Egypt. J. Chem. **Vol. 66**, No. 10 pp. 107 - 117 (2023)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Characterization, Encapsulation and Evaluation of the Newly Isolated Enterococcus Faecium as A Probiotic For Ruminants

Hoda S. El-Sayed[1], Shaymaa A. Ismail[2], Murad, H.A.[1], Asmaa G. Abu-El Khair[1], and Azzaz, H.H.[1]*

[1]*Dairy Science Department, National Research Centre, Dokki, Cairo, 12622, Egypt*
[2]*Department of Chemistry of Natural and Microbial Products, National Research Centre, Dokki, Giza, 12622, Egypt*

Abstract

The utilization of probiotics in ruminant diets has attracted a growing interest in providing various health and productive benefits. Enterococcus faecium characterization, encapsulation and evaluation as a potential probiotic for ruminants was the main aim of the current study. The isolated lactic acid bacterium (LAB) in this study was identified using MALDI-TOF MS technology and 16S ribosomal rDNA sequencing. The ability of the isolated LAB for producing antimicrobial agents as well as its tolerance to heat, acidity and bile salt was examined. Biomass production for the isolated strain using permeate basal media was conducted. For feeding purpose; the produced biomass was encapsulated using extrusion method by sodium alginate. The encapsulated biomass effects on rumen fermentation and feed degradation were evaluated using in vitro batch culture technique. The results indicated that the isolated strain which met the specifications of the ideal probiotic was Enterococcus faecium EGY_NRC3. The addition of the encapsulated E. faecium EGY_NRC3 to the ruminant ration led to higher degradability for the fiber fractions, dry matter (DM) and organic matter (OM) with higher production of total gases (TGP) and short chain fatty acids (SCFA) in the rumen in vitro. In conclusion; local production of LAB probiotic using milk permeate will lead to reduce the importation cost for probiotics and diminish the environmental pollution hazards.

Keywords: Enterococcus faecium, dairy animal, probiotics, encapsulation, rumen microflora, ammonia nitrogen and ruminal gas production.

1. Introduction

Shortage of feedstuffs and animals' lack of benefit from the available feedstuffs are the main problems facing animal nutritionists in Egypt [1]. To find effective solutions for these problems which have significant impacts on ruminant's productivity, many approaches have been given attention through time. Enrichment of the nutritional value of the agricultural by-products to be used as alternative feedstuffs through mechanical, chemical, or biological treatments is one of these approaches [2-3]. Also, the use of feed additives like feed enzymes [4], herbal and medicinal plants [5- 6], essential oils, algae and yeasts [7, 8], antibiotics [9], and probiotics [10] is another approach.

Nowadays, there is a great interest in the use of probiotics as live microbial feed supplement [11]. They can improve animal health via modulation of gastrointestinal microbial balance and activities [12]. Probiotics are broad and include a variety of bacteria (Enterococcus, Lactobacillus, Bifidobacterium and Bacillus) and yeasts which became commonly used as ruminant feed supplement10. Enterococcus is a genus of lactic acid-producing bacteria, that naturally inhabit the rumen and intestine of the ruminant, are easily cultivated and proliferate under aerobic or anaerobic conditions, therefore their preparations represent a good probiotic source for ruminants [12].

Enterococci are gram-positive, facultative anaerobic [13], capable of producing vitamin B12 [14], enzymes [15], and antimicrobial bacteriocin called enterocin [16]. Enterococcus faecium is commonly used in ruminant feeding as a probiotic due to its effectiveness in growth stimulation of rumen microflora and maintaining the activity of the ruminal lactate-utilizing bacteria which leads to the prevention of ruminal acidosis and increases the glucogenic propionate energy supply for the host animal [17]. Also, Enterococcus faecium plays a vital

*Corresponding author e-mail: hosam19583@gmail.com.; (Azzaz, H.H).
EJCHEM use only: Received date 21 September 2022; revised date 07 January 2023; accepted date 12 February 2023
DOI: 10.21608/EJCHEM.2023.163556.6995

role in the antagonism of the harmful bacteria in the rumen through the production of antimicrobial agents [18]. The action of Enterococcus faecium is depending on its availability and viability in the rumen, diet composition, and rumen environment [19].

Probiotics in general are very sensitive to gastrointestinal conditions and their microencapsulation has been considered the key tool to maintain their viability during feeding and their passage through the acidic conditions of the gut [20-23]. Microencapsulation provides various advantages including the utilization of simple equipment with low production cost in addition to the high encapsulation efficiency that reaches above 90% by applying the extrusion method using different coating materials [24]. Accordingly, the characterization, encapsulation, and evaluation of the newly isolated Enterococcus faecium as a potential probiotic for ruminants were the main aims of the current study.

2. Experimental

2.1. Microorganism: The used bacterial strain was isolated during an intensive course screening program concerned with the isolation of lactic acid bacteria from fresh milk and local homemade dairy products. Carbohydrate fermentation pattern [25] of the isolated strain, as well as its ability for the production of ammonia from arginine [26], was determined. In addition, identification of the isolate was carried out by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) as well as 16S ribosomal DNA (rDNA) sequencing. MALDI-TOF MS depends on the analysis of the total proteome in which a score more than 1.7 indicates genus identification and a score more than 2 is the confidence value at the species level. In 16S rDNA sequencing, the genomic DNA was initially extracted from a freshly prepared culture then PCR amplification of the segment of its 16S rRNA gene was carried out using the two primers, 8f (5'_AGAGTTTGATCCTGGCTCAG-3') and 1495R (5'_CTACGGCTACCTTGTTACGA-3'). On the base of the sequencing data, phylogenetic analysis was inferred using neighbor joining method and the phylogenetic tree was constructed using MEGA x.

2.2. Temperature tolerance: According to the Rajkowska method [27], the growth of the isolate was monitored at different temperatures (10-40 ºC) in which M17 broth medium was inoculated by 5 % (v/v) of 24 h old age activated M17 broth culture then the optical density was measured spectrophotometrically at 600 nm every 24 h up to 72 h. At 60 ºC, pour plate count [28] was used to enumerate its viable count after pre-incubation of the inoculated medium at that temperature for 30 min. The pre-incubation viable count was considered as the control in which the results were the average of two replicates.

2.3. pH, NaCl, and bile salt tolerance: The growth of the isolate was evaluated at different pH ranging from 2 to 9.6 according to the modified method of Wang *et al*., [29] in which the inoculated M17 broth medium (previously adjusted by 5 M HCl or NaOH to the desired pH) was incubated for 24 h at 30 ºC. Moreover, the growth in M17 broth supplemented with different concentrations of NaCl (0.5 to 6.5 %) and different concentrations of bile salt (1, 2 and 3 %) was estimated.

