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Antibacterial Analysis of Kefir Milk Supernatant and Aloe Vera Gel against Opportunistic Skin Pathogens

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Abstract

Opportunistic skin infections, which disrupt skin barriers in immunocompromised patients, cause over 50,000 deaths annually worldwide. With the rise of multidrug-resistant bacteria, medical facilities, pharmaceutical companies, and governments urgently seek solutions. Therefore, this study evaluates the antibacterial effects of Aloe Vera gel solution (AGS) and Kefir milk supernatant (KMS) against opportunistic bacteria causing skin infections. Pre-identified bacterial cultures were obtained from a lab, and were confirmed through Gram staining, biochemical assays (TSI, citrate utilization, urease, nitrate reductase), tube coagulase, catalase, and oxidase tests. AGS was prepared by mixing 1 mL of gel with 8 mL of sterile water, while KMS was obtained by fermenting kefir grains in milk centrifuging 5mL kefir and collecting the supernatant. Antimicrobial testing was conducted using Mueller-Hinton agar, with bacterial lawns matched with 05% McFarland standards exposed to 0.1 mL of AGS and KMS for 24 hours at 37°C, after that inhibition zones were measured. Gram-positive organisms included *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Bacillus subtilis*, *Corynebacterium diphtheriae* and *Staphylococcus epidermidis,* while gram-negative microbes included *Citrobacter freundii*, *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella oxytoca*, *Proteus mirabilis, Morganella morganii*, and *Pseudomonas aeruginosa*. The antimicrobial assay revealed that KMS exhibited effectiveness against *Streptococcus pyogenes, Enterobacter aerogenes, Escherichia coli*, and *Providencia stuartii* as indicated by inhibition zones of 19 mm, 15 mm, 13 mm, and 12mm respectively. AGS demonstrated antimicrobial activity against *Bacillus subtilis, Providencia stuartii,* and *Staphylococcus aureus*, with inhibition zones of 15 mm, 20 mm, and 15 mm, respectively. However, both KMS and AGS were found to be ineffective against *Staphylococcus epidermidis, Pseudomonas aeruginosa, Klebsiella oxytoca, Citrobacter freundii, Corynebacterium diphtheriae, Proteus mirabilis,* and *Morganella morganii.* The study demonstrates that KMS and AGS significantly inhibited skin opportunistic pathogens. Future research should optimize dosages to augment the efficacy and investigate the potential synergistic effects for resistant pathogens.

Keywords: Opportunistic pathogens, skin infections, Kefir Extract, Aloe Vera gel, Antibiotic resistance

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1. INTRODUCTION

Opportunistic pathogens are microorganisms that typically do not cause disease in healthy individuals but can lead to infections when the host's immune system is compromised [ ]. Skin infections caused by these pathogens are a significant concern, particularly among individuals with weakened immune systems due to conditions such as HIV/AIDS, diabetes, cancer, or those undergoing immunosuppressive therapies. Common opportunistic pathogens responsible for skin infections include S. aureus, particularly methicillin-resistant Staphylococcus aureus (MRSA), Pseudomonas aeruginosa, Candida spp, and certain dermatophytes. The prevalence of opportunistic skin infections varies widely based on the population and underlying conditions [1].

MRSA and P. aeruginosa are major concerns in healthcare settings, particularly for patients with compromised skin integrity. While MRSA is more prevalent in individuals with chronic wounds with prevalence rates ranging from 1% to 5% in the general population [2], P. aeruginosa frequently infects patients with burns or chronic ulcers, with infection rates reaching 20-30% in these groups [3]. Similarly, Coagulase-negative Staphylococci cause severe infections, particularly in those with indwelling medical devices. Staphylococcus aureus is a common cause of skin and soft tissue infections, with an estimated carriage rate of 11% in the general population, with prevalence rates varying by region, healthcare settings, and patient populations. In healthy individuals, S. aureus infections can cause conditions such as impetigo, cellulitis, and abscesses, which are typically manageable with prompt treatment. However, in immunocompromised patients, the prevalence and severity of S. aureus infections are significantly higher about 25% [4].

