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Protective effect of probiotic (*Lactobacillus casei*) against *Escherichia coli* causing diarrhea

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Abstract:

Based on the significant impact of *L. casei* against *E. coli* obtained from diarrheal case in several Baghdadi hospitals (Yarmouk and Mahmoudia), this study was conducted. Every sample that was grown on MacConkey agar, Eosine Methylene Blue, and Blood agar, as well as *E. coli*, was identify using Gram stain and biochemical testing. Forty-five isolates of the fifty diarrheal samples had positive *E. coli* results. Sensitivity tests were performed on the four *E. Coli* isolates that were obtained and came back positive *Lactobacillus casei* isolates were isolated and cultur in Man Rogosa Sharpe broth at 37°C for 24 hours, followed by a 24-hour incubation under CO2 (5–10%) and a reculture on MRS agar. The isolates were tested against E. coli using the agar well diffusion assay, and the diameter of the halo zone surrounding bacterial growths was measured.

Keywords:Lactobacillus casei; Escherichia coli; Probiotics; Diarrheal infections; Antibacterial activity

Introduction:

Gram-negative, mobile, facultatively anaerobe, and rod-shaped, *Escherichia coli* is among the most prevalent nosocomial pathogens that can cause bloodstream infections (BSI), stomach infections, and urinary tract infections. (Li *et al*., 2021). *E. coli* can grow quickly, with optimal conditions allowing for replication in approximately 20 min. Numerous gene editing systems have been established use *E. coli* as the bacterium, leading to the production of countless industrial products and enzymes. The genetic sequence of *E. coli* was one published in 1997, and since that time, over 4,800 genomes of *E. coli* have been sequenced. Its rapid growth characteristics make *E. coli* a valuable model for studying the evolution of microorganisms, and there is an ongoing long-term experimental evolution study that has included more than 50,000 generations (Tenaillon *et al* .,2016). recognized as a component of the intestinal flora, but it can be the source of human intestinal and extra intestinal diseases. Many strains of *E. coli* have been identified, leading to a variety of illnesses, which can range from mild gastroenteritis that resolves on its own to severe conditions like septic shock and kidney failure. Due to its virulence,

E. coli can evade the body's defense mechanisms and develop resistance to conventional antibiotics (Mueller & Tainter., 2023) . Antigenic typing of *E. coli* is conducted based on their profiles of somatic, flagellar, and capsular antigens. Over 180 O, 60 H, and 80 K antigens have been suggested (Wilczy nski *et al*.,2022). .The DEC pathotypes are divided into enteropathogenic , enterohemorrhagic, enteroaggregative, .enterotoxigenic ,and enteroinvasive *E. coli* based on their preferred sites of colonization by hosts, mechanisms of virulence, and the clinical signs and consequences that ensue (Gomes *et al.,* 2016). ETEC is frequently detected in food and water in places with poor sanitation, and it causes watery diarrhea in environments with minimal resources. To make a healthy individual sick, about 100,000,000 organisms must be consume. It is one of the most significant organism that causes diarrhea in travelers. In environments with minimal resources, ETEC also plays a major role in dehydrating diarrheal disease in infants and children. (Kotloff *et al* .,2013) Shiga toxins and heat-labile or heat-stable enterotoxins cannot be produced by EPEC, which are diarrheagenic of *E. coli* that can cause A/E lesions on the intestinal. Because these strains can form A/E lesions, they can adhere firmly to the intestinal epithelial cells' surface and induce localized lesions. (Mare *et al* .,2021). EPEC is commonly related to inducing intense watery diarrhea in infants from developing countries, as well as in adults who visit areas where bacterial diarrhea is common. In adults, diarrhea caused by EPEC manifests as watery stools (occasionally accompanied by vomiting) and is often associated with a mild fever. If not treated, this illness can last for as long as 120 days (Dupont., 2016). In both resource-rich and resource-limited areas, EAEC is the causal organism of both acute and chronic watery diarrhea. Lately, it has also been more frequently linked to traveler's diarrhea. (Mueller & Tainter., 2023). The STEC pathotype is still important and linked to human life-threatening illnesses, primarily hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC).. Shiga toxins are a collection of bacteria that generate one or more forms of Stx, also referred to as verotoxin or VT, and the STEC stand for verotoxigenic *E. coli*, or VTE. The STEC are significant in human healthcare because they lead to food- or water-borne bloody diarrhea (known as hemorrhagic colitis [HC]) and can result in a more severe condition, hemolytic uremic syndrome (HUS), which involves acute kidney failure, low platelet counts, and microangiopathic hemolytic anemia, with a higher prevalence in children under 5 years old and elderly individuals (Kim *et al*., 2020). Stx are divided into: stx1 and stx2, which are encoded on a prophage This practice should be avoided, since Shiga toxins 1 and 2 (Stx1 and Stx2) may also be generated in strains that exhibit other features associated with enteroaggregative *E. coli* (EAEC) (Frank *et al*., 2011; Mühlen and Dersch, 2020) .Attachment and Effacement (Intimin) The attaching and effacing (AE) pathogens, which include enteropathogenic E. coli (EPEC) and enterohemorrhagic *E. coli* (EHEC), constitute a noteworthy group of pathogenic *E. coli* that contribute significantly to global disease prevalence. The unique characteristics of A/E lesions involve the adjacent attachment of pathogens to the gut epithelial surface, the effacement of villi, and the rearrangement of actin filaments directly underneath the attachment site in the intestinal cells, resulting in the development of pedestal-like formations. This process activates intense actin