2.4. Stability of the isolated strain in simulated gastrointestinal juices: To simulate the gastric juice, pepsin was prepared at a final concentration of 3 g/L in saline solution then the pH was adjusted to 2.0 using concentrated lactic acid. In order to simulate small intestinal juice, pancreatin was added at a final concentration of 10 g/L to a sterile solution composed of (% w/v), 0.3 bile salts; 0.65 NaCl; 0.083 KCl; 0.022 $CaCl_2$; 0.138 $NaHCO_3$ then adjusted to pH 7.0 [30]. The tested isolate was activated in M17 broth inoculated with 5 % (v/v) and incubated for 48 h at 37 ºC. The cells were obtained by centrifugation of the activated culture at 5000 rpm for 15 min. The initial count of the tested strain was estimated by the pour plate method on an M17 agar medium incubated at 37 ºC for 48 h. The experiment was carried out in 15 mL falcon tubes in which 1 mL of cells were added to 9 mL of the prepared gastric solution and incubated for 2 h at 37 ºC. Aliquots of 1 mL were removed to assess resistance to gastric juices. The gastric solution was removed by centrifugation (4000 rpm/10 min) and replaced with 9 mL of intestinal solution that was incubated for 4 h at 37 ºC. After the incubation period, 1 mL was removed to assess resistance to intestinal juices. The stability counts were determined by the pour plate method on M17 agar medium incubated at 37 ºC for 48 h.

2.5. Auto-aggregation test: According to Collado *et al.,* [31], the isolate was cultivated in M17 broth for 24 h followed by centrifugation (10 min at 4500 rpm) in a sterilized centrifuging falcon and washing with a sterile 0.05 M phosphate buffer pH 7. After the cells were suspended in 4 mL of the same buffer and vortex for 20 sec, the absorbance was measured immediately at 600 nm and after 24 h incubation at 30 ºC. The auto-aggregation percentage was calculated according to the following equation:

$$\text{Autoaggregation (\%)} = [1-(A_{24}/A_0)] \times 100$$

Where, A_{24} is the absorbance after 24 h incubation period and A_0 is the absorbance at zero time.

2.6. Antibacterial activity: The antibacterial activity was determined using the well diffusion method according to Bali et al, [32] against *Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 14579, *E. coli* ATCC 8739 and *Salmonella Typhimurium* ATCC 14028. The bacterial cell suspensions matching a 0.5 McFarland standard were grown on nutrient agar at 37 ºC for 24 h. The supernatant of the isolated strain obtained after centrifugation of M17 pre-cultured medium incubated for 48 h at 30 ºC was examined in which 0.1 mL of the supernatant was placed in wells of 7 mm in diameter made in the agar plates with a sterile glass Pasteur pipette. The Ampicillin (10 µg /mL) was used as positive control for results comparison.

2.7. Antibiotic susceptibility test: The antibiotic susceptibility of the isolate was evaluated using different antibiotic discs including ampicillin (10 µg), cefadroxil (30 µg), erythromycin (15 µg), kanamycin (30 µg), streptomycin (10 µg), tetracycline (30 µg) and vancomycin (30 µg). Initially, the isolate was grown in M17 broth for 24 h and adjusted to around 10^5 CFU/mL then used for culturing M17 agar plates. Antibiotic discs were placed on the inoculated plates using sterile forceps and incubated at 37 ºC for 48 h. The inhibition zone around each disc was measured in mm.

2.8. Biomass production of the isolated strain using whey permeate: Different growth permeate basal media were formulated with the following composition (g/L permeate); Medium (1): Yeast Extract; 5.0, Peptone; 5.0 and Magnesium Sulfate; 0.5. Medium (2): Yeast Extract; 5.0, Peptone; 5.0, Magnesium Sulfate; 0.5, Ammonium Chloride; 3 and Ascorbic acid; 2.5. Medium (3): Yeast Extract; 5.0, Peptone; 5.0, Magnesium Sulfate; 0.5, Beef extract; 5 and Ascorbic acid; 2.5. The media were inoculated with 5 % (v/v) of 24 h old age activated M17 broth culture of the isolated strain then incubated at 37 ºC for 48 h followed by centrifugation at 6000 rpm for 15 min at 4 ºC and drying at 50 ºC. The un-inoculated media were used as a control.

2.9. Microencapsulation of the isolated strain by extrusion method using sodium alginate: Initially, sodium alginate was dissolved in distilled water at a concentration of 3 % (w/v) and sterilized by autoclaving at 121 ºC for 15 min. After that, a mixture of 100 mL sodium alginate and 25 mL of the isolate cells was homogenized using magnetic stirring for 10 min. The mixture was transferred into a measuring syringe of 5 mL (0.5 mm). After that, the mixture was extruded into the $CaCl_2$ solution (20 g/L w/v) under stirring for 30 min. The formed microcapsules were collected, washed with normal saline and stored in cooling for the next expermints [20]. Evaluation of the encapsulation efficiency (EE) was estimated in which one gram of the microcapsules was added to 9 mL of sterile 2 % (w/v) trisodium citrate solution and then vortex until completely broken of the microcapsules. The count of the isolate inside the broken microcapsules was detected by the pour plate method on M17 agar in which the plates were incubated at 37 ºC for 48 h anaerobically [30]. EE was calculated according to the following equation:

EE = Log N / Log No × 100

Where, N is the count of the isolated strain inside the microcapsules and No is the count of the free cells added to sodium alginate.

2.10. Impact of the addition of the microencapsulated isolate on ruminal fermentation characteristics (*in vitro*): Evaluation of the impact of the produced microencapsulated strain on rumen fermentation characteristics was carried out using the batch fermentation technique as described by Ismail et al., [33]. The incubation vessels (3 replicates for each treatment) were filled with 40 mL of a mixture of 1:3 (v/v) rumen fluids: buffer solution + 400 mg of the tested ration and separately supplemented with the microencapsulated strain at different levels (0, 1, 2 and 3 g/Kg of the tested ration) and then sealed and incubated for 24 h at 39 ºC. Each gram of the microencapsulated strain contains 1.2 x 108 CFU. The feed ingredients and the chemical composition of the tested ration were shown in table (1). After the incubation period, the vessels were filtered and the remaining residues were dried to constant weight to determine the dry matter (DM), organic matter (OM), neutral detergent fibre (NDF) and acid detergent fibre (ADF) digestibility. Rumen fluid pH, total gas production (TGP), ammonia NH3-N and short-chain fatty acids (SCFA) concentrations were determined as described by Ismail et al., [33].

