Detection of Microbial Contamination in Fast Food and the Efficacy of Citrus Leaf Extracts

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ABSTRACT

The World Health Organization (WHO) classifies foodborne illnesses as toxic or infectious disorders, with over 200 causal agents identified. A study was conducted on 50 samples of fast-food sandwiches from 10 local markets in Alexandria governorate, including liver, sausage, fries, falafel, and beans. Pathogenic strains of *E. coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, and *Bacillus cereus* were collected from Ain Shams University's Faculty of Agriculture in Cairo, Egypt. Pathogenic strains were isolated from fast food samples using selective media as a traditional method, and multiplex PCR method was used as a method for isolating microbes using genetic techniques. The Experimental Farm of the City of Scientific Research and Technological Applications in New Borg El Arab city, Egypt, was used to gather the leaves of *Citrus limon*, *Citrus sinensis*, and *Citrus unshiu*. In liver sandwich samples, almost 60% of the samples were infected by *Klebsiella pneumonia* or *Salmonella typhimurium* but only 40% of the sample tested positive to *Bacillus cereus*. In sausage sandwiches, *Staphylococcus aureus* and *Klebsiella pneumoniae* were found in 60% of samples, while 70% tested positive for *Bacillus cereus*. In falafel sandwiches, 50% were infected with *Staphylococcus aureus*, while 40% tested positive for *E. coli*, *Bacillus cereus*, and *Salmonella typhimurium*. The aqueous extract of citrus leaves showed outstanding capacity for inhibiting pathogenic microorganisms found in fast food samples, as the Minimum Inhibitory Concentration (MIC) of the tested citrus leaves extract ranged from 1.6 to 3.2 mg/ml against all the tested strains. Lemon leaf extract exhibiting the highest antibacterial activity with 1.6 mg/ml MIC and the highest antioxidant capacity with IC50 equal 47.96(µg/ml).

Key words: Multiplex PCR; detection; antimicrobial control analysis; contaminations; fast food.

INTRODUCTION

Foodborne illnesses can vary in severity ranging from a simple bout of gastroenteritis to potentially fatal neurological, hepatic, and renal disorders. It is estimated that around 30% of the population in high-income countries suffers from foodborne illnesses caused by microorganisms annually. Furthermore, microorganisms account for two-thirds of food-borne disease outbreaks, with roughly 250 different food-borne diseases identified. Animal products and poultry are the primary food sources for a variety of foodborne illnesses (Biswas *et al.,* 2008). Important microorganisms implicated in foodborne illnesses include *Salmonella species, Bacillus cereus, Escherichia coli, Klebsiella pneumonia*, and *Staphylococcus aureus*.

Public health organizations around the world are concern about maintaining food safety because of the increase in meals provided outside the home and the growing demand for ready-to-eat food (Newell *et al.*, 2010). The identification of any or all members of the Enterobacteriaceae as an indicator of food sanitary quality is a focus of attention for food experts. The presence of Enterobacteriaceae in meat an indicator of the presence of other pathogenic bacteria, which posed a concern to public health (Mira, 1989). The initial Enterobacteriaceae on meat is linked to the work surface used for food processing. Additionally, the presence of Enterobacteriaceae indicates that minced beef has been directly or indirectly infected in the intestines. The high total Enterobacteriaceae count seen in all types of beef dinners sold on the street may be caused by bacterial contamination from a variety of sources during inappropriate handling and marketing (Erkmen and Bozoglu, 2016). Fast food and other ready-to-eat meals, sold and prepared in public spaces, serve as the primary source of daily nutrition for millions of people. Uncooked foods, such as salads, are particularly vulnerable to contamination by pathogenic and spoilage microorganisms. Consumers who depend on this type of food are more concerned with its convenience than with its safety and sanitary features, and those who are not trained in proper food handling practices may perceive street food as being prepared in an unhygienic environment (Rane, 2011).

Food made at a cafeteria or similar establishment and consumed immediately is referred to as fast food. Uncooked foods, such as salads, are particularly vulnerable to microbial contamination by pathogenic and spoiling microbes in full restaurants and through vendors (Khater-Dalia *et al.,* 2013). While there is a

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lack of statistics on foodborne illnesses related to street food in many communities, microbiological studies in America, Asia, and Africa have revealed high bacterial counts and a high prevalence of foodborne bacterial pathogens linked to outbreak (Garin *et al.,* 2002).

Egypt, due to its rapidly growing population, is the most food-consuming country in the Arab world. Many restaurants serve traditional Egyptian food and rely heavily on local ingredients; approximately 25% of Egyptian consumers use restaurants or food delivery services (El Gamal *et al.*, 2018).

Traditionally, foodborne pathogens are identified by plating food samples on selective and non-selective media, followed by microbial counting. However, in other cases, culture-based methods cannot detect a living infection when the clinical indications are caused by cellular components (endotoxins) or metabolic products (enzymes, toxins). These methods are also time-consuming, costly, and require an enrichment phase. Consequently, faster molecular nucleic acid-based technologies are gradually taking the place of more traditional methods (Souii *et al.,* 2016). Multiplex PCR techniques, which integrate the improvement and amplification reaction phases into a single experiment, enable faster and more economical food-borne pathogen identification. In fact, multiplex PCR allows for the identification of many unique DNA markers in the same reaction under specific experimental conditions (Fratamico *et al.,* 2011). DNA probes can be tagged with different fluorescent dyes, which is different from a simple PCR process that cannot distinguish between different targets without markers and cross-talk. Here a quantitative PCR method can be used to identify different colors in a reaction mixture independently. For detection, however, spectrographs or independent channels with emission filters and narrow-band stimulation are needed (Zhao *et al.*, 2014).

The emergence of microorganisms resistant to drug therapy is a major public health concern. Many researchers are interested in developing modern antibacterial reagents since antibiotic-resistant bacteria becomes more prevalent and driving up healthcare expenses (Maiti *et al.,* 2014). Every year, a large amount of waste and byproducts are produced by fruits grown for agriculture. Trimming materials and juice production wastes from various industrial nutrition firms were among these wastes (El Gengaihi *et al.*, 2020). Plant extracts can be utilized as flavoring and preservation agents in the food industry. Plant extracts include high levels of phytochemical compounds and secondary metabolites that suppress pathogenic microorganisms (Morshdy *et al.,* 2022). Chlogenic acids are phenolic compounds produced by plants that are found in a variety of fruits, vegetables, spices, and other foods. Three key enzymes—Quinic acid hydroxyl cinnamonyl transferase (HQT), phenylalanine ammonia-lyase (PAL), and shikimic acid/Quinic acid—are mostly responsible for the synthesis of CGA. The concentration of CGA may be considerably increased by increasing the quantity of these enzymes (Yin *et al.,* 2021). The inherent antioxidant effects of CGA are attributed to its unique molecular structure, which consists of five active hydroxyl groups and one carboxyl group. In addition to eliminating superoxide anions and hydroxyl radicals and having a strong antioxidant effect, the phenolic hydroxyl structure rapidly interacts with free radicals to produce hydrogen free radicals (Miao and Xiang, 2020).