polymerization, leading to the creation of these pedestal-like structures (Martins *et al*.,2020). Enteroaggregative *E. coli* (EAEC) Enteroaggregative E. coli is the most commonly identified of diarrheagenic *E. coli*. EAEC is increas recognized as a novel gut pathogen that contributes to prolonged diarrhea and poor nutrition in youth and individuals with HIV in developed nations. It ranks as the following cause of traveler's diarrhea and is frequently responsible for acute diarrheal cases in both children and adults visiting emergency departments and hospital units across the USA. These bacteria are *E. coli* that do not produce heat-labile enterotoxin or Shiga toxins but adhere to cultured cells in self-aggregating formations referred to as "stacked bricks." Food serves as a means of transmission for EAEC infections, and notably, EAEC was found to be more commonly isolated from food sources compared to other bacterial pathogens, including ETEC, diffuse adherent *E. coli* (DAEC), and adherent-invasive *E. coli* (AIEC) are notable types. Although it is commonly found as a commensal organism, *E. coli* also serves as a significant pathogen in both humans and domestic animals (Nascimento *et al*.,2022). Diffusely adherent *Escherichia coli* is recognized as a diarrheagenic category of *E. coli* (DEC). Furthermore, recent research indicates that DEP has appeared as a initial factor of diarrheal illness in hospitalized youth. DAEC and EAEC are the most common pathogenic types, exceeding the prevalence of Salmonella and Shigella (Patzi-Vargas *et al.,* 2015). Globally, diarrheal illnesses continue to be a significant source of illness and rank as the eight leading cause of fatality among children under five in less developed areas (Ugboko *et al*., 2020) It has the ability to colonize and induce disease in locations beyond the intestinal ; these variants, known as extraintestinal pathogen are significant contributors to conditions such as wound infections, renal tract infections, peritonitis, pneumonia, meningitis, and septicemia. The ExPEC category features specific form , including uropathogenic *E. coli* (UPEC), neonatal meningitis-associated *E. coli* (Sora *et al*.,2021). The pathogenicity factors of uropathogenic *Escherichia coli* (UPEC) that be involved in the development of renal tract infections primarily consist of fimbriae that facilitate adherence and invasion of host cells, toxins that impact host cells, and systems for acquiring iron necessary for bacterial growth. Additionally, the production of enterohemolysin is considered a potential pathogenicity factor for enterohemorrhagic *E.coli* , also known as EHEC-hemolysin (EHEC-hly), which is identified by the presence of partial lysis zones on blood agar containing anticoagulant sheep red blood cells(Schwidder *et al*., 2019). Lipopolysaccharide is the primary constituent of the outer layer in Gram-negative bacteria, playing a key role in maintaining the bacterial structure and offering protection against various chemical attacks. LPS serves as the predominant antigen on the surface of most Gram-negative pathogen, accounting for as much as 80% of the outer membrane in organisms such as *E. coli* and Salmonella. This complex glycolipid features three main structural elements: lipid A, oligosaccharide, and the O Ag.Additionally, the components of LPS include the polysaccharide (O antigen), the oligosaccharide core, and lipid A(Avila-Calderon *et al.,* 2021). Gram-negative pathogen are encased by both an inner layer and an outer layer. The outer layer is distinctive due to its lipid composition and asymmetrical arrangement, with the inner leaflet comprising phospholipids,

