a) PCR result of *Salmonella typhimurium* targeting invA gene, (b) *Vibrio cholerae* targeting ompW gene, (c) *Escherichia coli* targeting uspA gene

Table 3: Isolated foodborne facultative anaerobic bacterial strains targeting specific gene primers

Isolation source	Gene Name			Species Identified	Product size
	invA gene	ompW gene	uspA gene		
Chicken peas	-	-	+	Escherichia coli	856bps
Coconut milk	-	-	+	Escherichia coli	856bps
Chicken peas	-	-	+	Escherichia coli	856bps
Tomato paste	-	-	+	Escherichia coli	856bps
Coconut milk	-	-	+	Escherichia coli	856bps
Tomato paste	-	-	+	Escherichia coli	856bps
Green peas	-	-	+	Escherichia coli	856bps
Fruit cocktail	-	-	+	Escherichia coli	856bps
Green peas	+	-	-	Salmonella typhimurium	284bps
Tomato paste	+	-	-	Salmonella typhimurium	284bps
Fruit cocktail	+	-	-	Salmonella typhimurium	284bps
Green peas	+	-	-	Salmonella typhimurium	284bps
Green peas	-	+	-	Vibrio cholerae	588bps

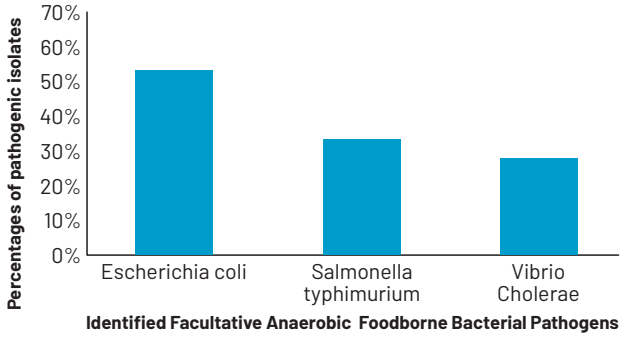

Figure 4: Frequency of molecular based identified facultative anaerobic foodborne bacterial pathogens from selected canned food

During biocontrol study by using *Lactobacillus plantarum* extracted cell free culture suspension showed inhibition activity against canned foods isolated facultative anaerobic bacterial pathogens Figure 5. *Lactobacillus plantarum* is a probiotic strain of lactic acid bacteria naturally appearing in human gut, which can modulate the immune system. Its immunomodulating properties are observed among others in decreasing the level of anti-inflammatory cytokines and it also inhibit the growth.

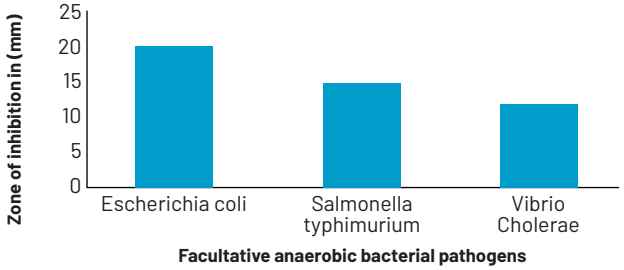

Figure 5: Biocontrol activity of Lactobacillus plantarum against identified facultative anaerobic foodborne bacterial pathogens in selected canned food

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DISCUSSION

The presence of foodborne pathogenic organisms in canned foods is a matter of significant concern due to the potential health risks they pose to consumers. Preserved food, when they processed and sealed correctly, can have a long shelf life and be a convenient source of nutrition. However, if any pathogenic microorganisms survive the canning process or contaminate the food during or after canning, they can multiply and cause foodborne illnesses when consumed. These illnesses can range from mild gastrointestinal discomfort to severe and life-threatening conditions. Vulnerable populations, such as young children, pregnant women, elderly individuals, and individuals with weakened immune systems, are at higher risk of developing severe complications from foodborne infections. Canned foods are designed to have a long shelf life without the need for refrigeration. If pathogenic organisms survive the canning process, they can multiply over time in the sealed environment and lead to illness when the food is eventually consumed. Unlike with fresh foods, consumers cannot rely on visual cues like spoilage or mold growth to identify the presence of harmful microorganisms in canned products. As a result, consumers may unknowingly consume contaminated food. During the study isolated strains resulted in the detection of *Escherichia coli, Vibrio cholerae* and *Salmonella typhimurium* from selected canned food by using biochemical and molecular techniques. Similar studies were performed in other research work they had also detected foodborne pathogens from canned food by PCR based techniques [12, 13]. Specific gene primers were used for the identification of foodborne facultative anaerobic bacterial pathogens from selected canned food products by PCR based technique reported that invA gene specifically present in n=30% *Salmonella typhimurium* and yields a product size of 284bp fragments [14, 15]. Whereas uspA genes was specifically present in n=62% *Escherichia coli* strains and yields a product size of 856 base pair fragments for the identification of *Escherichia coli* strains by targeting universal stress protein [16]. OmpW of *V. cholerae* is a 22 kDa outer membrane protein which is conserved, and has been used as a target gene for the detection and identification of *V. cholerae* in food samples [17-19]. Instances of foodborne illness outbreaks associated with canned foods can have serious public health consequences. In response, authorities may issue product recalls, leading to financial losses for manufacturers and a loss of consumer trust in the product. Governments and food safety agencies have established strict regulations and guidelines for the production and processing of canned foods to minimize the risk of contamination. Compliance with these regulations is crucial to ensure consumer safety. To prevent the presence of foodborne pathogenic organisms in canned foods, it is essential to implement proper food safety measures throughout the entire production process, from sourcing raw materials to sealing the cans. This includes maintaining hygienic conditions in processing facilities, using quality ingredients, employing effective heat treatment during canning to destroy pathogens, and conducting regular testing and monitoring for contamination. Consumers should also follow safe handling practices, such as checking for damaged cans or signs of spoilage before consumption in order to control the growth of foodborne pathogens, use of probiotics and prebiotics such as purified compounds of *Lactobacillus plantarum* is a good option as a bio-control agent against foodborne bacterial pathogens [20, 21]. Because they are capable to produce antimicrobial agents, such as bacteriocins, lactic acid and organic acid to inhibit or kill the growth of pathogenic foodborne microorganisms and are categorized as "Generally Regarded as Safe (GRAS)".

CONCLUSIONS

In selected canned food *Escherichia coli*, *Vibrio cholerae* and *Salmonellla typhimurium* had been found prevalently, and cell free suspension of *Lactobacillus plantarum* revealed the results as a good bio-control agent for isolated bacteria.

Authors Contribution

Conceptualization: KM, SAB
Methodology: SAB, HIS
Formal analysis: SB
Writing-review and editing: KM, SAB, SH, SB, HIS

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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