This study aims to isolate and identify pathogenic bacteria in fast food samples from the Alexandria Governorate using traditional microbiological and molecular methods. Additionally, the study will evaluate the antimicrobial efficacy of citrus aqueous extracts against the isolated pathogenic strains.

MATERIALS AND METHODS

1. Sample collection of fast-food sandwiches:

Fifty samples of fast-food sandwiches were collected randomly from 10 different local markets in Alexandria governorate. Ten sandwiches of each selected fast food such as liver (Kebda), Sausage, Fries, Falafel and beans (Ful medames) were purchased from different fast- foods vendors in Alexandria (Figure 1). The collected samples were assessed as soon as possible after being transported to the laboratory in a separate icebox under aseptic condition.

2. Strains of pathogenic bacteria:

Pathogenic strains of *E. coli* EMCC 1604, *Staphylococcus aureus* EMACC 1762, *Salmonella typhimurium* EMACC 1350, and *Bacillus cereus* EMACC 1080 were acquired from the Microbiological Resources Center (MERCIN) at Ain Shams University's Faculty of Agriculture in Cairo, Egypt. Bacterial strains were prepared and standardized to a concentration of 1×10^7 CFU/mL according to the method of Eldin *et al.* (2020).

3. Plant samples collection and extracts preparation:

The Experimental Farm of the City of Scientific Research and Technological Applications (SRTA City), New Borg El Arab city, Egypt, was where the leaves of *Citrus limon* (lemon), *Citrus sinensis* (orange), and *Citrus unshiu* (satsuma mandarin) were gathered. The plant leaves were dried in the shade for four days before being ground into a fine powder using a blender, extracted with deionized water as a solvent (1:20 w/v), centrifuged at 4000 x g for a total of ten minutes, and then filtered. A vacuum freeze dryer (Lyophilizer,

Model FDF 0350, Yangzhong, China) was used to lyophilize aqueous leaf extracts before storing them for later examination.

4. Preparing homogenized fast-food:

Sterilized scissors and forceps are used to chop the sample, and a sterilized spoon is used to mix it well. Ten milliliters of the produced samples were aseptically homogenized with ninety milliliters of sterile 0.1% peptone water using a mechanical shaker for a duration of one minute. To prepare the tenth fold serial dilutions, one milliliter of the preceding homogenate was mixed to nine milliliters of sterilized diluents (El-Fakhrany *et al.*, 2019).

5. Isolating the pathogenic bacteria using selective media from the collected fast-food samples:

Pathogenic bacteria were isolated using several selective mediums. To put it simply, *Salmonella typhimurium* was isolated on Brilliant Green Agar medium, while *Staphylococcus aureus* were isolated on Mannitol Salt Agar (MSA). According to Hamad *et al.* (2017), MacConkey medium was used to isolate E. *coli* and *Klebsiella pneumonia*, meanwhile Brilliance *Bacillus cereus* Agar was employed to isolate *Bacillus cereus* samples.

6. DNA extraction from bacterial cells:

According to Tiwari *et al.* (2012), the genomes of the bacterial isolates *Salmonella typhimurium*, *Bacillus cereus*, and *Klebsiella pneumonia* were isolated. The bacterial strains were cultivated in LB media for an entire night, and after shaking the culture at 200 rpm at 28ºC, two milliliters of the culture were centrifuged for five minutes at 10,000 rpm. For every sample, 1.5 milliliters of cetyl trimethyl ammonium bromide (CTAB) buffer (pH = 8) was added.

Figure 1. Photos of fast food sandwiches a. Liver (Kebda) sandwiches sample, b. sausage sandwiches samples, c. Falafel sandwiches samples. d. Beans (foul) sandwiches samples, e. Fries' sandwiches samples.

To every Eppendorf tube, 10 µL of β- Mercaptoethanol was introduced. Using a Vortex 37600 mixer (Thermolyne, USA), the mixture was vortexed extensively, and it was then incubated for 30 minutes at 65°C in a water path. After centrifuging the mixture for 15 minutes at 13,000 rpm, the supernatant was collected in a fresh Eppendorf tube, and the same amount of phenol was added. To get rid of the remaining phenol, the top layer was gathered in an Eppendorf tube and an equivalent volume of chloroform was added. Subsequently, cold isopropanol was added to precipitate DNA at -20°C for 20 minutes, and the mixture was centrifuged for 15 minutes at 13,000 rpm. After discarding the supernatant, 500 µL of 70% ethanol was added to the pellet, and it was centrifuged for five minutes at an 8.000 rpm. After discarding the supernatant, 70 µL of sterile buffer was added, and the gel electrophoresis process was carried out.

7. Agarose gel electrophoresis:

The extracted genomic DNAs of the three bacteria were examined on a 1% agarose gel that had been heated in a Tris-Borate EDTA (10X TBE) buffer using a microwave (Micro, Quartz Browner, USA) with Tris-base (108 g), boric acid (55 g), EDTA (9.3 g), and distilled water up to a liter (pH = 8). According to Sambrook *et al.* (1989), 950 mL dH_2O was used to dilute 50 mL of stock solution to create a 0.5X concentration. The electrophoresis apparatus (Biometra, USA) was used to load the agarose gel in TBE 0.5X. After staining with ethidium bromide, DNA was visible, and photos were taken with a gel documentation system (Alpha-chem Imager, USA).

8. Detecting pathogenic bacteria in fast- food samples using multiplex PCR:

Three distinct strains of human pathogen bacteria were found using particular primers. Table (1) displayed the amplified product's size, reference, and DNA nucleotide sequence for these primers. 12.5 µL of master mix, 1 µL of DNA (30 ng), 1 µL of each primer (10 p mol/µL), and 25 µL of sterile dH_2O were used in the PCR reaction. The PCR protocol was run as follows: a 3-minute initial denaturation at 95°C, 34 cycles of 94°C for one minute, one minute of annealing at 65°C, and One minute of extension at 72°C. A five milliliter PCR product was separated on a 2% (w/v) agarose gel electrophoresis in 0.5x TBE buffer following a final extension step at 72°C for 5 minutes. Using a DNA molecular length marker, the PCR product's molecular length was calculated. Ultimately, the gel was captured on camera with the Gel Documentation System (Hamad *et al.,* 2018a).