while the outer leaflet is made up of lipopolysaccharide (LPS), a highly negatively charged molecule that extends into the surrounding bacterial environment .In EHEC, outer membrane proteins (OMP) have been recognized as strong immunogens and play a crucial role in the bacteria's pathogenicity (Horne *et al.,* 2020). The antimicrobial susceptibility of *E. coli* sample was assessed following the guidelines set by the Antibiogram Committee for Microbiology/European Committee on Susceptibility Testing (CA-SFM/EUCAST) through the disk diffusion technique. The observed use of antimicrobials and the linked resistance patterns in farm animals vary globally, with European AMR surveillance data revealing significant vairation between countries in terms of their antimicrobial consumption and the prevalence of AMR in key indicator bacteria (Österberg *et al.,* 2016) Antibacterial resistance in *E. coli* is consistently most significant for antibacterial agents that have been utilized for the longest term in humans, like ampicillin. Nevertheless, over the last twenty years, there has been a notable rise in the emergence and propagation of cross-resistant pathogen , including pathogen that are resistant to more recent antibiotics such as fluoroquinolones and extended-spectrum cephalosporins (Silva *et al.,* 2020).

Probiotics as observed living , non-pathogenic bacteria that which aidin the host's well being when given in adequate quantities (Mishra &Acharya.,2021). In a generally healthy population, it's important to address safety profile concerns related to the use of living microorganisms. Many types of lactic acid payhogen , bifidobacteria, and yeasts, which account for the majority of present probiotic , are considered harmless for inclusion in foods and supplements because they come from genes and species with a safety record (Bourdichon *et al*., 2018). As a result, significant measures have been undertaken to create alternatives to antibiotic growth promoters (AGP) as feed addition, such as essential oil , fermentation liquid feed, organic acid, probiotic, and prebiotic. Research has indicated that probiotics can stimulate growth, thus improving animal production by boosting feed intake, conversion efficiency, and overall body weight (Dittoe *et al.,* 2018). In healthy individuals, the microbiota and host coexist in a symbiotic relationship that influences host health by controlling nutrition metabolism, defending against infections, and delivering signals to immune cells to enhance host physiology and immunity (Oniszczuk *et al*.,2021). Variability in health outcomes is often seen in clinical studies involving probiotics for dietary or supplement applications. Additionally, probiotics must operate effectively within the intricate microbiome, and the differences in individuals' microbiota present a challenge for achieving consistent probiotic effects across various populations. Recently, new methods for stratification and personalized nutrition have been suggested to enhance this situation (Veiga *et al.,* 2020). The most microorganisms Lactobacilli spp and Bifidobacteria. (Zhang *et al*., 2016). Particularly, *Lactobacillus* is a significant probiotic bacterium that plays key roles in immunomodulation in the intestinal mucosa. medical and practical research on probiotic *Lactobacillus* has show that these bacteria effective prevent and treatment antibiotic-associated diarrhea, traveler's diarrhea, and intestinal pathogen infections. Probiotics also have immunomodulatory effects and reduce inflammation in certain pathological

conditions like necrotizing enterocolitis and allergies. Rocha *et al*., 2017). Typically containing less than 50 % DNA G+C, lactobacilli are rod-shaped, gram-positive, facultatively anaerobic or microaerophilic, non-spore-forming, acid-tolerant, and catalase-negative bacteria. Because of their close phylogenetically and phenotypic relationships, *Lactobacillus casei, Lactobacillus paracasei*, and *Lactobacillus rhamnosus* are collectively referred to as the *L. casei* group. Members of this group share the same peptidoglycan types, are facultatively hetero fermentative, and contain 45–47 % DNA G+C. (Huang *et al.,*2018). The manipulation of gut flora is intricate and may lead to relationship between pathogen and the host; although probiotics are generally regarded as harmless , oral administration carries a risk of viable bacteria migrating from the gut tract to internal organs ,which can result in bacteremia and potential negative health effects (Durchschein *et al*., 2016; Yao *et al*., 2020).*L. casei* enhanced its capacity to adherence to mucin and intestinal epithelial cells in vitro after undergoing gut conditions, which may be linked to the intiation of Exopolysaccharides (EPS) synthesis. Proteomic analyses indicate that gastrointestinal stress prompted alterations in the expression of enzymes involved in EPS production, potentially accounting for the observed differences in the adhesion characteristics of lactobacilli. The prebiotic effect of EPS derived from *L. casei* strain has also been demonstrated. Prebiotics are characterized as substrates that are selective utilize by host oorganisms, providing a health promoting effect (Bengoa *et al*.,2021). The innate immune system can partially eradicate infections through various immune cells, including gamma delta T cells, natural killer cells (NKs), macrophages, and dendritic cells (DCs), which then present the pathogens to lymphocytes. Additionally, innate immune cells are capable of priming and enhancing the adaptive immune response through surface molecules and secreted cytokines. The adaptive immune system plays a crucial role in eliminating infections by activating cytotoxic T lymphocytes (CTLs) and humoral immune responses, as well as regulating the innate immune mechanism through feedback. In this overview, we will explore the cellular and molecular reaction and the roles of both the innate and adaptive immune systems in combating infections (Han *et al.*, 2020).

