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Research Article

ISOLATION AND IDENTIFICATION OF SOME PATHOGENIC AND CONTAMINATING BACTERIAL SPECIES OF MILK PRODUCTS IN HILLA CITY

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Abstract

The main objective of this study was to identify and classify various pathogenic and contaminating bacterial strains found in milk products, specifically focusing on cheese. A specimen of cheese that was found to be tainted was obtained and, after that, subjected to a sequence of dilution procedures. Subsequently, a portion of these dilutions was inoculated onto various growth media, followed by the implementation of several biochemical assays. Work continued on this project from the date of 22-11-2021/ 22-2-2022. Moreover, the pathogen bacteria species were isolated and identified by cultivating the sample on E. coli, Proteus mirabilis, Klebsiella, Salmonella, and Staph. Aureus medium. Initially, the sample underwent culturing on Eosin Methylene Blue (EMB) and MacConkey agar media to facilitate the identification of Escherichia coli (E. coli) and Klebsiella species. Additionally, Proteus bacteria were isolated and identified through culturing on a blood agar medium. Staphylococcus aureus (staph. aureus) bacteria were diagnosed using the Mannitol Salt agar medium, while Salmonella bacteria were identified on the Selenite-Sulfite agar (S-S-agar) medium. The laboratory tests conducted include the Indole test, Urease test, Simmons citrate test, Catalase test, Oxidase test, Coagulase test, and Fermentation test. Certain isolates exhibited negative results, while others displayed positive outcomes in the biochemical assays. Furthermore, an experiment was undertaken. The study examined the sensitivity of antibacterial agents against various bacterial strains. Results indicated that ciprofloxacin exhibited greater efficacy against Staphylococcus aureus, whereas Gentamycin and Amikacin demonstrated lower effectiveness. Conversely, all antibacterials above displayed equal efficacy against Escherichia coli. Additionally, Pseudomonas bacteria were found to be resistant to all tested antibacterials.

Keywords: Milk product, pathogenic bacteria, Antibiotic.

INTRODUCTION

Dairy products are foods rich in proteins, fats, minerals and vitamins, and they are of high nutritional value. These products are exposed to contamination with microorganisms such as bacteria and moulds during their collection, transportation, storage and handling [1,2]. This contamination is common in our country as the Milk is made in uncooled places for long hours and in the market under direct sunlight. Pollution may occur due to the growth of moulds on the surface of the Milk, which leads to the secretion of toxins in it [3]. Dairy products may contain many pathogens that are related to bacterial contamination of Milk and its derivatives, as most of these pathogens die through pasteurization, but there are a number of them that resist this process [4,5]. This leads to its growth and the formation of a thin, sticky layer known as the biofilm formed in laboratories and dairy products with the industrial biofilm, and among the bacteria that make up this membrane are *Staphylococcus*, *Bacillus, E.coli*, *pseudomonas, Proteus, klebsiella* isolates [6,7]. The biofilm is one of the virulence factors of pathogenic bacteria, as the bacteria gather inside extracellular polymers and the formation of cell structures in the form of a thin membrane that sticks to the surfaces. This membrane is formed in response to factors such as high density and environmental conditions unsuitable for production, storage and marketing processes [8]. The importance of entanglement in the production of biofilms is significant, and its significance extends to the isolates of Proteus bacteria [9,10].

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The proliferation of microorganisms in cheese is affected by various parameters, including moisture content, water activity, redox potential, aerobic or anaerobic conditions, pH, acidity, and salt level [11]. Bacterial contamination of cheese products can arise during both the processing and post-processing handling stages, particularly when adequate sanitation standards are not implemented. Spoilage bacteria in cheese can be derived from inferior-quality raw Milk. For instance, certain thermo tolerant bacterial species such as Bacillus, Clostridium, Lactobacillus, Microbacterium, Micrococcus, and Streptococcus can withstand heat treatment applied to milk and proliferate in specific types of cheese products [12, 13].

METHODOLOGY

Samples and Methods

Samples

1gm of cheese sample was taken from the place designated for selling cheese in Hilla, and a series of required dilutions were made, and it was grown on different media.