Table (1): Feed ingredients and chemical composition of the tested ration based on dry matter (g/kg)

Item	DM	OM	CP	CF	EE	Ash	NFE
C FM	**922.70**	**940.00**	**168.00**	**195.00**	**40.20**	**60.00**	**536.80**
Egyptian clover	**905.00**	**950.00**	**160.00**	**400.20**	**30.50**	**50.00**	**359.30**
Corn stalks	**930.00**	**932.50**	**15.00**	**481.20**	**17.40**	**67.50**	**418.90**
Control ration	**916.35**	**943.25**	**149.50**	**305.70**	**34.04**	**56.75**	**454.01**

CFM: Concentrate feed mixture, DM: Dry matter, OM: Organic matter, CP: crude protein, CF: crude fiber, EE: Ether extract, NFE: Nitrogen free extract

Control ration: 60% concentrate feed mixture (50% yellow corn + 15% soybean meal + 10% sunflower meal + 10% D.D.G.S + 15% wheat bran), 30% berseem (clover) hay and 10% Corn stalks

2.11. Statistical analysis: IBM SPSS Statistics for Windows (https://www.ibm.com/products/spss-statistics) was used for statistical analysis of the data using one way ANOVA test to compare means. Duncan's multiple range tests was used to test the significance among means at probability level of 0.05.

3. Results

3.1. Microorganism: The bacterial strain used in the present study was gram-positive catalase-negative cocci isolated from locally homemade cheese. The carbohydrate fermentation pattern of the isolated strain indicated its ability for the fermentation of glucose, lactose, and fructose. Moreover, it had been indicated to produce ammonia from arginine. MALDI-TOF MS identified the isolated strain as *Enterococcus faecium* with a confidence value of 1.83. Identification of the isolate was confirmed on the base of the results of 16S rDNA nucleotide sequencing that indicated its identity percentage of 99.37%. Phylogenetic analysis was inferred using the neighbor-joining method and the constructed phylogenetic tree was shown in Figure (1). The sequencing data were submitted to NCBI under the name *Enterococcus faecium* EGY_NRC3 and received accession number MW856656.

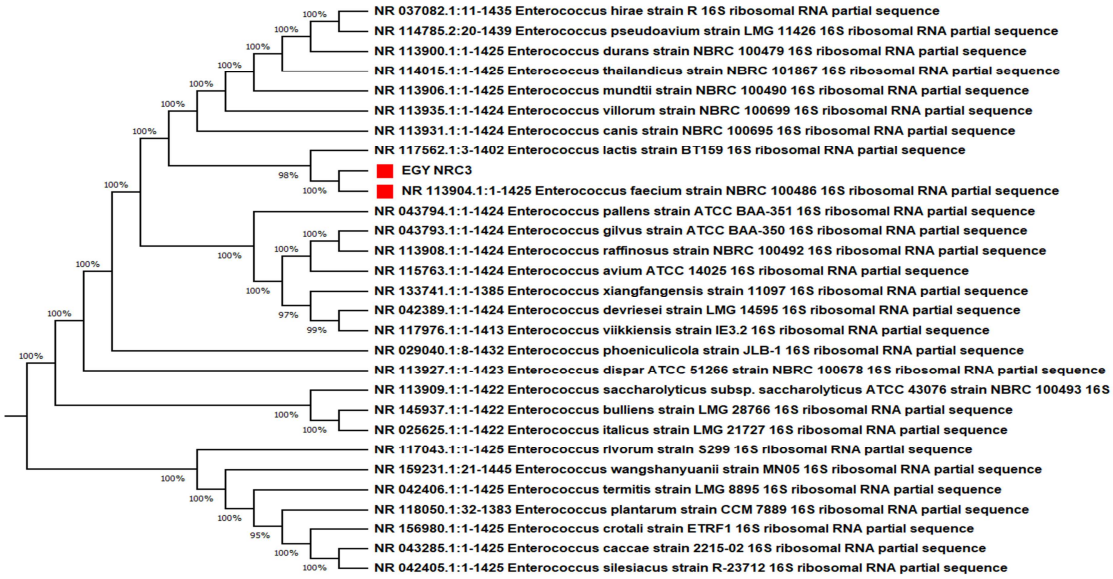

Figure (1): The phylogenetic tree for the isolated strain constructed using MEGA X

3.2. Temperature tolerance: The increase in the growth of the isolated strain after incubation at different temperatures for different incubation periods (24-72 h) was calculated by dividing the estimated optical density at 600 nm by the initial optical density. The results indicated the disability of the isolated strain to grow in low temperatures as shown in Figure (2). In addition, the viable count of the strain after pre-incubation at 60 ℃ for 30 min indicated the survivability of the strain with an observed decrease by 2.10 log cycles to reach 3.635 Log CFU/mL.

3.3. pH, NaCl and bile salt tolerance: The growth of the isolated strain at different pH, NaCl and bile salt concentrations were monitored. The results indicated that the strain could survive in M17 broth adjusted to different pHs ranging from 2 to 9.6 (Figure 3) but it could not survive in M17 broth supplemented with either high concentration of NaCl (4 and 6.5) or bile salt.

3.4. Stability of the isolated strain in simulated gastrointestinal juices: The stability of the isolated strain after exposure to gastrointestinal juices was shown in Figure (4). At the gastric solution, the initial count was recorded 9.60 Log CFU/g and at the gastric solution, a decrease was observed after 2 h to record 8.37 Log CFU/g. More declines in the count were observed after transferring the remained cells in the intestine juice to record 6.55 Log CFU/g after 4 h. The count was declined by about 3.00 log cycles in the gastrointestinal juices.

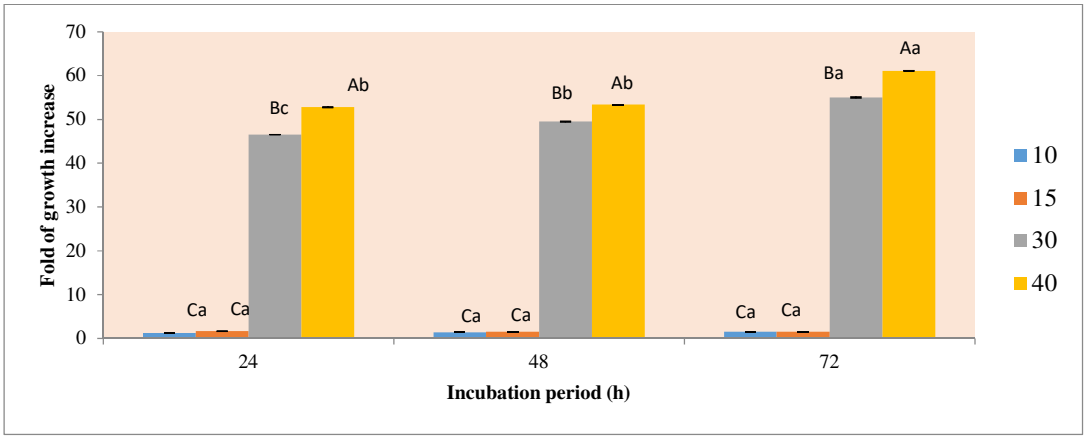

Figure (2): The effect of temperature and incubation period on the growth of the isolated strain