9. Minimum inhibitory concentration (MIC) calculation of tested extracts:

Descending concentrations were used to calculate the Minimum Inhibitory Concentration (MIC) of lyophilized lemon, orange, and tangerine leaf extracts against the strains of pathogenic bacteria that were identified, including *Staphylococcus aureus, E. coli, Bacillus cereus, Klebsiella pneumonia, and Salmonella typhimurium*. The well diffusion method, which involved lowering the leaf extract concentration, was used to calculate the minimum inhibitory concentration (MIC) and antibacterial activity of each extract (Weese *et al.,* 2004). The bactericidal activity of leaf extracts from lemon, orange, and tangerine was evaluated at different concentrations (1.6, 3.12, 6.25, 12.5, 25, 50, 75, and 100 mg/mL) after the extracts were diluted with sterile water. The MIC for every strain/extract was found after the created clear zones were measured and recorded (Weese *et al.,* 2004).

10. Radical scavenging capacity as assessed by the DPPH test:

According to Catarino *et al.* (2018) the efficiency of the lyophilized extracts to scavenge DPPH free radicals was evaluated. As a positive control, ascorbic acid was used. The values were expressed as IC50 (the concentration of lyophilized extracts inhibited 50% DPPH). Using a non - linear regression algorithm, IC50 values were compared to concentration plots.

The equation which was used to calculate inhibition% is:

$$\text{Inhibition (\%)} = \frac{\text{A of control - A of the sample}}{\text{A of control}} \times 100$$

where: A = absorbance

Table 1. Sequence of the specific primer of tested strains (Hamad *et al.*, 2018a)

Strain name	Tareget gene	Primer sequence 5`-`3	Amplicon size (bp)
Salmonella typhimurium	fimA	F:CCTTTCTCCATCGTCCTGAA R:TGGTGTTATCTGCCCGACCA	85
Klebsiella pneumonia	fimA	F:GTTTAACATTCAGCTGAA R:TAGGACCAATTGCCGTACCT	85
Bacillus cereus	hemolysin	F:CTGTAGCGAATCGTACGTATC R:TACTGCTCCAGCCACATTAC	185

11. Evaluation of the lyophilized plant leaves extract's phenolic compound profiles by HPLC:

The phenolic profile of the lyophilized extract was investigated using HPLC (Agilent 1260 infinite HPLC Series, Santa Clara, CA, USA). Using an Eclipse C18 column (4.6 mm× 250 mm i.d., 5 µm) at 40 °C, phenolic compounds were separated. Using a ternary linear elution gradient, the separation was performed using (A) HPLC-grade water with 0.2% H_3PO_4 (v/v) from Sigma-Aldrich, St. Louis, MO, USA; (B) methanol from Thermo Fisher Scientific, Waltham, MA, USA; and (C) acetonitrile from the same source (Thermo Fisher Scientific, Waltham, MA, USA).

The multi-wavelength detector was set at 280 nm and the mobile phase was applied at a flow rate of 0.9 mL/min. The injection volume utilized was about 5µL (Hamad *et al.*, 2018b).

12. Evaluation of the acceptance of lyophilized lemon leaves extract in liver (Kebda) sandwich:

A total of 10 qualified panelists conducted the evaluation in the Food Technology Department of the City of Scientific Research and Technological Applications in New Borg El Arab, Egypt. Lemon leaves extract was added to liver (Kebda) sandwiches that had been prepared using comparable techniques and ingredients to fast-food restaurants (2.5%-5%-7.5%-10% W/W) to liver not the sandwich. The extract was tested for its sensory impact to evaluate whether it might be used as a food ingredient. The samples were maintained at room temperature at 25 °C for 10 minutes before analysis. Panelists graded the Kebda sandwich according to the following criteria: color, odor, taste, texture, and overall acceptance (10 points/each item) on a scale of 1 to 10, with 10 being more accepted (Hamad *et al.*, 2022).

13. Statistical analysis:

The statistical analysis program "Co Stat 6.4" was used to do a one-way analysis of variance (one-way ANOVA) on all of the study's data. Duncan's multiple-range test was used to evaluate mean differences, and effects were deemed significant if they had a probability of $p<0.05$ (Duncan, 1955).

RESULTS AND DISCUSSION

1. Identifying the pathogenic bacteria on selective media from the collected fast food sandwiches samples:

During the current study, the collected fast-food sandwiches (liver sandwiches, sausage sandwich, Fries' sandwiches, Falafel sandwiches and bean sandwiches) were obtained from five different locations in Alexandria governorate, Egypt. Selective media is a standard procedure for detecting pathogenic microorganisms in biological samples. Data in Figure (2) reveals the presence of various bacterial strains in the selected fast-food sandwiches. For instance, in liver sandwiches, 30% of the obtained samples were infected with *Staphylococcus aureus*, while the contamination with *E. coli* and *Bacillus cereus* and were contaminated with *E. coli* and *Bacillus cereus* at a rate of 40%. Furthermore, the liver sandwiches showed highly contaminated with *Klebsiella pneumoniae* and *Salmonella typhimurium*, which infects 60% of the samples. On the other hand, the microbial infection was spread differently in sausage sandwiches.

Staphylococcus aureus and *Klebsiella pneumoniae* were found in 60% of the sausage sandwich samples where 70% of the samples tested positive for *Bacillus cereus*. Additionally, 50% of sausage samples contained *Salmonella typhimurium*, but only 30% tested positive for *E. coli* (Figure 2). In fact, the Fries' sandwiches are popular in Egypt. It contains usually fries with ketchup or mayonnaise or green salad. Six out of ten samples were infected with *Salmonella typhimurium* or *E. coli*. The *Staphylococcus aureus* spread impacted only four samples. The results indicated that, only three samples were infected with *Bacillus cereus*, while two were infected with *Klebsiella pneumonia* (Figure 2).

Falafel sandwiches is one of the most popular sandwiches in Egypt. Fifty percent of falafel samples were infected with *Staphylococcus aureus* while 40% of samples tested positive to *E. coli, Bacillus cereus and Salmonella typhimurium.* In addition, only 30% of the Falafel sandwiches samples tested positive for *Klebsiella pneumonia* (Figure 2). Finally, bean sandwiches (Ful medames) are also the most popular food in Egypt. Forty percent of the samples of bean sandwiches were infected with *Staphylococcus aureus* or, *Bacillus cereus.* On the other hand, 30% of the samples tested positive for *E. coli, Klebsiella pneumonia* and *Salmonella typhimurium* (Figure 2).

