Original Research

Journal of Advanced Veterinary Research
(2024) Volume 14, Issue 6, 926-929

Antibacterial effect of the probiotic candidate isolated from kishk sold in Upper Egypt

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ARTICLE INFO

Recieved: 03 March 2024

Accepted: 11 May 2024

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Keywords:

Kishk
Probiotics
Antibacterial effect
E. coli
Pseudomonas aeruginosa

ABSTRACT

Kishk is a traditional dry fermented dairy product prepared from a mixture of salted sour butter milk or yoghurt with wheat grains. The goal of this study was to evaluate the microbiological quality, together with isolation and identification of LAB from kishk and assessed their protective effects against *E. coli* and *Pseudomonas aeruginosa in vitro* and *in vivo*. One hundred samples of kishk were randomly obtained from farmers' houses and food stores in the Assiut governorate, Egypt. Total viable count and, total yeasts and molds were performed by plating the samples on selective media. Further, LAB were isolated on de Man Rogosa and Sharpe (MRS) agar and identified by biochemical tests. The antagonistic effect of the obtained LAB was evaluated against two of foodborne pathogens (*E. coli* and *Pseudomonas aeruginosa*) *in vitro* using well diffusion method and *in vivo* after inoculation in cow's milk. The average total bacterial and yeasts and molds counts were 8.13±0.09 and 2.92±0.16 $\log_{10}$ CFU/g, respectively. Based on phenotypical examination, LAB were identified and classified into three groups namely *Lactobacillus*, *Leuconostoc* and *Lactococcus*. Notably, LAB had more inhibitory activity against *E. coli* in comparison to *Pseudomonas aeruginosa in vitro*. However, there was no changes in the mean count of the tested organisms versus control group after inoculation in milk. This study revealed that kishk contain LAB bacteria that have antagonistic properties on *E. coli* and *Pseudomonas aeruginosa*. Hence, kishk could be a useful candidate for human health.

Introduction

Over last decades, probiotics have gained more attention and their application as functional ingredients in food, animal feed, and pharmaceutical products was spread worldwide (Gao *et al*., 2021). Probiotics are live microorganisms that induce a health benefit when added in sufficient amounts through improving the intestinal flora (Hill *et al*., 2014). Besides, probiotics could play a crucial role in some medical cases such as allergies, hepatic disease, *Helicobacter pylori* infections, urinary tract infections, lactose intolerance and cancer (Ejtahed *et al*., 2011). Moreover, the regular consumption of foods matrices contained probiotics can improve the immune system, therefore enhancing the body's resistance toward common infections and gastrointestinal illnesses (de Vrese *et al*., 2006; West *et al*., 2011).

Dairy industry is the largest food sector in which the highest number and diversity of probiotics are used for improving the product quality (Vargas-Ramella *et al*., 2021). Probiotics have been supplemented in several dairy products such as yogurt, cheese, fermented milk, ice cream, butter, cream, and infant formula (Granato *et al*., 2010; Kanmani *et al*., 2013; Lollo *et al*., 2013). Of note, probiotics are used during processing of dairy products as a starter culture alone or in combination with other starter as well as they may be added to the products after fermentation (Gao *et al*., 2021). Besides their health benefits, probiotics can improve the taste, aroma, and texture of the milk products (Vargas-Ramella *et al*., 2021).

Probiotics are a special group of bacteria called lactic acid bacteria (LAB) such as *Lactobacilli*, *Streptococci*, *Enterococcus* and *Bifidobacteria* that are widely distributed in nature and present normally in raw milk (Ogier and Serror, 2008; Yateem *et al*., 2008; Hati and Prajapati, 2022). These strains are used safely in food industry with proven efficacy for human health (Siró *et al*., 2008). Additionally, probiotics include some yeast strains from the *Saccharomyces* genus (Abid *et al*., 2022). LAB are non-spore forming Gram-positive bacteria that can ferment carbohydrates and produce lactic acid as the main by-product of the fermentation process. Importantly, such bacteria are acid and bile tolerant so they can survive in the gastrointestinal tract and confer their health benefits (Marco *et al*., 2017).