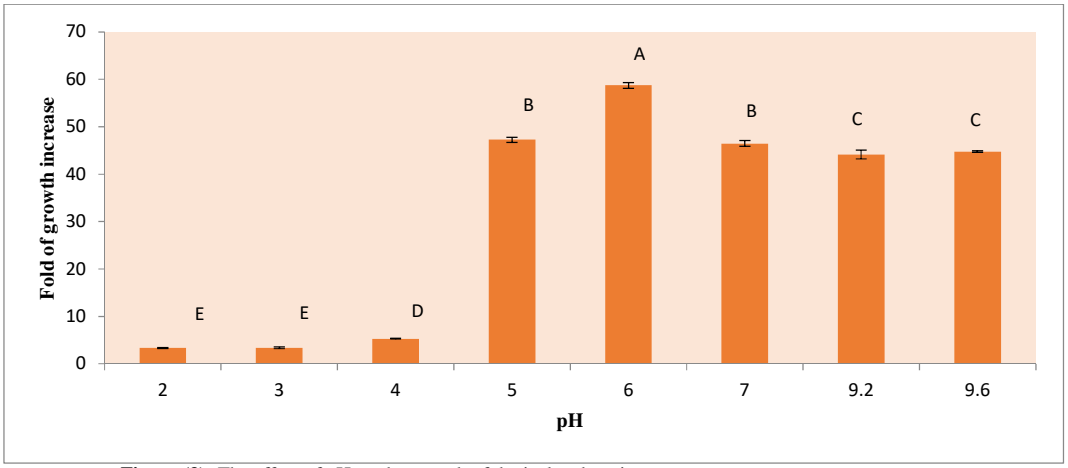

Figure (3): The effect of pH on the growth of the isolated strain.

Figure (4): ***In*** *vitro* viability of the isolated strain in simulated gastrointestinal conditions (Log CFU/g)

3.5. Autoaggregation: Cell aggregation is one of the most important phenotypic characteristics of the potentially probiotic strain. In the current study, the autoaggregation value estimated for the isolated strain was 85.77 %.

3.6. Antibacterial activity: The antibacterial activity of the isolated strain was evaluated against gram-positive and gram-negative bacteria. As illustrated in Table (2), Figure (5) the isolated strain exhibited broad antibacterial activity ranging from 13 to 18 mm depending on the pathogenic strain type.

Table (2): Antibacterial activity

Tested strain	Inhibition zone (mm)	
	Culture supernatant of the isolated strain	Ampicillin (10 µg)
Staphylococcus aureus ATCC 6538	17± 0.28[a]	19± 0.42[a]
Bacillus cereus ATCC 14579	18±0.33[a]	17±0.28[a]
E. coli ATCC 8739	13±0.29[c]	13±0.64[b]
Salmonella Typhimurium ATCC 14028	15±0.30[b]	14±0.14[b]

Means in the same columns with different superscripts differ, P < 0.05. ± MSD: Mean of standard deviation.
S: ≥17; I: 14–16 and R: ≤13. According to the CLSI and Eucast clinical microbiological zone diameter standards (mm) using ampicillin for comparison; Sensitive: ≥17; Intermediate: 14–16 and Resistant: ≤13.

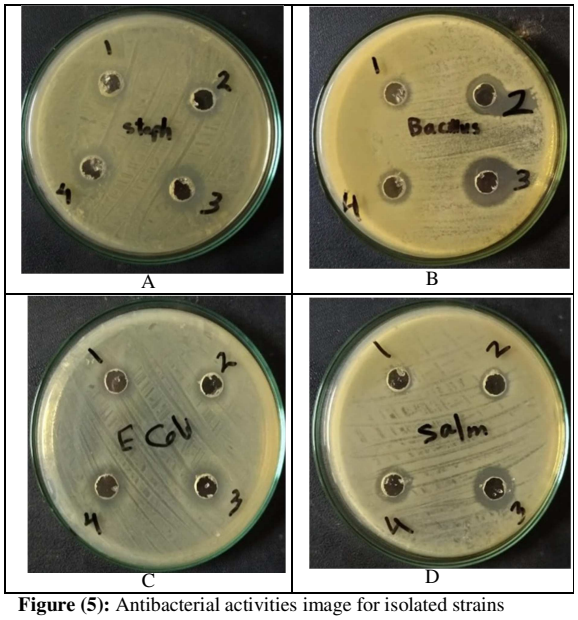

Figure (5): Antibacterial activities image for isolated strains against tested pathogen.
Note: Bacterial isolate No. 3 was the subject of the study of this research paper

3.7. Antibiotic susceptibility test: Antibiotic susceptibility of the isolated strain was examined and the result indicated that the susceptibility of the isolated strain to tetracycline (30 µg), ampicillin (10 µg), cefadroxil (30 µg), erythromycin (15 µg), and vancomycin (30 µg) with average inhibition zones ranged from 15 to 35 mm. In addition, it had been indicated to be resistant to kanamycin (30 µg) and streptomycin (10 µg) (Table 3).

Table (3): Result of the antibiotic susceptibility test

Antibiotics	Kanamycin (30 µg)	Streptomycin (10 µg)	Tetracycline (30 µg)	Ampicillin (10 µg)	Cefadroxil (30µg)	Erythromycin (15 µg)	Vancomycin (30 µg)
Inhibition zone (mm)	-	-	33 ± 0.33 ab	35 ± 0.59 a	29 ± 0.28 b	30 ± 0.30 b	15 ± 0.52 c

Means in the same columns with different superscripts differ, P < 0.05. ± MSD: Mean of standard deviation

3.8. Biomass production of the isolated *Enterococcus faecium* using whey permeates media: The isolated strain was able to grow well in the examined prepared media using whey permeate in comparison with standard M17 medium and the biomass produced (as dry cell weight) was shown in Figure (6). The produced biomass was recorded as 24.3, 23.5, and 23.7 g/L for media NO. 1, 2, and 3, respectively in comparison to 22.7 g/L for the M17 medium.

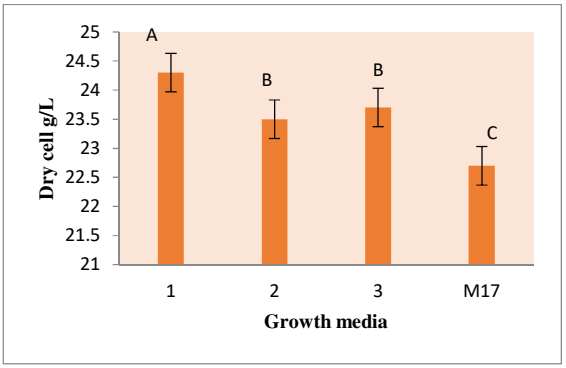

Figure (6): Biomass production of *Enterococcus faecium* NRC-3 using different whey permeate media.

