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### Occurrence of some probiotic bacteria in ewe's and goat's milk in Assuit City with regarding to genes responsible for production of bacteriocins.

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#### ABSTRACT

**S**mall ruminant's milk is recognized as one kind of nutritious food owed to its originality and medicinal values. 100 samples collected from raw ewe and goat milk (50 of each) reared in Assiut City, Egypt. Milk samples were screened for probiotics *Lactobacillus*, *Bifidobacteria* and *Enterococcus spp.* as well as their differentiation especially those harboring bacteriocin genes. On the other hand, milk samples were tested for the existence of *total aerobic bacteria* and *total coliforms*. Goat's milk showed higher existence of *total aerobic* and *total coliforms* count more than ewe's milk which showed higher existence of *Lactobacillus*, *Bifidobacteria* and *Enterococcus spp.* The study detected *Enterococcus faecium* as more in ewe's milk (40%) than goat's milk (25%) and tested for harboring bacteriocin genes (mes Y & Plantaricin E/F). Our study concluded that goat's milk had more total pathogenic parameters than ewe's milk, but ewe's milk had more probiotic lactic acid bacteria (LAB); *Lactobacillus*, *Bifidobacteria* and *Enterococcus spp.*, also, contained *Enterococcus faecium* strains harboring bacteriocin genes mes Y & Plantaricin E/F genes more than those of goat's milk. Both of them have its value and uses in dairy industry. Further studies are needed and caring produce of these types of milk and their products in Egypt and could be assessed in several models in a similar manner to what is studied with bovine milk.

#### INTRODUCTION

Ewe's and goat's milk are gaining worldwide interest for their nutritional and functional characteristics (Manis et al. 2023). They have high nutritive value comparing with bovine milk, it can be considered as a useful

strategy to stop the problems of poor nutrition and increasing the economic status of many developing countries in Africa and Asia (Guerreiro et al. 2013) especially countries in Medeterrarian region, where be manufactured getting wide variety of products providing con-

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sumers prebiotic substances or probiotic bacteria (Kondyli et al. 2012, Balthazar et al. 2017 and Verruck et al. 2019). But ovine milk has been implicated as an important source of infection owing to poor hygienic conditions (milking methods, subclinical mastitis and contamination during or post milking) (Aqib et al. 2019). So, to reduce the bacterial contamination continuous monitoring and improvement of the hygienic quality of milk by ensuring proper handling and production is important (Praja et al. 2023).

Genus *Lactobacillus*, together with *Bifidobacterium* species are most commonly used as probiotic, when be present in adequate amounts confer a health benefit (FAO/WHO, 2001). Some of the isolate of *Enterococci* exhibited probiotic activity against several food spoilage bacteria and food-borne pathogens (Nami et al 2019). Among food-associated lactic acid producing bacteria (LAB), *Enterococci* are the most controversial group and important discrepancies exist between studies about their function in foods (Ruiz et al. 2016) as it may have a relevant role in dairy fermentation due to their interesting proteolytic and lipolytic activities (Foulquié Moreno et al. 2006). Although, *Enterococcus faecium* has long been recognized for its probiotic benefits (Hu et al. 2019 and Popovic' et al. 2019), and *Enterococcus faecalis* may have antibacterial activity (El Halfawy et al. 2019, El-Sayed et al. 2019). Bacteriocins are considered as ideal candidates for several health care applications due to their limited range of activity and rapid degradability by proteolytic enzymes (Ahmed et al. 2023) So, Several previous studies have focused on the production of bacteriocins by gram-positive bacteria (*Staphylococcus aureus*) with lactic acid bacteria (Zommiti et al. 2016; Wyszynska and Godlewska, 2021).

While, our work aimed to screen the bacteriocin production from *Enterococcus faecium* and *Enterococcus faecalis* and to evaluate the small ruminant's milk through estimating the existence of *total aerobic bacteria* and *total coliforms* count as well probiotics bacteria as *Lactobacillus*, *Bifidobacterium* and *Enterococci spp*

## MATERIAL and METHODS

### Sampling:

A total of 100 samples of raw ewe and goat's milk from clinically healthy sheep and goat were collected from different farms located in Assiut City: 50 samples each, Before a manual milking, teats were carefully cleaned with cotton wool impregnated with 70% of ethanol. After, the three first streams of milk were discarded; udders and mammary secretions were examined for macroscopic signs of abnormality. The samples were collected in sterile tubes and then placed in isotherm cool box (4°C) and transported to the laboratory of food hygiene in animal health research institute at Assiut city. The samples were analyzed for their microbiological and hygienic quality as well as the prevalence of some probiotic bacteria.