Preparation of Reagents

Oxidase reagents: The oxidase reagents were made by directly dissolving 0.1 grams of tetra-p-paraphenylene diamine dehydrochloride in 10 milliliters of distilled water. The resulting solution was then stored in an opaque container to assess the bacterial capacity for oxidase enzyme production [14].

Catalase reagents: A dark container was used to store 3% of H2O2 to assess the catalase enzyme production capabilities of bacteria [14].

Kovac's reagents: The preparation involved dissolving 5 grams of P-dimethylamine benzyl aldehyde in 75 millilitres of amyl alcohol and adding 35 millilitres of concentrated hydrochloric acid. The Indole synthesis was detected using the method above [15].

Solutions

Normal saline solution: The solution dissolved 8.5 grams of sodium chloride (NaCl) in a small amount of distilled water. The volume was then adjusted to 1000 millilitres, and the pH was 7.2. The solution was sterilized using an autoclave at a temperature of 121 degrees Celsius for 15 minutes. Subsequently, it was stored at 4 degrees Celsius (15).

EDTA Solution: The solution dissolved 0.4 grams of EDTA in 12 millilitres of distilled water (D.W.). The pH of the solution was adjusted to 8 by adding 10 N sodium hydroxide (NaOH). Subsequently, the volume was increased to 20 millilitres to achieve a total concentration of 20 milligrams per millilitre [16].

Gram Stain Solutions: The specified microbiological protocols made the solutions. The solutions utilized in the study encompassed four distinct substances: crystal violet, iodine, pure alcohol, and safranine [17].

Preparation of Culture Media

A set of culture media was prepared by the manufacturer's guidelines and subjected to sterilization using autoclaving at a temperature of 121 degrees Celsius for 15 minutes.

MacConkey Agar Medium (pH:7.3)

The company has written the recommended methodology. Its primary purpose was to isolate the majority of Gram-negative bacteria and differentiate between bacterial isolates that ferment lactose and those that do not [18].

Blood Agar Medium (PH:7.1)

The blood agar medium was made by dissolving 40 grams of blood agar base in 1000 millilitres of distilled water. The media underwent autoclaving at a temperature of 121 degrees Celsius for 15 minutes. Subsequently, it was cooled to 50 degrees Celsius, following which a 5% concentration of fresh human blood was introduced. The medium above cultivated meticulous bacteria with specific growth requirements [14].

Nutrient agar medium

The nutrient agar medium was created by the specifications provided by the manufacturing business, with a concentration of 28 grams per litre. The utilization of this method encompassed a wide range of applications, including conducting general research, cultivating bacterial isolates, and activating them as needed [15].

Muller-Hinton agar medium

The Muller-Hinton agar medium was made according to the instructions provided by the manufacturer. The technique above was employed to assess antibacterial susceptibility [15].

Mannitol salt agar medium

The procedure involves the suspension of 111 grams of Mannitol Salt Agar in 1000 millilitres of purified water. Heat the medium until it is completely dissolved. The recommended method for sterilization is autoclaving at a pressure of 15 pounds per square inch and a temperature of 121 degrees Celsius for 15 minutes. If desired, the sterile Egg Yolk Emulsion (E7899) may be included in the solution post-autoclaving at a final concentration of 5% v/v. The user's text must be completed and provide information to rewrite academically [15].

Salmonella Shigella Agar (SS agar medium)

The components were suspended by adding a dehydrated powder to water, namely 63 grams of powder in 1000 ml of pure or distilled water. Heat the mixture while stirring regularly to ensure complete dissolution of the medium. It is advised to refrain from autoclaving or subjecting the medium to excessive heat, as overheating can compromise the medium's selectivity. The user's text must include information or content to be rewritten academically [19].

Eosin Methylene Blue (EMB) Agar Medium

Dissolve 36 grams of EMB Agar in 1000 milliliters of distilled water. Apply heat in order to achieve thorough dissolution of the medium. The recommended method for dispensing and sterilizing is autoclaving at a pressure of 15 pounds per square inch and a temperature of 121 degrees Celsius for 15 minutes. It is advisable to prevent excessive heat generation. The liquid should be cooled to a temperature of 50 °C and agitated to facilitate methylene blue's oxidation, restore its blue hue, and evenly disperse the flocculent precipitate [20].