In the present investigation, both liver and sausage samples had greater levels of infection than Falafel, Beans, and Fries samples. Several studies were done on fast food samples with meat or poultry products in different governorate in Egypt (Adam, 2009; Saad *et al.*, 2011 and Shaltout *et al.*, 2017). The focus of recent research has been on food poisoning illnesses caused by several pathogenic bacteria. Contamination in fast food is linked to various illnesses in people. During the food preparation and storage process, a number of workers have observed that hazardous germs including *Salmonella* and *Escherichia coli* can be present in raw meat and poultry and may migrate to other foods (Morris Jr and Vugia, 2021).

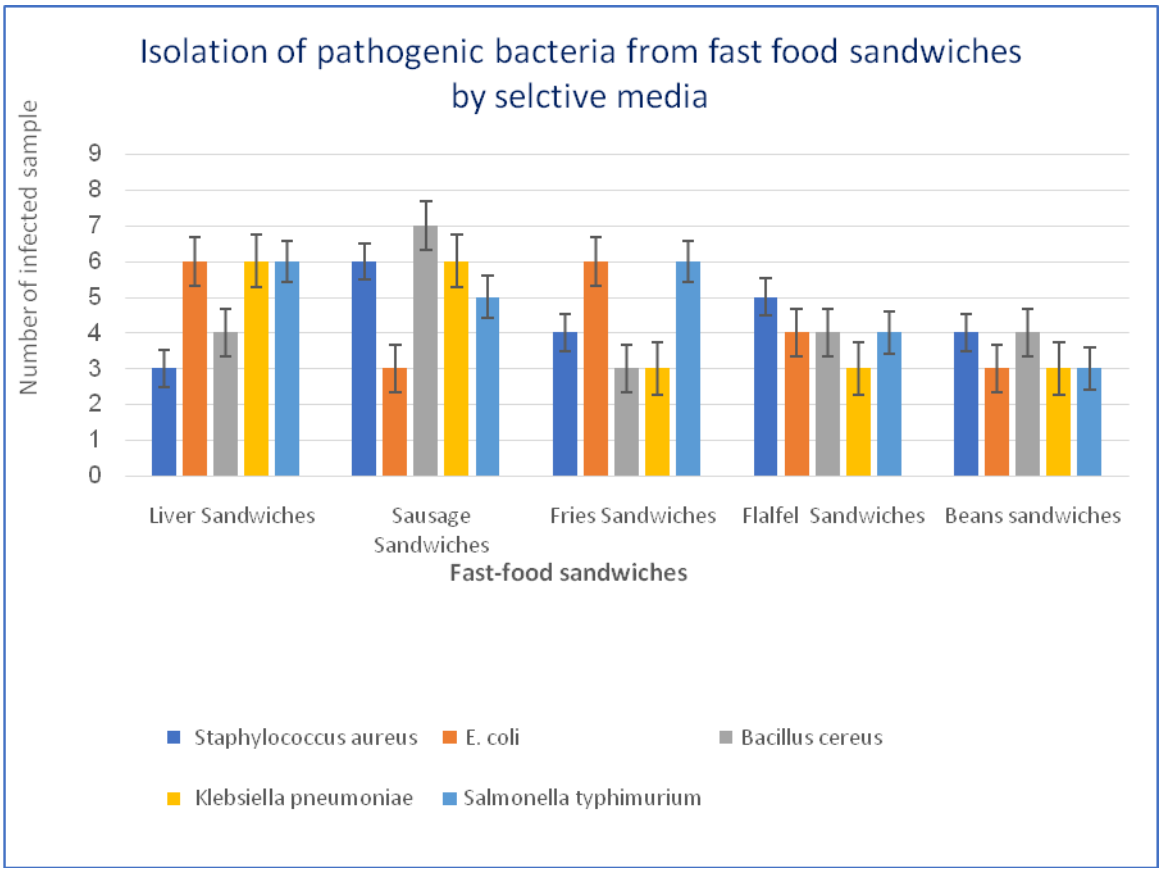

Figure 2. Isolation of pathogenic bacteria from fast food sandwiches using selective media

The current study's findings are consistent with those of prior research, since *E. coli* and *Salmonella typhimurium* were found in most of the samples from liver and sausage sandwiches. As Staphylococcus species invade food, they create heat-stable enterotoxins and other extracellular compounds such as that make the food harmful despite the fact it looks normal. These harmful compounds are linked to Staphylococcus infiltration (Bevilacqua *et al.*, 2017). Having them in food is a sign of both the food vendor's poor production practices and inadequate personal sanitation. They can tolerate elevated amounts of sodium chloride as well (Ruzante *et al.*, 2012 and Shaltout *et al.*, 2017).

In the current investigation, *Staphylococcus aureus* was found in all five analyzed sandwich samples, but to varying degrees. *Staphylococcus aureus* is a good indicator of the hygienic conditions used in the production and handling of meat and its products (Potter and Hotchkiss, 2001). The lowest percentage of *Staphylococcus aureus* occurrence was 30%, while the highest was 60%. Sausage and falafel samples were the highest, reaching 60% and 50%, respectively. This could be explained with low personal hygiene in food vendor (Shaltout *et al.*, 2017).

2. Detection of pathogenic bacteria in fast food sandwiches samples using multiplex PCR:

During the current study, the total DNA was isolated from each sample, then multiplex PCR was carried out. The results are presented in Figure (3). The specific primers of three pathogenic bacteria *Bacillus cereus*, *Klebsiella pneumonia* and *Salmonella typhimurium* were selected and used to detect the attacked microbes in the previous fast- food sandwich samples. The results showed that, in liver sandwich samples, almost 60% of the samples were infected by *Klebsiella pneumonia* or *Salmonella typhimurium* but only 40% of the sample tested positive to *Bacillus cereus.* These results were consistent with the selective media test results. In sausage sandwiches, 60% of the samples tested positive for *Bacillus cereus*, however only 50% tested positive for *Klebsiella pneumonia* or *Salmonella typhimurium*.