Pseudomonas spp. and Klebsiella spp., although found less frequently in healthy individuals, are major concerns in hospital settings, especially among immunocompromised patients. They can lead to severe complications such as sepsis, surgical wound infections, or burn wound infections. The prevalence of these infections ranges from 7.1% to 7.3% for Pseudomonas spp. and from 18.8% to 87.7% for Klebsiella spp [5] [6]. E. coli in immunocompromised patients can cause cellulitis, abscess formation, and, in severe cases, bacteremia. About 30-32% lead to systemic infections that are challenging to manage in immunocompromised patients [7]. Proteus spp. is also the causative agent of skin abscesses of the axilla in both healthy and immunocompromised patients [8]. Similarly, Enterobacter spp. and Citrobacter spp. also causehospital-acquired infections, including bloodstream infections, pneumonia, and complicated UTIs. Enterobacter spp., in particular, accounts for 1-5% of nosocomial bacteremia cases. Immunocompromised patients are at higher risk of severe complications, including sepsis, multi-organ failure, and increased mortality, due to these infections [9].

Antimicrobial agents have been used to treat these infections. . However, rising antibiotic resistance patterns are becoming a significant global health concern. Coagulase-negative Staphylococci, often implicated in nosocomial infections, have exhibited increasing methicillin resistance. Beta-hemolytic Streptococci, while traditionally susceptible to penicillin, are now showing resistance to macrolides and clindamycin. Staphylococcus aureus, particularly MRSA, remains a critical concern due to its resistance to multiple drug classes, including beta-lactams. Pseudomonas spp. and Klebsiella spp. have shown alarming resistance to carbapenems, leading to limited therapeutic choices and higher mortality rates. Enterobacter spp. and Citrobacter spp. are increasingly resistant to third-generation cephalosporins due to the production of extended-spectrum beta-lactamases (ESBLs) [10]. Bacillus spp., though less commonly pathogenic, has displayed resistance to vancomycin, raising concerns for patients with indwelling devices. E coli and Proteus spp. reveals the rising resistance to fluoroquinolones and beta-lactams. These resistance patterns are contributing to increased healthcare costs, prolonged hospital stays, and higher morbidity and mortality rates, underscoring the urgent need for robust antimicrobial stewardship and novel therapeutic approaches [11].

Given the alarming rise in antibiotic resistance, there is an increasing demand for alternative therapies. Probiotics, herbal remedies, and natural substances like Aloe Vera gel and kefir milk present promising solutions in this context. Both the kefir and aloe vera have long histories of use in traditional medicine for their therapeutic properties, including antimicrobial and anti-inflammatory effects. Aloe Vera is the most

widely used species of Aloe among more than 360 species in both traditional medicines. It comprises prostaglandins, gamma-linolenic acid glycoprotein, anthraquinone glycosides, and mucopolysaccharides [12]. Numerous researches indicated that this plant can used to heal wounds, burns, sunburns, and inflammatory skin conditions [13]. Furthermore, it can inhibit various skin pathogens including Staphylococcus aureus, Escherichia coli, Candida albicans, and Pseudomonas aeruginosa [14].

On the other hand, Kefir milk is a complex fermented dairy product created through the symbiotic fermentation of milk by lactic acid bacteria and yeasts contained within an exopolysaccharide and protein complex called kefir grain [15]. The antimicrobial activity of kefir can be attributed to the presence of proteolytic enzymes, organic acids such as lactic acid or acetic acid that cause pH reduction, CO2, bacteriocins, bioactive peptides, and hydrogen peroxide [16]. Previous studies have documented kefir in improving skincare properties, including reduced melanin synthesis, copper chelation, balancing cutaneous water, acne treatment [17], and exhibiting wound healing properties [18][19]. Regarding antimicrobial attributes, kefir has been extensively examined to provide antimicrobial action against gastrointestinal pathogens including E. coli, Salmonella, Campylobacter, Clostridium difficile, and Helicobacter pylori [20]. Despite their promising antimicrobial properties, the effectiveness of aloe vera gel and kefir against opportunistic skin pathogens has not yet been fully determined. Therefore, this study aims to address this gap and evaluate their potential impact against opportunistic skin pathogens

2. MATERIALS AND METHODS

2.1. Culture Collection

The microbiology laboratory at Jinnah University for Women provided one sample of each culture of opportunistic skin pathogens, which were cultured on nutrient agar plates. A single colony from each culture in nutrient broth and grown overnight at 37 °C and stored under refrigerated conditions until further use [21].