Laboratory diagnosis

Microscopic examination and colonial morphology: Identification of each main positive culture relied on the morphological attributes of a single colony, including colony size, shape, colour, type of pigments, translucency, edge, elevation, and texture. A Gram stain was employed to examine the cellular morphological characteristics of bacterial cells, encompassing Gram reaction, shape, organization, capsules, spores, and other relevant qualities.

Biochemical tests

Catalase test: Catalase is an enzymatic catalyst responsible for facilitating the decomposition of hydrogen peroxide into oxygen and water. The nutrient agar medium was inoculated with the chosen bacterial colonies and incubated at 37 ºC for 24 hours. Following incubation, the growth was carefully transferred onto the surface of a pristine slide using a wooden stick. A 3% hydrogen peroxide (H2O2) drop was introduced into the bacterial growth. The presence of gas bubbles signifies a positive outcome [14].

Oxidase test: This test's success relies on certain bacterial oxidase enzymes, which facilitate the transfer of electrons between electron donors inside the bacteria and a redox dye called tetramethyl-ρ-phenylene-diaminedihydrochloride. As a result of this electron transfer, the dye undergoes reduction and exhibits a distinct deep purple colouration.

A strip of filter paper was saturated with a small recently prepared reagent. Subsequently, the colony under examination was collected with a sterile wooden stick and gently spread across the surface of the filter paper. The presence of a good outcome was demonstrated by the emergence of a vivid and profound purple hue, observable within a time frame of 5 to 10 seconds [14].

Indole test: The peptone water medium tubes were infected with the colony of the bacteria under investigation and then incubated at a temperature of 37ºC for 18 hours. Following this incubation, a few drops of Kovac's reagent were introduced into the broth medium. The positive outcome of the experiment was determined by the presence of the red ring on the surface after agitation [21].

Citrate utilization test: The colony of the examined bacteria was used to inoculate the surface of the Simmons citrate slant medium, which was then incubated at a temperature of 37ºC for 1-3 days. The change in colour of the indicator from green to blue signifies the organism's ability to utilize citrate as the only source of carbon [21].

Urease test: Urease is an enzymatic catalyst that facilitates hydrolysis of the carbon-nitrogen bond present in amides, producing carbon dioxide, ammonia, and water. The urea-based agar was subjected to sterilization using an autoclave. Subsequently, it was cooled to a temperature of 50ºC, and urea substrate was introduced into the agar. The mixture was then put into sterile tubes. The agar was inoculated with a bacterial culture and incubated at 37ºC for 24 to 48 hours. Upon the breakdown of urea, the release of ammonia occurred, leading to an elevation in the pH of the medium. The pH shift was identified using a pH indicator, which exhibited a colour transition to pink in an alkaline environment. A pink medium was observed, indicating a positive result for the presence of urease. The absence of deep pink hue development was observed as a negative response.

Coagulase test: The test involves the utilization of rabbit plasma that has undergone vaccination with staphylococcus bacteria. The tube is subjected to incubation at a temperature of 37 °C for one hour and thirty minutes. The outcome is negative, with a duration of 18 hours. Conversely, if the outcome is positive, the plasma will undergo coagulation and manifest as aggregated fragments [22].

Antibiotic susceptibility testing

Disk diffusion test: The experiment was conducted with a sterile culture of a previously discovered bacterial strain. Determining the most effective Antibiotic for each bacterial isolate followed the suggested methodology outlined in the reference [23]. The inoculum for this experiment was created by introducing five isolated colonies cultivated on a blood agar plate into five millilitres of nutrient broth. The mixture was then incubated at 35 degrees Celsius for 18 hours. The resulting inoculum was subsequently compared to a (0.5) McFarland standard tube**.** A sterile swab was employed to collect an inoculum from the bacterial suspension, which was subsequently streaked onto a Mueller-Hinton agar plate and desiccated. In this experiment, the antibiotic discs were carefully positioned on the medium's surface at regular intervals using flamed forceps or a disc applicator. The samples were then incubated for 18 hours**.**

The measurement of inhibition zones was conducted by employing a ruler and then comparing the obtained results with the previously determined zones of inhibition as reported by [23].