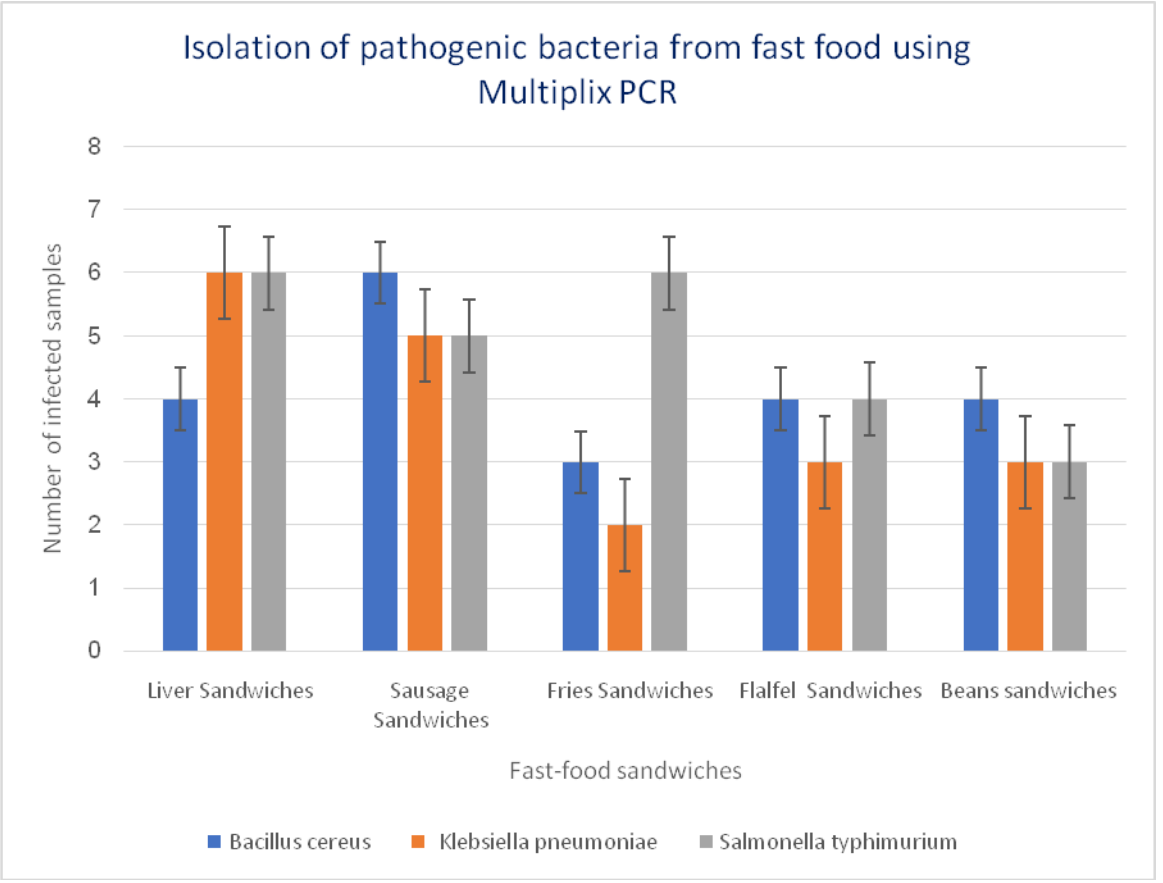

Figure 3. Isolation of pathogenic bacteria from fast food sandwiches using multiplex PCR

These findings partially support the selective medium result, as the multiplex PCR results were a bit lower (one sample lower) in both *Bacillus cereus* and *Klebsiella pneumonia*. Furthermore, in fries sandwich samples 60% of the samples tested positive for *Salmonella typhimurium.* Further, 30% of the samples tested positive for *Bacillus cereus*, whereas 20% tested positive for *Klebsiella pneumonia*. These findings aligned with the selective media test results. Falafel sandwich samples reveal a similar pattern of contamination as in the selective media test. Forty percent of the samples were contaminated with *Bacillus cereus* or *Salmonella typhimurium* where only 30% tested positive to *Klebsiella pneumonia.* At last, 30% of the bean sandwich samples found positive to *Klebsiella pneumonia* or *Salmonella typhimurium*, and 40% were positive for *Bacillus cereus*. These results for bean sandwich samples lined up with the selective media examination findings.

In food microbiology, Polymerase Chain Reaction (PCR) is one of the most popular identifying techniques for bacterial identification. However, certain inhibitors present in the contaminated food may hinder the PCR process. Furthermore, PCR is unable to distinguish between living and dead cells (Mandal *et al.*, 2011). On the other hand, according to Lee *et al.* (2014) inquiry, multiplex PCR proved a useful and informative method for identifying food-borne bacteria in Korean food that was ready to consume. In our case, the multiplex PCR results were comparable to the selective media results. This is consistent with Lee *et al.* (2014), as it demonstrates the importance of the multiplex PCR approach in detecting pathogenic microbes in fast food.

3. Antibacterial activity of citrus leaves extracts against the pathogenic bacterial strains detected strains in fast-food sandwiches:

A key technique to Figure out the lowest concentration of the compounds under test that will completely prevent and cease bacterial growth is the minimum inhibitory concentration (MIC). The antibacterial activity and MIC of lyophilized lemon, orange, and tangerine leaf extracts have been investigated versus *Staphylococcus aureus, E. coli, Bacillus cereus, Klebsiella pneumonia,* and *Salmonella typhimurium* pathogens using the well diffusion method. Results of lemon, orange and tangerine leaves extract

are provided in Tables (2, 3, and 4) respectively. The three extracts inhibited growth of the strains under investigation in varying levels. In a 100mg/100ml concentration of lemon, orange and tangerine leaf extracts against *E. coli*, the diameter of the inhibitory zone was 31, 25, and 18 mm, respectively. Furthermore, with the same quantity of lemon, orange, and tangerine leaf extracts versus *Staphylococcus aureus*, the inhibition zone diameters were 35, 27, and 23 mm, respectively. Likewise, with the same quantity of lemon, orange, and tangerine leaf extracts against *Salmonella typhimurium*, the inhibition zone diameters were 34, 27, and 25 mm, respectively. Additionally, with the same dose of lemon, orange, and tangerine leaf extracts vs *Klebsiella pneumonia*, the inhibitory zone diameter was 28, 27, and 28 mm, respectively. The inhibitory zone diameter for *Bacillus cereus* at the same quantity of lemon, orange, and tangerine leaf extracts was 36, 28, and 27 mm, respectively. Lemon leaf extract exhibits the strongest antibacterial activity against four of the total five tested bacteria, as demonstrated by previous results. Moreover, the MIC for lemon leaves extract was 1.6 mg/100mL for all the tested bacteria. The MIC of orange leaf extract was 3.12 mm/mL in *E. coli*, *Salmonella typhimurium*, and *Bacillus cereus*, while it was 1.6 mm/mL in *Staphylococcus aureus* and *Klebsiella pneumonia*. In the case of tangerine leaf extracts, MIC was 3.12 in all the strain except for *Klebsiella pneumonia* it was 1.6 mg/100mL.