### Microbiological examination:

Total Bacterial Count (TBC): carried out on plate count agar (APHA, 2004).

Coliforms count: enumerated by the most probable number (MPN) (FDA, 2002).

Enumeration, isolation and identification of *Lactobacillus spp.*: isolation on DeMan, Rogosa and Sharpe (MRS) plates (Difco™) and characterized by the methodology described in the Bergey's Manual of Determinative Bacteriology (Hammes and Hertel, 2009).

Enumeration, isolation and identification of *Bifidobacteria spp.* On Bifidobacterium media (Nebra and Blanch, 1999).

Enumeration, isolation and identification of *Enterococci spp.* (Maia et al. 2017)

Identification of *Enterococcus faecium* and *Enterococcus faecalis*: (Morrison et al. 1997 and Manero and Blanch, 1999).

Extraction of bacteriocin by growing the strains in MRS broth at 37°C for 48 hours, cells separated by centrifugation at 5000 rpm for 10 minutes. The pH of supernatant was adjusted to 5.5 and the bacteriocin activity in the supernatant was evaluated by agar well diffusion method (Ogaki et al. 2016) using *Staph.aureus* (reference strain) (NCTC No. 7447) it was obtained from High Quality Me-

dia unit (HQM) in Animal Health Research Institute in Dokki, Egypt.

PCR detection of some bacteriocin genes (Mesentericin Y and Plantaricin E/F) from the previous *Enterococci* isolates. This part was done in Research Laboratory for veterinary quality control on poultry production in Ani-

mal Health Research Institute, Dokki, Giza. The samples were performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Primers used were supplied from Metabion (Germany) are listed in **Table (A)**.

Table A. Primers, sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary Denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
<i>Mesentericin Y (mesY)</i>	ATGACGAA-TATGAAGTC TTAC-CAAAATCCATT TCC	186	94°C 5 min.	94°C 30 sec.	45°C 30 sec.	72°C 30 sec.	72°C 7 min.	Xiraphi et al., 2008
<i>Plantaricin E/F</i>	GGCATAGTTA AAATTCCCCC CAGGTTGCCG- CAAAAAAAG	428	94°C 5 min.	94°C 30 sec.	53.2°C 40 sec.	72°C 45 sec.	72°C 10 min.	Rizzello et al., 2014

**Statistical Analysis**

The statistical analysis was performed using programs GraphPadPrism 5.04 (GraphPad, Inc., San Diego, USA) and Statistical 12.0 (Dell, Inc., Tulsa, USA). The bacterial count

represented by mean±SD (standard deviation value). The data represented by using the Microsoft Excel Spreadsheet. Also Kolmogorov-Smirnov D test, Chi-square test and Fishers exact test

**RESULTS**

Table 1. Statistical analytical results of microbial count in the examined ewe's and goat's milk samples (n=50 each CFU/mL).

Samples	Ewe's milk N=50					Goat's milk N=50				
	Positive samples		Min.	Max.	Mean ±SD	Positive samples		Min.	Max.	Mean ±SD
	No.	%				No.	%			
TBC	50	100	1×10 <sup>1</sup>	9×10 <sup>5</sup>	13x10 <sup>3</sup> ± 16x10 <sup>2</sup>	50	100	2×10 <sup>1</sup>	3×10 <sup>7</sup>	23x10 <sup>3</sup> ± 2.1x10 <sup>3</sup>
Coli-forms**	34	68	>10	3×10 <sup>3</sup>	27.3x10 <sup>2</sup> ±4.3x10 <sup>2</sup>	32	64	>10	4×10 <sup>5</sup>	2x10 <sup>3</sup> ± 3.1x10 <sup>2</sup>

\*\*High significant statistical variation in *coliforms* count between Ewe's milk and Goat's milk (Kolmogorov-Smirnov D = 0.3725, p< 0.01)

Table 2. Occurrence of *Lactobacillus spp.* among the examined ewe's and goat's milk samples (n=50).

<i>Lactobacillus spp.</i>	Positive samples	
	Ewe's milk	Goat's milk
	40	36
<i>Lact.acidophilus</i>	4	4
<i>Lact.fermentis</i>	22	20
<i>Lact.bulgaricus</i>	4	3
<i>Lact.brevis</i>	2	2
<i>Lact.plantarum</i>	4	3
<i>Lact.casei</i>	4	4

Table 3. Occurrence of *Bifidobacteria spp.* among the examined ewe's and goat's milk samples (n=50).

