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ORIGINAL ARTICLE

Genetic and Morphological Effects of Lactobacillus Acidophillus on Some Virulence Factors of Proteus Mirabilis That Isolated From Diabetic Foot Ulcers

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ABSTRACT

The current study have been included 250 samples diabetic foot ulcer patients attending in Al-Diwaniyah Teaching Hospital and private clinics at a period of study from beginning October 2021 to February 2022, Isolates were identified by morphological form on blood agar and macConkey agar, traditional biochemical tests,then confirmed by Vitek 2 system and PCR was performed using a specific primer (16SrRNA) to identify the genus Proteus and distinguish it from the other Enterobacteria and bacteria.

Has been studied of the morphological and genetic inhibitory effect of probiotics on p. mirabilis and the results showed that Lactobacillus filters were more effective in the inhibitory effect on p. isolates mirabilis.

Molecular study demonstrated the effect of LAB on virulence factors through the disappearance of some bands during electrophoresis. The number of bundles in the molecular study of virulence factors (ureR, zapA, flaA, hmpA) decreased after treatment of Proteus terrestris with lactobacillus acidophillus.

Keywords: Proteus mirabilis, diabetes foot ulcers, Probiotic.

Research aims: We focus on knowing the phenotypic and genetic effect of probiotics on some virulence factors of Proteus mirabilis isolated from diabetic foot ulcers in Al-Diwaniyah Governorate.

INTRODUCTION

Diabetes mellitus is a silent illness that can lead to late chronic consequences like diabetic foot ulcers. Surgical debridement is used to treat this disease. Some surgeons utilize proteolytic agents after surgery to enhance surgical outcomes (Kardoust et al.,2021). DM is a chronic condition characterized by insufficient insulin synthesis or ineffective insulin utilization. In long-term untreated D.M., this results in increased blood sugar levels, causing damage to the heart, blood vessels, eyes, kidneys, and nerves (World Health Organization,2016). DM is a global pandemic of a chronic, progressive condition marked by a slew of complications that affect a wide range of physical systems. DFU are full-thickness wounds in the dermis below the ankle that have not healed or are healing slowly in persons who have had diabetes for more than three months. The three types of D.F.Us. are neuropathic, ischemic, and neuroischaemic ulcers (Armstrong et al., 2018).

Diabetic foot ulcers are a serious consequence of diabetes that increases the chances of amputation and death. The bacterial infection of diabetic foot ulcers with aggressive and resistant bacteria such as Proteus mirabilis significantly worsens the lesion and may not be treatable with standard therapies. It may be beneficial to develop novel techniques to target bacterial virulence in order to combat such diseases (Khayyat et al.,2021). Diabetes mellitus is a silent illness that can lead to late chronic consequences like diabetic foot ulcers. Surgical debridement is used to treat this disease. Some surgeons utilize proteolytic agents after surgery to enhance surgical outcomes (Kardoust et al.,2021).

A huge DM pandemic is sweeping the globe. According to the International Diabetes Federation, there are about 425 million diabetic individuals worldwide, with one out of every ten people having diabetes and one out of every two people going undiagnosed. (Toniolo et al.,2019). The exact incidence of DFU is unknown, although it is believed that 4-27 percent of DFU sufferers globally live in countries with different prevalence rates (Rosyid,2017).

The genus Proteus is a Gram-negative bacillus that belongs to the Enterobacteriaceae family. Members of the genus Proteus are wide spreads in the environment and the gastrointestinal tract of human and animals(Armstrong et al., 2018). Proteus species are found in wounds, particularly diabetic wounds (Hegazy, 2016).

A probiotic is a microbial dietary adjuvant that improves nutritional and microbial balance in the digestive tract while modifying mucosal and systemic immunity (A al-jeboury and Baker, 2008).

The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) officially defined probiotics as "live bacteria that, when administered in suitable proportions, exert a health benefit on the host" (Corcionivosch et al., 2010).