Table 2. Minimum inhibitory concentrations (MIC) and antibacterial activity of lemon leaves extract against detected strains in fast-food sandwiches samples in Alexandria

Strains	Aqueous extract (mg/100mL)						
	100	50	25	12.5	6.25	3.12	1.6
	Diameter of inhibition zone (mm)						
E. coli −	31±1.24^{a}	30±1.18^{b}	28±1.12^{b}	25±1.18^{b}	21±1.13^{b}	10±0.48^{b}	6±0.43^{b}
Staphylococcus aureus +	35±1.76^{b}	32±1.53^{b}	29±1.42^{b}	26±1.19^{b}	18±0.95^{b}	8±0.41^{b}	5±0.12^{b}
Salmonella typhimurium −	34±1.22^{b}	30±1.22^{b}	27±1.28^{b}	24±1.17^{b}	14±0.53^{a}	6±0.28^{a}	3±0.06^{a}
Klebsiella pneumonia	28±1.34^{a}	25±1.15^{a}	22±1.14^{a}	18±1.04^{a}	12±0.52^{a}	7±0.193^{a}	4±0.07^{a}
Bacillus cereus +	36±1.59^{b}	31±1.34^{b}	29±1.32^{b}	25±1.21^{b}	16±0.93^{a}	8±0.37^{b}	3±0.9^{a}

* Data in the same row between different inhibition zone diameter followed by different superscript letters differ significantly ($p < 0.05$).

Table 3. Minimum inhibitory concentrations (MIC) and antibacterial activity of orange leaves extract against detected strains in fast-food sandwiches samples in Alexandria

Strains	Aqueous extract (mg/100mL)						
	100	50	25	12.5	6.25	3.12	1.6
	Diameter of inhibition zone (mm)						
E. coli	25±1.12^{b}	19±0.85^{a}	19±0.65^{a}	12±0.46^{a}	9±0.44^{a}	3±0.14^{a}	0
Staphylococcus aureus	27±1.14^{b}	25±1.07^{b}	22±1.11^{b}	20±1.02^{b}	14±0.81^{b}	7±0.52^{b}	3±0.07^{b}
Salmonella typhimurium	18±0.75^{a}	20±0.94^{a}	17±0.73^{a}	12±0.62^{a}	8±0.38^{a}	2±0.09^{a}	0
Klebsiella *pneumonia*	27±0.98^{b}	24±1.31^{b}	22±1.18^{b}	20±0.56^{b}	13±0.55^{b}	5±0.11^{b}	2±0.08^{a}
Bacillus cereus	28±1.22^{b}	26±1.32^{b}	20±0.91^{b}	17±0.72^{b}	9±0.45^{a}	3±0.13^{a}	0

* Data in the same row between different inhibition zone diameter followed by different superscript letters differ significantly ($p < 0.05$).

Table 4. Minimum inhibitory concentrations (MIC) and antibacterial activity of tangerine leaves extract against detected strains in fast-food sandwiches samples in Alexandria

Strains	Aqueous extract (mg/100mL)						
	100	50	25	12.5	6.25	3.12	1.6
	Diameter of inhibition zone (mm)						
E. coli	18±0.82^{a}	15±0.78^{a}	12±0.46^{a}	10±0.46^{a}	7±0.28^{a}	3±0.16^{a}	0
Staphylococcus aureus	23±1.17^{b}	16±0.78^{a}	14±0.63^{a}	11±0.58^{a}	7±0.33^{a}	3±0.17^{a}	0
Salmonella typhimurium	25±1.24^{b}	21±1.05^{b}	17±0.96^{a}	12±0.57^{a}	9±0.43^{b}	4±0.21^{a}	0
Klebsiella *pneumonia*	28±1.52^{b}	25±1.2^{b}	23±1.08^{b}	18±0.92^{c}	10±0.47^{b}	6±0.28^{b}	2±0.09^{a}
Bacillus cereus	27±1.32^{b}	22±1.03^{b}	16±0.83^{a}	14±0.71^{b}	8±0.36^{a}	3±0.16^{a}	0

* Data in the same row between different inhibition zone diameter followed by different superscript letters differ significantly ($p < 0.05$).

Multiple studies have been conducted on the antibacterial effects of citrus species extracts. Lemon, orange, and tangerine leaf extracts demonstrated antibacterial activity against *Clostridium botulinum*, (Hamad *et al.*, 2023). In spite of testing the extracts on various strains, our results were consistent with Hamad *et al.* (2023). In another study conducted by Daniel *et al.* (2016), lemon leaf extract shown modest antibacterial efficacy against both gram-positive and gram-negative microorganisms. According to Daniel *et al.* (2016), lemon leaf extract is more effective against gram-positive bacteria than gram-negative bacteria. The results we obtained were partially consistent with Daniel *et al.* (2016), as lemon leaf extract exhibited stronger antibacterial activity against *Staphylococcus aureus* (gram-positive) than *Salmonella typhimurium* (gram-negative) and *Klebsiella pneumonia* (gram-negative). However, in our study, lemon leaf extract demonstrated the highest antimicrobial activity against *E. coli* (gram-negative).

4. Antioxidant activity of lemon, orange and tangerine lyophilized extract of leaves determined by DPPH method:

The DPPH test is a precise, dependable, and affordable technique to measure antioxidant radical scavenging activity. Table (5) demonstrates the antioxidant potential of the lyophilized lemon, orange, and tangerine extracted leaves. Ascorbic acid was selected as the reference antioxidant. All the tested extracts revealed a potential antioxidant activity as the IC50 of lyophilized lemon, orange, and tangerine extract were 47.96,54.41and 58.68 respectively where the IC50 for ascorbic acid was 34.98 µg/ml. Lower IC50 mean higher antioxidant capacity. Lemon leaves extract shows the higher antioxidant activity followed by orange and the lowest was tangerine. All the investigated extracts show antioxidant activity from the lowest concentration (10µg/ml) to reach over 90% of inhibition the DPPH free radical parts at 100 µg/ml.