<i>Bifidobacteria spp.</i>	Positive samples	
	Ewe's milk	Goat's milk
	27	18
<i>Bifido.bifidium</i> **	18	5
<i>Bifido.breve</i>	4	3
<i>Bifido.subtile</i> **	5	10
<i>Bifido.infantis</i>	0	0

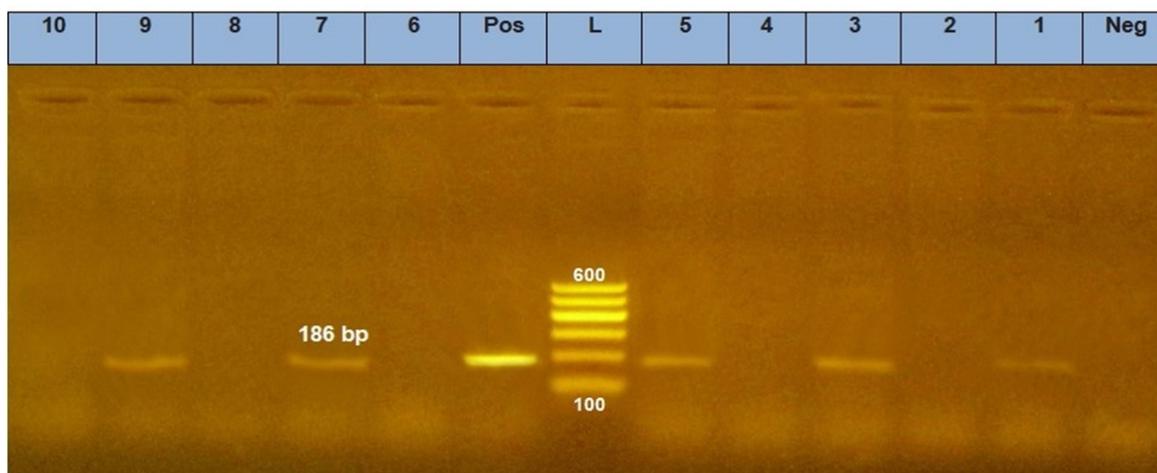
\*\* High significant statistical variation in *Bifido.bifidium* between Ewe's milk and Goat's milk (Chi-square = 6.537,  $p < 0.01$ )

\*\* High significant statistical variation in *Bifido. subtile* between Ewe's and Goat's milk ( Chi-square = 6.667,  $p < 0.01$ )

Table 4. Occurrence of some *Enterococci spp.* among the examined ewe's and goat's milk samples (n=50).

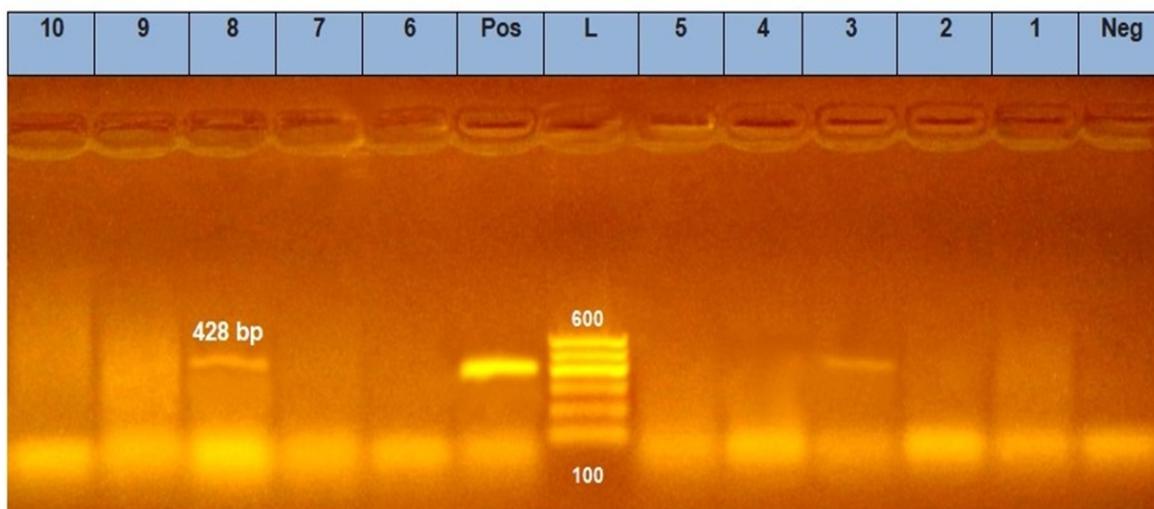
Types of samples	<i>Entero.faecium</i>				<i>Entero.faecalis</i>			
	ewe's milk		goat's milk		ewe's milk		goat's milk	
	No.	%	No.	%	No.	%	No.	%
Positive samples	10	20	5	10	4	8	2	4