Probiotics may have immunomodulatory effects in both viable and nonviable bacteria, suggesting that metabolic or secreted substances, as well as structural or cellular components, may be involved (Brochers et al., 2009). Several researchers and experts are currently focused on developing organically produced techniques to cure pathogenic causes of various diseases and infections in order to prevent the side effects and microbial resistance associated with most regularly used antibiotics and other chemotherapeutic medicines. Probiotics are one of these methods, which involves the use of safe microorganisms to inhibit harmful bacteria (Czerucka et al., 2007).

Probiotics, according to (Ammor et al., 2007), have a unique trait in terms of antibiotic resistance expression and transferability, which is important since there is worry about the dissemination of resistance determinants to human pathogenic and opportunistic bacteria.

The use of probiotics might be a viable alternative to the usage of antibiotics (Hou et al., 2015). By modifying the endogenous environment or the immune system, probiotics can have a direct or indirect influence on gut physiology (HA al-jebourysma,2010).

Lactobacillus treatment appears to minimize the recurrence incidence of infection, thus it's used to treat (Reid and Köhler, 2006). Lactobacillus acidophilus has a higher capacity for creating lactic acid, which is antibacterial and aids the body's defense against dangerous bacteria clinging to the intestinal mucosa (cell lining) (HA al-jebourysma , 2010).

MATERIAL AND METHODS

Collection of Samples: At a period of study from beginning October 2021 to February 2022, A total of 250 Swabs were taken from diabetic foot ulcer patients, with 80 of them testing positive for Proteus Mirabilis. Swabs were taken from patients at Al-Diwaniyah Teaching Hospital and private clinics . Sterile swabs with a carrier medium were utilized for this. They were planted right after the sample was taken for the purpose of diagnosis(Rajehwari et al.,2010).

Samples have been obtained Lactobacillus acidophilus from Department of Biology College of Science / Al-Mustanseria University, and Maintenance of LAB according to (Conteras et

al.,1997) in two ways Daily Working Cultures LAB and Stock culture, then used in this study.

Bacterial diagnosis: P. mirabilis bacteria were isolated from blood and MacConkey agar, which was cultivated aerobically at 37°C for 24 hours. To identify bacteria, biochemical tests such as oxidase, indole, citrate utilization, catalase, urease production, H2S formation, lactose fermented, Voges-proskauer reaction, Methyl red, Trible sugar iron test (TSI), and Simmon citrate were used (Hawkey, 2006; Forbes et al., 2016; Jacobsen et al., 2008), as well as Vitek2 and PCR (Bunyan and Albakery, 2021; Jaber and Almiyah., 2022)

Morphological Inhibitory Effect of LAB on proteus mirabilis

A On MRS Agar (solid medium): The inhibitory effect of lactic acid bacteria(Lactobacillus acidophilus) on Proteus mirabilis was determined according to (Silva et al., 1987).

B In MRS Broth (Liquid Medium): The inhibitory effect of lactic acid bacteria(Lactobacillus acidophilus) on Proteus mirabilis was determined according to (Vignolo et al., 1993).

Determination of Minimum Inhibitory Concentration of Lactobacillus LAB Concentrated Filtrate Against Proteus mirabilis Growth: Each concentrated filtrate was diluted differently in tubes containing sterile nutritional broth. The ratios were (10, 20, 30, 40, 50, 60, 70, 80, and 90%), giving each tube a final amount of 10ml. After that, each concentration was infected with a 0.1ml culture of Proteus mirabilis cultivated in nutrient agar and incubated for 24 hours at 37 degrees Celsius. After incubation, the development of the tubes was examined, and the minimum inhibitory concentration was calculated as the lowest concentration of the filtrate that prevented Proteus mirabilis from growing in the tubes.

Genetical inhibitory effect of LAB on the virulence factors of Proteus mirabilis: To evaluate the inhibitory properties of probiotics, we applied the procedure of (Alizadeh et al., 2018) with some modifications.