It is worth noted that the antimicrobial activity of probiotics recovered from dairy products has been increasingly demonstrated in several studies (Rushdy and Gomaa, 2013; Arqués *et al*., 2015; Saboori *et al*., 2022). Edalati *et al*. (2019) isolated LAB from raw camel's milk and assessed their antibacterial effects against two pathogenic bacteria (*S. aureus* and *E. coli*). The authors investigated that the isolated LAB bacteria have antagonistic properties on *S. aureus* (Edalati *et al*., 2019).

In Egypt, artisanal dairy products are usually manufactured by fermentation process, so they are valuable sources of LAB with unique probiotic features (Petrović *et al*., 2020; Grujović *et al*., 2022). Among these traditional probiotic foods in the Egyptian market today is kishk. It is a fermented homemade buttermilk-wheat mixture that is known as a wheat fermented milk. It was first manufactured in Upper Egypt during the Pharonic period (3200–322 B.C.) and is still produced today (Abou-Donia, 2008).

Kishk is a nutritious cuisine that has good preservation qualities at room temperature for long time without cooling. The extended shelf life of such traditional food may be due to the low moisture content (less than 10%), low pH of 4.2, and non-hygroscopic character (Atya and Khattab, 1985; Tamime and O'Connor, 1995). This food matrix is prepared by mixing parboiled wheat known as 'belila' with strained fermented buttermilk 'laban zeer' in a ratio of 2:1, respectively (Abd El Ghani *et al*., 2014). There is little available data that focused on the probiotic feature of this

ISSN: 2090-6277/2090-6269/

traditional product in Egypt.

To date, the *in vitro* antibacterial activity of kishk's probiotic against Gram-positive as well as Gram-negative bacteria has not been reported. Hence, the current study will focus on two of food-borne pathogens that implicated in several outbreaks of food poisoning. On the one hand, *E. coli* is a Gram-negative, facultative anaerobic and rod-shaped bacteria (Bialvaei *et al*., 2016). Although *E. coli* is a normal inhabitant in the intestines of healthy people and animals, some strains of this microbe are harmful causing health hazards in human. The pathogenic strains of such microbe are characterized by their ability to produce virulence factors like exotoxins (Goldwater and Bettelheim, 2012). On the other hand, *Pseudomonas* spp. is psychrophilic bacteria that leads to spoilage of dairy products at refrigeration temperature (Wang *et al*., 2018; Carminati *et al*., 2019). It can degrade milk proteins and fats through production of heat stable proteases and lipases, so induce common phenomena in milk such as bitterness, rancidity, and gelation (Jaspe *et al*., 2000; Yao *et al*., 2012). *Pseudomonas aeruginosa* is an opportunistic pathogen that implicated in lethal diseases such as sepsis and pneumonia. Furthermore, it can frequently exert different resistance to some antibiotics (Abd El-Baky *et al*., 2020).

Considering the pivotal role of the fermented dairy products and their probiotics on human health, recent research focused on the selection of new probiotics and studying their characteristics and their possible use, as well as their health effect. Hence, this study was conducted with the aim of LAB isolation from kishk and investigate the antibacterial activity of the kishk's probiotic on some of food poisoning bacteria (*E. coli* and *Pseudomonas aeruginosa*). The microbiological quality of kishk was evaluated in the present study.

Materials and methods

Samples collection and preparation for analysis

During April to June 2022, a total of one hundred samples of kishk were collected on a random basis at farmers' houses and food stores in Assiut city, Assiut governorate (Egypt). The kishk samples were transferred directly to the lab. at the Department of Food Hygiene, Safety and Technology, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt within 24 h for bacteriological analysis.

Microbiological quality assessment of kishk samples

Kishk samples were evaluated for quality through detection of total viable bacterial counts at 30°C for 24-48 h on Plate count agar (Oxoid M325, Basingstoke, Hampshire, UK) (Abd El Ghani *et al*., 2014) as well as yeasts and molds counts at 25°C for 3 - 5 days on Sabouraud Dextrose Agar (Oxoid CM0041) (Abd El Ghani *et al*., 2014). After incubation, the colonies developed on agar plates were counted. The counts were carried out in triplicate.