Photo 1. The amplified (mesY) gene of *Enterofaecalis* and *Enterofaecium* recovered from ewe's and goat's milk



Lane L: Molecular marker; Lane pos: Positive control; Lane Neg: Negative control; Lanes 2, 4, 6, 8, 10: negative for Mesentericin Y; Lane 1, 3, 5, 7, 9: positive isolates for Mesentericin Y

Photo 2. The amplified Plantaricin E/F gene of *Enterofaecalis* and *Enterofaecium* recovered from ewe's and goat's milk



Lane L: Molecular marker; Lane pos: Positive control; Lane Neg: Negative control; Lanes 1,2,4-7,9,10: negative for Plantaricin E/F; Lane 3,8: positive isolates for Plantaricin E/F.

### DISCUSSION

As the small ruminant's milk lacks detail evaluation, the present study aimed to assess the total *viable bacteria and coliforms*, that reflect its hygienic and environmental conditions at which the small ruminant's pasture as well as its probiotic volubility by assessment of *Lactobacilli*, *Bifidobacteria spp.* and *Enterococcus spp.* Where Table (1) showed the mean

values with standard deviation for total bacterial count and coliforms count existence as ( $13 \times 10^3 \pm 16 \times 10^2$ ,  $27.3 \times 10^2 \pm 4.3 \times 10^2$ , CFU/ml, respectively) for ewe's milk while higher results for goat's milk ( $23 \times 10^3 \pm 2.1 \times 10^3$ ,  $2 \times 10^3 \pm 3.1 \times 10^2$ , respectively). In addition, there was high significant statistical variation in *coliforms* count between Ewe's milk and Goat's milk (Kolmogorov-Smirnov  $D = 0.3725$ ,  $p <$

0.01). Our results in this study showed that all the examined samples were contaminated with aerobic plate count but the count was lower than (Morgan et al. 2003; Muehlherr et al. 2003 and Ombarak and Elbagory, 2017) who investigated the aerobic plate count was  $9.11 \pm 2.47 \times 10^6$  and  $2.04 \pm 0.91 \times 10^6$  CFU/ml for goat's and ewe's milk, respectively and the incidences of coliforms was similar to our findings which was 68.57% and 60%, for raw goat's and ewe's milk, respectively. In Egypt, the microbial quality of raw caprine's and ovine's milk may be affected by unhygienic milking procedures, bad handling, contaminated animals water supply, keeping environments, storage and transportation of milk (Chye et al. 2004, Hicham et al. 2009, Saad et al. 2013 and Bogdanovičová et al. 2016) but there is no evidence of health hazards from raw goat's or ewe's milk (Mcintyre et al. 2002). Presence of coliforms bacterial count in milk may be returned to the unsanitary conditions practices during milk manipulation (Kondyli et al. 2012). A reference according the Malaysians Food Act 1983 and Food Regulations 1985, the total aerobic bacteria concentration in milk, which safe for consumed should not exceed 5.0 log cfu/ml (Food Act 1983 and Food Regulations 1985 – Act 281, 2005) which means that our results detected that most of examined samples were safe.

The higher microbial counts in goat's than ewe's milk samples may be related to the anatomical structure differ between ewe's udder as it have higher, smaller udder with short rapidly closed teat canal than goat's one (Carretero et al. 1999), environmental surroundings, milk manipulation and feeding manner also have effect on microbial count (Eman et al. 2009 and Abo El-Makarem, 2016).

Our work also scopes on the economic important of using these types of milk in dairy industry especially in our developing country. So, we examine the samples for presence of probiotic bacteria Tables (1& 2) were showed the presence of different species from *Lactobacillus* and *Bifidobacterium spp.* isolated from ewe's and goat's milk. *Lactobacillus spp.* detected in ewe's milk (40 samples) in higher percentages than goat's milk (36 samples) and

vice versa with *Bifidobacterium spp.* (27 and 18, respectively). *Lact fermentis*, *Lact.bulgaricus* and *Lact.acidophilus* were the most isolated *Lactobacillus spp.* from milk samples and *Bifido.bifidium*, *Bifido.subtile* and *Bifido.breve* from *Bifidobacterium spp.* It is worth to be mentioned that was high significant statistical variation in *Bifidobacteria spp.* between Ewe's and Goat's milk (Kolmogorov-Smirnov D = 0.5185,  $p < 0.01$ ).