2.2. Confirmatory Tests

Prior to antimicrobial screening, the bacterial isolates were cultured on Blood Agar, MacConkey Agar, and Mannitol Salt Agar, followed by incubation under aerobic conditions at 37°C for 24hrs. A series of tests, including microscopy with Gram staining and biochemical assays such as Triple Sugar Iron (TSI), citrate utilization, urease activity, and nitrate reductase, were performed for identification. Tube coagulase testing, along with catalase and oxidase spot tests, was also conducted to confirm the identity of the isolated species.

2.3. MacFarland Preparation

To prepare a0.5 MacFarland standard, we mixed 0.05 mL of 1.175% barium chloride dihydrate ($BaCl_2 \bullet 2H_2O$) with 9.95mL of 1% sulfuric acid (H_2SO_4). Then, the turbidity of a resulting solution compared to a bacterial suspension containing approximately 1.5×10^8 CFU/mL [22].

2.4. Preparation of Aloe Vera Gel Solution (AGS) and Kefir Milk Supernatant (KMS)

The AGS was prepared by homogenizing 1ml Aloe Vera gel in 8 mL sterile water, resulting in a 11.1% (v/v) concentration of Aloe Vera gel in the solution. On the other hand, KMS was prepared in two steps (i) adding 2 gm. of kefir grains to 1L pasteurized cow milk for 24 hours of fermentation at room temperature (ii) 5mL of kefir (fermented milk) was centrifuged at 3000rpm for 10 min to obtain supernatant for antimicrobial assessment [ ]. The AGS and KMS were stored at 4°C in airtight containers to preserve their bioactive components. Both substances were kept for up to 1-2 weeks before testing [23].

2.5. Antimicrobial susceptibility testing of aloe vera and kefir milk

The molted sterile Muller-Hinton agar (MHA) (at 45 °C) was added to petri plates. For one hour, the gel was left on the plates for solidification. For organism inoculation, one swab was added in a MacFarland-matched culture and subsequently made a bacterial lawn. Using a flamed cork borer, wells measuring

10mm in diameter were created on the agar plate surface. Each well received approximately 0.1mL of the AGS and KMS, respectively. For 24 hrs, they were incubated at 37°C, and subsequently, the zones of inhibition were measured using a ruler [24].

.3. RESULTS AND DISCUSSION

Identification of Pathogens

The biochemical analysis identified various opportunistic pathogens: Gram-positive organisms isolated on blood agar included *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Bacillus subtilis*, all exhibiting beta hemolysis, while *Corynebacterium diphtheriae* and *Staphylococcus epidermidis* showed gamma hemolysis.

Gram-negative organisms isolated on MacConkey Agar included lactose fermenters such as Citrobacter freundii, Escherichia coli, Enterobacter aerogenes, and Klebsiella oxytoca, alongside non-lactose fermenters Morganella morganii, Proteus mirabilis, and *Pseudomonas aeruginosa*. The detailed biochemical profile is described in Table 1.

Antimicrobial Screening

Observations from the antimicrobial assays confirmed the efficacy of KMS against *Streptococcus pyogenes* (19mm), *Enterobacter aerogenes* (15mm), *Providencia stuartii* (12mm), and *Escherichia* coli (13mm). On the contrary, AGS also inhibited *Bacillus subtilis* (15mm), *Staphylococcus aureus* (15mm), *and Providencia stuartii* (20mm). However, both KMS and AGS were found to be ineffective against *Staphylococcus epidermidis, Pseudomonas aeruginosa, Klebsiella oxytoca, Citrobacter freundii, Corynebacterium diphtheriae, Proteus*

mirabilis and *Morganella morganii.* (Table 2).