Materials and methods:

Samples of collection from human:

Over the course of two months, 50 specimen were taken from human diarrhea of bot sex category from patients at two hospitals (Al-Yarmouk and AL-Mahmoudia) as well as from patients with clinical symptoms (diarrhea, and anorexia).

Isolation and detection of *E.coli*

A circuit of each liquid media was then streaked across the surface of MacConkey and blood agar, and the mixture was incubation for 24 hours at 37 C. Additionally, biochemical assays (IMVC) and TSI were performed.

Microscopic examination:

A sterile loop was used to pick each individual colony from the broth, and a smear was prepared on a microscopic slide, which was then fixed and stained using a Gram stain kit. The

arrangement, size, and shape of the cells were examintion under microscope with an oil immersion at 100X magnification (Murray *et al*., 2020). .

The antibiotic disks susceptibility testing:

The agar disc diffusion assay was used to test for antibiotic disc sensitive in accordance with (Bauer -Kirby *et al*.,1966) .as follows: The surface of the infected Muller Hinton agar plate was covered with the antibiotic discs. To guarantee full contact with the agar surface, each disc was gently pushed using heated and chilled forceps. After being inverted, the plates were kept in an incubator set to 37 C˚ for the whole night. Using a measuring caliper, the clear zones created by antimicrobial suppression of pathogen growth were measure in millimeters use the third chaplet: Techniques 43 Disc diffusion technique values were interpreted and classified as susceptible, intermediate, and resistant in accordance with the Clinical and Laboratory Standard Institute's criteria 2013 (Weinstein *et al*., 2019).

***Lactobacillus casei* prepration**

It was made by inoculating the MRS broth with 20 mg of commercial *Lactobacillus casei* powder and adding Tween 80 after 48 hours. The broth was then collected using a sterile loop, and the bacteria was cultivated on MRS agar to determine its identity and conformation.

Susceptibility test of *Lactobacillus casei* against *Escherichia coli*

The purpose of the agar well diffusion method was to assess how lactic acid suppressed the proliferation of *E. coli*. This approach was modified from (Bajpai *et al*.,2016).

1-*Escherichia coli* were cultured in nutrient broth.

2-In order to prepare cell-free supernatant .each probiotic (CNCM I-1572) was cultivated in MRS broth by adding Tween 80 for 24 h at 37°C. CFS was obtained by centrifuge the cultured at 1200 rpm for 20 min and sterilized by filtr using 0•20-µm porous membranes (Sigma, St. Louis, MO). Inhibition activity of CFS probiotic lactobacilli was investigated by well diffusion assay (Mami *et al*., 2008).

3. Following a 24-hour incubation period, transfer 0.1 ml of the nutrient broth containing *Escherichia coli* to the Muller Hinton agar. Using a swab ,evenly distribute the pathogen across the media. The plates were then incubated for two hours at 37°C. The dishes were then removed from the incubator and pierced multiple times (6 mm) with a sterile cork borer before the drilled pieces wereremoved.

4-100 µl of *Lactobacillus casei* extract was added to the holes in the agar, and the plates were placed in the refrigerator for two hours before being incubated for twenty-four hours at 370C. The diameter of the inhibitory region was then measured.

Result and Discussion

Isolation and detection of *Escherichia coli* from human suffering diarrhea.

Fifty specimen from human having diarrhea were examination. The pathogen isolation demonstrated that (50) of the samples were associated to the *Escherichia coli* (Table 4-1).