RESULTS AND DISCUSSION

Isolation and Identification of Proteus mirabilis: This study found that P. mirabilis was found in 80 percent (32 percent) of 250 swabs from diabetic foot ulcer patients, and that this bacteria could be detected using culture and biochemical testing. P. mirabilis appeared pale, yellow, and lactose nonfermenters when cultured on MacConkey agar, similar to P. mirabilis (Forbes et al., 2016). P. mirabilis growth on blood agar demonstrates swarming, fish odor, mucoid, and non-hemolytic colonies, all of which are consistent with the swarming phenomena (Al-Aabideen, 2005; Jaber and Almiyah., 2022).

P. mirabilis identification with the Vitek2 system indicated that all isolates were P. mirabilis, with a percentage of recognition ranging from 0% to 100%. (95 to 99 percent). This proportion was in line with expectations (Sung et al., 2000; Jaber and Almiyah., 2022)

The current study found that 80 isolates of bacteria P. mirabilis on the 16S rRNA gene, which represents the diagnostic gene for this bacterium, were used to execute the PCR technique to diagnose bacteria P. mirabilis using gene 16S rRNA. This result resembled the findings of (Schabereiter-Gurtner et al., 2001; Lu et al., 2000; Jaber and Almiyah., 2022), who used 16S rRNA for Proteus spp. identification.

Morphological Inhibitory Effect of LAB on proteus mirabilis

On MRS Agar (solid medium): The most efficient strategy for producing inhibitory metabolites against pathogenic p. mirabilis bacteria was propagating LAB isolates on MRS agar under anaerobic conditions, showed all LAB isolates had a significant inhibitory impact on Proteus mirabilis isolates, with an inhibitory zone of 18.5 mm after 24 hours of incubation. Furthermore, this LAB isolate was efficient against P. m. isolates and had the maximum inhibition zone for both incubation times (18 and 24 hr), After 24 hours of incubation.

In this technique,(Mahmood and Hameed, 2018; Al-Kafaji, 1992) discovered that employing MRS agar medium in evaluating the capacity of Lactobacillus isolates to create inhibitory compounds under anaerobic conditions produces satisfactory results.

The results showed that anaerobic culture of Lactobacillus isolates on MRS agar was an effective approach for producing inhibitory metabolites against the pathogens examined, Almost all LAB isolates produced inhibitory effect on P. m. isolates during a 24-hour incubation period than after an 18-hour incubation period. When extended incubation periods (30 hr and more) were performed on certain isolates, no variation in inhibition zone was seen or in some cases the inhibition zone was reduced. Because of this, The inhibitory zone diameter of the LAB varied (9 – 18.5) mm in diameter, gave an inhibitory effect after 18 hr, increased for 24 hr incubation periods, but no noticeable increase after more incubation periods, agreeing with (Udhayashree et al., 2012) who found that each LAB gave an inhibitory effect after 24 hr. But (Abd El-Gawad et al., 2005) was found that the inhibitory effect increased after (48 hr).

Although L .acidophillus had lesser antagonistic activity against proteus, it was more efficient against other bacteria. Because it is one of the variables impacting the influence of efficacy during the growing period, Lactobacillus effect results varies depending on the type or strain utilized within the same species. The antimicrobial, antibacterial, and product amount in the medium (Chaudhary et al., 2013).

After (24 hr) incubation, Lb. a. had the maximum inhibitory effect, which was attributable to acidophilin produced by Lb. a. (Frank and Marth, 1988). Gram-negative and gram-positive bacteria are both inhibited by LAB (Nigatu and Gash, 1994; Hindal and Ali, 2015). According to (Fang et al., 1996), LAB has a strong inhibitory impact on enteropathogenic bacteria. while this finding contradicts (Gilliland and Speck, 1977), who found that lactobacilli had a better antibacterial impact against Gram positive bacteria than Gram negative bacteria.