Recent study on comparable extracts used the DPPH assay to assess antioxidant activity, and the results were consistent with ours. Lemon extract in Hamad *et al.* (2023) experiment demonstrated more potent antioxidant activity than orange and tangerine extracts, however the calculating method was different. An additional investigation Ghafar *et al.* (2010) found that citrus samples have significant antioxidant activity comparable to or even superior to ascorbic acid, which had been used as a standard. According to Spada *et al.* (2008), several citrus samples demonstrated reasonable antioxidant activity when examined using the DPPH assay. Ascorbic acid was employed as a standard due to its distinctive antioxidant properties. Since ascorbic acid can remove reactive oxygen species and prevent the formation of free radicals, it is significant as an antioxidant (Wu *et al.*, 2008).

4. Phenolic compounds composition of lemon, orange and tangerine lyophilized extract of leaves assessed by HPLC:

The phenolic profile of the three extract was determine by HPLC. The phenolic composition of the samples was determined, and 19 compounds were identified quantitatively and qualitatively. The result is presented in Table (6).

Table 5. DPPH radical scavenging capacity of lemon, orange and tangerine lyophilized extract of leaves comparing with ascorbic acid as standard

Concentration (µg/ml)	Inhibition (%)			
	Ascorbic acid	Lemon Leaves extract	Orange Leaves extract	Tangerine leaves extract
10	15.22 ± 1.02^{a}	9.12± 0.47^{a}	7.22±0.36^{a}	6.70±0.34^{a}
20	29.17±1.31^{a}	18.92±0.96^{a}	15.67±0.762^{a}	13.24±0.64^{a}
30	42.38±1.56^{a}	34.16±1.54^{a}	28.12±1.14^{a}	25.56±1,15^{a}
40	57.17± 2.38^{a}	45.16± 2.01^{a}	36.17±1.29^{a}	32.15±1.43^{a}
50	72.14±4.92^{b}	52.14±1.89^{a}	47.15±2.27^{a}	43.14±1.67^{a}
60	89.04± 3.69^{b}	74.19± 2.86^{b}	55.13±3.08^{b}	51.12±2.35^{b}
70	92.11±4.12^{b}	86.02±4.03^{b}	69.76±3.22^{b}	64.92±3.23^{b}
80	95.02±4.76^{b}	91.56±4.45^{b}	89.76±2.84^{b}	79.95±3.62^{b}
90	97.24±5.34^{b}	94.14±4.23^{b}	92.13±3.82^{b}	89.11±3.42^{b}
100	99.67± 3.89^{b}	98.20±4.62^{b}	96.98± 4.68^{b}	92.52±3.92^{b}
IC_{50}(µg/ml)	34.98	47.96	54.41	58.68

*Data in the same column between different antioxidant activity (%) followed by different superscript letters differ significantly ($p < 0.05$).

Table 6. Phenolic compounds profile of lyophilized plants leaves extracts

Compound	Plants leaves extracts		
	Orange leaves	Lemon leaves	Tangerine leaves
	Conc. (µg/g)	Conc. (µg/g)	Conc. (µg/g)
Gallic acid	989.62	1182.41	1352.04
Chlorogenic acid	10790.10	8233.73	7761.21
Catechin	2388.79	2040.38	1532.46
Methyl gallate	27.65	617.32	64.79
Caffeic acid	55.75	239.94	167.64
Syringic acid	281.02	567.89	1002.08
Pyrocatechol	663.40	2075.88	833.44
Rutin	3259.56	1056.74	735.08
Ellagic acid	0.00	7018.64	744.17
Coumaric acid	0.00	0.00	0.00
Vanillin	342.10	174.31	160.16
Ferulic acid	1282.81	829.79	642.02
Naringenin	19810.64	6757.80	19969.70
Daidzein	0.00	31.17	259.35
Quercetin	191.66	105.20	25.00
Cinnamic acid	10.98	12.01	0.00
Apigenin	11.89	91.68	598.20
Kaempferol	49.70	31.47	0.00
Hesperetin	225.19	36.24	405.24

Naringenin (19810µg/g) was the most abundant phenolic substance identified in orange leaf extract, followed by Chlorogenic acid (10790µg/g), Rutin (3259µg/g), Catechin (2388µg/g), and Ferulic acid (1282µg/g). In lemon leaves extract, Chlorogenic (8233µg/g) acid was the most abundant compound followed by Ellagic acid (7018µg/g), Naringenin (6757µg/g), Pyrocatechol (2075µg/g), Catechin (2040µg/g), Gallic acid (1182µg/g), and Rutin (1056µg/g). In tangerine leaves extract**,** Naringenin (19969µg/g) was the most abundant compound followed by Chlorogenic acid (7761µg/g), Catechin (1532µg/g), Gallic acid (1352µg/g) and Syringic acid (1002µg/g)**.** The extracts which were tested in our study showed a potential antioxidant and antimicrobial activity. It was previously established that the *C. limon* flavonoids' antioxidant activity extended beyond their capacity to eliminate free radicals and consequently enhanced the antioxidant response of cells via activating the ERK/Nrf2 signaling pathway (Parhiz *et al.*, 2015). Chlorogenic acid was the most prominent component in all the studied extracts. Our body produces free radicals as a byproduct of many of its regular physiological processes.

The organs will begin to exhibit an aberrant spectrum of alterations if the quantity of free radicals beyond the range of antioxidant tolerance. Destabilization of the body's antioxidant system, or an imbalance between reactive oxygen species (ROS) and the antioxidant defense system, results to oxidative stress. Chlorogenic acid all-encompassing antioxidant mechanism can be summed up as follows: First, the poly- hydroxyl structure scavenges free radicals directly; second, it activates the anti-oxidant signaling pathway, controls the expression level of related genes, and increases anti-oxidant capacity; and third, it directly controls the activity of the endogenous oxidase system and related proteins (Cheng *et al.*, 2019).

Regarding both Gram-positive and Gram-negative bacteria, Chlorogenic acid exhibits significant inhibition properties. What follows simply describes the primary mechanisms: (1) disrupt the regular metabolic activities of bacterial cells, resulting in metabolic disorders within the cells; (2) interfere with normal cell cycle progression, thereby inhibiting the growth of microorganisms; and (3) destroy the structure of cell membranes, causing leakage of intracellular metabolites and triggering cell inactivation (Su *et al.*, 2019 and Wang *et al.*, 2022). Ellagic acid was found in lemon leaf extract at significant concentrations, about ten times that of tangerine leaf extract for the same molecule, but not in orange extract. Lemon extract was extract was revealing the higher antioxidant activity when it was measured by DPPH assay. The principles behind ellagic acid's diverse bioactivity mostly originate from its capacity to neutralize deleterious reactive oxygen and nitrogen species (RONS), a consequence of physiologic aerobic metabolism, as well as from its antioxidant and anti-aging properties.