Table 1. Confirmatory Tests for Opportunistic Skin Pathogens

Medium	Colony Appearance	Gram Reaction	Microscopy	Biochemical Tests	Organism Confirmed
Blood Agar	Beta Haemolysis	Positive	Cocci in clusters	Catalase Positive	*Staphylococcus aureus*
		Positive	Cocci in chains	Catalase Negative	*Streptococcus pyogenes*
		Positive	Scattered rods	Catalase Positive, Spore former	*Bacillus subtilis*
	Gamma Haemolysis	Positive	Rods (Chinese letters)	Catalase Positive, Non-spore former	*Corynebacterium diphtheriae*
Mannitol Salt Agar	Yellow colonies	Positive	Cocci in clusters	Coagulase Positive, Catalase Positive	*Staphylococcus aureus*
	Pink colonies	Positive	Cocci in clusters	Coagulase Negative, Catalase Positive	*Staphylococcus epidermidis*

MacConkey Agar	Non-lactose fermenting	Negative	Straight rods	Urease: Positive Oxidase: Negative TSI: K/A with gas, no H_2S Citrate: Positive Nitrate reductase: Positive	*Providencia stuartii*
	Lactose fermenting	Negative	Long rods	Urease: Negative Oxidase: Negative TSI: A/A with H_2S production Citrate: Positive Nitrate reductase: Positive	*Citrobacter freundii*
	Late-lactose fermenting	Negative	Rods	Urease: Negative Oxidase: Negative TSI: A/A with gas, no H_2S Citrate: Negative Nitrate reductase: Positive	*Escherichia coli*
	Non-lactose fermenting	Negative	Long rods	Urease: Positive Oxidase: Negative TSI: K/A with H_2S production Citrate: Positive Nitrate reductase: Positive	*Proteus mirabilis*
	Non-lactose fermenting	Negative	Rods	Urease: Positive Oxidase: Negative TSI: K/A with gas, no H_2S Citrate: Negative Nitrate	*Morganella morganii*

				reductase: Positive	
	Late-lactose fermenting	Negative	Rods	Urease: Negative Oxidase: Negative TSI: A/A with gas, no H_2S Citrate: Positive Nitrate reductase: Positive	*Enterobacter aerogenes*
	Sticky late-lactose fermenting	Negative	Rods	Urease: Negative Oxidase: Negative TSI: A/A with gas, no H_2S Citrate: Positive Nitrate reductase: Positive	*Klebsiella oxytoca*
	Non-lactose fermenting	Negative	Rods	Urease: Negative Oxidase: Positive TSI: K/NC (no sugar fermentation) Citrate: Positive Nitrate reductase: Positive	*Pseudomonas aeruginosa*

Table 2. Comparative analysis of KMS and AGS against skin opportunistic pathogens

Organism	Sensitivity	
	KMS	AGS
Staphylococcus epidermidis	Resistant	Resistant
Streptococcus pyogenes	19mm	Resistant
Staphylococcus aureus	Resistant	15mm
Pseudomonas aeruginosa	Resistant	Resistant
Klebsiella oxytoca	Resistant	Resistant
Enterobacter aerogenes	15mm	Resistant
Bacillus subtilis	Resistant	15mm
Providencia stuartii	12mm	20mm
Citrobacter freundii	Resistant	Resistant
Escherichia coli	13mm	Resistant
Corynebacterium diphtheria	Resistant	Resistant
Proteus mirabilis	Resistant	Resistant
Morganella morganii	Resistant	Resistant