3.9. *In vitro* study

3.9.1. Encapsulation of the isolated *Enterococcus faecium*: Initially, microencapsulation of the isolated *Enterococcus faecium* NRC-3 was carried out to maintain its stability and viability. The initial cell count before microencapsulation was 9.80 log

3.9.2. CFU/mL and the calculated percentage of microencapsulation efficiency was 96.89 %.

3.9.3. Effect of *Enterococcus faecium* NRC-3 addition level on feed degradability parameters: The data in Table (4) indicated that as the addition level of the isolate increased, the values of ruminal feed degradability parameters (DM, OM, NDF & ADF) gradually increased. There was a direct linear relationship between the isolate level addition and the ration digestion coefficients. However, there was no significant change was detected in the digestion values of DM, OM, NDF and ADF due to the addition of the isolate at 2 g or 3 g per Kg of the tested ration. Therefore, the addition of *E. faecium* NRC-3 at a level of 2 g/kg of feed dry matter will be recommended from an economical point of view.

Table (4): Effect of *Enterococcus faecium*NRC-3 addition level on feed degradability parameters

Supplementation level	DMD %	OMD %	NDFD %	ADFD %
Control	48.17^{c}	54.04^{c}	34.7^{c}	24.42^{c}
***E. faecium* (1 gm/Kg)**	50.82^{b}	56.69^{b}	37.35^{b}	27.07^{b}
***E. faecium* (2 gm/Kg)**	53.81^{a}	59.68^{a}	40.34^{a}	30.06^{a}
***E. faecium* (3 gm/Kg)**	55.88^{a}	61.75^{a}	42.41^{a}	32.13^{a}
MSD±	1.32	1.51	1.09	1.21

DMD%: dry matter degradability, OMD%: organic matter degradability, NDFD%: neutral detergent fiber degradability and ADFD%: acid detergent fiber degradability. ± MSD: Mean of standard deviation. Means in the same row with different superscripts differ, P < 0.05.

3.9.3. Effect of *Enterococcus faecium* NRC-3 addition level on feed fermentation characteristics: The data in Table (5) indicated an inverse relationship between the addition level of the isolate and the ruminal pH and CH_4 values. The control ration recorded the highest ruminal pH and CH_4 values, while the treated rations showed a gradual decline in ruminal pH values and CH_4 production with the increase of *E. faecium* NRC-3 addition level. In contrast, there was a positive (linear) relationship between *E. faecium* NRC-3 addition level and ruminal total gas production (TGP) volume and SCFA concentration. No significant change had been detected in the ruminal CO_2 production and NH_3-N concentration after *E. faecium* NRC-3 addition. There was no significant difference between the impact of the 2nd and the 3rd addition level of the isolate on ruminal pH value and SCFA concentration. The 3rd addition level increased significantly the ruminal TGP production when compared with the 2nd addition level. Therefore, the use of the third level of *Enterococcus faecium* NRC-3 will be excluded from an environmental point of view.

4. Discussion

In the current study, the potentiality of the isolated Enterococcus faecium as a probiotic for ruminants was investigated. Initially, the isolated strain was identified by MALDI-TOF MS in which the confidence value was 1.83, indicating a reliable identification of the genus with low confidence species identification. Furthermore, the identification of the isolate was confirmed by 16S rDNA and consequently the isolate named Enterococcus faecium EGY_NRC3. The ability of the isolate for producing antimicrobial compounds was in vitro evaluated against gram-positive (Staphylococcus aureus and Bacillus cereus) and gram-negative (E. coli and Salmonella Typhimurium) pathogens in which the results indicated a positive effect against all the tested strains. Among lactic acid bacteria, enterococci are capable for the production of various antimicrobial compounds including bacteriocin [16]. in comparison with Ampicillin (10 µg /mL), Staphylococcus aureus and Bacillus cereus (gram positive) were most significantly inhibited by the culture supernatant of the isolated strain. Whereas the effect of isolate strain against gram negative was low (E. coli) to intermediate as in case of S. Typhimurium [34].

Enterococci are widely spread in nature as they are commonly present within the human and animal intestinal microbiota in addition to dairy products, fermented vegetables and raw fruits [35]. Recently, they gained great attention for their exploration as probiotics for providing various health benefits [36]. In the current study, the isolated strain was able to survive at low pH values but it could not tolerate the existence of a high concentration of NaCl or bile salt. Additionally, its survival rate declined by about 3.00 log cycles through its existence in simulated gastrointestinal juices. Therefore, encapsulation of the isolated strain was examined by applying the extrusion method using sodium alginate in which the microencapsulation efficiency was 96.89 %. Microencapsulation had been estimated as an appropriate technique for saving the survival rate of probiotics without losing their viability for a long period [30].

For safety evaluation of the isolate, its antibiotic sensitivity was examined and the result indicated that the strain was sensitive to ampicillin and vancomycin that considered the commonly used antibiotics for the treatment of infections caused by Enterococcus sp. [37]. Toomey et al., [38] reported that only 4 of 10 strains of Enterococcus faecium were susceptible to vancomycin. Additionally, the antibiotic sensitivity test indicated that the isolated strain was resistant to kanamycin and streptomycin. This pattern was similar to that previously reported by Cho et al., [39].

Table (5): Effect of *Enterococcus faecium* NRC-3 addition level on feed fermentation characteristics

Supplementation level	pH	TGP	CH_4	CO_2	SCFA	NH_3-N
Control	6.43^{a}	123.47^{d}	12.58^{a}	79.20	1.37^{b}	11.26
***E. faecium* NRC-3 (1 gm/Kg)**	6.33^{ab}	129.88^{c}	11.89^{ab}	78.85	1.44^{a}	11.38
***E. faecium* NRC-3 (2 gm/Kg)**	6.27^{b}	134.67^{b}	10.65^{b}	77.02	1.45^{a}	11.53
***E. faecium* NRC-3 (3 gm/Kg)**	6.23^{b}	145.09^{a}	9.15^{b}	75.42	1.5^{a}	11.87
MSD±	0.09	8.36	1.91	8.93	0.09	0.65

TGP: total gas production, CH_4: methane production, CO_2: carbon dioxide gas production, SCFA: short chain fatty acids and NH_3-N: Ammonia- nitrogen. ± MSD: Mean of standard deviation

Means in the same row with different superscripts differ, $P < 0.05$.

The overall cost of any bioprocess is a major obstacle in its industrial application and the production media contribute about 40 % of the total cost [40]. Therefore, the utilization of industrial by-products attracted the research focus on the economic production of various biomaterials [41-42]. In the current study, the production of Enterococcus faecium EGY_NRC3 bacterial biomass using whey permeate was examined in comparison to the M17 medium (the standard selective medium for enterococci strains). Different nitrogen and salt supplementations whey permeate media were examined and the results indicated that all of the examined media were efficient for the bacterial growth with biomass production higher than that produced by using M17 medium (22.7 g/L), suggesting the applicability of whey permeate media for the economic production of the isolated bacterial biomass for extending its industrial application.