In MRS Broth (Liquid Medium): Lactobacillus isolates cultured in MRS broth were tested for their inhibitory impact. Lactobacillus inhibitory activity against pathogenic isolates was determined using the well diffusion technique. When utilizing Lactobacillus (Al-Kafaji, 1992) supernatant, the inhibition zone diameter ranged between (13-19) mm, maximum inhibition zone diameters reached (20) mm, which was higher than that recorded in solid medium. This could be due to the presence in MRS broth of a wide spectrum inhibitory effect against gram positive bacteria (Staphylococcus aureus, Bacillus subtilis) and gram negative bacteria (E. coli, Klebsiella spp., Proteus spp) (Gupta et al., 1998).

LAB isolates were cultured for (18, 24, and 48) hours in liquid medium to investigate the influence of incubation time.

The best inhibitory effect was achieved by Lb. a. the inhibition zone width reached (18 mm) against tested Proteus mirabilis isolates after a 24-hour incubation period. For Lb. a. isolates, increasing the incubation time to 48 hours had the least inhibitory impact.

The inhibitory impact of Lb. a. was best after 24 hours of incubation, not 48 hours. This might be because the inhibitory materials (Lb. acidophilin) lose action when produced outside the cells as the incubation period increases.

The inhibitory effect of Lb. a. against the tested isolate P. m. liquid medium for different incubation times (18, 24, and 48) hr is shown. The effect of concentrated filtrates on the tested isolate is also being investigated. Using a freeze-dryer, the filtrates of Lb. a. were concentrated thrice. The first and second folds of concentrated filtrates of Lb. a. have zone diameters of (21) and (23) mm, respectively, against P. m., whereas the third fold has the highest inhibitory effect after 24 hr incubation because all inhibitory substances were concentrated, and the zone diameter of Lb. a. against P. m. reached 30 mm. Because the inhibitory impact was weaker after 18 and 48 hours of incubation than after 24 hours, (Pfeiffer and Radler,1982) concluded that there is a link between the width of the inhibition zone and the concentration of inhibitory chemicals. On the other hand, (A al-jeboury and Baker,2008)

found that adding inhibitory compounds like bacteriocin and acidophilin in LAB enhanced the mortality of tested bacteria.

When comparing Lactobacillus isolates grown in MRS broth to those grown on solid medium, it was clear that MRS broth was a better stimulator for inhibitory product than MRS agar. This finding was consistent with the findings of a study (Fang et al., 1996), which found that MRS broth stimulated inhibitory effect against Gram positive (S. aureus) and Gram negative bacteria (E.coli ,Proteus spp.) Another researcher (Kubba, 2006) discovered that when liquid medium (MRS broth) was used to determine the effect of Lactobacillus on pathogenic bacteria, the best inhibitory effect was achieved.

Using the well diffusion approach, some of the strains examined exhibited high sensitivity to L. acidophilus. The findings are consistent with prior research (Reid and Burton, 2002), which found that Lactobacillus spp. isolates had probiotic properties that aid in health restoration and maintenance. The current findings are also consistent with those of (Abd El-Moez et al., 2008; Bokhari et al., 2017), who found strong activity of L.acidophilus as a probiotic in vitro against a variety of bacteria. (De Vuyst and Leroy, 2007) shown that lactic acid bacteria have a wide range of antimicrobial properties, and that antimicrobial generation by probiotic LAB contributes to gut health during in vivo interactions in the gastrointestinal tract.

This finding is consistent with a recent research (Koga et al., 1998) that found L. acidophilus strains to have inhibitory effect against a variety of bacteria. Our findings contradict a previous research (Chateau et al., 1993), which found that none of the Lactobacillus spp. could impede growth. Probiotics have been proven to protect against a number of diseases (Gupta et al., 1996; Stern et al., 2001). (Casey et al., 2007) found that lactobacilli had varying antibacterial activity, with just a few strains inhibiting all pathogens.

Determination of Minimum Inhibitory Concentration of Lactobacillus LAB Concentrated Filtrate Against Proteus mirabilis Growth: Serial dilutions of the three-fold filtrates of isolates Lb. a. were made to determine the MIC,s of the filtrates of LAB isolates that inhibit or minimize the growth of Proteus mirabilis. When obvious growth of this test bacterium was detected, the results in table(1) reveal that concentrations of 10% and 20% Filtrate had no influence on P. m. Filtrate concentrations of 30% and 40% resulted in little development of the test microorganisms.