Isolation of lactic acid bacteria (LAB)

According to the procedure described by Roberts and Greenwood (2003), 25 g of each of the kishk samples were transferred into a sterile mortar containing sterile white sand. These samples were mashed thoroughly in a stomacher (Seward®400) until complete homogenization. The isolation procedure was done according to De Man *et al*. (1960) with minor modifications. In brief, 25 g of the prepared samples of kishk were inoculated into 100 ml of de Man Rogosa and Sharpe (MRS) broth and incubated at 37°C for 24 h under anaerobic condition. Then, a loopful from the incubated tubes were streaked on MRS agar (Merck, Germany), and incubated at 37ºC for 24 h under anaerobic condition. Suspected colonies were phenotypically selected and picked up onto MRS agar slants, then incubated at 37ºC for 24 h before being subjected to identification.

Identification of isolates

All the obtained isolates were used to prepare smears on clean grease free microscopic glass slide and then stained with Gram's method of staining. The stained smear was observed under optical microscopy to check the bacterial morphology of the obtained isolates. The suspected LAB colonies were purified and identified using conventional biochemical tests as previously described (Harrigan and McCance, 1976). For instance, catalase test, production of acid from glucose, and growth at different temperatures were applied for the suspected colonies.

Determining the antibacterial activity of the isolated LAB using in vitro and in vivo tests

The antagonistic effect of LAB obtained from the current study was evaluated against two of foodborne pathogens (*E. coli* and *Pseudomonas aeruginosa*) *in vitro* using well diffusion method as previously described by Suresh *et al*. (2016) with some modifications. Firstly, LAB strains were enriched in MRS broth medium and incubated at 37°C for 24 h before directing the test. Then, 100 µL of the fresh bacterial culture having 10^6 CFU/mL was spread on MRS agar medium and left for 10 min to be absorbed. Further, one hundred µL of the overnight incubated cultures of *E. coli* or *Pseudomonas aeruginosa* were poured onto the wells (8 mm). Plates incubated overnight at 37°C, then the diameters of the inhibition zones were observed and measured in mm. The clear zone of more than 1 mm around wells was scored as positive (Jacobsen *et al*., 1999). Saline was used as a negative control. Each test was performed twice.

For evaluating the antibacterial activity of LAB against *E. coli* and *Pseudomonas aeruginosa in vivo*, fresh cow's milk, locally produced on the day of the experiment, was pasteurized at 63°C for 30 min. The prepared inoculum of LAB together with *E. coli* or *Pseudomonas aeruginosa* was added to the pasteurized milk in a count of 6 log CFU/mL. The inoculated milk was divided into three groups for further use as follows: part 1 is the control (contained LAB only), part 2 (contained LAB and *E. coli*) and part 3 (contained LAB and *Pseudomonas aeruginosa*). Then, the inoculated milk was stored in sterile glass containers in refrigerator (7°C). Samples were collected daily during storage and tested for *E. coli* and *Pseudomonas aeruginosa* counts on selective media of MacConkey agar (Oxoid CM0007B) (Younis *et al*., 2021) and Xylose Lysine Deoxycholate agar (XLD agar, Bio Lab, Budapest, Hungary) (Abdelsattar *et al*., 2021), respectively for a week. Each experiment was repeated in triplicate.

Statistical analysis

Experiments were done in triplicate to capture microbial variability. Descriptive statistics were calculated from the observed data with MS Excel (Microsoft Corporation). The statistical analysis performed consisted of mean comparison tests, univariate analysis of variance (ANOVA) followed by Tukey post-hoc test ($p < 0.05$) to evaluate significant differences in the concentration levels of *E. coli* and *Pseudomonas aeruginosa*.