Source	**Number. of total samples**	**Number. of *E.coli* isolate**
Diarrhea samples	50	45

Isolation *E.coli* on Blood agar:

The *E. coli* isolates were observed on blood agar to check for hemolysis after being incubated for 24 hours at 37°C. All the isolate formed colony that exhibited gamma-hemolysis clearly identifiable by examining the look of colonies that are large, round, gray, and damp. figure (3-1). This result agrees with ((Basavaraju& Gunashree, 2023).

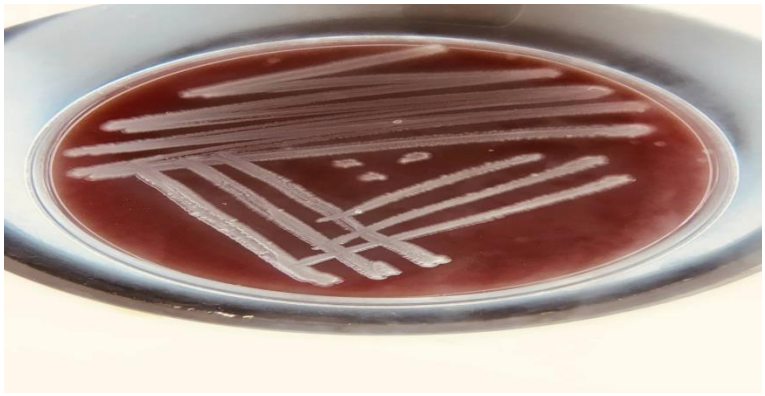

Figure (3-1): *E.coli* on Blood agar

Isolation of *E.coli* on MacConkey and EMB agars

All strains of *E. coli* produced a rose color with smooth borders that are convex and round on MacConkey agar can function to selectively cultivate gram-negative bacteria due to their fermentation of lactose, resulting in acidic by-compound that decrease the pH, causing the pH to turn rose . The secondary phase involved testing the *E. coli* isolate on Eosin Methylene Blue agar, which is utilized to differentiate *E. coli* from alternative Gram-negative pathogen, revealing a metallic green colony on the medium resulted in the formation of green metal pigments recognized as a round, slick colonies. Fig (3-2). The finding agree with (Alaa 2019).

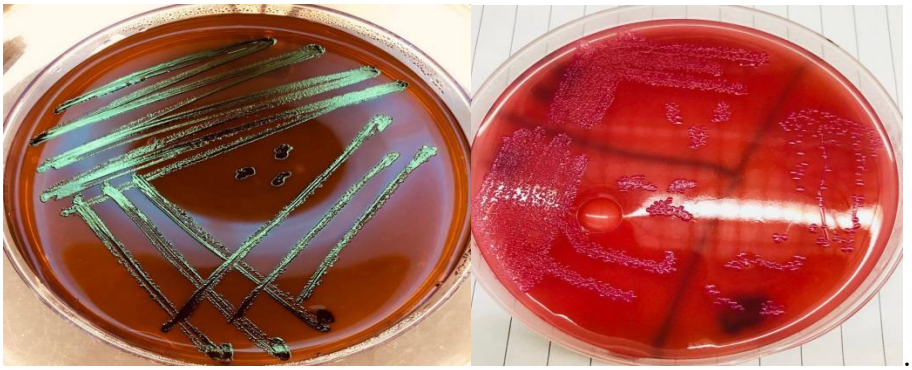

Figure (3-2): *E.coli* on Maconkey agar appearance pink color and EMB agar appearance as green metallic sheen colonie

Microscopic examination:

The *E. coli* isolate was observed under a light microscope as Gram-negative, shaped like short rods, non-spore forming, and appeared as single cells as well as in pairs figure (3-3).

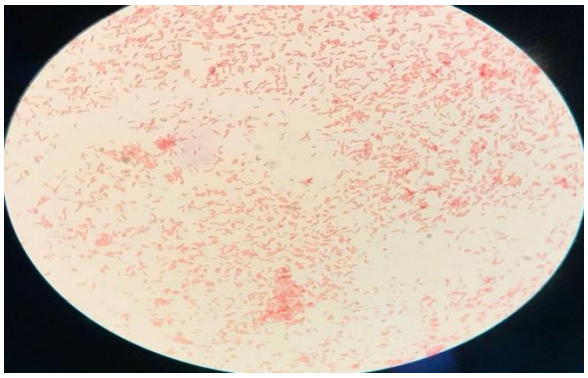

Figure (3-3): Microscopic section of *E.coli* under microscope appears as short rod, red color, single cell.