The purpose of the present study was to investigate the potential probiotic effect of the newly isolated E. faecium NRC-3 at three gradual levels on feed digestion in the rumen and the pattern of feed fermentation by rumen microorganisms. The in vitro batch culture technique was usually used to evaluate feed's dry matter and organic matter degradability in the rumen and indicate the metabolizable energy of feed [43]. In the current study, the improvement of feed degradability parameters (DM, OM, NDF, and ADF) by rumen microorganisms after the inclusion of E. faecium NRC-3 might be due to stimulation of rumen fermentation activity as a result of higher population and colonization of rumen microorganisms (especially fibrolytic bacteria) on feeds particles. In line with this suggestion, Izuddin et al., [44] reported that improvement of feed organic matter digestibility, gas and volatile fatty acid production in the rumen after lactic acid bacteria inclusion could be explained by the rise of the ruminal microbial population. Confirming current suggestions, Mamuad *et al.,* [12] observed that supplementation of *E. faecium* SROD to the diet increased the population of the cellulolytic bacteria, *R. flavefaciens* and *F. succinogenes*, and most fungal species in the rumen. The higher production of SCFA (acetate, propionate and butyrate) associated with *E. faecium* NRC-3 addition in the current study was reasonable because of the improvement of ruminal degradability for DM & NDF of the tested ration. It is well known that total volatile fatty acid (TVFA) production in the rumen is positively correlated with dry matter degradability [45]. Similarly, Mamuad *et al.,* [12] found that the addition of *E. faecium* SROD to the diet increased ruminal TVFAs concentration, and by increasing the inclusion level of *E. faecium* SROD, the acetate production increased. Also, Jiao *et al.,* [19] stated that the addition of *E. faecium* (LAB3) to high grain diets leads to a higher ruminal concentration of TVFA than control and suggested that *E. faecium* (LAB3) might support the growth of fibrolytic bacteria at pH 6.5.

In the present study, the gradual decrease of ruminal pH with the addition of *E. faecium* NRC-3 to the control was logical, because of the higher production of SCFA. Inconsistent with current data, lower pH values of the rumen liquor were observed with increasing the inclusion level of *E. faecium* SOLD in the diet than the control [12]. Mamuad *et al.,* [12] attributed this reduction in the ruminal pH to the increase in the production of organic acids like acetic and propionic acids by rumen microorganisms. In contrast, Jiao *et al.,* [19] stated that the addition of *E. faecium* (LAB3) to high grain diets had no significant impact on the ruminal pH *in vitro*. The lack of an effect on the ruminal NH_3–N concentration with the addition of *E. faecium* NRC-3 at all the additional levels could be explained through lower degradability of ration protein with the utilization of the resulted NH_3-N in the rumen for microbial protein synthesis directly [2]. Also, Mamuad *et al.,* [12] found that supplementation of *E. faecium* SROD at all inclusion levels didn't impact NH_3-N concentration in the rumen. The higher production of ruminal total gases with the addition of *E. faecium* NRC-3 to the ration was expected, because of higher DM, OM, NDF and ADF degradability of the treated ration. Inconsistent with our findings, Muck *et al.,* [46] reported that total gas production in the rumen was highly positively correlated with feed degradation rate. Also, Guo *et al.,* [45] reported that silage treated with *E. faecalis* showed higher ruminal gas production than that of the control silage. Mamuad *et al.,* [12] indicated that the supplementation of 0.1 % *E. faecium* SROD

increased the total gas production in the rumen *in vitro*, but decreased CH_4 and CO_2 production significantly compared to the control. According to Jiao *et al.,* [19]; the variance in the data of the different studies might be attributed to numerous factors including the kind and dose of lactic acid bacteria administered, feed ingredients and rumen environment and microbial diversity.

5. Conclusion

The newly isolated *Enterococcus faecium* NRC-3 showed typical characteristics as an ideal microorganism for probiotics production. In the current study, the use of cheese industry waste (permeate) as a substrate for the production of the isolated strain was applied for preparing an economically competitive probiotic product. Moreover, the addition of its encapsulated form to the ruminant ration *in vitro* succeeded in the alteration of rumen fermentation toward the production of more energy in the form of short-chain fatty acids and high biologically valued microbial protein.

6. Conflicts of interest

The authors have declared that no competing interest exists.

7. Formatting of funding sources

This paper is based upon work supported by the Science, Technology & Innovation funding Authority (STIFA) in Egypt through grant NO: 33413.

8. References

[1]. Azzaz, H. H, Aboamer, A.A., Alzahar, H., Hassaan, N. A. and Murad H.A., Effect of cellulases supplementation on milk yield and feed utilization by Baladi goats in early lactation. *Int. J. Dairy Sci.,* **15**, 48-53 (2020). https:// doi.org/10.3923/ijds.2020.48.53.

[2]. Azzaz, H. H, Farahat, E. S. A. and Ebeid, E. M. Effect of partial replacement of corn grains by date seeds on Rahmani ram's nutrients digestibility and Nubian goat's milk production. *Int. J. Dairy Sci.,* **12**, 266-274 (2017). https:// doi.org/ 10.3923/ijds.2017.266.274.

[3]. Azzaz,H.H, Aziz, H. A., Alzahar, H. and Murad. H.A. Yeast and *Trichoderma viride* don't synergistically work to improve olive trees by products digestibility and lactating Barki ewe's productivity. *J. Biological Sciences*, **18** (6), 270-279 (2018). https:// doi.org/10.3923/jbs.2018.270.279.

[4]. Mostafa S.A. Khattab, Azzaz H.H., Ahmed M. Abd El Tawab and Murad H.A., Production optimization of fungal cellulase and its impact on ruminal degradability and fermentation of diet. *Int. J. Dairy Sci*., **14**, 61-68 (2019). https:// doi.org/10.3923/ijds.2019.61.68.

[5]. Azzaz H.H., Farahat, E.S.A., Morsy,T.A., Aziz, H. A., Hadhoud, F. I. and Abd-Alla, M.S. Moringa oleifera and Echinacea purpurea as supplements for Rhamani lactating ewe's diets and their effect on rumen characteristics, nutrients digestibility, blood parameters, milk production, composition and its fatty acid profile. *Asian J. Anim. Vet. Adv.,* **11**, 684-692 (2016). https:// doi.org/10.3923/ajava.2016.684.692.

[6]. Fahim, N. H., Kholif A. E. and Azzaz, H.H. Fennel and ginger improved nutrient digestibility and milk yield and quality in early lactating Egyptian buffaloes. *Ann. Anim. Sci.,* **22** (1), 255–270 (2022). https://doi.org/10.2478/aoas-2021-0008.