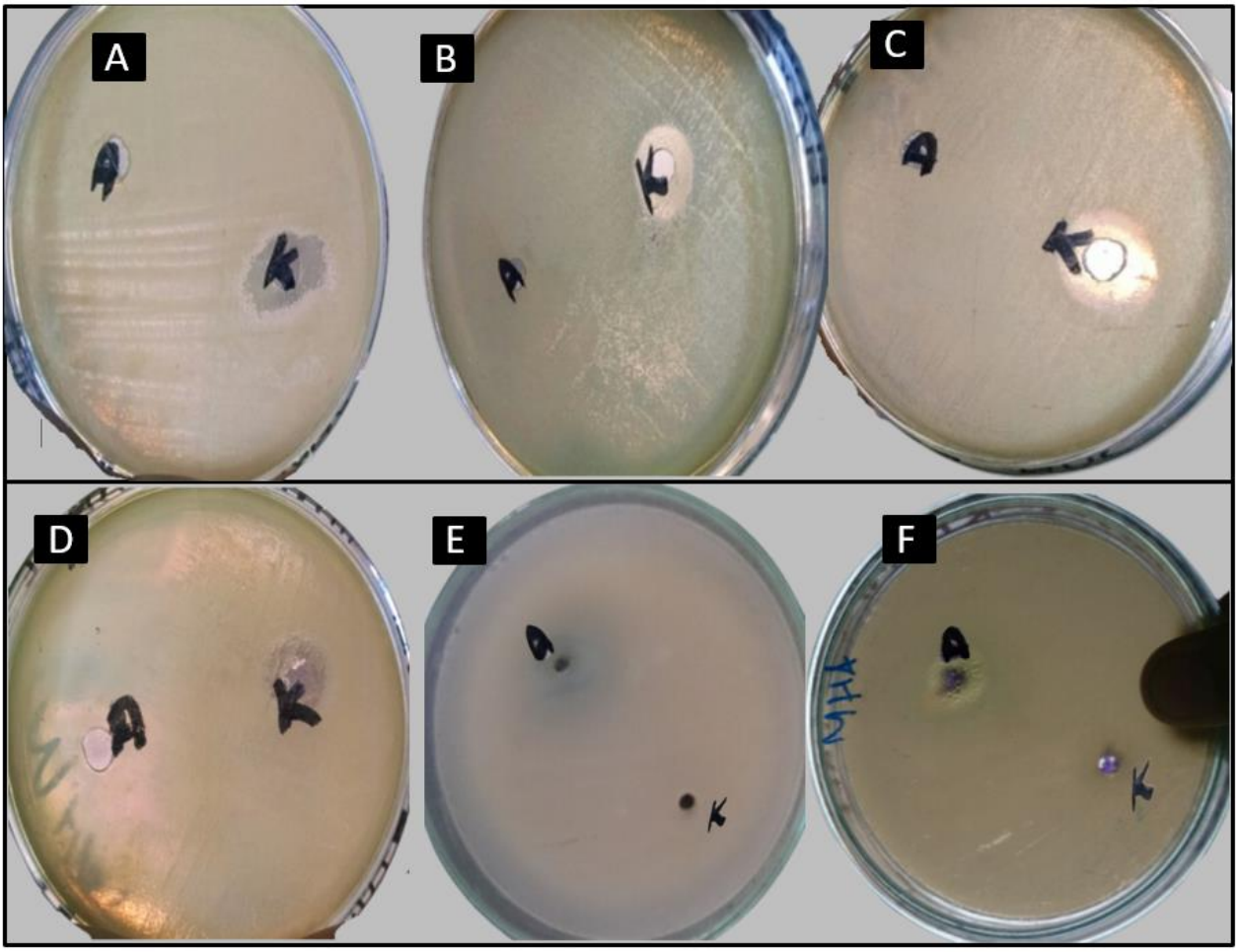

Figure 1. Antimicrobial screening of AGS (A) and KMS (K); A, *Escherichia coli*, B, *Enterobacter aerogenes,* C, *Streptococcus pyogenes*, D, *Providencia ustuartii*, E, *Staphylococcus aureus*, and F, *Bacillus subtilis.*