Results

Microbiological quality of kishk samples

Kishk samples were evaluated to determine the total viable bacterial counts and total yeasts and molds on agar plates. The obtained results revealed that the total bacterial counts were varied from 5.0 to 9.8 $\log_{10}$ CFU/g with an average of 8.13±0.09 $\log_{10}$ CFU/g. While the level of yeasts and molds was ranged from ≤ 1 to 4.01 $\log_{10}$ CFU/g with a mean count of 2.92±0.16 $\log_{10}$ CFU/g. Notably, the majority of samples (46%) showed that the total count of yeasts and molds was not detected at the detection limit ≤ 1 $\log_{10}$ CFU/g (Table 1). While the highest frequency distribution (44%) of the total bacterial count was ranged from 105 to 10^6 CFU/g

(Table 1).

Table 1. Frequency distribution of total viable count and total yeasts and molds in the examined samples of kishk.

Range (CFU/g)	Total viable count (%)	Total yeasts and molds (%)
$< 10^2$	3	46
10^{2-3}	7	33
10^{3-4}	5	21
10^{4-5}	21	-
10^{5-6}	44	-
10^{6-7}	15	-
10^{7-8}	3	-
10^{8-9}	2	-
Total	100	100

(-) means non-detectable

Identification of LAB from Kishk samples

A total of 100 isolates were randomly chosen from the collected samples. After phenotypical examination, all the obtained isolates were Gram-positive bacteria (cocci and bacilli). The isolates were classified into 87% rods and 13% cocci. These bacteria were identified and classified into three groups of LAB namely *Lactobacillus*, *Leuconostoc* and *Lactococcus* genera.

Antibacterial activity of the isolated LAB using in vitro and in vivo models

The inhibitory activity of the isolated LAB against two pathogenic bacteria (*E. coli* and *Pseudomonas aeruginosa*) was reported. For the *in vitro* investigation, the examined isolates had antagonistic effect against the examined bacteria. The inhibition was recorded as a positive result when the diameter of the inhibitory zone around the wells was 0.5 mm or more. LAB showed maximal inhibitory effect against *E. coli* in comparison to *Pseudomonas aeruginosa*. On the other hand, the *in vivo* examination for the antibacterial effect of the obtained LAB toward these pathogens in milk at refrigeration temperature did not show changes in the mean count of the tested organisms versus control group (Fig. 1).

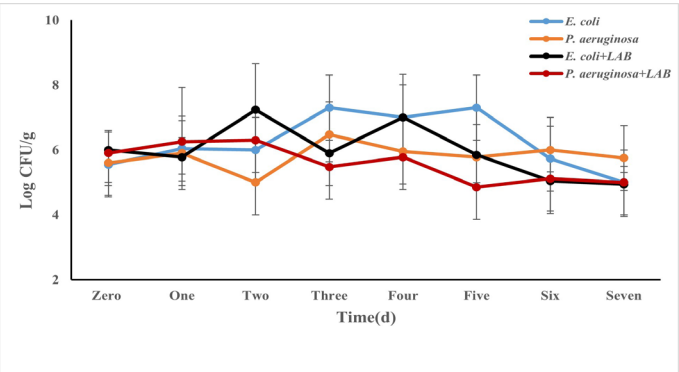

Fig. 1. Antibacterial activity of LAB obtained from kishk against *E. coli* and *Pseudomonas aeruginosa* after inoculation in cow's milk.

Discussion

Probiotic microorganisms that are beneficial to human health can be obtained through consumption of fermented dairy products. Herein, the present study assessed the microbiological quality of kishk and isolated then identified LAB in such food matrix. Furthermore, the antibacterial activity of the obtained LAB was examined toward some pathogens. We could prove that kishk is a rich dairy product with different kinds of probiotics that had protective effects against pathogenicity induced by *E. coli* and *Pseudomonas aeruginosa in vitro*.