Biochemical test results:

The biochemical examination yielded positive results for indole, showing a red ring after the addition of Kovac's reagent. Methyl red also turned red upon adding the methyl red reagent, and catalase produced bubbles after the introduction of H2O2. On the other hand, the tests for Voges-Proskauer, Simmons citrate, and oxidase returned negative results. In the Triple Sugar Iron (TSI) agar test, the results indicated an acid-acid (yellow-yellow) reaction on together the slant and the bottom, accompanied by gas bubbles but no production of H2S ,Figure(3-4). Which agree with (Mahe *et al*.,2021).

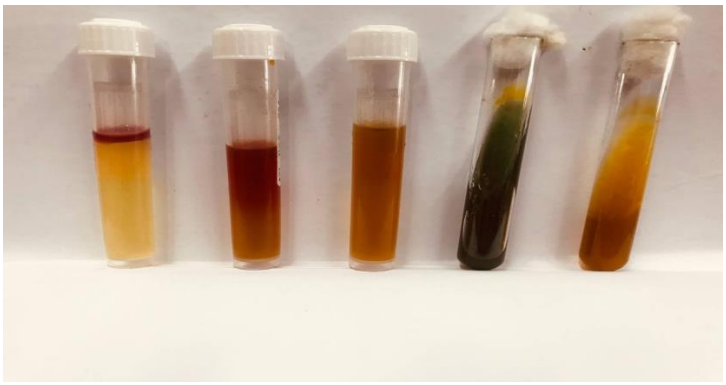

Figure (3-4) Biochemical test of *E.coli*

Antibiotic sensitivity of *E.coli* isolates:

Using the disk diffusion assay , fifty samples of *Escherichia coli* were analyzed for their sensitivity to ten different antibiotic discs. All of the tested *Escherichia coli* strains exhibited significant resistance effective toward Tetracycline, Ampicillin, Nalidixic acid, and Nitrofurantoin, while showing high sensitivity to Gentamicin, Ciprofloxacin, Chloramphenicol, and Ceftriaxone. Additionally, the isolates demonstrated intermediate susceptibility towards Cefotaxime, Amikacin figure (3-5).This results agree with (Silva *et al*., 2020). They discovered a significantly frequent occurrence of various classes of antimicrobial agents, including Penicillins, Aminoglycosides, Phenicols, Tetracyclines, and the combine of sulfamethoxazole-trimethoprim, in E. coli strains isolated from humans.

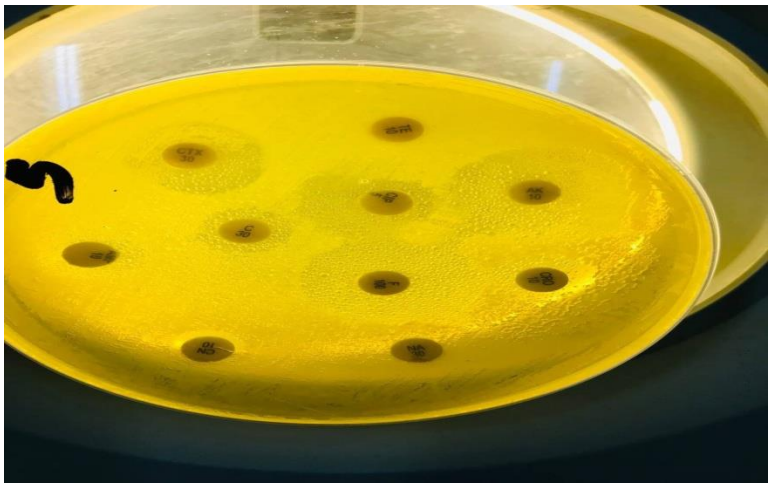

Figure (3-5): Antibiotic susceptibility test against *E. coli*

Identification of *Lactobacillus casei:*

Isolates of *Lactobacillus casei* were culture on MRS agar for 24 hours at 37°C with 5–10% CO2. The colories had a round, offwhite or milky appearance, with flat edges and a glossy, flat surface As showed in Figure (3-6). Which were in agreement with (Carvajal *et al.,* 2023) The bacterial isolates were analyzed in terms of culture characteristics and macroscopic findings. It was discovered that the majority of the isolates produced off-white pinhead colonies, which are indicative of *Lactobacillus* species.