Opportunistic skin infections are a significant concern, especially in healthcare settings and immunocompromised individuals. This study represents the first effort in Pakistan to elucidate the efficacy of Pakistan-origin KMS against various microbes, highlighting its potential as a promising alternative in wound care. In this study, KMS effectively inhibited *Streptococcus pyogenes*, *Enterobacter aerogenes*., *Escherichia coli*, and *Providencia stuartii*, as indicated by inhibition zones of 19 mm, 15 mm, 13 mm, and 12mm, respectively. Similarly, another study revealed the antimicrobial activity of *Lactobacillus kefiri* isolated from kefir milk against *Escherichia coli* and *Enterobacter spp.* (3.5 ± 0.8 × 107 and 4.8 ± 0.9 × 107) CFU count respectively [25]. Another study revealed that sap from Tibetan Kefir grains could inhibit the growth of *Staphylococcus aureus* and *Beta-Hemolytic Streptococcus spp. by* degrading their proteolytic enzymatic activity from 70,000kDa to 7000kDa [26].

In kefir milk, antimicrobial activity is due to the presence of bacteriocins that contribute to its antimicrobial effects against a wide range of skin pathogens [27]. Bacteriocin such as Nisin can form pores in the peptidoglycan layer of *Streptococcus pyogenes* or disrupts the outer membrane by interacting with lipid II molecules in *Escherichia coli*. The leakage of essential ions and the collapse of the electrochemical gradient across the membrane result in cellular dysfunction and death [28]. Kefir also produces small amounts of ethanol and acetaldehyde during fermentation, which can denature proteins and interfere with bacterial

metabolism. In *Enterobacter aerogenes* and *Providencia stuartii*, these metabolites interfere with cellular respiration and disrupt metabolic pathways, further weakening the bacteria's ability to survive in an acidic environment [29] [30]. In addition, Kefir's metabolites interfere with quorum sensing, the bacterial communication system that regulates gene expression for virulence and biofilm formation. In *Enterobacter aerogenes* and *E. coli*, disruption of quorum sensing could inhibit their ability to form biofilms and express virulence factors, making them more susceptible to the antimicrobial components in kefir [31].

AGS demonstrated inhibitory effects against *Bacillus subtilis, Providencia stuartii,* and *Staphylococcus aureus*, with inhibition zones measuring 15mm, 20mm, and 15mm, respectively. Similarly, a study revealed the antimicrobial property of aloe vera gel against *Bacillus cereus,* and *Staphylococcus aureus* with a zone of inhibition of 12.66 – 23.33mm [32] and 4mm respectively [33]. Tivari et al. reported antibacterial susceptibility of aloe vera gel against *S. aureus, and B. cereus* [34]. However, this is the first report indicating the *Providencia spp.* inhibition from aloe vera gel. The antimicrobial properties of Aloe vera gel are attributed to several bioactive compounds, including anthraquinones, saponins, and polysaccharides. Anthraquinones, such as aloin and emodin, disrupt bacterial cell membranes by increasing permeability. These compounds insert themselves into the lipid bilayer, causing destabilization and leading to leakage of cytoplasmic contents, which ultimately results in cell death. In *Staphylococcus aureus*, the thick peptidoglycan layer is targeted by anthraquinones, which disrupt membrane integrity. These compounds also inhibit DNA replication by intercalating into bacterial DNA, interfering with transcription and replication processes [35].

Saponins are glycosides that interact with cell membranes by binding to sterols and disrupting the lipid bilayer, leading to increased membrane permeability and eventual cell lysis. They form complexes with the bacterial cell membrane, causing structural defects that allow leakage of intracellular components. Gram-positive bacteria such as *Staphylococcus aureus*, and *Bacillus subtilis* are particularly susceptible to saponin-induced damage due to their relatively simpler cell membrane compared to Gram-negative bacteria. Their detergent-like properties can also damage bacterial spores in *Bacillus subtilis* [36].