[7]. Azzaz H.H., Ebeid H.M., Morsy T.A and Kholif S.M. Impact of feeding yeast culture or yeast culture and propionibacteria169 on the productive performance of lactating buffaloes. *Int. J. Dairy Science* **10** (3), 107-116 (2015). https://doi.org/10.3923/ijds.2015.107.116.

[8]. Kholif, A. E., Hamdon, H. A., Kassab, A. Y., Farahat, E.S.A., Azzaz, H. H., Matloup, O.H., Mohamed, A.G. and Anele, U. Y. Chlorella vulgaris microalgae and/or copper supplementation enhanced feed intake, nutrient digestibility, ruminal fermentation, blood metabolites and lactational performance of Boer goat. *J. Animal Physiology and Animal Nutrition,* **104**(6), 1595-1605 (2020). https://doi.org/ 10.1111/jpn.13378.

[9]. Azzaz H.H., Murad H.A. and Morsy T. A. Utility of Ionophores for Ruminant Animals: A Review. *Asian J. Animal Sciences*, **9** (6), 254-265 (2015). https://doi.org/ 10.3923/ajas.2015.254.265.

[10]. Azzaz, H.H., T. A. Morsy, and Murad, H. A. Microbial Feed Supplements for Ruminant's Performance Enhancement. Asian *J. Agricultural Research,* **10** (1), 1-14 (2016). https :// doi.org/ 10.3923/ajar.2016.1.14.

[11]. Murad H.A., Hosseany, E. N., Abd Elhamid, S. M., Abu-El Khair, A. G., Zahran, M. O. and Azzaz, H. H. Yeast biomass production from milk permeate with enrichment application of dairy animal's diets. *Int. J. Dairy Science,* **15**, 134-141(2020). https :// doi.org/ 10.3923/ijds.2020.134.141.

[12]. Mamuad Lovelia L. , Seon Ho Kim , Ashraf A. Biswas , Zhongtang Yu , Kwang-Keun Cho,Sang-Bum Kim , Kichoon Lee and Sang Suk Lee. Rumen fermentation and microbial community composition influenced by live *Enterococcus faecium* supplementation. *AMB Expr*, **9**, 123 (2019). https :// doi.org/ 10.1186/s13568-019-0848-8.

[13]. Li, Y., Liu, T., Zhao, M., Feng F, Luo W. and Zhong, H. In vitro and in vivo investigations of probiotic properties of lactic acid bacteria isolated from Chinese traditional sourdough. *Appl. Microbiol Biotechnol.,* **103**, 1893–1903 (2019). https :// doi.org/10.1007/s0025 3-018-9554-8

[14]. Li, P., Gu, Q., Wang, Y., Yu, Y., Yang, L. & Chen, J. V. Novel vitamin B12-producing *Enterococcus* spp. and preliminary in vitro evaluation of probiotic potentials. *Appl Microbiol Biotechnol.,* **101**, 6155–6164 (2017). https ://doi.org/10.1007/ s0025 3-017-8373-7

[15]. Sarantinopoulos, P., Andrighetto, C., Georgalaki, M. D,, Rea, M. C., Lombardi, A, Cogan, T. M., Kalantzopoulos,, G. and Tsakalidou, E. Biochemical properties of enterococci relevant to their technological performance. *Int Dairy J.,* **11**, 621–647 (2001). https ://doi.org/10.1016/S0958 -6946(01)00087 -5

[16]. Yang, E., Fan, L., Yan, J., Jiang, Y., Doucette, C., Fillmore, S. and Walker, B. Influence of culture media, pH and temperature on growth and bacteriocin production of bacteriocinogenic lactic acid bacteria. *AMB Express* **8**, 10. (2018) https :// doi.org/10.1186/s1356 8-018-0536-0

[17]. Pang, D. G., Yang, H. J., Cao, B. B., Wu, T. T. and Wang, J. Q. The beneficial effect of *Enterococcus faecium* on the in vitro ruminal fermentation rate and extent of three typical total mixed rations in northern China. *Livest Sci.,* **167**, 154–160 (2014). https ://doi.org/10.1016/j.livsc i.2014.06.008

[18]. Wang, C., Shi, C., Zhang, Y., Song, D., Lu, Z. and Wang, Y. Microbiota in fermented feed and swine gut. *Appl Microbiol Biotechnol*., **102**, 2941–2948 (2018). https :// doi.org/10.1007/s0025 3-018-8829-4

[19]. Jiao, P. X, Liu, F. Z, Beauchemin, K. A. and Yang, W. Z. Impact of strain and dose of lactic acid bacteria on in vitro ruminal fermentation with varying media pH levels and feed substrates. *Animal Feed Science and Technology*, **224**, 1-13 (2017). https://doi. org/10.1016/j.anifeedsci.2016.11.005.

[20]. Fayed, B., Abood, A., El-Sayed, H. S., Hashem, A. M. & Mehanna, N. S. H. A synbiotic multiparticulate microcapsule for enhancing inulin intestinal release and Bifidobacterium gastro-intestinal survivability. *Carbohydrate Polymers*, **193**, 137-143 (2018). https://doi. org/10.1016/j.carbpol.2018.03.068.

[21]. EL-Shafei, K., El-Gizawy, S. A., Olfat, S. B., Fatma, A. F., Sharaf, O. M., and Hoda, S. Comparative evaluation of the efficiency of the microencapsulation methods to improve the flavor production by *Leuconostoc mesenteroides* and *Streptococcus lactisdiacetilactis*. *Research Journal of Pharmacutical Biological and Chemical Sciences***, 7**(3), 1155-1167(2016).

[22]. El-Shafei, K., Elshaghabee, F. M. F., El-Sayed, H. S., and Kassem, J. M. Assessment the viability properties of *Lactobacillus casei* strain using labneh as a carrier. *Acta Scientiarum Polonorum Technologia Alimentaria*, **17**(3), 267-276 (2018). http://dx.doi.org/10.17306/J.AFS.2018.0583

[23]. Ismail, S. A., El-Sayed, H. S., and Fayed, B. Production of prebiotic chitooligosaccharide and its nano/microencapsulation for the production of functional yoghurt. *Carbohydrate polymers*, **234**, 115941(2020). http://dx.doi.org/ 10.1016/j.carbpol.2020.115941.

[24]. El-Sayed, S. M., Elaaser, M., and El-Sayed, H. S. Ameliorate the processed cheese production by functional microcapsules loaded with mustard seed extract and *Bifidobacterium bifidum*. *Biocatalysis and Agricultural Biotechnology*, 102221 (2021). https://doi.org/10.1016/j.bcab.2021.102221.