The growth of P. m. isolate was entirely suppressed at concentrations of 60 percent and higher of Lb. a. filtrate.

Table 1: Minimum Inhibitory Concentrations (MIC) of Lactic Acid Bacteria (LAB) against Proteus mirabilis isolate.

LAB isolates	MIC%								
	10	20	30	40	50	60	70	80	90
	+	+	+	+	-	-	-	-	-

+ = growth , – = no growth

Based on the above findings, 50 percent Lb. a. concentrations were chosen and reported as the MICs of the LAB filtrates against the growth of the test bacterial isolate P. m.

Genetical inhibitory effect of LAB on the virulence factors of Proteus mirabilis

Genetic detection of urease enzyme with (LAB):

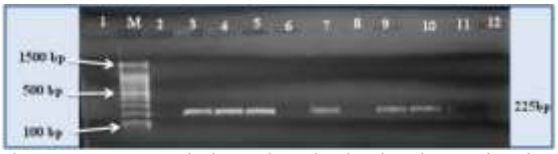

Figure 1: Agarose gel electrophoresis showing the results of UreR gene (225 bp) for bacteria P .mirabilis after treatment with probiotic (LAB), the Pits (1-12) Bacterial isolation with(LAB), M = marker (100-1500 bp).

in this study the proteus mirabilis produced ureR enzyme at 55 isolates(68.75%), while the isolates after treatment with LAB showed the isolates that produced ureR enzyme decreased to 20 isolates(30.74%).

Genetic detection of protease enzyme with (LAB):

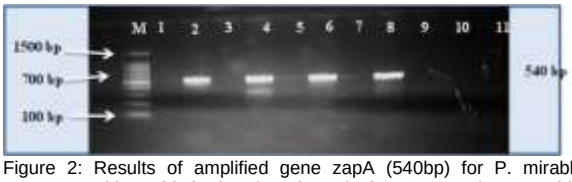

Figure 2: Results of amplified gene zapA (540bp) for P. mirablis after treatment with probiotic (LAB), using Single PCR .M (DNA Ladder 100-1500), (1-11)bacterial isolates.

in this study the proteus mirabilis produced zapA enzyme at 80 isolates(100%), while the isolates after treatment with LAB showed the isolates that produced zapA enzyme decreased to 30 isolates(37.5%).

Genetic detection of flagella gene with (LAB):

Figure 3: Results of amplified gene flaA (417 bp) for P.mirablis after treatment with probiotic (LAB), using Single PCR .M (DNA Ladder 100-1500), (1-15)bacterial isolates.

in this study the proteus mirabilis produced flaA enzyme at 80 isolates(100%), while the isolates after treatment with LAB showed the isolates that produced flaA enzyme decreased to 35 isolates(43.75%).

Genetic detection of hemolysine gene with (LAB):

Figure 4: Results of amplified gene hmpA (717 bp) for P. mirablis after treatment with probiotic (LAB),using Single PCR .M (DNA Ladder 100-1500), (1-11)bacterial isolates.

Table 2: The rate (%) of virulence factors in P. Mirabillis isolates from Diabetic Foot Ulcers after treatment with probiotic

The name of primers	Number of bands formed	%	Number of bands formed after treatment with probiotic	%	X2	P value
Hmp A	40	50.0	25	31.25	17.31	0*
Fla A	80	100.0	55	68.75	62.6	0*
Ure R	55	68.75	36	45	30.74	0*
Zap A	80	100.0	58	72.5	72.72	0*
Total						
X^2	90.25		14.54			
P value	0*		0.002*			

* Significant difference at P<0.05

in this study the proteus mirabilis produced hmpA enzyme at 40 isolates(50%), while the isolates after treatment with LAB showed the isolates that produced hmpA enzyme decreased to 15 isolates(18.75%).

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