Polysaccharides, especially acemannan, present in Aloe vera, have immunomodulatory and antimicrobial properties. They enhance the immune response, increasing the production of antimicrobial peptides and nitric oxide, which can further inhibit bacterial growth. In *B. subtilis*, polysaccharides like acemannan may inhibit spore formation or prevent the bacteria from transitioning between vegetative and spore states, impairing its survival [37]. Aloe vera gel contains phenolic compounds (e.g., p-coumaric acid) that possess antimicrobial properties by generating reactive oxygen species (ROS). ROS can damage bacterial DNA, proteins, and lipids, leading to oxidative stress and cell death. In *Providencia stuartii*, phenolic compounds impair cell permeability by interacting with cholesterol in cell membranes, creating pores and exhibiting toxic and hemolytic actions [38]. The inhibitory characteristic typically increased with the length of fermentation; this effect might be connected to the pH drop that was noted during the fermentation process. Kefir milk exhibits the strongest and most comprehensive antibacterial spectrum after a minimum fermentation period of 36–48 hours when the pH ranges between 3.71 and 3.81. However, the conventional kefir fermentation process typically lasts 18–24 hours at 25°C, during which the pH is observed to be between 3.94 and 4.05 [39]. In this study, kefir milk was fermented over a 24-hour period. However, the effects of extending the fermentation time have not been explored, presenting an opportunity for future research. Investigating longer fermentation periods could provide valuable insights into how the duration of fermentation influences microbial activity, bioactive compound production, and

overall health benefits of kefir milk. Such studies could enhance our understanding of kefir's potential therapeutic applications and optimize its use in various health interventions.

Nonetheless, both KMS and AGS were found to be ineffective against *Staphylococcus epidermidis*, *Pseudomonas aeruginosa, Klebsiella oxytoca., Citrobacter freundii., Corynebacterium diphtheriae., Proteus mirabilis,* and *Morganella morganii* in our experimental analysis. Similarly, a study found the resistance of *K. oxytoca* from Turkish kefir grains [40] and *S. aureus* and *P. aeruginosa* from kefir milk after 24 hours of incubation [41]. Another study found the resistance of *Coagulase-negative Staphylococcus, Citrobacter spp., Corynebacterium spp., Proteus mirablis,* and *Morganella spp* from aloe vera gel [42]. *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* produce biofilms, which are structured communities of bacteria encased in a protective matrix that impedes penetration of antimicrobial agents. The *Pseudomonas aeruginosa* utilize efflux pumps like *MexAB-OprM* to expel antimicrobial substances, while *Klebsiella oxytoca* and *Citrobacter freundii* produce extended-spectrum beta-lactamases (ESBLs) that degrade antimicrobial compounds. *Proteus mirabilis* alters the local pH through urease production, making the environment less hospitable for external agents, while *Morganella morganii* and Corynebacterium *diphtheriae* resist through beta-lactamase enzymes and thick cell walls, respectively, further reducing the efficacy of natural antimicrobial treatments. Together, these mechanisms limit the penetration and activity of bioactive compounds found in Kefir and Aloe vera and render them ineffective against these resilient pathogens [43].

Small sample analysis provides preliminary insights into the efficacy of the substances tested. However, the reliance on a single sample for each pathogen may limit the reliability and statistical significance of the results, as it does not account for variability among strains within each species. In addition, there is limited knowledge of how antimicrobial properties evolve during fermentation stages, and the synergistic effect on resistant pathogens is not observed. Future research should focus on optimizing formulations or combining these therapies to enhance efficacy and broaden their applicability across diverse wound types and patient populations. Moreover, exploring the molecular interactions between these natural compounds and bacterial resistance mechanisms could offer valuable insights for optimizing their antimicrobial properties [40].

4. CONCLUSION

The comparative analysis revealed that KMS proved effective against *Streptococcus pyogenes, Enterobacter aerogenes, Escherichia. coli*, and *Providencia stuartii,* while AGS significantly inhibited*Bacillus subtilis, Providencia stuartii,* and *Staphyloccous aureus*. These findings highlighted the potential of these solutions as natural, cost-effective alternatives in wound care, although further research is required to refine their formulations and application methods for improved clinical outcomes.

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6. CONFLICT OF INTEREST

All authors declared no conflict of Interest.

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