RESULTS AND DISCUSSION

Isolation and identification of pathogenic bacteria

The present investigation encompassed a representative sample of adulterated cheese obtained from a local cheese establishment in Hilla. In the region of Babil Governorate in Iraq, a significant number of research studies have been conducted on the issue of cheese contamination. In the country of Iraq, namely in the region of Salahaldin, there is a significant presence. This study aims to isolate and identify harmful bacteria, specifically E. coli and Klebsiella, by appropriate laboratory techniques. A study conducted by Alubady (2010) in Mosul demonstrated that the contamination rate reached 100%. The majority of these contaminations were found to be associated with Enterobacteriaceae, accounting for approximately 80% of the cases. Approximately 16.66% of the cases were found to be associated with staphylococci.

This investigation involved isolating and identifying various bacterial species from a contaminated cheese sample. The discovered bacteria included *Staphylococcus aureus, Escherichia coli, Salmonella, Klebsiella, and Proteus mirabilis*. Gram-negative bacterial species, including *Escherichia coli, Proteus mirabilis, Klebsiella, and Salmonella*, were identified by cultivating on different culture media using the streaking method. The media utilized in this study encompass blood agar, selective-salt agar, MacConkey agar, eosin methylene blue agar, and nutrient agar.

A Gram-positive bacterium, specifically *Staphylococcus aureus*, was obtained by cultivating on a medium known as Mannitol salt agar. After plotting the sample on Macconkey medium, single, dry, circular pink colonies appeared fermented with lactose sugar. These characteristics apply to *E. coli* bacteria. Figure (1A) shows that the EMB medium was also used as a diagnostic medium when the sample was plotted on the medium, so bright green colonies appeared. This proves the presence of *E. coli* bacteria after diagnosing it on Macconkey's medium. As shown in Figure (1B)

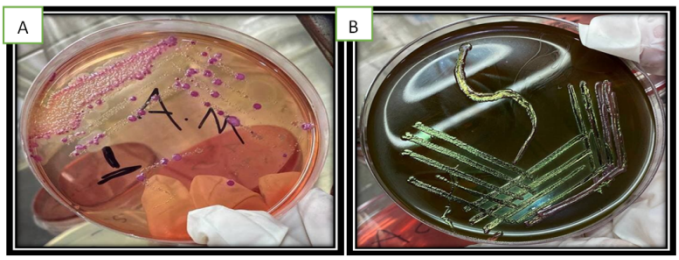

Figure 1. The growth of E coli on the different culture media: A-MaCconkey agar, B- EMB agar

Moreover, the sample was plotted on Macconkey medium and found pale, colourless, unfermented colonies of lactose. These characteristics apply to salmonella. As shown in Figure (2A), The sample was also planted on S.S. agar medium, and black colonies appeared, indicating the presence of typhoid bacteria (*salmonella*).

The reason for the appearance of *salmonella* bacteria in black colour on the medium is the presence of thiosulfate and iron citrate, and they have a role in revealing the ability of bacteria to produce H2S and thus, an interaction occurs between Fe and H2S; FeS produces iron sulfide, which has a role in the colour of black colonies. As shown in Figure (2B).

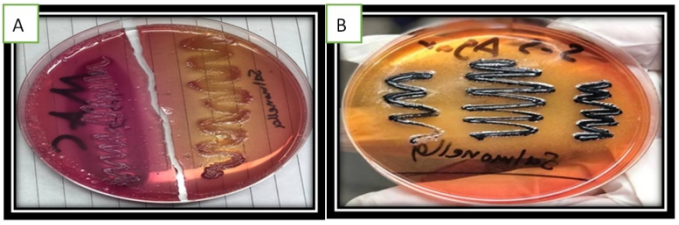

Figure 2. The growth of Salmonella typhi on the different culture media: A-MaConkey agar , B- SS agar

In addition, the material was grown on Macconkey's medium, forming colonies that exhibited a uniform violet colouration and possessed a wet and mucous consistency. The observed colonies were distinct from those of intestinal Escherichia coli bacteria and consequently identified as colonies of Klebsiella bacteria. As seen in Figure 3.