Table 7. Sensory evaluation of liver (Kebda) sandwich fortified with lyophilized lemon leaves extract

Sample	Color	Odor	Taste	Texture	Overall acceptance
Control	8.50±0.41^{b}	8.45±0.43^{a}	8.67±0.33^{b}	8.75±0.51^{b}	8.59±0.44^{b}
2.5%	8.63±0.45^{b}	8.52±0.49^{a}	8.72±0.46^{b}	8.74±0.42^{b}	8.65±0.41^{b}
5%	8.68±0.41^{b}	8.66±0.47^{b}	8.88±0.42^{b}	8.78±0.51^{b}	8.75±0.48^{b}
7.5%	8.52±0.38^{b}	8.76±0.49^{b}	8.68±0.43^{b}	8.66±0.45^{b}	8.65±0.39^{b}
10%	7.41±0.39^{a}	8.30±0.40^{a}	7.30±0.43^{a}	7.20±0.40^{a}	7.62±0.42^{a}

Data in the same column between different treatments followed by different superscript letters differ significantly ($p < 0.05$).

Normally, cellular antioxidant defenses and repair systems regulate the creation of reactive oxygen species (RONS), which is important in immunological defense, signaling, and the synthesis of energy from organic molecules (Genestra, 2007).

Excessive production of RONS leads to the breakdown of cells' detoxification systems and accumulation of RONS, which is a major factor in the development of oxidative stress (OS) and inflammation. It also causes irreversible damage to proteins, lipids, and DNA, which accelerates aging and certain degenerative disorders (Venkataraman *et al.,* 2013). This could explain why lemon extract exhibited better antioxidant activity than the other two extracts evaluated in our study.

5. Acceptability of liver (Kebda) sandwich fortified with lyophilized lemon leaves extract depending on sensory attributes:

Kebda (liver) sandwiches were prepared using the same recipes as food vendors. The lyophilized lemon leaf extract was applied to liver sandwiches in four concentrations: 2.5%, 5%, 7.50%, and 10% and the control sample were prepared in the same way but without the extract. The result is presented in Table (7). The results demonstrated an increase in overall sample acceptance at concentrations of 2.5%, 5%, and 7.5% lyophilized extract. 5% concentration of lemon leaves extract gave the highest overall acceptance in compare the other 3 concentration and the control. Lemon leaf extracts exhibited the highest antibacterial efficacy against the pathogenic strains examined. Usually lemon fruit juice (derived from a proper hand press of the lemon fruit) is added to liver sandwiches. This could explain why the four tested concentrations of lemon leaf extract were highly accepted.

CONCLUSION

This study highlights the significant public health risk posed by bacterial contamination in fast food. The results obtained in this study, whether from selective media analysis or molecular biology, showed that samples were contaminated with varying proportions of the studied strains especially *Salmonella typhimurium* and *Bacillus cereus*. While the results from both traditional and molecular methods largely aligned, some discrepancies were noted, which may be attributed to the inadequate hygiene practices in the food preparation facilities. The aqueous extract of lemon has proven their antimicrobial activity which is likely due to the high concentration of phenolic compounds, such as chlorogenic acid and ellagic acid, identified in the extract. This extract was also found to be sensory-acceptable to consumers. Lemon leaf extract, can be considered tools for reducing the impact of harmful microbes that can contaminate food. Their application could serve as a valuable supplement to proper hygiene and sanitation protocols, ultimately enhancing consumer safety.

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الملخص العربي

الكشف عن التلوث الميكروبي في الأغذية السريعة وفعالية مستخلصات أوراق الحمضيات

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تصنف منظمة الصحة العالمية (WHO) الأمراض المنقولة بالغذاء على أنها اضطرابات سامة أو معدية، مع تحديد أكثر من 200 عامل مسبب. أجريت دراسة على 50 عينة من شطائر الوجبات السريعة من 10 أسواق محلية في محافظة الإسكندرية، بما في ذلك الكبد والسجق والبطاطس المقلية والفلافل والفول. تم جمع السلالات المسببة للأمراض من الإشريكية القولونية والمكورات العنقودية الذهبية والسالمونيلا التيفية والعصية الشمعية من كلية الزراعة بجامعة عين شمس في القاهرة، مصر. تم عزل السلالات المسببة للأمراض من عينات الوجبات السريعة باستخدام وسائط انتقائية كطريقة تقليدية، واستُخدمت طريقة تفاعل البوليميراز المتسلسل المتعدد كطريقة لعزل الميكروبات باستخدام التقنيات الوراثية. تم استخدام المزرعة التجريبية لمدينة الأبحاث العلمية والتطبيقات التكنولوجية في مدينة برج العرب الجديدة، مصر، لجمع أوراق الحمضيات الليمون والبرتقال واليوسفى. في عينات ساندويتش الكبد، أصيب ما يقرب من 60٪ من العينات ببكتيريا كليبسيلا الرئوية أو السالمونيلا التيفية الفأرية ولكن 40٪ فقط من العينة كانت إيجابية لاختبار *Bacillus cereus*. في شطائر السجق، تم العثور على المكورات العنقودية الذهبية والكلبسيلا الرئوية في 60٪ من العينات، بينما كانت نتيجة اختبار *Bacillus cereus* إيجابية في 70٪. في شطائر الفلافل، أصيب50٪ بالمكورات العنقودية الذهبية، بينما كانت نتيجة اختبار *E. coli* و *Bacillus cereus* و *Salmonella typhimurium* إيجابية في 40٪. أظهر المستخلص المائي لأوراق الحمضيات قدرة كبيرة على تثبيط الكائنات الحية الدقيقة المسببة للأمراض الموجودة في عينات الوجبات السريعة، حيث تراوح الحد الأدنى للتركيز المثبط (MIC) لمستخلص أوراق الحمضيات المختبر من 1.6 إلى 3.2 ملغم/مل ضد جميع السلالات المختبرة. مستخلص أوراق الليمون يظهر أعلى نشاط مضاد للبكتيريا مع 1.6 ملغ / مل MIC وأعلى قدرة مضادة للأكسدة مع IC50 يساوي 47.96 (ميكروغرام / مل).

الكلمات المفتاحية: تفاعل البوليميراز المتسلسل المتعدد؛ الكشف؛ تحليل التحكم بالمضادات الميكروبية؛ التلوث؛ الوجبات السريعة.