[25]. MacFaddin, J. F. (2000). Biochemical tests for identification of medical bacteria, 3rd ed. Lippincott Williams & Wilkins, Philadelphia, PA (2021). https://www.microbiologyresearch.org/content/journal/ijsem/10.1099/00207713-31-1-108 https://doi.org/10.1099/00207713-31-1-108

[26]. Mulaw, G., Sisay Tessema, T., Muleta, D., and Tesfaye, A. In vitro evaluation of probiotic Properties of lactic acid bacteria isolated from Some traditionally fermented rthiopian food products. *Int J Microbiol*., **25**, 7179514 (2019). doi: 10.1155/2019/7179514.

[27]. Rajkowska, K. and Kunicka-Styczyńska A. Probiotic properties of yeasts isolated from chicken feces and kefirs. *Pol J Microbiol*., **59**, 257–263 (2010). https://doi.org/10.3389/fnut.2020.00027.

[28]. Azmi, A. F. M. N., Mustafa, S., Hashim, D. M., and Manap, Y. A. Prebiotic activity of polysaccharides extracted from Gigantochloalevis (Buluhbeting) shoots. *Molecules*, **17**(2): 1635-1651(2012). https://doi.org/ 10.3390/molecules17021635.

[29]. Wang J, Xing Z, Tang W, Zheng Y and Wang Y. Isolation, identification, and potential probiotic characterization of one *Lactococcus* from Kefir grain. Food Sci., Biotechnolology, **24**, 1775–80 (2015). https://doi.org/10.1007/s10068-015-0231-8.

[30]. Mahmoud, M., Abdallah, N. A., El-Shafei, K., Tawfik, N. F., and El-Sayed, H. S. Survivability

of alginate-microencapsulated *Lactobacillus plantarum* during storage, simulated food processing and gastrointestinal conditions. *Heliyon*, **6**(3), e03541(2020). https://doi.org/10.1016/j.heliyon.2020.e03541.

[31]. Collado, M. C., Meriluoto, J., and Salminen, S. Adhesion and aggregation properties of probiotic and pathogen strains. *Eur. Food Res. Technol*. **226**, 1065–1073 (2008). https://doi.org/10.1007/s00217-007-0632-x

[32]. Bali V, PS Panesar and MB Bera,. Isolation, screening and evaluation of antimicrobial activity of potential bacteriocin producing lactic acid bacteria isolate. Microbiol J., **1**: 113-119 (2011). **DOI:** 10.3923/mj.2011.113.119

[33]. Ismail S. A., Azza M. Abdel-Fattah, Mohamed A. Emran, Azzaz H. H., Mamdouh S. El-Gamal and Amal M. Hashem,. Effect of partial substitution of ration's soybean meal by biologically treated feathers on rumen fermentation characteristics (*in vitro*). *Pakistan Journal of Biological Sciences*, **21**(3), 110-118 (2018).https://doi.org/10.3923/pjbs.2018.110.118.

[34]. Anonymous CLSI (Clinical and Laboratory Standards Institute), Zone diameter and minimal inhibitory concentration (MIC) Standards (2015).

[35]. Byappanahalli, M.N., Nevers, M.B., Korajkic, A., Staley, Z.R. and Harwood, V.J., Enterococci in the environment. *Microbiology and Molecular Biology Reviews*, **76**(4), 685-706 (2012). https://doi.org/10.1128/MMBR.00023-12.

[36]. Anjum, J., Zaidi, A., Barrett, K. and Tariq, M., A potentially probiotic strain of *Enterococcus faecalis* from human milk that is a virulent, antibiotic sensitive, and nonbreaching of the gut barrier. *Archives of Microbiology*, **204**(2), 1-18 (2022). https://doi.org/10.1007/s00203-022-02754-8.

[37]. Favaro, L., Basaglia, M., Casella, S., Hue, I., Dousset, X., de Melo Franco, B.D.G. and Todorov, S.D., Bacteriocinogenic potential and safety evaluation of non-starter *Enterococcus faecium* strains isolated from home made white brine cheese. *Food microbiology*, **38**, 228-239 (2014). https://doi.org/10.1016/j.fm.2013.09.008.

[38]. Toomey, N., Bolton, D. and Fanning, S., Characterisation and transferability of antibiotic resistance genes from lactic acid bacteria isolated from Irish pork and beef abattoirs. *Research in microbiology*, ***161***(2), pp.127-135 (2010). https://doi.org/10.1016/j.resmic.2009.12.010.

[39]. Cho, S., Barrett, J.B., Frye, J.G. and Jackson, C.R., Antimicrobial resistance gene detection and plasmid typing among multidrug resistant enterococci isolated from freshwater environment. *Microorganisms*, ***8*** (9), 1338 (2020). https://doi.org/10.3390/microorganisms8091338.

[40]. Ismail, S. A. and Emran, M. A., Direct microbial production of prebiotic and antioxidant chitin-oligosaccharides from shrimp byproducts. *Egypt J, Aquat Biol Fish*, **24**(4), 181-195 (2020). https://doi.org/10.21608/EJABF.2020.98021.

[41]. Hassan, A. A. and Ismail, S. A. Production of antifungal N-acetyl-β-glucosaminidase chitinolytic enzyme using shrimp byproducts. *Biocatal Agric Biotechnol.*, 102027 (2021). https://doi.org/10.1016/j.bcab.2021.102027

[42]. Ismail, S.A., Nour, S.A. & Hassan, A.A., Valorization of corn cobs for xylanase production by *Aspergillus flavus* AW1 and its application in the production of antioxidant oligosaccharides and removal of food stain. *Biocatalysis and Agricultural Biotechnology*, **41**, 102311(2022). https://doi.org/10.1016/j.bcab.2022.102311.

[43]. Aboul-Fotouh G.E., G.M. El-Garhy, Azzaz, H. H., A.M. Abd El-Mola and G.A. Mousa. Fungal cellulase production optimization and its utilization in goat's rations degradation. *Asian J. Anim. Vet. Adv*., **11**, 824-831 (2016). https://doi.org/10.3923/ajava.2016.824.831.

[44]. Izuddin W. I., Teck Chwen Loh, Anjas Asmara Samsudin, Hooi Ling Foo. In vitro study of postbiotics from *Lactobacillus plantarum* RG14 on rumen fermentation and microbial population. R. Bras. *Zootec.,* **47**:e20170255 (2018). https://doi.org/10.1590/rbz4720170255 .

[45]. Guo G, Shen C, Liu Q. et al. (2020). The effect of lactic acid bacteria inoculums on in vitro rumen fermentation, methane production, ruminal cellulolytic bacteria populations and cellulase activities of corn stover silage. *J Integr Agric.* **19**, 838–847 (2018). https://doi.org/10.1016/S2095-3119(19)62707-3.

[46]. Muck R E, Filya I. & Contreras-Govea F E. Inoculant effects on alfalfa silage: *In vitro* gas and volatile fatty acid production. *Journal of Dairy Science*, **90**, 5115–5125 (2007). https://doi.org/10.3168/jds.2006-878.