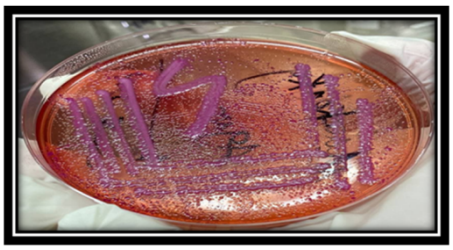

Figure 3. The growth of *Salmonella typhi* on MaConkey agar

Furthermore, the diagnostic culture media employed included a blood agar medium, facilitating observing swarm formation in bacteria, including Proteus mirabilis. As depicted in Figure 4.

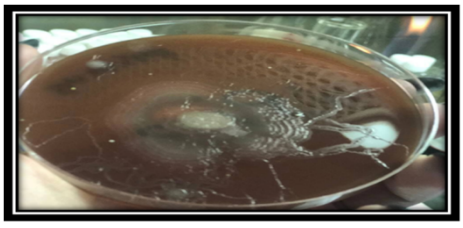

Figure 4. *Proteus miribilis* on Blood agar

Furthermore, a specimen was subjected to diagnosis through cultivation on a medium known as Mannitol Salt Agar. This resulted in the emergence of yellow colonies, indicating a shift in the pH of the medium from basic to acidic due to the fermentation of Mannitol sugar. This characteristic is commonly observed in Staphylococcus aureus, As depicted in Figure 5.

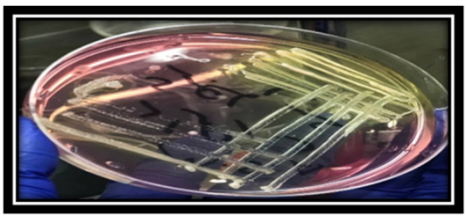

Figure 5. *Staph.aureus* on mannitol salt agar

Biochemical characterization

The initial isolate exhibited negative results for Citrate and Coagulase but positive results for Indol consumption, catalase activity, oxidativease activity, Urease production, and H2S production. These findings suggest that the isolates are likely derived from Escherichia coli. Several isolates were recognized as Proteus mirabilis and exhibited good results for citrate utilization, urease activity, catalase production, and hydrogen sulfide (H2S) production. However, these isolates tested negative for indole production, oxidase activity, and coagulase production. In addition, The isolates exhibited positive results for Salmonella identification through tests for indole, urease, oxidase, and coagulase but were positively identified through citrate, catalase, and H2S tests show in table (1) [26, 27]. This investigation revealed that the predominant intestinal bacteria identified were *E. coli, Salmonella spp., and Klebsiella spp.* Prior research has also indicated that coliform bacteria are more prevalent in milk and milk containers due to their abundance in animal hosts and the surrounding environment [28, 29]. The primary microbiological hazards linked to ingesting dairy products derived from unpasteurized Milk or cow's Milk that has been contaminated post-pasteurization are *E. coli and Salmonella spp*. This is particularly prevalent in underdeveloped nations with inadequate hygiene protocols. The user did not provide any text to rewrite [30].

In summary, this study presents findings indicating that milk and dairy products are vulnerable to several pathogens, such as *Salmonella spp., S. aureus, E. coli, Klebsiella pp., and Proteus spp.* Several factors contribute to the occurrence and abundance of pathogens in Milk and its products. These factors include the size of the farm, the number of livestock present, the level of hygiene during the milking process, the practices followed in farm management, the sanitation measures employed during processing, post-processing, and transportation, and the geographical location and season. The user's set of numbers consists of three elements [31, 32, 33]. Milk and dairy products can be contaminated by antimicrobial-resistant (AMR) microorganisms that originate from several sources, posing a possible danger of contamination. The sources considered in this study include the animals themselves, filthy milk containers, persons involved in milk handling, and airborne dust and droplets that may be present during production and processing [33].