In the current study, the microbiological evaluation revealed that the examined samples of kishk contained high level of total viable count (with an average of 8.13±0.09 $\log_{10}$ CFU/g). Similarly, Abd El-Razik *et al.* (2016) noticed that the total bacterial counts in different Kishk samples were ranged from 5.15 to 7.50 log CFU/g. This high bacterial load may be attributed to processing of kishk from raw milk and unpasteurized ingredients in farmers' houses. On the other hand, Abd El Ghani *et al.* (2014) collected kishk samples from four governorates (10 samples, each) in Upper Egypt and found that the total viable counts in the tested samples were 3.35±0.10, 3.37±0.12, 2.96±0.49 and 2.36±0.24 for A, B, F and G provinces respectively. These differences in the obtained findings may be due to the total number of the examined samples in the present study and in the previous ones, and the used protocol. In contrary to the total bacterial count, total yeasts and molds counts in the examined samples were low in most of the examined samples. The current investigation was in agreement with those determined previously by Abd El Ghani *et al.* (2014). Detection of yeasts and molds in kishk samples could be attributed to the aerial contamination during making and handling of such product (Abd El Ghani *et al.*, 2014).While, Kebary *et al.* (2014) and Abd El-Razik *et al.* (2016) could not detect yeasts and molds in the examined kishk samples.

Probiotic dairy products have beneficial effects on human health such as prevention of diarrhea and constipation, increase in the effectiveness against *Helicobacter pylori* infection, preservation of oral health, cholesterol lowering, enhancement of mineral absorption (Yerlikaya, 2014; Gao *et al.*, 2021)). In addition, these food matrices may be a good way to enhance the blood lipid profile and improve antioxidant defences in human body (Moura *et al.*, 2016). In this study, we were able to detect and isolate different kinds of LAB such as those of the *Lactobacillus*, *Leuconostoc* and *Lactococcus* genera. The obtained findings could be supported by an investigations by Atya and Khattab (1985) and Abd El Ghani *et al.* (2014). In contrast, Awad *et al.* (2013) found that all of the identified species were belong to the genus *Lactobacillus*.

Herein, the current report revealed that probiotics isolated from kishk had inhibitory effect on *E. coli* and *Pseudomonas aeruginosa* in the *in vitro* model. LAB could produce antimicrobial metabolites such as bacteriocins, diacetyls, lactic acid and hydrogen peroxide. These metabolites have an antimicrobial activity against many pathogenic and food spoilage bacteria (Marteau *et al.*, 1990; Edalati *et al.*, 2019; Ibrahim *et al.*, 2021). Edalati *et al.* (2019) reported that most of LAB isolated from camel's milk had an inhibitory effect on *S. aureus* subsp. *aureus* PTCC 1431, while only few had an inhibitory effect on *E. coli* ATCC 25922. However, we found that the highest antibacterial impact was obvious against *E. coli* in comparison to *Pseudomonas aeruginosa*. Similarly, *Lactobacillus* spp. showed highest antibacterial activity against *Pseudomonas aeruginosa* but it was found to be inactive against both Staphylococcus aureus and *E. coli* (Younis *et al.*, 2021). Interestingly, despite the strong antagonistic effect of LAB against *E. coli* and *Pseudomonas aeruginosa in vitro* in the current study, they did not exert any bactericidal effects toward such pathogens when inoculated in cow's milk. This may be due to milk components particularly protein that could interfere with the antibacterial effect of probiotics on the examined pathogens. Overall, although kishk has good microbiological and physicochemical qualities at ambient temperature, it could be contaminated with yeasts and molds during processing. Moreover, kishk is a fermented dairy product rich in probiotics that had inhibitory effects against some pathogens.

Conclusion

Kishk is a popular dairy product known as fermented milk-wheat mixture in Upper Egypt. The obtained results in the present study recommend kishk as a source for the isolation of potentially probiotics. They were identified as *Lactobacillus*, *Leuconostoc* and *Lactococcus* spp. which may have antagonistic properties against some pathogens (*E. coli* and *Pseudomonas aeruginosa*) *in vitro*. Since kishk is widely consumed, it is essential to use good quality raw ingredients and establish an affective food safety system during its preparation for the safety of consumer.

Conflict of interest

The authors declare that they have no conflict of interest.

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