In addition, there was high significant statistical variation in *Bifido.bifidium* isolates between Ewe's and Goat's milk (Chi-square = 6.537,  $p < 0.01$ ) and high significant statistical variation in *Bifido.subtile* isolates between the both types of milk (Chi-square = 6.667,  $p < 0.01$ ).

Bifidobacteria are considered the primary probiotic bacteria associated with milk and dairy products (Rodrigues et al. 2011). Raw ewe's and goat's milk is a good source for isolation of wild lactobacilli which are able to bring unique processing properties in development of dairy products, cheeses or fermented dairy products (Miroslav et al. 2014) and there are many studies isolated lactic acid bacteria (LAB) from Ewe's and Goat's milk (Chen et al. 2020; Tanaka et al. 2023). So, In Egypt, deeply in need for new economic sources of probiotic products manifested in sheep and goat milk production is nearly not used. The beneficial effects of probiotic consumption include antibacterial activity (El Halfawy et al. 2019)

Among the Lactic Acid Bacteria (LAB), *Enterococcus spp.*, which reported to goodwill to produce antimicrobial compounds including bacteriocins, bacteriocin - like substances, or metabolites with antibacterial activity, which made them potential candidates for biopreservative agents for a wide range of food products, promotion of human and animal health by improving the intestinal microbial balance and is now being considered as a probiotic trait (Franz et al. 2011, Yang et al. 2014 and Bali et al. 2016).

*Entero.faecium* could be detected in (20 and 10%) and *Entero.faecalis* (8 and 4%) in ewes and goats' milk, respectively (Table 4). while, The isolated *Entero.faecium* and *En-*

*terofaecalis* strains in this study investigated for bacteriocin production by the antibacterial activity of it through extraction of bacteriocin by cold centrifugation and using the supernatant (which contain bacteriocin) against *Staph.aureus* by well diffusion method. We choose *Staphylococcus aureus* because it is a significant and costly public health concern since it may enter the human nourishment chain and usually causing foodborne illness (Liu et al. 2022). Also, milk and milk products are known to be a source of *S. aureus* contamination whether they are collected from dairy animals or from food handlers carrying the organism because of poor individual cleanliness (Bingol et al. 2012). Five strains from ewe's milk and five from goat's milk were bacteriocin producers then these strains subjected to PCR for detection the presence of some bacteriocin genes as *Mesentericin Y (mesY)* and *Plantaricin E/F* and found that 5 strains of *Enterofaecium* have *mesY* (3 isolated from ewe's, 2 from goat's milk origin), one of them have both genes (returned to goat's milk) and one have only *Plantaricin E/F* (from ewe's milk) while, no strain of *Enterofaecalis* have any of both genes.

*Enterococcus spp.* carries many bacteriocin-related genes that PCR array allowed quick and easy identification of the presence of these genes (Henning et al. 2015). *Plantaricin E/F* containing natural substances against foodborne pathogenic, as well as spoilage bacteria has raised considerable interest for their application in food preservation (Todorov, 2009). Also, *mesY* bacteriocin genes provide antimicrobial activity and may also be useful in activity against spoilage and pathogenic organisms in select food applications. Many strains of *Enterococcus spp.* encode more than one bacteriocin, although it remains to be seen which ones might be actively expressing bacteriocin proteins (He´chard et al. 1992 and Henning et al. 2015)

It is now relatively simple to use specific PCR screening techniques to determine whether bacteriocin genes are present in the bacterial isolates especially when multiple species are present in the samples (Kubašová et al. 2020).

## CONCLUSION

It is concluded that small ruminant raw milk samples contained different types of probiotics which carry a lot of benefits to human in both health and economic importance. Goat's milk had more total pathogenic studied parameters than ewe's milk and ewe's milk had more probiotic LAB; *Lactobacillus*, *Bifidobacteria* and *Enterococcus spp.* and contained *Enter. Faecium* strains harboring bacteriocin genes *mesY* & *Plantaricin E/F* genes more than those of goat's milk.

It is recommended that further intensive studies should be carried out for the production of dairy products from these valuable economic types of milk to fulfill the needs of local markets.

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