Table 1. Results of biochemical tests of some pathogenic and contaminating bacterial species of milk products

Bacteria	Indol	citrate	Ureaes	Catalase	Oxidase	Coagulase	H2S
E.coli	+	-	+	+	+	-	+
Proteus mirabilis	-	+	+	+	-	-	+
klebsiella	-	+	+	+	+	-	+
Staph. aureus	+	+	+	+	-	+	-
Salmonella	-	+	-	+	-	-	+

The user's content needs more information or context to be rewritten academically. Therefore, it is crucial to address and control the prevalence and spread of multidrug-resistant microorganisms that can be transmitted via animal or animal-derived products, such as Milk and meat. Furthermore, it is crucial to maintain the effectiveness of currently available antibiotics.

Antibiotic sensitivity towards the Pathogenic Bacteria Isolated from Milk products

Furthermore, this study involved an assessment of bacterial susceptibility to antibiotics. To evaluate this, three specific antibacterial agents, namely ciprofloxacin, Amikacin, and Gentamycin, were administered to staphylococcus bacteria. The bacterium aureus exhibits greater sensitivity to the antibiotic ciprofloxacin than Amikacin and Gentamycin. Conversely, *E.coli* bacteria demonstrate equivalent effectiveness in response to ciprofloxacin, Amikacin, and Gentamycin, indicating that E.coli is susceptible to these antibacterial agents. As depicted in Figures 5A and 5B, the researcher employed pseudomonas bacteria in the study. Notably, this strain of bacteria resisted various antibacterial agents, including ciprofloxacin, Amikacin, and Gentamycin. As depicted in Figure 5C.

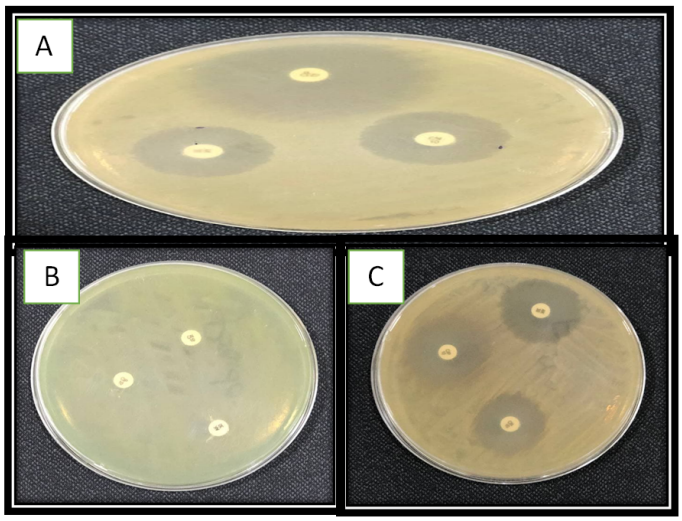

Figure 5. Antibacterial activity of Some antibiotics on pathogenic bacteria Isolated from Milk product, A: *Staphylococcus aureus, B: E coli, C: Pseudomonas*

Several prior investigations conducted in various regions across the globe have documented elevated numbers of multidrug-resistant (MDR) bacteria [34, 35, 36], as well as bacteria that produce extended-spectrum beta-lactamases (ESBLs) [37, 38]. The elevated levels of resistance identified in this study may be attributed to the improper utilization of antibiotics within dairy farming practices. The findings indicate that the utilization of these antibiotics is prevalent throughout the geographical scope of the study. The presence of considerable levels of drug resistance presents a noteworthy public health concern due to the challenging nature of treating foodborne outbreaks and the fact that this group of multidrug-resistant organisms (MDRs) within the food supply serves as a reservoir for disseminating resistance genes. The prevalence of antibiotic resistance rates for affordable and commonly accessible antibiotics is a cause for concern among low-income groups residing in developing nations, such as Ethiopia, due to the restricted accessibility and exorbitant expenses associated with freshly created pharmaceuticals [39 ,40].

Conclusion

Certain pathogenic bacteria have the potential to infect cheese, particularly when it is stored in unsanitary and unhygienic environments.

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