Figure (3-6): *Lactobacillus casei* colonies on MRS agar

Microscopical examination:

outcome of microscopic analysis of *Lactobacillus casei*, which showed up as non-spore-forming, Gram-positive bacilli with a long or short rod shape and chain They possess a robust peptidoglycan layer that enables them to retain crystal violet after being washed with ethanol, resulting in a blue stain from the crystal violet. As showed Figure (3-7). These results agree (Hendronoto *et al* .,2017) and have been extensively utilized as competitive exclusion agents to combat pathogenic bacteria, and it has been proposed that they provide additional advantages to host tissues, and they have been characterized as probiotic bacteria.

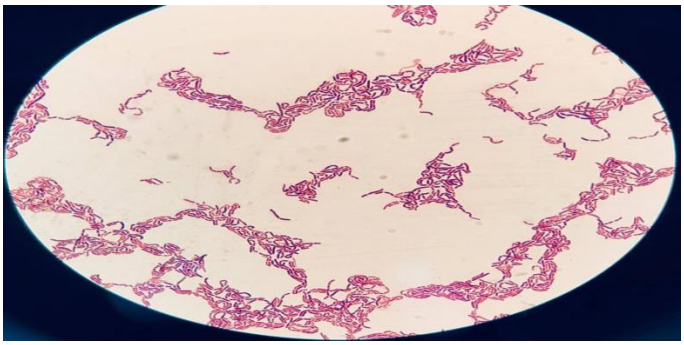

Figure (3-7): *Lactobacillus casei* under microscope

Suppressive effects of *E. coli* against *Lactobacillus casei* growth ex vivo

The concentration of *Lactobacillus casei* (1.5×10^8 CFU/ml) determined the mean zone width of inhibition (Figure 3-8). The outcome demonstrated the growth-inhibitory impact of *Lactobacillus casei* bacteria on the development of *E. coli* isolated from diarrhea- by showing that *E. coli* did not grow surrounding the wells containing *Lactobacillus casei* Which were in agreement with (Abolfazl *et al.,*2015). In their research, they discovered that four species of Lactobacillus, namely *L. fermentum, L. paracasei, L. plantarum, and L. rhamnosus,* exhibited antimicrobial effects against diarrheagenic *E. coli*..

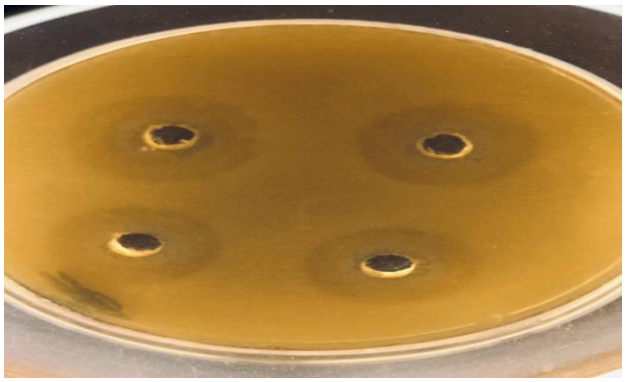

Figure (3-8): Inhibitory effect of *Lactobacillus casei* against *E. coli* in Muller Hinton agar.

Conclusions

1. This study's findings revealed that *Lactobacillus casei* has a pronounced inhibitory effect on the growth of *Escherichia coli* isolate from patients with diarrhea, highlighting its potential as a natural antimicrobial agent .
2. *E. coli* isolate were effectively detection using selective media (MacConkey, EMB, Blood agar), microscopy, and biochemical testing, with a high occurrence rate (45 out of 50 samples).
3. The *E. coli* isolates exhibited significant resistance to commonly prescribed antibiotics such as Tetracycline and Ampicillin, underscoring the necessity for natural alternatives like probiotics.
4. The well diffusion assay demonstrated that the cell-free supernatant from *L. casei* exhibited a significant inhibition zone, indicating its potent antibacterial effects against pathogenic *E. coli* .

5. This study suggests that the probiotic *Lactobacillus casei* may serve as a valuable preventive and treatment strategy for diarrheal illnesses, especially those linked to antibiotic-resistant strains of *E. coli.*

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Declaration of Competing Interest:

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

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